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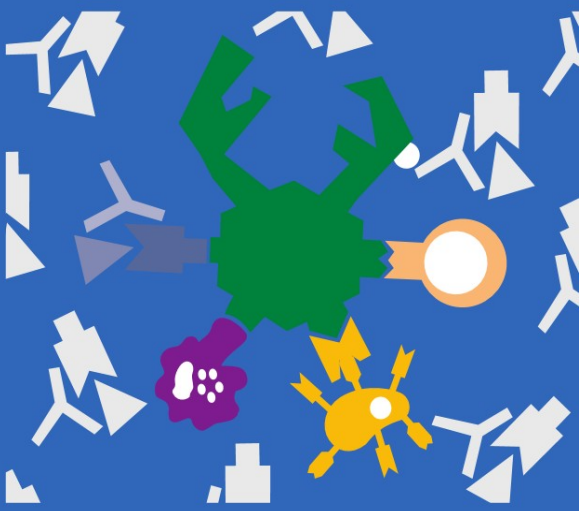
Editor

From Molecular to Modular Tumor Therapy

Tumors are Reconstructible Communicatively Evolving Systems



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Tumors are Reconstructible Communicatively Evolving Systems

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Therapy-Derived Systems Biology: A Pragmatic Communication Theory

Chapter 1
Bridging Theory and Therapeutic Practice: From Generalized Disease Models to Particular Patients

Albrecht Reichle

Abstract The traditional problem of the poor presentability as well as diagnostic and therapeutic practicability of individual patient care is still unresolved. Biomodulatory therapies for metastatic tumors bring transparency into tumor systems by breaking into a tumor's holistic communicative world, and by dissecting the tumor for practical purposes, such as attenuation of tumor growth, in comprehensible evolutionary processes. Biomodulatory therapies show that the holistic communicative structures of a tumor are now an experimentally and therapeutically accessible entity: Communication within systems—which is self-content to some degree—works with the implicit understanding that (1) the validity and denotation of particular systems objects (proteins, cells etc.) is always context-dependent, (2) the validity and denotation of the systems objects may be therapeutically redeemed by systems-immanent communication rules, which are determined by descriptively accessible communicative systems textures including intersystemic exchange processes. The difference between theory and practice may be decisively attenuated (1) by giving reductionistically derived systems features an internal communicative context (formal-pragmatic communication theory), (2) by introducing a novel and scientifically accessible perspective, i.e. the tumor's 'living world', which is defined as a tumor's holistic communicative world, and (3) finally by binding the systems features to tumor-immanent evolutionary processes (modularity of biochemical and cellular processes, rationalization of tumor functions).

The newly discovered tumor-associated systems architectures, which are built on the capability of tumor systems to modularly rearrange the validity and denotation of systems objects, clearly differ from the reductionistically derived systems comprehension: (1) Communicatively-derived systems structures offer new insights into evolutionary processes, promoting tumor development and expansion into the 'metabolism' of tumor evolution. (2) Based on the perception of a systems participator, we ultimately leave behind typical reductionistically derived teleological

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systems features. (3) Both, reductionist and holistic understanding are exerted to reproduce a situational stage of tumor disease: Differential perspectives of therapeutic interaction are entangled with various levels of knowledge and consecutively with different therapy strategies.

Keywords Personalized tumor therapy • Communication theory • Metastatic tumor • Tumor models

1.1 Introduction

The traditional problem of the poor presentability as well as diagnostic and therapeutic practicability of **individual patient care** is still unresolved. Applied science subsumes particular tumor features in general patient models without attending to individual, evolutionary-developed systems patterns in metastatic tumor disease.

For a patient as an **individual**, no difference exists between the patient as a general and as a particular person. In the present context, the term 'general patient' refers to the biochemical, cellular, and organ unity or, in other words, to the empiric patient among many other patients with identical reductionistically derived characteristics. The particular patient, on the other hand, is characterized by distinct individual and even particular therapeutically accessible features (e.g. via the tumor's Achilles' heel).

When the knowledge about a patient is generalized and projected into a unique cohort – meaning that one patient is the representative of an entire patient population, – the general oncologic knowledge meets the **nude identity of the tumor patient** as a formal prerequisite of the coherency of the physicians' conceivability. If the knowledge about a disease is empirically derived, i.e. based on the view of clinicians, the internal nature of the disease is perceived as foreign as its external nature, namely that of a whole patient population with distinct biological stigmata.

If differentiation between the accepted situational notion and the 'transcendentally' true notion of an individual disease ceases, that means disease perception under idealized conditions of a 'homogeneous' patient cohort, we are unable to explain, why we can reflexively learn and improve our own knowledge and standards in patient care.

We may not accept our notions about an individual patient – which are always only locally and time-dependently justified – to be true in an objective sense.

The conflict between intelligible, classifiable model diseases and an individually emerging disease needs to be overcome by **contextualist diagnostic and therapeutic approaches**. Scientific ambition for objectivity in the comprehension of metastatic tumor diseases is marked by the search for intersubjective agreements. Scientists present data sets and applied tumor models generated either by sophisticated technologies (e.g. 'omics') and mathematically reprocessed data or by the pure availability of drugs for combinatory use (combination of 'historical' standard therapies with novel therapy principles). Subsequently, these data sets are incommensurable, resulting in divergent comprehensions of metastatic tumor diseases and finally in the call for novel 'ontologies'.

The present book aims at leading the reader away – in a scientifically accessible manner – from the daily conflicts between **theory and practice** and between **the generalized and individual tumor patient**, so that more personalized diagnostic and therapeutic strategies can be developed for controlling metastatic tumor disease:

- First, recording the systems concept of tumor biology based on rather different sciences (biochemistry, cell biology, and medical oncology) in form of the functional world of single tumor-associated cell types (tumor microenvironment and tumor cells) and respective biochemical processes (with the main focus on inflammation) including their potential contribution to communication
- Then, giving reductionistically derived systems features an internal communicative context (formal-pragmatic communication theory)
- Finally, binding the systems features to tumor-immanent evolutionary processes (modularity of biochemical and cellular processes, rationalization of tumor functions)

As shown, the difference between theory and practice may be decisively attenuated by introducing a novel and scientifically accessible perspective, i.e. **the tumor's 'living world'**, which is defined as a tumor's holistic communicative world. Addressees and receivers of communicative processes are the systems objects of a tumor, i.e. molecules, pathways, cellular organelles, cells, and the host's organs. The texture of a tumor's 'living world' consists of structured systems-wide contexts.

Communication within systems – which is self-content to some degree – works with the implicit understanding that

- The validity and denotation of particular systems objects is always context-dependent (integration of addressees, receivers of communication, including their signals) and subjected to contingency programming.
- The validity and denotation of the systems objects may be therapeutically redeemed by systems-immanent **communication rules**, which are determined by descriptively accessible **communicative systems textures** including **inter-systemic exchange processes**.

The texture of a tumor's 'living world' allows the implementation of a 'big functional world' inside small tumor networks, if modular tumor architectures are successfully rearranged by **biomodulatory tumor therapies** (modulators of transcription factors, low dose metronomic chemotherapy, Imides, histone deacetylase inhibitors, etc.) to attenuate tumor growth with **modest toxicity**.

That way, the **conflict** between context-disrupting claims for generalized diseases with their attributed reductionistically derived features and the availability of situational patient-derived tumor-associated features **may be resolved**. Therapeutically emerging tumor-associated features in form of action- and therapy-relevant yes/no statements mirror the therapeutic facts at an involved organ site. Objective tumor response or stable disease resulting from communicative interference with tumor systems is mediated by biomodulatory therapy approaches.

The holistic communicative concept of tumors described in a **formal pragmatic communication theory** does not give in to a generalized, commonly used 'homogeneous' tumor model (which hardly includes the individuality of a tumor disease, despite the general assumption of individually varying tumor evolution). Additionally, this holistic concept does neither agree with the frequently valueless subjectivity of individual diagnostic and therapeutic decisions nor with a circular concluding teleology (e.g. tumor cell selection comprehended as the competitive 'survival of the fittest' in the Darwinian sense).

At first sight, the fact seems rather daunting that all systems processes are subjected to a continuous contingency programming on the basis of tumor-immanent, partly autonomous and, therefore, individually evolving processes. However, when we therapeutically meet the challenges presented by a tumor's 'living world', we may achieve **therapy-derived systems interpretation** including individual but also classifiable processes linked to distinct **situational, stage- and tumor type-associated evolutionary developments**.

The newly discovered tumor-associated systems architectures, which are built on the capability of tumor systems to modularly rearrange the validity and denotation of systems objects, clearly differ from the reductionistically derived systems comprehension:

- The holistic communicative structures of a tumor are now an experimentally and therapeutically accessible entity.
- Communicatively-derived systems structures offer new but not teleologically preconceived insights into evolutionary processes, promoting tumor development and expansion into the '**metabolism'** of **tumor evolution**.
- The holistic communicative view allows a more abstract systems perspective of tumors.
- Based on the perception of a systems participator, we ultimately leave behind typical reductionistically derived teleological systems features (i.e. tumor-associated angiogenesis, immunology, inflammation, coagulation etc.).
- Both, reductionist and holistic understanding are exerted to reproduce a situational stage of tumor disease: **Differential perspectives of interaction** are entangled with **various levels of knowledge** and consecutively with **different therapy strategies**.

Tumor-associated evolutionary processes exclusively lie in a communicatively-linked molecular and cellular world. Biomodulatory tumor therapies bring transparency into the holistic communicative system by breaking into a tumor's 'living world' and by dissecting the tumor for practical purposes, such as attenuation of tumor growth, in **comprehensible evolutionary processes**.

Knowledge about these processes may finally **bridge theory and practice** in a novel appreciation of **tumor pathophysiology** and in novel biomodulatory-based **study designs** (adaptive trial designs). Systems-related read-out parameters derived from cellular **secretome analytics, molecular imaging techniques**, **and comparative systems analytics of different tumor types and systems stages** are urgently needed to describe modular, evolutionary developing tumor architectures and intersystemic exchange processes.

At the end of this short introduction, I want to thank all authors for their excellent contribution and their willingness to implement their contribution into the conceptional context of this book. Ms Schoell, I want to thank for her excellent linguistic support.

Biomodulatory therapy approaches, realized in multiple multi-center phase II trials in cooperation with many colleagues, represent the basis for describing tumor systems. These studies could only be carried out with the support of others convinced of the 'alternative' therapy approach in contrast to current emancipatory interests.

The ideas for these novel biomodulatory tumor therapies were based on the intent to palliatively treat systemically pre-treated patients with metastatic tumors. These studies would have been impossible without the tremendous support of a meanwhile retired colleague, Dr. Bross, my colleagues at our and external departments, and various supporters from the pharmaceutical industry: Thank you very much indeed.

I would like to express my gratitude to Dr. Witz for giving me the opportunity to publish in his book series focusing on tumor microenvironment.

The book and its contributions have been conceptionally structured to introduce the reader to evolutionary tumor systems but also to open up perspectives that may be derived from novel systems considerations.

Chapter 2
Tumor Systems Need to be Rendered Usable for a New Action-Theoretical Abstraction: The Starting Point for Novel Therapeutic Options

Albrecht Reichle

Abstract A tumor system not only consists of diverse cell types but also comprises all components of action insofar that these components are oriented in terms of diverse cell types. Thus, it is necessary to decode paradox situations of cellular rationalization, deformation, and communication processes or, in other words, to uncover inconsistencies within tumor cell compartments or distinct topologies of aggregated action effects. Here, a theory may be helpful that discharges into an action-theoretical abstraction and simultaneously includes evolutionary tumor developments. In an evolutionary process, tumor cells may exploit the whole extent of the rationalization features of stroma cells to implement the functional diversity of systems behavior aimed at maintaining homeostasis and robustness in tumor systems. The introduction of genomic/non-genomic systems-directed therapeutic approaches may allow both, the uncovering of systems topologies of aggregated action effects and the broadening of therapeutic options via systems-directed approaches. (1) Tumor systems biology is now turning into a **scientific co-subject**. (2) Developing **action-theoretical systems terms** with the corresponding conceptual equipment may contribute to the classification of tumor subsystems. (3) Systems-directed therapies may **meet new therapeutic requirements**, which might help to create therapeutic approaches that are specifically designed for the demand of tumor stages, corresponding systems stages. Therefore, patients would probably not have to be selected according to age and/or co-morbidities because of known adverse toxicities of standard therapies (maximal tolerable doses). In contrast, therapies may meet the (individual) tumor system's characteristics by a systems-orientated selection of biomodulatory acting agents. As shown, toxicities may be modest [56].

Keywords Tumor systems • Modularity • Rationalization • Metastatic tumor • Robustness • Personalized tumor therapy • Biomodulatory therapy • Metronomic chemotherapy • Transcriptional modulation

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2.1 Explorative Considerations (The 'Now')

Cancer represents the largest genetic experiment ever conducted: Distinct acquired genetic lesions are not distributed at random in tumor cells, **despite the high variability of cancer causes**, the **heterogeneity** of observed genetic aberrations, and the **divergence** of morphologic characteristics of diverse tumor types. The non-random distribution of genetic aberrations might be explained by the fact that cancer-associated dysregulated transcription factors must still collude in a life-maintaining manner for cancer (stem) cell self-renewal, for proliferation, and for the build up of a cellular infrastructure suitable for tumor promotion [1]. As a main characteristic, cancer (stem) cells must be able to contribute to an evolutionary process. In subsystems, such as angiogenesis, inflammation must be activated and coordinated to allow expansive tumor growth. Stroma cells in the immediate vicinity are ultimately challenged, either functionally within their 'living world' (differentiation, trans-differentiation, dedifferentiation, apoptosis) or by the newly developing systems context characterized by the rationalization or the deformation of cellular functions and the acquisition of new cell types [2]. Vice versa, the function as a tumor (stem) cell is cooperatively determined by the adjacent microenvironment [3]. Many cellular functions associated with invasion and metastasis are often not constitutively expressed by carcinoma cells, but rather transiently in response to contextual signals that tumor cells receive from their stromal microenvironment [4]. Therefore, the simultaneous modeling of both stroma and tumor cell functions may open up new therapeutic perspectives in cancer therapy [5]. The communicatively designed tumor microenvironment is integrated into an evolutionary process. Thereby, it acquires cells from blood circulation and subjects cells to rationalization processes to establish new systems behavior: stroma cells from a formally organized functional status within the previous functional 'world'. Conversely, experimental data support the assumption that stroma cells even impose pressure on tumor cells to change or keep functions. Ultimately, stroma cells with molecular aberrations may contribute to malignant conversion [8].

The change in systems complexity induced by a developing tumor interferes with the affected organ and may destroy not-regenerative cell inventories. Thus, this change not only alters previous ways of interactions among organ-associated cells but also considerably affects the communicative infrastructure of rationalized forms of communication within an affected organ. It is necessary to simultaneously decode paradox situations of cellular rationalization, deformation, and communication processes, i.e. to uncover inconsistencies within tumor cell compartments by means of a theory that includes the evolutionary development of a tumor as well as its biologic history in order to increase therapeutic options with systems-directed approaches.

2.2 Methodological Approach

2.2.1 Theory of Communicative Interactions in Tumor Compartments

Three competing research approaches are applied regularly. As required by methodology, these approaches have to virtually dissect the coherence of systems and the functional 'world' of distinct cell systems.

2.2.2 Structural Differentiation

Classic methodology is comparatively classifying. The theoretical core is formed by assumptions about the structural differentiation of cells (histopathology) in functionally specialized systems of interaction. These assumptions are sufficient for supporting the observation that the structural integrity of tumor compartments needs to be maintained to sustain appropriate tumor-stroma-cell communication for tumor progression [9]. Thereby, functional considerations are not sufficiently separated from structural ones in such a way that the disposed concurrence between methodological strategies may unfold.

The likely importance of this conceptual separation was shown by Karnoub: Mesenchymal stem cells must pass through an 'educational' process to act as cells promoting metastatic process [10,11]. Investigations into evolutionary processes of tumor development discharge this theory of structural differentiation into a more theoretically oriented model that includes systems functions [9].

Considering the functional aspects of morphologic changes, Dvorak [12] developed the basic principles of this action-theoretical concept by comparatively characterizing similarities between wound healing processes and tumor growth, thereby including morphological data (structural differentiation). Although morphologically based, the introduction of an evolutionary view has allowed a systems therapeutic approach that recalls the famous remark of Dobzhansky [13]: 'Nothing in biology makes sense except in the light of evolution'.

Tumor-associated changes in cellular structures are currently reconstructed in all intersections: More recently, much attention has been drawn to cellular stroma components that are suspected of promoting cancer progression, such as the composition of lymphocytic tumor infiltrates, fibroblasts, macrophages, and other inflammatory cells, immunosuppressive cells called myeloid-derived suppressor cells (MDSCs), and mesenchymal stem cells. Analytically attained data about these cell types allow a one-dimensional conception of the total process of structural differentiation: A distinct function is unidirectionally coupled to cellular structure.

Thus, the process of structural differentiation may not be designed as a multidimensional process, i.e. a decoupling of systems and a functional 'world' of tumor cell systems. Mediated by newly structured mediator-guided subsystems, the decoupling

process during tumor development may have a decisive influence on the (still) structured differentiated functional 'worlds' of cell systems in an affected organ.

From different methodological viewpoints, the total extensiveness of tumor pathology may be highlighted only now and in such a way that would be desirable for the development of one (individual) tumor therapy with a broadened basis. However, the conceptual equipment is neither available for action-theoretical abstractions and systems-associated tumor stages nor for functional classifications based on an adequate differentiation between

1. Synchronous structural differentiations of the functional 'world' of tumor-associated cell systems
2. The spin-off of functional systems that are differentiated via chemokines and cytokines as well as the interior differentiation of these cell systems (e.g. accumulation of regulatory T-cells, mesenchymal stem cells)
3. The differentiation processes induced by tumor (stem) cells, which simultaneously dedifferentiate differentiated cellular functional areas (rationalization of functions) in terms of a colonization of the functional 'world' of organ tissues (metastatic process), simultaneously facilitating the integration of new cellular elements from the peripheral blood **(mobilization, trafficking)**

2.2.3 Rationalization

A further competitive research approach exclusively investigates the rationalization of functional systems in the course of evolutionary growth complexity during tumor development and tumor spread under the aspect of different **purposes**. The aspect of rationalization may be elucidated by the analytically defined functional spectrum (references) of fibroblasts [14] or macrophages within a cellular system: Macrophages and other inflammatory factors do more than just foment angiogenesis in tumors [15], i.e. they actively aid cell movements that produce metastases, thereby calling tumor cells to the vessels. On the other hand, they may act as tumor-antigen presenting cells for tumor control [16,17]. This out- lined functional 'world' of macrophages gives an impression of rather divergent options of rationalizations within a systems context [18]. Therefore, ambitious efforts are currently under way to retrain tumor-associated macrophages. The higher the involvement of evolutionary processes, the higher the accessibility of 'socialization' processes of tumor and stroma cells by systems-theoretical analyses. This 'socialization' may neither be intuitively nor exclusively realized by the reconstruction from the tumor cell site, as it is commonly the case [6]. Necessary changes of the point of view and method should be conducted accurately without the confusion of paradigms. The increasingly higher organization of a tumor cell system during tumor growth results in the development of systems perspectives, in which the functional 'world' of distinct cell types is featured as a component of the respective systems 'world' [7]. Systems organizations are gaining a kind of autonomy by neutralizing separation towards previous cellular functions or by the assignment of new functions. Thus, distinct

cell types obtain **systems-immanent functions** and become indifferent to other 'socialization' processes. This development characterizes the mediator-associated separation of developing tumor-adjacent macrophages from immuno-suppressive tumor promoting cells to weapons that destruct tumors [19].

Stroma cells are either present in affected organs or develop after the trafficking of bone marrow-derived mobilized cells out of circulation [20]. The implementation of a new form of integration (rationalization) of these stroma cells allows an evolutionary advancement of the systems complexity with the remodeled rationalization of cellular functions: The diversified resources of tumor growth-promoting cytokines are distributed among rather different stroma-associated cell types (redundancy). Thus, different rationalization processes are conceivable without the systems deprivation of an essential growth-promoting mediator if a cell system would functionally drop out due to new systems-related differentiation processes [21]. The clue of this finding is that distinct systems functions, such as inflammation, may be maintained despite the change in cellular composition during tumor development. Furthermore, these observations underline the necessity of an action-theoretical abstraction.

2.2.4 Deformation

A third research approach, originally advanced by Loewenstein [22], focused on the evolutionary process of tumors with regard to the functional aspects of increasing complexity. More recent observations have followed a similar line, i.e. growth factors make cancer cell cancerous, and otherwise, if carcinoma cells are deprived of signals from the stroma compartment, they may revert to an earlier phenotype state, in which they do no longer display the traits of high-grade malignancies [23]. The question remains, how do they communicate?

With an exclusively functional consideration, the systems-associated constrictions of cellular functions, which take place in cell systems during evolution, are misplaced from the perspective of an observer on the level of communication by tethering inter-systemic exchanges at imbalances in communication. Thereby, the importance of the identity-threatening deformation of cell systems is withdrawn, as it is appreciated from a participator's perspective: Tumor-associated stroma cells may even be driven into apoptosis by systems characteristics: In a figurative sense, they are neutralized by the system [24].

2.2.5 Resulting Observation Levels

Pathologic systems-biological processes in cancer may be reported from different observation levels:

1. In Loewenstein's view, pathologic cancer processes are predominantly mirrored in deficient cell-to-cell communication [22].

2. The initial source of observation may also be an altered systems-associated cell composition [25].
3. Distorted functions of single cell systems within the tumor microenvironment [24–27]: Deformations.

In tumor systems biology, diverse 'wound healing' processes, such as inflammation and angiogenetic processes, have been identified as factors independent of the viewpoint of observation.

2.2.6 Approach to an Action-Theoretical Systems Term: The Scientist as a Subject of the System

Each of the three research approaches and viewpoints described bring about the **separation of subject and object**. In other words, none of the three approaches considers it necessary to uncover the object: **A tumor's systems biology is also a scientific subject**, a co-subject of the scientist that interests not only as an approach for observation, description, and explanation of cellular behavior. Even more, it serves as a communication partner, for instance via biomodulatory therapies, and thus as an approach of hermeneutic comprehension. This approach represents a scientifically new aspect for understanding tumor biology, implicating a decisive broadening of therapy options that arise from the evolutionary consideration of tumor development [5].

2.2.7 Tumor Systems Need to be Rendered Useable for a New Action-Theoretical Abstraction

The constitution of this new kind of consideration about the **objects of interest** an action-theoretically derived (therapy-related) systems theory is different from the exclusively analytic/empiric systems terms that derive from results generated by functional genomics/proteomics in tumor systems biology**.**

2.2.8 Assignment of Systems-Theoretical and Action-Theoretical Inconsistencies

The systems concept in tumor biology is introduced by a systematic recording of the functional 'world' of single cell types including their potential contribution to communication.

The change from the perspective of an observer to that of a participator is justified by the action-theoretical description of a system in biomodulatory therapies [5].

Thus, a new frame for action may be launched for new systems-directed therapies, which may affect tumor growth by regulatory activities and thereby modulate functions of subsystems that could be found ubiquitously or in distinct tumor groups and different tumor stages. This concept has been outlined especially for metastatic stages [5].

2.3 Conceptual Equipment

Behavior dispositions, behavior reactions, behavior releasing stimuli. In a cell system, we have to differentiate between the reactions of a cell system on mediators, the addressing of reactions to other cell systems, and the addressing of another cell system calling out the response. A system of fundamental terms (behavior dispositions, behavior reactions, behavior-releasing stimuli) permits the separation of cellular behavior from observable events. Thus, tumor systems may be rendered usable for a new functional systems classification, the starting point for new therapeutic options. Behavior dispositions may have a great impact on tumor growth. This assumption is underlined by the claim that attempts at determining metastatic tumor properties should focus on genes and proteins that confer the responsiveness of a primary tumor cell to stroma cells, rather than on genes and proteins that directly mediate the cellular phenotypes of invasive metastasis [10].

Denotation and identity of a cell or a cell system. Intercellular relations within the tumor compartment are reconstructed from the perspective of distinct cell systems, which represents the most frequently used reconstruction. Here, the notion of rules comes into play. The application of a rule induces the assignment of symbols (e.g. pathway structures) and the assignation of an identical denotation and validity.

For the introduction of functional aspects into tumor pathology, it is important to note that the denotation of cell systems does not necessarily derive from the identity of the object, for instance morphology, which may be identified as an identical cell system by a different observer.

Macrophages, fibroblasts in tumor stroma, and their multifaceted functional stages represent an exceptional example: Their identity comprises diverse realizations of functions within different systems conditions, which means that identity is not based on observable invariance but on **intercellular validity**. Vice versa, the identity and validity of rules are related between cell systems (Fig. 2.1).

Role structure between cell systems. Obviously, standardized anticipation of distinct behavior seems to exist, considering the constitution of a growth-promoting microenvironment based on distinct tumor (stem) cell functions. Nevertheless, new communication pathways may be initiated that are related to the new functional ‘world’ of tumor cells. However, cell system A does not know, whether it adheres to a rule, or if is exposed to the susceptibility of cell system B or to the ability to reach consensus (educational processes). Educational effects have been observed in tumor systems [10].

Autonomy. A typical feature of the establishment of tumor systems is that their formation empirically depends on the specific prerequisites of a host's organism. Also from an empirical viewpoint, subsystems may develop certain autonomy (for example, inflammation and cancer-associated autoimmunity). Although tumor systems may not exist beyond a social cellular system, just the same as subsystems without a tumor system, these subsystems may vary independently to some extent and could contribute to border-line histology (Fig. 2.1). Additionally, cell systems may not constitutively generate functions, which may also be transiently acquired by 'education' for a small time frame [10].

Subsystems may be independent to a certain degree, i.e. they do not feature characteristics as invariable references, must steadily advance **contingent relations** to one another, and are not fixed to invariant features of developmental stages. Contingency programming may adapt interactions via adhesive interactions with stroma cells, stroma proteins, and growth factors [28]. However, relations of subsystems are predetermined by their affiliation to a common action system. Subsystems are forming environments for one another, but in a regulated trade-off.

Reproduction. Each action system presents itself as an area of reciprocal interpenetration of subsystems. Each of these subsystems is specialized in reproducing basic functions facilitating tumor promotion. The distinct reproductive function of tumor (stem) cells is underlined by molecular-pathologic data showing that molecular aberrations in the primaries determine tumor biologic behavior, for instance, early or late metastatic spread as well as metastatic sites [29,30].

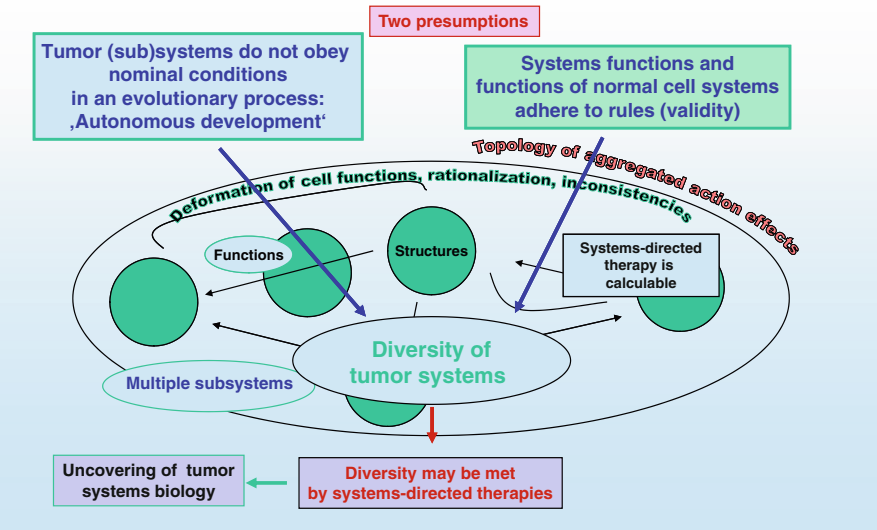

Fig. 2.1 Systems-directed therapies may integrate action-theoretical systems terms (theory) and biomodulatory therapy-derived comprehension (experimental part) of tumor-associated subsystems (e.g. inflammation, angiogenesis…), thereby uncovering and meeting diversity of tumor systems

Evolutionary processes. Basically, tumor development is comparable with an evolutionary process, during which single cell systems acquire to a greater or lesser extent (area of application within the communicative exchange) diversified complexity (for example, integration of extensive inflammation during metastatic spread). Cell systems experience pathologic deformations in case of inconsistencies between the functional 'world' and the systems 'world' and may even be driven to apoptosis. Now, in the mirror of evolutionary processes, the functional 'world' of cell systems may be recognized under systems-therapeutic conditions and vice versa [5,31] (Fig. 2.1).

2.3.1 Sensitive Assessment Tools

Clinical phenomenology, hermeneutic observation of systemic exchange of information during evolution, and systems-targeted therapies represent action-orientated research approaches (Fig. 2.2). How may systems pathologies be conceptually characterized?

Robustness, stability, and homeostasis of a tumor system describe how a subsystem is controlled during biomodulatory therapies or evolutionary processes [32]. By means of biomodulatory therapies, the following observations within phase II trials on different metastatic tumor types indicate therapy-related alterations of tumor **robustness, stability, and homeostasis** in a therapeutically relevant way:

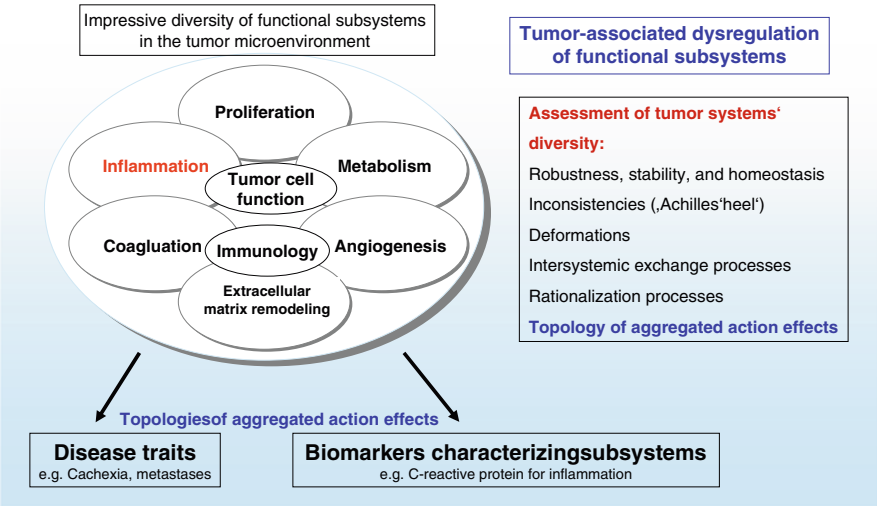

Fig. 2.2 Assessment tools of systems biology are rationalization processes, inconsistencies, deformations, altered inter-systemic communication, and topology of aggregated action effects. The more exact systems biology may account for the objects of interest studied by means of these tools, the more it may justify the use of systems-biological research approaches

1. Stable shaping of and focusing on the tumor system's organization (very delayed objective response)
2. Significant modulation of tumor-associated disease traits, for instance, inflammation, ECOG status, paraneoplastic syndromes (biomodulation-derived biomarkers)
3. Biomodulatory activity depending on the metastatic organ site in castrate-resistent prostate cancer (tumor-stroma specificity as expected from the known differential behavior of various cell types within tumor compartments and varying stroma cell compositions at different metastatic sites)
4. The predominant site of progression at the original localization of metastases (hints for impact on metastatic processes) [5]

Changes of systems characteristics, cumulative activities (positron emission tomography, PET), and biomarkers (e.g. C-reactive protein) were recorded by monitoring functions and components of subsystems (for instance, inflammation, angiogenesis, etc.).

Inconsistencies. Pathologies arising from 'social' interactions of cell systems may not be matched with **nominal conditions**. This circumstance has to be met from a systems-therapeutic view: Systems-immanent pathologies may emerge as **inconsistencies**, in which communicative networked interactions between cell systems may be involved. 'Fallacies', 'self-delusions', or 'instigations' may objectively apply force to organisms [8,20–24,33]. Misleading communicative contributions are provoked by an interactive communication praxis (for instance, tumor-associated autoimmune phenomena), which depends on the areas of application (conspirative behavior of a body's own normal cells), thereby limiting the operational praxis and response repertoire of cell systems. 'Fallacies' may occur as communicative processes that are to limited extent critically appreciated by neighboring cells. Markert phrased the presumption that 'very little cell differentiation is truly autonomous in vertebrate organisms' [31]. However, tumor cells may exploit the whole extent of stroma cell autonomy to implement the functional diversity of systems behavior, which is mirrored in highly diversified rationalization, deformation, and communication processes aimed at maintaining homeostasis, stability, and robustness of tumor systems. These systems characteristics may be mapped in distinct topologies of tumor systems- aggregated action effects. A way to uncover these aggregated action effects are biomodulatory therapy approaches [5].

'Fallacies' are likely to play an important role in cancerogenesis and progression as well as in the development of benign tumors. Vice versa, inconsistencies offer an operational range for systems-directed therapeutic approaches [5,17,19,23,34,35].

Furthermore, the interference of inconsistencies could also explain the durable and sometimes rapid therapeutic responses observed in highly vascularized tumors such as angiosarcomas and renal clear cell carcinomas. These responses also occur in pronounced inflammatory tumors, for example, in Langerhans' cell histiocytosis. Inconsistencies targeted with genomic/non-genomic biomodulatory therapy approaches could bring about a collapse of overstressed hyperactive communication systems that maintain distinct functional stages [5]. Also self-depictions arising as tumor-associated autoimmune phenomena may be controlled by biomodulatory

therapy approaches [36]. An impressive example for self-depiction during tumor initiation seems to be the autoantigen-triggered evolution of chronic lymphocytic leukemia (CLL) [33].

Deformations: Abstractions of inconsistencies in which networked cell systems may be involved, thereby discharging in paradox pathologies, may arise as deformations of cell systems including their functional spectrum. Other paradox processes may be uncovered by analyzing rationalization processes. Paradox processes can be of such quality that a systematic congestion caused by rationalization of the functional 'world' of tumor-associated stroma cells may result in an overload of communicative infrastructures (for instance, Langerhans' cell histiocytosis). Paradox processes may be monitored by analyzing the diversification of rationalization or deformation processes, or, in extreme cases, apoptotic cell death [24].

Functional pathologies become evident because of the interactive communication praxis of cell systems assigned to areas of application: spontaneous tumor necrosis may also be understood as functional pathology. Here, the tumor microenvironment may not maintain or advance the originally constituted system in an evolutionary context. Additionally, no controlled degradation takes place after damage of systems functions. In case of tumor (stem) cells, the identity of the denotation and the object itself is never the same (quiescent, tumor-promoting phase). Therefore, 'deformation' of a tumor (stem) cell may also result from a neutralization process (in contrast to active controlling, for example, immunologically).

As the importance of a tumor cell in the role of a tumor-promoting cell is critically influenced by the tumor-associated microenvironment, targeting of tumor (stem) cells via microenvironment seems to be therapeutically promising [3,5,37]. The fact that a cancer (stem) cell must be promoted by a number of inflammatory conditions, particularly in the metastatic stage of cancer disease, fits with the successful use of anti-inflammatory therapy components in the systems-targeted treatment strategy presented recently [5].

Metastatic spread may be promoted by a series of rather different cell systems invading the tumor compartment. Despite the presence of cancer cell dissemination in different organ sites, release from dormancy and growth are selective for particular organ sites and depend on stroma composition but not on one singular cancer cell-driven process [29,30].

Intersystemic exchange processes. The complimentary reciprocal activity, which subsystems may generate for one another, may be analyzed as currents of inter-systemic exchange. Therefore, from a therapeutic point of view, the systems-biological model does not specify whether a 'wound healing mechanism' has to be suppressed or stimulated to achieve tumor control: Inflammation control as well as stimulation of inflammation may control tumor growth, immuno-suppression, and immune stimulation [5,34]. Contradictory decisions could be associated with the same capacity to achieve tumor control in a distinct tumor type. Thus, the questions arising are: which therapeutic approach would be easier to put into practice, which is likely to be more compatible with other therapeutic approaches, and which is the most tolerable approach with regard to side effects.

2.3.2 *Action-Oriented Research Approaches: Broadening of the Therapeutic Spectrum (Individualized Therapy)*

Topology of aggregated action effects. Detection of **inconsistencies** between the action status of a cell type and the systems organization within a tumor engross the insights into the pathophysiological organization of important functional elements and constellations discharging into a distinct topology of aggregated action effects [5]. Characteristic constellations may be ubiquitously found in rather different tumor types (for example, highly 'pro-angiogenic' 'inflammatory' tumors) and, therefore, beyond a specific tumor type or its distinct organization of subsystems (Fig. 2.3). Consecutively, a broad repertoire of biomodulatory therapy approaches targeting the **functional status** of cell systems **or cell communication** should be available for targeting functional pathologic (individual) constellations at low toxicity levels. Concerted modulation of transcriptional networks via peroxisome proliferator-activated receptor (PPAR) alpha/gamma agonists, interferon-alpha, glucocorticoids, PPAR-delta antagonists, metronomic low dose, angiostatic and immunomodulatory acting chemotherapy have shown a wide activity in metastatic tumor control, even the capability for remission induction [5,38–41]. The cellular microenvironment may even modulate via orphan receptors a set of transcription factors characterizing 'stemness' of tumor cells, e.g. Okt 3/4 genes [42–45]. Do systems complexity and the myriad of reductionist therapeutic approaches targeting tumor or stroma cells precede the simplicity of biomodulatory treatment strategies?

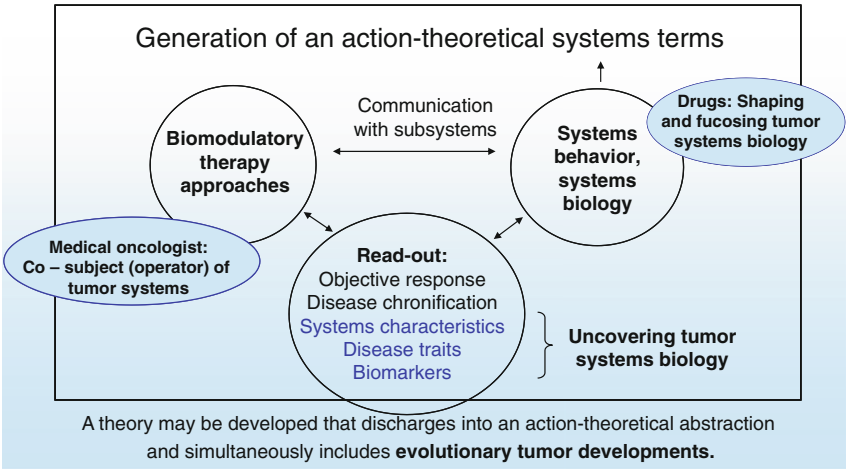

Fig. 2.3 Practical and emancipatory interests in therapies integrated in the coherence of science bring together the constitution of new objects of interest (therapy-derived systems biology) and their pragmatic application, here in form of biomodulatory therapy approaches. Biomodulatory derived changes in the tumor may demerge individually moving processes within the tumor tissue into more easily elusive constellations

The repertoire of drugs abruptly expands with the introduction of systems-therapeutic concepts, as (1) substances with unintended indication, such as drugs modulating the transcriptional networking, may be introduced [46,47]. (2) Contrary to the molecular genetic heterogeneity of tumor cells, tumor growth-promoting systems promise a high grade of similarities (for example, angiogenesis and inflammation). Therefore, a similar repertoire of drugs might be available, which target and regulate corresponding tumor-associated subsystems mirrored by biomarkers [48]. (3) Targeting functionally defined subsystems seems to become of increasing interest, as subsystems may be exclusively functionally defined in a systems context but simultaneously linked to alternating structural systems [21]. Targeting functional systems structures opens up a new therapeutic window favoring concerted biomodulatory strategies. (4) Beyond that, it should be possible to abstract traditionally described subsystems: Drugs with biomodulatory activity as (nuclear) transcription factors regularly have an activity profile far above the capacity of hermeneutic comprehension [5]. Transcriptional networking may have a decisive regulatory impact on tumor promotion, for instance, on the angiogenic switch or on tumor stem cell behavior [37]. Indeed, the abdication of hermeneutic comprehension was a prerequisite of modern science. To what extent is comprehension necessary for describing tumor biology from an action-theoretical view (Fig. 2.4)?

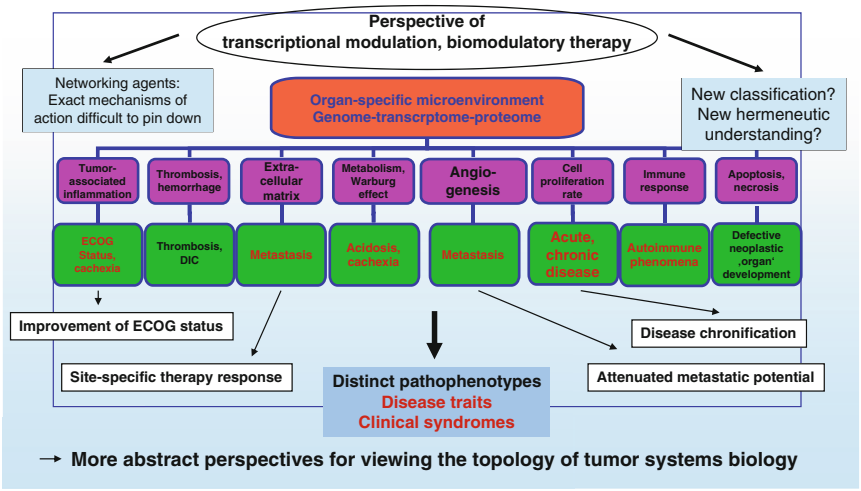

Fig. 2.4 Systems-biological approaches are open for the detection of new networking interactions (experimental part). Thereby, the context of discovery (modulation of tumor associated disease traits, biomarkers) has to be consistently separated from the context of justification (rational for a biomodulatory therapy approach). The currently established genomic/non-genomic biomodulatory therapies may lead to novel and more abstract perspectives for viewing the topology of tumor systems biology

1. From a different point of view, subsystems are also action and functional systems (genome, transcriptome, proteome [pathways], cellular, extra-cellular microenvironment, tumor [stem] cell, tumor-associated disease traits). By no means do they accentuate only arbitrary systems. The classification of subsystems has not only a theoretical but also a practical impact, as the benchmarks of the systems correspond to the components of which functional sequences are composed.
2. Systems-biological approaches are open for the detection of new networking interactions (experimental part). Thereby, the context of discovery (modulation of tumor-associated disease traits, biomarkers) has to be consistently separated from the context of justification (rational for a biomodulatory therapy approach).
3. Basically, a hermeneutic comprehension of action mechanisms within familiar observation levels is no prerequisite in respect of the multi-fold coregulative and the cell-specific activities of (nuclear) transcription modulators in different cell systems. In contrast, the currently established genomic/non-genomic biomodulatory therapies may lead to novel and more abstract perspectives for viewing the topology of tumor systems biology [5].

2.4 Discussion: Critical Reflection on Tumor Systems Biology (The 'Then')

The uncovering of tumor systems requires more than analytical approaches, for instance, the use of research approaches, such as phenomenology (including case reports, description of therapy-associated side effects), hermeneutic understanding, theory of evolutionary processes, and systems-directed therapies.

Assessment tools of systems biology are rationalization processes, inconsistencies, deformations, altered intersystemic communication, and topology of aggregated action effects. These tools are only now emerging in their constellation during tumor development (in different tumor types and stages) as a decoupling of systems and the functional 'world' of cell systems. The more exact systems biology may account for the objects of interest studied (for instance, the topology of aggregated action effects) by means of these tools, the more it may justify the use of systems-biological research approaches (Fig. 2.5).

Currently, the instruments for merging different scientific directions for systems-theoretical considerations are missing. Basic research is predominantly technology-oriented, aligning itself with the dichotomy of structure- and function-analytical problems. Closer collaboration between academic institutions and biotech and pharmaceutical industries will be required to facilitate research on systems-biological processes [49].

A tumor system as a system of action consists not only of diverse cell types but comprises all components of action insofar that these components are oriented in terms of diverse cell types, the system's objects. Cumulative knowledge, though

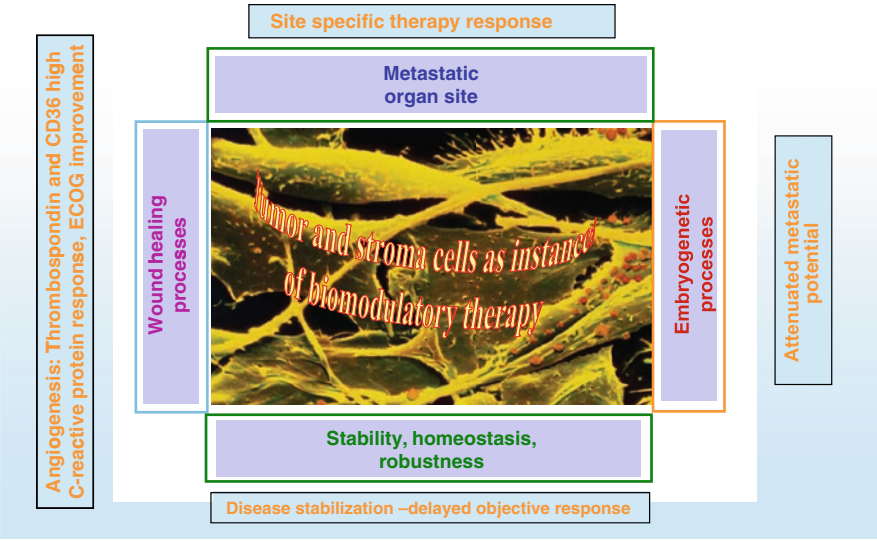

Fig. 2.5 Multifaceted shaping and fucosing of the tumor's organization is the result of multitargeted biomodulatory therapy approaches including stimulatory and regulatory agents with pleiotropic activity and known poor or no monoactivity within the respective tumor type: Dexamethasone, pioglitazone, interferon-alpha, COX-2 inhibitor, metronomic low-dose chemotherapy

scientifically acquired, is more specifically a complex of meanings symbolized within distinct references to different cell types: References are dissipating from the view of a participator (systems biology as a co-subject of the scientist) and cellular functions are anticipated as rationalization processes. The diversity of rationalization processes is based on the intercellular validity of communication rules and might be generally an explanation for the large amount of cases in which cell cultures or animal models cannot be transferred into clinical praxis.

An action-theoretically oriented tumor model diversifies therapeutic instruments by uncovering new systems qualities that may be targeted by broadening therapeutic options by the introduction of biomodulatory approaches. Now, therapies may be guided by monitoring (new) functional pathophysiological processes (biomarkers): If biomodulatory therapies remove differential cell or systems functions involved in metastatic progression, the metastatic process may be inhibited as shown in our systems-directed genomic/non-genomic therapeutic approaches [5] (Fig. 2.6).

Therefore, the most important task is to look for common systems features ('topologies', inconsistencies) within different tumor types to get action-theoretically guided classifications of distinct tumor-associated evolutionary systems processes. Furthermore, classification is essential, as classification is the basic language of medicine and systems organizations across different tumor types, which need to be clearly defined. The uncovering of common features in different tumor types is only the beginning: Lymphomas could soon be classified according to their activation of inflammatory signaling pathways [50], common stroma gene expression sets may

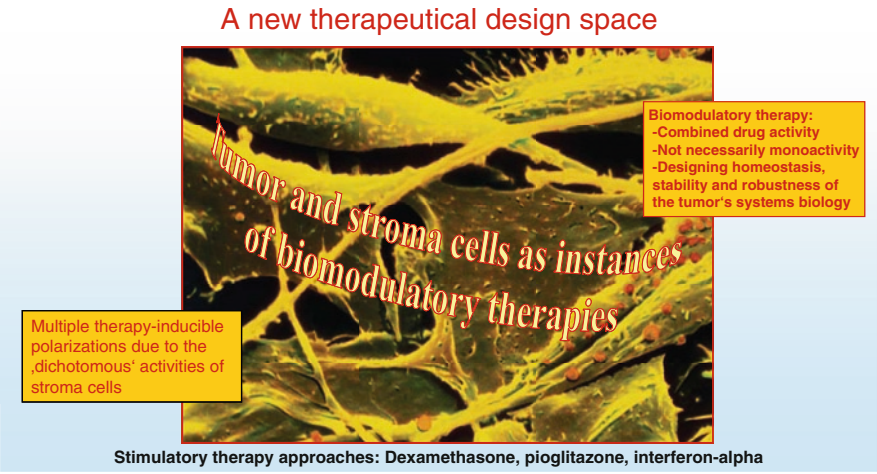

Fig. 2.6 Cellular functions of neighbouring stroma cells are decisively influenced by the tumor cells. The stroma cell proportion within the tumor compartment is highly sensitive for biomodulatory therapy approaches due to the dynamic character and the context dependent dichotomous activities of stroma cells

be detected in response to tumor invasion [51], neoplasias may be classified according to their responsiveness towards combined modulation of transcriptional networking [5], and so on. Another attempt may be the formulation of stroma scores, which still seems to neglect functional system aspects [25].

Action-theoretical systems-terms may additionally contribute to the classification of tumor subsystems via new biomarkers: The method to uncover action-theoretical systems terms is now pioneered from bedside to bench. Clearly defined and distinctive functional systems similarities could be the basis for administering a specific repertoire of (biomodulatory) medications during distinct functional tumor systems stages. The functional status of different systems constellations may be monitored by respective biomarkers. This perspective allows a new comprehension of individualized therapy. Especially the time-sensitivity of a therapeutic approach may be addressed.

In the near future, biomodulatory therapy approaches of metastatic tumors could be methodological tools of an **individualized tumor therapy**: In contrast to 'causal' therapeutic approaches aiming at the blockage of aberrant tumor-associated pathways by a restricted repertoire of highly specific drugs, multiple potential modulators (activators and deactivators) of transcriptional processes are available for biomodulatory therapy approaches. According to our experience, monoactivity of a single transcription modulator is no prerequisite for its successful use and the combined administration activity of all modulators could be followed by respective biomarkers. Close monitoring would further allow us to choose other modulator combinations in cases of weak interactivity to facilitate an objective tumor response [5].

The introduction of sophisticated technologies, such as microarray analyses, pathway analysis in cancer and stroma cells, and accompanying translational

research, has caused some fundamental biological understanding of complex cell interactions associated with important therapeutic implications [52,53]. Analytically and empirically obtained data are important, including the myriad of prognostic markers: But the systems perspective offers the opportunity of weighing constellations as well as pathophysiologically important elements for taping new treatment strategies! A striking difference is visible in the **pragmatic function**, which generated data in different scientific areas. Here, we can combine therapeutically derived information on systems biology to establish systems-biological models. Information may be generated on three levels: Biomodulatory processes, tumor response (traditionally tumor shrinkage), and side effects on the level of the whole organism. Systems-biological considerations may pave the way via new sources of prognostically relevant biomarkers that are representative for subsystems to convey transparency of systems-analytical accessible systems topologies, which may be targeted by (biomodulatory) genomic/non-genomic systems-orientated therapies.

Systems-directed therapies could meet rather new therapeutic requirements. Studying systems biology may help to create therapeutic approaches specifically designed for the demand of tumor stages, corresponding systems stages, and involved organ sites. In this context, the clinical discussion about the appropriate clinical study endpoint is coiled up again: Chronification of metastatic disease or induction of complete remission? Some types of cancer can be held in check by means of stroma by causing cancer cells to behave more like normal cells [5,54].

An important consequence may arise from the cumulative knowledge about mostly unidirectionally analyzed cellular systems interactions on the one hand and the accumulation of results of action-theoretically defined systems terms on the other hand: Patients would probably not have to be selected according to age or comorbidities or both because of known adverse toxicities of empirically evaluated ‘standard’ therapies (maximal tolerable doses) as in case of administering systemic and exclusively reductionist therapies. On the contrary, therapies may meet the (individual) tumor’s systems characteristics by a systems-orientated selection of biomodulatory acting agents. As shown, toxicities may be modest [55]. Therefore, therapies could ‘come’ to the patient.

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Chapter 3
Principles of Modular Tumor Therapy

Albrecht Reichle and Gerhard C. Hildebrandt

Abstract Nature is interwoven with communication and is represented and reproduced through communication acts. The central question is how may multimodal modularly acting and less toxic therapy approaches, defined as modular therapies, induce an objective response or even a continuous complete remission, although single stimulatory or inhibitingly acting drugs neither exert monoactivity in the respective metastatic tumor type nor are they directed to potentially 'tumor-specific' targets. Modularity in the present context is a formal pragmatic communicative systems concept, describing the degree to which systems objects (cells, pathways etc.) may be communicatively separated in a virtual continuum, and recombined and rededicated to alter validity and denotation of communication processes in the tumor. Intentional knowledge, discharging in reductionist therapies, disregards the risk-absorbing background knowledge of the tumor's living world including the holistic communication processes, which we rely on in every therapy. At first, this knowledge constitutes the validity of informative intercellular processes, which is the prerequisite for therapeutic success. All communication-relevant steps, such as intentions, understandings, and the appreciation of messages, may be modulated simultaneously, even with a high grade of specificity. Thus, modular therapy approaches including risk-absorbing and validity-modifying background knowledge may overcome reductionist idealizations. Modular therapies show modular events assembled by the tumor's living world as an additional evolution-constituting dimension. This way, modular knowledge may be acquired from the environment, either incidentally or constitutionally. The new communicatively defined modular coherency of environment, i.e. the tumor-associated

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microenvironment, and tumor cells open novel ways for the scientific community in 'translational medicine' (Reichle A, Hildebrandt GC (2009) Principles of modular tumor therapy. Cancer Microenviron 2(Suppl 1):227–37).

Keywords Modularity • Tumor systems biology • Formal-pragmatic communication theory • Evolvability • Modular communication • Robustness • Metastatic tumor

3.1 Introduction

Nature is interwoven with communication and is represented and reproduced through communication acts. As communication is a process covering all cell communities, also those in tumor tissues, it seems to be difficult to imagine that particularly cancer diseases originate from an equipollent cell only. Therefore, considerations about communication processes within the tumor compartment have to start with the central question whether an equipollent, communicatively structured tumor microenvironment is necessary rather than individual cells causing specific cancer diseases.

Single molecular changes in cancer cells, as specific as they may be, only lead to the development of specific malignancies, when they actively communicate on a subcellular level to finally alter cellular behavior and when adjacent cell types acknowledge the communicated information in a sense the originator intended. This communicative act must allow and must be responsible for the reorganization of well-established normal tissue. Further, in view of the differential steps of communication, the cell community in tumor tissue, which is represented as a holistic communicative system, is also a critical part determining the functionality (quiescent, tumor-promoting phase) of cancer (stem) cells and the development of cancer disease.

Consecutively, tumor development may be described as pathological communication processes on the tissue, the cellular, and the molecular level. Complex biochemical networks are mediators of cellular communication and, considering the multiplicity of tumor-associated communication processes we should include the sub-cellular complexity of biochemical networks as a target into novel concepts of therapeutic approaches.

Transcription factors with their concerted activity are central regulators of subcellular communication processes. Their complex integration into the sub-cellular context is best characterized by their often chimera-like function, equivalent with their communicative integration within networks, which constitute multifold systems functions within the tumor tissue. Dependent on distinct circumstances (the often unconsidered 'background'), they may exert cell type-dependent opposing biological effects. Consequently, a major challenge is to elaborate how single communication processes acquire validity and distinct denotations on the background of numerous input signals discharging into specific biological responses that control tumor evolution.

Up to now, frequently used tumor therapies aim at blocking distinct communication processes involved in tumor promotion, for instance, by changing the denotation of a distinct communication-associated pathway in tumor or stroma cells or by directly targeting and eliminating the bulk of tumor cells (monoclonal antibodies). Successful examples of 'magic bullets' (Paul Ehrlich) in standard clinical care in hematology are, for instance, tyrosine kinase inhibitors in chronic myelocytic leukemia and monoclonal CD20 antibodies in B-cell lymphomas [1,2]. The underlying idealizations with regard to the manner of how to use therapeutically relevant changes in denotations of 'tumor-specific' pathways refer to a well-rehearsed coherency of interactions that should fulfil practical and, at best, tumor-specific functions. Therefore, therapeutic approaches in tumor therapy are predominantly designed in a reductionist way [1].

Previous modes for therapeutically modifying communication processes in metastatic tumors included, for instance, the use of small molecules, monoclonal antibodies, or cellular therapies. The modes were based on the intentional comprehension of these communication processes [1], presuming what distinct communicating cells generally (i.e. under generalized conditions) insinuate with a signal used in a given situation. This way of generalizing validity of an addressed signal distracts from the often situatively complex biochemical conditions that make a signal valid in the first place. Context-related changed validity of transcription factors and consecutively altered denotations are exceptional examples.

The dimension validity of a communication process is introduced by formal communication theories that are trying to assume circumstances under which a communication process is or becomes valid. Although acknowledgement of validity is a prerequisite of communication processes, the functional and structural premises for redeeming validity are commonly discussed to a far lesser extent, if not neglected altogether [3–5].

The communication theory developed in this paper is anchored in observations derived from controlled clinical trials on the use of a combination of biomodulatory acting drugs (= systems-directed therapies) in a broad variety of metastatic tumors [6]. Reductionist considerations may not explain how multimodal, less toxic systems-directed therapies are able to induce an objective response, even a continuous complete remission, although single stimulatory or inhibitingly acting drugs (i.e. modulators of transcription factors) do neither exert mono-activity in the respective metastatic tumor type and nor are they directed to potentially 'tumor-specific' targets [6]. As an explanation for the activity of these biomodulatory therapy approaches, we introduced a new communication-technically paraphrased term as target for the cumulative functional activity of systems-directed therapies known as tumor-specific 'topologies of aggregated action effects' [6]: Systems-directed therapies may primarily neglect tumor-related activities that seem to be operationally induced by the division of function, such as inflammation, neoangiogenesis, Warburg effect, immune response, extra-cellular matrix remodeling, cell proliferation rate, apoptosis, and coagulation effects. From a systems perspective, these differential activities present themselves as an enhancement of complexity [6]. Their presenting character turns out to be primarily communicative, as shown in the methodological discussion.

Communication-technical considerations will be helpful to uncover mechanisms of action of modularly designed therapy approaches and to conceptualize how this novel way of treatment modulates sub-cellular and cellular communication. At first, these considerations involve a theory relating to communicative aspects of socially linked cell communities, such as the tumor compartment. The theory is also supported by observations derived from a unique pattern of modular therapies administered in a broad variety of metastatic tumors [6].

This theory leads to the question how communication processes may be initiated (therapeutic aspect) in the context of the basic components of the communicative 'metabolism', which foster natural or therapeutically adjoined but implicitly evolutionary-linked tumor development. Induction of novel validity in informative cellular or intercellular communication processes by modular events may be an important mechanism promoting tumor evolution or treatment.

3.2 Methods: A Formal-Pragmatic Communication Theory

Clinical results used to support the formal-pragmatic communication theory refer to recently published data [6].

3.2.1 Definition of the Tumor's Living World as a Holistic Communicative Unit

Exemplarily for cellular transcription factors, their context-dependent and cell type-specific transcriptional activity illustrates the meaning of the term modularity. The activity is mirrored on a cellular level by the multi-functionality of, for instance, macrophages or fibroblasts.

Modularity in the present context is a formal-pragmatic communicative systems concept, describing the degree and specificity to which systems' objects (cells, pathways, molecules, e.g. transcription factors, etc.) may be communicatively separated in a virtual continuum, reassembled and rededicated (e.g. co-option) to alter validity and denotation of communication processes. This concept refers to possible interactions between the systems objects in a tumor as well to the degree to which the communicative rules of the systems architecture (for establishing validity and denotation) enable or prohibit the focus on validity and denotation. Systems objects acquire the features of symbols, which are rich in content and which are able to acquire novel references by rearranging validity and, consecutively, denotation. Tumors consist of modules, which become a scientific object by communicatively uncovering the tumor's living world (defined as the tumor's holistic communicative world) with biomodulatory and therefore modularly designed events (for instance biomodulatory therapies).

Modularity implicitly imparts a certain degree of evolvability to systems by allowing specific modular features (i.e. modular communicative networks) to undergo changes with regard to validity and denotation of systems objects without substantially altering the functionality of the entire communicative system (holism of the tumor's living world): The systems 'metabolism' modularly and non-randomly changes validities and denotations of biochemical and biological processes. Modularly induced evolutionary steps advance the classic definition of evolvability as the capacity of an organism or a biological system to generate new heritable phenotypes [7] by evolvability within the tumor's living world.

3.2.2 Situative Objectivation of the Tumor's Living World

We, and the smallest living units, i.e. socially interconnected cell communities, are 'born' to communicate. To describe intercellular communication features, we are constrained to terms borrowed from appraising interpersonal relations: Cell systems are getting instigated, educated, reeducated, and attracted, and addressed cells may even be subject to fallacies [8–12]. These few samples, describing different modes of agreement by an addressee or an addressing cell unit, show communication processes that are more than the appreciation of signals independent of the level of communication. Prerequisite for the following discussion is that we assign a single cell communication competence on the background of its genetic repertoire.

Communication processes with their occasionally complex facets of appreciation and generation of agreement might be considered constitutive in nature. However, the question arises whether differentially designed and therapeutically aligned communication procedures, such as modular therapy approaches, have the ability to objectify interrelations and communication structures between basically communicatively associated and evolutionary developing cell communities, such as tumors. If so, a second and now situative objectivation could be generated besides the intentionally acquired previous context-dependent knowledge.

Addressing the question which background communication processes may be initiated in tumors first, for instance, to alter the validity and denotation of transcriptional processes, requires a clarification of the single steps of communication from an intentional point of view (communication theory). In a second step, we have to explain the background which principally allows the commonly used reductionist therapy approaches to uncover the so far frequently unconsidered risk-absorbing background 'knowledge'. This knowledge reassures systems robustness as illustrated by recovery from reductionist therapeutic interventions for tumor control. Tumor's robustness may be specifically responsible for poor therapeutic outcome, and robustness may absorb severe therapy-induced toxicities in a patient's organism.

How may the social organization of a tumor be possible? If modular events, similar to modular therapy approaches, tie the holistic communicative activity of a tumor, a 'social' action theory could be derived, which may objectify the 'metabolism' of

evolving evolutionary systems. An analysis of the prerequisites for communicative action seems to be necessary to exploit the dimension of the living world's background, which cross-links and stabilizes larger cell communities, such as tumors.

3.2.3 Formal-Pragmatic Theory About Denotation of a Communication Process

A formal-pragmatic theory about the denotation of a communication process may establish an internal interrelation of denotation and validity.

Intention is inherent to all messages, also in those of intercellular communication. The understanding of a signal or a more complex message by the addressed cell is a prerequisite for the requested appreciation of a message.

Appreciation is a normative notion, dominant and rich in content, which reaches out to the understanding of, for instance, transcriptional cascades, which may be context-dependently assessed as a 'grammatical' phrase. The understanding of a cellular signal, which has been perceived as valid, is not equivalent with the appreciation of an addressed intention (agreement, disagreement, refusal, etc.). Signals, which are perceived as valid and valid signals should be differentiated.

If appreciation is established, for example, in an agreement, both sites of an intercellular communicative exchange have to accept the respective communication process as appropriate. Appreciation assesses the intercellular acknowledgement of the validity of a basically criticizable intercellular communication process.

Denotation issues cannot be completely separated from validity issues. The denotation-theoretical question 'what does it mean to understand a communication process cannot be isolated from the question under which circumstances a communication process may be considered to be valid.'

3.2.4 Perception of Validity

A cell would not know what it means to understand the denotation of a communication process, if it did not know how to help itself to agree on something with other cells. The prerequisites for communicative comprehension via transmitters, ligands, cytokines, and hormones, etc. may already appreciate that the communicative activity, which may be established with their help, is directed to the comprehension of a transmitted message. That means, as long as a 'tumor cell' does not find a comprehensive cellular surrounding or may not traffic suitable cell types in its adjacent surroundings, it may not function as a tumor cell. Therefore, also disabling comprehension within communication pathways may be a therapeutic aim.

The communicative activity of many molecules and communicative structures is context-dependent with regard to the validity and denotation within a communication

process; for instance, single NF-kappaB signaling pathway can perform multiple biological functions even in the same clonal populations. This phenomenon may be assessed for many transcriptional processes [13–17]. The communication process itself may be hedged by highly variable cellular communication architectures (synapses, gap junctions, receptors, pathways, transcription factors, acetylation modifiers, etc.).

3.2.5 Novel Idealizations: Therapeutically Relevant Redemption of Validity

A method for redeeming the therapeutic validity of communication processes by administration of modular therapies requires idealizations that are present in the living world of a tumor (holistic communicative activity of a tumor). These idealizations exclusively unfold their effectiveness within tumor-associated communication processes. Cells have access in form of explicit knowledge on the background of their (epigenetically modified) genetic repertoire. Thus, as our idealizations reach communication competence, the cells' explicit knowledge, which relies on idealizations (theme-dependent context knowledge), and the risk-absorbing knowledge of the tumor's living world (mediating robustness and systems context) compete in the range of the background knowledge about the tumor's living world [18].

At first, this background knowledge about the tumor's living world represents scientifically none-thematized, situative, speculative, horizon-knowledge. We implicitly rely on this risk-absorbing knowledge in every therapeutic intervention. The background knowledge covers the many assumptions we silently make based on a speculative horizon.

The background knowledge about the living world is subjected to conditions of scientific comprehension: Intentional ways fail to describe risk-absorbing knowledge, in which context-dependent knowledge about commonly administered reductionist therapy approaches is rooted, and the network of the holistic communicative activities turns out to be the medium through which the tumor's living world is mirrored and generated.

In an evolutionary developing tumor system, the idealizing potency lies in the therapeutic anticipation of physicians: Communicative actions (modular therapeutic interventions) are now an element of a cycle process, in which the physician is likewise a product of current knowledge and tradition. Therefore, tumor systems biology may not be generally interpreted in context-free explanations [6].

Holistic character of communication. Each communication initiated activity is linked via communication-technical relations with many other communication-initiated activities. The knowledge about a communication technique (modular therapy) is interwoven with the knowledge about the behavior of the communicatively uncovered living world of a tumor.

3.3 Implementation of the Formal-Pragmatic Communication Theory

Exploitation of background knowledge about the tumor's living world: Disrupting the holistic communicative thicket.

A formal-pragmatic communication theory is provided to explain the therapeutic efficacy of drug combinations characterized by exclusively combined biomodulatory activity and no or poor mono-activity.

3.3.1 Clinical Results Supporting a Formal-Pragmatic Communication Theory

If modularly designed therapies particularly target communicatively linked systems, i.e. their modularity as represented by a distinct systems response (e.g. attenuation of inflammation), modularity should be indicated by unique systems-associated biomarkers. Vice versa, identical modular systems should be accessible for different biomodulatory designed therapy approaches because of the tumor- or situation-dependent variation of cellular promoters of modular systems [17,19].

As shown (chapter 12 and 13), modular systems architecture of metastatic tumors could be uncovered by a small set of biomodulatory therapies. Differentially designed therapy modules were able to uniquely induce a response in serum C-reactive protein (CRP) levels of patients across a broad variety of metastatic tumors (Fig. 3.1): the observed CRP response preceded or was closely linked to clinical

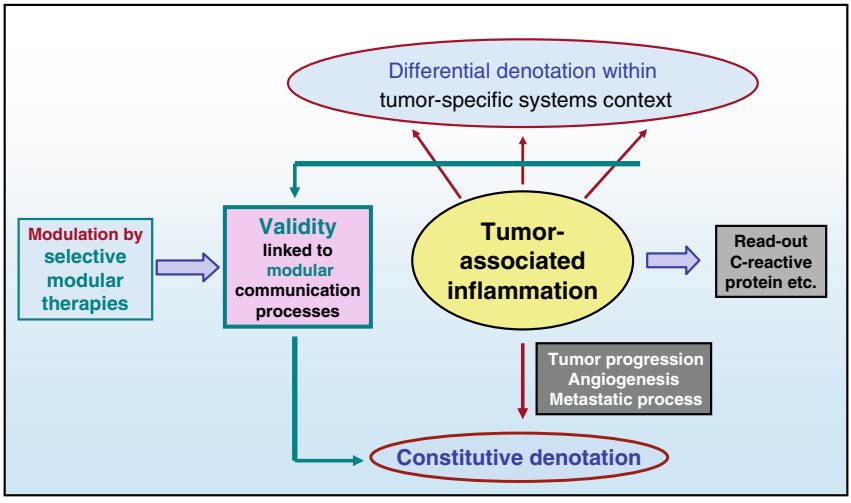

Fig. 3.1 Shaping and focusing systems' communication: disrupting the holistic thicket (Principles of modular tumor therapy)

tumor response (stable disease >3 months, partial remission, or complete remission). This demonstrates that tumor-promoting pro-inflammatory processes are differentially accessible from a communication-technical point of view and differentially constituted in their modularity. Nevertheless, CRP may serve as a unique modularly-linked systems marker to early show the efficacy of these therapies [6].

Most cells within the tumor compartment are constrained to respond to administered modular therapies: targeted molecules are ubiquitously available and partially constitutionally expressed, particularly certain receptors targeted with their respective stimulatory ligands, such as the glucocorticoid receptor, and peroxisome proliferator-activated receptor alpha/gamma. Consequently, many cell systems are included in processes, which may modify modularity and consecutively evolvability. Clinically, this kind of activity is supportively reflected by tumor responses, which occur within a strongly delayed time frame following biomodulatory therapies [6].

Stage-specific and tumor-specific dysregulation of PPARgamma and COX-2 expression in tumor cells are now well established in a broad variety of tumors [20].

Tumor-associated dysregulation of transcription factors (modular communication-technical background) in tumor and stroma cells may be addressed by biomodulatory therapies, such as low-dose metronomic chemotherapy in combination with or without transcriptional modulators (dexamethasone, interferon-alpha, cyclooxygenase-2 inhibitor (PPARdelta), and pioglitazone) [6].

High PPARgamma expression was shown to be representative for the possibility to achieve modular response (improved survival) with different therapeutic approaches (metronomic low-dose chemotherapy plus or minus pioglitazone and rofecoxib) [20]. Notably, metronomic chemotherapy does not even directly target PPARgamma expression, and clinical response to therapy is not linked to inflammation control [21]: therefore, differential modular systems may be targeted to achieve clinical response.

Therapeutic systems-directed interactions mediated by modular therapies may basically interfere within the horizon of living worlds of organisms constituted elsewhere and its organs as well as with tumors. Therapeutic specificity may be achieved by the possibility of modifying the tumor's holistic communication system without significant organ-related side effects, as indicated by a large series of clinical trials [6].

3.3.2 Translation of Clinical Results in a Formal Communication Theory

Translated into a formal communication theory, administered biomodulatory therapies do not directly alter denotations of distinct pathways, such as reductionist designed 'targeted' therapy approaches, but redeem novel validity of modularly induced informative communication processes embedded into the tumor's living world. Modularity is shown to be a specific systems feature, which may be operationally uncovered and defined by distinct biomodulatory drug combinations.

At first, from a clinical point of view, the question how validity is redeemed with biomodulatory approaches on a molecular or cellular basis seems to be of minor importance, whereas particularly the 'know that', the normative communication-linked question is therapeutically critical because of the possibility of bringing about therapeutically relevant yes or no statements.

With regard to the 'know how', direct blocking of proinflammatory signaling pathways by the administered biomodulatory therapies may be excluded as the only explanation for the clinically observable effects. Therefore, decisive changes in the prerequisites of validity of, for instance, pro-inflammatory processes have to be suggested. Changes of validity are implicitly linked with changing denotations of communicative processes, such as the attenuation of tumor growth.

One molecular basis could refer to the cell type-specific combinatorially and dynamically shaped validity and denotation of protein complexes involved in cellular communication networks: NF-kappaB signal transduction pathways may regulate contradictory cellular responses in different cell types and, as recently shown, even within the same clonal population (i.e. cell proliferation versus differentiation and survival, immunity, and inflammation). Controlling factors of the function of NF-kappaB signal transduction pathways involve time, cellular conditions, and external circumstances [17]. However, specifically the latter are insufficiently understood, and this particular background knowledge could be uncovered by biomodulatory therapies on both a cellular and a tissue level.

At this point, the quantitative and qualitative assessment of biochemical processes in a systems context comes into play to prove and advance the formal-pragmatic communication theory on a biochemical level. This way, computational models on the whole tumor tissue's cell-type-specific 'omics' data could be rooted in direct systems biological observations, which may be derived from modular interventions (therapy approaches). Up to now, the direct assignment of communication-relevant validity and denotation modulating biochemical processes in distinct cell types is only fragmentarily assessable.

For therapeutical purposes, inflammation is often symbolized by the classical pro-inflammatory cytokines IL-6, IL-1, and TNFalpha, irrespectively of the cellular sources releasing these cytokines and the cell types calling out for response [22]. However, modular therapy approaches, which include the risk-absorbing, validity modifying background knowledge into the therapeutic calculus, may overcome these reductionist idealizations as all communication relevant steps (intention, understanding, appreciation of messages) and the differential tumor-associated promoters of communication may be simultaneously modulated (Fig. 3.2) [6].

3.3.3 Explication of a Formal-Pragmatic Communication Theory

The claims for redeeming novel therapeutic validity are not only directed towards therapeutic success but also tailored on the relation of communication to the objective

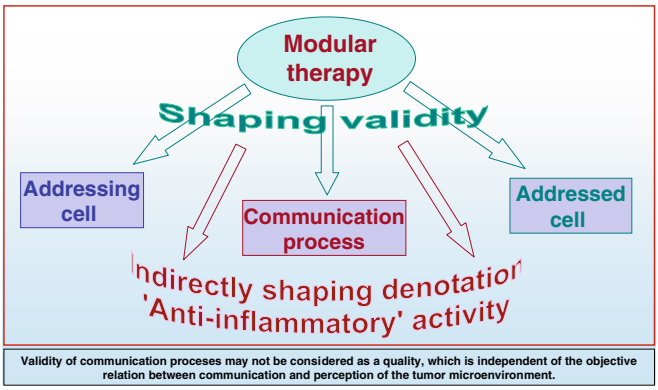

Fig. 3.2 Validity of communication processes may not be considered as a quality, which is independent of the objective relation between communication and perception of the tumor microenvironment

features of the tumor compartment, the evolutionary developing modularity of a tumor, as tumor-associated pro-inflammatory processes, for example, are differentially integrated into the modular architecture (Fig. 3.1).

Modularity may allow the retrospective establishment of spaces for evolutionary developments if modular events (therapy) are implemented. Simultaneously, the background of the tumor-associated living worlds loses its action-guiding function as consensus-warranting evolutionary driven resource. The communicative interaction structures are now the objects of an actor (physician), who brings about distinct reactions in tumor processes, characterized by specification of tumor systems' denotations via redeeming novel validity (Fig. 3.1).

Objectivation of the tumors' living world Modular therapies may be the communicative medium for establishing novel validity of communication-driven processes within the tumor's living world by the rearrangement of protein complexes, altered release of mediators, etc. (Fig. 3.1). Modular therapies may supplement propositional aspects of communication, i.e. the presence of the tumor's living world by normative aspects, namely by therapy-derived yes or no statements ('know that'): Assigned to the function of transcription factors, the changing 'background' may critically determine their validity and denotation in a situation-related manner.

Sustainability of modular therapy. Besides the possibility for redeeming novel validity (for instance inflammation control), modular therapy approaches are characterized by sustainability as indicated by frequently observed late objective tumor response [6].

Communicative systems architecture. The matter of validity of intercellular communication processes may not be considered anymore as a matter detached from the objective relation between communication and knowledge about cellular behavior. From a therapeutic view, the possibility for redeeming validity marks the change from the 'know how' to the 'know that': Knowledge about the tumor and

communicative knowledge (modular systems) are integrated into one another. Therefore, therapeutic options about clinically relevant modular communication techniques are linked with the knowledge of how the communicatively accessible living world really behaves (communicative systems architecture).

Function of modular communication. The therapeutic modulation of validity is aimed at achieving novel denotations of communication processes [17]. The dimensions' denotation and validity are internally tightly related within communication processes. The function of modular communication is to configure the coherence between validity and denotation. Thereby, novel denotations may be therapeutically tailored via modulation of validity processes (e.g. tailoring validity of pro-inflammatory processes for tumor control). Mediators of these communication processes are communication-related molecules, pathways, protein complexes, etc., whose denotation may be situative exchangeable to some degree or is subject to decisive modifications in a non-random communicative tumor systems context embedded in the tumor's living world.

Specificity of redeemed communicative validity. Specific conditions of compliance for redeeming validity on the site of the tumor's living world constitute relations between communication technique (specified modular therapy approaches) and distinct tumor-associated situation-engraved systems stages. Modular therapies in different metastatic tumor types show a high grade of specificity for redeeming novel validity via modular therapy elements [6].

Differentially redeemed validity of modular events (therapy approaches) represents the convergence point that facilitates (clinically) important yes or no statements. Not until then does the communicative situation allow a second objectivation of the tumor by uncovering the tumor's living world. Modularly changing validity and denotation of components of the tumor's living world represent the dimensions fostering evolutionary processes in tumor development, for example, the link between tumor-associated inflammation and tumor progression.

Tumors constitute a solitary world with an internal context. This solitary world is represented by highly specific topologies of aggregated action effects. As indicated by moderate systemic toxicity profiles of the administered modular therapies, these action effects obviously need to be clearly separated from those appearing in a normal organ context.

Systems-related biomarkers, such as C-reactive protein in serum or PPARgamma expression in tumor cells, may guide modular therapies. Corresponding systems changes may be closely linked to clinical response after modular therapy. Therefore, the redemption process of a novel therapy-guided validity may be followed early in the therapeutic process by indicators specifically associated with functional changes in single systems features. Interestingly, the validity of prognostic markers in malignant tumors can change with the tumor stage as demonstrated for COX-2 expression and PPARgamma expression in melanoma cells [20].

Tumors are integrated systems. Randomized trials clearly indicate that tumors may be described by communicatively integrated and interwoven systems: In melanoma, both metronomic chemotherapy and pioglitazone plus rofecoxib independently develop clinical systems-directed activities and even seem to act

synergistically [21]: Tumor-specific topologies of aggregated action effects may be specifically targeted with differential modular approaches to enhance therapeutic efficacy as tumors are composed by various modular elements, which are drawn into inter-systemic exchange processes (possible synergism).

The modularity of a tumor is an independent tumor characteristic. As described, the modular systems concept does not follow the classic systems perception of functional pathophysiology. It is exclusively communication-derived and guided by redeeming novel validity through modular therapy approaches. Besides histology or molecular pathology, the modularity of a tumor is an independent tumor characteristic [6]: Tumors are additionally represented in a modular communicative architecture. The modular architecture of tumor-associated cell systems is directly embedded in the holistic totality of the tumor's living world.

Modular therapy approaches may be designed tumor-specifically and stage-specifically (Table 3.2). The advantage of a modular view of therapeutic interventions is the situative reference in topologies of aggregated action effects. The therapeutic value of the topologies of aggregated action effects lies in the presentation character of current communicative circumstances.

Evolutionary reconstruction of tumor-associated systems. Redeeming validity is tailored on the relation of modular communication to the objective features of the tumor compartment, the reconstructible evolutionary (modular) systems, for example, indicated by differential impact of pro-inflammatory processes within the tumor system [6]. Modular events (therapies) serve as a prerequisite for the reconstruction of the tumor's living world, in which cells are symbolic communicative figures with – to some degree – exchangeable references connected by modular structures: Consecutively, communicatively derived systems may be described by rationalization processes, deformations, and intercellular exchange [6].

'Metabolism' of evolution. How may new systems properties emerge? The possibility for redeeming novel validity shows the modulation of validity as an important evolutionary promoter (the 'metabolism' of evolution). The formal-pragmatic communication theory is able to establish modular coherency between environmental tumor cell-associated and microenvironment-associated communication processes as well as a modularity-based evolvability of systems.

Reproductive structures. As the most meaningful reproductive structure we commonly suggest the genetic repertoire. Modular therapies now show that modular events, assembled by the tumor's living world, seem to present an additional evolution-constituting dimension, which primarily lies within the limits of the genetic repertoire. Additionally, also the heritable inventory might be evolvable.

Table 3.2 Modular therapies

• Combined transcriptional modulation
• Metronomic chemotherapy
• Epigenetically modulating drugs
• Combine therapies including biomodulatory acting drugs without or with poor monoactivity (indication discovery)
• Combination with reductionist approaches?
• Sequential modular–reductionist therapies?

This way, modular knowledge may be either incidentally or constitutionally acquired from the environment.

Cell communities and cells constitute themselves, alternating in a close modular response to informative processes. Therefore, modular communication is usable as an internal systems-relevant and environmental communication mode: The evolutionary link between two different 'worlds' may be successfully constituted by a formal pragmatic communication theory.

3.4 Discussion

The living world of malignant tumors creates the term opposite to those idealizations, which originally constitute scientific knowledge.

'Commonly', W. Kolch remarked, 'we try to find out the function of a system by disassembling it and measuring the activity of isolated components. This approach is very successful in characterizing the individual parts but very limited in reconstructing the function of a system as a whole' [23], suggesting that the systems concept as antithesis to reductionist concepts remains fully consistent with reductionist scientific approaches.

A holistic communication-based model termed the tumor's living world now opposes reductionist systems approaches. This world is uncovered by redeeming validity of communicative tumor processes through the implementation of modular knowledge on the cellular and external environment (for instance for therapeutic requirements): The tumor's entire communicative system is subjected to modular interventions pursuing the integration of complex biochemical systems processes. In the first half of the twentieth century, the biologist Spemann already characterized evolutionary systems in a communicative context: 'Reciprocal interactions may play a large role, in general, in the development of harmonious equipotential systems [24].

Modular therapies represent an alternative therapeutic solution compared to reductionist designed approaches. 'Systemic' therapies in a reductionist sense are designed by combinations of modifiers of pathways, which are more or less tumor specific, and their rationale is usually based on analytics of pathway signatures [25].

In modular therapies, the communicative complexity of tumors, i.e. the multifold divisions in functions and structures, mirrors the modularly structured totality of tumor-specific communication processes. The present model, a formal-pragmatic communication theory, may now explain the therapeutic efficacy of exclusively biomodulatory acting drug combinations (stimulatory or inhibitory acting drugs, which do not exert mono-activity in the respective metastatic tumor type and are not directed to potentially 'tumor-specific' targets) in a modularly and evolutionary context. These findings recall the famous remark of Dobzhansky, 'nothing in biology makes sense except in the light of evolution' [26].

The important new step in our novel concept of understanding tumor biology and tumor evolution is the introduction of the tumor's living world as a holistic and therefore self-contained communication process in its idealization, in which external,

communication-guiding interferences (modular knowledge) may be implemented to differentially focus on the coherency of the communication-technically, all important dimensions validity and denotation. Now, mostly generalized tagged references derived from context-dependent knowledge about single communication-mediating cells, molecules, or pathways may be virtually neglected for communication-technical purposes [6]. These systems objects may be perceived as symbols in a continuum, rich in content, whose validity and denotation may be exchangeable but not at random.

This way, the tumor's living world is turning into a scientific object that becomes accessible for experimentally or therapeutically designed modular approaches for uncovering the tumor's modularity. This modularity is defined by a distinct communicative architecture but also by the way how modularity has been communicatively uncovered.

Inclusion of prepositions for validity, which are present in the living world, and the implicit interplay of validity and denotation, which may be focused on modular events, afford transparency, how evolutionary processes may be first induced in the range of their molecular-genetically defined backbone. Imposed modular acting events, such as modularly designed therapies, may induce significant modular response in socially linked cell systems (prerequisite) and may foster space for evolutionary development by redeeming novel validity. This space may be biochemically assessable by the multiple varying biological functions of, for example, transcription factors [17]. Following modular events, molecular-genetic alterations might occur additionally.

As a holistic process, the therapeutically relevant acquisition of the 'language' of communicative intercellular processes followed by its transformation into a hypothesis creating activity on the basis of clinical results (derived from modularly designed therapy approaches) may give hints on the 'metabolism' of evolutionary tumor development. Supported by the possibility of redeeming novel validity of communicative processes with modular events, a possible mechanism to promote a tumor's evolutionary development may be simultaneously changing validities of communicative processes mediated by the systems objects. The procedure is closely linked to the differential development of novel denotations of the systems objects: via communication-relevant processes, systems objects are acquiring novel references within the holism of the tumor's living world without first substantially altering the functionality of the entire communicative system.

In analogy to modular therapy approaches, constitutional and incidental modular events from the tumor microenvironment or from the macroenvironment could be critically involved in modularly promoting tumor development or growth. Differentially designed modular therapy approaches should specifically meet a tumor's living world on corresponding steps of tumor development and should allow situation-linked insights in modular architecture (comparative uncovering of a tumor's modular architectures) [27].

Commonly used context-dependent knowledge is shown to underestimate the impact of risk absorbing prepositional background knowledge for pragmatic therapeutic purposes. The combination of modest changes in therapeutic design, i.e. the

introduction of biomodulatory therapies, seems to make a major difference in the experimental efficacy of evaluating systems on a communication level.

We may retranslate modularly induced functional changes in tumors into intentional knowledge by comparatively reconstructing novel communication-linked processes on a biochemical basis to

1. Prove the formal-pragmatic communication theory by an intentional and computational idealization [28,29]
2. Advance reductionist knowledge for novel reductionist therapy approaches, which may be used in parallel or subsequentially

Generally, the new communicatively defined modular coherency of the macroenvironment, i.e. the tumor-associated microenvironment, and the tumor cells open novel ways for the scientific community in 'translational medicine'.

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3.4.1 Glossary

3.4.1.1 Co-option

Reuse of existing genetic components, metabolic reactions, or signaling modules in diverse biological systems, such as tumors, for instance, discharging in the evolution of patterns of dysregulated transcription factors.

3.4.1.2 Evolvability

The capacity of an organism or a biological system to generate new heritable phenotypes. Therapeutically modularly induced evolutionary steps advance this definition: Modularity may allow retrospectively established spaces for primarily none heritable evolutionary developments, if modular events (therapy) are implemented.

3.4.1.3 Modularity

In the present context, modularity is a formal pragmatic communicative systems concept, describing the degree and specificity to which systems objects (cells, pathways, etc.) may be communicatively separated in a virtual continuum and recombined and rededicated to alter the validity and denotation of communication processes in the tumor.

3.4.1.4 Modular Communication (Therapies)

The function is to configure the coherence between the validity and denotation of communication processes. Modular therapies may supplement prepositional aspects of communication, i.e. the presence of the tumor's living world by normative aspects, namely by therapy-derived yes or no statements ('know that').

3.4.1.5 Risk-Absorbing Background Knowledge

This knowledge constitutes the validity of informative intercellular processes, which is the prerequisite for therapeutic success. Background knowledge about the tumor's living world is subjected to other conditions of scientific comprehension: Intentional ways fail to describe risk-absorbing knowledge, in which context-dependent knowledge about commonly administered reductionist therapy approaches is rooted. After this second objectifying step (physicians as operators of tumor systems), the network of the holistic communicative activities turns out to be the medium through which the tumor's living world is mirrored and generated.

3.4.1.6 Tumor's Living World

The living world comprises the tumor's holistic communication processes, which we rely on in every therapy. The living world of morphologically defined tumor cell systems creates the term opposite to those idealizations, which originally constitute scientific (intentional) knowledge. The living world is uncovered by r deeming the validity of communicative tumor processes by implementing the modular knowledge of cellular and external environments (for instance for therapeutic requirements). Only with experimental or therapeutic experiences (modular therapies) is the tumor's living world separated into categories of knowledge, for example, into modular systems. Specific conditions of compliance for redeeming validity constitute relations between communication technique (specified modular therapy approaches) and distinct tumor-associated situation-engraved systems stages.

3.4.1.7 Reconstruction of Tumor-Associated Systems

Redeeming validity is tailored on the relation of modular communication to the objective features of the tumor compartment, the reconstructible evolutionary (modular) systems.

3.4.1.8 Robustness

The inherent property of a system to maintain normal performance despite external and internal perturbations.

3.4.1.9 Separated or Separating 'Social' Tumor Systems

The possibility for redeeming novel validity by modular therapies is indicative for the existence of biologically separated or separating 'social' systems, i.e. in our context, metastatic tumors: Tumors constitute a solitary world with an internal context.

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Part II
Tumors Share Common Processes During Tumor Evolution: Communicative Aspects of a Situation's Interpretation for Creating Systems-Directed Therapies

Chapter 4
Cancer and Coagulation; Focusing on Tissue Factor and Heparanase

Yona Nadir

Abstract Cancer patients have a pro-thrombotic state due to the ability of cancer cells to activate the coagulation system and to interact with haemostatic cells, thus tilting the balance between pro- and anti-coagulants. Tissue factor (TF), the main initiator of blood coagulation, is a transmembrane receptor that is expressed constitutively in tumors. TF also plays a role in cellular signalling, contributing to tumor growth and metastasis. The only known endogenous modulator of blood coagulation initiated by TF is tissue factor pathway inhibitor (TFPI) – a plasma Kunitz-type serine protease inhibitor. Growing evidence suggest involvement of tumor derived substrates, including heparanase, in activation of the coagulation system. Heparanase is an endo-β-D-glucuronidase that cleaves heparan sulfate chains on cell surfaces and in the extracellular matrix, activity that closely correlates with cell invasion, angiogenesis and tumor metastasis. Recently we demonstrated that heparanase is involved in the regulation of the hemostatic system. Heparanase was found to up-regulate the tissue factor and interact with TFPI on the cell surface, leading to dissociation of TFPI from the cell membrane and increased cell surface coagulation activity. Taking into account the prometastatic and pro-angiogenic functions of heparanase, its overexpression in human malignancies and abundance in platelets, its involvement in the coagulation machinery is an intriguing novel arena for further research. Thus, inhibition of factors participating in blood coagulation may potentially reduce thrombotic complications and tumor growth.

Keywords Cancer • Coagulation • Tissue Factor • Tissue Factor Pathway Inhibitor • Heparanase

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Abbreviations

TF	Tissue factor
PC	Protein C
PS	Protein S
TFPI	Tissue factor pathway inhibitor
VEGF	Vascular endothelial growth factor
TNF α	Tumor necrosis factor alpha
FGF	Fibroblast growth factor
PDGF	Platelet derived growth factor
EGR1	Early growth response 1
MAPK	Mitogen-activated protein kinase
JNK	c-*jun* Terminal NH_2-kinase
PKC	Protein kinase C
NF	Nuclear factor
ECM	Extracellular matrix
TGF	Transforming growth factor
HUVEC	Human umbilical vein endothelial cell
MMP	Matrix metalloproteinase
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
TSP-1	Thrombospondin-1
GPI	Glycosyl phosphatidylinositol
AML	Acute myeloid leukemia
CLL	Chronic lymphatic leukemia
CML	Chronic myeloid leukemia
ALL	Acute lymphoblastic leukemia
LMWH	Low molecular weight heparin

4.1 Introduction

Cancer patients have a pro-thrombotic state because of the ability of cancer cells to activate the coagulation system and to interact with hemostatic cells, thus tilting the balance between pro- and anticoagulants [1]. Over expression of tissue factor (TF), cancer procoagulant – a cysteine protease that activates factor X, and acquired activated protein C resistance [2], are thought to be the main factors for coagulopathy in malignant disorders. Additionally, drugs used in cancer patients are contributing to the hypercoagulable state [3]. The best characterized substance with a direct pro-coagulant activity is TF, a transmembrane receptor that is constitutively expressed in tumors, i.e. human leukemias, lymphomas, adenocarcinomas and sarcomas [4]. TF also plays a role in cellular signaling, contributing to tumor growth and metastasis [4, 5]. The only known endogenous modulator of blood coagulation initiated by TF is tisue factor pathway inhibitor (TFPI) – a

plasma Kunitz type serine protease inhibitor [6, 7]. Growing evidence suggest involvement of tumor derived substrates, including heparanase, in activation of the coagulation system.

4.2 Tissue Factor (TF)

Blood coagulation is a host defense system that maintains the integrity of the high-pressure closed circulatory system. To prevent excessive blood loss, the hemostatic system, which includes platelets, vascular endothelial cells, and plasma coagulation proteins, is recruited. The main initiator of blood coagulation is TF.

4.2.1 TF Structure and Expression

TF is a 47-kDa transmembrane protein expressed in both vascular and nonvascular cells. The TF gene is located on chromosome 1 and is 12.4 kb in length consisting of six exons [8]. The protein is composed of 263 amino acid residues. An N-terminal domain of 219 amino acid residues is the dominant component of the protein and is oriented extracellularly. In addition, a short hydrophobic domain of 23 amino acids represents the transmembrane region and a short 21-residue C-terminus represents the cytoplasmic domain. Although specific glycosylation sites have not been established, tissue factor has multiple potential N- and O-linked sites [8]. In the vessel wall, TF is constitutively expressed in subendothelial cells such as vascular smooth muscle cells leading to rapid initiation of coagulation when the vessel is damaged [3]. In contrast, endothelial cells and monocytes do not express TF under physiological conditions; as a consequence, there is no appreciable contact of cellular TF with the circulating blood. In response to various stimuli, however, TF expression and activity can be induced in these cells, as well.

4.2.2 TF and the Coagulation System

In vitro the generation of thrombin and the formation of a fibrin clot propagate through two separate pathways, the intrinsic pathway and the extrinsic pathway (Fig. 4.1). In vivo, the coagulation cascade is usually initiated as soon as TF comes into contact with circulating activated factor VII (VIIa), resulting in the formation of TF-FVIIa complex. The TF-VIIa complex activates factor IX, which in turn activates factor X; alternatively, factor X is directly converted to factor Xa by TF-FVIIa. In complex with factor Va and calcium, factor Xa catalyzes the conversion of prothrombin to factor II – thrombin, thereby leading to factor I – fibrin formation, platelet activation, and, ultimately, generation of a thrombus. Several of these

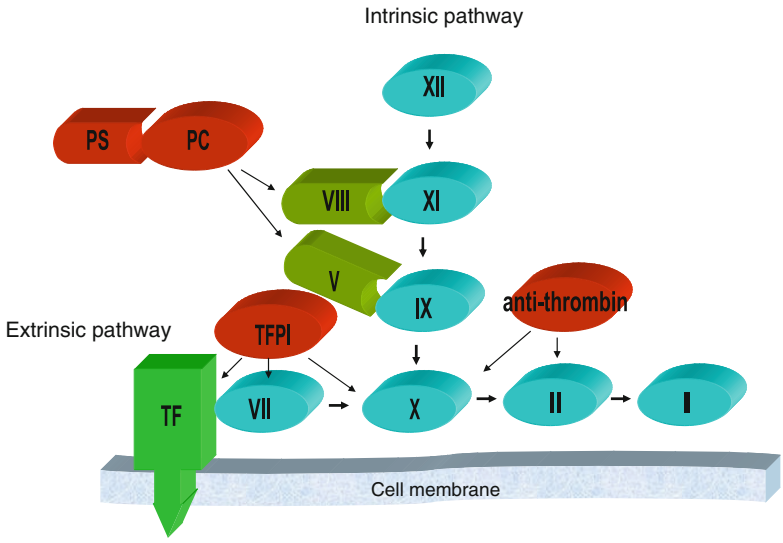

Fig. 4.1 The coagulation system. The intrinsic pathway is initiated with the activation of factor XII. The extrinsic pathway is initiated with the formation of the complex tissue factor (TF) and factor VIIa. Protein C (PC), protein S (PS), anti-thrombin and tissue factor pathway inhibitor (TFPI) are the system inhibitors

activated proteases, including factor IXa, factor Xa, thrombin, and the TF-VIIa complex itself, can convert factor VII to VIIa in an auto-feedback loop.

The majority of TF resides in various intracellular compartments, predominantly in the Golgi. Tissue factor at the cell surface is localized in cholesterol-rich lipid rafts and extensively colocalized with caveolin-1 [9]. FVIIa binding to TF induces the internalization of TF. Of interest, TF-FVIIa complex formation at the cell surface leads to TF mobilization from the Golgi with a resultant increase in TF expression at the cell surface. This process is dependent on FVIIa protease activity [9].

The extent of TF protein induction in vascular cells does not always correlate well with TF activity [10, 11]. One possible explanation is the concomitant secretion of TFPI, the endogenous inhibitor of TF. Another possible reason is the distribution of TF in several cellular compartments [11, 12]. Biologically active TF is indeed located at the cell surface, whereas intracellular TF constitutes a pool that is only released upon cell damage. A combination of tumor necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF) favors cell surface over intracellular distribution as compared with stimulation with either agonist alone, suggesting a complex regulation of the cellular distribution of TF [11]. Discrepancies between TF protein expression and activity can further be accounted for by the induction of a functionally inactive form of TF at the cell surface, termed latent or encrypted tissue factor. Expression of encrypted TF enables a cell to rapidly increase TF activity in response to certain stimuli without the need for de novo protein synthesis. De-encryption of TF has been observed secondary to changes in intracellular calcium levels, alterations in membrane phosphatidylserine expression, or modifications in the quaternary structure of TF [13]. Hence, the relative

contribution of TF protein induction, cellular localization, and structural modification appears to determine the net procoagulant effect elicited by a given mediator.

4.2.3 Increased TF Expression in Tumors

Up-regulation of TF gene expression appears to be characteristic of malignant cells and normal host cells responding to inflammatory or remodeling signals (e.g., endothelial cells, monocytes, macrophages, and fibroblasts). TF shares homology with members of the cytokine receptor superfamily [14]. Therefore, it is not surprising that cytokines and growth factors generated by inflammatory and malignant cells induce TF expression. Among these are interferon-γ [15], TNF-α [16], interleukin-1β [17], CD40 ligand adhesion molecule [18], serotonin [19], histamine [10], thrombin [20], oxidized LDL [21], C-reactive protein [22], angiotensin II [23], fibroblast growth factors (FGF) [24], platelet-derived growth factor (PDGF) [25], VEGF [26], and also, endotoxin [27] and hypoxic conditions [28]. Inappropriate expression of TF alters the behavior of cells. Cancer cells transfected with TF exhibit a more malignant phenotype both in vitro and in vivo compared to the parent cell lines [29, 30]. Increased TF expression has been detected in a variety of human tumors, including glioma [31], breast cancer [32, 33], non-small cell lung cancer [34, 35], leukemia [36a, 36b], colon cancer [37], and pancreatic cancer [38]. Elevated TF expression in tumors has been correlated with unfavorable prognostic indicators, such as increased angiogenesis, advanced stages of disease, and the multidrug resistant phenotype [39], that contribute to poor survival rates in cancer patients.

4.2.4 TF and Angiogenesis

TF appears to play a critical role in both physiologic and pathologic angiogenesis. It is well established that TF deficiency in transgenic mice causes embryonic lethality by day 10.5 due to impared vascular integrity and abnormal development of the yolk sac [40]. A similar histopathology associated with lethality occurs with VEGF deficient embryos [41], suggesting that TF and VEGF regulate similar functions. The switch to an angiogenic phenotype requires a shift in balance between endogenous proangiogenic and antiangiogenic factors that regulate vessel growth and development. In colorectal cancer, for example, increased TF positivity in higher grade tumors has been correlated with increased vascular density and VEGF expression, as well as the clinical stage of colorectal cancer and angiogenesis [37]. Similar correlations between TF expression, VEGF expression, and microvessel density have also been found in non-small cell lung cancer [35] and breast cancer [32]. Tissue factor and VEGF have also been found to be colocalized in tumor cells of human lung and breast cancer specimens [42]. Analysis of several human breast cancer [42] and melanoma [43] cell lines revealed a significant correlation between the level of synthesis of VEGF and TF in vitro. Subcutaneous inoculation of a high

TF and VEGF-producing melanoma cells into mice with severe combined immunodeficiency yielded highly vascular tumors in vivo [43]. A similar experiment with a low TF and VEGF-producing cells produced relatively avascular tumors in vivo. However, when a low TF and VEGF-producing melanoma cells that had been transfected with the full-length TF DNA was used in these experiments, vascular tumors grew and expressed high levels of both TF and VEGF. These studies support the hypothesis that TF regulates VEGF synthesis and contributes to tumor angiogenesis [43].

TF and VEGF participate in a vicious cycle of clot formation and tumor growth. Not only does TF induce VEGF, but the converse also holds true since VEGF in turn up-regulates the expression of TF on endothelial cells by activating the early growth response-1 gene (EGR1) [44]. Decreased phosphatidyl 3-kinase (PI3-K) activity concurrent with increased p38 and Erk-1/2 mitogen-activated protein kinase (MAPK) activity induce up-regulation of TF expression by VEGF in tumor-related endothelial cells [45]. Differential signaling pathways may control TF-induced regulation of VEGF during physiologic and pathologic angiogenesis. Using human fibroblasts, it was reported that TF-induced production of VEGF required the binding of activated factor VII to TF and subsequent generation of activated factor X and thrombin [46]. However, in some malignant melanoma cell lines, TF-mediated regulation of VEGF is regulated independent of clotting via activation of the cytoplasm tail of TF, rather than via the ligand-binding extra-cellular domain [43].

4.2.5 TF Signaling

Signal transduction pathways regulating TF induction in endothelial cells involve the MAP kinases p38, p44/42 (ERK), c-*jun* terminal NH_2-kinase (JNK), and protein kinase C (PKC) [10, 16, 47, 48]. These signal transduction molecules stimulate the TF promoter by activating transcription factors such as AP-1, nuclear factor (NF)-κB, and EGR-1 [18, 48, 49],ultimately resulting in upregulation of TF mRNA [21, 49–51].

Unlike MAP kinases or protein kinase C, the PI3-kinase pathway negatively regulates endothelial TF expression; as a consequence, inhibition of PI3-kinase or its downstream mediators increases TF expression [45, 47, 50, 52].

4.2.6 Blood-Borne TF

Tissue factor is not only present in vascular cells or leukocytes but can also be detected in the bloodstream, referred to as circulating or blood-borne TF [53]. This form of TF is mainly associated with microparticles [54] originating from endothelial cells, vascular smooth muscle cells, leukocytes, or platelets [55, 56]. In addition, TF containing microparticles are released from atherosclerotic plaques [54].

Monocytes and platelets are known to exchange microparticle-bound TF [57]. Because megakaryocytes, the bone marrow precursors of platelets, do not express TF, it is likely that this exchange represents a mechanism through which platelets are loaded with TF. In addition to carrying microparticle-derived TF, activated platelets induce tissue factor expression in human endothelial and smooth muscle cells, presumably by releasing soluble mediators such as serotonin and PDGF [58]. Aggregating platelets thus induce a positive feedback loop that enhances local TF concentrations through two mechanisms and may be important for thrombus formation and/or propagation.

Recently, an alternatively spliced form of TF has been discovered, which is soluble, circulates in the blood, and exhibits procoagulant activity [59]. Cytokines stimulate its expression and release from endothelial cells [60]. Alternatively spliced TF is not bound to microparticles and appears to represent a distinct form of circulating TF; as such, it may have an important role in thrombus propagation [60]. Alternatively spliced human TF contains most of the extracellular domain of TF but lacks a transmembrane domain and terminates with a unique peptide sequence [59]. Studies on blood-borne TF imply that activation of coagulation, contrary to the traditional belief, may be initiated and propagated without contact of the blood with the extravascular space. The importance of blood-borne versus vessel wall-associated TF is currently a subject of debate [61–63]. One study described that TF from leukocyte-derived microparticles importantly contributes to thrombus propagation in an animal model of thrombosis [61], whereas another study identified vessel wall-derived TF as the primary mediator driving thrombus formation after vascular injury [62]. It is also controversial whether physiological concentrations of circulating TF can exhibit clot-forming activity in vivo [63]. Thus, the relative contribution of soluble TF, microparticle-bound TF, and vessel wall-associated TF to initiation and propagation of thrombosis requires further studies.

4.3 Thrombin

Thrombin is a multifunctional serine protease that has a crucial role in blood coagulation.

Thrombin was reported to be involved in angiogenesis. It enhances VEGF protein synthesis and secretion in normal and malignant cells [64], participates in release from subendothelial extracellular matrix (ECM) of biologically active basic FGF and transforming growth factor beta (TGF-beta)[65], induces increased expression and secretion of angiopoietin-2 from human umbilical vein endothelial cells (HUVECs) [66] and induces HUVEC proliferation [67]. Matrix metalloproteinases (MMPs) take part in degradation of ECM components. Thrombin was shown to be involved in the regulation of various MMPs via the thrombin proteinase activated receptor (PAR) family [68]. Interestingly, the phenotype of mouse embryos that lack prothrombin closely resembles the pathology seen in TF knockout mice [69, 70].

4.4 Tissue Factor Pathway Inhibitor (TFPI)

TFPI is a potent direct inhibitor of factor Xa, and in a factor Xa dependent fashion, produces inhibition of the factor VIIa-TF complex. TFPI can also inhibit TF/VIIa directly [71]. In addition, TFPI causes internalization and degradation of TF-FVIIa complexes on the cell surface [72]. In vivo studies demonstrated that TFPI blockes angiogenesis and tumor growth [73, 74].

4.4.1 TFPI Structure and Expression

The TFPI molecule is a ~46 kDa protein consisting of three tandem Kunitz-type protease inhibitor domains. Its first Kunitz domain appears to bind factor VIIa in the factor VIIa-TF complex and its second Kunitz domain is required for binding to factor Xa [75]. Heparan sulfate proteoglycans (HSPGs) on the cell surface are directly associated with TFPI and act as the uptake and degradation receptor for TFPI-factor Xa complex (Fig. 4.2) [76].

There are three pools of TFPI in vivo: the majority of TFPI is bound to the vascular endothelium, approximately 10% is associated with lipo-proteins in the plasma and a smaller portion is present in platelets. In addition to endothelial cells, other cells can synthesize TFPI, including mesangial cells, smooth muscle cells, monocytes, fibroblasts, and cardiomyocytes [77–79]. Mechanisms that regulate TFPI gene expression are largely unknown. Interestingly, PDGF and bFGF, two potent angiogenic mediators, have been reported to induce TFPI expression by vascular smooth muscle cells, while inflammatory mediators such as IL-1 and TNFα had no effect [80, 81].

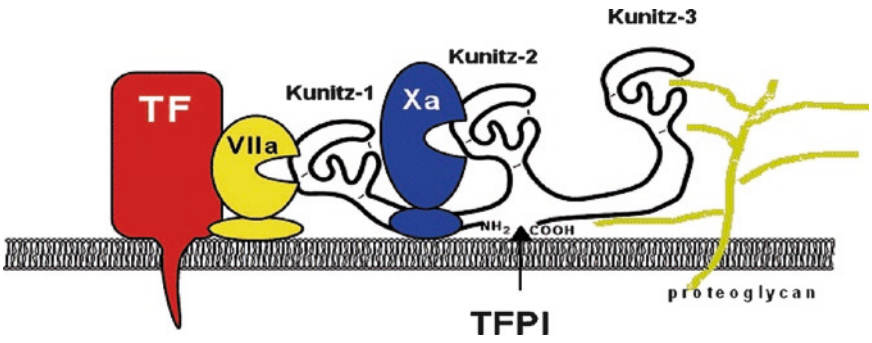

Fig. 4.2 TFPI structure. TFPI is composed of three kunitz domains. The first binds factor VIIa-tissue factor complex, the second binds factor Xa, and the third and c-terminal associates with the cell surface via heparan sulfate proteoglycan

4.4.2 TFPI in Blood and Cells

The normal concentration of TFPI in human plasma is approximately 100 ng/mL [82]. In the blood stream, TFPI exists in a free form and also in lipoprotein-assosiated form [83]. Stored TFPI is released into the plasma from platelets by activation and from endothelial cells by the action of heparin suggesting heparan-sulfate proteoglycan cell surface binding sites [84–86]. Proteoglycan receptors that are known to bind TFPI are the transmembrane-anchored ryudocan/syndecan 4 [87] and the glycosyl phosphatidylinositol (GPI)-anchored glypican 3 [88]. Thrombospondin-1 (TSP-1) that accounts for about 25% of the protein within platelets α-granules was found to interact with TFPI and acts to localize it "as molecular bridge" to surfaces within the extravascular space, where it can efficiently down-regulate TF-initiated coagulation after vascular injury [89]. Studies examining the sub-cellular localization of TFPI in HUVEC indicated that TFPI-binding proteoglycans are present in caveolae, a caveolin-coated invaginations that perform transport and signalling functions affecting cell growth, apoptosis, and angiogenesis [6].

4.5 Heparanase

Heparanase is an endo-β-D-glucuronidase capable of cleaving heparan sulfate (HS) side chains at a limited number of sites, yielding HS fragments of still appreciable size (~5–7 kDa) [90, 91]. Heparanase activity has long been detected in a number of cell types and tissues. Importantly, heparanase activity correlated with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence of HS cleavage and remodeling of the extracellular matrix (ECM) barrier [92, 93]. Similarly, heparanase activity was implicated in neovascularization, inflammation and autoimmunity, involving migration of vascular endothelial cells and activated cells of the immune system [92–94]. A single human heparanase cDNA sequence was independently reported by several groups [95–98]. Thus, unlike the large number of proteases that can degrade polypeptides in the ECM, one major heparanase appears to be used by cells to degrade the HS side chains of HSPGs. Expression of heparanase is restricted primarily to the placenta, keratinocytes, platelets and activated cells of the immune system, with little or no expression in connective tissue cells and most normal epithelia [92, 93]. Up-regulated expression of heparanase was noted in essentially all human tumors examined, inflammation, wound healing and diabetic nephropathy [92–94]. During embryogenesis, the enzyme is preferentially expressed in cells of the developing vascular and nervous systems [99].

4.5.1 Heparanase Structure

The heparanase gene (~50 kb) is located on human chromosome 4q21.3 [100]. The gene is expressed as 5 and 1.7 kb mRNA species, generated by alternative splicing.

The 5 kb form contains 14 exons and 13 introns, whereas in the short form the first and 14 exons have been spliced out. Only one gene has been shown to encode for a protein with heparanase activity [95–98]. Sequence analysis revealed that heparanase is highly conserved, with similar sequences found in human, rat, mouse, cow, chicken, mollusks and zebra fish [92, 99]. The gene has not been identified in drosophila and C. elegans. The human *heparanase* cDNA contains an open reading frame that encodes a polypeptide of 543 amino acids with a molecular weight of 61.2 kDa. The active heparanase purified from placenta, platelets and various cell lines was found to lack its N-terminal 156 amino acids, suggesting post-translational proteolysis of the heparanase polypeptide [97, 101]. In fact, active heparanase was subsequently reported to be a heterodimer consisting of a 50 kDa subunit (Lys^{158}–Ile^{543}) associated non-covalently with an 8 kDa peptide (Gln^{36}–Glu^{109}). The intervening 6 kDa peptide (Ser^{110}–Gln^{157}) is excised by proteolysis [102–104]. Based on the predicted amino acid sequence, the 50 kDa subunit of human heparanase contains six putative *N*-glycosylation sites. Although glycosylation was not required for enzyme activity, secretion of heparanase was regulated by glycosylation [105]. The sequence also contains a 35-amino acid N-terminal signal sequence (Met^{1}–Ala^{35}), and a C-terminal hydrophobic domain (Pro^{515}–Ile^{534}). Heparanase has been shown to be related to members of the clan A glycosyl hydrolyses (GH-A) [106]. Protein sequence alignment approaches in combination with secondary structure predictions indicated that heparanase contains sequences that are homologous to families 10, 39 and 51 of the GH-A, especially in terms of the active-site regions [106]. This clan of enzymes uses a general acid catalysis mechanism for the hydrolysis of glycosidic bonds. The mechanism requires two critical residues, a proton donor and a nucleophile, both of which appear to be conserved in heparanase at Glu^{225} and Glu^{343}, respectively [106]. Site-directed mutagenesis of these residues completely abolished heparanase activity, indicating that heparanase uses a catalytic mechanism characteristic of GH-A glycosyl hydrolyses [106].

4.5.2 Pro-angiogenic Properties

HSPGs are prominent components of blood vessels, and HSPG degrading enzymes have long been implicated in a number of angiogenesis-related cellular processes. A critical early event in the angiogenic process is degradation of the subendothelial basement membrane (BM), followed by endothelial cell (EC) migration toward the angiogenic stimulus. Similar to its involvement in tumor cell dissemination, it is conceivable that by degrading HS in the BM, heparanase may directly facilitate EC invasion and sprouting. Indeed, heparanase expression by bFGF-stimulated, bone marrow-derived EC was demonstrated by RT-PCR [107]. Immunohistochemistry of tumor specimens revealed heparanase staining of EC in capillaries, but not mature blood vessels [107, 108]. Moreover, by releasing HS-bound angiogenic growth factors (i.e., bFGF, VEGF) from the ECM [109], heparanase may indirectly facilitate EC migration and proliferation [107, 108, 110]. In fact, given the multitude

of biological mediators that are sequestered by HS in the ECM [111], heparanase activity liberates a number of active molecules that may act cooperatively or synergistically to promote neovascularization. Moreover, HS fragments released by heparanase from the cell surface stimulate the mitogenic activity of bFGF [107] and possibly other pro-angiogenic factors. Heparanase also releases growth factor-HS saccharide complexes from cell surfaces, although it has not been demonstrated whether such 'liberated' complexes are more active than counterparts that remain attached to membrane HSPGs.

4.5.3 Pro-metastatic Properties

The clinical significance of the enzyme in tumor progression emerges from a systematic evaluation of heparanase expression in primary human tumors. Immunohistochemistry, *in situ* hybridization, RT-PCR and real time-PCR analyses revealed that heparanase is up regulated in essentially all human tumors examined. These include carcinomas of the colon [108, 112], thyroid [113], liver [114], pancreas [115, 116], bladder [117, 118], cervix [119], breast [120], gastric [121, 122], prostate [123], head and neck [124, 125], as well as multiple myeloma [126], leukemia and lymphoma [127]. In most cases, elevated levels of heparanase were detected in about 50% of the tumor specimens, with a higher incidence in pancreatic (78%) and gastric (80%) carcinomas, and in multiple myloma (86%). In all cases, the normal looking tissue adjacent to the malignant lesion expressed little or no detectable levels of heparanase, suggesting that epithelial cells do not normally express the enzyme. In several carcinomas, most intense heparanase staining was localized to the invasive front of the tumor [117, 122, 124], supporting a role for heparanase in cell invasion. Furthermore, patients that were diagnosed as heparanase-positive exhibited a significantly higher rate of local and distant metastasis as well as reduced post-operative survival, compared with patients that were diagnosed as heparanase-negative [112, 116, 117, 122, 126]. Collectively, these studies provide a strong clinical support for the pro-metastatic function of heparanase. Interestingly, patient's survival was noted to correlates not only with heparanase levels, but also with its localization. In addition to localization in the cytoplasm, heparanase was also noted to assume nuclear localization, demonstrated by cell fractionation [128], and by immunostaining of cultured cells [128] and tumor biopsies [121, 129]. Interestingly, nuclear localization was correlated with maintained cellular differentiation [129] and favorable outcome of patients with gastric [121, 129] and head and neck [130] carcinomas, suggesting that heparanase is intimately involved in gene regulation. Whether gene transcription and maintained cellular differentiation is due to direct interaction of heparanase with the DNA, or is a consequence of heparanase-mediated nuclear-HS degradation is yet to be demonstrated. In addition, heparanase up regulation in primary human tumors correlated in some cases with tumors larger in size [114, 120, 122], and with enhanced micro vessel density [112, 114, 118, 126], providing a clinical support for the pro-angiogenic function of the enzyme.

4.5.4 Non-enzymatic Functions

Applying heparanase that lacks enzymatic activity due to point mutations (Glu^{225}, Glu^{343}) in its active site, it was noted that heparanase exerts also non-enzymatic activities, independent of its involvement in ECM degradation and alterations in the extracellular microenvironment associated with angiogenesis, cell survival, and migration. For example, cell surface expression of enzymatically inactive heparanase elicits a firm cell adhesion, reflecting an involvement in cell–ECM interaction [131]. Moreover, as described below, inactive heparanase enhances Akt signaling and stimulates PI3K- and p38-dependent endothelial cell migration and invasion [132]. It also promotes VEGF expression via the Src pathway [133]. At present, no information is available on protein domains responsible for the non-enzymatic functions of the heparanase molecule, nor on the putative heparanase receptor that appears to mediate these effects.

4.5.5 Hematopoetic Cells and Heparanase

Heparanase activity has been detected in several types of normal hematopoetic cells, including neutrophils, megakaryocytes, and activated lymphocytes, and may mediate their extravasation during inflammatory and immune response [134]. Heparanase expression pattern in hematologic proliferative disorders was investigated. In mononuclear cells derived from various leukemia, heparanase RNA was expressed in 14 of 15 acute myeloid leukemia (AML). In contrast, all 33 chronic lymphatic leukemia (CLL), 7 of 8 chronic myeloid leukemia (CML), and six of eight acute lymophoblastic leukemia (ALL) patients showed no detectable expression of heparanase mRNA[127]. This study proposes that heparanase expression is associated with the acute myeloid leukemias [127]. A recent study indicates that myeloma cells express high levels of heparanase detected by immunohistochemistry and activity assay. Expression of heparanase in multiple myeloma appears to play a direct role in enhancing bone marrow microvessel density, implying that heparanase plays a role in regulating the growth and progression of myeloma [126].

4.5.6 Inhibition of Heparanase by Heparins

Anti-coagulant activities of cell surfaces have been predominantly attributed to HS [135, 136], which are composed of repeating hexuronic and D-glucosamine sulfated disaccharide units. HS have been shown to exert anticoagulant activities on cells, on ECM and in tissues, due to their catalysing function for protease inhibition by antithrombin and subsequent complex formation [135–137].

Moreover, cell surface HS can facilitate the catabolism of coagulation factors such as factor VIII [138]. Other coagulation inhibitors such as TFPI also associate with the luminal face of endothelial cell plasma membrane via HS [76]. HS are also important constituents of the sub-endothelial basement membrane, where they cross-link various components, e.g. laminin and collagens, thereby contributing to the integrity of the blood vessel wall [139]. HS, unfractionated heparin and other heparin derivatives have been investigated as heparanase inhibitors, and some of them exerted anti-metastatic activity in animal models [140]. Both the type of the polysaccharide backbone and degree of sulfation seem to affect the heparanase inhibiting activity of sulfated polysaccharides [141, 142]. However, different heparin preparations display significantly different anti-heparanase activity [141, 142] indicating that this activity is also dependent on more subtle structural features. Recently, heparanase strong affinity to heparins was utilized in vitro to reverse heparins effect. Heparanase was shown to reverse the anti-coagulant activity of unfractionated heparin on the coagulation pathway as well as on thrombin activity. In addition, heparanase abrogated the factor X inhibitory activity of low-molecular-weight heparin (LMWH). The pro-coagulant effects of the non-active heparanase were also exerted by its major functional heparin-binding peptide [143].

4.5.7 Heparanase and TF

TF is constitutively expressed in various cell types, including pericytes adjacent to the vessel wall, but absent from blood cells and endothelial cells. This localization is crucial for hemostasis since it prevents a direct contact between TF and the circulating blood. Immunohistochemical studies revealed that many tumors express high levels of TF, raising the possibility of TF role in the pathogenesis of cancer [1]. We have demonstrated that heparanase over-expression in human leukemia, glioma, and breast carcinoma cells results in a marked increase in TF levels verified by immunoblot and real-time PCR analyses [144]. Likewise, TF was induced by exogenous addition of recombinant heparanase to tumor cells and primary endothelial cells, induction that was mediated by p38 phosphorylation and correlated with enhanced procoagulant activity. TF induction was further confirmed in heparanase over expressing transgenic mice and, moreover, correlated with heparanase expression levels in leukemia patients [144]. Recently, heparanase was found to exert also non-enzymatic activities, independent of its involvement in ECM degradation and alterations in the extracellular microenvironment [145]. For example, inactive heparanase enhances Akt signaling and stimulates PI3K- and p38-dependent endothelial cell migration and invasion [132]. It also promotes VEGF expression via the Src pathway [133]. We added another example for the multiple non-enzymatic functions of heparanase, indicating an important involvment of heparanase in haemostasis. We propose that heparanase up-regulation in leukemias can facilitate

disease progression not only by promoting cellular invasion, traditionally implicated with heparanase activity, but also by enhancing TF expression and blood coagulation, positioning heparanase as a valid target for the development of novel therapeutics for solid and hematological malignancies.

4.5.8 Heparanase and TFPI

TFPI is a plasma Kunitz-type serine protease inhibitor and the only known endogenous modulator of blood coagulation initiated by TF [6, 7]. TFPI concentration in plasma is increased in patients with acute myocardial infarction [146, 147]. There are also reports on the plasma levels of TFPI in relation to disseminated intravascular coagulation [148] and to other diseases, such as diabetes mellitus [149], renal diseases [150], and cancer [151, 152]. Recently we demonstrated that exogenous addition or over expression of heparanase by transfected cells results in release of TFPI from the cell surface and its accumulation in the cell culture medium [153]. Importantly, the *in vitro* studies are supported by elevation of TFPI levels in the plasma of transgenic mice over-expressing heparanase. Moreover, increased levels of TFPI have been noted in the plasma of cancer patients [151, 152], reflecting, possibly, induction of heparanase expression and elevation of its plasma levels revealed by a newly developed ELISA assay [154]. In HUVEC and tumor derived cell lines, release of TFPI from the cell surface correlated with enhanced TF-mediated coagulation. This effect was evident already 30 min following heparanase addition, and prior to the induction of TF [144] or TFPI expression. Thus, heparanase enhances local coagulation activity by two independent mechanisms: induction of TF expression [144], and TFPI dissociation from the cell surface [153]. Both functions require secretion of heparanase, but no enzymatic activity. The underlying mechanism is apparently release of TFPI due to its physical interaction with the secreted heparanase, as clearly evident by co-IP experiments, reflecting a functional interaction between heparanase and a membrane protein. Extracellular accumulation of TFPI upon heparanase addition suggests that following their interaction, the complex TFPI/heparanase dissociates from the plasma membrane and accumulates extracellularly.

Elevated levels of heparanase may be generated locally upon degranulation of neutrophils, mast cells and platelets [134], further facilitating blood coagulation at the site of platelet activation. Heparanase upregulation is noted in essentially all primary human tumors examined, correlating with reduced post operative survival and poor prognosis [155, 156]. Cancer patients often display a pro-thrombotic state due to the ability of tumor cells to activate the coagulation system. Over-expression of TF and acquired activated protein C resistance were suggested as main factors for coagulopathy conditions in malignant disorders [2]. Hemostatic function of heparanase, executed by inducing TF expression and releasing TFPI from the endothelial cell surface, provides a mechanism by which heparanase contributes to tumor complication, in addition to its established pro-angiogenic and pro-metastatic activities [155, 156].

4.5.9 A Model for Interaction Between Heparanase, TF, and TFPI

Platelets and tumor cells have abundant amount of heparanase [145]. Activation of the coagulation system, including platelet activation occurs in malignant and angiogenic processes [157]. Heparanase released from activated platelets and tumor cells, may, according to our findings, interact on the cell surface with TFPI and induce up-regulation of TF and TFPI in the cell. Heparanase-mediated release of TFPI from the cell surface together with its induction of TF up-regulation, render the cell surface highly pro-coagulant. Heparanase may also form complexes with TFPI and circulate in the plasma, possibly binding to endothelial cells and other intravascular components i.e. platelets and microparticles. As both heparanase and TFPI use HS as low-affinity receptors, a competition between these two proteins in binding to HS is expected, but was not found in our work to play a significant role in TFPI release. These aspects are depicted in Fig. 4.3.

Taken together, data support the notion that heparanase is a modulator of blood coagulation, and suggest a novel mechanism by which heparanase regulates TF and TFPI levels in endothelial and cancer cells. The elevation of heparanase levels in human tumors together with the pro-thrombotic state of most neoplasms, suggest a

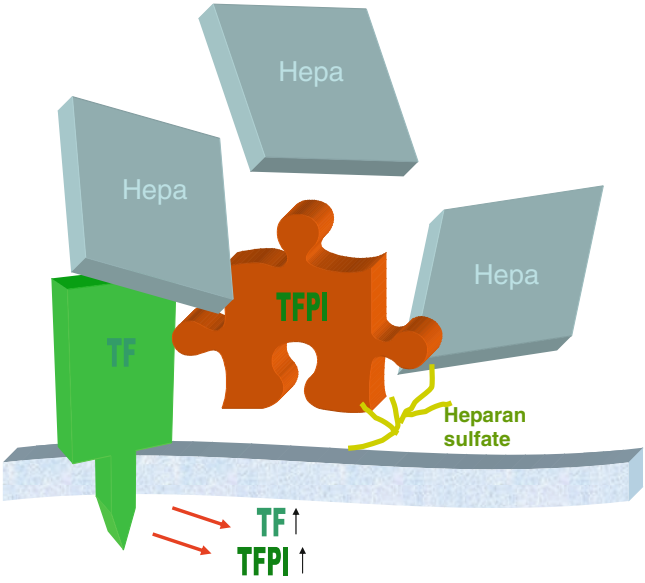

Fig. 4.3 A model of the interaction between heparanase (Hepa), TF, and TFPI. Heparanase interacts on the cell surface with TFPI and induce up-regulation of TF and TFPI in the cell. Heparanase-mediated release of TFPI from the cell surface together with its induction of TF up-regulation, render the cell surface highly pro-coagulant. Heparanase may also form a complex with TFPI and circulate in the plasma

possible clinical relevance of the procoagulant function of heparanase. Targeting domains of heparanase that mediate its enzymatic activity-dependent and independent functions may prove beneficial for patients with cancer and pro-thrombotic conditions.

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Chapter 5
The Role of Mesenchymal Cells in Cancer: Contribution to Tumor Stroma and Tumorigenic Capacity

Ofer Shoshani and Dov Zipori

Abstract Mesenchymal stromal cells were first isolated from the bone marrow, where they serve as a component of the tissue microenvironment. These cells provide a physical support for the other cells of the tissue; i.e., the hemopoietic cell lineage, and further participate in the formation of bone structures. Most importantly, stromal cells regulate the growth and differentiation of hemopoietic stem cells. The mesenchyme is not specific to the bone marrow: such cells are found body-wide, and serve similar regulatory functions. By the same token, the mesenchymal stroma contributes to tumor formation by providing regulatory signals. In addition, the stromal cells themselves may undergo transformation, and subsequently form tumors. This chapter discusses these two major aspects of stromal cell involvement in the tumorigenic process.

Keywords Tumor • Tumorigenesis • Stroma • Mesenchyme

5.1 Introduction

The bone marrow is a unique environment, harboring many cell types, which are arranged in an elaborate tri-dimensional structure. Originally, this compartment was found to be the origin of hemopoietic cells, as shown in the experiments of McCulloch and Till [1]. However, other cellular constituents of the bone marrow were disregarded until the groundbreaking experiments of Friedenstein et al [2]. In this work, fibroblastic cells derived from the bone marrow showed bone-forming capacity, and more importantly, were able to create an ectopic bone marrow environment in vivo. The cells belonging to this fibroblastic population were given many

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different designations, including osteoprogenitor cells, fibroblastoid cells, stromal cells, colony forming unit-fibroblasts (CFU-F), mesenchymal cells and finally, mesenchymal stem cells/multipotent stromal cells/mesenchymal stromal cells (MSCs). The exact in vivo origin of MSCs is not certain, and their definition is based mainly on their in vitro growth properties and capacity to differentiate. The derivation of continuously growing stromal cell populations from the bone marrow revealed the heterogeneity of this population [3–5]. Among the many different cell types discovered were fibroblasts, adipocytes, endothelial cells, osteogenic cells, and more, all with distinct morphologies. Clonal populations of such stromal cells were shown to have the potential to differentiate into three cell types: adipocytes, osteocytes and chondrocytes [6]. However, this multipotency is not shared by all cultured mesenchymal cells, as they exhibit marked heterogeneity (Fig. 5.1). Although some of these cells are multipotent, others have diminished potential. Initially, stromal cells were considered to be structural entities, scaffolding the compartment in which hemopoiesis occurs. This underestimation is slowly being abandoned, as more functions of

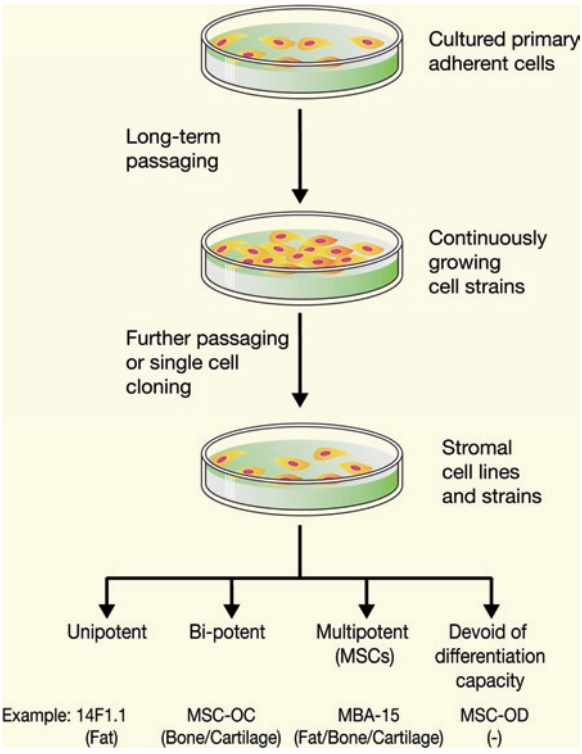

Fig. 5.1 The heterogeneity of MSC populations: seeding of bone marrow cells and derivation of independent MSC cell strains reveals extreme heterogeneity in differentiation potential. While some populations are multipotent, others have a decreased number of differentiation options or otherwise lack differentiation capacity. 14F1.1: a pre-adipogenic cloned cell line; MBA-15: a long-term cultured cell line; MSC-OC: a primary MSC cell strain; MSC-OD: a primary MSC cell strain. The cell lines and primary MSCs are all of bone marrow origin

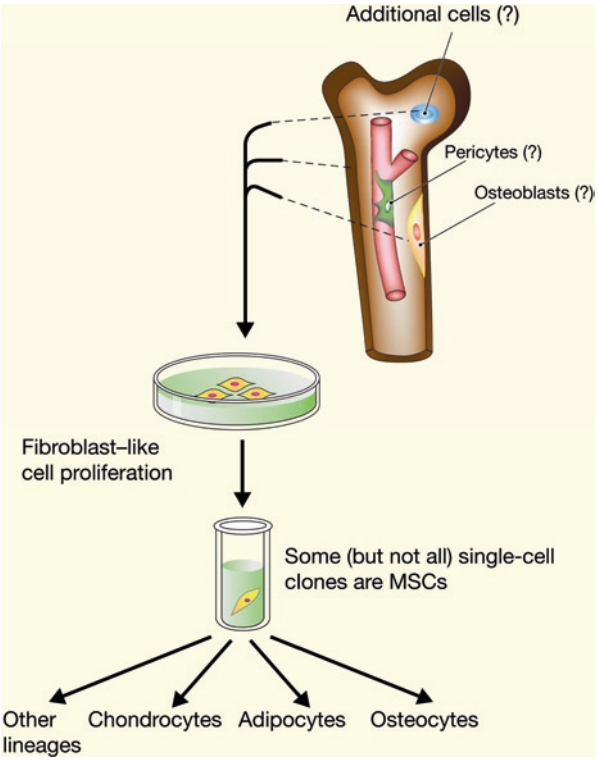

Fig. 5.2 In vivo origin of MSCs in the adult: several cell types have been suggested to be the in vivo precursors of cultured MSCs. However, the only well-established definition of these cells is based on their capacity to differentiate, at the single clone level, into several cell lineages

these cells are discovered. Tissue culture work revealed that these cells are capable of creating conditions which allow long-term maintenance of hemopoiesis [7]. The molecular mechanism responsible for this stromal function of MSCs has not been completely resolved, and to date, it is not possible to induce long-term maintenance of hemopoietic stem cells, alongside their differentiation, in the absence of supportive descendents of MSCs. It is clear, however, that stromal cells contribute to the process by serving as a docking site for stem cells, by expression of adhesion molecules, extracellular matrix components, chemotactic signals and differentiation-inducing cytokines. Most importantly, these cells restrain differentiation and allow self-renewal by expressing differentiation antagonists [8–12]. It was also demonstrated that MSCs possess immuno-modulatory functions, such as T cell suppression [13,14]. Such immunosuppressive properties were found to be independent of MHC allogeneity in mice, and dependent on cell-cell contact as well as soluble factors released by MSCs. In addition, MSCs carry different immune system-related molecules such as toll-like receptors (TLRs) [15], T cell receptors (TCRs) [16,17], and B cell receptor components [18].

In past years, much knowledge has accumulated regarding MSCs; however, fundamental issues are still left in the dark. Most importantly, these cells are not known to possess unique surface markers, which could make it possible to identify them in vivo. A plethora of markers have been suggested as possible MSC markers (reviewed in [19]). Recent studies suggest a CD146 positive phenotype to human MSCs, which were identified in vivo as adventitial reticular cells (ARC) [20] or otherwise pericytes [21]. In the mouse, similar cells were identified in vivo as being PDGFRα^{+}Sca-1^{+} cells [22]. Clearly, no consensus exists as to the exact origin and nature of the cells grown in culture as MSCs (Fig. 5.2). The standard method for deriving MSCs is by negative selection (i.e., removing other cells, such as CD11b macrophages). However, certainty regarding the success in derivation of MSCs is reached only after these cells are grown in culture and tested for their capacity to differentiate into at least three cell types: adipocytes, osteocytes and chondrocytes. MSCs are not unique to the bone marrow and actually exist in other body compartments as well, such as adipose tissues, ears, cord blood, placenta and many more. They therefore represent a multipotent progenitor population which is tissue non-specific, and exhibits body-wide distribution [19].

Even though much is left to be explored, MSCs are considered for cell therapy for a plethora of human diseases, due to their known beneficial characteristics; i.e., their differentiation potential and immunological properties. MSCs were found to have homing properties to injured tissues and tumor sites. This homing capacity prompted laboratories around the world to look at MSCs as a possible treatment for wound healing. In addition, their tumor homing capacity enables their use as cellular vehicles artificially expressing anti-tumor proteins.

5.2 Current Status of Pre-clinical Attempts and Clinical Trials Using Isolated MSCs

The only stem cell type which is currently being used successfully in the clinic worldwide, is the hemopoietic stem cell (HSC). In fact, even this cell type is not used as a homogenous population. Rather, a mixture of rare long-term repopulating HSCs is transplanted alongside a majority of progenitor cell populations which have a much more limited engraftment potential. The use of other stem cell populations in the clinic, such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), attracts much attention and hope, but to date faces several obstacles, including moral issues, immunological incompatibility between the cells and the potential recipient, and the threat of possible tumor formation by these cell types. From these viewpoints, MSCs are a better candidate cell type since they present no moral difficulties, and are abundant and easy to obtain from any individual, irrespective of age. Thus, these cells could be used in an autologous manner, circumventing the issue of immune barriers.

Currently there are approximately 100 clinical trials conducted using mesenchymal cells, according to the Clinical Trials website of the United States National Institute of Health (http://clinicaltrials.gov). These trials take advantage of the already

known beneficial properties of MSCs, their ability to differentiate into different cell types and thus regenerate affected tissues, and their ability to modulate immune responses. Autologous and allogeneic transplantation of MSCs is used in trials for conditions such as: diabetic foot [23], graft versus host disease (GvHD), multiple sclerosis, graft rejections, articular cartilage defects, multiple system atrophy, bone fractures, Crohn's disease, systemic lupus erythematosus (SLE), homozygous familial hypercholesterolemia, type 1 diabetes mellitus (T1DM), chronic obstructive pulmonary disease (COPD), myocardial infarction, liver cirrhosis, osteogenesis imperfecta [24], Parkinson's disease, osteoarthritis and stroke. A recent paper reviewed MSCs in clinical applications with the emphasis on renal and cardiovascular applications [25]. An additional study demonstrated the capacity of MSCs to restore cardiac function in chronic ischemic cardiomyopathy [26]. This was done in female swine injected with male donor MSCs. After engraftment, heart function parameters showed improvement. In addition, infarct sizes declined. This study showed that MSCs differentiated into cardiomyocytes, vascular smooth muscle and endothelial cells. The possibility that cell fusion rather than differentiation accounts for the observation, was not explored.

MSC therapy for GvHD is under extensive review by the scientific community, as there are high hopes that the immuno-suppressive properties of MSCs could aid in this condition. Unfortunately, the use of MSCs for GvHD therapy is not straightforward. Pilot studies conducted using injection of MSCs into patients with steroid-refractory severe acute GvHD showed no or very limited therapeutic effect [27,28]. However, one study did show beneficial effect for the use of MSCs in treating steroid-resistant severe acute GvHD, as observed by a significant decrease in mortality of treated patients in a 2-year follow-up [29]. In another report, MSCs were shown to lose their immunosuppressive potential after allotransplantation [30]. MSC immunosuppression is possibly conveyed by inhibiting T cell proliferation; however, MSCs do not affect T cell effector properties [31].

The participation of MSCs in wound healing was described in several studies. One mode of action by which MSCs execute this activity is by releasing paracrine factors. Among these factors are specific chemokines which are responsible for the recruitment of macrophages and endothelial cells to the wounded site, thus hastening the healing process [32]. This kind of role played by transplanted cells is referred to as a "trophic effect", implying that the MSCs participate in the process of tissue regeneration by supplying signals that modulate tissue organization, but do not fulfill a progenitor cell role by differentiating into new tissue cells that correct the damage inflicted by the disease. In contrast to this trophic effect, other studies suggest that MSCs aid in wound healing by their ability to transdifferentiate into multiple skin types. MSCs were shown to migrate to wound sites in a mechanism involving the use of the chemokine receptor CCR7. Upon systemic engraftment of green fluorescent protein (GFP)-positive MSCs, pan-cytokeratin, CD31 and α-SMA-positive-GFP cells were detected in the wound, contributing to wound repair [33].

Overall, great effort is put into assessment of the efficiency of MSC use for the therapy of human diseases. It is too early to say how prevalent the use of these cells will become, in view of conflicting information obtained from both pre-clinical

animal models as well as from clinical trials. Nevertheless, the huge effort put into this issue promises that sufficient information will be obtained soon, which will determine the feasibility of practical use of this cell type.

5.3 Homing and Engraftment of MSCs Following Transplantation

In order to use MSCs for targeted cell therapy, they should first be shown to have the ability to home into specific compartments and to perform this function at high efficiency. However, many reports show poor MSC engraftment, and only a low percentage of the transplanted cells can be found in the recipients. MSCs have the ability to home into the bone marrow, which is one of the sources of these cells in an adult. Surprisingly, it was shown that even though MSCs do have such a property, they start losing it as early as 24 h after culturing, and completely lose their homing and engraftment capabilities after 48 h of culture ex vivo [34]. A major issue in MSC research is therefore to find ways to increase their engraftment potential. An additional issue would be the targeting of these cells to specific sites. Normally MSCs are found scattered all through the organism and any clinical use would require good targeting strategies.

Different approaches showed promising potential in re-establishing homing capacity in MSCs. It was found that by treating MSCs with a cocktail of five cytokines: IL-6, IL-3, SCF, Flt-3 ligand and HGF, the cells home to the bone marrow more significantly. Infusing cytokine-treated MSCs into irradiated mice, resulted in faster recovery of hemopoiesis, and a higher degree of chimerism. Apparently, the use of the cytokine cocktail resulted in rapid accumulation of internal CXCR4 on the cell membrane, which made the cells more sensitive to its ligand SDF-1, thus improving migration into the bone marrow [35]. A 1-day exposure of human MSCs to a low oxygen concentration (1%) resulted in an increase in expression of CX3CR1 and CXCR4, which enhanced their engraftment in vivo. In addition, hypoxic conditions cause murine MSCs to express membrane-type (MT)1-MMP, which apparently play an important role in their migration and ability to form capillary-like structures [36,37]. Growing MSCs in hypoxic conditions resulted in lesser differentiation capacity compared to normoxic conditions; however, once returned to normoxic conditions, differentiation capacity was restored [37]. Yet another approach used to increase mouse MSC homing is by genetically modifying them to transiently express α4 integrin (CD49d), which forms a heterodimer with endogenous β1 integrin (CD29). Together, these molecules cause the cells to adhere to VCAM-1 and fibronectin in vitro. In addition, the modified MSCs were found to populate the bone marrow of syngeneic mice more than tenfold 5 weeks after transplant, compared to unmodified MSCs [38]. Human MSCs were successfully manipulated and subsequently homed into bone marrow of mice by engineering their CD44 membrane protein using sialofucosylation [39]. This enzymatic procedure turned their CD44 into a hematopoietic cell E-selectin/L-selectin ligand

(HCELL), which confers tropism on bone compartments. Dynamic real-time microscopy showed that sialofucosylated MSCs exhibited tethering and rolling interactions, adherence and infiltration into sinusoidal vessels.

These studies show that one may be able in the future to modify MSCs in a manner that will increase their engraftment significantly and direct them to preferred body locales. However, it is still unclear to what extent MSCs migrate in vivo and why they lose this capacity so rapidly following culture. A preferable research direction would therefore be an attempt to maintain MSCs in culture under conditions reminiscent of their in vivo niche and by that, prevent the decline in their biological functions, including migratory processes. In addition, much effort is still required to unravel the molecular basis of MSC migration, which is still vague. In this context, one study showed the potential of untreated human MSCs to adhere to endothelial cells by coordinated rolling and adhesion in a P-selectin-dependent manner. P-selectin-/- mice showed significantly less MSC rolling and adhesion to vessel walls [40].

5.4 MSCs as a Double-Edged Sword: Do They Support Tumor Cell Growth or Are They Safe for Use in Tumor Ablation?

One of the first studied functions of MSCs was their capacity to support the in vitro survival of HSCs. This survival and growth-promoting activity is not restricted to HSCs. Rather, MSCs similarly support the growth of tumor cells (Fig. 5.3). Moreover, the former section indicated that hypoxia enhances MSC migratory functions. As tumor environments are known to be hypoxic, it is suspected that such conditions may cause MSCs in tumor sites to promote angiogenesis and enhance tumor growth

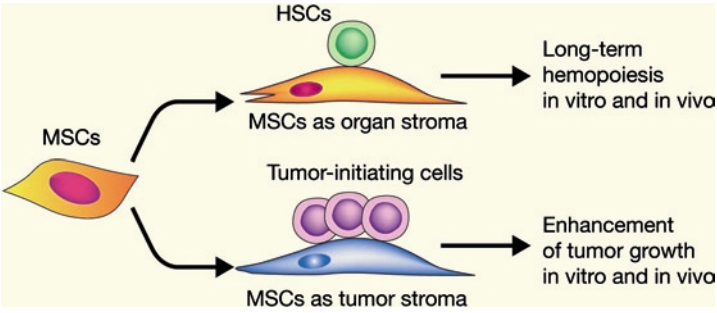

Fig. 5.3 The mesenchyme serves as a docking site and a microenvironment for survival and growth: ample evidence shows that in vivo HSC maintenance is dependent on the organ stroma. Similarly, tumor development and expansion is mesenchymal stroma-dependent. These in vivo phenomena have been reproduced in vitro: HSCs survive in vitro in the presence of stroma, and tumor cell survival and proliferation is markedly promoted by cultured stromal cells

[36]. This phenomenon may be part of the mechanism that tumors use to recruit cells that enhance their growth. Cultured stromal cells do indeed enhance the in vitro growth of carcinomas [41]. A role of monocyte chemotactic protein-1 (MCP-1) was established as a migration stimulator secreted from breast tumors. This molecule was shown to increase MSC homing to the tumor site, and blocking this molecule with a specific antibody resulted in a 37–42% decrease in homing ability [42].

5.4.1 MSCs in Tumor Promotion

Several lines of evidence demonstrate a role for MSCs in the promotion of tumor growth. In a bilateral tumor-bearing mouse model for breast carcinoma, one of the tumors was irradiated unilaterally with a low dose of radiation (1–2 Gy). This was followed by a transfusion of luciferase-expressing MSCs. After 48 h, luciferase-labeled MSCs accumulated more significantly in the irradiated tumor, than in its contralateral counterpart. The question remains whether this tropism is beneficial for clinical prognosis [43]. Human MSCs secrete VEGF and support endothelial differentiation in vitro. This was reproduced in an in vivo study using mice bearing human orthotopic pancreatic cancer xenografts. These mice were treated with a systemic administration of 4×10^5 lentivirally-marked GFP human MSCs. Following treatment, a twofold increase in blood vessel density was observed within the tumors [44]. The participation of MSCs in tumor fibrovascular network formation is implied by their attributes, which resemble tumor-associated fibroblasts (TAFs). It was shown that long-term conditioning of MSCs with tumor conditioned media resulted in the secretion of familiar tumor-associated fibroblast proteins, such as TGF-β, VEGF and IL-6. Thus, tumor-associated fibroblasts might originate from MSCs localized to the tumor tissue. In addition, co-injection of human MSCs with Skov-3 tumor cells (50:50) resulted in expedited tumor growth, which necessitated the sacrifice of the mice 70 days prior to regular tumor growth (Skov-3 alone) [45]. MSCs were shown to localize to breast carcinomas, and possibly act as tumor-associated stroma [46,47]. Human MSCs were co-injected subcutaneously with weak metastatic breast carcinoma cells MDA-MB-231 and MCF-7/Ras. This resulted in an increase in local tumor growth and increased metastatic potential, as observed by higher metastatic nodules found in the lungs of the mice. Apparently, the tumor cells stimulate the secretion of CCL5 from the MSCs, which in turn, increase tumor cell motility, invasion and metastasis. Interestingly, this effect was reversible: tumor cells were separated from the MSCs after inoculation and tumor formation, and re-injected into mice. The new tumors which formed showed regular metastatic properties, which indicated that once the MSCs were absent, these cells ceased to be "educated" to become motile [48]. Another chemokine implicated in breast cancer metastasis is CCL2. MSCs co-cultured with breast cancer cells were shown to secrete this chemokine, and blocking this secretion resulted in a 21–50% decrease in tumor formation. Interestingly, when MSCs were induced to differentiate into osteoblasts, the secretion of CCL2 was significantly increased [49].

Multiple myeloma (MM) models show that MSCs are essential players in the development of the disease. Apparently, MSCs secrete IL-6, which is a myeloma growth factor, and this results in increased tumor growth, once plasmacytoma cells are co-injected together with MSCs. Interestingly, MSCs also secrete activin A, which is a tumor suppressor growth agent. However, it appears that in multiple myeloma, the basal level of activin A secretion is overwhelmed by the secretion of IL-6, which stimulates tumor growth. The inclusion of stromal cells in a mouse plasmacytoma inoculum injected subcutaneously resulted in promotion of tumor growth. This could be blacked by the co-injection of the IL-6 antogonist, activin A [50–53]. Myeloma cells were shown to secrete a Wnt inhibitor, Dickkopf-1, which prevents MSCs from differentiating into osteoblasts, thus hastening osteolytic lesion formation. Treatment of MSCs with the Wnt signaling activator 6-bromoindirubin-3'-monoxime (BIO), rescued these cells from the osteoinhibitory state. In addition, such treatment resulted in a decrease in IL-6 secretion from MSCs, which in turn resulted in a reduction of myeloma cell proliferation [54]. Recent findings indicate that human MSCs derived from MM patients are abnormal. They show diminished capacity to support hemopoiesis, and differentiate into osteoblasts. Gene expression profiles of MM-derived MSCs, compared with MSCs derived from healthy subjects, revealed already known factors such as IL-6 and DKK1 to be overexpressed. Moreover, new soluble factors were found and one of them, GDF15, was found to induce dose-dependant growth of MOLP-6, which is a myeloma cell line [55]. Thus, MSCs enhance tumor growth both by serving as stromal support that enables tumor expansion and by enhancing angiogenesis, which is again crucial for tumor growth and spread.

5.4.2 MSCs in Tumor Inhibition

The "beneficial" effects that MSCs have on cancer progression, growth and spread suggest that one should be very careful when considering the use of MSCs in the clinic. Is it possible that these cells would enhance tumor growth or induce the appearance of dormant tumors? Yet many experimental attempts point to the opposite: Human MSCs can serve as cellular vehicles for anti-tumor drugs (reviewed in [56]). Several publications indicate that these cells have tropism to tumor sites, and if genetically modified to secrete anti-tumor agents, are successful in abrogating tumor growth. Melanoma cells co-injected with interferon-β overexpressing MSCs resulted in formation of significantly smaller tumors, and prolonged life expectancy in treated mice [57]. In a mouse myeloma model established by injection of KMS-12-BM cells, genetically modified MSCs, which express osteoprotegerin, were able to reverse osteoclast activation and reduce bone loss caused by the disease [58]. Human MSCs also show promising results as cell therapy vehicles for the treatment of gliomas. They were shown to home to the glioma site and increase animal survival when expressing interferon-β [59], S-TRAIL [60] and Delta24-RGD [61]. One possible molecule involved in the ability of human MSCs to home to glioma

sites is matrix metalloproteinase one (MMP-1) [62]. A strong inhibition of lung metastases in a C-26 lung metastasis model was achieved by targeted delivery of NK4, an antagonist of hepatocyte growth factor (HGF), using a genetically modified mouse MSC. These cells were injected via the tail vein, migrated to tumor sites, inhibited tumor-associated angiogenesis and induced apoptosis of tumor cells, thus significantly prolonging the survival of treated mice [63]. Murine MSCs also show potential in fighting melanoma in a mouse model. Direct inoculation of MSCs into tumor sites resulted in the abrogation of tumor growth. It is apparent that MSCs have cytotoxic effects, as tumors cells underwent apoptosis. Furthermore, it was shown that MSCs inhibit angiogenesis in vitro, as well as in vivo, in a concentration-dependent manner, thus affecting blood supply to tumor sites and inhibiting tumor growth. When as many as 10^6 MSCs were injected directly into tumor sites, tumor growth was inhibited, and histological analysis revealed lower vascular density in the tumors [64]. One possible route for inhibition of tumor cell proliferation was demonstrated in vitro by administering human MSC-conditioned media to MCF-7 and H7402 human hepatoma cells. Such treatment resulted in a significant decrease of tumor cell proliferation, as observed by a decrease of up to 10% less BrdU incorporation. As the mRNA levels of NF-κB were downregulated, it was proposed that this molecule is involved in the depression of tumor cell proliferation mediated by MSCs [65]. A recent study showed that repeated infusions of $Lin^-CD44^{hi}Sca1^-cKit^+CD34^-$ mouse MSCs were able to significantly reduce progression to low-grade gastric dysplasia in *Helicobacter felis*-infected mice, possibly by inhibiting Th-17 related pro-inflammatory activity [66].

Apparently, the use of MSCs as a means to suppress tumor growth should be carefully examined for each type of malignancy, due to their supportive stroma functions. Nevertheless, ample evidence from a variety of experimental systems point to the potential of MSC use in the targeting and destruction of tumor tissue.

5.5 MSCs as Tumor-Initiating Cells: Does MSC Transplantation Pose a Threat in Terms of Cancer Formation?

Recent publications show that mouse and human MSCs possibly harbor tumorigenic potential. However, there is no consensus regarding this issue, and some investigators maintain that these cells are safe, particularly following a limited number of passages. Some stromal cell lines, which have been long-term passaged in vitro, are still non-tumorigenic [67]. The lack of agreement on the subject of MSC tumorigenic potential calls for further investigation. Tumorigenicity of MSCs, when detected, is probably due to cell transformation under culture conditions. These conditions impose great stress on cells that experience removal from their natural niche and exposure to unfavorable conditions. The latter could

potentially drive MSCs into crisis, followed by transformation. Culture is imperative for cell expansion and at this point cannot be avoided, so that sufficient amounts could be injected into patients. Therefore, the possibility that MSCs would undergo malignant transformation in vitro is realistic, and should be examined carefully. The following text will discuss the different aspects of MSC tumorigenicity.

5.5.1 In Vitro Senescence of MSCs

Cell senescence is a well-known phenomenon that occurs following in vitro cell culture. This process is related to accumulating DNA damage in the cells, possibly due to oxidative stress and formation of reactive oxygen species (ROS). In addition, factors such as donor age, cell plating density and serum constituents have an impact on the evolution of this process. These factors have an impact on telomere length and cell cycle dynamics. Possibly, the cells which undergo senescence cease to proliferate, and are slowly outnumbered by transformed cells, which adopt the capability to withstand the different environment present in culture. This might account for the reports on MSC tumorigenicity. It appears that murine MSCs undergo senescence at a much earlier stage than their human counterparts. This might be due to the fact that DNA damage control is stricter in human cells as compared to mouse cells (reviewed in [68]) (Fig. 5.4).

Epigenetic changes might be involved in the development of cell senescence. In human MSCs, cell senescence occurs after 2–3 months of culture, although apparently this process starts from the first passage onwards [69]. Overall methylation levels seem to be maintained; however, specific CpG methylations change significantly especially in genes related to cell differentiation and development (homeobox genes). There is a possibility that such epigenetic transitions might be

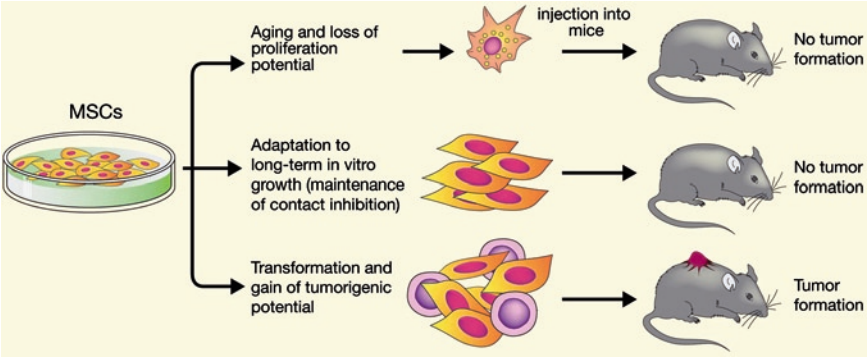

Fig. 5.4 Alternative fates of MSCs in culture: Senescence, long-term growth or transformation and acquisition of tumorigenic potential

responsible to transformational events. Thus, chromosomal aberrations might not be sufficient for the transformative process, which possibly requires modifications in DNA methylations. It should also be taken into account that cells with no chromosomal aberrations might be tumorigenic, solely based on their epigenetic profile, which changes under in vitro conditions [70].

5.5.2 Aneuploidy and Chromosomal Aberrations in Cultured MSCs

Murine MSCs show systematic chromosomal aberrations, independent of mouse strain and culturing methods. From early passages (P2), most of the MSCs show polyploid DNA, mainly near-tetraploid. Importantly, it was also shown that rat MSCs show loss of genomic stability and gain of chromosomal aneuploidy as early as following a single passage [71,72]. Clinical use of MSCs necessitates their production in a manner which will maintain their safety. A recent study reports that 5/20 human MSC cultures at the first passage, show random aneuploidy. It was concluded, however, that the aneuploidy did not result from the culture process; rather, it was donor dependent. In addition, the research found no selective growth advantage for such aneuploid cells. However, this possibility was not investigated further since only one more passage was examined. All MSCs derived in this study became senescent, and failed to undergo cellular transformation [73]. An additional study examining ten different human MSCs in long-term cultures (up to 25 passages), revealed no chromosomal aberrations, and no telomere-maintaining mechanisms, events that might lead to cell transformation. Importantly, no genetic instability was diagnosed in human adipose stromal cells (up to 35 population doublings) [74]. However, it was found that other cell types, such as primary chorionic villus cells, gain genomic instability in culture in the form of tetraploidy, following eight to ten population doublings (PDs). This emphasizes the dependence on cell type when looking at culture-induced genomic instability [75]. An additional indication for the safety of human MSCs was demonstrated in a study comparing them to rhesus MSCs. This study showed that human MSCs maintain an intact genome even after 30 population doublings, in contrast to rhesus MSCs which acquire aneuploidy and tetraploidy under the same conditions. Interestingly, rhesus MSCs show 70% tetraploidy/10% aneuploidy at passage (P)30, and this ratio shifted towards aneuploidy at P90 (40% aneuploidy/40% tetraploidy). Both cells, however, failed to form any tumors once injected subcutaneously into immune-deficient mice [76]. One important study examined the tumorigenic potential of human MSCs derived from children diagnosed with idiopathic thrombocytopenic purpura (ITP) and autoimmune neutropenia. In this study, normal levels of p53, RB, p16 and H-RAS were detected, as well as undetectable hTERT activity. In addition, these cells maintained normal karyotypes, and did not form tumors upon transplantation into immnodeficient SCID mice [77].

5.5.3 MSC Tumorigenicity

Cultured mesenchyme may maintain a normal growth phenotype, including contact inhibition and lack of tumor formation even after prolonged culture periods [3]. However, ample evidence shows that murine MSCs are able to undergo cellular transformation (Fig. 5.4), in contrast to data found in reports on human MSCs. One study compared the ability of cells from both origins to generate osteosarcoma lesions in the lungs of intravenously injected animals. The authors demonstrated that mouse cells rapidly acquire numerical chromosomal abnormalities at P4, as compared to the normal phenotypes of P7 human MSCs. Upon injection into immune-deficient mice, only the mouse MSCs formed osteosaroma-like lesions in the lungs. It is important to mention, in this context, that the use of human MSCs against the background of the mouse model is problematic, and might not disclose the true nature of these cells [78]. In a study conducted with murine MSCs, alarming evidence for their tumorigenicity was brought to light. It appears that murine MSCs accumulate chromosomal aberrations as early as on the first passage in culture. Upon systemic delivery of MSCs from passages as early as P29, they formed fibrosarcomas in immune-deficient mice. Apparently, MSCs underwent cellular senescence at passages 2–5, as they exhibited an enlarged and flattened morphology, in addition to a slower proliferation rate. After this stage, the culture overcame the crisis and started to show an acquired proliferation capacity. Already after one passage, the cells showed only 50% normal karyotype (40, XY), and as passage number increased, chromosomal instability in the form of aneuploidy was also growing [79]. In an independent study aimed at analyzing the benefits of the use of genetically modified mouse MSCs, it was unexpectedly found that the mice developed tumors in lungs and extremities following systemic infusion of cells [80]. The researchers highlight the fact that the original non-modified MSCs exhibited abnormal cytogenetics, and formed sarcomas after systemic administration. The sarcomas showed clonal evolution of cytogenetic properties. Additional MSC cultures did not necessarily form tumors, even when containing elaborate cytogenetic abnormalities. This indicates that not all aberrations are hazardous. Importantly, beside the original MSC culture, which had confirmed tumorigenic potential, in ten more additional cultures, no tumorigenic potential was observed, emphasizing that MSC tumorigenicity is a relatively low-frequency event [80]. Murine MSCs which formed osteosarcomas were examined, and were found to be aneuploid, harboring translocations and homozygous loss of the cdkn2 region. CDKN2A/p16 protein expression was identified in 88 osteosarcoma patients, showing a correlation with the results obtained from the mouse model, implicating MSCs and cdkn2 expression in malignant transformation [81]. A model for age-related tumorigenesis in mice is suggested, by showing that MSCs spontaneously accumulate point mutations in p53, and express embryonic factors in a fashion resembling naturally occurring fibrosarcomas in aged mice [82]. The mechanisms underlying sarcoma development are reviewed in [83]. Interestingly, sarcomas are generated in two distinct pathways: the first involves specific well-characterized translocations which are essential for the pathogenesis of the disease and are used in clinical

diagnostics. The second pathway, however, is less straightforward, showing complex random karyotypes with severe genetic and chromosomal instabilities. The cell origin of this cancer is still unknown; however, it is possible that MSC transformation might account for the random chromosomal instabilities type of sarcoma.

As MSCs are considered for clinical therapy in tissue repair, the tumorigenic potential following engraftment of such cells mounted on a bioscaffold was assessed. Evidently, tumors formed only in allogeneic or immune-suppressed mice, and did not depend on scaffold material. In addition, the expansion of CD4+ CD25+ T regulatory cells was observed, which suggests that MSCs are able to suppress the host's anti-tumor immune response [84].

The safety of human MSCs started becoming questionable as accumulating data implicated these cells as having tumorigenic potential. The identification of transformed cell populations in human MSCs came to light. These cells exhibited an increase in telomerase activity, chromosome aneuploidy and translocations, and formed aggressive tumors upon injection into NOD/SCID mice [85]. Apparently, human MSCs (from adipose tissue), transform in a spontaneous manner. This process occurred at 4–5 months of culture, after the normal expansion period for clinical use (6–8 weeks) of these cells (ten different samples studied). Transformed cells had a higher expression of c-myc compared to pre-senescent cells, which might be the reason that these cells bypassed senescence. In addition, transformed cells exhibited extensive chromosomal abnormalities, and formed tumors upon injection i.v. into irradiated mice (38/38 mice with tumors). In contrast, pre senescent cells showed no tumorigenic potential [86]. Human MSCs were shown to undergo spontaneous malignant transformation in culture at a transformation rate ranging between 40% and 50%. These cells avoided cell senescence via a transformative event, which allowed their rapid proliferation and formation of epitheloid tumors upon engraftment in mice [87]. A two-stage model for the transformation of human MSCs is proposed. In the first step, the cells adjust themselves to circumvent senescence by overexpression of c-myc and repression of p16. Then, telomere shortening causes cell crisis and hastens the process of cell selection, enabling only transformed cells to continue growing by stabilizing telomere changes [88]. Of note is the finding that human fetal neural stem cells transplanted in an ataxia telangiectasia (AT) patient, led to the formation of tumors, 4 years after the procedure. Biopsy revealed that the tumors were glioneuronal and of donor origin. This data suggests that all stem cell therapy should be taken with caution [89].

5.6 Possible Mechanisms Underlying MSC Tumorigenicity: Chromosomal Instability – Culprit or Savior?

The use of MSCs for therapy requires their expansion ex vivo for a prolonged time, thus exposing these cells to environmental stress, which could potentially turn them into transformed cells. Current modes of application are using karyotypic analysis to ensure that only cells which harbor a normal karyotype are used for therapy.

An early model for the genetic evolution of human solid tumors has been suggested. In this model, cells acquire tetraploid DNA content, followed by random structural chromosomal abnormalities and aneuploidy. These chromosomal structural changes might result in activation of growth-promoting genes, which will give rise to cell selection [90]. Although the reasons for aneuploidy and polyploidy of MSCs in culture and cells in vivo might be different, it appears that these events strongly correlate with cancer pathogenesis.

An extensive, recently published review underscores the path by which cells are able to acquire aneuploidy, and in turn tumorigenicity [91]. Although it is apparent that in some cases aneuploidy has beneficial effects, in most cases, however, aneuploidy results in poor prognosis. One way genomic instability can develop, is by the generation of centrosome amplification. Such an event can result from centrosome fragmentation or overduplication in diploid cells, or alternatively, due to cell tetraploidization (as DNA synthesis is coupled to centrosome doubling). Tetraploid cells can arise in three distinct pathways [92]: cell fusion, cytokinesis failure, and mitotic slippage. It is arguable whether tetraploid cells should be considered as transformation-prone. On the one hand, the aberrations in such cells, which normally cause diploid cells to die, might occur unnoticed, as they have an extra set of chromosomes. On the other hand, the extra set of chromosomes might prove to be beneficial, as tumor suppressor genes are also duplicated. Thus, there is a favorable outcome for near-triploid neuroblastoma patients over diploidy [93], and in Down's syndrome, extra chromosome 21 suppresses tumor prevalence in this population, due to overexpression of the chromosome 21-resident gene DSCR1 (calcineurin inhibitor). Thus, polyploidy might entail beneficial properties in the appropriate context [94]. It is worth mentioning that polyploid cells occur naturally in the living body, in cells such as hepatocytes [95], megakaryocytes [96] and myocytes [97].

In the case of centrosome amplification, there is an increasing risk for multipolar spindle formation during mitosis. Such an event might lead to unequal distribution of maternal DNA in the two or more daughter cells. In most cases, such daughter cells will not be able to grow. However, on rare occasions, they might be proliferative and presumably tumorigenic. Multipolar spindle formation is a rare event, and thus, cannot explain observed rates of chromosomal instability in solid tumors. In addition, cells with centrosome amplification use preventative modes to avoid multipolarity, possibly by centrosome clustering [98]. Thus, a mechanism linking extra centrosomes to chromosomal instability was proposed [99]. This mechanism shows that supernumerary centrosome numbers cause chromosome lagging during mitosis, even in bipolar mitotic spindles, due to formation of an intermediate multipolar spindle.

Tetraploid cancer cells undergo apoptosis, unless p53 is inhibited [100]. Thus, changes in p53 expression during MSC culture might partially account for their potential tumorigenicity. Evidently, the loss of p53 in p21-deficient mouse MSCs leads to tumorigenicity and increased chromosomal content, compared to p53 wild-type cells [101]. Interestingly, it was found that human epithelial cancer cells are able to suppress p53 in neighboring fibroblasts [102]. Another report demonstrated that the absence of p53 promotes osteogenesis in MSCs [103]. This piece of information might explain the occurrence of osteosarcoma formation reported after

injection of MSCs. There are additional molecules which may convey tetraploidization and tumorigenesis in MSCs, one of them is Aurora-B. Several types of cancers show increased expression of this protein, and its overexpression in murine epithelial cells makes them tetraploid and enables them to significantly promote mammary epithelial cancers [104]. This might suggest a role for tetraploidy in tumorigenic events; however, it is possible that Aurora-B overexpression results in other unseen events which in turn lead to tumorigenicity.

5.7 Summary

MSCs may contribute to cancer formation by two general mechanisms. One entails the activity of MSCs as tumor stromal cells (often called cancer-associated fibroblasts (CAFs) (Fig. 5.5). Human tumors do not develop beyond a few millimeters unless supported by a stromal meshwork. This is not different from the requirement of any normal tissue and organ for supportive and regulatory stroma. Tumors do not differ in this respect from normal tissues. Although it is often suggested that the tumor stroma differs markedly from normal stroma, this issue still requires substantiation. Ample amounts of data shows that tumor growth and spread is enhanced by progeny of MSCs. In this respect, MSCs should be targeted and eliminated within tumors, in order to cause tumor regression. The limited success of anti-angiogenic treatments may be due to the presence of MSCs within tumors that could re-initiate angiogenesis. In contrast to this supportive effect of MSCs, some investigations point to possible inhibitory functions of these cells in their normal, unmanipulated state. Extensive

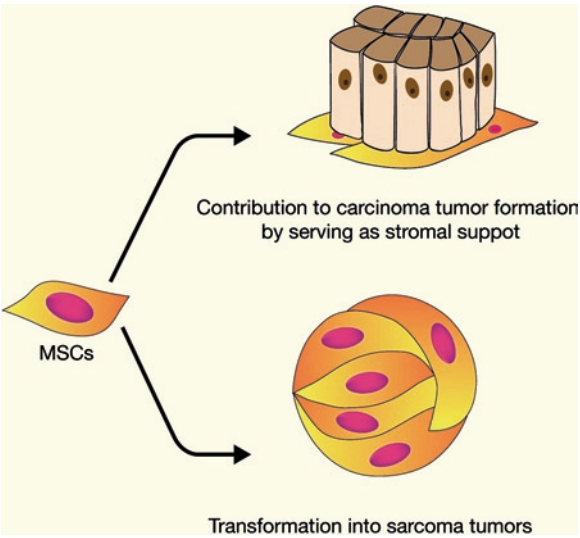

Fig. 5.5 MSCs contribute to tumor formation in alternative and basically different modes: MSCs may serve as stromal components of the tumor, essential for its development. However, MSCs may undergo malignant transformation and become tumor-initiating cells

information relates to the capacity of MSCs to serve as vehicles that carry therapeutic molecules into tumors. This relates to the tendency of MSCs to home preferentially to sites such as the bone marrow and tumor microenvironments. Correct genetic modifications of MSCs that would promote their specific migration and homing capacities are needed for further development of this therapeutic modality. It is also imperative to find ways to maintain MSCs in culture under conditions that allow the maintenance of their original properties, which are often lost upon culture.

The second major way by which MSCs may contribute to tumor formation is by gaining tumor-initiating capacities (Fig. 5.5). Although several studies demonstrate lack of tumorigenic potential of both mouse and human MSCs, other studies indicate a high propensity of mouse MSCs towards malignancy, and a milder but yet significant capacity of human MSCs to form tumors. This is associated to genetic instability entailing aneuploidy and chromosomal aberrations. Although it might be expected that such occurrences would provide a solid molecular basis for MSC malignant transformation, this is not the case. It still has to be determined how far such changes are deterministic. Indeed, cells that have undergone extensive genetic changes often maintain a non-tumorigenic phenotype.

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Chapter 6
Shaping Tumor Associated Macrophages: The Role of NF-κB

Robin Soper and Thorsten Hagemann

Abstract Tumor associated macrophages (TAMs) are known to form a large part of many human and murine tumors. These TAMs have been programmed by the tumor microenvironment and interact with other cells within the tumor leading to increased tumor growth, survival, invasion and metastasis. While TAMs are tumor supportive, "classically" activated macrophages are polarized to be tumoricidal. Signaling through the transcription factor, nuclear factor kappa B (NF-κB) has been shown to regulate many diverse genes and is heavily involved in inflammation and immunity and as such it has been shown to play a key role in the determination of macrophage function.

Keywords Tumor-associated macrophage • NFkB, • Tumor microenvironment • Phenotype • Cytokines

6.1 Introduction

The link between chronic inflammation and cancer is not a recent concept, with the original observation of the presence of leukocytes in tumors being made by Virchow in 1863 [1]. The implications of this observation were not seized upon at the time and it is only relatively recently that this area has attracted significant interest as a target for anticancer therapies. It has been demonstrated that the presence of chronic inflammation is associated with a greater cancer risk at the inflammatory site [2]. Furthermore malignant cells have been shown to release factors that recruit inflammatory cells and promote the generation of an inflammatory environment. In many cancers macrophages comprise a large component of this leukocyte infiltrate [3,4].

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6.2 Macrophage Polarisation

Macrophages are plastic cells that play a multitude of roles as determined by their physiological environment and anatomical location [5]. They are recruited as monocytes from the bloodstream into healthy tissue and upon arrival in the tissue they differentiate into macrophages. They can also be recruited by chemoattractants released from the site of inflammation, injury, infection or malignancy and differentiate in response to the microenvironment that they encounter. This microenvironment directs the macrophage towards particular phenotypes ranging between the polar extremes of M1 and M2 (Fig. 6.1) [6]. Macrophages of the M1 phenotype are referred to as "classically" activated and occur in response to interferon-γ and microbial products. They are characterized by high major histocompatability complex (MHC) molecule expression and elevated proinflammatory cytokine release (such as interleukin-12), inducible nitric oxide synthase (NOS2) upregulation and are highly capable of killing pathogens and tumor cells. At the other pole, M2 or "alternatively" activated macrophages are responsible for curbing the immune response, clearing cell debris and promoting tissue remodeling and angiogenesis [7]. The M2 phenotype can be further subdivided according to the activating stimuli and function [8]. Stimulation by IL-4 and IL-13 leads to M2a, with M2b arising in response to combined exposure of either IL-1R or TLR agonists in conjunction with immune complexes. Both M2a and M2b promote Th2 type responses and have immunoregulatory roles. Interleukin-10 exposure leads to the M2c phenotype, which is concerned with suppression of the immune response and tissue remodeling. These M2 macrophages are characterized by low MHC molecule expression, increased anti-inflammatory IL-10 release and expression of scavenger receptor

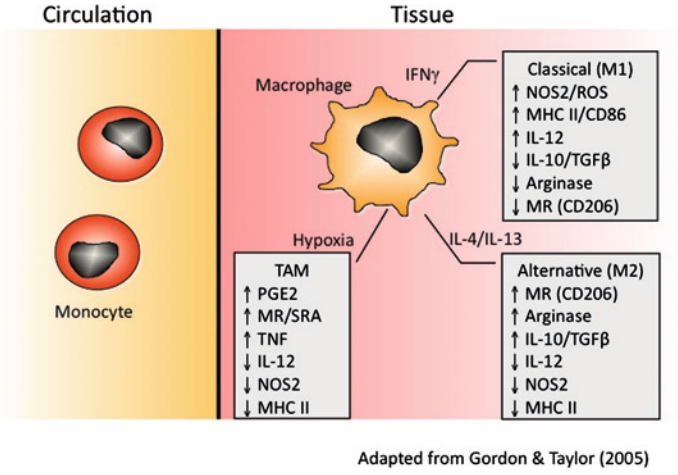

Fig. 6.1 Macrophage polarisation in response to the microenvironment. Upon recruitment from blood stream and entry into the tissue, monocytes differentiate into macrophages in response to the local environment. This environment directs the macrophages towards a particular phenotype associated with a range of molecular characteristics

(SR-A) and mannose receptor (MR) [6]. They also demonstrate upregulation of arginase-1, Fizz1, Ym1 and macrophage galactose-type C-type lectin-2 (Mgl2) transcription [9]. One of the defining phenotypical differences between the M1 and M2 phenotypes is the production of IL-12 and IL-10. Interleukin-12 stimulates a strong immune response, causing the activation of natural killer (NK) cells and the production of interferon-γ and interleukin-2 by Th1 cells. Interleukin-10 on the other hand stimulates activation of the signal transducer and activator of transcription (STAT)3 pathway and inhibits STAT1, suppressing IL-12 production and consequently IFN-γ release. It further inhibits the production of other proinflammatory cytokines through increased RNA degradation.

6.3 Tumor Associated Macrophages: An Alternative Macrophage Phenotype

Solid tumors are comprised not only of malignant cells but also nonmalignant stromal cells [10]. These nonmalignant cells are not merely innocent bystanders, indeed the interactions between the various stromal cells themselves and the malignant compartment has a profound effect on cancer growth, progression, metastasis and angiogenesis [11]. Leukocytes account for a large proportion of the nonmalignant stroma, comprising up to 50% of the total tumor mass. The composition of the leukocyte population is also a key factor in determining the clinical outcome. A high proportion of T lymphocytes correlates with better prognosis in a number of tumor types [12,13] yet a high macrophage density is indicative of a poor prognosis in the majority of tumors [14]. Indeed both pharmacological (through the use of bisphosphonates) and transgenic depletion of macrophages has been demonstrated to have a potent inhibitory effect on tumor progression in a number of murine tumor models [15]. Tumor associated macrophages (TAMs) are recruited to the tumor site as monocytes by chemokines like CCL2, SDF1 and VEGF and differentiate in response to the tumor microenvironment. As such they are more similar to the M2 phenotype, having low expression of MHC II and IL-12, and elevated production of IL-10, VEGF and PGE_2. This may be an oversimplified view of TAMs however and in keeping with their plastic nature there are reports of TAMs with a more proinflammatory phenotype during the early stages of tumor initiation, which adopt a more immunosuppressive phenotype as the tumor progresses [16]. There have been many investigations looking at “re-educating” or targeting TAMs for destruction. The phosphatase SHIP has been implicated in inhibiting the polarization towards an M2 phenotype and experiments using macrophages from ship−/− mice showed them to have dimished NO production in response to LPS and be arginase-1 high [17]. Futhermore, tumors in these mice grew much faster [17]. Depletion of macrophages through the use of clodronate containing liposomes lead to decreased tumor burden and metastasis [15]. Macrophages have also been targeted in a mouse model of ovarian cancer through attaching a saporin toxin to a SR-A specific antibody, resulting in macrophage depletion and reduced tumor burden [18]. A similar targeting method was employed using a DNA vaccine against legumain,

which is highly expressed on TAMs. This provoked a strong CD8+ T-cell response against the TAMs and resulted in decreased tumor burden, angiogenesis and metastasis [19]. Finally, through the use of an IL-10 receptor-specific antibody and CpG oligodeoxynucleotides, TAMs were switched from an M2 to an M1 phenotype and this resulted in a rapid reduction in tumor size [20]. It can be clearly seen from these examples that targeting TAMs and macrophage polarization can potentially be a highly successful way of promoting antitumor activity. With this in mind it is understandable that a great deal of interest has been shown in investigating the pathways that regulate this polarization process as well as pathways that support the protumor functions of TAMS. A major pathway in this process has been shown to be NF-κB.

6.4 The NF-κB Signaling Pathway

The NF-κB family consists of five proteins: NF-κB1 (p105/p50), NF-κB2 (p100/p52), Rel A (p65), Rel B and c-Rel [21]. While it is only the Rel proteins that contain the transcription activation domains, all NF-κB family members possess a Rel Homology Domain (RHD) which contains a nuclear localisation sequence (NLS) and is used in dimerisation, association with members of the IκB family and binding to the NF-κB DNA target sites. Through numerous combinations of hetero and homodimers, the NF-κB family can differentially regulate a large array of biological responses. NF-κB dimers are retained in the cytoplasm by association with IκBs through RHD binding, preventing nuclear localisation. Activation of NF-κB signalling occurs by stimulation of three main pathways (Fig. 6.2). Inflammatory cytokines and pathogen associated molecular patterns (PAMPs) lead to activation of the IKK complex via the Toll-receptor/IL-1 receptor and TNF receptor families. This IKK complex is a heterotrimer consisting of two kinases, IKKα, IKKβ and a regulatory subunit, IKKγ (NEMO), which is required to link the IKK complex to upstream signals. Activation of this complex leads to the phosphorylation of the IκB (at Ser32 and Ser36 on IκBα and equivalent sites on other IκBs) by IKKβ, causing polyubiquitination (on Lys21 and Lys22 of IκBa or equivalent sites on other IκBs) and degradation by the 26S proteosome [22]. The other catalytic subunit, IKKα, has been shown to be dispensible for this process. This frees the NF-κB dimer (most commonly p50/p65), which translocates to the nucleus where it can bind to sequence specific DNA binding sites leading to the transcription of particular target genes [23]. This is known as canonical activation. Alternatively, factors such as lymphotoxin B, BAFF, RANKL and CD40L can induce a different NF-κB pathway through binding their receptors. This pathway involves the activation of an IKKα homodimer in response to the activation of NF-κB inducing kinase (NIK) [24,25]. The activated IKKα homodimer phosphorylates NF-κB2 (p100) at the C-terminus. NF-κB2 can be viewed as having an inbuilt IκB and upon phosphorylation of the two C-terminus sites, ubiquitination and partial degradation, yields p52 leading to nuclear translocation. This p100/p52 is most commonly found in association with Rel B and its

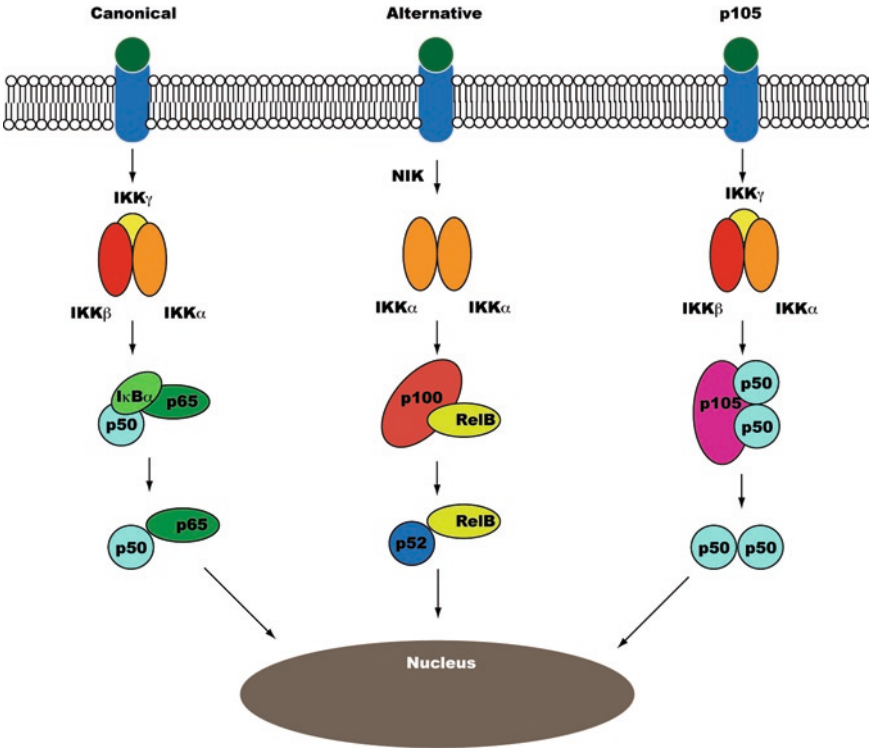

Fig. 6.2 The three pathways of NF-κB activation. In the canonical pathway, the IKK complex is activated leading to the phosphorylation and ubiquitination of IκBa, freeing the p50/p65 heterodimer to enter the nucleus and induce gene transcription. The alternative pathway is regulated by NIK and the activation of the IKKa homodimer, which targets the p100 partial degradation to p52 leading to nuclear translocation. The p105 pathway uses the same IKK complex as the canonical pathway to induce phosphorylation and complete degradation of the p105 freeing the p50 homodimer to enter the nucleus

nuclear translocation leads to the activation of a different set of genes from Rel A. This is known as alternative activation. The third NF-κB pathway is concerned with the nuclear translocation of p50 homodimers and is referred to as the p105 pathway [26]. When this pathway is inactive, a p50 homodimer is retained in the cytoplasm associated with p105. Proteolytic processing of p105 occurs by two separate mechanisms. Constitutive processing of p105 leads to the production of p50 monomers that can bind RelA and c-Rel and becomes part of the canonical pathway. Agonist induced proteolytic processing occurs in response to stimuli such as IL-1, TNF-α and LPS and leads to the phosphorylation of p105, at Ser927 and Ser932, by the IKK complex. This causes ubiquitination of the p105 and its total degradation by the proteosome. The p50 homodimer is then free to translocate to the nucleus. The binding of p50 homodimers to transactivation sites blocks these sites but does not induce transcriptional activation unless they are associated with BCL-3. BCL-3 is a member of the

IκB family but distinct from the other family members it contains a transactivation domain. Depending on the context it can act as either a transcriptional activator or repressor [27]. It is almost exclusively found in the nucleus where it can form complexes with p50 and p52 homodimers. The effect that BCL-3 has on NF-κB activity is largely determined by post-translational modification. Ubiquitylation of BCL-3 is a requirement for its nuclear localization and deubiquitylation of BCL-3 displaces p50 and p52 homodimers from the promoter regions, allowing transcriptionally active NF-κB dimers to bind. The other key post-translational modification of BCL-3 is phosphorylation, when BCL-3 is both ubiquitinylated and phosphorylated it forms transcriptionally active complexes with p50 and p52 homodimers. However, with the exception of cyclin D1, the genes that are regulated by this process have yet to be characterized. If the BCL-3 is ubiquitinylated yet unphosphorylated it stabilizes the homodimers causing inhibition of NF-κB activity, a process that has been hypothesised to induce tolerance.

The activation of the canonical pathway leads to the increased transcription of genes that are important to the innate immune response, such as adhesion molecules, proinflammatory cytokines, chemokines and enzymes associated with the production of anti-apoptotic and inflammatory mediators. The release of proinflammatory cytokines such as IL-1β and TNF-α generates a positive feedback loop leading to further activation of NF-κB. Genetic deletion of NF-κB family members causes a number of functional defects. Macrophages derived from Rel B−/− mice overproduce IL-1β, produce normal levels of IL-6, IL-10 and IL-12 yet are unable to produce TNF-α [28]. TNF-α production is similarly impaired in c-Rel−/− macrophages however IL-12 levels are also diminished in these cells [29,30]. It should be noted that an element of redundancy exists between various NF-κB molecules with various components being able to compensate for each other [31]. This has been demonstrated in comparisons between RelA knockout mice and RelA, c-Rel double knockout mice, where c-Rel was shown to reduce the impact of TNF-α induced apoptosis that occurred in RelA deficient mice [23]. While IKKβ−/− leads to an embryonic lethal condition in mice as a result of liver apoptosis this could be overcome if TNFR1 was also knocked out [32]. Furthermore using cell selective IKKβ deletion it was shown that IKKβ protects macrophages from LPS induced apoptosis [33], with IKKβ deleted cells being much more susceptible to apoptosis. The use of IKKα mutants, where two point mutations have been performed to yield an inactivatable form of IKKα, has shown that IKKα has a role in regulating proinflammatory responses in macrophages by phosphorylating p65 and increasing its rate of nuclear turnover and clearance [34]. The absence of IKKα kinase activity results in prolonged inflammatory responses.

In malignant cells themselves, the aberrant activation of the NF-κB pathway is associated with numerous effects that promote tumorigenesis. They can become independent of exogenous growth factors, resistant to apoptosis, non-responsive to growth inhibition, immortalized and capable of inducing angiogenesis [35]. Furthermore it can also induce more aggressive tumor types, capable of metastasis and invasion. This can occur in response to genetic mutation or microenvironmental factors such as hypoxia, reactive oxygen intermediates (ROI) and proinflammatory cytokines. Indeed it is believed that cytokines like TNF-α and IL-6 derived from

macrophages at sites of chronic inflammation contribute to these processes through activation of NF-κB and STAT3 pathways.

While NF-κB is a key player in regulating immune and inflammatory responses, it cannot be viewed in isolation from other inflammatory pathways. Indeed maximal expression of many inflammatory factors can only be obtained by a combination of transcription factors [36]. The Janus kinases/Signal Transducers and Activators of Transcription (JAK/STAT) pathway is another family that can coordinate with NF-κB in order to optimize the transcription of target genes [9]. Upon activation, receptor associated JAKs are tyrosine phosphorylated, this in turn leads to tyrosine phosphorylation of receptor associated STAT causing dimerisation and subsequent nuclear translocation. Having entered the nucleus, the STATs bind to specific regions of the target genes resulting in the activation of gene expression. The level of transcriptional activation is regulated by serine phosphorylation of the STAT protein under the control of mitogen activated protein kinases (MAPKs) or mTOR [37]. Both STAT1 and NF-κB activation, along with some MAPK-dependent AP-1 binding as well as other transcription factor binding is required for the maximal expression of NOS2 and IL-12p40 [38,39], clearly demonstrating the requirement for cooperation between the signaling pathways.

6.5 NF-κB and Macrophage Polarization

That NF-κB plays a major role in the determination of macrophage phenotype is without doubt. Unsurprisingly however, the precise role it plays in the promotion and maintenance of the TAM phenotype is difficult to elucidate, being likely to have multiple effects, which will no doubt vary in response to the context. A number of studies have been carried out to investigating the role that various components of the NF-κB pathway play in regulating the TAM phenotype. These studies have used cells with alterations to the NF-κB pathway on a genetic level.

It has been shown that the inhibition of IKKβ has a profound effect on tumor number and size in a mouse model of colitis associated cancer (CAC) [40]. In this model, mice are injected with the carcinogen azoxymethane (AOM) and this is followed by oral dosing of dextran sodium sulphate (DSS), which disrupts the intestinal barrier bringing enteric bacteria into contact with lamina propria macrophages causing chronic colitis. The combination of these insults leads to the initiation of tumors. In this study IKKβ is deleted solely in cells that express LysM (i.e. myeloid cells) using a cre/lox system. Deletion of IKKβ in the macrophages resulted in a decrease in tumor number and tumor size. It was hypothesized that this effect was largely due to a decrease in IL-6 secretion by the macrophage compartment as IL-6 increases proliferation of transformed epithelial cells. This idea was further strengthened by the observation that neutralizing IL-6 receptor antibodies had a similar effect on tumor size and number [41]. A similar story was found in a chemically induced hepatocellular cancer (HCC) model where HCC is achieved following administration of diethylnitrosamine (DEN) [42]. In this model DEN induced necrosis of hepatocytes and these necrotic bodies are

then recognised by Kuppfer cells (resident liver macrophages). Necrotic cell death stimulates the Kuppfer cells to release proinflammatory cytokines, such as IL-6 and promotes a proinflammatory environment. This causes "compensatory proliferation", stimulating hepatocytes to enter the cell cycle and, if those hepatocytes have DEN-induced oncogenic mutation(s), promotes malignant growth. Loss of IKKβ function in hepatocytes promotes this process as NF-κB signalling inhibits both necrotic and apoptotic cell death. However, the concurrent deletion of IKKβ in Kuppfer cells decreased the tumor burden 16-fold [42]. The decreased release of proinflammatory cytokines was shown to work on two levels: firstly as a direct effect on the "compensatory proliferation" of hepatocytes but it was also apparent that stellate cells responded to an unidentified factor produced by the Kuppfer cells causing them to release a powerful hepatocyte growth factor.

The effect of inhibition of IKKβ in macrophages has also been investigated using the ID8 ovarian cancer model [43]. In this model ID8 ovarian cancer cells are injected into the peritoneum representing late stage ovarian cancer where the malignant cells have spread to and are engaged in colonizing the peritoneum. During this study, the model was established for 7 weeks at which time bone marrow derived macrophages were adoptively transferred into the peritoneum. These macrophages were either wildtype, had been transfected with an IKKβ dominant negative virus to induce knockdown of IKKβ or mock transfected. In a further 2 weeks the mice injected with the IKKβdn macrophages showed a hugely reduced tumor burden compared with all other groups. This was repeated using TAMs taken from established tumors and yielded the same results. Studies have shown that IKKβ inhibits the activation of STAT1 leading to a decrease in NOS2 expression [44]. Many studies have shown that NO donors induce apoptosis in and are cytotoxic to tumor cells in vitro [45–47]. Indeed in vitro studies undertaken demonstrated that IKKβdn macrophages had increased tumoricidal capabilities, an effect that could be rescued using the NOS2 inhibitor 1,400 W [43]. However, this was discounted as an explanation for the decreased tumor burden observed in vivo as there was only a transient increase in peritoneal NO levels following adoptive transfer of IKKβdn macrophages, with levels returning to normal within 24 h, whereas the decrease in tumor burden persisted for much longer. Analysis of the TAM phenotype in the ascites showed that the IKKβdn macrophages had a M1-like phenotype with an IL-12high, IL-10low, TNF-αlow profile when compared with their wildtype and mock transfected counterparts. Interleukin-12 is a factor with a known ability to recruit natural killer (NK) cells and in accordance with this, an increase in the number of NK cells found in the peritoneum was observed. The use of IL-12p40 neutralizing antibodies in vivo "rescued" this effect, demonstrating it to be as a result the elevated ascitic levels of IL-12. These experiments were also repeated using the LysM-cre/lox system described above. These data clearly show that signaling through NF-κB is vital to maintaining the TAM phenotype and for promoting their tumor supporting role. In order to elucidate the factor responsible for activating NF-κB signaling, a similar ID8 adoptive transfer model was employed, this time using macrophages deficient in receptors and adaptor proteins upstream of NF-κB. Adoptive transfer of mice with IL-1R−/− or

MyD88–/– macrophages, but not those with TLR2 or TLR4 deletion, resulted in markedly less tumor burden. This strongly indicates interleukin-1 to be one of, if not the, NF-κB activating factor(s) in the system.

Another approach used to investigate the role of NF-κB signaling in controlling macrophage phenotype has been the use of p50–/– mice. It has been found that high amounts of p50 NF-κB homodimers are localized in the nucleus of TAMs [48]. These p50 NF-κB homodimers are capable of binding to transcription sites but they do not contain subunits with transcription activation domains (like the Rel proteins). As such, their binding does not induce gene transcription and their presence there merely prevents the binding of Rel containing NF-κB dimers resulting in an inhibition of NF-κB signaling. The result of this, regarding TAMs, is the promotion of the IL-12low IL-10high phenotype [49]. In a model of murine fibrosarcoma, p50–/– mice showed a significantly reduced tumor growth [49]. The same result was achieved using p50–/– bone marrow chimeras indicating that the cells responsible for this event come from the haematopoietic compartment. When these TAMs were isolated from the tumor and treated with LPS/IFNγ in vitro, they were shown to be of the M1 phenotype, with upregulated expression of genes encoding IL-12p40 and downregulation of IL-10. This indicates that the inactivation of NF-κB signaling leads to the promotion of TAM phenotype.

While these studies using IKKβ knockdown or cell-specific deletion and those employing p50–/– both altered the TAM phenotype towards IL-12high IL-10low it appears that in mechanistic terms they contradict each other. However, that may be a premature conclusion to make and there are a number of factors that must be considered. Given the level of redundancy that exists between the members of the NF-κB family, the possibility that there may be some level of compensation for the permanent loss of p50 by another member of the family cannot be discounted and it would be difficult to predict the outcome of such an event. Furthermore, as the effect of p50 and p52 homodimers is also under the control of BCL-3 regulation, which can result in NF-κB transactivation inhibition or activation, the absence of p50 homodimers is likely to have a profound effect on this system. It has also been demonstrated, in a TLR mediated inflammatory system, that p50–/– macrophages have elevated expression of IL-12p40 [50]. In this system it was shown that this event was not mediated through direct NF-κB signaling rather MAPK instead. The induction of *c-fos*, under the control of ERK1/2, inhibits the expression of IL-12p40. The activation of ERK1/2 is the culmination of a MAPK signaling cascade initiated by tumor promoting locus 2 (Tpl-2) kinase. These macrophages do not express detectable levels of Tpl-2 kinase because p50/p105 is required for its stabilization. As such the absence of p50/p105 prevents *c-fos* mediated IL-12p40 repression. It is interesting to note that these macrophages also demonstrated a requirement for p50/p105 in mounting an immunosuppressive effect in response to IL-10 [51]. It is clear to see that the effects of p50 knockout are potentially wide ranging and not exclusively limited to traditional NF-κB pathways. Also, as previously mentioned, a number of NF-κB dependent genes require the binding of other transcription factors as well for maximal expression.

Concurrent binding of STAT1 is required for the full transcription of NOS2 and IL-12p40. It was proposed by Hagemann et al. [43] that part of the mechanism of action of IKKβ in maintaining the TAM phenotype was through the repression of STAT1 phosphorylation and upon deletion of IKKβ this inhibition was blocked resulting in greater STAT1 activation. This proposed mechanism for the upregulation of some of the M1 type target genes diminishes the requirement for downstream NF-κB signaling meaning that a vestigial amount of IKKβ activity following viral knockdown may be sufficient to allow target gene transcription in conjunction with STAT1.

While TLR2 did not affect the level of tumor burden in the ID8 ovarian cancer model used by Hagemann et al. [43], a study by Kim et al. [52] employing a mouse model of lung metastasis did demonstrate a role for TLR2 signaling. The extracellular matrix proteoglycan, versican activated macrophages through TLR2, inducing TNF-α release which lead to greatly increased metastatic growth. Similarly, TLR4 deletion was shown to have no effect on tumor burden in this system. However, following chemotherapy and radiotherapy, patients with a TLR4 loss of function allele relapse more quickly than patients with the normal allele [53,54]. This was shown to involve recognition of high-mobility-group box 1 (HMGB1) by TLR4 on dendritic cells and activation of MyD88. These examples demonstrate the degree of contradiction between different systems, further complicating the elucidation of the role of individual components of a signaling pathway.

6.6 Crosstalk Between Hypoxia Inducible Factor and NF-κB

TAMs have been shown to accumulate in areas of tumors that are poorly vascularised. As such these areas are hypoxic (of low oxygen tension) and lead to the adaption of TAMs to hypoxia. This occurs through the upregulation of pro-angiogenic and hypoxia inducible genes, such as VEGF, CXCL8, βFGF and glycolytic enzymes [10]. The transcription of many of these genes is under the control of HIF-1 and HIF-2 [55]. Experiments performed using HIF-1α conditional knockouts showed that the absence of HIF-1α leads to a marked decrease in macrophage motility and invasiveness. Furthermore, HIF-1α has also been demonstrated to upregulate CXCR4 expression and CXCL12 potentially explaining its effect in the function and location of TAMs, tumor cells and stromal cells. While hypoxic conditions stabilize HIF-1α and protect it from proteosomal degradation, HIF-1α is also found in response to certain proinflammatory cytokines, LPS and other stimuli under conditions of normal oxygen tension. It has been suggested that this is due to NF-κB regulating HIF-1α on a transcriptional level, which has been demonstrated under hypoxic conditions [56]. Further evidence to support this proposal comes from studies using macrophages from IKKβ−/− mice where hypoxic conditions were shown to upregulate NF-κB activity with a consequential upregulation of HIF-1α [57]. The absence of NF-κB signaling in IKKβ−/− macrophages led to decreased levels of HIF-1α protein.

6.7 Concluding Remarks

As demonstrated here the role that NF-κB plays in the shaping of the macrophage response works on many levels and also varies depending on the stage of the tumor. This functional plasticity is very apparent when considering the requirement of NF-κB target gene transcription in the production of inflammatory cytokines such as TNF-a and IL-6 particularly in early stages of tumor initiation, whereas high levels of p50 homodimers appear to be important in maintaining the immunosuppressive TAM phenotype found in more established tumors. Therapeutic interventions with inhibitors of the NF-κB pathway will also need to take into account this plasticity as well as being tailored to the tumor type. The differing effects of IKKβ inhibition is clearly demonstrated in comparisons between the AOM/DSS colitis associated cancer model and the DEN hepatocellular carcinoma model. In both of these models deletion of IKKβ in the macrophage compartment had an inhibitory effect on tumor progression, however in the DEN model inhibtion of IKKβ signaling in the hepatocytes led to them becoming more susceptible to necrosis, causing the release of more proinflammatory cytokines and aiding tumor progression. In the AOM/DSS model IKKβ deletion in the enterocytes was shown to decrease the incidence of tumors through the induction of apoptosis of pre-neoplastic progenitors. Clearly, effects of this kind would need to be taken into account when attempting to modulate NF-κB function and potentially methods to target the macrophages specifically would have to be employed. It is clear that further investigation into the activity of NF-κB in TAMs at different stages in tumor development is necessary and warranted. While investigations carried out to date have shown a crucial role of NF-κB in TAM function and consequent tumor progression, there has been limited research on the interaction between members of the NF-κB family and other signaling pathways in TAMs. This information could provide greater understanding of how the plasticity of TAMs is modulated and lead to new therapeutic interventions.

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Chapter 7
The Metabolic Achilles Heel: Tumor Cell Metabolism as Therapeutic Target

Eva Gottfried, Katrin Peter, and Marina P. Kreutz

Abstract Many years ago Otto Warburg observed that tumor cells exhibit an increased glycolysis even in the presence of oxygen and he stated that this metabolic shift to glycolysis represents "the origin of cancer cells" [1,2]. His observation has gained new attention during the last years and many reports show that there is a molecular basis for the so-called "Warburg effect". Furthermore it is clear right now that not only the glucose metabolism but also many other metabolic pathways e.g. the amino acid metabolism, the lipid metabolism and the adenosine metabolism, are altered in the tumor cell and that these changes represent possible target structures for cancer therapy (Table 7.1). In this article we review recent findings and aspects of the metabolic alterations of tumor cells with a special focus on the implications for the immune response in the tumor environment.

Keywords Tumor metabolism • Warburg • Immune escape

7.1 Tumor Glucose Metabolism: The Warburg Phenotype

In contrast to normal differentiated cells that mainly rely on oxidative phosphorylation, most cancer cells primarily use aerobic glycolysis for energy production. The link between cell metabolism and cancer was first described many years ago by Warburg and is now known as "Warburg effect" [1,2]. This "glycolytic phenotype" of solid malignant tumors is characterized by an upregulation of glycolytic enzymes such as pyruvate kinase, hexokinase and lactate dehydrogenase (LDH). Tumor cells

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Table 7.1 Overview of metabolic changes and their impact in the tumor environment

Dysregulation: Gene/Oncogene/ tumor suppressor gene	Target (genes)	Metabolites	Effects
Mitochondrial mutations, loss p53	Mitochondrial genome SCO2, NFκB	ROS	– Decreased respiration – Apoptosis resistance
Myc	Glucose metabolism Glutamine metabolism	Lactate Glutamate	– Tumor proliferation – Immunosuppression
Hypoxia/HIF (VHL)	Glucose metabolism VEGF, VEGFR, COX	Lactate, VEGF PGE2	– Tumor proliferation – Immunosuppression – Angiogenesis
Raf/Ras	NFAT, NFκB, STAT3 (mitochondria) Glucose metabolism		– Tumor proliferation
STAT1/3	IDO	Kynurenine 3-HAA	– Immunosuppression
NFκB **NFAT**	COX	PGE2	– Tumor proliferation – Myeloid suppressor cells/arginase

Cyclooxygenase (COX), hypoxia-inducible factor (HIF), prostaglandin (PGE2), nuclear factor of activated T-cells (NFAT), 3-hydroxyanthranilic acid (3-HAA), vascular endothelial growth factor receptor (VEGF-R), synthesis of cytochrome c oxidase (SCO2), indoleamine 2,3-dioxygenase (IDO), von Hippel-Lindau (VHL), nuclear factor-kappa B (NFκB), Reactive oxygen species (ROS)

have been shown to express predominantly the M2 isoform of pyruvat kinase (PKM2) [3]. PKM1 and PKM2 are different splicing products of the same mRNA transcript [4]. PKM2 exists in dimeric and tetrameric forms and the dimeric form predominates in tumors [5]. PKM2 expression seems to be necessary for aerobic glycolysis and provides a growth advantage for tumor cells in vivo [3]. In addition, LDH and hexokinase are of crucial importance for tumor cell proliferation as inhibition of LDH results in the stimulation of mitochondrial respiration and a reduced proliferation in vitro and in vivo [6]. Furthermore tumor tissues show an increased expression of glucose transporters (GLUT). Accordingly, tumor cells are characterized by an increased uptake of glucose and the positron emission tomography (PET) exploits this feature of tumor cells for tumor diagnosis and staging. Glucose is metabolized via glycolysis and its endproduct lactate is secreted in cotransport with protons, which in turn lowers the pH of the tumor environment. A low pH is characteristic for the tumor milieu and local acidification has positive effects on extracellular matrix degradation and migration of tumor cells. Therefore Gatenby et al. proposed an "acid-mediated tumor invasion model" where an altered glucose metabolism leads to acidification of the tumor milieu which in turn allows tumor cells to form invasive cancers [7]. In addition, work by Mueller-Klieser and colleagues has nicely shown that high lactate levels in the primary lesion of human head and neck tumors, cervix carcinoma and rectal carcinomas correlate with incidence of distant metastases [8–10]. Therefore it seems that the

glycolytic phenotype of tumor cells represents a growth advantage and may represent an important basis for tumor progression and metastatic spread.

In addition to glycolysis, recent research demonstrated that the pentose phosphate pathway is augmented in some tumors. It converts glucose to ribose for nucleic acid synthesis and also leads to lactate generation. The non-oxidative part of the pentose phosphate pathway is controlled by transketolase enzyme reactions and the expression of the transketolase TKTL1 predicts cancer patient progression and survival [11,12].

Dysfunction of mitochondria is considered to represent a major factor contributing to the so-called "Warburg effect" in tumor cells. Mitochondria possess their own genome which codes for proteins required for oxidative phosphorylation. Alterations in mitochondrial DNA have been reported in various types of cancer such as breast, ovarian and colorectal cancer but their functional significance for tumor development needs to be addressed in further studies [13]. In line with the Warburg hypothesis, Cuezva and colleagues have shown that kidney, colon and breast carcinomas exhibit a repression of the ß-catalytic subunit of the mitochondrial ß-F1-ATPase concurrent with an increase in glyceraldehyde-3-phosphate dehydrogenase [14,15]. This is of special importance for colon carcinoma chemotherapy as down-regulation of mitochondrial F1F0-ATP synthase is linked to drug resistance against 5-Fluorouracil [16].

Mitochondria play an important role not only in energy metabolism but also for radical oxygen species generation and apoptosis. As several agents used in clinical studies, like paclitaxel or vinblastine, target mitochondria via caspases or other regulatory elements in the apoptotic machinery [17] outcome of anticancer therapy and drug resistance is linked to the (dys)function of mitochondria [16].

7.2 Amino Acid Metabolism in Cancer: Increased Glutaminolysis and Expression of IDO and Arginase in the Tumor Environment

Besides the glucose metabolism, the amino acid metabolism is altered in tumors because growing tumors require a continuous supply of both essential and non-essential amino acids for anabolic macromolecule synthesis. Glutamine is the most abundant amino acid in the body and serves as "nitrogen shuttle" as it contains two nitrogen side chains. It has been proposed that tumors act as "glutamine traps" as high rates of glutamine uptake are characteristic for many tumor cells. The increased uptake of glutamine and its flow to glutamate or lactate has been termed "glutaminolysis" and seems to be an important feature of transformed cells [5,18]. The increased turnover of glutamine is in part based on the higher activity and expression of glutaminase, the first enzyme in glutamine metabolism [19]. Accordingly, cancer patients exhibit lowered plasma glutamine levels but elevated glutamate concentrations [20]. Glutamate and lactate are secreted by tumor cells and both metabolites have been shown to suppress T cell activity in vitro [21,22].

Dysregulation of glutamine metabolism is not the only characteristic change in amino acid metabolism that impairs the immune system. Alterations in tryptophan and arginine metabolism in tumor cells and tumor-infiltrating myeloid cells also play a fundamental role in modulating the immune response.

Indolamine 2,3-dioxygenase (IDO) is a tryptophan catabolizing enzyme which is overexpressed in many cancers, e.g. melanoma, colon and renal cell carcinoma [23], and exists in two isoforms, IDO1 and IDO2 [24]. IDO catalyzes the conversion of tryptophan to kynurenine and is the first enzyme in the pathway that leads to the de novo generation of nicotinamide adenine nucleotide (NAD). NAD is an important cofactor required for several energy-producing catabolic reactions and a cofactor for sirtuins, a specific class of deacetylases relevant for transcriptional regulation [25]. Deprivation of the essential amino acid tryptophan represents an antimicrobial defense mechanism but also suppresses the proliferation of different T cell subsets [26].

Arginine levels are regulated by two enzymes. Arginase (ARG) hydrolyzes arginine to ornithine and urea, whereas nitric oxide synthase (NOS) oxidizes arginine to citrulline and nitric oxide (NO). Ornithine is the precursor for polyamines (putrecine, spermidine and spermine) synthesis, naturally occurring alylamines that are essential for cell growth. Polyamine concentrations and biosynthetic enzyme activities (e.g. ornithine carboxylase/ODC) are high in tumor cells compared to their normal counterparts and represent attractive structures for anti-cancer therapy [27]. The expression of ARG and NOS and its isoforms seem to differ between man and mice. Contrary to mice that express iNOS and ARG-1 in tumor-associated macrophages, this holds not true for human macrophages. In humans, ARG-1 is expressed in granulocytes, whereas human tumor cells have been reported to express ARG-2, and iNOS [28]. Recently it has been shown that human myeloid suppressor cells in renal cell carcinoma are a subpopulation of polymorphonuclear cells that deplete arginine by releasing ARG-1 from intracellular granules [29]. In mouse lung carcinoma the same authors demonstrated that ARG-1 is regulated via Cyclooxygenase 2 (COX-2) expression as pharmacological inhibition of COX-2, but not COX-1, blocked ARG-1 induction [30].

7.3 Alterations in Tumor Lipid Metabolism: COX Expression and Ganglioside Production

Arachidonic acid metabolites, so called prostanoids, including prostaglandins and thromboxanes, are synthesized by **COX-1/2** [31]. PGE2 can stimulate cell proliferation and motility and suppresses apoptosis of colorectal cancer cells [32]. Cyclooxygenases show an altered expression in many cancer entities. While COX-1 is constitutively expressed in almost all tissues, its isoenzyme COX-2 is induced by certain inflammatory cytokines and oncogenes and is primarily found in tumors [31]. Overexpression of COX-2 is associated with a poor prognosis in breast cancer and rapid disease progression in ovarian cancer [33,34].

Other lipids synthesized and shedded by tumor cells are **gangliosides** [35,36]. Gangliosides represent a family of complex glycosphingolipids with sialic acid residues being responsible for the formation of cell lipid membrane domains [37]. Several

tumor entities, such as neuroblastoma, retinoblastoma, melanoma, hepatocellular carcinoma, squamous cell carcinoma, colon carcinoma and lymphoma are known to display an aberrant ganglioside composition [38]. Hypoxia has been shown to induce an aberrant expression of gangliosides in cancer cells [39]. Gangliosides can be shedded from the membrane and thereby gain access to the circulation [38]. Elevated levels of gangliosides are found e.g. in the plasma of patients with neuroblastoma [36] and can enhance tumor growth indirectly by protecting tumor cells from host immune destruction. They suppress T cell function, induce T cell apoptosis [40,41] and impair the antigen-presenting function of human dendritic cells [42].

7.4 Adenosine Accumulation in the Tumor Environment

Multiple cell types release adenine nucleotides in the form of ATP, ADP, and AMP. These are rapidly metabolized by surface ectoenzymes like ecto-5'-nucleotidase (CD73) to adenosine [43,44]. Adenosine is an endogenous purine nucleoside that is constitutively present in the extracellular milieu at low concentrations. A considerable increase of the extracellular adenosine concentration has been reported for hypoxic tissues, which are found in solid tumors [45]. Accordingly, HIF-1 has been shown to regulate the CD73 in intestinal epithelial cells [46]. CD73 is also expressed on the surface of tumor and immune cells [47,48] and elevated activity is found in breast carcinoma [49], gastric cancer [50], pancreatic cancer [51], and glioblastoma [47]. Accelerated metabolism of AMP into adenosine in the tumor environment lowers the level of AMP and could contribute to the diminished activity of AMP-activated protein kinase (AMPK), an important endogenous inhibitor of the mammalian target of rapamycin (mTOR) pathway. Aberrant activation of mTOR is characteristic for many tumors and discussed as a possible therapeutic target in cancer [52].

Elevated levels of adenosine could also result from an increased intracellular adenosine production by dephosphorylation of AMP by cytosolic 5'-nucleotidase [53] or a disturbed degradation of adenosine to inosine, catalyzed by intracellular adenosine deaminase (ADA). ADA has been considered as a marker of malignancy and decreased ADA activity has been found in several carcinomas, including colon carcinoma [54].

Methylthioadenosine phorsphorylase (MTAP) is an enzyme of the polyamine metabolism which is expressed constitutively in most normal cells and tissues [55]. MTAP catalyzes the degradation of 5'-deoxy-5'methylthioadenosine (MTA), a byproduct of the polyamine metabolism, to adenine and methylthioribose-1-phosphate, which are thereupon converted to adenosine and methionine. In many different tumors like malignant melanoma [56], osteosarcoma [57], leukemia [58], endometrial adenocarcinoma [59], non-small cell lung carcinoma [60] and breast cancer [61] a decreased expression of MTAP is found compared to the normal tissue. This leads to an accumulation of MTA in the tumor environment. In case of malignant melanoma the loss of MTAP expression results in a higher invasive potential [62], leading to the hypothesis that loss of MTAP expression might contribute to metastasis of malignant melanoma [63].

7.5 Molecular Background of Metabolic Alterations in the Tumor Environment

7.5.1 Oncogenic Transformation and Hypoxia Lead to Metabolic Alterations

Overexpression of tumor oncogenes and loss of tumor suppressor genes represent the molecular basis for the development of cancer. Many of these genetic alterations are directly linked to metabolic changes in the tumor cell.

Genetic alteration or loss of **p53**, one of the most frequently mutated genes in cancer, modulates the balance between respiration and glycolytic pathways. P53 activation leads to increased mitochondrial respiration by inducing the expression of synthesis of cytochrom c oxidase 2 (SCO2). Accordingly, p53-deficient cells show a decreased oxygen consumption and increased lactate production and SCO2 seems to be the one important mediator of this effect [64]. Furthermore loss of p53 leads to activation of the **NFκB** pathway and thereby upregulates GLUT3 expression [65]. Loss of p53 also causes mitochondrial DNA depletion and altered mitochondrial reactive oxygen homeostasis [66]. Recently, Vander Heiden and coauthors proposed that highly proliferating cells switch to glycolysis because mitochondria are needed as synthetic organelles to supply components for the generation of nucleotides and phospholipids for new cell structures [67]. In the light of this paper, tumor cells concomitantly experience a "glycolytic switch" as well as a "mitochondrial switch". Mitochondria take a turn from a catabolic to an anabolic organelle and tumor cells rescue their energy metabolism through an accelerated glycolysis for NADH and ATP generation.

Oncogenic transformation does not only decrease the mitochondrial activity of tumor cells but can directly accelerate glycolysis. Activating mutations in the phosphoinositol 3-kinase (PI3-K), or deletion of phosphatase and tensin homolog (PTEN), a PI3-K antagonist, lead to the activation of its downstream effector Akt and are commonly observed in cancer cells. Constitutive Akt activity induces the transformed cell to accelerate their glucose uptake and stimulates aerobic glycolysis [68]. Maintenance of the oncogenic Akt kinase activity seems to be required for the aggressive tumor cell phenotype as disruption of Akt1 results in delayed tumor growth and reduced lung metastasis in a mouse model ErbB2-induced mammary tumorigenesis [69]. In addition, Akt-transformed cells are impaired in their ability to use ß-oxidation in response to glucose deprivation which results in glucose addiction [70]. Akt is also an important downstream effector of other oncogenes like Ras [71].

In human glioblastoma cells, **Ras** inhibition resulted in downregulation of HIF-1 and several genes associated with glycolysis like Glut-1 and LDH A. Accordingly, glycolysis was inhibited and cell death induced [72]. Yun et al. showed recently that tumor cell lines with KRAS or BRAF mutations upregulate the glucose transporter GLUT1 and mutant cells exhibited enhanced glucose uptake and glycolysis [73] Activating mutations in **BRAF** are found in many colorectal and pancreatic tumors

and also in melanomas. In metastatic melanoma it has been shown recently that oncogenic BRAF activates NFAT signaling. As NFAT is an important regulator of COX-2 this leads to higher COX-2 expression in metastatic melanoma cells [74]. In addition, Ras/Raf-1 activation induces NF-κB activation which in turn induces COX-2 [75]. In human lung carcinoma cells triggering of the NF-κB pathway via inflammatory mediators like TNF also induced COX-2 [76]. COX-2 in turn can induce the expression of arginase 1 in myeloid suppressor cells [30].

Dysregulated expression of the **myc** oncogene occurs in about 30% of human cancers and c-myc overexpression regulates mitochondrial glutaminolysis and triggers cellular addiction to glutamine as bioenergetic substrate [77,78]. Furthermore, myc leads to an upregulation of glycolytic enzymes, like LDH A [79]. Oncogenic myc also collaborates with hypoxia inducible factors, **HIF1** and **HIF2**, to create the metabolic phenotype that is described as Warburg effect [80]. HIF transcription factors are dimers composed of two subunits, HIF1alpha (or HIF2alpha, respectively) and HIF1beta. HIF is stabilized in response to low oxygen tension (**hypoxia**) which is characteristic for the tumor milieu as a result of decreased microcirculation in the tumor tissue [81]. HIF induces the transcription of more than 70 genes via hypoxia response elements (HRE) in the respective promoters or enhancers, e.g. VEGF, Flt-1 (VEGF-R), Glucose transporter-1 (Glut-1), LDH, monocarboxylate transporter 4 (MCT-4) involved in lactate transport, carboanhydrase IX and COX-2 [82,83]. In addition to hypoxia, oncogenic transformation can also induce HIF independent of the presence or absence of oxygen [84]. In renal cell carcinoma, mutations in the von Hippel Lindau (VHL) gene lead to a stabilization of HIF as the VHL protein is important for the degradation of HIF which in turn induces the expression of HIF responsive genes.

Myc interacts with HIF but also with a variety of other factors, e.g. BIN1, a possible tumor suppressor. Bin1 expression is reduced in many human tumors e.g. melanoma, breast cancer and prostate carcinoma [85,86] and loss of **Bin1** induces the STAT1 and NF-κB-dependent expression of IDO [87]. Recently it has been shown that acetylation of **STAT3**, a transcription factor which is upregulated in many human malignancies, promotes the transcription of IDO in dendritic cells [88]. In addition, IDO is regulated by IFNγ and other inflammatory mediators [89]. Accordingly, human activated T cells modulate IDO expression in breast and kidney cell lines via IFNγ [90].

These data show that tumor metabolism and metabolism of tumor-infiltrating immune cells is under the control of hypoxia as well as oncogenes and tumor suppressor genes.

7.6 Impact of Tumor Metabolism on Immune Cell Function

There is increasing evidence that the altered metabolism of tumor cells, e.g. increased glycolysis or differences in the amino acid metabolism, modulates immune cell function. Tumors are infiltrated by a variety of immune cells including macrophages, dendritic cells, myeloid suppressor cells, regulatory CD4+ T cells and other T cell populations. Many studies suggest that tumor progression, metastasis

and the clinical outcome of malignancies are regulated based on the composition and activation status of the immune cell infiltrate in the tumor.

Lactate accumulation and acidification modulate immune cell function: Accelerated glycolysis leads to accumulation of **lactate** and acidification of the tumor environment. It has been reported that extracellular lactate in wounds stimulates macrophages to secrete vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-beta), both known to be immunosuppressive factors [91]. In addition, data from Shime et al. demonstrated recently that lactic acid regulates transcription and secretion of IL-23, a tumor-promoting cytokine [92]. Douvdevani et al. described that a low pH and high lactate concentration of peritoneal dialysis fluids are inhibitory for macrophage/monocyte TNF and IL-1beta release [93]. In contrast, acidosis seems to improve antigen presentation by dendritic cells and induces neutrophil activation [94,95]. We and others have shown that tumor-derived lactic acid strongly inhibits both the differentiation of monocytes to dendritic cells [96,97] and the activation of T cells [22]. Recent data indicate that functional inhibition of immune cells may be related to the uptake of lactate from the tumor environment which results in an inhibition of immune cell glycolysis [98].

Influence of amino acid metabolism on myeloid and lymphoid cells: Lactate is also a possible end product of glutaminolysis, another important hallmark of tumor cells. In addition, glutaminolysis results in lowered plasma **glutamine** levels but elevated glutamate concentrations in the sera of tumor patients. High glutamate concentrations inversely correlate with the proliferative response of cancer patients' T cells in vitro [99]. This effect could be mediated via the Glutamate receptor mGlu5R that is constitutively expressed on T cells [100]. Beside an increased level of glutamate in the tumor environment, T cell suppression could also be the results of glutamine depletion as tumors compete with the host cells for circulating glutamine.

Similarly, it has been described that tumor cells and infiltrating myeloid cells express elevated levels of tryptophan (IDO) and L-arginine metabolizing (arginase) enzymes that deplete **tryptophan and arginine** in the tumor environment but also in the periphery. Accordingly, patients with infections or malignant disease have been reported to have low tryptophan concentrations in serum/plasma [101]. Interestingly, decreased serum tryptophan concentrations predict poor prognosis in melanoma patients [102]. As melanoma is known as an immunogenic tumor, these data suggest immunosuppression by tryptophan deprivation because T cell activation results in an increased demand for this amino acid. Several in vitro data show indeed that accelerated tryptophan metabolism and the accumulation of metabolites like **3-hydroxykynurenine and 3-hydroxyanthranilic acid** (3-HAA) lead to immunosuppression. Combined effects of tryptophan deprivation and tryptophan catabolites result in down-regulation of the TCR zeta-chain in murine CD8+ T cells [103] and 3-HAA inhibits the proliferations of human CD8+ T cells in vitro [104]. Furthermore, arginine depletion also inhibits T cell activation in the tumor environment. Myeloid suppressor cells accumulate in many tumors and express arginase which depletes arginine from the environment [105,106]. In summary, both, depletion of amino acids and accumulation of specific amino acid metabolites locally blocks T cell proliferation in the tumor environment.

7.7 Tumor-Derived Lipids Suppress Immune Cell Activity

Prostaglandins are involved in immunosuppression. High COX-2 expression by tumor cells leads to an increased production of prostaglandins which upregulate IDO expression in tumor-associated dendritic cells [107,108]. This leads to the generation of a specific subtype of immunosuppressive tolerogenic dendritic cell and in turn to the expansion of regulatory T cells [89,109].

Other tumor-derived lipids like **gangliosides** impair the maturation and migratory activity of Langerhans cells [42], whereas neuroblastoma-derived gangliosides were found to inhibit the differentiation, maturation, and function of DC [110]. In addition, gangliosides purified from squamous cell carcinoma downregulated the expression of components of the antigen-processing machinery of DC [111]. Accordingly, Caldwell reported that DC incubated with gangliosides are deficient in the expression of costimulatory molecules and were unable to induce a normal T cell response [112]. Disruption of NFκB activation may contribute to the inhibition. Gangliosides are also found in the supernatant of several tumor cell cultures and are able to inhibit the differentiation of hematopoietic cells, as measured by the formation of erythroid and myeloid colonies from CD34+ precursors [113]. In summary, tumor-derived lipid metabolites such as prostaglandins and gangliosides have a potent inhibitory capacity on DC, at least in vitro.

7.8 Immunosuppression by Adenosine

Adenosine is known to have a general immunosuppressive effect and anti-inflammatory properties on different types of immune cells. It acts by binding to four different types of G-protein coupled cell surface molecules, termed the A_1, A_{2a}, A_{2b} and A_3 adenosine receptors [114].

Adenosine influences a wide range of T lymphocyte responses. It inhibits T cell proliferation as well as expression of cytotoxic effector molecules [115,116] and T cells show a reduced secretion of proinflammatory cytokines [117]. Furthermore, adenosine modulates the function of dendritic cells dependent on their adenosine receptor expression profile [118]. It enhances the secretion of IL-10 but inhibits secretion of IL-12 by dendritic cells and also by monocytes and macrophages [119–121]. Since the balance between both cytokines regulates the development of T helper cells and determines the induction of an effective immune responses against tumor cells, adenosine-induced IL-10 and suppression of IL-12 could be important for the immune suppression in the tumor environment [122]. In addition, the cytotoxic activity as well as the production of inflammatory cytokines by natural killer cells (NK cells) is decreased [123].

In contrast to adenosine, which has been investigated in several studies, little is known about the effects of 5'-methylthioadenosine **MTA** on immune cells. MTA has been described as an inhibitor of inflammation, since MTA inhibits the secretion

of TNF and activation of NFκB [124–126]. Inhibition has been attributed to a block of LPS induced gene transcription via disturbed histone methylation by MTA [127]. The anti-inflammatory effect of MTA has also been observed for T cells, since MTA suppresses T cell activation, the expression of proinflammatory cytokines and increases the expression of IL-10 [128].

MTA also inhibits lymphocyte proliferation as well as the secretion of IgM and IgG by peripheral blood lymphocytes [129,130]. MTA has also been demonstrated to inhibit natural killer cell mediated cytotoxicity [131]. As many effects of adenosine and MTA overlap this suggests similar signaling mechanisms of both molecules e.g. via adenosine receptors.

7.9 Tumor Metabolism as Therapeutic Target

The alterations in tumor cell metabolism, such as accelerated glycolysis, glutaminolysis and fatty acid metabolism, represent attractive targets for the development of anti-cancer drugs.

7.10 Inhibition of Tumor Glycolysis

Early after Warburg's observation that tumor cells show major differences in glucose metabolism, some attempts focused on the inhibition of tumor glucose metabolism as cancer treatment [132]. These studies used **2-deoxyglucose (2-DG)**, a non-metabolizable glucose analogue and inhibitor of hexokinase, the enzyme that catalyzes the initial step during glycolysis. This approach has gained new attention during the last years [133] and in addition new drugs have been developed such as **3-bromopyruvate (3-BrPA)**, another hexokinase inhibitor [134]. 3-BrPA and 2-DG reduced liver tumor growth in a rabbit and a rat model, respectively [135–137]. Both drugs increased the efficacy of chemotherapeutics (adriamycin, paclitaxel, doxorubicin and vincristine) in vitro and in a non-small cell lung carcinoma and osteosarcoma mouse model [138,139]. Inhibition of glycolysis by 2-DG or 3-BrPA also sensitizes acute lymphoblastic leukemia cells to glucocorticoids [140,141]. Furthermore, 2-DG leads to radiosensitization only in tumor cells expressing wild-type p53 but p53 deficient cells were more sensitive to 2-DG treatment alone [142]. Similar results were obtained for LKB1, another tumor suppressor and an upstream mediator of mTOR. As shown recently, loss of LKB1, increases the sensitivity of non-small cell lung cancer to 2-DG [143].

Clotrimazole, an antifungal azole derivative, induces the dimerization of 6-phosphofructo-1-kinase (PFK). Dimers of PFK are less active than tetramers and thereby clotrimazole inhibits the enzyme activity which results in a decreases glycolytic flux [150]. Acetylsalicylic acid, a non-specific COX inhibitor and anti-inflammatory

drug, also inhibits PFK in vitro [151]. These findings link the mechanism of action of non steroidal anti-inflammatory drugs (NSAID) to the altered glucose metabolism found during inflammation and in tumors.

7.11 Targeting the Glucose Uptake

Tyrosine kinase inhibitor Imatinib (Gleevec), reverses the Warburg effect in BCR-ABL positive chronic myeloid leukemia cells by switching cell metabolism from glycolysis to glucose oxidation. It has been proposed that the antiproliferative and proaoptotic effect may in part be mediated by reduction of glucose uptake and lactate secretion [149]. Another approach to target glucose metabolism are Hsp90 inhibitors such as 17-allylaminogeldanamycin, which promote HIF-1a degradation and thereby have profound effects on tumor growth.

mTOR is a threonine kinase belonging to the phosphoinositide kinase related kinase family and common downsteam effector of PI3-K/PTEN/Akt as well as Ras/Raf pathways. mTOR inhibitors are already established anticancer drugs [144]. Wei and colleagues described a decreased glucose uptake in glioblastoma cells of mice treated with rapamycin implicating a link between the mTOR pathway and glucose metabolism [145]. HIF regulation through mTOR inhibition could be one possible explanation as HIF regulates several glucose-metabolism associated genes. Accordingly, the mTOR inhibitor temsirolimus, inhibits HIF-1a expression and transcriptional activation of the HIF-target gene VEGF in breast cancer cell lines [146].

7.12 Acceleration of the Mitochondrial Activity

Another drug that modulates glucose metabolism is **dichloroacetate** (DCA) which is used in the treatment of congenital lactic acidosis in children [147]. It targets mitochondrial pyruvate dehydrogenase kinase (PDK) [148] which phosphorylates and inhibits the pyruvate dehydrogenase complex (PDC). PDC catalyzes the conversion of pyruvate to acetyl-CoA and is an important control point in glucose and pyruvate metabolism. DCA downregulates PDK and thereby leads to activation of PDC, which induces a shift from glycolysis to glucose oxidation. The growth inhibition of tumor cells as well as induction of apoptosis was shown in vitro and in a nude rat model (148].

7.13 Modulation of Tumor Lipid Metabolism

COX-2 overexpression is found in many tumors and therefore lipid metabolism is another potential target for tumor therapy [31]. Already in the 1990s, it was reported that regular use of non-specific COX inhibitors like aspirin was associated with a

decreased tumor incidence of colon, breast and lung carcinoma indicating a protective effect of NSAIDs [152]. Since then, several studies showed a heterogeneous risk reduction for the incidence of several tumor entities [152,153]. In one of the first randomised studies, treatment with the non-specific COX inhibitor indomethacine prolonged survival of patients with metastatic tumors [154]. In addition, indomethacine and the selective COX-2 inhibitor celecoxib increase the radiosensitivity of tumors [155,156].

Furthermore, clinical studies have demonstrated an effect of combination therapies with COX-2 inhibitors and the PPARγ (Peroxisome proliferators-activated receptor γ)-agonists pioglitazone in combination with low dose chemotherapy in glioma [157] and melanoma [158], which is in line with in vitro data showing that PPARγ -agonists inhibit proliferation and induce apoptosis in several tumor cell lines [159,160]. Our own data show that pioglitazone also modulates the mitochondrial activity of prostate tumor cells and thereby inhibits tumor cell proliferation [161].

7.14 Rescuing Anti-tumor Immune Response

COX-2 inhibition: COX-2 overexpression leads to an increased production of prostaglandins in the tumor environment which has a strong impact on immune cell differentiation and activation. Prostaglandins are important for maturation of dendritic cells and upregulate IDO mRNA expression in vitro [107]. In line with these data, peritumoral dendritic cells in different carcinoma coexpress IDO associated with elevated prostaglandin levels [108] suggesting that prostaglandins also influence IDO expression in vivo. Recently, Chung and colleagues have nicely shown that IDO-expressing dendritic cells expand autologous regulatory T cells (Treg) [109]. Treg are known to suppress antitumor response in mouse models and accumulation of Treg is described in different cancer tissues, e.g. colorectal cancer or melanoma [162,163]. Accordingly, Celecoxib-treated tumor bearing mice show a decreased expression of IDO and the accumulation of Tregs was reduced. This was correlated to a reduction tumor size and metastasis [164]. Direct targeting of IDO via downregulation of IDO2 with siRNA, also generated antitumor immunity in vivo in a murine bladder tumor model [165]. Furthermore inhibition of IDO also potentiates cancer chemotherapy in breast cancer models [87,166].

Phosphodiesterase-5 inhibitors (sildenafil): Not only IDO but also arginase (ARG) has immunosuppressive functions through the depletion of the amino acid from the tumor environment. In tumor-bearing mice it was shown, that myeloid derived suppressor cells (MDSC) are directly involved in the suppression of immune responses in cancer [167]. MCSC express ARG-1 and efficiently deplete arginine form the surrounding medium. One strategy for tumor therapy is to target the suppressive activity of MDSC by phosphodiesterase-5 inhibitors (sildenafil), [168]. Sildenafil is known to downregulate ARG-1 and inducible NOS2 expression in MDSC and restored the T-cell proliferation, enhanced in vivo intratumoral T-cell infiltration and reduced tumor growth [168].

Modulation of adenosine metabolism: Another potential target is given by the adenosine metabolism. Extracellular adenosine monophosphate (5'AMP) is metabolized to adenosine by ecto-5'-nucleotidase CD73 expressed on tumor cells and tumor-infiltrating Treg. This results in the accumulation of adenosine which is known to suppress T cell proliferation and cytokine production [43,48]. Using adenosine receptor antagonists like caffeine, or targeting the A2 receptors by siRNA treatment, can reactivate T cell activity and **rescue anti-tumor immune responses** [169,170].

7.15 Summary and Concluding Remarks

About half a century after Warburg's observation that the glucose metabolism is altered in tumor cells, it is quite clear that these metabolic alterations are indeed important for tumor development and progression. But the glucose metabolism is only one piece of the tumor metabolome puzzle. Amino acid metabolism, lipid metabolism and adenosine metabolism are also adapted to fulfill the tumors needs for energy and building blocks for new cell structures. Furthermore there is increasing evidence that the altered tumor metabolism is directly linked to tumor cell transformation and the overexpression of oncogenes or the loss of tumor suppressor genes are key regulators of the accelerated glycolysis and glutaminolysis in tumors. The complex network of tumor-derived metabolites also leads to local immunosuppression and may thereby facilitate tumor progression and metastasis. Targeting tumor cell metabolism is therefore not only an approach to kill the tumor cell directly but could possibly also overcome some limits of immunotherapy.

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Chapter 8
Could Be Systems-Directed Therapy Approaches Promising in Glioblastoma Patients?

Oliver Grauer and Peter Hau

Abstract Glioblastoma bears one of the most severe prognoses of human cancers. At this time, the available therapy is directed against specific pathological mechanisms of these tumors, as proliferation, angiogenesis, or immunosuppression. This approach neglects systems biology: Several compartments as tumor, local precursor, immuno- and endothelial cells, the extracellular matrix and intratumoral vessels communicatively interact with the host's organs. The development of a tumor is a dynamic process, comparable to a developing organ, and is highly dependent on interactions between different structures and compartments within the tumor. Large-scale unbiased assays will be needed to investigate the specific molecular and cellular patterns of each individual glioblastoma. Most likely, new models will be individually compiled in the future work-up of glioblastomas, generating information for the setup of a multi-targeted personalized concept approaching the systems biology of glioblastoma. These new approaches include advanced in vivo models using engineered animals and in silico models based on bioinformatic methods. Interventions will influence all levels of tumor biology, including the genetic, epigenetic, proteomic, and metabolomic level. First publications aim to define targets for treatment using systems biology approaches. In our opinion, a clinically meaningful improvement will only be possible with interventions that are multi-targeted and consequently inhibit glioma-initiating cells, enhance local antitumor immune responses, and target the most relevant molecular mechanisms responsible for tumor cell proliferation and invasion. This review will focus on the most prevalent and malignant primary brain tumor of men, glioblastoma, which is notorious for its therapy resistance to classical treatments.

Keywords Glioblastoma • Stem cell niche • Therapy resistance • Systems biology • Personalized concepts

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8.1 The Target: Glioblastoma

Glioblastoma (GBM) is among the most deleterious diseases of man [1]. Reliable epidemiological data are only available for the United States of America (USA). Glioblastoma represents about 20% of the estimated 40,000 new cases of primary tumors of the central nervous system (CNS) and 65% of glial brain tumors diagnosed in the USA each year (Central Brain Tumor Registry of the United States, www.cbtrus.org). The most relevant prognostic factors are age, WHO Performance Score at diagnosis, the methylation status of the O6-methylguanin-DNA-methyltransferase (MGMT) promoter, and mutations of isocitrate-dehydrogenase-1 (IDH-1) [2,3].

Therapy comprising debulking surgery, concomitant radio-chemotherapy and adjuvant chemotherapy with temozolomide (TMZ) prolongs the median overall survival after initial diagnosis to only about 14 months at this time [4,5]. A 5-year analysis revealed an overall survival of 27.2% at 2 years, and 9.8% at 5 years for patients treated with combined radio-chemotherapy [6]. The molecular evaluation of these data has disclosed a subgroup of patients with methylation of the MGMT promoter, with a more favorable prognosis [6,7]. Importantly, patients with a methylated MGMT promoter and a WHO Performance Score of 0 have a 66% probability of survival at 2 years [3]; this is a first step towards a personalized therapy in a distinct cohort of glioblastoma patients to severely increase prognosis in this genetically characterized subgroup. However, cures are still never reached in these patients.

8.2 Therapy Resistance in Glioblastoma

The discussed standard regimens all bear the problem of a 100% relapse rate, usually within 1 year after start of therapy. This disappointing response pattern is caused by several factors including a lacking systems biology view of the disease and the failure of newly designed therapies targeting specific molecular events, tumor-intrinsic factors or treatment-induced resistances, which are based on the robust pathophysiology of GBM.

8.3 Insufficient Activity of Targeted Agents in Monotherapy

Molecular profiling of glioma has revealed crucial signaling pathways driving the malignant behavior of glioblastoma. Nodal mutations constituting master drivers of glioblastoma initiation and progression have not been described yet, though first promising candidates are discussed [8].

Therapeutic approaches targeting a singular disease-associated molecular event have been disappointing so far. A classic example of a non-successful targeted approach is the resistance of most GBM patients against EGFR-targeted drugs [9].

EGFR is highly expressed in glioblastoma, partly in its truncated form, but targeting EGFR has produced virtually no value in patients with glioblastoma.

8.4 Glioblastomas' Intrinsic Resistance

Major tumor-intrinsic reasons for low efficacy of chemo- and targeted therapy against glioblastoma are poor blood-brain barrier penetration of cytostatic agents especially in the therapeutically relevant periphery of the tumor node [10], expression of drug efflux pumps (multidrug resistance genes), and the expression of resistance-associated enzymes such as O6-methylguanin-DNA-methyltransferase. Resistance against classical chemotherapeutics, e.g. alkylating agents, is pronounced in glioma cancer stem cells (G-CSC) [11].

The complex system of tumor-intrinsic resistance aggravates drug delivery (immunotherapy, targeted therapies, and cytotoxic drugs) and may consecutively modulate tumor sensitivity.

8.5 Resistance Induced by Treatment

Induced (extrinsic) resistance may rapidly occur in glioblastomas. Multidrug resistance proteins such as multiple drug resistance (MDR-1) and multidrug resistance protein (MRP) can be induced by chemotherapeutics [12,13–14].

A novel mechanism of resistance against chemotherapeutic agents may develop during administration of bevacizumab, an antiangiogenic agent, by consecutively decreasing vascular density of the tumor [15,16]. Bevacizumab generates unusual patterns of response, as documented with MR imaging, resembling "pseudo-responses" by 'normalization' of blood vessels and therefore the blood-brain-barrier with a decreased penetration of gadolinium into the brain parenchyma [17–23].

8.6 Consequences of Therapy Resistance

Intrinsic and extrinsic therapy resistance leads to a largely unresponsive tumor phenotype in the majority of patients with glioblastoma. Therefore, it will be of utmost importance to develop markers for early response and resistance to overcome the robustness of GBM's tumor system by adaptive trial designs.

Commonly, targeted approaches are aimed at defined solitude tumor cell-associated structures. However, it becomes clear that mono-targeted approaches or approaches neglecting the 'conspiratory' activity of the adjacent stroma remain merely ineffective in glioblastoma. Pleiotropic acting drugs with the capacity to target simultaneously several cellular compartments of the GBM, sometimes unintendently, are the most efficient and promising ones, as detailed below.

Understanding systems biology in GBM appears pivotal for the development of new combined, in the first place biomodulatory therapeutic strategies with modest toxicity [24]. Only then it will be possible to develop personalized multi-targeted treatment approaches leading to long-term disease chronification or even cure in GBM.

8.7 Systems Biology in Glioblastoma

Systems' robustness: Considering the robustness of GBM's systems biology that translates into a high resistance against any single agent therapeutic approach, systems-directed combined therapies might be the most promising strategy to perpetuate significant improvement in glioblastoma.

First, systems biological diagnostic approaches have to analyze the factors constituting the 'intrinsic' systems robustness of GBM meticulously. Finally, this analysis has to be advanced on a personalized basis, as the individual tumor consists of an ever distinct array of molecular patterns with critical communicative nodes, though systems-derived subgroups may be uncovered [25,26]. From the list of individual molecular-physiological changes, the hierarchy of central (nodal) events has to be defined for systems-related similar subgroups of GBM as well as for individual cases, aimed to systematically address the communicative tumor systems architecture as a whole.

The communicative aspect of systems: The knowledge of the complex cross-talks between the compartments of the pre-tumor and tumor niche is of utmost importance to understand the complex system of glioblastoma development. A cascade of mutations affecting genes that control cell growth, apoptosis, angiogenesis, and invasion, has been described [27,28] (Figs. 8.1 and 8.2). Therefore, the simultaneous modeling of tumor cells, microenvironment and their interactions with the tumor host may be most promising for the treatment of GBM [29]. This includes the combination of therapies that inhibit proliferation and invasion of tumor cells, target angiogenesis, tumor-associated inflammation, or reconstitute the local immune response [30]. Targeting of tumors via the adjacent microenvironment seems to have potential as well [31,32]. A paradigmatic paper of Hoey et al. [33] using a pair of human antibodies against transplanted human cancer stem cells and mouse antibody against mouse intratumoral vessels did show synergistic effects, if they are delivered simultaneously. This provides a strong rationale for targeting both the tumor cells and the microenvironment in a system biology approach.

8.8 Pathophysiology of Glioblastoma as Therapeutic Target

Main pathophysiological features of glioblastomas, as of most tumor entities, are tissue invasion that is enhanced by remodeling of the extracellular matrix, insensitivity to growth inhibition, evasion of apoptosis, self-sufficiency of growth signals,

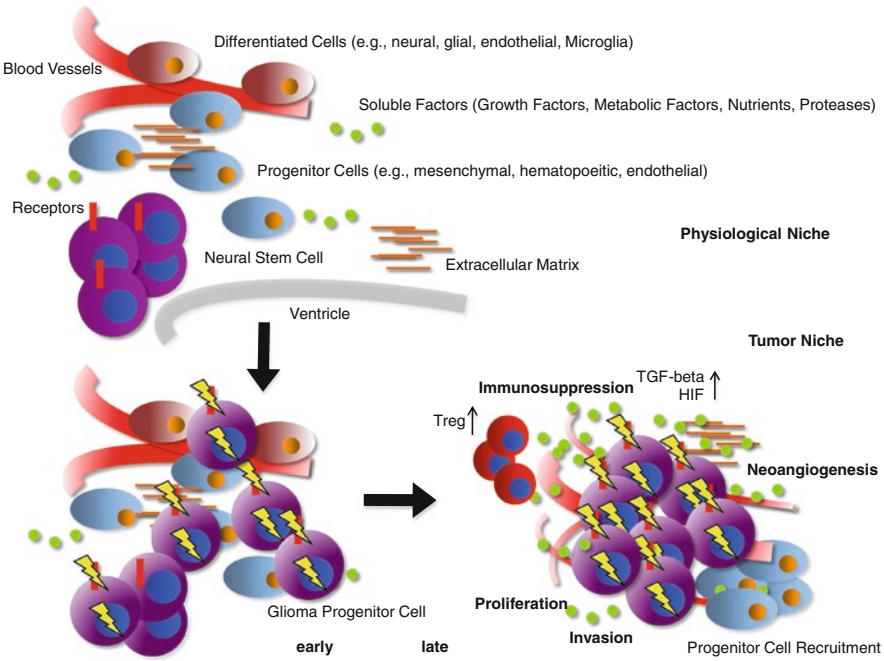

Fig. 8.1 The glioblastoma microenvironment and its development from a normal stem cell niche. The development of the tumor niche is not well understood. A possible scenario may be the following: The physiological niche consists of several compartments that are well balanced. In early development of the tumor niche, a mutation, possibly of a growth factor receptor, leads to unrestricted proliferation of a physiological stem cell. Later on, growth factor balance gets deranged, with a marked upregulation of several factors including TGF-beta and HIF. This leads to local immunosuppression and angiogenesis as the driving events for the development of a full-blown tumor

limitless replication, sustained angiogenesis, and an inflammatory environment. A theory, based on GBM pathophysiology, including systems biological knowledge about glioblastomas, which are allowing direct translation into clinical diagnostic and therapeutic approaches does not exist at this time. Further more, it is currently not possible to classify these mechanisms in an order of importance within a systems biological context. Future research from a systems biology point of view will have to elucidate the hierarchy of these mechanisms.

8.9 Glioblastoma Cells with Stem Cell Function

Glioblastoma cells with stem cell function (G-CSC), glioblastoma progenitor cells or glioblastoma initiating cells, are suggested to be the ancestor of the full-blown tumor in patients with glioblastoma due to their self-renewal capacity and limitless proliferative potential [34]. Recently, integrated genomic analysis has revealed

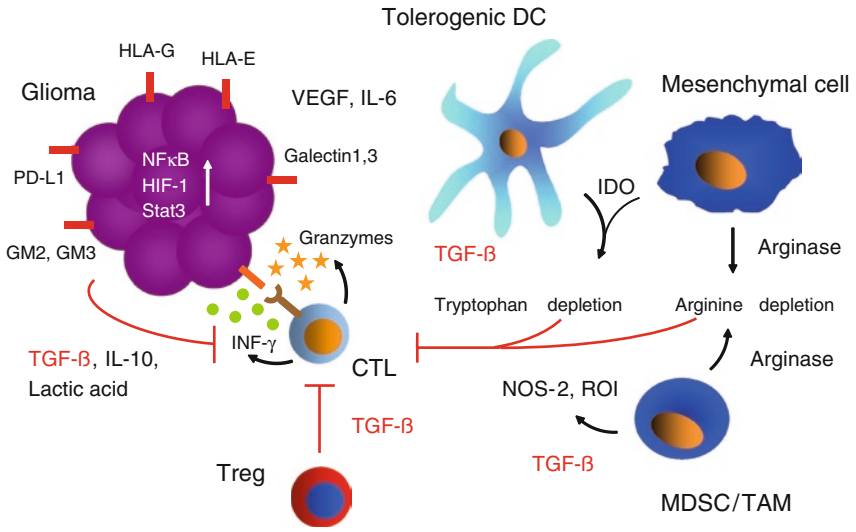

Fig. 8.2 The immunosuppressive network within malignant glioma. Within the tumor, effector cells such as cytotoxic T cells (CTL) are exposed to high concentrations of immunosuppressive factors including cytokines, such as TGF-beta or IL-10, tumor-cell derived metabolites such as lactic acid, and enzymes, such as indoleamin-2,3-dioxygenase (IDO), Arginase, NOS-2 or Reactive Oxygen Intermediates (ROI), that are either produced by the tumor cells or different subpopulations of immune suppressor cells that are attracted to the tumor site or generated within the tumor microenvironment, such as mesenchymal cells, tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), dysfunctional dendritic cells (DC) and regulatory T cells (Treg). In addition, immuno-inhibitory surface molecules such as HLA-G or HLA-E, PD-L1, galectins and minor brain gangliosides (GM2, 3) expressed on tumor cells contribute to the immune escape of malignant glioma. Many of these events are orchestrated by TGF-beta that plays a crucial role in setting up the immunosuppressive microenvironment in malignant gliomas. Moreover, master transcriptional factors such as NF-kB, HIF-1 and Stat3 are constitutively expressed in malignant glioma

several clinically relevant subtypes of GBM. The described GBM features are dependent on the applied methodological approaches. In an approach using the database of the cancer genome atlas project, PDGFRA, IDH-1, EGFR, and NF1 were identified as the driving genes that define the mentioned subtypes [35]. The classification divides GBM into a proneural, neural, classical, and mesenchymal subtype which each have distinct prognoses. In addition, the respective genetic profile can closely be related to normal brain cell types, suggesting a transition of normal cells to G-CSC. Others have published similar classifications [25]. We have used a 24-gene signature to distinguish two subgroups of GBM with a proneural signature resembling fetal neural stem cells and a mesenchymal signature similar to adult neural stem cell lines [36]. The GMB subtypes can further be divided by using available stem cell markers, and they are characterized by distinct expression profiles concerning extracellular matrix molecules and several signaling pathways, e.g. that of transforming growth factor-beta2 (TGF-beta2) [36]. Evidence suggests that the origin of G-CSC from normal precursor cells may provide a new subclassification for GBM. However, the discussed results will have to be integrated, further.

As migration is the first apparent step towards differentiation of NSCs, dysregulated migration features may lead to the release of proliferation control and to tumor formation [37]. Alternatively, dysregulated extrinsic factors within the niche might lead to uncontrolled proliferation of stem cells and tumorgenesis [38]. Histological and ex vivo cell culture studies suggest that physiological stem cells lie within a vascular niche in which endothelial cells regulate stem cell self-renewal [39–41].

8.10 The Glioblastoma Stem Cell Niche

The tumor niche in glioblastoma can be hypothesized to consist of a disorganized microenvironment compiled of cellular (several precursor cells, e.g. mesenchymal, endothelial and hematopoetic) and non-cellular components, where glioblastoma stem cells (G-CSC) preferentially seed and develop. A series of recent studies has brought evidence that blood vessel alterations support G-CSC development [15,42] and maintenance [43,44]. Recent data convincingly demonstrate that targeting the vascular components of the niche can lead to the eradication of G-CSCs, thereby providing comprehensive data on the importance of compartmental interactions within these tumors [33,42]. Similar to the normal NSC niches in the subventricular zone, the G-CSC niche may provide regulated signals necessary to maintain the undifferentiated state of G-CSC, thereby preserving their self-renewing and multifunctional capacities [45,46].

Tumor cells preferentially home at the vascular basal lamina [47–49]. Recent data show that several genes are differentially expressed in vessels of glioblastomas in comparison to normal brain vessels [50] and therefore, may be responsible for a dysfunctional G-CSC promoting microenvironment. Overwhelming evidence indicates that hypoxia regulates angiogenic properties [51], and that bone marrow-derived precursor cells contribute to the growth of endothelium-lined vessels at the vicinity of tumor masses [52]. These processes are regulated by numerous pro-angiogenic and anti-angiogenic growth factors [53]. Vascular endothelial growth factor (VEGF), which is induced by hypoxia inducible factor (HIF-1) in hypoxic areas and derives from tumor cells as well as endothelial progenitor cells, induces blood vessel formation [54] and directly regulates tumor cell invasiveness [55]. These data convincingly suggest that antiangiogenic agents should be included in the treatment of GBM. Moreover, G-CSC seems to be dependent on and promoted by an hypoxic niche [56].

The soil of the tumor niche is the **extracellular matrix** (ECM). GBM have a distinct ability to infiltrate the brain parenchyma and, by means of ECM modification and expression of proteases [49,57], disrupt the extracellular matrix that inhibits motility of normal cells. A number of extracellular matrix proteins as hyaluronic acid, chondroitin sulfate proteoglycans [58] and tenascin [59] have been characterized for their ability to modulate the migration of glioma cells [60–72], NSC [63] and G-CSC [64]. These proteins constitute further possible targets of a systems biology approach to GBM.

8.11 Key Regulators of the Tumor Niche

The tumor niche represents a closely regulated tumor-stroma-interaction, which is influenced by several adjacent compartments. Recent data have shown that agonists of a nuclear transcription factor, peroxisome proliferator-activated receptor gamma (PPARgamma) agonists, induce growth arrest and apoptosis in GBM cells, G-CSC cells and spheres [65]. The fate of G-CSC may be influenced driving these cells into an oligodendroglial differentiation [66]. This is specifically interesting as the role of PPARgamma agonists extends beyond inhibition of proliferation, including effects such as induction of apoptosis [67–72]. Furthermore, invasion can be reduced by treatment with PPARgamma agonists [68,73]. Several of these effects may be transduced by inhibition of TGF-beta mediated effects [74]. The described effects may be further enhanced by combination of PPARgamma agonists with other targeted approaches as HMG-CoA reductase inhibition [75] or retinoic acid receptor antagonism [76]. In a pilot trial combining low-dose metronomic chemotherapy with a PPARgamma agonist and an inhibitor of COX-2, a subset of patients demonstrated impressing responses with long-term stabilizations of their disease [77]. It remains to be elucidated which molecular markers can predict responses to such combined biomodulatory regimen.

Soluble factors, e.g. TGF-beta and HIF-1 are among the important regulators of the tumor niche. A recent study of human gliomas suggests that bone morphogenic proteins that are niche-derived regulators of neural stem cell fate might also regulate the differentiation status of G-CSC [78]. It is intriguing to speculate that proteins from the large TGF-beta family regulate neural stem cell self-renewal, and that defects in this regulation might induce a transition of neural stem cells to tumor stem or progenitor cells [37]. A recent publication describes that TGF-beta and LIF regulate the self-renewal capacity of patient-derived tumor stem cells, but not of normal human neural progenitors [79]. The induction of LIF is Smad-dependent and activates the JAK-STAT pathway. Therefore, TGF-beta and LIF may be addressed as attractive therapeutic targets [29,79–81].

8.12 Tumor Metabolism

The tumor metabolism has recently become one of the most intensely investigated topics in tumor biology. The Warburg effect describes a phenomenon where glycolysis is performed despite a sufficient level of oxygen (aerobic glycolysis) [89,90]. It is known for long that aerobic metabolism provides one of the key events in the progression of solid tumors [82]. Persistence of aerobic glycolysis is a characteristic of cancer cells [82] and is strongly regulated by several oncogenic proteins, e.g. myc, p53 and HIF-1 [83–86]. HIF-1 induces the expression of several enzymes involved in glycolysis, including lactate-dehydrogenase A (LDH-A), the enzyme

converting pyruvate to lactate [87]. LDH-A, in turn, also modulates TGF-beta [57], a known enhancer of GBM invasion.

The shift toward aerobic glycolysis increases the production of lactic acid with a decreased pH of the pericellular space [88], leading to apoptosis of non-tumor cells [89,90] and invasion of malignant cells into the parenchyma following a front of acidic microenvironment [84]. Lactate activates HIF-1, VEGF-A and VEGF-R1 [91] as well as proteolytic enzymes [57,92,93], which allow tumor cells to enter into the brain as well as to home along the basal lamina of the brain capillaries [94]. HIF-1 is a crucial factor controlling neovascularization, glucose metabolism, survival and tumor invasion [45,87]. Together, these results established a yet underestimated link between metabolic and molecular events which may be a major driver of tumor progression.

8.13 Tumor-Associated Inflammation in GBM

Tumor-infiltrating leukocytes, in particular tumor-associated macrophages (TAM), are prime regulators of cancer inflammation [95]. TAMs accumulate preferentially in hypoxic regions of tumors and promote tumor progression by secretion of angiogenic factors, proteases, growth factors, motility factors and pro-inflammatory mediators [96,97]. In GBM, a wide range of TAM have been observed that express TREM1 (triggering receptors expressed by myeloid cells-1) [98,99]. Engagement of TREM1 stimulates macrophages to secrete pro-inflammatory cytokines and chemokines, such as IL-8, MCP-1, TNF-α and IL-1 [100]. TREM1 expression in macrophages is regulated by NF-kB at the transcriptional level [101]. These data strongly emphasize the contribution of NF-kB pathway activation in bridging tumor-associated inflammation and tumor promotion and progression of GBM.

Constitutive NF-kB activation may be either promoted by genetic alterations or by microenvironmental signals, including hypoxia, cytokines, and Reactive Oxygen Intermediates (ROI) [102,103], and induces several cellular alterations associated with tumorgenesis and more aggressive phenotypes, including insensitivity to growth inhibition, resistance to apoptotic signals, immortalization, angiogenesis and tissue invasion [104]. Constitutive NF-kB activity has been reported from various glioma cell lines and primary cultures from tumor tissue [105].

8.14 Proliferation Behavior

The mechanisms responsible for switching tumors from dormancy to proliferation are not well understood, but are an example for a systemic coordinated interaction of tumor and stroma cells. A recent publication suggests that dormant tumors undergo a stable genetic reprogramming during their switch to a fast-growing

phenotype. In a genomic analysis, a consensus signature was found across solid tumors including glioblastoma, with angiogenesis being the most significantly affected functional gene category. The switch was associated with a down-regulation of angiogenesis inhibitors and regulation of several classes of genes connected to invasion, establishing a strong correlation between dormancy, angiogenesis and proliferation [106].

8.15 Invasion

Clinical recurrence of malignant gliomas is closely associated with a rapid infiltration of tumor cells into the surrounding healthy brain parenchyma [107]. The overexpression of TGF-beta is associated with upregulation of matrix metalloproteinases type 2 and 9 (MMP-2 and MMP-9), of molecules of the extracellular matrix [57,108], and of integrins [57], a large family of cell surface receptors that connect cells to extracellular matrix proteins and act as signal transducers [48]. Integrins facilitate extracellular matrix dependent organization of the cytoskeleton and activation of intracellular signaling that is required for the regulation of cell adhesion and migration [109].

HIF-1 is an additional crucial factor regulating invasion [87]. A number of proteins involved in detachment and invasion including: vimentin, fibronectin, keratins, matrix metalloproteinase 2 (MMP-2), cathepsin D, and urokinase plasminogen activator receptor are HIF-induced [110]. HIF-1 also induces the loss of E-cadherin, a key player in cell adhesion and epithelial-mesenchymal transition [45].

8.16 Angiogenesis

The developing vasculature delivers nutrients and provides a vascular niche for glioblastoma development and maintenance. Treating CD133+ glioblastoma cells with bevacizumab blocks their ability to induce vessel formation in vitro, and to induce tumors in a nude mouse model [15].

The formation of new blood vessels by capillary sprouting is governed by molecular interactions between vascular cells and components of the extracellular matrix. The role of TGF-beta in angiogenesis involves upregulation of angiogenic factor expression like VEGF derived from vascular endothelial cells and glioma cells and of bFGF, tissue proteases (e.g., MMP-2, MMP-9) and extracellular matrix proteins [111,112]. High levels of VEGF provide the tumor with a pro-angiogenic and immunosuppressive environment [111]. Hypoxia activates hypoxia-inducible transcription factors (HIFs) [45], that function as master switches to induce expression of angiogenic factors, including VEGF. In hypoxia, the HIF-1 subunits become stabilized and activate transcription of target genes [113] including VEGF [114], thereby inducing marked angiogenesis.

8.17 Local Immunosuppression

HIF-1α and TGF-beta have also been identified as key factors in gliomas promoting the release of chemoattractants that orchestrate the recruitment of different kinds of immune cells to gliomas [115–118]. However, the generation of tumor-specific immunity is generally prevented by local immunosuppressive factors, particularly TGF-beta which blocks both the innate and adaptive arm of the immune system [119,120]. TGF-beta strongly inhibits the generation of cytotoxic T-cells (CTLs) and lymphokine-activated killer (LAK) cells and suppresses the cytolytic activity of CTLs and other effector cells such as macrophages, NK cells and LAK cells by reducing pore-forming proteins (e.g., perforin and granenzyme B) and by suppressing the release of pro-inflammatory cytokines and cytotoxic mediators (e.g., INF-γ, TNF-alpha and NO) [121,122]. TGF-beta also inhibits both proliferation and differentiation of T-helper type 1 (Th1) cells, important players in antitumor immunity [138], and promotes the conversion of naïve CD4+ T cells into immunosuppressive CD4+ regulatory cells (Treg) [124,125]. In addition, B cell activation, proliferation and secretion of immunoglobulins are markedly impaired by TGF-beta [126]. Abundant evidence further documents that the differentiation, maturation and function of dendritic cells (DCs), professional antigen-presenting cells, is profoundly suppressed by TGF-beta [127–129]. These dysfunctional, tumor-conditioned DCs induce either suppressive Treg or T-cell unresponsiveness [130–132]. Similarly, TGF-beta was also shown to stimulate the differentiation of myeloid precursor cells to myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM) that accumulate in gliomas and markedly inhibit DC function, and T cell and NK cell responses [133–136] (Fig. 8.2). Altogether, these data clearly indicate that HIF-1α and TGF-beta play a central role in glioma-mediated immunosuppression.

8.18 Pathophysiology-Based Therapy in Glioblastoma

8.18.1 Diagnostics Promoting Systems Comprehension

Compiling large-scale molecular knowledge about aberrant transcriptional networks with adequate methods is in its beginnings [8]. High-throughput methods as microarrays detecting molecular-genetic aberrations and gene dose [137–139], protein arrays using several kinds of antibody based methods [140–144], and mass spectroscopy for the investigation of metabolism [145], will probably yield the most objective results of changes in the systems biology of glioblastomas. Besides these methods, imaging methods as magnetic resonance spectroscopy (MRS) can be used to cluster several subtypes of glioblastomas, potentially defining distinct groups for treatment [146]. At this time, results from large-scale analyses influence the molecular-biological classification of GBM [147] and elucidate single promising targets.

To further specify complex systems developments in GBM [148], bioinformatics are adducted to create new mathematical models on the basis of results experimentally derived from different observation levels (genetic, epigenetic, proteomic, metabolomic) [149,150]. Of importance, the development of a tumor is a dynamic process, comparable to a developing organ, and is initially highly dependent on the interactions between different structures and compartments within the tumor niche. Interventions, either intrinsic (e.g. by the tumor itself or the host) or extrinsic (e.g. therapy) will influence all levels of GBM's tumor biology, including the genetic, epigenetic, proteomic, and metabolomic level.

To improve the translation of such screening techniques into the clinical setting, and in view of the associated high costs, a panel of the most important pathogenetic events of glioblastomas should be defined and evaluated with the most adequate techniques. For example, clustered analysis from high-throughput assays could be combined with genetic or immunohistochemical analysis of prognostic (e.g.: MGMT, IDH-1, EGFR, p53, PTEN) and pathogenetic markers (e.g.: VEGF, bFGF, TGF-beta: angiogenesis; Tenascin-C: invasion) to establish personalized diagnostic and therapeutic patterns [151].

Recent regimen use shot-gun strategies as cytostatic drugs or targeted approaches without a specific pre-screening of target expression in the individual patient. First approaches screening for individual genetic, proteomic and metabolomic patterns of each patient in an unbiased way have been published [137–145], but are not translated into standard therapy yet. Recent technological approaches to detect genetic and molecular-genetic aberrations (high-density oligonucleotide arrays, and next-generation sequencing technologies within the human cancer genome atlas project) have revealed several potentially therapy-relevant molecules, e.g. IDH-1 [152,153]. Similar approaches are now transduced to the proteomic [154,155] or metabolomic [156,157] level. Such efforts could be translated into a personalized therapeutic concept fusing diagnostic and therapy planning in a single step [158].

Genomic screening: The sum of genetic aberrations build up a cellular infrastructure supporting tumor promotion [159]. Genomic screening analyses are commonly used for detection of putative targets [160–161], or screens for miRNAs targeting disease-relevant gene expression, on a merely computational basis [162]. Most likely, new models will be individually compiled in the future work-up of glioblastomas, generating information for the setup of individualized concepts [158]. These new models include advanced in vivo models using engineered animals and in silico models based on bioinformatic methods [163]. This approach allows for hypothesis generation and data integration in both the experimental and clinical settings.

Molecular imaging could also be used for the follow-up to individualize treatment regimens. 1H (proton)-MRS ratios can discriminate tumor and necrosis [164]. The median apparent diffusion coefficient (ADC) is higher in necrosis as compared with both tumor and mixed tumor and necrosis [165]. Both O-(2–18F-fluoroethyl)-L-tyrosine (FET) and 11C-methly-L-methionine (MET)-positron emission tomography (PET) have been used to distinguish tumor progression from reactive lesions

induced by treatment. In a study in patients with glioma WHO grade II to IV receiving several treatment modalities, a positive predictive value of 84% was found for FET-PET [166].

8.19 Targeting the Invasive Feature

Tumor cells in glioblastomas consist of functionally heterogeneous either proliferative or invasive cell fractions, as well as core and peripheral tumor cells expressing divergent anti-apoptotic mechanisms. Much evidence suggests an inverse correlation between proliferation and invasion both in vitro and in vivo as detected by cDNA microarray technology [61,167]. During invasion, glioma cells may be relatively resistant to cytostatic drugs, as these agents are dependent on cell division and therefore proliferation. Consequently, it has been shown that invading cells are resistant to induction of apoptosis, correlating to a shift in the expression of apoptosis-regulating genes [167]. In addition, overexpression of pro-survival genes as Bcl-2 promotes the invasion of glioma cells in vitro [168]. The expression of SF/HGF inhibits apoptosis of migrating GBM cells and confers resistance to cell death [169], and EGFR signaling acts anti-apoptotic [170]. Therefore, migration not only induces anti-apoptotic effects but also enhances survival pathways as PI3-K/Akt [171]. It can be speculated that the inhibition of invasion would enhance the susceptibility to cytostatic agents. However, no specific anti-invasive agents have been approved so far; therefore, this hypothesis has only been challenged in vitro at this time [171].

Attractive therapeutic strategies to target the tumor microenvironment are inhibition of aberrant NF-kappa B activation in glioblastoma or inhibition of hypoxia inducible factor-1 (HIF-1), especially in combination with cytotoxic drugs or anti-angiogenic agents [172,173].

8.20 Targeting Angiogenesis

Similar links have been detected between anti-angiogenic treatment and invasion. Antiangiogenic therapy seems to increase the invasive properties of glioma cells. Early in vitro results [174] have recently been verified by observations from human high-grade glioma trials using bevacizumab, where an increased FLAIR-enhancement suggesting increased invasion has been observed using magnetic resonance imaging (MRI) [17,22,23]. Both the results from in vitro as well as in vivo studies recommend a combined use of anti-angiogenic and ant-invasive modalities. Therefore, considering the lack of available anti-invasive agents, it seems urgent to develop clinically applicable anti-invasive therapies to allow combinations of these with anti-proliferative and/or anti-angiogenic drugs.

Some agents initially developed for specific cellular targets have been shown to have pleiotropic off-target effects that could probably enhance their clinical efficacy. Cilengitide is a selective inhibitor of integrins on endothelial cells with a predominant antiangiogenic effect, but has a bi-modal biological effect as it develops anti-invasive properties on tumor cells as well. The substance is under investigation within several clinical protocols [18,175–177]. However, its efficacy as a monotherapy approach in relapse of glioblastoma is only moderate [177].

The antiangiogenic single-target agent bevacizumab, a humanized antibody against VEGF-A (vascular endothelial growth factor A), is the only mono-target approach with considerable clinical efficacy and gained approval for relapsed glioblastoma in the USA: The approved regimen combines bevacizumab with irinotecan [178], but several alternative regimen have been tested using bevacizumab as monotherapy [179] or combined with other cytotoxic agents, i.e. temozolomide or nitrosoureas.

8.21 Targeting Immunosuppressive Features

An approach to overcome glioma-induced tolerance mechanisms involves e.g. targeting immunosuppressive mediators within the tumor microenvironment [185] (see Fig. 8.2).

Many different specific inhibitors have successfully been studied in preclinical models to break immune resistance of malignant gliomas [186,187]. The most advanced in clinical application is a phosphorothioate-modified antisense oligonucleotide which is complementary to the mRNA encoding TGF-beta2 (tarbedersen) and is currently tested in a phase III trial vs. systemic standard chemotherapy (temozolomide or BCNU) after promising results in phase I/II trials [29].

In addition, inhibitory cytokine signaling molecules (e.g., Stat3) are known to be constitutively activated in several human glioma cell lines, promoting tumor cell growth and survival [189]. Selective inhibitors of Stat3 have been evaluated in murine glioma models and were shown to activate intratumoral macrophages and microglia, induce apoptosis in glioma cells, and inhibit tumor growth [190,191].

Interestingly, multikinase inhibitors such as sorafenib and sunitinib have been shown to promote phospho-STAT3 dephosphorylation. Moreover, both agents modulate the tumor immunological microenvironment by reducing the immunosuppressive function of myeloid-derived suppressor cells and the development of Treg. Therefore, both sorafenib and sunitinib may be used to reverse immune suppression and as a potentially useful adjunct for enhancing the efficacy of immune-based cancer therapy [191–194]. First promising examples of combinations of peptide vaccination with cytostatic agents have been published [195,196].

8.22 Multi-Targeted Treatment

System oriented therapy in GBM in our opinion necessitates a **multi-targeted treatment** approaching the nodal pathophysiological events of each individual tumor. Multi-targeted approaches target several molecular mechanisms in parallel. Multi-targeted effects can be induced by a single agent within multiple cell types of the tumor compartment, or by a composition of mono-targeted agents. However, a multi-targeted approach not automatically targets the nodal pathophysiological mechanisms of GBM. Therefore, a multi-targeted approach that is relevant under the view of systems biology of GBM should be based on a specific analysis of the pathophysiologically relevant communicative components of the respective tumor.

Multi-target inhibitors as sorafenib, cediranib [197,198], and sunitinib (against VEGFR1-3, PDGFR-a/b, FLT-3, c-KIT and RET [199]) are in the clinical development for GBM to address systems biological considerations. Therapeutic regimen integrating several modes of action, as the combination of cilengitide plus temozolomide (EMD 121 974-011, EORTC 26071-22072 [177]), cediranib plus CCNU (D8480C00055; recruiting), EGFR-targeted vaccination and temozolomide [195], bevacizumab plus irinotecan (Genentech trial; [179]), or imatinib plus hydroxyurea [200] have been evaluated or are currently under investigation (Table 8.1).

Other single-target substances as inhibitors of protein kinase Cß [180–183], mTOR inhibitors [184], inhibitors of EGFR [201–203], PDGFR [200,204,205] and others that demonstrated only marginal effects in patients with relapsed

Table 8.1 Published glioblastoma clinical trials in adults constituting systemic approaches. Trials combining cytostatic with mono- or multi-targeted drugs are emphasized. The efficacy evaluation is given for the combination, neglecting possible efficacy of the single-substance comparator arm, if applicable. If several studies exist for the same combination, the most recent or most powered trial is listed. The degree of efficacy is evaluated using the historical meta-analysis data of Wong et al. [210], where PFS-6 was 15% for GBM, whereas the median PFS was 9 weeks

Author	Year	Targeted agent	Target	Classical agent	Efficacy
[179]	2009	Bevacizumab	VEGF	Irinotecan	Positive (as single agent)
[202]	2009	Erlotinib	EGF-R	Temozolomide	Pilot trial
[200]	2009	Imatinib	bcr-abl	Hydroxyurea	Negative
[201]	2008	Erlotinib	EGF-R	Carboplatin	Negative
[211]	2008	Gefitinib	EGF-R	Temozolomide	Phase I
[195]	2008	Peptide Vaccination	Immune system	Temozolomide	Pilot trial
[212]	2008	Thamidomide	Angiogenesis	Irinotecan	Modest
[213]	2007	Thalidomide	Angiogenesis	Temozolomide	Negative
[214]	2005	Celecoxib	COX-2	Irinotecan	Modest
[215]	2002	Marimastat	MMP	Temozolomide	Negative

VEGF = vascular endothelial growth factor; EGF-R = endothelial growth factor receptor; COX-2 = cyclooxygenase 2; MMP = matrix metalloproteinase. The overview shows that multi-targeted approaches are not automatically relevant modulators of the robust pathophysiological therapy resistance of GBM

glioblastoma should be investigated within adequate combination therapies to evaluate their potential for systemic GBM therapy.

The given examples can be extended to other teleological derived treatment modalities. In consequence, a combination of cytostatic, anti-invasive and anti-angiogenic, anti-inflammatory drugs combined with agents which are suggested to reconstitute the local immune system [206,207], could further enhance therapy efficacy.

8.23 Approaches for Personalizing GBM Therapy

Recent regimen use shot-gun strategies as cytostatic drugs or targeted approaches without a specific pre-screening of target expression in the individual patient. First approaches screening for individual genetic, proteomic and metabolomic patterns of each patient in an unbiased way have been published [137–145], but are not translated into standard therapy, yet. Recent technological approaches to detect genetic and molecular-genetic aberrations (high-density oligonucleotide arrays, and next-generation sequencing technologies) have revealed several potentially therapy-relevant molecules, e.g. IDH-1 [152,153]. Similar approaches are now transduced to the proteomic [154,155] or metabolomic [156,157] level. Such efforts could be translated into a personalized therapeutic concept fusing diagnostic and therapy planning in a single step [158].

8.24 Outlook

The challenge will be to correlate diagnostically compiled informative tumor patterns with specific tumor-associated disease traits or with therapy response depending on the tumor's functional systems status. Bioinformatic approaches may be helpful that allow defining individual informative tumor patterns based on a possibly handy range of methods to select personalized therapies and to predict response.

We have to notice that huge systems biological knowledge based on a reductionist derived scientific horizon represents only one side of a medal: Redemption of the situative identity of systems objects (comprising either proteins, signaling pathways, or single cell types) is an interactive communicative process, which necessitates redemption of the objects' validity and denotation by steadily evolving 'surroundings' during tumor progression. Now the tumor system is advancing to a holistic communicative system, which is accessible for novel kinds of therapies, so called biomodulatory therapies [208, 209]. That means, the other side of the medal opens a second scientific horizon and offers the opportunity to approach systems issues from two scientifically completely different sites, as differential perspectives of interaction with tumor systems are entangled with various horizons of knowledge (chapter 1, 26) [208, 209].

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Part III
Systems-Relevant Molecular and Cellular Targets: Implementation of Modular 'Knowledge'

Chapter 9
Functional Impacts of Signal Integration: Regulation of Inflammation-Related Transcription Factors by Heterotrimeric G Proteins

Wendy Wing Shan Yeung, Maurice Kwok Chung Ho, and Yung Hou Wong

Abstract Oncogenic mutations of G proteins and G protein-coupled receptors (GPCRs) have been identified in various endocrine tumors for almost 20 years. Chronic inflammation contributes to tumorigenesis by the induction of cytokine and chemokine production and leukocyte infiltration. Many inflammatory mediators and chemoattractants elicit their effects by stimulating specific GPCRs. The subsequent activation of various G proteins often results in the modulation of transcription factors via complex signaling networks. Human herpesviruses can even resort to hijacking such control by making their own constitutive GPCRs that eventually lead to the development of Kaposi's sarcoma. Increasing evidence indicates that inflammation-related transcription factors such as STAT3 and NFκB are common effectors of converging streams of G protein signals, which further signifies the importance of G protein-mediated regulations of inflammatory actions and tumorigenesis. This chapter aims to review the regulations of transcription factors mediated by G proteins and the biological relevance of cross-communications between different signaling cascades.

Keywords G proteins • Signal transduction • Inflammation • Cancer

Abbreviations

A_1R, $A_{2A}R$, $A_{2B}R$, A_3R	Types 1, 2A, 2B and 3 adenosine receptors
Akt	Protein kinase B
AP-1	Activator protein-1
AT_1R	Type 1 angiotension II receptor

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ATF-2	Activating transcription factor-2
ASMC	Airway smooth muscle cells
Bax	Bcl-2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
bFGF	Basic fibroblast growth factor
BK_1R, BK_2R	Types 1 and 2 bradykinin receptors
BLT_1, BLT_2	Types 1 and 2 leukotriene B_4 receptors
C5a	Complement 5a
cAMP	Cyclic AMP
CaM	Calmodulin
CaMKII	Calmodulin kinase II
CBP	CREB-binding protein
CCR	CC chemokine receptor
C/EBPδ	CCAAT/enhancer binding protein δ
CHO	Chinese hamster ovary cells
COX-2	Cyclooxygenase-2
CREB	Cyclic AMP-responsive element binding protein
c-Src	Cellular-sarcoma
CXCR	CXC chemokine receptor
DAG	Diacylglycerol
Egr-3	Early growth response-3
EGF	Epidermal growth factor
EP4	Prostaglandin E4 receptor
Epac	Exchange proteins directly activated by cAMP
EPRAP	EP4 receptor-associated protein
ERK	Extracellular signal-regulated kinase
fMLP	*N*-formyl-methionyl-leucyl-phenylalaine
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte/macrophage colony-stimulating factor
G protein	Heterotrimeric guanine nucleotide binding regulatory protein
GPCR	G protein-coupled receptor
GRPR	Gastrin-releasing peptide-preferring receptor
H_1R, H_2R	Types 1 and 2 histamine receptors
HCMV	Human cytomegalovirus
hBD-3	Human β-defensin-3
HEK293	Human embryonic kidney 293 cells
HEL	Human erythroleukemia cells
hIP	Human prostacyclin receptor
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular cell adhesion molecule-1
IFN-γ	Interferon-γ
IκB	Inhibitor of κB
IKK	Inhibitor of κB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase

IP_3	Inositol trisphosphate
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
KSHV-GPCR	Kaposi sarcoma herpesvirus-encoded G protein-coupled receptor
LHSCC	Laryngeal and hypopharyngeal squamous cell carcinomas
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
mAKAP	Muscle A kinase-anchoring protein
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage-colony stimulating factor
Mcl-1	Myeloid cell leukemia sequence 1
MEK1/2	Mitogen-activated protein kinase kinase 1/2
MIP-1α	Macrophage inflammatory protein-1α
MIP-1β	Macrophage inflammatory protein-1β
MMP	Matrix metallopeptidase
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κB
NPC	Nasopharyngeal carcinoma
p38	p38 mitogen-activated protein kinase
PAF	Platelet-activating factor
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-activated receptor
PDK-1	3′-phosphoinositide-dependent protein kinase-1
PGE2	Prostanglandin E2
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PIP2	Phosphatidylinositol bisphosphate
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
RyR2	Ryanodine receptor 2
SST_2R, SST_4R	Types 2 and 4 somatostatin receptors
STAT	Signal transducer and activator of transcription
Tac1	Tachykinin 1
TIMP-1	Tissue inhibitor of metalloprotease-1
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
TXA_2	Thromboxane A_2 receptor
Tyk2	Tyrosine kinase 2
US28	Viral chemokine receptor US28
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells.

9.1 Introduction

Chronic inflammation is one of the major pathological bases manifesting the development of gastric cancers, hepatitis and hepatocellular carcinoma, cervical cancer, ulcerative colitis and colorectal cancer [1]. Microbial infections, viral infections and autoimmune responses can lead to chronic inflammation-associated cancer formation. Human herpesviruses, such as human cytomegalovirus (HCMV) and Kaposi sarcoma herpesvirus (KSHV) are known to be associated with tumorigenesis and tumor progression. HCMV infection potentiates malignancies of colon cancer and malignant glioma [2,3]. KSHV was initially discovered from Kaposi's sarcoma lesion of an AIDS patient [4]. It was subsequently discovered that KSHV contributed to the pathogenesis of KS, primary effusion lymphoma [5] and lymphoproliferative disorder multicentric Castleman's disease. Emerging evidence shows that herpesvirus infection interferes or inhibits host cell immune defense and maintains a tumor-promoting microenvironment by expressing virulent homologues of host cell proteins that disturb normal cell cycle progression and leads to apoptosis of the host cells. For example, cellular growth and transformation are induced by viral-encoded homologues of cytokines, chemokines or chemokine receptors [6]. The constitutive expression of viral chemokine GPCRs triggers prolonged activation of G protein signaling and eventually becomes the major inputs for chronic leukocyte infiltration and cancer development. GPCRs can serve as proto-oncogenes since overexpression of various wild type GPCRs can transform cells in the presence of their specific ligands. Mutations on GPCRs may result in constitutive signaling and oncogenesis [7]. Naturally occurring mutations in GPCRs have been identified in human tumors [8,9].

Stimulation of GPCRs triggers the activation of Gα and release of Gβγ complex, with both components capable of regulating downstream effectors. According to the amino acid identities and their functional similarity, Gα subunits are classified in four major families ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$). $G\alpha_s$ and $G\alpha_i$ family members are initially known as the activators and inhibitors, respectively, of adenylyl cyclases to modulate the intracellular cAMP level. cAMP is important for the regulation of protein kinase A (PKA) activity and cAMP-sensitive guanine nucleotide exchange factor Epac1 and Epac2 [10]. $G\alpha_q$ family members ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{16}$) stimulate phospholipase Cβ (PLCβ) isoforms which hydrolyze the membrane phospholipid phosphatidylinositol bisphosphate (PIP_2) to release diacylglycerol (DAG) and inositol trisphosphate (IP_3). These two secondary messengers subsequently stimulate Ca^{2+} mobilization and protein kinase C (PKC) signaling pathways [11]. Rho GTPases have been identified as downstream signaling mediators of $G\alpha_{12/13}$ and regulate cytoskeletal rearrangement as well as gene transcription [12]. The Gβγ complexes are nowadays recognized as independent functional compartments regulating a whole repertoire of signaling and transcriptional events [13].

Interestingly, all four families of Gα subunits have been identified as proto-oncogenes with transforming and tumor-promoting properties in vitro and in vivo. The expression of constitutive active mutant of different Gα subunits is capable of

inducing neoplastic transformation in NIH3T3 cells. Oncogenic mutations have been mapped to the genes encoding Gα subunits in various human endocrine tumors. GTPase-deficient mutations of $G\alpha_s$ that lead to sustained activation of adenylyl cyclases have been found in human pituitary tumors. In human ovarian tumors, constitutively active mutants of $G\alpha_s$ and $G\alpha_{i2}$ were found [14]. Besides, expression of a dominant negative $G\alpha_{i2}$ protein inhibits the growth of murine melanoma cell line CL19, this further supports that Gα subunits are involved in tumor formation [15]. Mice deficient in $G\alpha_{i2}$ induce ulcerative colitis, which is a form of inflammatory bowel disease, and lead to colon adenocarcinomas development [16]. Recently, oncogenic mutations on $G\alpha_q$ have been identified in blue naevi and ocular melanoma of the uvea [17]. The mutations are exclusively found in the GTPase domain and result in sustained activation of $G\alpha_q$. $G\alpha_{12}$, also known as the *gep* oncogene, induces neoplastic transformation via STAT3 [18]. Recent findings reveal a significant correlation between $G\alpha_{12}$ transcripts and nasopharyngeal carcinoma (NPC) lymph node metastasis. Knockdown of $G\alpha_{12}$ in NPC cells shows a decrease in cell migration, invasion and a reversal in fibroblastoid morphology [19]. G protein signals often converge at some common downstream molecules such as mitogen-activated protein kinases (MAPK), signal transducer and activator of transcription (STAT) and nuclear factor κB (NFκB) and these signaling molecules are associated with inflammation and oncogenesis (Fig. 9.1). This chapter reviews the current knowledge about the regulations of various transcription factors by the four families of Gα subunits and their associated Gβγ complexes. Different aspects of signal integration between G proteins will also be discussed.

9.2 G protein-Mediated NFκB Regulation in Inflammation and Cancer

NFκB is one of the major regulatory factors involved in the development of inflammatory diseases and cancer and it represents a valuable therapeutic target for drug discovery. The NFκB transcription factor family has five members (p65, p50, p52, c-Rel and RelB) in the mammalian system. Mice deficient in the p65 subunit is embryonic lethal whereas mice that lack other NFκB members show dysfunction in immune responses. NFκB activity is highly activated at the sites of inflammation and in most human cancers. NFκB induces the expression of pro-inflammatory cytokines in immune cells, whereas it appears to control apoptosis in tumorigenic cells. Various inflammatory mediators have been shown to signal via different GPCRs to stimulate NFκB activation and gene transcription. Therefore, a thorough understanding on the molecular mechanisms between GPCR and NFκB may help to combat inflammatory diseases and cancer.

The canonical NFκB signaling cascade requires the activation of inhibitor of κB kinase (IKK) complex. IKK is composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ). Activated IKK phosphorylates inhibitory

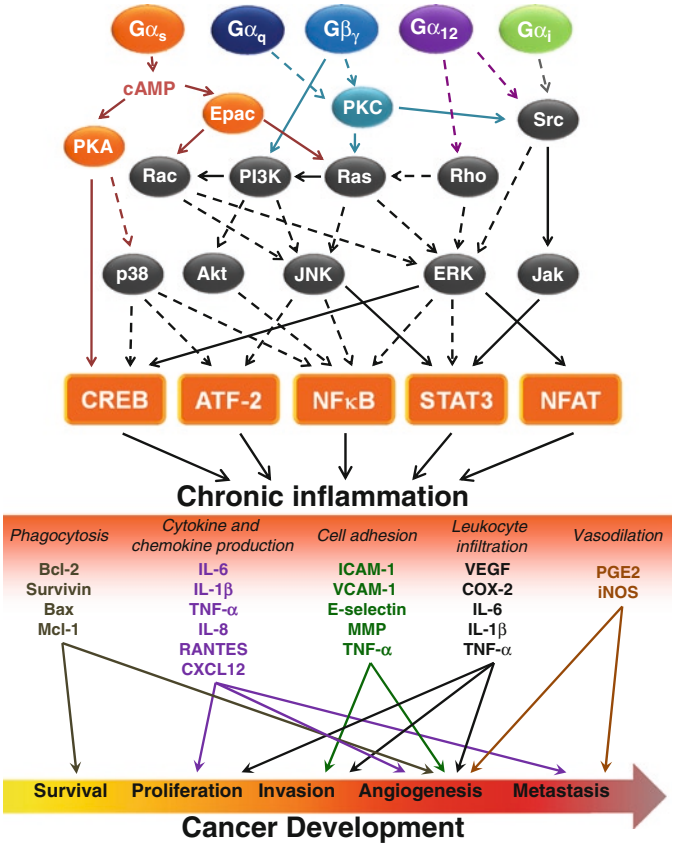

Fig. 9.1 Transcriptional regulation mediated by heterotrimeric G proteins in inflammation and cancer development. Activation of G protein-coupled receptors stimulates the activity of proto-oncoproteins, like Ras and Src, and subsequently activates various MAPKs to modulate transcription factors such as CREB, NFκB, STAT3, NFAT and ATF-2. These transcription factors induce the gene expressions that promote inflammatory responses. The secreted pro-inflammatory cytokines and chemokines further activates various transcription factors and upregulates gene expressions involved in tumor cell survival, proliferation, invasion, angiogenesis and metastasis

IκB proteins at Ser^{32} and Ser^{36} and promotes polyubiquitination of IκB at Lys^{21} and Lys^{22} and subsequently leads to its degradation by 26S proteasome [20]. This results in the activation and nuclear translocation of NFκB homodimers or heterodimers, which then binds to specific DNA to induce NFκB-dependent gene transcription that includes cytokines (interleukin (IL)-1, IL-2, IL-6, IL-10, IL-12, IL-17, IL-21, and IL-23), interferon-γ, transforming growth factor β, tumor necrosis factor (TNF-α) and TNF-related apoptosis-inducing ligand (TRAIL), chemokines (IL-8, IP-10, MIP-1α, MCP-1, RANTES, and eotaxin), adhesion molecules (ICAM-1, VCAM-1, E-secretin) and inflammatory enzymes (5-lipoxygenase, cyclooxygenase (COX)-2), and inducible nitric oxide synthase (iNOS) [21]. Among these, it has been suggested that IL-12, TRAIL and IFN-γ are important for anti-tumor immunity

whereas TNFα, IL-6 and IL-17 promote tumor growth [22]. It has been reported that one of the binding site for NFκB is located upstream of H_4 receptor gene [23]. The $G_{i/o}$-coupled histamine H_4 receptor is predominantly expressed in immune cells and its expression fluctuates upon inflammatory stimuli [24]. Mice deficient in histamine H_4 receptor exhibit decreased allergic lung inflammation [25]. Histamine H_4 receptor is induced by sepsis and is transcriptionally controlled by NFκB. Activation of H_4 receptor results in the development of sepsis-induced splenic apoptosis by counteracting the anti-apoptotic effect of NFκB [26].

At the sites of inflammation, numerous inflammatory mediators are produced exogenously by infection or endogenously by the cells at inflamed sites and leukocytes. A number of viral infections can lead to chronic inflammatory events which are achieved by hijacking the host machinery of chemokine signaling. For example, the Kaposi's sarcoma herpesvirus (KSHV) encodes a GPCR for chemokines and results in constitutive transactivation of NFκB and inflammatory mediator production [27]. NFκB activation induced by GPCR agonists, such as bradykinin, thrombin, histamine, adenosine, prostaglandins, chemokines and chemoattractants, has been documented in various cell types including immune cells and several cancer cell lines (Table 9.1). Numerous studies have demonstrated that multiple intermediates are involved in regulating GPCR-mediated NFκB activity. Protein kinase C (PKC) is one of the important downstream molecules of GPCR-induced NFκB activation (Table 9.1). It has been shown that PKC isoforms play critical roles in inflammatory responses [28] and cancer development [29]. Aberrant regulation of PKC isoforms has been reported in several malignancies and is linked to cancer progression. PKC has become a potential therapeutic target for cancer treatment [29]. PKC is known to activate ERK signaling cascades via the Ras/Raf/MEK pathway [30]. Ras/Raf/MEK/ERK is one of the key pathways involved in mitogenic signaling activated by GPCRs and GPCR-induced ERK activation is involved in the regulation of NFκB activity [31](Table 9.1). Dysregulation of the ERK pathway is commonly found in several human cancers and mutations on the upstream kinases can stimulate constitutive ERK activation independent of growth factors and promote tumor formations [32]. In addition to PKC and ERK, other signaling molecules such as c-Src, PI3K, JNK and p38 have been demonstrated to modulate GPCR-mediated NFκB activation.

Various gene expressions induced by GPCRs are dependent on NFκB activity. As summarized in Table 9.1 Pro-inflammatory cytokines including IL-1β, TNF-α, and IL-6 are upregulated upon stimulation of specific GPCR ligands, such as bradykinin, chemokines, chemoattractants, prostacyclin and thrombin, in various cell types. TNF-α and IL-1β promote inflammatory responses and stimulate tumor cell growth, invasion and angiogenesis [33]. It has also been demonstrated that IL-1β and TNF-α induce bradykinin receptor expression through the activation of NFκB and JNK and p38 MAPK pathways in osteoblasts and fibroblasts [34]. Expressions of chemokines (IL-8, MCP-1, MIP-1), which elicit the cellular responses via GPCRs, are induced by angiotensin, bombesin, chemoattractants, histamine, prostacyclin, prostaglandin and thromboxane receptors in a NFκB-dependent manner. The production of cytokines and chemokines provides a feedforward loop to further activate diverse signaling cascades

Table 9.1 Regulation of NFκB activity by GPCRs

GPCR category	Receptor subtype	Coupled G proteins	Possible intermediates	Regulated NFκB target genes	Cell/tissue type	References
Adenosine	A_1	$G\alpha_{i/o}$, $G\alpha_{16}$	c-Src, PKC, Ras, Raf-1, MEK1/2, ERK	–	Lymphoblastoma reh cells	[102]
Angiotensin	AT_1	$G\alpha_q$	–	MCP-1, M-CSF, E-selectin, ICAM-1, VCAM-1, iNOS, MMP9, PPARα, PPARγ	Vascular smooth muscle cells (VSMC cells)	[103–106]
Bradykinin	BK_2	$G\alpha_{i/o}$, $G\alpha_q$	PLC, PKCδ, RhoA	IL-6, IL-1β	Various fibroblasts, epithelial cells	[107–109]
	BK_2	$G\alpha_{i/o}$, $G\alpha_q$	PKC, Ras, Raf-1, ERK	COX-2, PGE2	Epithelial cells, astrocytes, osteoblasts	[37–39]
	BK_1, BK_2	$G\alpha_{i/o}$, $G\alpha_q$	PKC, PI3K, MEK1/2, ERK, p38	MMP-9	Neutrophils, astrocytes	[110–112]
Bombesin		$G\alpha_q$	–	IL-8, VEGF	Prostate cancer cells	[113]
Chemokine	CXCR2	$G\alpha_{i/o}$	PKCζ, Akt, IKK, p38	Bcl-2, survivin	Melanoma and prostate cancer cells	[114]
	CXCR4	$G\alpha_{i/o}$	PI3K, MEK1/2, ERK	IL-6	Osteosarcoma cells, lung cancer cells, microglia	[115–117]
	CXCR6	$G\alpha_{i/o}$	PI3K, PDK-1, Akt	TNF-α	ASMC	[118]
	CCR1	$G\alpha_{i/o}$, $G\alpha_{14}$, $G\alpha_{16}$	Raf-1, MEK1/2, PLC, PKC, CaM, CaMKII, c-Src	–	Osteosarcoma cells, monocytes, HEK293 cells	[94,119]
	CCR2	$G\alpha_{i/o}$	PKC, Ca^{2+}	IL-6, ICAM-1	Human tubular epithelial cells, VSMC cells	[120,121]
	KSHV-GPCR	$G\alpha_q$, $G\alpha_{13}$	RhoA	IL-1β, IL-6, IL-8, TNF-α, MCP-1	NIH3T3 Fibroblasts	[27,122]

Chemoattractant	C5a	$G\alpha_{i/o}$	–	PAI-1, IL-8	Immune cells	[123–125]
	fMLP	$G\alpha_{i/o}$	PI3K, PKC, ERK	IL-1α, IL-1β, IL-6, IL-8, iNOS	Immune cells	[123,126,127]
	BLT_1, BLT_2	$G\alpha_{i/o}$	ERK, JNK, ROS	IL-6, MCP-1, TNF-α	Monocytes	[128,129]
Histamine	H_1	$G\alpha_q$	Ca^{2+}, PKC, Raf, MEK, ERK	GM-CSF, IL-8, IL-6	Epidermal keratinocytes	[130]
Prostacyclin	hIP	$G\alpha_s$, $G\alpha_{16}$	cAMP, PKA	TNF-α, IL-1α, IL-6, IL-12, IFNγ, IL-4, IL-10, IL-13, MIP-1α, MCP-1	Dendritic and T cells	[131,132]
Prostaglandin	EP_4	$G\alpha_s$, $G\alpha_{i/o}$	EPRAP, p105	MIP-1β	Macrophages	[133]
Somatostatin	SST_2	$G\alpha_{i/o}$, $G\alpha_{14}$	PKC, CaMKII, ERK, c-Src	–	Pancreatic carcinoma AR42J cells	[134]
Thrombin	PAR-1	$G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_{13}$	p38, PKC, PI3K	ICAM-1, E-selectin	Endothelial cells, fibroblasts	[135–138]
				IL-6, G-CSF	Fibroblasts	[139]
			PKC, c-Src, EGFR, MEK1/2	COX-2	VSMC cells	[40]
Thromboxane	TXA_2	$G\alpha_q$, $G\alpha_{13}$	PKC	MCP-1	HUVEC	[140]

and induce inflammatory responses. During inflammation, leukocytes migrate to the sites of inflammation, roll along the endothelium surface and then adhere firmly to the endothelium. Selectins play crucial roles in leukocyte rolling whereas leukocyte adhesion to the endothelium is dependent on the expression of cell adhesion molecules [35]. It has been shown that activation of GPCRs (AT_1R, CCR2 and PAR-1) induces gene expression of cell adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, through NFκB pathway in endothelial cells, epithelial cells and vascular smooth muscle cells (Table 9.1). COX-2 is an important enzyme for the synthesis of lipid inflammatory mediators including prostaglandins and prostacyclins from arachidonic acid. Dysregulation of COX-2 and prostaglandin expression have been found in many cancers such as colon, lung, breast, pancreas, and head and neck cancers [36]. Reports have demonstrated that COX-2 expression can be regulated by GPCR-mediated NFκB stimulation. Treatment of bradykinin results in an elevation in COX-2 and PGE_2 expression through NFκB dependent pathways [37–39]. In vascular smooth muscle cells, thrombin-induced COX-2 production is dependent on NFκB activity [40]. Collectively, there is considerable evidence to show that GPCRs regulate inflammatory-related gene expressions in part through NFκB signaling.

9.3 The Modulation of STAT Activity by Heterotrimeric G Proteins

In mammals, seven family members of signal transducer and activator of transcription (STAT) proteins have been cloned: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. Initially, STAT proteins were discovered by the studies of signaling cascades of cytokine receptors for interferons and IL-6. Cytokine receptors have no intrinsic tyrosine kinase activity and its tyrosine kinase activity is activated by receptor-associated cytosolic Janus kinase (Jak) family kinases. Upon stimulation of cytokines or growth factors, their cognate receptors undergo dimerization and stimulate Jaks. The activated Jaks subsequently recruit and phosphorylate STAT proteins and lead to the homo- or hetero-dimerization of STAT proteins. The activated STAT dimers translocate into the nucleus and induce gene transcription. The regulation of STAT proteins (particularly STAT3) is linked to inflammation and inflammatory-related tumorigenesis. Using genetic modified mice, it was found that the intestinal epithelial-cell-specific STAT3 ablation increases the susceptibility of the animals to chemically-induced epithelial damage and mucosal inflammation. In contrast, STAT3 hyperactivation promotes tumor incidence and growth [41]. STAT3 is constitutively active in several human cancer cells and tumor-associated leukocytes and it is important for cell proliferation and survival [42]. It has been documented that STAT3 activation in tumor cells enhances the ability of the tumors to evade the immune system by suppressing immune responses [43]. KSHV-encoded G protein-coupled chemokine receptor (KSHV-GPCR) constitutively activates the PLC/PKC signaling pathway, leading to constitutive phosphorylation of STAT3 at Tyr^{705} and cell transformation [44].

The lessons for viral GPCRs suggest that STAT3 can be activated by native GPCRs upon ligand binding (Table 9.2). Early studies showed that the activation of angiotensin II receptor (AT_1R) leads to the phosphorylation of STAT1, 2 and 3 [45,46]. Since then, ample evidence on GPCR-induced STAT3 activation has been reported. Stimulation of GPCRs, including adrenergic, bradykinin, chemokines, chemoattractants, histamine, prostacyclin, somatostatin and thrombin receptors, results in STAT3 phosphorylation and its transcriptional activation (Table 9.2). Jaks (Jak2/3 and Tyk) are now known to be involved in GPCR-mediated STAT3 activation in additional to cytokine receptor-mediated responses. STAT3 activation induced by adrenoceptor, BK_2R, C5aR, H_1R, hIPR and SST_4R is mediated through MAPK pathways. GPCR-induced STAT3 activation triggers gene expressions. Activation of α_1- or β-adrenoceptor results in the induction of IL-6 expression in fibroblasts [47–49]. Moreover, treatment of angiotensin II leads to the release of IL-18Ra, tissue inhibitor of metalloprotease-1 (TIMP-1), matrix metallopeptidase 2 (MMP2) and vascular endothelial growth factor (VEGF) in a STAT3-dependent manner in various cell types [46,50–54]. GPCR-induced up-regulation of other inflammatory mediators, such as antimicrobial peptide human β-defensin-3 (hBD-3), IL-13, TNF-α, iNOS and MMP9, has been reported and the production of these mediators are dependent on STAT3 activity (Table 9.2).

As the immediate signaling partners of GPCRs, G proteins and their activation are essential for the regulation of various STAT isoforms. All four families of Gα subunits can activate STAT3 through multiple downstream molecules [18,55–57]. MAPKs (ERK, JNK) and Src tyrosine kinases appear to be required for STAT3 phosphorylation induced by all four families of Gα subunits. Jak is important for $G\alpha_s$-, $G\alpha_{16}$- and $G\alpha_{12}$-induced STAT3 activation whereas PI3K is involved in $G\alpha_s$ and $G\alpha_{12}$-activated STAT3 activity. Other signaling molecules, such as Rap, Ral, PKA, Rac1, PLCβ, PKC, CaMKII and PDGFα, constitute of a complex signaling network for G protein-mediated STAT3 activation [13] (Fig. 9.1).

STAT1 activity is known to oppose STAT3-dependent pro-carcinogenic inflammatory responses. The differential regulation of STAT proteins in immunity suggests that STAT proteins are potential therapeutic targets for anti-tumor drug development. Regulations of STAT1 activity by G protein signaling have been observed as well. In the human neuroblastoma SH-SY5Y cell line, IL-6 induces the expression of the G_i-coupled μ-opioid receptor and this up-regulation is dependent on STAT1 and STAT3, but not NFκB [58]. GPCR activation by specific ligands can result in an up-regulation of STAT1 activity. It has been shown that stimulation of prostacyclin, histamine and chemokine receptors triggers the activation of STAT1 [59–61]. In addition, the constitutively active $G\alpha_{16}QL$ stimulates STAT1 phosphorylation as well as STAT1-dependent c-fos gene transcription in HEK293 cells [56]. Multiple signaling intermediates are required for $G\alpha_{16}QL$-mediated STAT1 activation including PLCβ, c-Src, Jak and ERK. Treatment of lysophosphatidic acid (LPA) inhibits epidermal growth factor-mediated STAT1 activation and the inhibitory effect is dependent on PKC. GPCR agonists such as bradykinin and ATP also elicit similar inhibitory effects on STAT1 activation [62].

Table 9.2 Regulation of STAT3 activity by GPCRs

GPCR category	Receptor subtype	Coupled G proteins	Possible intermediates	Regulated STAT3 target genes	Cell/tissue type	References
Adrenergic	α_1	$G\alpha_q$		IL-6	Fibroblasts	[48]
	α_{1A}	$G\alpha_q$	p38, EGFR	–	PC12 cells	[141]
	β_2	$G\alpha_s$	PKA, c-Src, Raf-1, MEK1/2, Ras, JAK2/3, Rac1, JNK, PI3K	–	HEK293 cells	[56]
	β	$G\alpha_s$	cAMP	IL-6, C/EBPδ	Cardiac fibroblasts, myocytes	[47,49]
Angiotensin	AT_1	$G\alpha_q$	JAK2, Tyk2	IL-18Rα	VSMC cells	[45,46,53]
	AT_1	$G\alpha_q$		TIMP-1	Proximal tubular epithelial cells, fibroblasts	[51,54]
			JAK	MMP2, VEGF	Gastric cancer cells	[52]
Bradykinin	BK_2	$G\alpha_q$	Tyk2, ERK	–	Vascular endothelial cells	[142]
Chemokine	CXCR4	$G\alpha_{i/o}$	Jak2	–	Mesenchymal stem cells	[143]
	CCR1, CCR4, CCR5	$G\alpha_{i/o}$	Jak2/3	–	T cells	[61,144]
	CCR2B	$G\alpha_{i/o}$	Jak2	–	HEK293 cells	[145]
	CCR2	$G\alpha_{i/o}$			Murine peritoneal marcophages	[146]
	KSHV-GPCR, CXCR2	$G\alpha_{i/o}$	Jak2	–	NIH3T3, human microvascular endothelial cells	[44,147]

Chemoattractant	C5a	$G\alpha_{i/o}$	Jak2, MEK1/2, Raf-1, CaMKII, ERK	–	HEL, HEK293 cells, neutrophils	[57,95,148]
	fMLP	$G\alpha_{i/o}$	Jak, Raf-1	–	HEK293 cells, neutrophils	[148,149]
Histamine	H_1R	$G\alpha_q$	Jak, ERK	hBD-3	Keratinocytes	[150]
	H_1R, H_2R	$G\alpha_s$, $G\alpha_q$	Jak2/3	IL-13	T helper cells	[151]
Prostacyclin	hIP	$G\alpha_s$	PLC, PKC, c-Src, Jak, ERK, JNK	–	HEL cells	[95]
Somatostatin	SST_4	$G\alpha_{i/o}$	ERK	–	CHO cells	[152]
Thrombin			Jak2	TNF-α, iNOS	Rat microglia	[153]
	PAR1	$G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_{13}$	Jak	MMP9	Human dermal fibroblasts	[154]

9.4 Interaction Between NF-κB and STAT3 in Inflammatory Responses

Both NFκB and STAT3 have been shown to play central roles in inflammation and inflammation-related tumorigenesis and anti-tumor immunity by regulating the expression of an overlapping subset of genes that are involved in proliferation, survival, angiogenesis and invasion. Signal integration between the two transcription factors has been documented. STAT3 promotes NFκB nuclear accumulation by p300-mediated acetylation of RelA in cancer cells and tumor-associated hematopoietic cells [63]. Numerous pro-inflammatory and oncogenic genes can be induced by both STAT3- and NFκB-dependent transcription. Pro-inflammatory mediators which are downstream of NFκB, such as COX-2, IL-6, IL-11, IL-17 and IL-23, can lead to activation of Jak/STAT pathways and feed forward cancer inflammation [64]. STAT3 inhibits the expression of NFκB-dependent gene transcription in immune responses that regulate the microbial infections and tumor growth [42]. In tumor associated macrophages, STAT3 inhibits NFκB-induced anti-carcinogenic cytokine (IL-12) expression and induces the production of pro-carcinogenic cytokine, IL-23 [43]. STAT3 interacts with NFκB to inhibit NFκB activity and suppresses the IL-1β- or LPS/IFN-γ-mediated iNOS promoter in mesangial cells [65].

9.5 Other Transcription Factors Regulated by Heterotrimeric G Proteins

In addition to NFκB and STAT activation, other transcription factors are also involved in the regulation of inflammatory responses in various cell types such as cyclic AMP-responsive element binding protein (CREB), activator protein-1 (AP-1) and nuclear factor of activated T-cells (NFAT). CREB has been implicated in asthmatic inflammation, ischemic brain inflammation and cancer development. CREB activity can be modulated by cAMP and PKA. Activation of $G\alpha_s$ stimulates adenylyl cyclases and produces cAMP to activate PKA which phosphorylates CREB at Ser^{133} in the nucleus. Ser^{133} phosphorylation of CREB is critical for its transcriptional activity and can be stimulated by MAPK pathways as well [66]. The phosphorylated CREB in turn binds to p300/CREB-binding protein (CBP) and modulates gene transcription [67]. $G\alpha_s$-mediated CREB activation is important for the development of endocrine tumors. In pituitary somatotroph cells, transfection of activated $G\alpha_s$ stimulates CREB phosphorylation as well as transcriptional activation. In H1299 human lung cancer cells, over-expression of constitutively active $G\alpha_s$ results in an increase in CREB phosphorylation and transcriptional activation, as well as enhancement of γ-ray-induced Bak expression and modulation of apoptosis induced by H_2O_2 and γ-rays [68].

Table 9.3 Regulation of other inflammation-related transcription factors by GPCRs

GPCR category	Receptor subtype	Coupled G proteins	Possible intermediates	Regulated transcription factors	Regulated gene expression	Cell/tissue type	References
Adenosine			p38	CREB	–	Macrophages	[155]
	A_{2A}	$G\alpha_s$	cAMP, PKA	CREB	–	Monocytes	[156]
	A_{2B}	$G\alpha_s$	cAMP, PKA, p38	CREB	–	CHO cells	[157]
	A_3	$G\alpha_{i/o}$	PI3K, ERK	CREB	Bcl-2, Bad	Rat heart	[158,159]
Adrenergic	β_2	$G\alpha_s$	–	AP-1, C/EBP, CREB	IL-6	Cardiac fibroblasts	[160]
	β_3	$G\alpha_s$	p38, PKC	CREB, ATF-2	–	Adipocytes	[75]
	β_2	$G\alpha_s$	mAKAP, RyR2, PKA, Calcineurin	NFATc	–	Cardiac myocytes	[80]
	β	$G\alpha_s$	PKA, p38,	AP-1	IL-6, IL-11	Osteblasts	[161]
Angiotensin			Ca^{2+}	NFAT	–	VSMC cells	[81]
	AT_1	$G\alpha_q$	–	CREB	BKB2R	Mouse inner medullary collecting duct cells, VSMC cells	[162,163]
Bradykinin	BK_2	$G\alpha_q$	Ca^{2+}, CaMKII, PKC	CREB	COX-2	SH-SY5Y neuroblastoma, human ASM cells	[164,165]
Bombesin		$G\alpha_q$	Ca^{2+}, calcineurin	NFAT	COX-2	Colon carcinoma	[166]
			Ca^{2+}, ERK, p38	AP-1	COX-2	Intestinal epithelial cells	[167]

(continued)

Table 9.3 (continued)

GPCR category	Receptor subtype	Coupled G proteins	Possible intermediates	Regulated transcription factors	Regulated gene expression	Cell/tissue type	References
Chemokine	US28, ORF74	$G\alpha_q$, $G\alpha_{12}$	PLC, PKC, calcineurin, p38, MEK1	CREB, NFAT	–		[72]
	KSHV-GPCR	$G\alpha_q$, $G\alpha_{13}$	–	AP-1	bFGF	NIH3T3 cells	[27]
	CXCR4	$G\alpha_{i/o}$	–	CREB	Mcl-1	Megakaryocytic cells	[168]
	CXCR4	$G\alpha_{i/o}$	PI3K, PKC, p38, ERK	CREB	Tac1	Non-tumorigenic breast cells	[169]
	CXCR4	$G\alpha_{i/o}$	ERK	AP-1	MMP13	Basal cell carcinoma, chondrocytes, LHSCC	[170–172]
	CCR5	$G\alpha_{i/o}$	–	NFAT	IL-2	T cells	[173]
Chemoattractant	C5a	$G\alpha_{i/o}$	PI3K, ERK	CREB	Bcl-2	Neutrophils	[174]
	C5a	$G\alpha_{i/o}$	JNK, p38	AP-1	Oncostatin M	Macrophages, monocytes	[175]
	fMLP	$G\alpha_{i/o}$	MEK, calcineurin	NFAT	MIP-1β, MCP-1	Human mast cells	[176]
	BLT	$G\alpha_{i/o}$	PI3K, ERK	–	Mcl-1, Bax	Neutrophils	[177]
			–	AP-1	–	Monocytes	[178]

Histamine	H_1R	$G\alpha_q$	Jak, ERK	AP-1	hBD-3	Keratinocytes	[150]
	H_1R	$G\alpha_q$	–	NFAT	MCP-1, IL-6	HUVEC	[179]
Neuropeptide Y	Y_1R	$G\alpha_{i/o}$	JNK, p38	ATF-2	–	SK-N-MC cells	[76]
Platelet-activating factor		$G\alpha_{i/o}$, $G\alpha_q$	–	CREB	MMP2, MT1-MMP	Melanoma cells	[70]
Prostaglandin	EP2	$G\alpha_s$	cAMP, PKA	CREB	COX-2	Pancreatic cancer cells, keratinocyte, MDCK cells	[180–182]
Somatostatin	SST_1	$G\alpha_{i/o}$	–	CREB	Calcitonin	Medullary thyroid carcinoma	[183]
Thrombin		$G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_{13}$	Ca^{2+}	NFAT	–	VSMC cells	[81]
			p38	ATF-2	IL-8, MCP-1	HUVEC	[184]
Thromboxane	TXA_2	$G\alpha_q$, $G\alpha_{13}$	PKC	AP-1	MCP-1	HUVEC	[140]

In fact, other GPCRs and Gα subfamilies have also been demonstrated to trigger CREB activation and regulate the gene transcription (Table 9.3). In rat intestinal epithelial cell line, activation of $G\alpha_q$ stimulates COX-2- and CREB-dependent transcriptional activity via p21-activated kinase/MAPK kinase kinase 6/p38 signaling cascade [69]. Stimulation of G_q-coupled PAF receptor induces MMP2 and membrane type 1-MMP release in CREB-dependent pathway in metastatic melanoma cells [70]. HCMV encodes four GPCRs (US27, US28, UL33 and UL78) that show high homology to human chemokine receptors. The viral chemokine receptor US28 exhibits constitutive activity and has been shown to induce cell transformation and production of VEGF via $G\alpha_q$, Gβγ, p38 and ERK in NIH3T3 cells [71]. It has also been found that viral chemokine receptors US28 from human cytomegalovirus and ORF74 from human herpesvirus 8 are constitutively active. Both receptors stimulate CREB and NFAT activation through multiple intermediates including $G\alpha_i$, PLC, PKC, calcineurin, p38 and MEK1 [72].

The transcription factor activator protein 1 (AP-1) is a heterodimer composed of c-jun, c-fos, and activating transcription factor (ATF) subfamilies. A number of GPCR ligands have been reported to activate AP-1 activity and induce release of pro-inflammatory mediators (Table 9.3). ATF-2 is activated by inflammatory stimuli via JNK and p38 MAPK pathways [73]. In ATF-2 mutant mice, a reduction in adhesion molecules and cytokine production is observed upon the addition of lipopolysaccharide (LPS), anti-CD3 antibody or coxsackievirus B3 infection [74]. GPCR-induced ATF-2 activation has been reported. G_s-coupled β_3-adrenergic receptor-induced IL-6 production is dependent on p38, PKC signaling and via activation of transcription factors CREB and ATF-2 in adipocytes [75]. Co-treatment of G_i-coupled neuropeptide Y_1 and G_q-linked muscarinic acetylcholine M_1 receptors leads to an additive effect on ATF-2 phosphorylation in SK-N-MC cells [76].

NFAT, is a family of important transcription factors, which regulates the expressions of inflammatory genes. Cytosolic NFAT is activated through dephosphorylation by calcineurin and activated NFAT translocates into the nucleus [77,78]. Immunosuppressive drugs such as FK506 and cyclosporine A are calcineurin inhibitors and they can suppress NFAT activity [79]. Regulation of NFAT activity by heterotrimeric G proteins has been documented (Table 9.3). Stimulation of G_s-coupled β_2-adrenergic receptor induces NFATc activation via PKA and calcineurin [80]. In vascular smooth muscle cells, thrombin and angiotensin II stimulate NFAT-dependent transcriptional activity. Co-stimulation of G_q-coupled receptor agonists and platelet derived growth factor-BB results in a synergism in NFAT activation via Ca^{2+} [81]. Bacterial superantigens, such as Staphylococcal enterotoxin, activate $G\alpha_{11}$ to trigger activation of PLCβ/PKC, Ca^{2+} mobilization, ERK1/2 activation, translocation of NFAT and NFκB and IL-2 production in human primary T cells [82]. $G\alpha_{12/13}$-mediated NFAT activation has been demonstrated in cardiac fibroblasts [83,84]. The involvement of Gβγ complex in the regulation of Wnt/Frizzled-mediated NFAT activity has also been reported [85,86].

9.6 Functional Impacts of Signal Integration

At the inflammation sites or tumors, cells are exposed to multiple exogenous stimuli. Extensive intracellular signal integrations are expected to occur in response to the inflammatory stimuli or during cancer development. Cross-talks between signals from different Gα subfamilies are apparently important for the regulations of inflammation-related transcriptional activities. An interesting demonstration of such signal integration has been performed in COS-7 cells co-expressing G_s-linked dopamine D_1 receptor (D_1R) and G_q-linked gastrin-releasing peptide-preferring receptor (GRPR). Despite that D_1R or GRPR agonist alone induces JNK activity, D_1R activation results in an inhibitory effect on JNK activity triggered by GRPR stimulation [87]. In human SK-N-MC neuroepithelioma cells, co-stimulation of endogenous G_i-coupled neuropeptide Y_1 and G_q-linked muscarinic acetylcholine M_1 receptors triggers a synergistic activation of ERK and CREB phosphorylation [76]. Sphingosine 1-phosphate synergistically potentiates thrombin-activated tissue factor expression in endothelial cells via NFκB activation and the induction of Egr-1 expression [88]. In Jurkat T cells, activation of G_i-coupled CXCR4 potentiates the activation of Egr-3 induced by G_s-coupled β_2-adrenergic receptor or G_q-coupled platelet-activating factor receptor [89]. Cooperative effects between receptor tyrosine kinases and GPCRs have also been demonstrated [90]. Co-stimulation of EGF and G_i-coupled receptor results in a synergistic JNK activation which involve Src, PI3K, Ca^{2+}/calmodulin and Rac [91]. Simultaneous applications of prostaglandin E2 and TNF-α synergistically triggers the expression of amphiregulin and promotes the growth and migration of colon cancer cells [92]. In NIH3T3 fibroblast cells, G_i-coupled somatostatin receptor subtype 2 (SST_2R) potentiates cell apoptosis induced by TNF-α through upregulation of TNF-α receptor expression and enhancing TNF-α-mediated downstream signaling including NFκB and caspase activation as well as JNK inhibition [93].

Some GPCRs can in fact couple to multiple G proteins to trigger diverse signaling events. Human monocytic THP-1 cells express CCR1, $G\alpha_{14}$ and $G\alpha_{16}$ endogenously. Although CCR1 is known as a $G_{i/o}$-coupled receptor, Lkn-1-induced NFκB activation is insensitive to pertussis toxin pretreatment, suggesting that CCR1 may couple to $G\alpha_{14}$ and $G\alpha_{16}$ to trigger NFκB activation [94]. Stimulation of human prostacyclin receptor activates $G\alpha_s$ and $G\alpha_q$ proteins simultaneously to induce STAT3 phosphorylation and transcriptional activation in HEL cells [95]. In HEK293T cells, BK_2R-activated ERK2 and transcriptional activity of Elk-1 are dependent on $G\alpha_q$-mediated PKC and $G\alpha_i$-driven Ras activation [96].

Receptor cooperativity in inflammatory responses has also been documented. Coexpression of protease-activated receptor 2 (PAR2) and Toll-like receptor 4 complex results in a synergistic activation of NFκB-mediated inflammatory response in HEK293 cells [97]. In human colon epithelial cell SW620, a synergistic IL-8 production is observed upon co-stimulation with PAR2 agonist and LPS [97]. G_q-coupled $P2Y_6$ receptor activation potentiates LPS-stimulated IκB phosphorylation and degradation and NFκB activation in murine J774 macrophages [98].

9.7 Future Perspectives

Regulations of NFκB and STAT signaling pathways clearly play pivotal roles in inflammatory diseases and tumor promotion. Recently, several inhibitors targeting on IKK/NFκB and STAT3 have been developed to increase the efficacy of conventional anti-tumor therapies and showed promising results in pre-clinical models [99,100]. It will be exciting to know whether these inhibitors can usher new avenues for treating inflammatory diseases and cancer without inducing severe side effects in the clinical trials. A rather unexpected fact is that mutation on the genes encoding NFκB and STAT have not been found in tumors, whereas naturally occurring mutations on GPCRs as well as Gα subunits have already been identified in different human cancers. Many GPCRs are overexpressed in different cancer cells and may facilitate tumor formation upon activation by specific ligands. Infections by a number of viruses which encode constitutively active GPCRs can propel the development of various cancers [101]. Further studies on the transcriptional regulations by GPCR-G protein axis of signals will provide invaluable clues to delineate the physiological consequences caused by over-reactive GPCRs or G proteins, or those pathogenic viral infections which express cytokines, inflammatory mediators and constitutively active GPCRs. Accumulating evidence suggests that the activation of GPCRs causes autocrine- or paracrine-based chronic stimulation of various transcription factors to promote cancer formation (Fig. 9.1). Thorough investigations on the signaling network between GPCRs and transcription factors will ultimately lead to a better understanding on tumor development and facilitate the discovery of target-specific anti-inflammatory and anti-cancer drugs.

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Chapter 10
Molecular Cross-Talk Between Nuclear Receptors and Nuclear Factor-κB

Ilse M.E. Beck, Guy Haegeman, and Karolien De Bosscher

Abstract Nuclear receptors can function as ligand-activated transcription factors but can even so cross-talk with other transcription factors. In this respect, NF-κB, a central regulator of both inflammation and tumorigenesis, can cross-react with and is negatively affected by these nuclear receptors. In current medicine, the nuclear receptor ligands for the glucocorticoid receptor form still the mainstay for treatment of inflammation-based afflictions. However, also other nuclear receptor ligands can affect inflammatory processes. In this respect, the cross-talk of various nuclear receptors with each other has been given renewed attention in recent literature. We will discuss the cross-talk of nuclear receptors with NF-κB and each other in the context of the attenuating control of inflammatory and tumor-promoting mechanisms, using the well described glucocorticoid receptor as a focal point.

Keywords Inflammation • NF-κB • Nuclear receptor (NR) • Cross-talk • Glucocorticoid receptor (GR) • Peroxisome proliferator-activated receptor (PPAR) • Estrogen receptor (ER) • Androgen receptor (AR) • Progesterone receptor (PR) • Liver X receptor (LXR) • Vitamin D receptor (VDR) • Orphan receptor

Abbreviations

AF	Activation function
AMPK	AMP-activated protein kinase
AP-1	Activator protein-1
APOC3	Apolipoprotein C-III
ARE	Adenylate-uridylate (AU)-rich element
ATF	Activating transcription factor

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Bcl	B-cell lymphoma
Brg	Brahma-related gene
Brm	Brahma
C	Carboxy
cAMP	Cyclic adenosine monophosphate
CAR	Constitutive androstane receptor (NR1I3)
C/EBP	CCAAT enhancer-binding protein
CBP	CREB-binding protein
CC10	Clara cell secretory 10 kDa protein
cdc37	Cell division cycle 37 protein
Cdk	Cyclin-dependent kinase
c-FLIP	Cellular-FLICE inhibitory protein
ChIP	Chromatin immunoprecipitation
CK2	Casein kinase 2
COUP-TFII	Chicken ovalbumin upstream promoter-transcription factor II (NR2F2)
COX-2	Cyclo-oxygenase-2
CREB	cAMP-responsive element-binding protein
CRM1	Chromosome region maintenance, synonym: exportin1
Cyp3a4	Cytochrome P450, subfamily IIIA, polypeptide 4
DBD	DNA-binding domain
Dexras1	DEX-induced Ras1
Dok-1	Downstream of tyrosine kinase 1
DRIP205	Vitamin D receptor-interacting protein complex component (MED1)
DUSP	Dual specificity phosphatase
eNOS	Endothelial nitric oxide synthetase
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor (NR3A1, NR3A2)
ERE	Estrogen response elemnt
ERK	Extracellular signal-regulated kinase
ERR	Estrogen-related receptor (NR3B1, NR3B2, NR3B3)
ELKS	Protein rich in amino acids E, L, K and S
FKBP	FK506-binding protein
FXR	Farnesoid X receptor (NR1H4)
GC	Glucocorticoid
GILZ	GC-induced leucine zipper
GR	Glucocorticoid receptor (NR3C1)
GRE	Glucocorticoid response element
H3	Histone H3
H4	Histone H4
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HNF-4	Hepatocyte nuclear factor-4 (NR2A1, NR2A2)
Hsp	Heat shock protein
ICAM	Intercellular adhesion molecule
Ifit1	Interferon-induced with tetratricopeptide repeats 1

IFN	Interferon
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthetase
IP-10	Interferon-inducible protein of 10 kDa
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinase
KO	Knock-out
LBD	Ligand-binding domain
LPS	Lipopolysaccharide
LXR	Liver X receptor (NR1H2, NR1H3)
MAPK	Mitogen-activated protein kinase
MEKK	MKK kinase, synonyms: MKKK, MAPKKK, MAP3K
MHC	Major histocompatibility complex
MK	MAPK-activated protein kinase
MKK	MAPK kinase, synonyms: MEK, MAPKK, MAP2K
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
MR	Mineralocorticoid receptor (NR3C2)
MSK	Mitogen-and stress-activated protein kinase
NCoR	Nuclear corepressor
NEMO	NF-κB essential modulator, synonym: IKKγ
NGFIB	Nerve Growth factor IB (NR4A1)
NF-κB	Nuclear Factor-κB
NIK	NF-κB-inducing kinase
nGRE	negative GRE
NLS	Nuclear localization signal
NOR1	Neuron-derived orphan receptor 1 (NR4A3)
NR	Nuclear receptor
Nurr1	Nuclear receptor related 1 (NR4A2)
PAI-1	Plasminogen activator inhibitor type 1
PGC-1	PPARγ coactivator-1
Pin1	Protein NIMA(never in mitosis gene a)-interacting
PKA	Protein kinase A
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor-α (NR1C1, NR1C2, NR1C3)
PR	Progesterone receptor (NR3C3)
P-TEFb	Positive transcription elongation factor b
PXR	Pregnane X receptor (NR1I2)
RA	Retinoic acid
RANKL	Receptor activator of NF-κB ligand
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RIP	Receptor-interacting protein

Rel-HD	Rel homology domain
RNA Pol II	RNA polymerase II
SGK	Serum and glucocorticoid-inducible kinase
SHP	Small heterodimer partner (NR0B2)
SLAP	Src-like adaptor protein
SLPI	Secretory leukocyte protease inhibitor 1
SMRT	Silencing mediator for retinoid and thyroid-hormone receptors
SOCS	Suppressor of cytokine signalling
SP-A	Surfactant protein A
SRC	Steroid receptor coactivator
SUMO	Small ubiquitin-related modifier
SWI/SNF-	Switching of yeast mating type/sucrose non-fermenting
TA	Transactivation domain
TAK1	TGF-activated kinase 1
TAB2/3	TAK-binding protein
TANK	TRAF family member-associated NF-κB activator
TBK1	TANK-binding kinase 1
TLR	Toll-like receptor
TNF	Tumor necrosis factor-α
TNF-R	TNF-receptor
TR	Thyroid hormone receptor (NR1A1, NR1A2)
TRADD	TNF-R-associated death domain
TRAF	TNF-R-associated factor
Trip6	Thyroid receptor-interacting protein 6
TTP	Tristetraprolin
VCAM	Vascular cell adhesion molecule
VDR	Vitamin D receptor (NR1I1)
ZDF rat	Zucker diabetic fatty rat

10.1 Introduction

In ancient times, inflammation was described by its typical characteristics: rubor/redness, dolor/pain, calor/heat, tumor/swelling, ultimately leading to loss of function of the organ or tissue. Although inflammation serves an inherent advantageous purpose, i.e. removing damaging agents and restoring tissue structure and function, a rapid clearance of the inflammation is advisable, as the unfavourable chronic inflammation harms the body. Furthermore, inflammation has also been described to play a role in the ontogenesis of cancer and cardiovascular diseases [1–4].

In 1935, Kendall and Reichstein isolated and identified the natural ligand for the glucocorticoid receptor (GR), cortisone [5], although the glucocorticoid receptor itself was not cloned until 1985 [6]. More than a decade later, steroidal hormones were acknowledged for their anti-inflammatory activities, more specifically in

rheumatoid arthritis [5]. As such, hydrocortisone and other glucocorticoids (GCs) effectively suppress the immune system and halt inflammation-associated symptoms, but these exogenously administered GCs also display marked pleiotropic effects in the regulation of protein, lipid and carbohydrate metabolism, stress homeostatic regulation, reproductive processes, growth and brain functions such as memory and behaviour [7–9]. These widespread effects of GCs lie at the basis of the feared and detrimental side effect profile of a chronic therapy with GCs, comprising osteoporosis, diabetes, cataracts, a fat redistribution leading to a typical moon face and hunchback, skin thinning and muscle wasting and emotional instability [10, 11].

Despite these adverse effects, GCs still remain the preferred treatment to combat acute inflammatory disorders and chronic autoimmune and inflammatory afflictions (e.g. rheumatoid arthritis, asthma, systemic lupus erythematosis, inflammatory bowel disease, …), to suppress the immune system and thus prevent graft rejection of transplant patients [12, 13]. Additionally, because of their apoptosis-modulating abilities, GCs are also applied since a long time in the treatment of certain lymphomas [14–18].

10.2 Nuclear Factor-κB (NF-κB): A Central Player

On a molecular level, the transcription factor nuclear factor κB (NF-κB) plays a pivotal role in the onset and propagation of inflammation, and also in cancerogenesis [19–21, 22–29]. A rigorous knowledge of how this transcription factor can affect inflammation and cancer on a molecular level, is thus key to understand the windows of opportunity via which nuclear receptors intervene to halt NF-κB activation and activity.

In an inflammatory context, the heterodimeric transcription factor NF-κB can drive the transcription of cytokines, chemokines, growth factors, lipid-derived mediators, cell adhesion molecules and peptides. In turn, these targets can fuel the inflammatory loop by once again activating NF-κB, and selectively drive inflammatory processes such as localized hyperaemia, exudation of plasma, diapedesis or leukocyte migration, or containing the inflamed site by fibrosis [30–33]. Moreover, NF-κB is also an important player in tumorigenesis [28]. The activation of NF-κB can indeed initiate the transcription of genes coding for anti-apoptotic proteins, e.g. c-FLIP and Bcl-x_L; and growth factors; e.g. VEGF [1–4]. Futhermore, NF-κB's ability to activate the promoter of interleukin-6 (IL6) is deemed an essential characteristic for its role in cancerogenesis [34, 35].

The transcription factor family of NF-κB comprises five members and is characterized by a N-terminal Rel-homology domain (Rel-HD), present in all five members, which is responsible for the proteins' DNA binding, dimerization and interaction with IκB (inhibitor of NF-κB). The NF-κB p65 (RelA), RelB and c-Rel contain a C-terminal transactivation domain, whereas NF-κB1 (p50/p105) and NF-κB2 (p52/p100) do not. The latter two can be proteasomally cleaved to yield

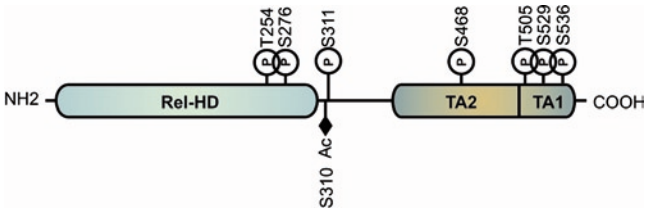

Fig. 10.1 Structural properties of NF-κB p65. The posttranslational modification sites for phosphorylation and acetylation are indicated. Abbreviations: Rel-HD, Rel-homology domain; TA, transactivation domain; P, phosphorylation; Ac, acetylation

NF-κB p50 and NF-κB p52, respectively. However, NF-κB1 nor NF-κB2 contains a transactivation domain. Of these five NF-κB transcription factors, the heterodimer NF-κB p65-p50 is most commonly researched, in particular the transactivation domain-containing NF-κB p65 (Fig. 10.1) [33, 36, 37].

The classical activation of NF-κB can be triggered by a wide range of stimuli, among which cytokines, viruses, oxidative stress, phorbol esters, lipopolysaccharide and B- and T-lymphocyte activation [38–40]. However, these activation pathways ultimately all activate the IκB kinase (IKK) complex. The canonical or classical activation of the NF-κB p65-p50 dimer by tumor necrosis factor-α (TNF) is a widely used model for NF-κB activation (Fig. 10.2). Upon binding of TNF to its receptor, TNF-R, this receptor trimerizes and attracts adaptor proteins, such as TRADD, TRAF2 and MEKK3, which relay the activation signal to the activation of the IKK complex and the mitogen-activated protein kinase kinases (MKKs) [36, 37, 41, 42].

The unactivated NF-κB p65-p50 heterodimer, residing in the cytoplasm, is bound to the inhibitory IκB molecule, masking the NF-κB's nuclear localization signals (NLSs). The activated, i.e. phosphorylated IKK complex, can phosphorylate this IκB and thus target IκB for ubiquitination and 26S proteasomal degradation [36, 37, 41]. This IKK complex consists of the catalytically active IKKα and IKKβ and an IKKγ/NEMO scaffold, assisted by the transient complex members Hsp90, ELKS and cdc37 [43–46]. In particular, IKKβ can phosphorylate IκB [47, 48]. The subsequent degradation of IκB frees the NF-κB subunits, unmasking their NLSs and thus allowing transport of these proteins into the nucleus. Once activated, NF-κB can bind to specific recognition sequences (κB sites) in the DNA and as such control the transcriptional activity at the proximal transcription start site [36, 37]. As DNA is wound in chromatin, NF-κB-dependent transcription is also regulated by chromatin condensation/decondensation processes via either variable histone modifications (phosphorylation, acetylation, methylation,…) or ATP-dependent enzymes [33, 49–55].

The activation of the MKKs results in the phosphorylation and downstream activation of the mitogen-activated protein kinase (MAPKs): extracellular regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK); which in turn can activate another layer of kinases among which the mitogen- and stress-activated protein kinase (MSK) [42]. Interestingly, the MAPK cascade, and especially MSK, is

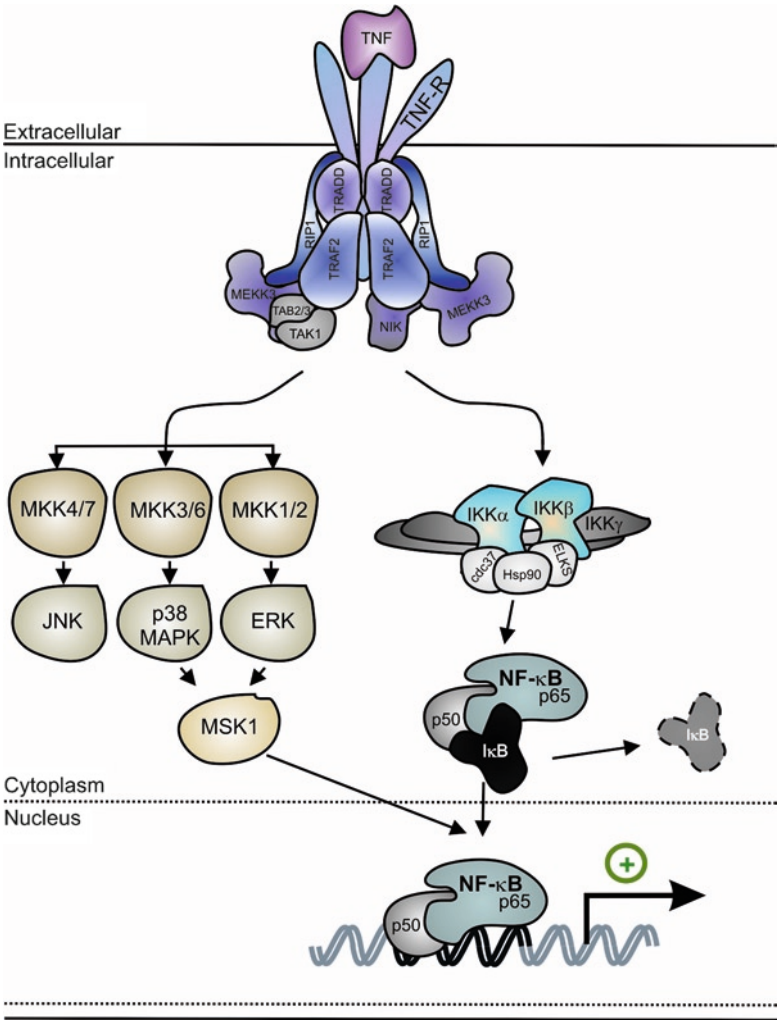

Fig. 10.2 TNF activation pathway: NF-κB and MAPK activation. TNF-R activation by TNF leads to the trimerization of the receptor and the subsequent recruitment of TRADD, RIP1 and TRAF2. Subsequently, via signaling through MEKK3, TAK1 and/or NIK, the liganded TNF-R can activate the IKK complex and various MAPK signaling cascades, culminating in the activation and modulation of NF-κB. Abbreviations: TNF, tumor necrosis factor; TNF-R, TNF-receptor; TRADD, TNF-receptor-associated death domain; TRAF, TNF-receptor-associated factor; RIP, receptor-interacting protein; TAK, TGFβ-activated kinase; TAB, TAK-binding protein; NIK, NF-κB-inducing kinase; MEKK, MAPK kinase kinase; MKK, MAPK kinase; JNK, c-Jun N-terminal kinase; MAPK, Mitogen-activated protein kinase; ERK, Extracellular signal-regulated kinase; MSK, Mitogen- and stress-activated protein kinase; NF-κB, Nuclear factor κB; cdc37, cell division cycle 37 protein; Hsp90, Heat shock protein 90; ELKS, Protein rich in amino acids E, L, K and S; IKK, IκB kinase; IκB, Inhibitor of NF-κB

involved in the posttranslational control of NF-κB [56–59]. Not only the MAPK cascade, but also a vast array of other kinases, such as IKKα, IKKβ, TBK1, CK2, PKCζ, and PKA, can phosphorylate various Ser residues of NF-κB p65 (Fig. 10.1), fine-tuning its activity (duration, intensity), location, DNA-binding status, and interactions with cofactors and IκB (reviewed in [60–62]). Furthermore, NF-κB p65 function can also be regulated by acetylation and SUMOylation [62–66]. Together these posttranslational modifications tightly modulate the cellular actions of NF-κB.

A plethora of literature indicates a mutually antagonistic cross-talk between NF-κB and nuclear receptors [67, 68]. In the following review article, we will discuss cross-talk mechanisms of several nuclear receptors, with an emphasis on the widely researched glucocorticoid receptor, with NF-κB signalling in the above discussed context.

10.3 Nuclear Receptors: The Road to Relief

The evolutionary conserved nuclear receptors (NRs) comprise a superfamily of ligand-dependent transcription factors, which are divided into subgroups on the basis of their ontogeny [69]. These cytoplasmic and nuclear receptors can be activated by their specific ligands: steroid hormones (such as glucocorticoids, estrogens, progesterone, mineralocorticoids, androgens, vitamin D3, ecdysone, oxysterols and bile acids), retinoic acids, fatty acids and prostaglandins. Upon ligand binding and activation, these NRs form homo- or heterodimers and thus regulate specific gene transcription repression and activation via a variety of mechanisms. In short, nuclear receptors can either bind specific promoter DNA sequences (i.e. response elements), or either bind and affect the activity of other DNA-bound factors, such as NF-κB, activator protein-1 (AP-1), cAMP-responsive element-binding protein (CREB), interferon regulatory factor 3 (IRF3) or signal tranducer and activator of transcription (STAT), without direct binding of the NR to the DNA [70]. So far 48 NRs have been identified in man and these can be divided according to structure, ligand and ontogeny in seven subfamilies or classes. For most NRs, the ligands were identified and usually form an integrated part of its name. Interestingly, a subgroup of NRs of which the ligands have not yet been found, i.e. orphan NRs, exists, e.g. nerve growth factor IB (NGFIB) and nuclear receptor-related 1 (Nurr1). Advances in the ligand search for these orphan receptors has identified fatty acids as ligands for PPAR and oxysterols as ligands for LXR. However, for some of these orphan receptors, it was hypothesized that ligands for these NRs simply do not exist, as structural data showed a lack of ligand-binding pockets, and that these NRs thus possibly operate as ligand-independent transcription factors.

The research into the control of inflammation and cancer most prominently features the glucocorticoid receptor (GR) and some of its subgroup co-members. The GR, or NR3C1, belongs to class 3 of the nuclear receptors, together with the mineralocorticoid receptor (MR), the estrogen receptor (ER), the estrogen-related

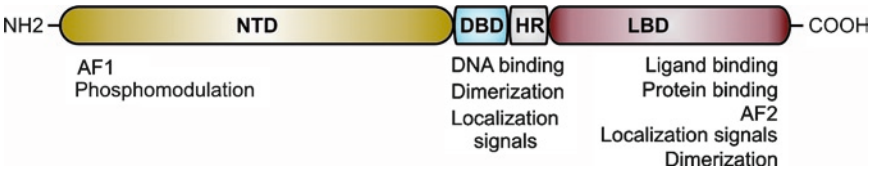

Fig. 10.3 Basic structure of nuclear receptors. The nuclear receptors comprise, read from N-terminal to C-terminal side, an N-terminal domain, a DNA-binding domain, hinge region and finally a ligand-binding domain. Below, the allocated functions are mentioned below the diagram. Abbreviations: NTD, N-terminal domain; AF, activation function; DBD, DNA-binding domain; HR, hinge region; LBD, ligand-binding domain

receptor (ERR), the progesterone receptor (PR), and the androgen receptor (AR) [71]. These NRs can interact with NF-κB and especially GR, AR and ER are researched in the combat against inflammation and cancer. Interestingly, also the fatty acid receptor peroxisome proliferator-activated receptor (PPAR) and the cholesterol sensing liver X receptors (LXR) have been given renewed attention in this inflammatory context [72, 73].

Nuclear receptors are characterized by a common structural organization (Fig. 10.3). The N-terminal domain contains a transactivation function AF-1, is most commonly targeted for posttranslational phosphorylations, and is highly variable among NRs. The adjacent DNA-binding domain (DBD) is implicated in NR dimerization and of course DNA binding via its D-loop zinc finger motifs. The C-terminal domain of the NRs share NLSs, a ligand-binding domain (LBD), protein binding sites and a second transactivation domain AF-2 [69, 71]. The activity of all NRs are regulated by posttranslational modifications affecting their localization, activity, half-life and interactions [60, 74–77].

Inactive Type I NRs, such as GR, MR, AR, PR and ER, are withheld in the cytoplasm in a ligand-receptive state, by their association with a chaperoning complex, which masks their NLSs. These chaperoning complexes can comprise Hsp90, Hsp70 and a plethora of immunophilins, such as FKBP51, FKBP52 or cyclophilin 40. However, in one cell not all GR-chaperoning complexes are to be considered identical, adding yet another layer of complexity onto the NR regulatory mechanisms [78]. These cytoplasmic NRs need a ligand stimulus to change conformation, shed their chaperoning complex and subsequently travel into the nucleus. However, neither the unactivated cytoplasmic state, neither the activated nuclear state should be considered as a fixed condition. NRs are highly dynamic in space and time and both liganded and unliganded NRs can shuttle rapidly between cytoplasm and nucleus [79–85]. Furthermore, the GR is constantly in motion even within the nucleus, constantly sensing the changing cellular environment [86–90]. In the nucleus, these receptors can bind onto their specific hormone recognition DNA sequences, affect transcription via binding or tethering onto other DNA-bound transcription factors, or affect signalling cascades which operate upstream in the transcription factor-activating machinery of e.g. NF-κB (Fig. 10.4) [60, 70, 91, 92].

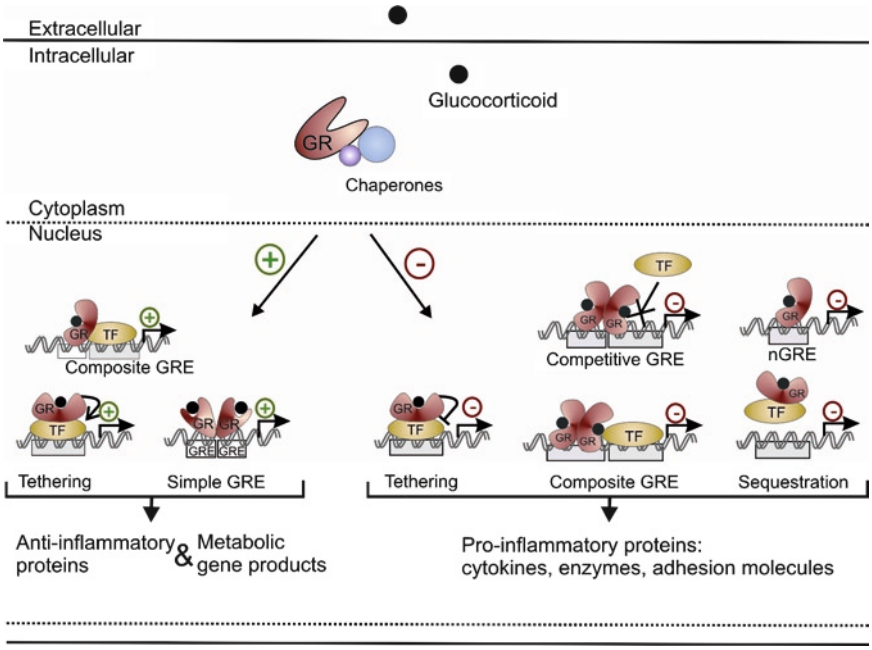

Fig. 10.4 Glucocorticoid receptor activation and repression mechanisms. The unliganded, unactivated GR resides in the cytoplasm. Its complexation with chaperone proteins keeps the GR in a ligand-receptive state. The GC-acivated GR translocates into the nucleus where it can give rise to positive and negative transcriptional effects via a variety of mechanisms. GC-mediated promoter activation can originate from the DNA binding of a GR homodimer on a GRE, from a GR:transcription factor tethering mechanism or from a coordinated DNA binding of a GR:transcription factor complex onto a so-called composite GRE. The latter two mechanisms can also mediate GC-regulated negative transcriptional effects. Furthermore, GCs can prevent or halt transcription via competition or sequestration mechanisms or possibly also, via direct DNA binding of a GR monomer on a negative GRE (nGRE). Abbreviations: GR, glucocorticoid receptor; GC, glucocorticoid; GRE, GC response element; nGRE, negative GRE, TF, transcription factor

As MR and GR have a close phylogenetic relation, it is not surprising that MR can be activated by both GCs and mineralocorticoids. Furthermore, both GR and MR can bind to the same response elements [93]. Although GCs can also occupy the ligand-binding pocket of MR, interference of GCs in MR signalling is limited due to their differential binding affinities and MR's topical restriction. Namely, whereas GR is ubiquitously expressed, MR is known to be expressed only in the epithelial cells of kidney, salivary glands and colon, and non-epithelial cells of brain and heart [94–96]. Furthermore, 11beta-hydroxysteroid dehydrogenase 2, which can metabolize cortisol to the inactive metabolite cortisone, is present in these typical mineralocorticoid target tissues [97]. Overall, activated GRs and MRs target a distinct set of genes [98].

Other nuclear receptors (type II and type III), such as retinoic acid receptors RAR/RXR and PPAR, are constitutively nuclear and bound to their DNA response element, regardless of the presence of their cognate ligands. In the unactived state,

interaction of these NRs with corepressors, e.g. silencing mediator for retinoid and thyroid-hormone receptors (SMRT), nuclear corepressor (NCoR) and histone deacetylases (HDACs), negatively controls their activity [99, 100]. Conversely, when ligands bind to these NRs, the consequent conformational change of the receptors invoke the derepression of this corepressor:NR complexes [101] and the subsequent attraction of coactivators with histone acetyl transferase (HAT) activity, such as cAMP-responsive element-binding protein (CBP), p300 and/or steroid receptor coactivators, SRC-1. This model in which corepressors are interchanged for coactivators upon receptor activation is denominated as the 'cofactor exchange' model [102, 103]. PPAR, like LXR and other NRs, can heterodimerize with the RXRs.

In recent years, the presence and regulation of the chromatin environment of DNA promoter sequences has been taken into account when researching the effects NRs can exert on their target promoters. In this respect, condensed chromatin is associated with a low transcription rate, while decondensed/relaxed chromatin constitutes a transcription-facilitating environment. Most often, the latter state also features histone phosphorylation, e.g. by MSK1 or IKKα, and acetylation, e.g by CBP or p300, in which the modulation of histone tails results in a relaxation of the chromatin [51, 104–110]. As such, the interaction of HDACs with the inactive PPARs or RAR/RXRs, and the interaction of the HAT-containing CBP with active NRs can be understood in this chromatin regulation template [109, 111].

All NRs use the plethora of nuclear corepressors and coactivators to implement and co-regulate their transcriptional effects. The corepressors or coactivators can bind onto the C-terminal LBD of the NRs via conserved LXXLL (X, any amino acid) motifs [112, 113]. The known coactivators which can be recruited by NRs are chromatin-modifying proteins (e.g. the ATP-dependent chromatin remodelling SWI/SNF-complex constisting of Brg-1 or Brm), members of the p160 family (e.g. SRC-1, SRC-2) and p300 or CBP, but also molecular scaffolds that allow the assembly of cofactor complexes (e.g. PPARγ-coactivator-1 (PGC-1)). Coactivator molecules such as CBP, p300 and SRC-1 modulate the activity of the transcription apparatus through their HAT activity [101, 109, 110, 112, 114]. Specificity in ligands and NRs is reflected in a preferred coactivators recruitment profile [115–119]. However, the distinction between corepressor and coactivators is in a cellular context not rigidly distinguished and thus a coactivator can function as a corepressor, depending on the cellular context [120, 121]. Therefore, it is more correct to talk about 'cofactors'.

Furthermore, multiple activating cofactors can be recruited in a combinatorial or cyclic manner [122–124]. For instance, ER recruitment onto the estrogen-stimulated pS2 promoter is preceded by p300 and SRC1 promoter association and histone acetylation. When observing longer time frames, a cyclic and dynamic recruitment of both receptor and coactivators can be observed for this ER-stimulated promoter [124]. In contrast, the AR-mediated PSA promoter stimulation is accompanied by a gradual increase and subsequent decrease of receptor and cofactor recruitment [122, 125]. The work of Hager et al. showed that GCs implemented a cyclic on/off promoter loading for the GR on a MMTV promoter [126]. The accessibility of GR to its promoter-imbedded binding sites can be GC-inducible or constitutive [90]. These cyclic recruitment mechanisms of both steroid hormone receptors and cofactors,

can be seen in the framework of the 'sensing' cell. By constantly scanning for changes in the presence of ligand, the quantity of receptor or cofactor, the posttranslational modulation of the NRs and cofactors the cell allows changes to be sensed rapidly, but also allows these changes to rapidly impact ongoing mechanisms.

In an inflammatory context, this rapid sensing for both the NR and NF-κB status can modulate their cross-reactions, as we will discuss below. This cross-talk of NRs with NF-κB can result in either a cooperative enhancement or a transrepression of gene expression. As ligand-activated NRs and activated transcription factors both reside in the nucleus, this cross-talk is deemed to be a nuclear event. However cytoplasmic NR-regulated events may also contribute to the NRs' interference with NF-κB function [127–129].

10.3.1 NF-κB and the Glucocorticoid Receptor, GR

The glucocorticoid receptor is transcribed from one gene, but hardly constitutes a homogeneous population. Cell cycle regulation of its transcription, alternative splicing, different transcription start sites and different translation start sites lie at the basis of a heterogeneous population of GRs [130–134]. The GRα is most commonly researched, but GR is actually expressed as a cohort of – from long to short – GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2 and GRα-D3, which all originate from different translation start sites. The alternatively spliced GRβ is considered a dominant-negative for GRα function and might play a role in GR resistance, in which the patient is refractory to GC therapy. Overall, different cells might express a distinct pattern of GR isoforms and these isoforms display different characteristics in e.g. localization, transactivation, transrepression and apoptosis-inducing capacities,… [74, 134, 135]. Taken together with the heterogenous population of phosphomodulated GRs and the diverse GR chaperoning complexes, these distinct expression patterns for GR might explain cell and tissue specificity of particular GR mechanisms and responses.

This 'diversification' story continues when contemplating the anti-inflammatory mechanisms of the GRs. This mechanism of GR consists of different layers, which have most likely not all been characterized at present. Currently, its anti-inflammatory multi-mechanism comprises the transactivation of promoters of inflammation-repressing proteins, the destabilization of pro-inflammatory mRNAs and the transrepression of NF-κB-dependent gene transcription of a variety of genes by several mechanisms [60, 67, 91, 92]. These mechanisms can be both target gene- and cell type-dependent.

10.3.1.1 Transactivation of Promoters of Inflammation-Repressing Proteins

The GR-mediated transcriptional activation can be regulated via different mechanisms. The binding of a homodimerized GR to an inverted repeat GC response

element (GRE), constitutes the classic paradigm for GR-mediated transactivation (Fig. 10.4) [136], but might comprise only a small part of all GC-induced transcriptions [92, 137–139]. Other known GR transactivation mechanisms rely on tethering mechanisms in which GR binds to another DNA-bound transcription factor, e.g. CREB and activating transcription factor 1 (ATF1) for glutamate synthetase gene expression [140, 141], without associating with DNA itself, or on composite GREs in which DNA-bound GR cooperates with a DNA-bound transcription factor to induce promoter activation, e.g. GR association with C/EBP promoting DUSP1 gene transcription (Fig. 10.4) [70, 92, 142–144]. Experiments with a GR dimerization mutant, GR*dim*, which is incapable of fuelling the classic GRE-mediated transactivation has sparked the general belief that GR transactivation mechanisms are predominantly responsible for the detrimental GC side effect profile, while GR transrepression mechanisms form the basis for the anti-inflammatory and NF-κB-repressing effects [11, 145, 146]. Recent advances, however, have nuanced this model: GR-invoked transrepression can also contribute to certain side effects and GR activation mechanism can also induce anti-inflammatory mechanisms [137, 146]. Yet, this dichotomous model still forms the basis for many investigations [145, 147].

Although many of the GR-transactivated genes, either via non-classical or via classical GRE mechanisms, play a role in the plethora of functions of the GR and thus possibly the GC-associated side effects, some of these transactivated genes have marked anti-inflammatory actions [137]. However, the contribution of these GR transactivated anti-inflammatory proteins to the total anti-inflammatory mechanism of GR, remains a matter of debate. Total translation inhibitors could in some cases diminish the NF-κB-repressing effects of activated GR, but could not completely abolish its negative interference with NF-κB-driven gene expression [148–155]. Most likely, the anti-inflammatory 'weight' of each GC-induced anti-inflammatory protein should be researched individually and cell-specifically.

In this regard, the GC-mediated upregulation of IκBα was discovered almost 15 years ago. Mechanistically, the TNF-depleted, cellular stock of IκBα is replenished by its GC-induced transcription, and this restocked IκBα should promote the dissociation of DNA-bound NF-κB p65 and expedite its subsequent return to the cytoplasm [128, 156, 157]. However, the upregulation of IκBα by GCs, and the concomitant sequestration mechanism, appears to occur quite cell-specifically [92, 148]. Moreover, GCs do not seem to affect the NF-κB occupancy at κB sites in ICAM or IL8 promoters [158–161].

Other GC-induced genes with noted anti-inflammatory functions are GILZ, DUSP1, lipocortin, SLPI-1, IL10, the IL1 receptor decoy type II, Dexras1, Dok-1, SLAP, p11/calpactin binding protein, thymosinβ-4-sulfoxide, CC10, β-adrenergic receptors, SOCS1, SGK1 and tristetraprolin (TTP) [137, 146, 162–166]. The implications of the induction of most of these genes, and their anti-inflammatory mechanisms have been recently reviewed in Clark et al. [137].

DUSP1, a dual specificity phosphatase, can dephosphorylate MAPKs at T and Y residues [167]. This dephosphorylation of especially p38 and JNK MAPKs and to a lesser extent ERK MAPK leads to their deactivation [167–171]. The expected decrease of p38 MAPK activity levels in GC-incubated cells, is challenged by the

apparently paradoxal finding that prolonged GC exposure of lymphoid cells can on the contrary induce p38 MAPK activation [172]. Further research into the cell specificity and implications of this matter for NF-κB function is warranted. Furthermore, also other GR-mediated mechanisms can target these kinases (see below).

As a consequence of the GC-induced DUSP1 production, the actions of this phosphatase can contribute to the GC-mediated transrepression of various pro-inflammatory genes [173, 174]. In that respect, DUSP1 knock-out (KO) mice display a weakened GR transrepression of inflammatory gene transcription. However, these mice retain their sensitivity to anti-inflammatory mechanisms, confirming that the GR anti-inflammatory mechanism works via multiple factors and pathways [168, 169].

The GC-induced protein GILZ (GC-induced leucine zipper) can also deactivate ERK MAPK, via interfering with the phosphorylation and activation of the upstream kinase Raf-1, thus compromising the subsequent activations of MKK1/2 and ERK1/2 MAPK [164, 166, 175, 176]. Furthermore, GCs can dissociate the Raf-1:Hsp90 association, thus weakening the activation of Raf-1's downstream targets [177, 178]. However, the role of GILZ in the above mechanism has not yet been researched. Additionally, GILZ can target the activity of NF-κB and AP-1 via direct binding, and thus attenuate the expression of pro-inflammatory genes [164, 166, 179–182].

The GC-induced production of SOCS1 [162, 183–186] might play a role in the proteasomal degradation of NF-κB p65, as ubiquitination of NF-κB p65, targeting it for degradation, is mediated by the E3-ubiquitin ligase SOCS1. As expected, clearance of cellular DNA-bound NF-κB p65 causes transcriptional termination [187]. However, SOCS1 could possibly compete for binding to NF-κB p65 with the nuclear peptidyl-prolyl isomerase Pin1 [188, 189]. The conformational changes in NF-κB p65 elicited by binding of Pin1 contributes to stabilization of NF-κB p65's active, nuclear conformation [188]. Because GR stimulates SOCS1 transcription and GR can also bind to SOCS1 [190], it would be interesting to investigate the role of GCs in the switch between NF-κB:Pin1 and NF-κB:SOCS1 binding and its possible implications in GR's anti-inflammatory mechanism.

Interesting to note is the recent finding that in lung epithelial cells, stimulated GR can also cooperate with activated NF-κB to induce the transcription of the TLR2 gene [191, 192]. This Toll-like receptor, TLR2, signalling pathway, eventually initiating pro-inflammatory gene transcription, can even be induced by a synthetic GC. Mechanistically, this involves an association of GR with PI3K. Nevertheless, also under these conditions, GCs ultimately repress AP-1 and NF-κB transcriptional activity [192].

10.3.1.2 Destabilization of Pro-inflammatory Gene mRNA

The GC-mediation induction of DUSP1, GILZ and TTP and the deactivation of p38 MAPKs, and thus its downstream kinase targets, can contribute to the destabilization of pro-inflammatory gene mRNAs. These mRNAs of often cytokines and chemokines,

are characterized by adenylate-uridylate (AU)-rich elements (AREs) at the 3′-untranslated end [193]. The GC-induced TTP can contribute to this destabilization by binding to these ARE-containing mRNAs and thus prompting their exonuclease-mediated degradation [194, 195]. In the context of an inflamed cell, TTP function is attenuated via its phosphorylation by the p38 MAPK-activated kinase MK2, and ARE-containing transcripts such as cyclo-oxygenase-2 (COX-2) mRNA and TNF mRNA are thus stabilized [196–202]. Conversely, as GCs can diminish p38 MAPK activity levels via different mechanisms, GCs preclude TTP phosphorylation. Moreover, GCs can increase the TTP expression and protein levels [137, 163, 203, 204]. In support, knockout, knockdown and short hairpin-based studies of TTP showed that TTP significantly contributes to the GC-induced decrease in TNF mRNA quantities [163, 203]. Combined, GCs can thus contribute to the destabilization of ARE-containing transcripts, such as TNF mRNA [137, 146, 150, 154, 205–207]. As a feature in the GR negative feedback mechanism of downregulation, also the GR mRNAs are subjected to a similar mechanism [208]. In conclusion, the GC-induced destabilization of ARE-containing mRNAs, in combination with the GC-mediated transrepression mechanisms, ensures a rapid elimination of cellular pro-inflammatory gene transcripts.

10.3.1.3 Transrepression of NF-κB-Dependent Gene Expression

A variety of GR-repressing mechanisms can be discerned (Fig. 10.4) [67, 92]. Ligand-activated GR can repress transcription via direct DNA binding onto so-called negative GREs or nGREs, via competitive DNA binding onto or in close proximity of another transcription factor-binding site, or via DNA binding together with another transcription factor on a composite GRE. The described sequestration model, however, appears to play no distinct role in the GC-mediated repression of NF-κB-driven gene expression. Yet, the ‘tethering’ mechanism is considered prototypical in the GR-mediated inhibition of NF-κB-driven transcription. Thus, GR can modulate NF-κB-regulated gene expression via a direct GR:NF-κB interaction, or additionally via perturbing the signalling cascade of kinases toward NF-κB activation, and/or via altering the composition of the proinflammatory gene promoter-bound enhanceosome.

Direct GR:NF-κB Association

A direct interaction of GR with the transcription factor NF-κB was reported already 15 years ago [209]. Mapping of the interacting domains via mutation studies, revealed GR association with the Rel-HD and the C-terminal transactivation domain of NF-κB [148, 210]. The association of these C-terminal domains of NF-κB p65 with GR appears to be key to accommodate GR transrepression on NF-κB-regulated gene transcription [148, 209, 211]. Conversely, from a GR viewpoint, this GR:NF-κB association involves specifically the zinc finger region of

the GR DBD [159]. Nevertheless, GR DNA binding in itself is not required to accommodate this GR:NF-κB interaction [209, 212]. However, chromatin immunoprecipitation (ChIP) assays showed that GR binds proximal to DNA-bound NF-κB [158, 159, 213, 214]. The latter two arguments combined, point towards a 'tethering' mechanism. A last mechanistic item on GR:NF-κB association revolves around the NF-κB cofactor thyroid receptor-interacting protein 6 (Trip6). Knockdown of this Trip6 and interaction studies suggest that this LIM domain-containing Trip6 could function as a necessary recruitment platform to accommodate GR:NF-κB binding, but also to allow the GR's repressive effects on NF-κB p65-driven gene expression [214].

Typically, this tethering mechanism is mirrored by a reciprocal NF-κB-mediated repression of GR/GRE-driven gene transcription [209, 215, 216].

In this respect, protein kinase A (PKA) has a quite controversial role. This kinase can contribute to the activational NF-κB S276 phosphorylation, which promotes its association with the coactivator CBP [217, 218]. Conversely, NF-κB S276 phosphorylation appears to be necessary to accommodate NF-κB-mediated repression of GRE-regulated promoters and GR can associate with PKAc [127]. Surprisingly, NLS-defective NF-κB and GR mutants, which thus localize to the cytoplasm, still support GR-mediated NF-κB transrepression and NF-κB-mediated GR transrepression, arguing for a mutual antagonistic cross-talk of GR and NF-κB in the cytoplasm. However, as both GR and NF-κB extensively shuttle between cytoplasm and nucleus [81, 83, 84, 219–221], it cannot be excluded that these mutants also shuttle and could thus possibly, as would be expected, relay their transrepression mechanisms in a nuclear setting. Although this cytoplasmic mechanism might contribute to the GR-regulated mechanism of NF-κB transrepression, experiments using the nuclear Gal4-p65 S276A and S276C mutants confirm the involvement of nuclear GR-mediated transrepression events aimed at halting NF-κB-dependent transcription [216].

Modulation of Activational NF-κB Signalling Cascades

The activated GR can modulate the activity of several kinases involved in signalling toward pro-inflammatory gene transcription and NF-κB activation or modulation (recently reviewed in [60]). As mentioned above, NF-κB is extensively regulated via posttranslational modifications, and of these the phosphomodulation of NF-κB is particularly well researched.

First the GR can negatively affect MAPK function via DUSP1 and GILZ upregulation (see Section 10.3.1.1). Activated GRs can additionally cross-talk with JNK and its upstream regulators, ultimately inhibiting the function of downstream targets c-Jun, ATF-2 and Elk-1 [222, 223]. Possible mechanistic interventions are: a direct interaction of GR with JNK [222, 224], or a direct association of GR with MKK7 [224], and an inhibition of MEKK1:Hsp90 interaction [177]. Interestingly, the deactivating effects of GR on the JNK MAPK culminates in the recruitment of inactive JNK MAPK, together with GR, to DNA-bound AP-1 on e.g. the c-jun gene

promoter [224–226]. Conceivably, an analogous mechanism could exist for activating NF-κB p65 kinases and NF-κB p65. However, currently, no such mechanism has been reported.

Downstream of the p38 and ERK MAPKs, which are in itself subjected to a variety of GC-mediated effects, lie the MAPK-activated kinases (MKs) [42]. As such, also the p38 MAPK- and ERK-activated MSK1 [59, 227–231] is influenced by GC actions. This nuclear kinase plays an important promoting role in pro-inflammatory gene transcription via CREB S133, ATF1 S63 and NF-κB S276 phosphorylation and transactivation [56, 58, 59, 227, 230] and via histone H3 S10 phosphorylation, thus provoking a local, transcription-facilitating chromatin relaxation [105, 106, 232]. The phosphorylation of NF-κB S276 promotes CREB-binding protein (CBP) and p300 binding [56, 58, 217, 218]. Additionally, MSK-mediated H3 phosphorylation creates a platform for 14–3–3 binding, and combined this situation promotes heterochromatin protein HP1γ dissociation and RNA polymerase II (RNA Pol II) recruitment [233–236]. As such, the combined administration of GCs and MSK1 inhibitors causes an additive repressive effect on NF-κB-regulated gene expression [237]. Although activated GR does not affect the MSK1 phosphorylation or activity status, GCs can target the MSK1 localization by inhibiting its recruitment to pro-inflammatory gene promoters and by driving a part of the total cellular MSK1 from its nuclear 'home' to the cytoplasmic outskirts via a GR- and CRM1-dependent mechanism, associated with a GC- and MSK1 activity-dependent interaction of GR and MSK1 [238]. Consequently, H3 S10 phosphorylation at these gene promoters is abolished, overall NF-κB p65 S276 phosphorylation is attenuated and pro-inflammatory gene transcription is halted [58, 237, 238]. In this respect, further mechanistic studies into the GR-MSK1 interaction and the GC-mediated MSK1 export could unveil new GC-mediated mechanisms. Nevertheless, experiments using Compound A, a selective GR modulator which does not support GRE-mediated transcription, but can drive repression of NF-κB-regulated gene expressions, already showed that the GR-provoked translocation of MSK1 can be placed in the context of the grand, multifactorial mechanism of GR-mediated transrepression of NF-κB-mediated transcription [238].

The kinase complex comprising IKKα and IKKβ, which is essential to the degradation of IκBα and the subsequent release of NF-κB [36], performs also additional roles in the NF-κB 'machinery'. Firstly, IKKα and IKKβ can phosphorylate NF-κB p65 at S536 [239–241], and thus contribute to NF-κB's activity level, most likely via promoting the association NF-κB S536ph with p300 [64]. Moreover, the IKK-mediated phosphorylation of NF-κB reduces its binding affinity for IκBα, and thus also counteracts the sequestration model for gene repression [240, 242]. Secondly, IKKα promotes the binding of NF-κB onto specific gene promoter sites [243]. And lastly, activated IKKα can translocate into the nucleus, and transduce local H3 S10 phosphorylation, similar to MSK1, thus facilitating pro-inflammatory gene expression [107, 108]. No effect of GCs on NF-κB S536 phosphorylation have been reported, but GCs can regulate IKKα promoter occupancy and H3 S10 phosphorylation. These latter two events were both inhibited at the SP-A promoter by a GC stimulus [213].

In a recent publication, IKKα phosphorylation and activation was elicited via the subsequent inductions of PI3K and the serum and glucocorticoid-inducible kinase (SGK). These events lead to p300 phosphorylation, an increase in NF-κB activation and eventually a marked rise in NF-κB-driven gene transcription [244]. However, GCs can possibly influence this pathway as the cellular quantities of SGK1 can be augmented by GC treatment [245–247]. Of note, aldosterone, the ligand for MR, can activate NF-κB in the cortical collecting duct via SGK1 signalling, while GCs can still attenuate this NF-κB activation [248]. In light of the recent findings in IKK complex mechanistics, an exhaustive study about the effects of activated GR on the IKK complex functions, its activation pathways and its (anti)-inflammatory implications would be advisable.

GR Targeting the Enhanceosome

In the above section some kinases (MSK, IKK), which form an intricate part of the NF-κB signalling pathway, were also affected in their gene promoter recruitment characteristics. The composition of the enhanceosome which is assembled onto active NF-κB-dependent promoters can be intrinsically modulated by a ligand-activated GR in various manners.

The prototypical example of a GR-targeted enhanceosome, could be found in the publications of the group of Yamamoto [159, 160]. Upon the activation of the promoters of IL8 and ICAM1, a pre-initiation complex (PIC) and RNA Pol II is recruited to these promoters, and the C-terminal domain of this polymerase is subsequently phosphorylated at S2 and S5. The former RNA Pol II S2 phosphorylation is necessary to allow transcription and is mediated by the co-recruited cyclin-dependent kinase Cdk9 of the positive transcription elongation factor-b (P-TEFb) complex, comprising Cdk9 and CylinT1 [159, 249, 250]. This transcription elongator complex P-TEFb is recruited onto a DNA-bound NF-κB p65 protein, which needs to be phosphorylated at S276, and this binding is evenso necessary for NF-κB-driven transcription [251]. However, a ligand-activated GR can compete with P-TEFb for binding to NF-κB p65. If in this competition binding of GR is favoured over binding of P-TEFb, IL8 gene transcription is attenuated and phosphorylation of RNA Pol II S2 is halted [159, 160]. However, this mechanism operates in a gene promoter-specific manner, as the NF-κB-regulated IκB gene promoter-occupying enhanceosome is not regulated in a similar way [160]. Of note, GCs do not alter the composition of the PIC. Taken together with the above mentioned effects of GCs on the NF-κB p65 S276 phosphorylator MSK1, it appears that GR intervenes at different points in the chain of recruitment events which culminate in the transcription of the IL8 gene [159, 160, 238, 251].

From another perspective, NF-κB does not always function as a transcription factor, but can also function as a – most likely tethering – cofactor. The group of Glass [252] described a IRF3-driven promoter activation of Ifit1, IP-10 and a recombinant IRF3 promoter, in which NF-κB p65 binds to the DNA-bound IRF3

in a toll-like receptor TL4/TLR9-stimulated cell. Glucocorticoids negatively interfere with this transcription via evoking a competition model, but only in a TLR4/TLR9-stimulated context and not in a TLR3-induced cell. Mechanistically, activated GR then competes with IRF3 for direct binding to NF-κB p65. However, as GR has a greater affinity for NF-κB p65 than IRF3 does, GR prevails in this competition model and thus transcription of the IRF-3:NF-κB-driven gene is inhibited [252]. Interestingly, the kinase TBK1 can regulate the activating phosphorylations of both NF-κB S536 [241] and IRF3 phosphorylations [253, 254]. Recently, McCoy et al. revealed that GCs can negatively affect the phosphorylation and activity of this TBK1 in a TLR3- and TLR4-stimulated cellular context [255]. The GC-mediated repression of TBK1 function and thus IRF3 activity [255], could hence contribute to the described IRF3:NF-κB cofactor:GR competition model [252]. Combined, these two GC-regulated mechanisms inhibit IRF3-driven gene expression of e.g. RANTES [252, 255–257].

The assembly of cofactors surrounding DNA-bound NF-κB p65 can also alter under the influence of GCs. These cofactors often have modulating capacities, e.g. acetyl or methyl transferase activity, which they exert on either other proteins of the enhanceosome, e.g. NF-κB, or on the extruding histone tails of the chromatin. This plethora of histone tail modifications assemble into the 'histone code'. This code can define the chromatin condensation/relaxation status, the accessibility of transcription factor binding and the likelihood of transcription from a given promoter [51].

In that respect, GCs can attenuate histone H4 K8 and K12 acetylation via a combined mechanism. These steroids can diminish the HAT activity of CBP, while enhancing the transcription of the histone deacetylase HDAC2, directing these HDAC2's to NF-κB:CBP complexes and steering HDAC1 to, e.g. the SP-A gene promoter [213, 258, 259]. Furthermore, GCs decrease the H3 and H4 acetylation levels at the promoters of the SP-A and IL8 genes [213, 260]. These CBP- and HDAC-based mechanisms all contribute to a GC-diminished transcription of NF-κB-regulated genes [213, 258, 259]. However, activated GR does not compete with NF-κB for a limited cellular amount of cofactors (CBP/p300 or SRC-1), as was shown via overexpression and analyses with cofactor-interacting defective GR mutants [49, 214, 216, 261–263]. GCs can also increase the dimethylation of local H3 K9 at the SP-A gene promoter, which is associated with transcriptional repression [213]. Combined with the above discussed, GC-mediated effects on the H3 S10 phosphorylating kinases MSK1 and IKKα, we conclude that the GR-affected enhanceosome is clearly reflected in a changed chromatin environment.

Of note, in overexpression studies with SRC-1, SRC-2, and/or the comodulator SRC-1 and TIF-2 Associated Modulatory Protein (STAMP), the resulting increase in the fold repression for GR-mediated inhibition of NF-κB-driven gene expression of IL8 does point to a possible role for these coregulators [120, 264]. We advise studies in a more endogenous setting via knock-down and KO studies and/or ChIP analyses to resolve the role of these factors in the GR transrepression mechanism which targets NF-κB-driven gene expression.

In conclusion, the GC-mediated transrepression of NF-κB-driven gene transcription operates via a stimulus-, gene- and cell-specific, multifactorial mechanism. The basis of this gene- and cell-specificity is captured in the varying cellular cofactor and transcription factor concentrations and activities, the different and specific gene promoter sequences and its intrinsic transcription factor binding sites and the distinct local histone code and chromatin condensation state.

10.3.2 NF-κB and the Peroxisome Proliferator-Activated Receptors, PPAR

The PPAR subfamily of NRs comprises a PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3) and is differentially expressed in distinct tissues. These transcription factors become active upon induction with their cognate ligands, i.e. fatty acid derivates or fibrates, and can form a heterodimer with RXR. The PPAR family NRs play a role in lipid and glucose metabolism, cell proliferation and apoptosis, but also display marked anti-inflammatory effects [129, 265–268]. As such, PPARα ligands mediate anti-atherogenic activities and contribute to controlling obesity-induced hepatitis [269–271]. PPARα ligands, but not PPARγ ligands, can attenuate IL1-stimulated IL6, prostaglandin and COX-2 production in human aortic smooth-muscle cells. Moreover, activated PPARα can restrain the inflammatory response in aortic smooth-muscle cells and diminish plasma acute-phase protein quantities in the vascular wall [72, 272]. Nevertheless, also PPARγ ligands can repress NF-κB mediated transcription, e.g. iNOS and MMP9 in macrophages [273]. Furthermore, PPARγ ligands have a beneficial effect on intestinal epithelial cell inflammation [274]. Also, PPARβ/δ can repress NF-κB activity in adipocytes [275]. Of note, PPARβ/δ can stimulate tumor growth. In this respect, selective activation of PPARβ/δ in non-small cell lung cancer cells was associated with an increase in NF-κB p65 DNA binding and protein levels, a decrease in IκBα gene expression and a marked inhibition of transcription of the known tumor suppressor: phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [276]. Conversely, PPARα and PPARγ have anti-tumorigenic effects in a variety of cancer cells [276–278].

Like GR, also PPARα and PPARγ can inhibit NF-κB- and AP-1-mediated gene transcription [72, 279, 280]. Mechanistically, these nuclear PPARs interfere with NF-κB via a multifaceted mechanism. As for GRs, PPAR-mediated transrepression of NF-κB driven gene expression is mirrored by a reciprocal repression mechanism. As such, activated NF-κB can inhibit the PPAR response element-driven promoter activity, independent of the promoter context [72].

A PPARα-dependent stimulation of IκBα expression and the resulting diminished NF-κB DNA binding, has been suggested to play a role in the PPARα-mediated NF-κB-repressive mechanism. This gene induction would occur via a necessary recruitment of DRIP205 (also known as MED1) to the κB-adjacent Sp-1 site in the IκBα gene promoter. Although PPARα ligand incubation does

not influence IKK activity or IκBα degradation in primary human hepatocytes [280], these ligands can attenuate IKK activity, IκBα phosphorylation and the IκBα degradation rate in human umbilical vein endothelial cells (HUVECs) [281]. As for GR, the contribution of PPAR-induced IκBα is not considered to be essential to PPAR's overall NF-κB-repressive mechanism and is highly cell-specific. However, it could prove interesting to investigate whether also GR-, ER- and AR-mediated IκBα promoter activation would necessitate DRIP205.

Additionally, recent findings of Okayasu et al. [281] indicate that PPARα activation can stimulate AMP-activated protein kinase (AMPK) and thus entice the phosphorylation of its downstream targets Akt and eNOS. Moreover, knockdown studies and pharmacological inhibition experiment showed that AMPK is a critical factor in PPARα-mediated transrepression of NF-κB-driven gene expression in mouse endothelial (SVEC4) cells [281]. Conversely, PPARβ/δ can decrease AMPK phosphorylation [282]. It would be interesting to investigate whether AMPK is also involved in PPARγ- or GR-mediated repression of NF-κB, and thus whether this AMPK-based mechanism is shared by various NRs.

PPARγ ligands can provoke a NF-κB segregation mechanism; transcriptionally active NF-κB is extruded to the cytoplasm [283]. Also, PPARα ligand stimulation was associated with a decreased nuclear translocation rate of NF-κB p65 [129]. However, to date, the mechanism by which PPARs can affect the NF-κB p65 localization has not been described and might operate cell-specifically.

Notably, the PPARγ agonist 15d-PGJ(2) can also negatively affect NFκB function without actually needing the PPARγ receptor. This 15d-PGJ(2) can covalently modify critical cysteine residues in IKKβ and the DNA-binding domain of NF-κB. As a result, IKKβ activity and NF-κB DNA binding is compromised and ultimately these events lead to a decrease in NF-κB-driven gene expression [284–286]. Moreover, 15d-PGJ(2) can also lead to a mitochondria-dependent apoptosis via a NF-κB-dependent mechanism [287]. Furthermore, it appears that PPARγ function is under the control of a negative regulatory feedback loop, as lipopolysaccharide (LPS) stimulation of macrophages leads to an NF-κB-dependent decrease in PPARγ mRNAs [288]. Of note, A20, an NF-κB-induced inhibitor of IKK complex activation [289], was recently indentified as an inducer of PPARα gene transcription. This increase in cellular PPARα is pivotal to the A20-mediated protection against oxidative necrosis in an ischemia/reperfusion injury model [290].

The receptor PPARβ/δ operates distinctly different from PPARγ and PPARα, albeit that PPARβ/δ also has NF-κB-modulating effects. In vivo studies comparing Zucker diabetic fatty (ZDF) to lean rats revealed that PPARβ/δ expression levels and PPAR DNA-binding activity in white adipose tissue of ZDF rats was reduced. Concomitantly, IL6 gene transcription and NF-κB DNA binding was enhanced, which originated from this decreased PPARβ/δ function. Activation of the PPARβ/δ indicated that this receptor can prevent LPS-induced ERK activation and althus impede NF-κB activation in adipocytes. In vivo, ZDF rats and PPARβ/δ KO mice showed a constitutively increased ERK phosphorylation [275].

The combination therapy hypothesis proposes that the combination of two therapeutic agents, and the resulting additive effects, allows to use lower dosages of each of these agents. Thus combination of these lower dosed agents could limit the associated side effects [291]. In this respect, combining PPARγ and GR ligands to combat inflammatory afflictions results indeed in an additive anti-inflammatory effect on a specific subset of TLR-stimulated gene inductions. Mechanistically, this additive repression most likely originates from the association of PPARγ with the corepressor NCoR, and from combined GR- and PPARγ-mediated targeting of NF-κB [252]. Similarly, combining PPARα agonists and GCs results in an additive transrepression of NF-κB-driven gene expression. Furthermore, this additive effect was appropriately reflected in an additive inhibition of endogenous IL6 mRNA and protein production [292]. Recent findings in PPARα KO mice in various murine models, suggest that PPARα could also be a contributing factor in the GR-mediated NF-κB repressive mechanism itself [293–295]. However, the precise mechanistics of this role were not yet defined. Notably, a trimeric combination therapy of PPARγ agonists, GCs and COX-2 inibitors is currently used in the treatment of hormone-refractory prostate cancer [296].

Interestingly, PPARα agonists actually counteract classic GRE-regulated transcription of recombinant vectors and endogenous genes, such as GILZ. A ChIP analysis of the promoter of GILZ revealed that PPARα agonist incubation can abolish GR promoter occupancy and diminish RNA Pol II recruitment. The concomitant nuclear association of activated GR:PPARα necessitates the PPARα DBD and LBD [292]. An in-depth analysis of the mechanistic basis of this cooperative and antagonistic cross-talk of PPARα and GR is currently lacking. As these PPARα agonists can also halt GC-initiated transcription of key metabolic regulators, such as glucose-6-phosphatase, the PPARα-GR combination strategy might constitute an efficacious anti-inflammatory therapy with a reduced GC-mediated side-effect profile. Physiological experiments, indeed, confirmed that a GC-elicited deterioration of hyperinsulemia in high-fat diet-fed mice could be countered by the addition of the PPARα agonist fenofibrate [292].

Interestingly, also the combinatorial use of PPARγ and RXR agonists in chondrosarcoma cells elicited an additive anti-inflammatory effect, as was exemplified for MMP1 and MMP13 gene transcription. These effects were accompanied by an increase in PPARγ gene promoter occupancy and a cross-SUMOylation of the PPARγ:RXR heterodimer [297]. In an earlier report by Pascual et al. [99], ligand-dependent SUMOylation of PPARγ in macrophages was reported to direct this PPARγ to NCoR:HDAC3 complexes, residing on inflammatory gene promoters. The addition of PPARγ to these complexes prevents cofactor exchange, thus prevents NF-κB:cofactor complex recruitment and thus precludes NF-κB-mediated promoter stimulation [99, 298]. This SUMOylation at PPARγ K77 appears to be essential to the repression mechanism by which PPARγ halts NF-κB-driven gene expression [100].

In conclusion, these trimeric cross-talk mechanisms between two NRs (GR:PPAR or PPAR:RXR) and NFκB holds promise for a new, efficient therapeutic strategy with possibly a more beneficial effect profile. However, further research into the cell type specificity and molecular basis of these combinatorial mechanisms is warranted.

10.3.3 NF-κB and Liver X Receptor, LXR

The Liver X receptors, LXRα (NR1H3) and LXRβ (NR1H2), are activated by oxysteroles, i.e. oxygenated cholesterol derivatives, and can thus sense cellular cholesterol homeostasis. Furthermore, LXRs can also function as an anti-inflammatory and anti-atherogenic regulator [299, 300]. Recently, LXRs were shown to have anti-proliferative capacities in breast cancer cells. In that context, they can inhibit ERα gene transcription [301].

In an inflammatory model induced by bacterial pathogens, activation of the LXRs results in a decrease of NF-κB-dependent cytokine production of IL1β, IL6, iNOS, MCP-1, MMP9, COX-2 and TNF [302–306]. LXRα/LXRβ KO mice are also more susceptible to bacterial infection [299].

A direct or indirect LXR-mediated repressive effect on NF-κB-regulated transcription was shown using an NF-κB-driven reporter gene [306]. Furthermore, LXR agonists elicited a hampered IκBα degradation in murine splenic B-lymphocytes, suggesting a delayed NF-κB p65 translocation [307]. However, in macrophages, NF-κB DNA binding, as assessed by electrophoretic mobility shift assay (EMSA) analysis, was not affected by activated LXRs [308]. The LXR-mediated inhibition of LPS-induced TNF gene transcription was also associated with a decrease in p38 MAPK phosphorylation [305]. Analogous to the PPARγ:SUMO1 link, LXRs are SUMOylated via SUMO2/3 and subsequently directed to distinct pro-inflammatory gene promoters where these SUMOylated LXRs lock down NCoR corepressor complexes at these inflammatory gene promoters [298, 309].

Similar to the combination of PPARα or PPARγ agonists with GCs, combining LXR agonists with GCs results in an additive anti-inflammatory effect on TLR3-stimulated IP10, Ifit1 and iNOS expression and on LPS-stimulated TNF and iNOS expression [252, 310]. To date, the mechanistic basis for this additive repression has not yet been elucidated. Additionally, LXR agonists can also increase PPARα mRNA levels and protein production in the duodenum, jejunum, and ileum, but not in the liver [311].

10.3.4 NF-κB and the Estrogen Receptor, ER

The estrogen receptor (ER) subgroup comprises two distinct receptors ERα (NR3A1) and ERβ (NR3A2), both of which can activate or repress gene transcription [312]. Activated ERs homodimerize or heterodimerize upon ligand binding, translocate to the nucleus and can regulate gene transcription via direct DNA interaction, in this case on an estrogen response element (ERE) or via tethering mechanisms on other DNA-bound transcription factors. Alternatively, estrogens can also bind a membrane-associated estrogen receptor and thus relay its so-called non-genomic effects. These non-genomic events, by definition, are not dependent on gene transcription. Rather, these events include direct estrogen effects on cytoplasmic and nuclear proteins,

e.g. kinase signalling cascades, altering the function of these proteins and thus indirectly modulating gene transcriptions [313–315].

Functionally, estrogens have an outspoken role in reproduction. However, these steroidal hormones have also been reported to have a function in the regulation of cardiovascular, skeletal, central nervous and the immune systems [316–319]. The cross-talk of activated ERs with NF-κB can have cell-specific effect on inflammation and the mechanistics and implications thereof will be discussed below. In aggressive hormone-refractory cancers, the absence or loss of ER function was linked to a constitutively active NF-κB and MAPKs and the resulting elevated cytokine and growth factor levels [320–323]. Furthermore, the negative effects of estrogen on NF-κB-driven cytokine production, in particular IL6, correlate with prevention of age-related disorders, e.g. post-menopausal rheumatoid arthritis, and tumorigenesis [34, 324–327].

Similar to the GR, ER can also directly interact with NF-κB in the nucleus thus imposing its negative effect [328–331]. To allow these negative effects of ER on NF-κB-mediated transcription, an intact ER DBD and NF-κB p65 Rel-HD is required [327, 329]. It appears that particulary ERα, rather than ERβ, is involved in the estrogen-mediated repression of NF-κB signalling [332, 333]. However, cell-specific effects may be at play here, as estrogens, via most likely ERβ, have also been reported to attenuate NF-κB p65 nuclear translocation in peritoneal macrophages of endometriosis and thus to diminish iNOS expression levels [334, 335]. In in vitro experiments in several cell types using EMSA analyses, estrogens appeared to block NF-κB DNA binding and thus negatively affected cytokine production [327, 330, 336–340]. The earlier discussed segregation model in which a nuclear receptor can stimulate IκBα gene expression has also been reported to be mediated by ERs [336, 341]. Of note, the cellular IκBα concentrations are higher in ER-positive breast cancer cells than in ER-negative breast cancer cell line. Alternatively, Cvoro et al. [121] revealed a cofactor switch model with interesting players in U2OS-ERα cells. Namely, unliganded ERα was recruited onto the TNF gene promoter together with c-Jun, NF-κB p50, NF-κB p65, CBP and Hsp90 in response to a pro-inflammatory signal and unliganded ERα can thus be considered a coactivator. However, ligand stimulation of ERα inhibits TNF gene transcription via switching the coactivator complex ERα:CBP:Hsp90 for the cofactor SRC-2, which acts as a corepressor in this context. Nevertheless, the gene promoter occupancy for c-Jun, NF-κB p50 and NF-κB p65 is not affected [121]. Similarly, ligand-activated ERα recruitment can displace CBP, but not NF-κB p65, from the gene promoter of MCP-1 (monocyte chemoattractant protein-1) and IL8 in MCF7 breast cancer cells. Conversely, the IL6 gene promoter association of NF-κB p65, CBP and the p300/CBP-associated factor, p/CAF, is diminished upon the recruitment of activated ERα [342]. Although the fact that estrogen cannot effect NF-κB p65 recruitment to the TNF, MCP-1 and IL8 gene promoter seems in conflict with the earlier EMSA analyses and the ChIP results for the IL6 gene promoter, most likely, the overall mechanism by which ERs can negatively affect NF-κB-mediated gene repression is multifaceted and can comprise both mechanisms in different cell types and on different gene promoters [343, 344].

Recent research has added a new facet to the ER-mediated NF-κB-transrepressive mechanism; PPARα appears to play a role in the anti-inflammatory activity of estrogens as the efficacy of estrogens to attenuate lung inflammation and mechanistically to inhibit NF-κB activation is compromised in PPARα KO mice. Interestingly, PPARα also appears to contribute to the estrogen-induced upregulation of ER gene expression [345]. As a similar PPARα-contributing mechanism is suggested for GR's anti-inflammatory mechanism [293], it would be interesting to investigate the role of PPARα in other NR-mediated mechanisms.

Next to the slower genomic effects of ER, depending on gene transrepression or transactivation mechanisms, literature covering the non-genomic effects of estrogens, which can manifest themselves in a matter of seconds, adds on new insights [346]. As such, estrogens can elicit a diminished phosphorylation of p38 MAPK and NF-κB DNA-binding affinity, ultimately resulting in a normalization of the cytokine production in several inflammation models [340, 347]. Conversely, ER can increase ERK MAPK activity and thus activate NF-κB, resulting in a promoter-specific activation of the anti-oxidants Mn-superoxide dismutase and gluthathion peroxidases in MCF-7 cells [348, 349]. As apparently activated ER can cell-specifically impact p38 MAPK and ERK MAPK activity, it would be interesting to investigate whether this could indirectly repress the MSK1-mediated NF-κB phosphorylation. In general, estrogens can impact several kinase signalling pathways, which may indirectly impact NF-κB and NF-κB-driven gene expression. However, as these non-genomic effects ultimately also impact transcription, this mechanistic classification is challenged.

As for GR, the activation of ERα/ERβ and NF-κB features a reciprocal repression mechanism in a variety of cell lines. Activation of NF-κB via different pro-inflammatory signals can thus repress the activation of ERE-regulated gene promoters [350–352]. However, not all cross-talk between ER and NF-κB results in mutual antagonism. Gene promoter-specific cooperation of ER and NF-κB has been reported, e.g. for transcription of the serotonin 5HT1A receptor gene [353]. Furthermore, not all cell lines are susceptible to estrogen-mediated inhibiton of NF-κB. Murine fibroblasts and rat smooth muscular cells cells, for instance, do not display an inhibition of NF-κB-mediated transcription in response to estrogens, most likely due to the lack of a functional ER [354, 355]. Notably, in Jurkat cells and human peripheral blood T cells, activated ERβ seems to be able to activate NF-κB activity [331]. In murine splenocytes estrogens can also lead to an activation of NF-κB and upregulate certain NF-κB-driven genes, e.g. interferon IFNγ, via activation and recruitment of Bcl-3 to the gene promoters [335]. Clearly, the cross-talk between NF-κB and ER is both cell- and gene promoter-specific [335, 353–357] and thus these specificities should advisably be researched when investigating new ER:NF-κB cross-talk mechanisms.

As mentioned above, the ER-negative hormone-refractory breast cancers are characterized by a constitutively active and DNA-bound NF-κB, while ER-positive tumors lack active NF-κB [323]. In a whole, this observation supports a role for ER-mediated inhibition of NF-κB signalling in cancer. As also expression of the ERs itself can be stimulated via an ERE-dependent mechanism, MAPK and NF-κB

activation in breast cancer is associated with the downregulation of ER via a reciprocal repression mechanism [358, 359]. Furthermore, cofactors play a particular and cell-specific role in ER response mechanisms (reviewed in [317, 360, 361]). In that respect, the ER-mediated regulation of the lifetime of the oncogenic SRC-3 forms an important recent finding. SRC-3 is sequentially phosphorylated and poly-ubiquitinated, in which the sequential modulations of SRC-3 serve as a 'transcriptional time clock' controlling the activation and functional lifetime of SRC-3 [362]. Furthermore also the localization and solubility of this SRC-3 appears to be regulated by phosphorylation events and SRC-3:ERα interactions [363]. Whether and how the activation of NF-κB could impact these mechanisms is currently not known.

10.3.5 NF-κB and the Androgen Receptor, AR

The androgen receptor (NR3C4) forms the cognate receptor for testosterone. Like GR, also AR can directly interact with NF-κB, albeit weakly, mediating its mutually antagonistic cross-talk mechanisms. Androgen-activated AR can stimulate androgen response element-mediated transcription, while attenuating NF-κB-driven gene expression of e.g. IL6. Reciprocally, activated NF-κB can halt androgen response element-regulated promoter activity [364]. The latter process appeared to involve AR's N-terminal domain from 297 on and the DBD [365].

In endothelial cells, AR-mediated repression of NF-κB activity was reported to regulate a negative effect on the transcript levels of VCAM1, ICAM1, IL6, MCP-1, CD40, TLR4, PAI-1, and COX-2 [366]. Although the IκBα upregulation model, with concomitant sequestration of inactivated NF-κB p65, was also suggested to occur in AR:NF-κB cross-talk [367], most likely, this mechanism is limited to select cell types, as investigations in COS-1 cells revealed no androgen-mediated IκBα gene transcription [365]. The AR:NF-κB mutually antagonistic cross-talk has also been suggested to occur via a competion for limited amounts of the cofactor CBP [368]. However, this general competition model lacks gene promoter-specificity as the cofactor CBP is utilized by a plethora of genes.

Interestingly, flutamide, a non-steroidal anti-androgen can decrease cytokine production, reportedly via a decrease in NF-κB DNA binding. Moreover, pharmacological inhibition of ERα indicated that this anti-androgen-driven mechanism of cytokine repression could be (in part) mediated via ER-regulated mechanisms [369]. However, androgens could also activate NF-κB and augment COX-2 and iNOS production in cerebral arteries, and overall exacerbate neuroinflammation [370].

Similar to ER, the AR gene can be driven by its own ligand. In accordance with the reported reciprocal repression, NF-κB can thus repress the gene transcription of AR [371–374]. As such, TNF-activated NF-κB p65 and the B-myb transcription factor is recruited to the AR gene promoter, together with a HDAC1:SMRT:mSin3A corepressor complex in androgen-sensitive cancer cells. These transcription factors can interact *in* cis at a composite genomic element, resulting in a decreased AR expression in androgen-dependent LNCaP human prostate cancer cells. Conversely,

in androgen-independent cells, TNF-activated NF-κB does not result in an AR downregulation and did not direct NF-κB:B-myb nor the HDAC1:SMRT:mSin3A corepressor complex to the AR gene promoter [374]. In contrast, a κB site in the AR promoter would actually be responsible for NF-κB-driven production of AR in androgen-sensitive prostate cancer cells [373]. Further research would be necessary to elucidate this apparent paradox. Additionally, increased AR levels have been associated with cancer progression to an androgen-independent prostate cancer and thus anti-androgen cancer therapy resistance [374].

Functionally, androgens can attenuate IL6 protein production in bone marrow-derived stromal cells. Furthermore, the AR-mediated decrease in IL6 protects the bone from IL6-regulated osteoclastogenesis [375, 376]. Additionally, androgens can elevate the osteoprotegerin (OPG) mRNA levels in osteoblasts without affecting the RANKL mRNA levels [377]. The resulting increase in the OPG/RANKL ratio is indicative for a decrease in bone metabolization. Similar bone-protective mechanisms have also been reported for estrogens (recently reviewed in [378]).

The use of androgens or anti-androgens in cancer treatments should be considered for each cancer specifically, as androgen depletion can attenuate normal and cancerous prostate growth, while testosterone may cause proliferation and apoptosis. In AR-negative prostace cancer cells, NF-κB was constitutively active [379]. However, in DU145 AR-negative, hormone-refractory prostate cancer cells, extracellular androgens can activate a membrane-associated AR and thus downregulate PI3K/Akt and NF-κB activity, induce pro-apoptotic genes, such as FasL, and increase caspase-3 and Bad protein activity [380]. In AR-expressing prostate cancer cells, androgens decreased NF-κB translocation and activity. Consequently, NF-κB-driven gene-expression of the anti-apoptotic Bcl-2 and IL6 was diminished [381].

10.3.6 NF-κB and the Progesterone Receptor, PR

The progesterone receptor (NR3C3) is transcribed of a single gene but can exist as multiple isoforms: PR-A, PR-B and a truncated PR-C. Activation of the progesterone receptor, can instigate inhibition of NF-κB-driven gene expression via a direct association of PR with NF-κB [364]. As expected, activated NF-κB also manisfests a reciprocal repression onto PR-stimulated gene promoters [149, 215, 382, 383].

As PR is mostly expressed in breast and endometrium, PR can function in the maintenance of pregnancy and the near term transformation of uterine quiescence into a uterus in labour. Right before parturition, surfactant protein of the fetal lung can activate fetal amniotic fluid macrophages. These cells subsequently activate NF-κB in the uterine wall and induce COX-2, IL6 and other inflammatory cytokine production. These proteins can contribute to the uterine wall contractility and thus to the parturition [383–385]. Furthermore, activated NF-κB can act via the reciprocal repression mechanism to counteract PRE-regulated promoter activation of genes which are involved in maintenance of pregnancy [386]. Recently, a role in the switch from pregnant to labouring uterus has been identified for the PR-C isoform.

Activation of NF-κB, results in an increase in expression of this PR-C isoform. As this PR-C is a truncated DNA binding-deficient isoform and can furthermore attenuate PR-B DNA-binding and transactivation, this event can augment the progesterone insensitivity of the myometrium [383, 386].

However, PR has also described functions in immunosuppression and tumorigenesis. For instance, in human leukocyte cells, activated PR plays an important role in the attenuation of cytokine gene expression [382, 387]. Furthermore, PR can play a protective role in breast cancers, which feature a high level of NF-κB activation and the concomitant induction of inflammatory cytokines via NF-κB (reviewed in [388]).

10.3.7 NF-κB and the RARs, RXRs, RORs

The retinoic acid receptors, RARα (NR1B1), RARβ (NR1B2) and RARγ (NR1B3), can be activated via stimulation with vitamin A or related compounds. Alternatively, the retinoid X receptors, RXRα (NR2B1), RXRβ (NR2B2) and RXRγ (NR2B3), can bind to retinoids, which structurally resemble vitamin A. Additionally, the adopted orphan receptors, RAR-related orphan receptor RORα (NR1F1), RORβ (NR1F2) and RORγ (NR1F2) sense for cholesterol or all-trans retinoic acid. Due to their ligand resemblance, we will discuss these receptors together. The RXRs can heterodimerize with subfamily 1 nuclear receptors including the RARs, but also with other NRs, like the constitutive androstane receptor (CAR; NR1I3), the farnesoid X receptor (FXR; NR1H4), liver X receptors (LXRs; NR1H3 and NR1H2), PPARs, the pregnane X receptor (PXR; NR1I2), thyroid hormone receptors (TRs, NR1A1 and NR1A2), and the vitamin D receptor (VDR; NR1I1). In contrast, RORs appear to bind DNA as a monomer [389, 390].

Interestingly, a LPS-stimulated increase in TNF, IL6, IL1α and IL1β transcript levels could be gene-specifically lowered by the addition of a RXR-specific ligand, but not a RAR-specific ligand in hepatic macrophages. This diminishing mechanism incorporates posttranscriptional effects, as RXR activation could destabilize the TNF mRNAs [391]. The basis or extent of this mechanism in RXR regulation is currently unknown. Furthermore, overexpression of RORα can impose a decline in the levels of TNF-induced IL6, IL8 and COX-2 transcripts. Mechanistically, the RORα can decrease NF-κB p65 translocation and in vitro DNA binding, and increase IκBα gene transcription [392]. However, these RORα-mediated effects on NF-κB function may only make out a small part of ROR's anti-inflammatory mechanism, as knowledge about the mechanisms of these RORs is currently slim.

However, NF-κB does not seem to envoke a reciprocal repression on RAR-regulated gene transcription, as exemplified by the following model. The RAR is constitutively bound to the DIF2 gene promoter. Transcription of DIF2, a gene involved that is involved in monocytic differentiation in acute promyelocytic leukemia cells, can be modestly increased by the addition of all-trans retinoic acid (RA), which instigates the release of a promoter-bound corepressor complex.

However, incubation with TNF boosted the RA-mediated induction of DIF2 gene transcription via the recruitment of NF-κB [393]. A similar synergistic stimulation with TNF and RAR ligands was reported for the expression of the polymeric immunoglobulin receptor, which reportedly plays a role in the increase in mucosal immunity [394].

10.3.8 NF-κB and the Thyroid Hormone Receptor, TR

The thyroid hormone receptors, TRα (NR1A1) and TRβ (NRA2), can both be activated via thyroid hormones. Alternative splicing can give rise to TRα1, TRα2, TRβ1 and TRβ2 isoforms. Although these TRs function mainly in the regulation of metabolism, nevertheless, cross-talk of these TRs with the transcription factor NF-κB has been described [69].

The expression of TRs itself is under the control of NF-κB. Namely, activated NF-κB can diminish TRα1, TRα2 and TRβ1 transcripion in vitro and in vivo in various inflammatory contexts [395, 396]. Furthermore, the NF-κB-mediated decrease in TRβ1 transcript levels, in turn, results in a repressed deiodinase type 1 gene transcription [396]. As the latter gene product plays an important role in the catabolization of thyroid hormones from T4 to the more active T3, NF-κB activation most likely results in a decreased cellular TR response. However, further investigations into the implications of these events are deemed necessary.

We would also like to mention that thyroid-stimulating hormones can initiate IL6 release from human adipocytes via a necessary NF-κB activation and can increase IL6 mRNA gene induction in CHO cells [397]. The predominant function of these thyroid-stimulating hormones is control of the release of TR-binding thyroid hormones. Whether and how this IL6-targeting mechanism could affect thyroid hormone release and activity is currently unclear.

10.3.9 NF-κB and the Vitamin D Receptor, VDR

The vitamin D receptor, VDR (NR1I1), can be activated by vitamin D and is closely related to the below discussed pregnane X receptor (PXR) (see Section 10.3.10). The activated VDR heterodimerizes with RXR in the nucleus and can positively or negatively affect gene expression. Of note, GCs can dimish the expression of the VDR gene and vitamin D can fuel the transcription of its own receptor. Functionally, the VDR has been implicated in the regulation of mineral metabolim, but also inflammation and cancer [398].

VDR ligands can affect the immune system by impeding dendritic cell maturation and inhibiting the development of a T helper type 1 (Th1) T-cell response. In these dendritic cells, the VDR ligand 1α,25(OH)D3 or synthetic D3 analogs could suppress the expression of the NF-κB family member, RelB, via gene promoter binding of a VDR:RXRα heterodimer and a corepressor complex comprising

HDAC3 [399, 400]. RelB is pivotal to the differentiation and maturation of dendritic cells. For the Th1 T-cell response, VDR ligands such as 1,25-dihydroxyvitamin D3 can repress the IL12 gene transcription in macrophages and dendritic cells, possibly by downregulating NF-κB activity [401].

Alternatively, 1,25-dihydroxyvitamin D3 can reduce the NF-κB p50 protein levels in activated lymphocytes, as well as the NF-κB in vitro DNA-binding and transcriptional activity [402]. Nevertheless, in old VDR KO mice the NF-κB mRNA levels were reduced in comparison to old wt mice [403]. In human keratinocytes and peripheral blood mononuclear cells, 20-hydroxycholecalciferol, a metabolite of vitamin D3, appeared to diminish NF-κB p65 translocation, activity and DNA binding and augment IκBα gene expression and protein production in a VDR-dependent manner [404, 405]. In contrary, in human proximal tubular kidney cells, a synthetic vitamin D analogue could diminish TNF-stimulated RANTES gene transcription and protein production without affecting IκBα phosphorylation and degradation or NF-κB p65 translocation and activity. Here, the repressive effects of activated VDR, are attributed to a diminished NF-κB p65 binding to the RANTES gene promoter together with a direct VDR:NF-κB p65 association [406]. Furthermore, in human colonic cancer cells, activation of VDR results in a diminished NF-κB p65 S536 phosphorylation and hampered the IL1β-stimulated IκBα degradation, culminating in a decreased IL8 gene transcription [407]. Taken together, VDR agonists can attenuate the transcription of various NF-κB-mediated genes, albeit via cell-specific mechanisms.

As expected, VDR:NF-κB cross-talk features a reciprocal repression mechanism in which activated NF-κB can diminish VDR-driven gene expressions [408]. Mechanistically, this inhibition is associated with a VDR:NF-κB interaction and a decrease in VDR associaton with the coactivators SRC-1 at VDR-driven gene promoters [409].

10.3.10 NF-κB and Other Nuclear Receptors

The farnesoid X receptor, FXR (NR1H4), can sense the cellular environment for oxysteroles and is closely related to the LXRs. This FXR plays an important role in hepatoprotection and can also inhibit NF-κB activity in the hepatic inflammatory response. Exemplary, FXR KO mice suffer from intense hepatic inflammation and the spontaneous formation of liver tumors. Furthermore, these mice show an increased responsiveness to a LPS stimulus, as measured by COX-2, iNOS, IP10 and IFNγ transcript levels [410]. In vascular smooth muscle cells, FXR ligands can diminish the IL1β-mediated induction of iNOS and COX-2 gene transcription. As such, activation of FXR can counteract vascular inflammation. Evenso, these receptors can mediate anti-atherogenic effects [411].

The small heterodimer partner (SHP) (NR0B2) is an orphan NR of which the expression is induced with FXR ligands. Like FXR, also this NR can inhbit NF-κB activity, as assessed via an NF-κB-driven reporter gene assay in vascular smooth muscle cells [411].

The pregnane X receptor (PXR) (NR1I2) can be activated via a wide array of ligands, and plays a role in the clearance of xenobiotics. This PXR can inhibit LPS- and TNF-mediated activation of an NF-κB-driven recombinant promoter [412]. Furthermore, investigations with PXR KO mice showed that activated PXR can indeed inhibit various NF-κB-regulated gene transcriptions [413]. Functionally, the PXR:NF-κB cross-talk could account for the PXR-mediated protection against inflammatory bowel disease and liver fibrosis [412, 413].

As the drug metabolizing capacity of the body is decreased by a pro-inflammatory stimulus, an involvement of NF-κB in this event was suspected. Clearance of xenobiotics is co-regulated by the PXR-induced cytochrome P450 family member Cyp3a4. However, recent findings indicate that the reciprocal repression of activated NF-κB on PXR-driven gene expression, may account for the loss of Cyp3a4 mRNA expression in an inflammatory setting. In that respect NF-κB directly interacts with PXR, inhibits PXR:RXR binding and PXR:DNA binding onto the cyp3a4 gene promoter [414]. The NF-κB-regulated expression of cytochrome P450, also a drug-metabolizing enzyme, can be counteracted by various nuclear receptors such as CAR, GR, PXR, RXR, PPAR, FXR, and LXR [415].

The hepatocyte nuclear factor-4, HNF-4α (NR2A1) en HNF-4γ (NR2A2), are adopted orphan nuclear receptors wich can be activated by fatty acids. As such these receptors are mostly expressed in liver and play an important role in liver development. HNF-4α and NF-κB are opposing transcription factors in the transcriptional regulation of the apolipoprotein C-III (APOC3) gene in hepatic cells. As such, activated HNF-4α transfers APOC3 promoter activation, whereas TNF-activated NF-κB decreases APOC3 gene transcription via attenuating HNF-4α DNA binding and transactivation functions [416]. Further investigations into this HNF-4α:NF-κB cross-talk is necessary to clarify its role in liver development and functional maintenance.

The chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII, NR2F2) is an orphan NR, which regulates various aspects of metabolism. In a model of adenovirus type 12-mediated tumorigenesis, the COUP-TFII-regulated repression of MHC class I transcription plays a major role in its phenotype. At this quiescent gene promoter NF-κB cannot bind and COUP-TFII, in association with HDACs, acts as a resident repressor. Although a TNF stimulus can augment the promoter occupancy of NF-κB p65, TNF cannot impact the lack of transcription due to a persistent histone deacetylation and HDAC4:COUP-TFII recruitment to the promoter [417]. COUP-TF-II can also cross-talk with the GR via a necessary direct association. In that respect, activated GR enhances COUP-TFII-mediated promoter activation of e.g. phosphoenolpyruvate carboxykinase, an important enzyme in gluconeogenesis. Nevertheless, COUP-TFII hampers GR transactivation mechanisms [143]. As such, the cross-talk of COUP-TFII with GR may function to coregulate metabolism.

Possibly, the COUP-TFII:GR and COUP-TFII:NF-κB interactions could suggest a trimerized cross-talk mechanism; albeit most likely under restricted conditions. To date, no such report was made. Overall, the implications of these COUP-TFII-based cross-talk mechanisms deserve additional research.

Lastly, the NR4A family comprises nerve growth factor IB, NGFIB (NR4A1, also known as Nurr77), nuclear receptor related 1, Nurr1 (NR4A2), and neuron-derived orphan receptor 1, NOR1 (NR4A3). These receptors have no known ligands and are considered to be ligand-independent transcription factors.

Gene expression of pro-inflammatory cytokines and chemokines, among which IL1β, IL6 and IL8 can be diminished via overexpression of NGFIB, Nurr1 or NOR1, in human atherosclerotic lesion macrophages [418]. In Jurkat cells, overexpression of NGFIB results in the decline of IL2 promoter activation, reportedly via the inhibition of NF-κB [419]. Additionally, NGFIB can diminish the NF-κB activity in HEK293 cells, as assessed via reporter gene analyses [420]. Also Nurr1 can inhibit NF-κB activity. A direct association between the two transcription factors at specific inflammatory gene promoters, leads to the recruitment of a CoREST corepressor complex and thus halts pro-inflammatory gene transcription in microglia and astrocytes [421]. In contrast, Nurr1 can cooperate with NF-κB p65 to enhance expression and secretion of IL8 from synovial tissues [422].

In apoptosis research, NGFIB overexpression in HEK293 cells promoted resistance to apoptosis via an elevation of NF-κB activity and the subsequent gene expression of the anti-apoptotic cIAP-1 [423]. Furthermore, activation of the thromboxane A(2) receptor leads to an increased expression of Nurr1 and is associated with enhanced lung cancer cell proliferation [424]. Currently, the (possible) role of NF-κB in this mechanism has not been elucidated. In short, cross-talk between NGFIB and NF-κB can play a role in both inflammation and cell fate.

Of note, the expression of NR4A NRs can be induced by inflammatory stimuli, via an NF-κB-dependent pathway in macrophages, thus installing a negative feedback loop [425]. In support, in synovial tissue Nurr1 mRNA is elevated in an NF-κB- and CREB-dependent manner. The specific NF-κB-binding site in its promoter can recruit either p65-p50 heterodimer or p50 homodimer NF-κB protein complexes [426]. Additionally, in Leydig cells, NGFIB promoter activation may be regulated via both NF-κB p50 and C/EBPβ transcription factor functions [427]. However, confirmation via ChIP assay is currently lacking for this NGFIB stimulation mechanism. TNF-stimulated NF-κB p65 can impede the transactivation of NGFIB and thus hamper steroidogenic gene expresson in these same cells [428].

Interestingly, NR4A NRs and particularly NGFIB can also cross-talk with the GR via a direct interaction via their DBDs. In that respect, the CRH-stimulated expression of POMC, a precursor of ACTH, is coregulated by GCs and particularly NGFIB. Whereas NGFIB can promote POMC gene transcription, recruitment of GR to this promoter inhibits its activity [429].

However, as orphan receptor have no (identified) ligand, these receptors cannot be activated exogenously and research in this field often relies on overexpression and knockdown/knockout approaches. If a ligand could be ascertained, this could open up a new perspective on the function of these orphan NRs. However, it also remains possible that these NRs are simply not ligand-dependent.

10.4 Conclusions

Current cross-talk between NRs and NF-κB encompasses the most important effects of steroids on inflammation. As evident from this review, various NRs can combat pro-inflammatory gene expression. Moreover, when scrutinizing the mechanism of one well-researched NR, e.g. GR, we see that this anti-inflammatory, NF-κB-targeting mechanism is built up in different layers of gene promoter- and cell-specific mechanisms. Possibly, future research into the mechanisms of the other NRs will reveal new NF-κB-modulating mechanisms.

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Chapter 11
The Biomodulatory Capacities of Low-Dose Metronomic Chemotherapy: Complex Modulation of the Tumor Microenvironment

Urban Emmenegger, Annabelle Chow, and Guido Bocci

Abstract The cyclic administration of conventional (i.e., maximum tolerated dose [MTD]) chemotherapy targets primarily the tumor cell population. In contrast, chemotherapeutics used at lower doses but on a more frequent basis, and without treatment-free breaks, preferentially affect the tumor vasculature. This so-called low-dose metronomic (LDM) form of chemotherapy administration can be considered as a complementary and/or alternative form of antiangiogenic therapy to the use of targeted antiangiogenic agents such as antibodies or small molecule drugs that interfere with vascular endothelial growth factor (VEGF) pathways. However, it becomes increasingly clear that LDM chemotherapy affects also aspects of the tumor microenvironment other than angiogenesis such as immune responses. Herein, we summarize the complex effects of LDM chemotherapy on the tumor microenvironment, with special emphasis on angiogenesis. We also compare the effects of LDM versus MTD chemotherapy. Finally, we outline how pharmacogenetic characteristics of the tumor host and microenvironment may be exploited in the future to predict response to LDM therapy.

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11.1 Introduction

In 2000, Hanahan and Weinberg delineated six hallmarks of cancer, including tumor cell intrinsic properties such as limitless replicative potential, self-sufficiency in growth signals and insensitivity to antigrowth signals as well as apoptosis-inducing stimuli [50]. Cancer cells are also characterized by their propensity to invade neighboring normal tissue and disseminate to remote organs, and by supporting the formation of a tumor-intrinsic vascular network that is interconnected with the vascular network of the host. Other important aspects of the tumor microenvironment that facilitate successful tumor growth have recently been highlighted and comprise the capability of cancer cells to undermine mechanisms of immuno-surveillance [65] and to capitalize on cancer-related inflammation [20,77].

More than 30 years ago, Folkman described the need for access to the vascular system of the tumor host both as an Achilles' heel of neoplastic growth and a treatment target [36]. In the meantime, interfering with the tumor vasculature has been validated as a successful anticancer strategy. In fact, in a number of phase III trials of advanced stages of colorectal, lung, breast, kidney and liver cancers, the use of targeted antiangiogenic agents such as the monoclonal antibody bevacizumab (which targets VEGF A) and small molecule VEGF receptor tyrosine kinase inhibitors (RTKI; e.g., sunitinib and sorafenib) resulted in improved overall and/or progression free survival [61]. By inhibiting the growth of new blood vessels, antiangiogenic agents deprive tumor cells from access to oxygen and nutrients, and impair the removal of toxic metabolites. However, the biological impact of VEGF pathway inhibitors is more complex than simply impairing the expansion of the tumor vasculature. They can affect the function of existing blood vessels, inhibit the mobilization and intratumoral recruitment of various bone-marrow derived, proangiogenic cells (e.g., endothelial cell precursors and various types of myeloid cells), and shape antitumor immune reactions by facilitating the differentiation of dendritic cells [28].

Antivascular effects are not a unique property of targeted antiangiogenic agents. Indeed, most chemotherapeutics can affect the tumor vasculature in various ways [79]. Moreover, angiogenesis inhibition is one of the major consequences of LDM chemotherapy, i.e., the frequent – often daily – extended administration of small doses of conventional chemotherapeutic drugs without major breaks [62].

Herein we will summarize the current understanding of the antiangiogenic basis of metronomic chemotherapy scheduling. Furthermore, we will compare the complex effects of LDM versus MTD chemotherapy on aspects of the tumor microenvironment other than angiogenesis, such as immune responses.

11.2 Conventional Chemotherapy: Beyond Cytotoxic Effects

Rapidly proliferating cells are exquisitely sensitive to the effects of chemotherapeutic agents given in a conventional schedule, i.e., intermittent administration at the MTD. This is reflected through the antitumor effects of chemotherapy and the

commonly seen side effects that involve normal host tissues with high cellular turnover, such as the hematopoetic system (i.e., myelosuppression), intestinal mucosa (i.e., gastrointestinal side effects) and hair follicles (i.e., hair loss) [37,62].

Endothelial cells are amongst the most rapidly proliferating cells within the tumor microenvironment [37]. Thus, they are expected to be susceptible to the effects of chemotherapeutic agents [59,79]. Indeed, a broad range of vascular side effects are testimony for the antivascular activities of MTD chemotherapy [100]. However, various treatment-induced adaptive changes may explain why these antivascular effects are mitigated and hence are not considered to represent a major mechanism of antitumor activity of MTD chemotherapy (Fig. 11.1). First, MTD chemotherapy has been shown to induce the expression and secretion of proangiogenic factors such as VEGF by tumor cells [70,80,81,96]. These factors can support neoangiogenesis in the chemotherapy-free break period. Furthermore, they can render endothelial cells relatively resistant to the effects of chemotherapeutics [113]. Second, a number of chemotherapeutics are capable of mobilizing bone-marrow derived endothelial cell precursors, which then incorporate into the tumor vasculature or promote the acute repair of affected vascular structures and foster vascular expansion in a paracrine manner [101,104]. This acute surge of endothelial cell precursors appears to be mediated among others by the

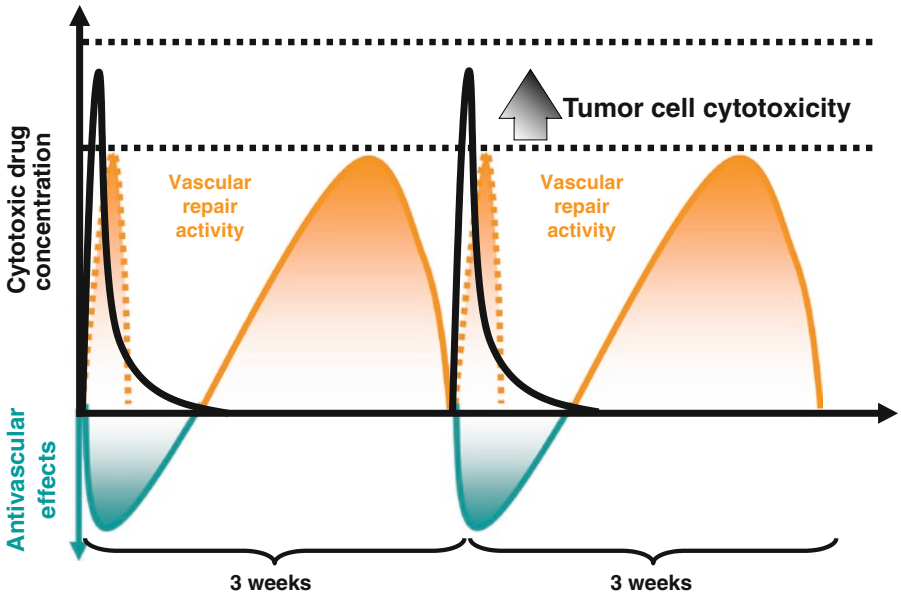

Fig. 11.1 Conventional MTD chemotherapy affects tumor cells directly and via antivascular effects. However, the antivascular effects are counteracted by the early mobilization and intratumoral recruitment of bone marrow derived endothelial cell precursors, and treatment-induced secretion of proangiogenic factors by tumor and/or stromal cells. Furthermore, during hematological recovery, another wave of endothelial cell precursors and other types of bone marrow derived cells is integrated into the tumor vasculature and microenvironment. Overall, the net effect of MTD chemotherapy on the tumor vasculature may be proangiogenic under certain circumstances

granulocyte-colony stimulating factor (G-CSF) [105]. Thus, inadvertently the practice of using G-CSF as an adjunct for certain MTD chemotherapy regimens to facilitate hematological recovery possibly could boost vascular repair and recovery. Third, a second wave of endothelial cell precursors and other proangiogenic bone marrow derived cells is mobilized during hematological recovery in the chemotherapy-free break period [5]. A successful way to impair these repair processes and to enhance the chemotherapy-related antivascular effects is the combined use of MTD chemotherapy with targeted antiangiogenic drugs such as bevacizumab, or with LDM chemotherapy [60,102].

11.3 Low-Dose Metronomic Chemotherapy

11.3.1 Principles

The use of LDM chemotherapy as an antiangiogenic treatment strategy is based on three major principles (Fig. 11.2):

1. Chemotherapeutic agents impair endothelial cell proliferation and induce endothelial cell apoptosis at significantly lower doses than needed for the same effects in cancer cells [10]. Although the detailed mechanisms of the exquisite chemosensitivity of endothelial cells remain to be fully elucidated, several explanations have been forwarded. Whereas cancer cells must acquire the capability to withstand high levels of genomic instability and DNA damage during

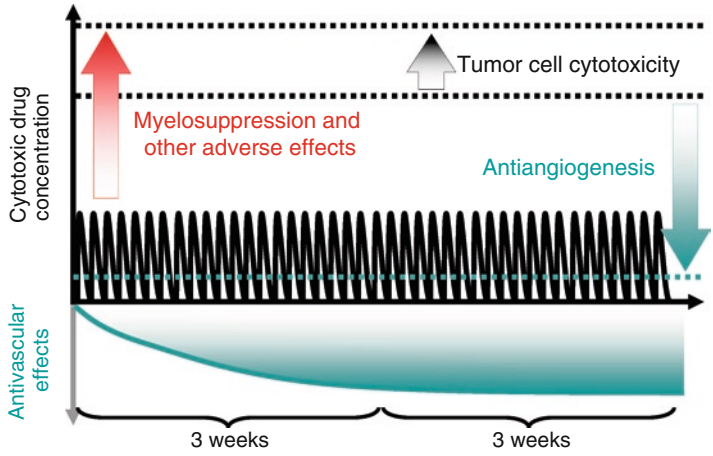

Fig. 11.2 Chemotherapeutic drugs affect endothelial cells at significantly lower doses than cancer cells or other rapidly proliferating cells such as hematological precursors. Thus, frequently applied but relatively low doses of chemotherapeutics result in significant antiangiogenic effects, which can be achieved without high-grade toxic side effects and the need for mandatory treatment interruptions

tumorigenesis, this is not a prerequisite for tumor endothelial cells [72]. Furthermore, endothelial cells may be sensitized to the effects of cytotoxic drugs by two distinct mechanisms. First, it has been shown that chemotherapeutics such as cyclophosphamide (CPA), various microtubule binding drugs, irinotecan and 5-fluorouracil (5-FU) as well as 5-FU precursors induce thrombospondin-1 (TSP-1) in endothelial and/or tumor cells [8,9,21,49,73,74,84,122]. Studies by Zhao et al. have implicated the p38 MAPK pathway as well as the transcription factor Egr-1 in TSP-1 induction by 5-FU [122]. TSP-1, a potent endogenous inhibitor of angiogenesis, facilitates apoptosis in tumor endothelial cells expressing the CD36 receptor [115,118]. Thus, elevated TSP-1 levels may selectively lower the apoptotic threshold of endothelial cells. Apoptosis in endothelial cells is further facilitated by the induction of Fas receptor following exposure to non-cytotoxic doses of CPA, cisplatinum, taxanes and doxorubicin [88,118], and upregulation of Fas ligand by TSP-1 [115]. Moreover, TSP-1 also exerts antiangiogenic effects by binding and sequestering VEGF [46].

2. Since endothelial cells are so exquisitely sensitive to a broad range of cytotoxic agents, antiangiogenic effects can be achieved with chemotherapy doses that are unlikely to affect the viability of cancer cells directly. This also explains why antiangiogenic doses of chemotherapeutic agents do not result in high-grade adverse effects typically seen with MTD chemotherapy such as grade 3–4 myelosuppression [1,13,14,16,18,23,31,38,39,71,93,120].
3. The latter implies that LDM regimens can be administered over prolonged periods of time without mandatory treatment-free breaks.

Experimental evidence to support the concept of LDM chemotherapy was first reported in 2000. Browder et al. showed that below-MTD doses of CPA administered every 6 days produced more sustained antiangiogenic effects than conventional every 3-week MTD CPA administration [15]. LDM CPA and docetaxel regimens have been shown to be effective even in tumor models with acquired resistance to MTD CPA and docetaxel chemotherapy, respectively [15,57], hence giving credit to the notion that mechanisms other than direct tumor cell cytotoxicity may account for the antitumor effects of LDM protocols. In fact, the antiangiogenic nature of LDM protocols is supported by a number of preclinical findings: LDM regimens reduce microvessel density, induce endothelial cell apoptosis that precedes tumor cell apoptosis, impair tumor perfusion as assessed in magnetic resonance imaging (MRI) studies and result in sustained tumor hypoxia [8,15,32,64,121]. Besides activities directed towards locally residing tumor endothelial cells, LDM therapy also impairs the mobilization and viability of endothelial cell precursors [103].

Proangiogenic factors such as VEGF or basic fibroblast growth factor also promote endothelial cell survival and confer relative chemotherapy resistance [113]. Thus, high levels of proangiogenic molecules may contribute to intrinsic or acquired resistance to LDM therapy [14,27]. Conversely, the beneficial effects of LDM therapy can be enhanced when combined with targeted antiangiogenic agents such as the anti-VEGF receptor 2 antibody DC101 [64], antiangiogenic

RTKI (e.g., sunitinib and imatinib [86], axitinib [74] and semaxinib [8]), or when combined with TSP-1 peptide derivatives [118]. Such combinations usually show much greater anti-tumor efficacy than LDM chemotherapy alone or targeted antiangiogenic monotherapy. Moreover, other approaches have been explored, including the combination of metronomic schedules with hypoxic cell cytotoxins, such as tirapazamine [32], or MTD chemotherapy [86,102]. However, only limited preclinical data is available on LDM regimens combining various chemotherapeutics administered in a LDM manner, despite the increasing use of such combinations by oncologists. Indeed, a few pioneering preclinical in vitro and in vivo studies have focused on the association of the 5-FU precursor tegafur-uracil (UFT) and CPA in a metastatic model of human breast cancer [83], or irinotecan/oxaliplatin/5-FU in a model of human colorectal cancer [35], in order to facilitate a rational rather than empirical development of such associations.

11.3.2 Clinical Applications

The concept of LDM chemotherapy has been rapidly embraced by oncologists [30], and is emerging as a complementary or potentially alternative antiangiogenic treatment strategy to VEGF pathway inhibitors. Benefits of LDM therapy have been demonstrated in a number of phase II clinical trials of a broad range of malignancies at advanced stages [1,13,14,16,18,23,38,39,43,62,71,93,117,120]. Furthermore, metronomic regimens using UFT significantly improved overall survival in patients with early stage breast and lung cancers in randomized phase III trials [58,116]. Several phase III trials have been initiated to further study the use of LDM chemotherapy in early breast cancer [87], and advanced breast and colorectal cancer (www.clinicaltrials.gov, NCT01131195 and NCT00442637).

Grade 3 and 4 adverse effects are rarely observed with LDM regimens, in sharp contrast to MTD chemotherapy, and to a lesser degree compared to targeted antiangiogenic agents such as bevacizumab and RTKI [1,13,14,16–18,23,38,39,43,62,71,93,120]. Consequently, the majority of patients tolerate LDM regimens over prolonged periods of time without treatment interruptions. In addition, combining LDM therapy with targeted antiangiogenic agents does not appear to increase the risk of adverse effects compared to targeted antiangiogenic monotherapy [23,39]. Often, LDM therapy involves outpatient-friendly oral regimens by using orally available alkylating agents (e.g., CPA, trofosfamide), 5-fluorouracil precursors (e.g., UFT, capecitabine) and microtubule binding drugs (e.g., vinorelbine). If off-patent drugs such as CPA are applied, the cost of such regimens is considerably less than treatment with targeted antiangiogenic therapies [11,22].

As far as the clinical documentation of antiangiogenic effects of LDM therapy is concerned, we note the absence of validated markers, as is the case for the field of antiangiogenic therapies in general [4,54,56,99]. DCE-MRI studies showed a reduction of tumor vessel permeability and blood flow in patients with various

advanced malignancies subjected to daily treatment with 500 mg capecitabine po bid, and 400 mg celecoxib po bid [108]. Moreover, after 2 months of therapy increased numbers of apoptotic circulating endothelial cells were associated with improved progression-free survival in patients with advanced breast cancer treated with LDM CPA and methotrexate [76]. However, the baseline number of circulating endothelial cells, and baseline levels or treatment-induced changes of circulating endothelial cell precursors were not associated with treatment response. Similarly, the results of analyses of circulating pro- and antiangiogenic markers at baseline – or changes thereof during LDM therapy – for predictive purposes are not conclusive across a number of published clinical studies [1,14,16,18,19,63]. Recently, Fontana et al. have proposed an alternative clinical approach to the direct quantification of circulating endothelial cells and their precursors [38]. The levels of circulating VE-cadherin (VE-C) RNA, an endothelial-specific transcript, were evaluated through quantitative reverse transcription-PCR analysis of whole blood as an indirect measurement of bone-marrow-derived circulating endothelial cell progenitors (as previously demonstrated by Rabascio et al. [89]). In metastatic prostate cancer patients responding to LDM CPA, celecoxib and dexamethasone therapy, VE-C mRNA levels were significantly lower than in non-responders.

While the combined analysis of circulating endothelial cell precursors and markers of bone-marrow toxicity have been used to define the optimal biological LDM dose of a given cytotoxic agent in mice [103], the low number of endothelial cell precursors in humans is among the reasons why such an approach cannot be easily translated clinically [6]. Thus, in the absence of validated biomarkers to guide metronomic dosing, an operational definition of LDM therapy may comprise the use of chemotherapy doses that can be applied for extended periods and without a need for treatment interruptions, i.e., that result in grade 3–4 adverse effects only in a small minority of patients, if at all [14,68]. Although flat dosing is commonly applied in clinical trials of LDM therapy, an individualized gemcitabine dosing strategy described by Takahashi et al. suggests that the ‘individualized maximum repeatable dose’ can vary significantly among patients [109]. As far as drug administration frequency is concerned, mathematical modeling suggests that daily – or even more frequent – dosing is superior to less frequent drug administration [48]. Aside from uncertainties about optimal dosing and scheduling, another potential limitation of the metronomic concept is the delayed onset of antitumor effects. Thus, LDM monotherapy should not be considered in situations of rapid tumor progression [14,71].

11.4 Low-Dose Metronomic Chemotherapy: Beyond Antiangiogenic Effects

It is not without precedent that the mode of chemotherapy administration can affect the mechanisms of action of chemotherapeutic agents. Indeed, preferred 5-FU incorporation into RNA during bolus administration is distinct from preferential incorporation into DNA when applying infusional 5-FU regimens. This

Table 11.1 Modulation of the tumor microenvironment by MTD versus LDM chemotherapy

	Maximum tolerated dose chemotherapy	Low-dose metronomic chemotherapy
Strategy	Maximal tumor cell kill, cytotoxicity	Antiangiogenesis, cytostasis
Primary target	Tumor parenchyma	Tumor vasculature
Dose	Maximum tolerated doses	Non-cytotoxic doses
Schedule	Cyclic administration, mandatory treatment-free periods	Frequent administration, continuous dosing
Side effects	Grade 3–4 common	Grade 3–4 rare
'Collateral' effects	Induction of **proangiogenic** factors, mobilization and recruitment of bone-marrow derived proangiogenic myeloid cells and endothelial cell precursors	**TSP-1** induction, induction of **endothelial cell Fas** and **FasL** expression
	Immunosuppression, facilitated antigen presentation	Immunostimulation (depletion of regulatory CD4+CD25+ T-cells, dendritic cell stimulation and maturation)
	Prothrombotic effects	–
	–	**Anti-Hif-1α activity**

TSP-1: thrombospondin-1; Fas/FasL: CD95 receptor/ligand; Hif-1α: hypoxia-inducible factor 1α

could explain the absence of complete cross-resistance between these regimens [107]. However, both bolus and infusional 5-FU are used at MTD doses. Thus, these 5-FU regimens intend to target tumor cells directly whereas LDM chemotherapy appears to affect primarily angiogenesis. However, there is growing evidence that LDM regimens can also directly affect tumor cells and other cellular tumor elements. In the following, we will discuss some of these postulated non-antiangiogenic effects of LDM regimens, which are summarized in Table 11.1.

11.4.1 Hypoxia-Inducible Factor 1α Inhibition

LDM regimens have been shown to decrease tumor oxygenation [32]. Although severe treatment-induced oxygen deprivation contributes to the anti-tumor effects of LDM and other antiangiogenic therapies, hypoxia also mediates adaptive responses that eventually might support treatment-refractory disease progression. The hypoxia-inducible factor 1α (Hif-1α) pathway is centrally involved in such adaptation [90]. Interestingly, cytotoxic agents such as topoisomerase 1 inhibitors impair the translation of Hif-1α at non-cytotoxic doses [91]. Furthermore, non-cytotoxic doses of doxorubicin affect the DNA binding of Hif-1α [69]. In other words, the LDM use of such agents may increase the hypoxic stress in tumors and at the same time undermine adaptive survival mechanisms. Finally to mention that Hif-1α inhibition is involved in reduced mobilization of proangiogenic bone-marrow derived cells and as such can reinforce the antiangiogenic effects of LDM doxorubicin [69].

11.4.2 TSP-1 Induction

Increased TSP-1 secretion by endothelial and/or tumor cells as a consequence of LDM therapy has been described as an important mediator of the antiangiogenic effects of such regimens [9,49]. However, TSP-1 has been implicated in many more processes such as tissue differentiation and response to injuries, regulation of inflammation and immune response, bone mineralization, and coagulation as well as fibrinolysis, all of which could have an impact on tumor progression and/or therapeutic resistance [12,114]. In fact, the expression of the TSP-1 receptor CD36 is not only restricted to endothelial cells. For instance, CD36 can also be found on macrophages/monocytes [119], and TSP-1 has been implicated in macrophage recruitment during wound healing [82]. Similarly, LDM CPA therapy induced TSP-1 secretion appears to be involved in the recruitment of macrophages into regressing glioblastoma xenografts [25]. However, the role of macrophages in the tumor context are complex and can involve both tumor promoting and inhibiting effects [78]. TSP-1 has also been shown to bind to CD47 on T-cells, which induced naive or memory CD4+CD25– T cells to become suppressive [44]. Furthermore, TSP-1 interacts directly with a number of extracellular matrix proteins found in tumors [110]. It remains to be demonstrated how these diverse TSP-1 effects contribute to the antitumor effects seen with LDM regimens.

11.4.3 Immunomodulation

The effects of chemotherapeutic agents on tumor antigen presentation and cellular effectors of the immune system depend on the type of cytotoxic agent, the dose as well as the schedule used. In most instances, MTD chemotherapy is considered to reduce the number and impair the function of immunological effector cells [67,123]. On the other hand, chemotherapy-related tumor cell destruction may facilitate antigen presentation and immunological memory generation.

LDM regimens using the alkylating agents CPA and temozolomide have been shown to reduce the number of immunosuppressive CD4+CD25+ regulatory T-cells in rodent models, whereas such a phenomenon is not seen with MTD treatment schedules [2,41]. LDM CPA also depletes CD4+CD25+ regulatory T-cells in humans with advanced malignancies, and restores T- and NK-cell effector functions [42].

Various topoisomerase inhibitors and antimicrotubule agents such as vinblastine and taxanes can promote dendritic cell maturation, survival and proliferation at sub-cytotoxic doses [111]. When using the ovalbumin-transduced EL4 tumor model, the intratumoral injection of vinblastine results in clonal expansion of ovalbumin specific T effector cells [112].

LDM CPA administration was superior compared to MTD CPA therapy if combined with specific antitumor immunotherapy in a mouse melanoma model [51]. Although both regimens reduced the number of tumor specific cytotoxic T-cells, the reduction occurred more slowly in the LDM CPA treated mice.

In addition, LDM CPA therapy spared CD8+ T memory cells. The use of combined chemo-immunotherapy in a patient with castration-resistant prostate cancer confirms the feasibility of such an approach in humans and suggests a potential clinical benefit [97].

11.4.4 Lack of Pro-Thrombotic Activity

While the risk of thrombotic events is generally increased in patients with malignancies [34], the use of MTD chemotherapy further increases this risk [47]. Thromboembolic complications are also among the more common side-effects of targeted antiangiogenic agents [17]. This could explain why the risk of thromboembolisms can be even further augmented when MTD chemotherapy is combined with targeted antiangiogenic agents [66]. In contrast, the use of LDM regimens does not appear to elevate the risk of thromboembolic events [1,13,16,18,23,38,39,71,93,120]. In fact, Ma et al. have shown in vitro that lowering the concentration of chemotherapeutic drugs such as gemcitabine and cisplatinum results in reduced pro-coagulatory activity [75]. Furthermore, the pro-coagulatory effects of targeted antiangiogenic agents may be attenuated by concomitant low-dose chemotherapy under certain circumstances.

11.5 Low-Dose Metronomic Chemotherapy: The Pharmacogenetic Perspective

The role of the tumor microenvironment as predictor of response to antitumor therapies is being increasingly emphasized. Individual genetic traits of patients could have a central role in responses to chemotherapy or antiangiogenic strategies by modulating the secretion of proangiogenic factors or endogenous angiogenesis inhibitors. For instance, a recent study focused on the IL-8 gene and its genetic variants in order to evaluate their influence on response to LDM CPA and bevacizumab therapy in patients with recurrent ovarian cancer [98]. The results suggest that the IL-8 251A/T polymorphism may be a molecular predictor of response to such therapy. However, the validation of specific polymorphisms that are predicting response to LDM regimens is a complex process, which will need to involve both preclinical and clinical studies. It is highly desirable that pharmacogenetic studies within LDM clinical trials will evaluate both genotype and phenotype in correlation with clinical outcome. At the present time, LDM chemotherapy is mainly explored as a palliative treatment strategy after numerous lines of standard chemotherapy. This aspect should be considered when planning and executing pharmacogenetic studies as part of LDM chemotherapy trials. The following paragraphs summarize relevant aspects of pharmacogenetic studies focused on the LDM treatment strategy. At least the following three specific issues should be addressed:

11.5.1 How to Integrate Pharmacogenetic Investigations into Metronomic Phase II/III Clinical Trials?

Pharmacogenetic analyses within LDM chemotherapy trials could be conducted as integral part of large randomized phase II/III trials or as independent studies focused on the validation of specific genetic determinants. While the first approach may be helpful to find new pharmacogenetic determinants (i.e., by sequencing numerous genes directly involved in the metabolism and mechanism of action of drugs used in LDM protocols such as CPA), the second approach is instrumental to corrobate statistical correlations between selected single nucleotide polymorphisms (SNPs) or haplotypes and clinical end-points. Such studies can be conducted both in a prospective and retrospective way.

11.5.2 What Is the Most Effective Pharmacogenetic Strategy to be Used?

Two types of approach have been defined in recent years in order to set up pharmacogenetic studies, i.e., the "candidate gene approach" and the "whole genome SNP approach". The first one involves a priori SNP selection (maximally 3–5) regarding a gene of interest in order to confirm a hypothesis, e.g., that the IL-8 251A/T SNP may represent a suitable candidate to predict response to LDM CPA plus bevacizumab therapy. The second approach is much more costly by investigating 100,000 SNPs but may reveal unexpected correlations. To move pharmacogenetics of LDM chemotherapy into clinical practice, the "pyramidal model" proposed by Johnson et al. [55] could be followed. The required steps from early data to clinical application include (i) the initial sequencing of the candidate genes (e.g., IL-8 gene), (ii) in vitro studies (e.g., functional analysis of IL-8 polymorphisms), (iii) proof of concept clinical studies (e.g., IL-8 SNP analysis in ongoing and planned LDM trials), (iv) SNP analysis in relevant patient population, (v) studies aimed at documenting a sufficient degree of variability of given SNPs in order to be predictive clinically, and (vi) comparison of pharmacogenetically guided versus standard patient care.

11.5.3 How to Decide About Candidate Genes to be Investigated?

Candidate genes for pharmacogenetics of LDM therapy should not be restricted to genes implicated in angiogenesis, such as VEGF, VEGF receptor-2 and IL-8, but also genes involved in the metabolism of chemotherapeutic drugs. As an example, the biotransformation of CPA involves a 4-hydroxylation activation step carried out by several cytochrome P450 (CYP) isoforms, including 2B6, 3A4, and 2C9. Cytochrome P450 2B6 is the most important isoform in this respect, and the liver

is the main organ of this rate limiting reaction resulting in the active metabolite 4-hydroxy-CPA [33]. Thus, SNPs that could modulate the enzymatic activity of the aforementioned CYPs may heavily alter the response to LDM CPA therapy. Furthermore, the frequency of specific SNPs may dictate the sample size needed for pharmacogenetic studies that are appropriately powered for statistical analyses. As an example, to investigate the VEGF-A 936C/T SNP, we should consider that the frequency of 936T is around 16% [95].

11.6 Outlook

Clinical strategies targeting aspects of the tumor microenvironment such as angiogenesis, osteoclast activity [24] and immunity [26] have been shown to be beneficial even though the advancement is only incremental to date. Especially the successful application of antiangiogenic therapies has taught us a few lessons:

1. Targeting a single aspect of the tumor microenvironment such as angiogenesis is unlikely to result in a 'seismic' shift in antitumor efficacy [106]. In fact, the clinical impact of antiangiogenic monotherapy is very modest in most tumor types, combining antiangiogenics with cytotoxic therapy does not appear to increase the overall survival by more than a few months, and the cure rate of early and late malignances is not increased when applying antiangiogenics. However, preliminary studies suggest that the simultaneous administration of agents affecting different aspects of the tumor microenvironment is feasible and may be beneficial [7,92–94]. This remains to be studied in more detail in randomized clinical trials.
2. Antiangiogenic agents usually need to be given continuously for prolonged periods of time for maximal efficacy. While this creates new challenges (e.g., treatment adherence, in particular if oral drugs are used) [85], this also points to one of the major shortcomings of conventional chemotherapy, i.e., the need for treatment-free breaks for patients to recover from side-effects. Indeed, while MTD chemotherapy affects many processes in the tumor microenvironment, including angiogenesis, these effects are usually short-lived. Even worse, the acute changes inflicted by MTD chemotherapy may elicit adaptive responses that eventually can undermine the initial antitumor effects [40]. Although antiangiogenic therapies were considered to be less susceptible to acquired resistance than conventional tumor therapies, this has not been confirmed (pre)clinically [3,59]. Therapeutic resistance remains a major obstacle in the era of antiangiogenic therapies, but the underlying mechanisms appear to be clearly distinct from classical cytotoxic drug resistance. A better understanding of these mechanisms will hopefully allow to delay if not to circumvent such resistance in the not so far future.
3. The use of microenvironment targeting agents results in cytostatic rather than cytotoxic effects. This challenges the way treatment response and resistance are defined and monitored.

LDM chemotherapy is distinct from both conventional chemotherapy (Table 11.1) and other antiangiogenic therapies in clinical use or testing [45,53]. First, the antitumor effects of LDM chemotherapy are usually more subtle and often delayed. However, this might be beneficial in that adaptive mechanisms are less violent [40]. Second, due to its excellent safety profile compared to MTD chemotherapy and – to a lesser degree – to targeted antiangiogenic agents, LDM chemotherapy can be administered over prolonged periods of time without mandatory treatment interruptions. Third, although the full extent of the pleiotropic effects of LDM therapy remains to be elucidated, the broad range of activities could be superior under certain circumstances compared to the use of the highly specific, targeted agents with a very narrow spectrum of antitumor effects [52]. In spite of all these promises, certain aspects of LDM therapy need further refinement. Metronomic dosing and scheduling are largely empirical to date. We lack insights into which cytotoxic drug(s) to choose for metronomic purposes in a given patient. Moreover, there is a lack of predictive markers of response.

Nonetheless, 50 years after initial CPA trials the modified use of old-fashioned, 'dirty' drugs like CPA has revealed new and unexpected secrets. The golden anniversary of CPA and other cytotoxic agents [29] could be the starting point of many more exciting revelations to come.

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Part IV
Tumors are Evolvable Modular and Rationalized Systems: From Molecular to Modular Tumor Therapy

Chapter 12
Systems Biology: A Therapeutic Target for Tumor Therapy

Albrecht Reichle and Thomas Vogt

Abstract Tumor-related activities that seem to be operationally induced by the division of function, such as inflammation, neoangiogenesis, Warburg effect, immune response, extracellular matrix remodeling, cell proliferation rate, apoptosis, coagulation effects, present itself from a systems perspective as an enhancement of complexity. We hypothesized, that tumor systems-directed therapies might have the capability to use aggregated action effects, as adjustable sizes to therapeutically modulate the tumor systems' stability, homeostasis, and robustness. We performed a retrospective analysis of recently published data on 224 patients with advanced and heavily pre-treated (10–63%) vascular sarcoma, melanoma, renal clear cell, cholangiocellular, carcinoma, castration-resistent prostate cancer, and multivisceral Langerhans' cell histiocytosis enrolled in nine multi-center phase II trials (11 centers). Each patient received a multi-targeted systems-directed therapy that consisted of metronomic low-dose chemotherapy, a COX-2 inhibitor, combined with one or two transcription modulators, pioglitazone +/– dexamethasone or IFN-alpha. These treatment schedules may attenuate the metastatic potential, tumor-associated inflammation, may exert site-specific activities, and induce long-term disease stabilization followed by prolonged objective response (3–48%) despite poor monoactivity of the respective drugs. Progression-free survival data are comparable with those of reductionist-designed standard first-line therapies. The differential response patterns indicate the therapies' systems biological activity. Understanding systems biology as adjustable size may break through the barrier of complex tumor-stroma-interactions in a therapeutically relevant way: Comparatively high efficacy at moderate toxicity. Structured systems-directed therapies in metastatic cancer may get a source for detecting the topology of tumor-associated complex

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aggregated action effects as adjustable sizes available for targeted biomodulatory therapies (Reichle A, Vogt T (2008) Systems biology: a therapeutic target for tumor therapy. Cancer Microenviron 1:159–170)

Keywords Low-dose metronomic chemotherapy • COX2 • PPAR • Dexamethasone • Interferon-alpha • Systems biology • Metastatic tumor • Melanoma • Sarcoma • Angiosarcoma • Castration-resistent prostate cancer • Renal clear cell carcinoma • Cholangiocellular carcinoma • Langerhans' cell histiocytosis

12.1 Introduction

Unlike laws of nature, causal relations between initiating processes of tumor development are not anchored in an invariance of nature. Therefore, molecular and cytogenetic aberrations at initial diagnosis are generally heterogeneous in both tumors and single tumor types. Invariance within the tumor process may be observed during tumor progression. In interaction with normal human tissue, tumor cells use processes according to laws of nature to build up a favorable infrastructure for proliferation. In 1986, Dvorak interpreted for the first time these laws of nature as tumor-associated 'wound healing' mechanisms such as angiogenesis, inflammation, immunology, remodeling of the extracellular matrix, specific changes in cell metabolism and coagulation, and altered behavior in proliferation [1–7]. Accordingly, tumors may be figuratively conceived as 'never healing wounds'. With this interpretation, Dvorak addressed the systems biology of a tumor in a contemporary context. Up to now, a tumor's systems biology has rarely presented a target for a systematic approach in cancer treatment.

The dysregulated systems biology of a tumor may commonly not be understood mono-causally or explained context-free. The tumor's systems biology intents on a dysbalance between interfering functional elements in a way that conditioning and conditioned tumor-promoting elements (e.g. wound healing mechanisms) behave reciprocally also under therapeutic aspects.

The dysregulation of wound healing mechanisms is reflected in tumor-associated disease traits (e.g. tumor-associated inflammation, ECOG performance status, thrombophilia, and tumor-associated auto-immunity) and on the molecular level in the dysregulation of (nuclear) transcription factors, both in tumor and neighboring stroma cells (see chapter 22). Transcription factors regulate in a concerted action distinct gene cascades and consecutively important cell functions for survival. Their cooperative interaction is also important for the survival of tumor cells.

In seven published phase II trials, we combined modulators (ligands) of (nuclear) transcription factors (pioglitazone, dexamethasone, interferon-alpha, cyclooxygenase-2 (COX-2) inhibitors) with the aim to suppress tumor-associated inflammation [8–15]. Corticosteroids are known for their anti-inflammatory activity; interferon-alpha at low doses (3.0–4.5 MU three times a week) shows both anti-inflammatory and angiostatic activity as well as the antidiabetic drug

pioglitazone (peroxisome proliferator-activated receptor (PPAR)-alpha/gamma agonist) [16–18]. Besides its anti-inflammatory activity, the COX-2 inhibitor also exerts an anti-proliferative via suppression of the PPAR-delta expression [19]. The efficacy of the anti-inflammatory therapy approach was controlled by the measurement of C-reactive protein (CRP) levels in serum.

To enhance the therapeutic efficacy, a second wound healing mechanism was therapeutically targeted: neoangiogenesis. Metronomic low-dose chemotherapy with either trofosfamide or capecitabine may enhance the important antiangiogenic factor thrombospondin-1 in serum with simultaneously negligible cytotoxic activity of the respective drugs [20]. The present therapeutic approach – a combination of anti-inflammatory, angiostatic and immunomodulatory therapy – is primarily directed against invariant mechanisms embedded in the laws of nature that are generally important during tumor progression. Therefore, treatment efficacy may be expected to some degree, independently of the tumor type.

The summary of recently published data on combined anti-inflammatory and angiostatic therapy approaches in metastatic cancer may support the 'wound healing' hypothesis from a therapeutic view. Firstly, we want to show with our data from seven clinical trials that different antiinflammatory approaches are not only clinically efficacious and safe but show a moderate toxicity profile and may even induce continuous complete remission in combination with angiostatic therapies. Secondly, we are going to demonstrate according to the observed typical response characteristics that our therapeutic approaches have primarily biomodulatory rather than classic cytotoxic activity. Thirdly, we have introduced combined anti-inflammatory and angiostatic approaches for the therapy of metastatic tumors. The combined activity may even induce continuous complete remission.

The summarized results of the presented biomodulatory therapy approaches in different metastatic tumors contradict the paradigm that for the most part only drug-mediated blockades of more or less tumor-specific aberrant pathways may induce tumor response, a paradigm which is supported by an overwhelming number of clinical data.

12.2 Patients and Methods

12.2.1 Selection of Metastatic Diseases

We performed retrospective analyses of recently published data from our study group on patients with advanced and heavily pre-treated tumors (Table 12.1). According to our chosen therapeutic approaches – a combined anti-inflammatory and angiostatic therapy – we selected (1) tumors with high vascular density such as vascular sarcomas and renal clear cell carcinomas (RCCC), (2) a highly inflammatory tumor type, i.e. chemo-resistant multivisceral Langerhans' cell histiocytosis, and (3) tumors with a known inflammatory component at least in the metastatic stage (melanoma, cholangiocellular carcinoma, and castration-resistent prostate

Table 12.1 Combined targeting of (nuclear) transcription factors

			Receptor agonist/antagonist				
Tumor type	Metronomic chemotherapy	No. of patients	PPAR α/γ agonist[a]	PPARδ antagonist[b]	Glucocorticoid[c]	IFN-α[d]	Publications
Kaposi sarcoma	Trofosfamide	1	+	+	–	–	Arch Dermatol, 2004
Angiosarcomas	Trofosfamide	6	+	+	–	–	Cancer, 2003
Sarcomas I	Trofosfamide	21	+	+	–	–	Cancer, 2004
Melanoma I	Trofosfamide	19	+	+	–	–	Cancer, 2004
Melanoma II Arm A		35	–	–	–	–	Melanoma research, 2007
Arm B		32	+	+	–	–	
Langerhans' cell histiocytosis	Trofosfamide	2	+	+	–	–	Br. J. Haematol, 2005
Renal clear cell carcinoma I	Capecitabine	18	+	+	–	–	Biomarker insights, 2006
Renal clear cell carinoma II	Capecitabine	33	+	+	–	+	Biomarker insights, 2006
Castration-resistent prostate cancer	Capecitabine	36	+	+	+	–	Lancet oncology, 2006 ASCO abstract, 2007
Cholangiocellular carcinoma	Capecitabine	21	+	+	–	–	Tumor microenvironment prague, 2004 (medimond)

PPAR peroxisome proliferator-activated receptor

[a]Pioglitazone

[b]Selective COX-2 inhibitor

[c]dexamethasone

[d]interferon-α

Basic treatment considerations

Furthermore, anti-inflammatory approaches were selected according to known effects of dexamethasone in hormone

cancer (CRPC). All patients were enrolled in phase II trials, and melanoma patients additionally participated in a randomized phase II trial.

12.2.2 Patients' Characteristics

The local ethics committee approved study protocols, and patients were required to provide written informed consent before enrolment. Patients presented were recruited between February 2001 and July 2006 in seven phase II trials including one randomized phase II trial in metastatic melanoma. Patients with advanced bidimensionally measurable neoplasias, either systemically pretreated or not, who experienced disease progression and who had a life expectancy of more than 3 months were eligible for the studies. Controlled brain metastases were no exclusion criteria. The remaining inclusion criteria are indicated in the respective publication.

12.2.3 Basic Treatment Considerations

Treatment schedules were intended to achieve disease stabilization in metastatic neoplasias of different origin with uniform biomodulatory treatment principles and to limit therapy-related toxicity in advanced tumor stages. All patients received a combined anti-inflammatory and angiostatic therapy consisting of (1) metronomic low-dose chemotherapy (trofosfamide or capecitabine), (2) COX-2/PPAR (peroxisome proliferator-activated receptor)-delta blockade (rofecoxib or etoricoxib) combined with (3) one or two transcription modulators, i.e. pioglitazone (peroxisome proliferator receptor alpha/gamma agonist) +/−dexamethasone or pioglitazone +/− IFN-alpha (Table 12.1) [8–15].

12.2.4 Anti-Inflammatory Therapies

We have chosen drugs with transcriptional activity in the field of inflammation control: glucocorticoids (dexamethasone 0.5–1.0 mg daily), interferon-alpha (3–4.5 MU three times a week), and the glitazone pioglitazone (45–60 mg daily).

Also the administered coxibs (rofecoxib 12.5–25 mg daily or etoricoxib 60 mg daily) may express transcriptional activity by the inhibition of PPAR-delta. The transcriptional modulators used are all multifunctional modulators that may not only achieve specification of their activity by nuclear receptor cross-talk [21–23] but may also have important receptor dependent (genomic and non-genomic) as well as independent (non-genomic) activities [17,18,24].

Furthermore, anti-inflammatory approaches were selected according to known effects of dexamethasone in castration-resistent prostate cancer and interferon-alpha (at high-doses) in metastatic renal cell carcinoma. Interferon-alpha was used at a dose

level for angiostatic activity, i.e. at very low doses. In metastatic RCCC, we selected in a second consecutive trial an anti-inflammatory approach with presumably enhanced anti-inflammatory capacity: pioglitazone, coxib, and additionally interferon-alpha [12]. In CRPC, a combination of two activators of nuclear transcription factors (pioglitazone and dexamethasone) has been introduced [14].

A randomized phase II trial (metastatic melanoma) evaluated the additional effects of anti-inflammatory therapy in addition to metronomic low-dose chemotherapy on progression-free and overall survival (combined antiinflammatory/angiostatic versus angiostatic approach) [9]. In the trials melanoma I, sarcoma I, and vascular sarcomas, we introduced a 14 day lead-in phase with antiinflammatory therapy only (pioglitazone plus rofecoxib or etoricoxib) [8,9].

12.2.5 Angiostatic Therapies

Angiostatic therapy consisted of metronomic low-dose chemotherapy, either 50 mg oral trofosfamide (Baxter) administered continuously two or three times daily or 1 g/m^2–1 g absolute oral capecitabine (Roche) administered twice per day.

12.3 Systems Biology: A Therapeutic Target for Tumor Therapy

12.3.1 Treatment Schedules

Patients were centrally randomized for the Melanoma II trial. Arm A received 50 mg oral trofosfamide (Baxter) administered continuously three times daily from day 1+, Arm B of trofosfamide in the same dosage plus continuously 60 mg oral pioglitazone (Takeda) and 25 mg oral rofecoxib (MSD) once daily starting with day 1+. Treatment was continued until disease progression was documented or for a maximum of 6 weeks after confirmation of CR. Following disease progression, a crossover from Arm A to B was allowed.

Patients treated in the vascular sarcoma trial (including one patient with Kaposi sarcoma), in the Melanoma I, Sarcoma I, and Langerhans cell histiocytosis trials received Arm B-therapy as described above. Melanoma and sarcoma patients had a lead-in phase with anti-inflammatory therapy alone over 14 days. Patients in the RCCC study I received 1 g/m^2 oral capecitabine (Roche) administered twice daily from day 1+, 60 mg oral pioglitazone (Takeda). Patients enrolled before November 2004 also received 25 mg oral rofecoxib daily, whereas patients enrolled after November 2004 were given 60 mg oral etoricoxib daily instead, starting with day 1+. Patients in study II (RCCC II) received additionally 4.5 MU IFN-alpha subcutaneously, three times per week, from day 1+. Patients with cholangiocellular carcinoma were treated with the schedule of RCCC I.

12.3.2 Combined Targeting of Wound Healing Processes

In all studies, we selected transcriptional modulators including those of nuclear transcription factors with the aim to control tumor-associated inflammation. For metastatic melanoma, we performed a randomized phase II trial to directly study the impact of inflammation control on progression-free and overall survival. A historic comparison (RCCC I/II) shows the impact of weak versus strong control of tumor-associated inflammation on progression-free and overall survival in renal clear cell carcinoma. In castration-resistent prostate cancer, published data from metronomic cyclophosphamide and dexamethasone treatment are available for a historical comparison [25].

12.4 Pre-Treatment Evaluation Is Indicated in the Respective Publications

12.4.1 Evaluation of Efficacy

Response and toxicity were evaluated in patients who had a follow-up duration of ≥3 weeks. Objective tumor responses were identified using the World Health Organization (WHO) criteria (vascular sarcomas, sarcomas, melanomas) or RECIST criteria (RCCC and CRPC).

12.5 Modulation of Tumor-Associated Disease Traits

12.5.1 ECOG Status: ECOG Performance Status Was Routinely Monitored

Monitoring of CRP Serum CRP levels were measured in follow-up to evaluate the incidence of systemic inflammatory response in metastatic tumors dependent on the tumor histology and to determine the intensity of the inflammatory response as well as the time of inflammation response in relation to objective tumor response.

As part of an exploratory retrospective analysis, PFS and OS were evaluated separately for two groups of patients: (1) CRP responder: Patients with normal range CRP levels throughout the first 6 weeks of treatment and patients with elevated CRP levels, who responded with an at least 30% decrease within the first 6 weeks of treatment (two consecutive measurements at least 14 days apart). (2) CRP non-responder: Patients with ≤30% decline or increasing CRP levels in two consecutive measurements 14 days apart within the first 6 weeks of treatment. Patients receiving a lead-in phase with anti-inflammatory therapy were monitored for CRP at study inclusion and in a 14 day interval.

12.5.2 Metastatic Sites

On the background of the discussion, whether combined biomodulatory therapies have any tissue specificity, i.e. are dependent on the cellular tumor-stroma composition at an organ site, we analyzed the response dependent on the localization of the metastatic organ sites. To assess whether an anti-inflammatory/angiostatic treatment approach has any impact on the metastatic spread during progression, we analyzed the metastatic sites after progression on study medication.

12.5.3 Statistics and Data Analysis

Primary endpoints in all trials were PFS and treatment safety. Analysis of treatment safety was restricted to patients receiving study medication, analysis of the tumor response to patients who were treated for at least 3 weeks. The overall response rate was defined as percentage of patients with confirmed CR or PR. SD was defined as no tumor progression (<25%) during a 6 months treatment interval. Response duration was calculated from randomization or study inclusion to the date of first observation of progressive disease (PD) or death. Progression-free survival was defined as the interval between the beginning of treatment and disease progression. Survival duration was calculated from randomization or study inclusion. Survival distributions were generated using the Kaplan-Meier method. Survival analyses were performed on eligible patients, the full analysis set (FAS) and on the intent-to treat (ITT) population (defined as all randomly assigned patients). In addition, the Fisher exact test and the "Student t"-test were used to identify significant associations between clinical and biologic variables.

12.6 Results

In total, 224 patients with metastatic cancer from eleven centers and various medical specialties including urology, dermatology, gastroenterology, and hematology/oncology were treated within seven trials: The intention was to show the efficacy and tolerability of a combined anti-inflammatory (pioglitazone plus coxib) and angiostatic therapy (trofosfamide or capecitabine) in advanced tumor stage and in a high number of refractory cancer (10–63%). More detailed patient characteristics may be found in the respective publications [8–15].

All trials were initiated as palliative therapies. Therefore, it is remarkable that we could observe objective response (3–48%) and continuous complete remissions independent of the tumor type (vascular sarcoma, RCCC, melanoma, castration-resistent prostate cancer, cholangiocellular carcinoma, and Langerhans' cell histiocytosis) in all treatment groups (except RCCC I) (Table 12.2).

Table 12.2 Combined targeting of angiogenesis and inflammation: efficacy

	Response			
Tumor type	No. of patients	Partial response (%)	Complete response (%)	cCR (%)
Sarcomas I	21	19	16	5
Angiosarcomas	6	17	33	17
Melanoma I	19	10	5	0
Melanoma II Arm B	35	9	3	3
Langerhans' cell histiocytosis	2	–	100	100
Renal clear cell carcinoma I (no IFN-α)	18	0	0	0
Renal clear cell carinoma II (plus IFN-α)	33	35	13	6
Castration-resistent prostate cancer	36	28	6	6
Cholangiocellular carcinoma	21	24	5	5

Table 12.3 Progression-free/overall survival with combined angiostatic plus anti-inflammatory therapy

	Treatment		Median Progression-free/overall survival (months)		
Trial	Angiostatic	Anti-inflammatory	Pretreated patients (%)	Trial	Historical control (first-line)
RCCC I	Capecitabine	Pio/Rofe	39	4.7/16.2	
RCCC II	Capecitabine	Pio/Eto/IFN-α	21	11.5/25.6	11.0/na(for sunitinib)
CRPC	Capecitabine	Pio/Eto/Dexa	39	3.6/14.4	na/17.5 (for taxotere)
Melanoma II					
Arm A	Trofosfamide	–	63	1.2/8.2	na/5.6 (for DTIC)
Arm B	Trofosfamide	Pio/Rofe	60	2.0/18.8	–
Cholangiocellular carcinoma	Capecitabine	Pio/Rofe	10	2.0/8.0	PR plus stable disease 20–73%

Pio – pioglitazone; Rofe – rofecoxib; Eto – etoricoxib; RCCC – renal clear cell carcinoma; CRPC – hormone refractory prostate cancer; na – not available

Median progression-free survival as the primary endpoint in all trials is listed in Table 12.3. Interestingly, despite of the inclusion of systemically pre-treated patients at a high percentage (10–63%), the PFS rate is comparable to the respective rate achieved in first-line therapy (trial RCC II, Melanoma II, and cholangiocellular carcinoma). In metastatic melanoma (Melanoma II), metronomic low-dose chemotherapy with trofosfamide seems to be even equivalent to standard DTIC treatment in a historical comparison [26–28].

12.7 Tailored Modeling of Tumor-Associated Disease Traits

Overall, five different tumor-associated disease traits were followed within each trial with biomodulation-derived biomarkers: (1) Changes in the ECOG status, in (2) serum CRP levels, (3) the resolution of paraneoplastic syndromes, (4) objective tumor response at single metastatic organ sites, and (5) the dissemination of metastatic disease at tumor progression (metastatic spread).

12.7.1 ECOG Performance Status

ECOG performance status could be improved in all trials (19–100%). As expected in the Melanoma II trial, no ECOG improvement was observed within treatment arm A (without anti-inflammatory therapy) (Table 12.4). An improvement of the performance status due to inflammation control was possible on the basis of a very low rate of grade III toxicities in all trials (Table 12.5).

Table 12.4 Tumor-associated inflammation in metastatic cancer

Trial	Frequency of CRP elevation >10 mg/L (%)	CRP >30% response (% patients)	Significance of CRP response during 2–6 weeks on treatment	Improvement of ECOG status (% patients)	Progression-free survival and overall survival
Renal clear cell carcinoma I	72	69	p = 0.32	22	Significant improvement of PFS and OS in RCCC II (non randomized)
Renal clear cell carcinoma II	100	100	p = 0.0005	24	
Castration-resistent prostate cancer[a]	28	11	p = 0.67	30	
Melanoma I	81	88	p = 0.004	19	
Melanoma II					
Arm A	87	6	p = 0.52	0	Significant improvement of overall survival (CRP responder)
Arm B (randomized)	100	69	p = 0.0007	27	
Sarcoma	79	74	p = 0.006	28	
Angiosarcoma[a]	100	100	–	–	
Langerhans' cell histiocytosis	100	100	–	100	

[a] Resolution of paraneoplastic syndromes: lupus erythematodes, hypoglycaemia

Table 12.5 Toxicities WHO Grade 3 (no Grade 4 toxicities) within all seven trials (n = 224 patients)

Toxicity	No. of patients (%)	Trial	Toxicity related to the following drug
Cushing syndrome	1 (0.4)	CRPC	Dexamethasone
Depression	1 (0.4)	RCCC	Interferon-alpha
Hand-Foot-Syndrome	5 (2.2)	CCC, CRPC	Capecitabine
Hematotoxicity	14 (6.2)	All trials	Metronomic chemotherapy
Edema	5 (2.2)	All trials	COX-2 inhibitor
Nausea/Vomiting	3 (1.3)	All trials	

12.7.2 Paraneoplastic Syndromes

The anti-inflammatory activity of the chosen treatment schedules was additionally shown by the resolution of paraneoplastic syndromes: Hypoglycemia and lupus erythematodes respectively [29,30].

12.7.3 Serum CRP Level in Follow-Up

The incidence of elevated CRP levels (>10 mg/L) at study inclusion differed considerably between the different tumor types (Table 12.4). In groups with consistently elevated CRP levels (RCCC, melanoma, sarcoma, Langerhans' cell histiocytosis), a significant CRP response (>30%) was observed during the lead-in phase with anti-inflammatory therapy alone or during 4–6 weeks of combined treatment. Thus, efficacy of an anti-inflammatory therapy could be sufficiently followed in metastatic diseases with constitutive systemic inflammatory response (Table 12.4).

A CRP response indicated stable disease or objective response in most patients; however, few patients experienced progressive disease (6%) despite of a CRP response. Therefore, CRP response indicates a tailored modeling of a tumor-associated disease trait but CRP assessment should not be used as a tumor marker. In CRPC, a CRP decrease was always paralleled by PSA response, whereas CRP response and/or ECOG improvement preceded objective responses by months (3.1–8.6 months) in all other trials with the exception of individual patients with vascular sarcomas [8]. Due to the observed objective tumor responses to anti-inflammatory therapy in diseases without initial systemic inflammatory reaction such as CRPC, localized inflammatory tumor-associated processes have to be suggested as basis for the observed objective tumor responses [31].

12.7.4 Impact of Anti-inflammatory Therapy

The efficacy of an anti-inflammatory therapy alone has already been shown in a randomized comparison in advanced cancer [32]. We can now extend the experiences on

anti-inflammatory therapy: (1) Anti-inflammatory therapy adds further benefits to angiostatic low-dose chemotherapy by a significant improvement of OS in metastatic melanoma, although the objective response rates in both treatment arms did not significantly differ (randomized melanoma phase II trial, Melanoma II), and (2) the intensity of an anti-inflammatory approach, as indicated by the extent of CRP decrease in serum, may have significant impact on outcome (sequentially performed RCC trials I/II).

12.7.5 Intensification of Anti-inflammatory Therapy

Two kinds of intensification of anti-inflammation were tested including a second transcriptional modulator, i.e. dexamethasone (CRPC) or interferon-alpha (RCCC II) (Table 12.1). The addition of low dose interferon-alpha to pioglitazone and COX-2 inhibitor dramatically increased the control of tumor-associated inflammation and consecutively improved the tumor response as well as the survival rate (historical comparison). These results demonstrate that strong inflammation control may be an important prerequisite for the response in metastatic, non-resectable RCCC. In CRPC, dexamethasone showed very modest anti-tumor activity. However, the addition of a glitazone (plus coxib) resulted in a high response rate, interestingly even up to the achievement of complete remission. Due to the poor monoactivity of capecitabine in CRPC, most activity of the schedule might be related to the anti-inflammatory approach.

12.7.6 Combined Transcriptional Modulation

The combined use of transcription modulators for inflammation control in CRPC (dexamethasone, pioglitazone, and coxib) and in RCCC II (interferon-alpha, pioglitazone, and coxib) – and glitazones plus coxib in all the other tumor types – seems to improve outcome in comparison to historical controls or is at least equivalent but with less therapy-related toxicity. Except for the monoactivity of metronomic low-dose chemotherapy in advanced melanoma and presumably in angiosarcomas, all other treatment components, i.e. interferon-alpha at very low dose-levels, pioglitazone, coxibs, and low-dose dexamethasone have very modest or none monoactivity at all in the respective tumor types [33–38]. Exclusively their combination paves the way for objective responses via transcriptional cross-talks.

12.7.7 Angiostatic Therapy

Metronomic low-dose chemotherapy showed a significant activity in the randomized melanoma trial (melanoma II) (Table 12.2). Recently published data disclosed that the second drug capecitabine has a rather modest activity in CRPC [33] when administered in a nearly metronomic manner. Thus, most clinical

effects in CRPC may be related to a combined anti-inflammatory activity. This observation is supported by unpublished data indicating objective responses after a change to metronomic low-dose treosulfan (250 mg twice daily) in patients with progressive CRPC on study medication. In cholangiocellular carcinoma, anti-inflammatory and angiostatic effects cannot be separated and assessed in correlation to historical data.

12.7.8 Metastatic Sites and Response

To evaluate tumor-stroma-specific activities of the administered drugs, we studied whether specific single metastatic organ sites respond predominantly to combined biomodulatory therapy. An organ-specificity of combined anti-inflammatory and angiostatic activity could be observed in CRPC: in bone lesions, resolution or >50 regression (scintigraphy) of metastatic lesions could be observed, whereas only minor responses or stable diseases were diagnosed in all other metastatically involved organs.

12.7.9 Metastatic Sites at Progression

Overall, 76% of the patients within the Melanoma trial II, RCCC trial II, and CRPC trial were systematically studied for metastatic sites at tumor progression. Interestingly, 67% of these patients had no additional metastatic organ sites at the time of progression, but local tumor progression or additional metastasis in the organ involved originally. This finding could indicate an attenuation of metastatic spread by the combined antiinflammatory and angiostatic approach. Probably because of the short median progression-free survival in Melanoma II, no significant differences could be found between the two treatment arms concerning metastatic spread at progression. The treatment and response characteristics support biomodulatory mechanisms of action: (1) No or poor single agent activity of each administered drug, (2) a very moderate toxicity profile during long-term drug administration up to 26 months, (3) very delayed objective responses, (4) improved overall survival without an increase of response rate (randomized Melanoma trial), (5) significant modulation of tumor-associated disease traits, e.g. inflammation, ECOG status, paraneoplastic syndromes, (6) activity depending on the metastatic organ site in CRPC, and (7) predominant site of progression at the original localization of the metastases.

12.8 Safety Profile

The toxicity profiles of the presented biomodulatory approaches are modest as reflected in a low rate of WHO grade >2 toxicities and no grade 4 toxicities (Table 12.6). Thus, the desirable therapeutic effects could be achieved by

Table 12.6 Combined targeting of angiogenesis and inflammation: patients with progressive disease

Tumor type	No. of patients	Targeted (nuclear) transcription factors	Progressive disease (no. of patients %)
Sarcomas I	21	PPAR α/γ, PPAR δ	4 (19)
Angiosarcoma	6	PPAR α/γ, PPAR δ	0
Melanoma I	19	PPAR α/γ, PPAR δ	4 (21)
Melanoma II Arm B	35	PPAR α/γ, PPAR δ	6 (17)
Langerhans' cell histiocytosis	2	PPAR α/γ, PPAR δ	0
Renal clear cell carcinoma I	18	PPAR α/γ, PPAR δ	9 (50)
Renal clear cell carcinoma II	33	PPAR α/γ, PPAR δ via IFN-α receptor	2 (7)
Castration-resistent prostate cancer	36	PPAR α/γ, PPAR δ glucocorticoid receptor	5 (14)
Cholangiocellular carcinoma	21	PPAR α/γ, PPAR δ	0

Receptor ligands: PPAR α/γ – agonist, PPAR δ – antagonist (COX-2 inhibitor), dexamethasone, interferon-alpha PPAR – peroxisome proliferator-activated receptor

minimizing side effects, even by improving the ECOG status before objective tumor response will be achieved. Because of the low rate of grade 3 toxicities, long-term drug administration up to more than 2 years was possible (median time on study medication 3.6 months (range 0.5–26.0). The low rate of toxicities > grade 2 might be related to the fact that each drug is not administered at a maximal tolerable dose, even not at a dose level where mono-activity may be observed.

A second important point for safety evaluation is the question whether activating biomodulators may promote tumor activity. The stimulatory therapy with transcriptional modulators (interferon-alpha, PPAR-alpha/gamma agonist, dexa- methasone) did obviously not enhance the percentage of patients with continuously progressive disease compared to standard therapies in the individual tumor types.

12.9 Discussion

The uniform treatment schedules presented were initially chosen to facilitate disease stabilization in patients with advanced and pre-treated cancer with less toxic agents. Surprisingly, it turned out that these treatment schedules have the capacity to induce objective responses (3–48%) and, in individual patients, even continuous complete remissions in every tumor type mentioned. Furthermore, they may induce OS rates, which compare with established standard first-line therapies.

With respect to the multi-facetted activities of the administered drugs (anti-proliferative, angiostatic, antiinflammatory, metabolic activity, immunomodulatory), and their differential cell-specific activities, the exact mechanisms of

action of the selected drug combinations are difficult to pin down [17,18,39]. The studied drug combinations are interacting with the systems biology of the different cell types at regulatory sites and have both genomic and non-genomic activity.

With the exception of individual patients suffering from vascular sarcoma, responses to therapy occurred much delayed and three phases were observed: (1) Inhibition of further tumor progression, (2) prolonged disease stabilization by 3.1 months to a mean of 8.6 months, followed by (3) objective responses. In some tumor types, response to therapy could be monitored by a serum parameter, C-reactive protein, indicating the tailored modeling of a tumor-associated disease trait, namely inflammation. Systemic tumor-associated inflammation, however, was no prerequisite for objective tumor response to a combined anti-inflammatory therapy approach as shown in castration-resistent prostate cancer that has a very low incidence of systemic inflammatory events [31].

On the basis of these observations, we now postulate tumor-associated inflammation as both a pathophysiologically important element and a therapeutic target but without presupposing causal relationships between inflammation and tumor progression. On the contrary, the prerequisites for our clinical observations, i.e. the multifaceted regulatory activities of the single administered drugs and the differential responses of the multiple cell types within the tumor compartment, reveal the relations of conditioned and conditioning tumor-promoting moments as reciprocal on the basis of pathophysiologically important interacting elements (e.g. inflammation, angiogenesis, and tumor cell proliferation). The still 'indistinct' but regulatory activity profile of the administered drugs and the favorable therapy results of the uniform treatment concept in a broad variety of different tumor types strongly support our hypothesis that tumor growth may be successfully attenuated by targeting the tumor system's biology simultaneously at multiple regulatory sites, e.g. (nuclear) transcription factors.

Pathologic systems biological processes in cancer may be reported from different observation levels: (1) In Loewenstein's view pathologic cancer processes are predominantly mirrored in a deficient cell-cell communication [40]. (2) The initial source of observation may also be an altered systems-associated cell composition, and (3) distorted functions of single cell systems within the tumor microenvironment [1,3,6]. Inflammatory processes have been identified to be involved in tumor systems biology independently of the viewpoint of observation.

One aspect is getting of growing systems-therapeutic interest since normal adult and cancer stem cells may be detected by selective expression of the transcription factor Okt-4 [41,42]: Inflammation plays a critical role on all virtual stages of tumor development, tumor initiation, promotion and progression [43]. The inhibition of gap junctional communication has been identified as an important mechanism by which inflammatory processes affect cancer development: Cancer cells exist in two forms, those that do not express connexin (gap junction genes), and those that express connexin genes but that gap junction function has been rendered non functional by oncogenes/loss of tumor suppressor genes [44]. Here the use of agents to turn on critical genes, i.e., such as the connexin genes

seems to be important [45–47]. That the cancer stem cell must be promoted by a number of inflammatory conditions, particularly in the metastatic stage of cancer disease (cachexia!) fits with the successful use of anti-inflammatory therapy components in the present systems-targeted treatment strategy [48].

Conventional therapy methods commonly neglect the complexity of the tumor compartment. They mainly target the molecular-genetically highly variable tumor cell, whose variability is explained by the complexity of the tumor development. By blocking a pathological signaling pathway with a small molecule or an antibody, the whole tumor system should be destroyed, synonymously with the assumption that tumor development could result from a single causative principle. Furthermore, combining cytotoxic therapy elements guided by the simple availability of drugs buys moderately enhanced efficacy at a simultaneously enhanced toxicity profile, as shown by many studies.

A lead back to a final first principle that may be therapeutically targeted to eradicate metastatic cancer is generally not permitted, in particular in knowledge of the multi-facetted activity profile of the administered biomodulatory agents. However, instead of such a lead back to a first principle, we have to deal with multiple and various constellations of elements (aggregated action effects), one of which – in our case – is tumor-associated inflammation. The constellation of elements has to be broken down to its single moments, but, simultaneously, we have to understand the relationship between one another rather than separately adding one to another and thereby neglecting the importance within the complex constellation. The principle therapeutic difficulty lies in this point.

The therapeutic components chosen directly address this difficulty based on the hypothesis that the combined activity of regulatory but pleiotropic agents, particularly transcription modulators (besides the angiostatic approach), may shape the tumor's organization, e.g. the 'wound healing' mechanisms, by attenuating simultaneously multiple activities involved in tumor growth such as angiogenesis, anti-inflammation, and proliferation. This hypothesis is supported by seven treatment-related characteristics: (1) No or poor single agent activity of each administered drug (predominantly combined regulatory activity) when given alone, (2) a very moderate toxicity profile during long-term drug administration (presumably no dose-response relationship), (3) very delayed objective responses (stable shaping and focusing of the tumor system's organization), (4) improved overall survival without an increase of the response rate in arm B of the randomized Melanoma II trial (biomodulatory activity), (5) significant modulation of tumor-associated disease traits, e.g. inflammation, ECOG status, paraneoplastic syndromes (biomodulation-derived biomarkers), (6) activity depending on the metastatic organ site in CRPC (tumor-stroma-specificity as expected from the known differential behavior of the various cell types within the tumor compartment, and the varying stroma cell compositions at the different metastatic sites), and (7) predominant site of progression at the original localization of the metastases (hints for impact on metastatic processes). Preclinical data on the action of COX-2 inhibitors and PPAR alpha agonists are already revealing antimetastatic activity [49,50].

Even if metronomic chemotherapy has any cytotoxic activity in the classic sense, the response characteristics do not support a response behavior as usually found in response to pulsed chemotherapy.

The clinical efficacy of the combined anti-inflammatory and angiostatic approach in different tumor types reveals preserved regulatory elements for targeting 'wound healing' processes with transcriptional regulators (biomodulatory agents) in tumor and adjacent stroma cells. (1) The favorable clinical results achieved with a small repertoire of transcriptional modulators indicate a constitutive dysregulation of distinct transcription factors, which – on the other hand – seems to be paradoxically linked to the heterogeneous tumor-associated molecular-genetic aberrations depending on the tumor type [51]. (2) The combined genomic/non-genomic therapy approach specifically shapes the organization of the tumor-stroma-interaction. (3) The clinically combined activity of (nuclear) transcription factors in the RCCC II and CRPC trial give sufficient clinical evidence for a crosstalk between drug-activated/deactivated transcription factors.

The focus on the systems biology of a tumor as the original target of cancer therapy necessitates biomarkers that indicate stable response in the field of tumor-associated disease traits or tumor-associated phenomena such as inflammation, angiogenesis, coagulation, and metabolism. Rather than the primary or "classic" markers for tumor response including tumor shrinkage or decrease of tumor markers, this new group of markers reflects efficacious biomodulation. However, we are aware of the limitation that some of these tumor-associated phenomena mirroring tumor biomodulation are sometimes difficult to follow on a systemic level. They can not be uniformly interpreted across tumor entities as demonstrated in our example of CRPC in comparison to other tumors, when inflammation seems to be quite differently integrated in the tumors' pathophysiology: PSA decline was paralleled but not preceded by a CRP decline in CRPC, whereas in other tumor types including RCCC decrease of CRP or ECOG performance improvement preceded tumor response.

In the immediate presence and future, biomodulatory therapy approaches of metastatic tumors could be methodological tools of individualized tumor therapy: In contrast to 'causal' therapy approaches aiming at blocking aberrant tumor-associated pathways by a restricted repertoire of highly specific drugs, multiple potential modulators (activators and deactivators) of transcriptional processes are available for biomodulatory therapy approaches. According to our experiences, mono-activity of a single transcription modulator is no prerequisite for its successful use and their combined administration activity could be followed by respective biomarkers. Close monitoring would further allow us to choose other modulator combinations in cases of weak interactivity to facilitate objective tumor response.

Finally, the constitutive dysregulation of transcriptional activity is shown to be an important target for biomodulatory therapy approaches in metastatic cancer. Biomodulation in metastatic tumors provides tools for recognizing patterns in therapy-associated events via biomodulation-derived biomarkers. Thereby, it enables (1) the shaping of the tumor system's organization and (2) the uncovering

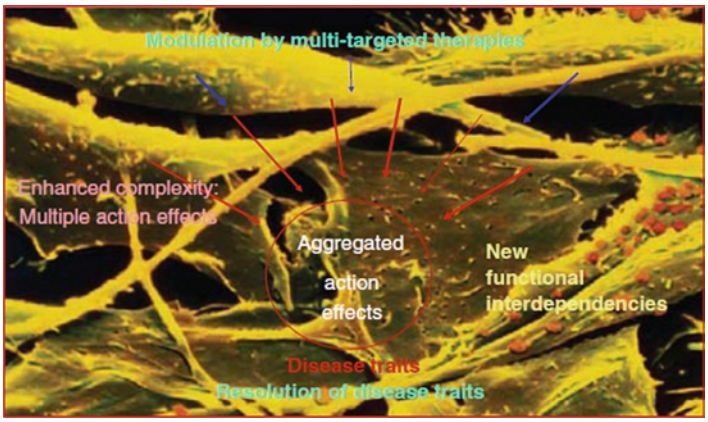

Fig. 12.1 The differential response patterns within our clinical trials indicate the therapies' systems biological activity. Understanding systems biology as adjustable size may break through the barrier of complex tumor-stroma-interactions in a therapeutically relevant way: Comparatively high efficacy at moderate toxicity. Structured systems-directed therapies in metastatic cancer may get a source for detecting tumor-associated complex aggregated action effects as adjustable sizes available for targeted biomodulatory therapies

of endogenous sources such as transcription factors and their crosstalks for managing growth behavior by counterbalancing the tumor systems' biology.

Our seven published phase II trials on combined targeted therapy of tumor-associated wound healing mechanisms, e.g. inflammation and neoangiogenesis, have shown that using an approach for understanding systems biology as adjustable size, we may break through the barrier of complexity of tumor-stroma-interactions in a therapeutically relevant way (Fig. 12.1). For a targeted modulation, elements such as inflammation and neoangiogenesis are available, which are dysregulated on the basis of acquired chromosomal aberrations. Biomodulation of systems biological processes facilitate comparatively high efficacy at moderate toxicity.

General interpretations of the tumor's systems biology may not be performed in context-free explanations. The requirements of application (therapy schedule, tumor type) and the number of surrogate markers define the way the interpretation is conducted. Additionally, they define the hermeneutic understanding of extremely complex cellular interactions correspondingly to the chosen picture, the wound healing mechanisms. In the present case, this means the following: Naturally, the administered drugs, particularly the transcriptionally active modulators, have still an insufficiently illuminated spectrum of activities, which may be even dependent on the cell type. General interpretations concerning the systems biology do not obey the same categories of refutation as general theories and remain per se open for discussion. The logic of an explanation of the tumor's systems biology is the result of a connection between a hermeneutic understanding (wound healing mechanisms) and the causal explanation (e.g. co-regulatory activity of transcription factors).

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Chapter 13
The Comparative Uncovering of Tumor Systems Biology by Modularly Targeting Tumor-Associated Inflammation

Albrecht Reichle and Gerhard C. Hildebrandt

Abstract So far, tumors have been assumed to defy experimental therapeutic access from inside in a comprehensive and reconstructive way (systems view) and to only comply with reductionist knowledge with regard to biochemical pathways.

Our main aim was the uncovering and reconstruction of tumor systems structures mediating tumor-associated inflammation (eight phase II trials, two of them randomized). Thus, we comparatively analyzed anti-inflammatory activities and clinical response induced by continuously administered biomodulatory treatment modules (module M: metronomic low-dose chemotherapy; module A: pioglitazone plus etoricoxib; module A+M; module A+M/+: plus second transcriptional modulator [interferon-alpha or dexamethasone]) in the metastatic stages of different types of tumors (266 patients; 54% systemically pre-treated; metastatic melanoma, sarcoma, renal clear cell carcinoma, castration-resistent prostate cancer, gastric cancer, and Langerhans' cell histiocytosis).

Tumor-specific and stage-specific therapeutic accessibility of inflammation-related processes to induce response in all tumor types indicate a constitutive spin-off of new systems functions during metastatic processes. Furthermore, this accessibility shows the differential integration of inflammation into the context-dependent 'living world' of tumor compartments that is marked by tumor-specific and subtype-specific rationalization processes: Inflammation-related activities are communicatively promoted and differentially adapted during tumor evolution. Empirically, differences may be detected in the modalities of developing evolutionary systems and in the acquired functional impact of inflammation-related systems. Biomodulatory therapies, administered as fixed modules, may contribute to the discovery and understanding of novel regulatory systems in tumor biology.

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This study highlights the claim for validity of therapeutic inflammation control as an important prerequisite for tumor control on the basis of action-relevant yes or no statements that generate facts on-site in tumors via biomodulatory therapy modules.

Keywords Systems biology • Metastatic tumors • Modularity • Rationalization • Robustness • Biomodulatory therapy • Transcription factors • Metronomic chemotherapy • Inflammation

Abbreviations

PPAR	Peroxisome proliferator-activated receptor
COX-2	Cyclooxygenase-2
Module M	Metronomic low-dose chemotherapy
Module A	Pioglitazone plus etoricoxib or rofecoxib
Ifn-a	Interferon-alpha
ECOG	Eastern cooperative oncology group performance status
CRP	C-reactive protein
CRPC	Castration-resistent prostate cancer
RCCC	Renal clear cell carcinoma
LCH	Langerhans' cell histiocytosis
CCC	Cholangiocellular carcinoma
RCCC I	Trial RCCC I
RCCC II	Trial RCCC II
SD	Stable disease
PR	Partial remission
CR	Complete remission
CCR	Continuous complete remission

13.1 Introduction

Reductionist considerations are commonly used to create new therapy approaches. The reductionist concept is based on the attempt to reduce complex intracellular and intercellular interactions of tumor diseases to one single cause or at least only to a few causes or distinct hierarchies to build up cause-effect-chains as a rationale for therapy planning. The targeting of these suggested causes is presumed to result in the eradication or at least attenuation of tumor disease.

Aberrantly expressed genes and their respective gene products in tumor cells serve as exceptional causal targets of cancer therapy. An important and clinically approved example is Philadelphia-positive chronic myelocytic leukemia. The Philadelphia translocation encodes for a chimeric protein of increased tyrosine kinase activity, which can be targeted by a small molecule (TKI; tyrosine

kinase inhibitor). This way, presumably life-long tumor control can be achieved in more than 60% of patients.

However, we frequently have to face **two major therapy-relevant problems** in cancer disease: Cancer cells often develop multiple chromosomal aberrations during tumor evolution, meaning that multiple aberrations are functionally integrated in a tumor cell by networking. This phenomenon may be therapeutically met by uncovering more complex molecular signatures. The second therapeutic challenge is the close communicative network between tumor and stroma cells, which has not yet been adequately addressed.

Therefore, tumor models need to be developed that address the communicatively linked functions within a tumor compartment: Seemingly confusing networks within tumors may be considered for therapeutic purposes as a well-structured holistic communicative network, gathering all informative processes mediated by proteins, cytokines, etc. The input of a communicatively linked and modularly structured background is now responsible for differentially redeeming validity and denotation of all systems objects, the proteins, i.e. transcription factors, cell functions, and pathways [2·3].

Such systems are self-content: Modular changes do not necessarily implicate the loss of a systems' functionality, if functions are rearranged or even if new systems functions may spin off, such as systemic inflammatory processes during the metastatic stage of tumor disease. Systems are becoming evolvable.

An exceptional example in this context is the transcription factor NF-kappaB, which acquires even opposing functions depending on developmental stage, cell type, and organ site. Even more, NF-kappaB may develop differential functions within one clonal population [4].

If multiplicity of functions linked to distinct systems objects (i.e. proteins, pathways, etc.) is available, these functions should be shapeable by biomodulatory therapy approaches. Therefore, we may suggest that novel therapy-relevant targets lie in the communicative architecture of tumor systems.

Based on this novel pragmatic communication theory, two major questions should be asked:

1. Does **modularity** constitute a big world inside small world networks? If yes, it should be possible to implement modular 'knowledge' with respective biomodulatory therapy approaches [5].
2. Are **biomodulatory therapies** sufficient to induce tumor response at all? If yes, biomodulatory therapies represent a methodological approach to comparatively uncover – now with normative statements – the tumor's modular systems structures and the modular activity of cells promoting distinct systems functions.

How does modular therapy work? When implementing reductionist therapy approaches we are used to inhibit communication-related pathways, i.e. signaling pathways. Modular therapies evolve the informative background, which redeems validity and denotation of tumor-associated objects (Fig. 13.1). Biomodulatory therapies may simultaneously alter the behavior of the addressing as well as the addressed cell, for example by promoting the addressed cell not to acknowledge the received signal (chapter 14, 26).

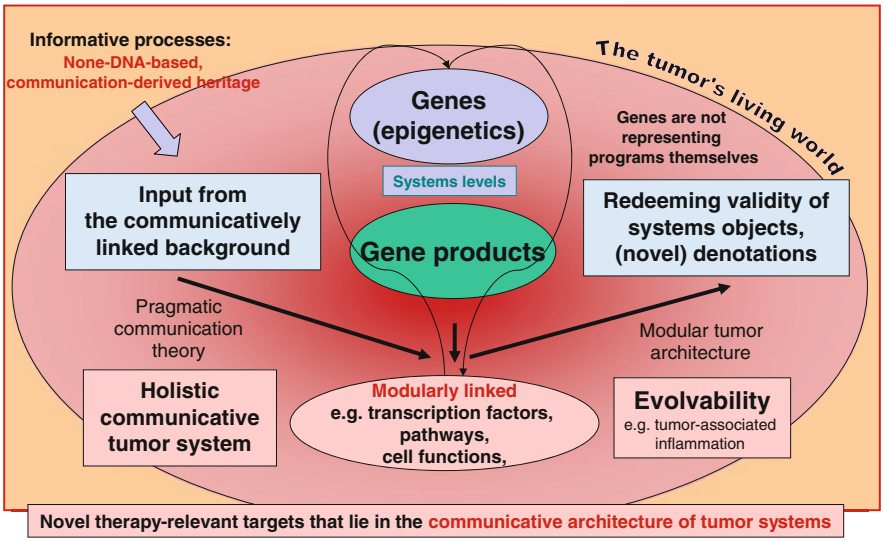

Fig. 13.1 Tumors allow experimental therapeutic access from inside in a comprehensive and reconstructive way (systems view) via modular (biomodulatory) therapy approaches and may be described as evolutionary developing systems. Modular therapies evolve the informative background, which redeems validity and denotation of tumor-associated objects. Therapeutically accessible pathologies may derive from the decoupling of functional cellular and systems 'world' and can be targeted by modular therapy approaches

Rationalization processes serve as further targets for biomodulatory therapies (chapter 2). The functional spectrum of distinct cell types within the tumor compartment is limited despite the commonly observed huge plasticity and may be challenged by the required systems-associated functions directed at the systems objects. These profiles of requirements may lead to discrepancies, which can be described as inconsistencies, Achilles' heels, deformations, or missing intersystemic exchange processes. Additionally, we have to expect that different patterns of cell types within a tumor compartment may promote particular functions, such as inflammation, in a tumor type-dependent manner.

Finally, we can state our hypothesis on the mechanisms of action of modular therapy: Tumor-associated inflammation is frequently observed during metastatic stages. Inflammation seems to be associated with tumor progression, neoangiogenesis, and metastatic processes. However, as shown in the present data analysis, differential accessibility of tumor-associated pro-inflammatory processes by modular therapy approaches suggest that – rather than in a uniform fashion – it seems to be differentially integrated into the context of tumor systems.

Modular therapies consist of stimulatory and inhibitory acting drugs. Monoactivity of a single drug is no prerequisite. Drug targets may be ubiquitously available structures and are not necessarily presented by specific proteins coded by mutated genes. Modular therapies may shape the validity of informative processes associated with tumor-associated inflammation aiming at attenuating tumor growth. C-reactive protein may serve as an easy systems-related read-out parameter [2].

The present re-evaluation of previously published clinical trials mainly aims at showing the modular integration of tumor-associated inflammation and rationalization processes within the tumor context by means of novel developed methodologies for targeting tumor systems. The methodological instruments are structured biomodulatory therapy approaches shaping the validity of communicative processes. Three questions in particular were pursued within the present data analysis:

1. May we describe systems-mediated rationalization processes with normative therapy-derived statements?
2. Are systems stage-specific modular therapies available, which may be guided by biomarkers?
3. May biomodulatory therapies, administered as fixed modules, contribute to discover and understand novel regulatory systems in tumor biology, for instance tumor-associated inflammation?

13.2 Methods

Our basic experimental plan was to show that differential control of tumor-associated inflammation may lead to attenuation of tumor growth in different metastatic tumor entities [2, 6].

Between 2001 and 2008, we included 266 patients with metastatic neoplasms (castration-resistent prostate cancer [CRPC], renal clear cell carcinoma [RCCC], sarcoma, melanoma, multivisceral Langerhans' cell histiocytosis [LCH], and gastric cancer) in eight clinical trials [2, 6–14]. Most tumors had been systemically pre-treated (54%) to prove the activity of biomodulatory therapy modules in a palliative setting. Two trials (melanoma and gastric cancer) were randomized, and their clinical outcome has been reported recently [6, 12].

As backbone of our all-oral, multi-pronged, and modularly acting therapy schedules, we used daily metronomic low-dose chemotherapy in all tumor types. As anti-inflammatory axis of treatment, we added pioglitazone as an agonist of the nuclear transcription factor peroxisome proliferator-activated receptor alpha and gamma and coxib (rofecoxib or etoricoxib). To enhance the anti-inflammatory activity of the treatment schedule, we added low-dose interferon-alpha in patients with hormone-refractory prostate cancer dexamethasone and in renal clear cell carcinoma (Figs. 13.2 and 13.3).

Patients of two trials were randomized (metastatic melanoma [12], metastatic gastric cancer [6]) into arms comparing metronomic chemotherapy with or without anti-inflammatory therapy. For renal clear cell carcinoma, two sequential trials were conducted with escalating anti-inflammatory activity of the treatment schedule. All trials and their design have been published as indicated in Table 13.1.

Metronomic low-dose chemotherapy has a pleiotropic activity profile and predominantly acts in an angiostatic and immunomodulatory manner [15–17].

We used stimulatory and inhibitory acting drugs to attenuate tumor-associated inflammation and angiogenesis. The single doses of anti-inflammatory therapy are

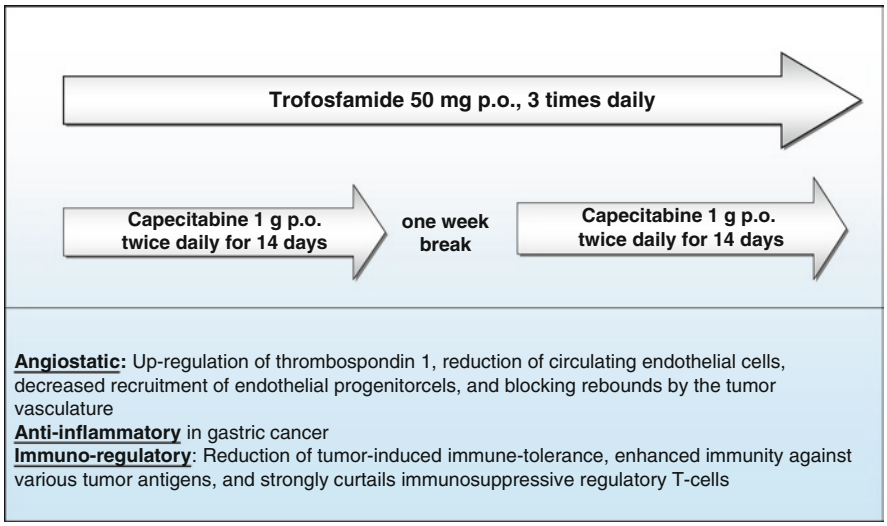

Fig. 13.2 Angiostatic therapies: Metronomic low-dose chemotherapy

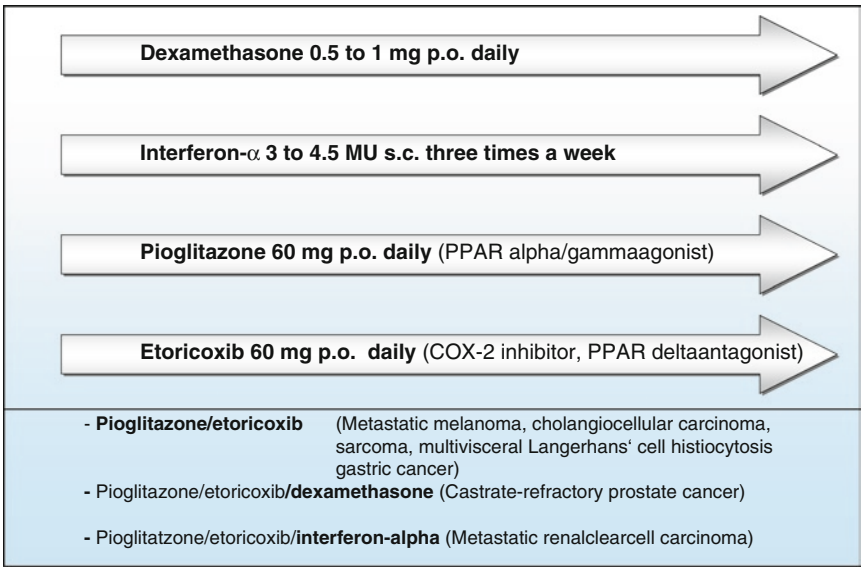

Fig. 13.3 Tumors' systems biology: dysregulation of (nuclear) transcription factors

indicated in Table 13.1. All therapy-relevant targets are ubiquitously distributed across different cell types within the tumor compartment.

Therapeutic modules: Biomodulatory acting fixed drug combinations characterized by combined systems-directed activity may be more precisely described as therapy modules. Modules are biomodulatory elements mediating regulatory activities within a tumor compartment by targeting tumor cells and adjacent stroma

Table 13.1 Therapy Modules

	Module A (lead-in)	Module M	Module A/M	Module A/M plus dexa	Module A/M plus interferon-a
Melanoma[a, c] (randomized)	+	+	+	–	–
Gastric cancer[a, b] (ran.)	–	+	+	–	–
RCCC[b, c, (i)] (sequential)	–	–	+	–	+
CRPC[b, c, d]	–	–	–	+	–
Sarcoma[a, c]	+	–	+	–	–
LCH[a, c]	–	–	+	–	–

A (anti-inflammatory)[c] = piolitazone 60 mg daily plus rofecoxib 25 mg daily or etoricoxib 60 mg daily; M (metronomic) = trofosfamide[a]50 mg thrice daily, or capecitabine[b]1 g/m^2 or 1 g absolute twice daily for 14 days every 3 weeks; Dex = dexamethasone[d] 0.5 or 1 mg daily; Interferon-alpha[(i)] 3 or 4.5 MU thrice weekly

cells as well as their dynamic functions (communication). Therapeutic activity does not necessarily apply to the mono-activity of a single drug but rather to synergistic regulatory processes, which may be cumulatively initiated by an action-oriented therapeutic approach.

C-reactive protein: C-reactive protein (CRP) was continuously monitored at respective study visits to uncover possible links between systems-directed modulation of tumor-associated inflammatory processes and clinical or objective tumor response, progression-free survival (PFS), and overall survival (OS). Thus, **situation- and stage-specific** background knowledge on systems behavior in an individual tumor disease could be collected.

Tumors were monitored for infection-related CRP elevation to be distinguished from systemic tumor-associated inflammatory processes.

13.2.1 Tumor-Specific and Stage-Specific Therapeutic Accessibility of Inflammation-Related Processes

The activity of treatment modules is described by means of situation-related (systems-stage-dependent) systems explanations [2, 5] based on **therapy-derived** normative yes or no statements. Of special interest were

1. The anti-inflammatory activity of the modules in the respective tumor types
2 The time course of CRP response and clinical tumor response
3. The suitability of theoretically derived systems terms for assessing the tumor's systems behavior (intersystemic exchange, rationalization processes, inconsistencies) as well as the modular activity of biomodulatory therapy approaches [2]

May biomodulatory therapies, administered as fixed modules, contribute to the discovery and understanding of novel regulatory systems in tumor biology? To answer this question, **tumor systems biology** is reconstructed as indicated by the

response behavior to standardized biomodulatory therapy modules (Fig. 13.1). The following **modalities were used for assessing tumor systems behavior**:

1. **Inconsistencies** may be therapeutically met, if an approach leads to a rapid response by hitting the main weakness of a tumor system (Achilles' heel). Paradox processes, such as weaknesses, may develop on the basis of a systematic congestion caused by rationalizing the functional 'world' of tumor-associated stroma and tumor cells. This rationalization results in an overload or restriction of communicative infrastructures or in a decoupling of systems and the functional world of cell systems.
2. In an evolutionary process, tumor cells may exploit the whole extent of **rationalization** features of both stroma and tumor cells to implement the functional diversity of systems behavior aimed at maintaining homeostasis and robustness in tumor systems. Differential biomodulatory accessibility of tumor-associated inflammatory processes for mediating clinical tumor response is indicative for corresponding differential integration of tumor-associated inflammatory processes into a tumor's systems context.
3. Disturbances in **intersystemic exchange processes** are suggested in case of low sensitivity of CRP responses to predict clinical response.

13.2.2 Statistics and Data Analysis

Primary end point of all trials was PFS. Secondary endpoints included objective response rate, OS, toxicity, and C-reactive protein response in serum.

For the present evaluation, clinical response was defined as stabilization of progressive disease for at least 3 months (tumor progression <25%), objective tumor response, and partial or complete remission as indicated in the respective publications [2]. Progression-free survival was defined as the interval between the beginning of treatment and disease progression. Survival duration was calculated from randomization to treatment or study inclusion. Survival distributions were generated by means of the Kaplan-Meier method.

C-reactive protein levels were dichotomously separated into normal CRP (<10 mg/dl) and elevated CRP (≥10 mg/dl). CRP response was defined as decrease of >30% from baseline within 4–6 weeks of treatment for every cancer entity except gastric cancer (>50% decrease). All trials or single treatment arms were comparatively re-evaluated with regard to anti-inflammatory activity (CRP response) and clinical response. If available, PFS and OS rates were compared with regard to single or combined modules. In a second step, we evaluated the predictivity of anti-inflammatory response for clinical response **(sensitivity and specificity)**.

The Fisher exact test and the "student t"-test were used to identify significant associations between clinical and biologic variables.

13.3 Results

Altogether, 266 patients were enrolled into eight phase II trials. Clinical outcome has been recently reported in detail [1, 2].

C-reactive protein in metastatic tumors: Variable profiles of tumor-associated **systemic inflammation** were empirically detectable depending on the metastatic tumor type: Systemic inflammation frequently occurred in most metastatic tumors studied (72–100%), including cholangiocellular carcinoma (CCC) (100%), but only in about one third of metastatic CRPCs (28%) [2].

CRP response behavior: 98% of CRP responders could be detected during the treatment interval of 4–6 weeks. All administered modules turned out to have the capacity for attenuating tumor-associated inflammation, although efficacy was not uniformly distributed between diseases: Metronomic chemotherapy induced CRP responses in 67% of patients with gastric cancer, module A/M in 92% of patients with melanoma. In contrast, module M induced poor CRP response in melanoma as did A/M in RCCC (Table 13.2). Module A mediated no additional response in gastric cancer. **The addition of a second transcriptional modulator** in patients with RCCC was paralleled by increased frequency of CRP response (Table 13.2) and a steep decline of base-line CRP levels after 4–6 weeks of treatment (Table 13.3). CRP responses were already observed during the 14-day lead-in phase with module A in some patients with angiosarcoma (83%) and melanoma (23%). CRP response to therapy occurred independently of the detected frequency of systemic inflammation in the respective tumor types (Table 13.2).

In CCC, systemic inflammation was related to tumor-associated cholangitis and responded to anti-microbial therapy in 90% of patients. Only 8% of all other patients received concomitant anti-microbial therapies.

13.3.1 CRP Response as Predictor for Clinical Tumor Response

CRP none-response to biomodulatory therapy was consistently associated with high predictivity for missing clinical response independent of the tumor type. This association strengthens the concept of tumor-associated inflammatory processes

Table 13.2 C-reactive protein responder and therapy module/disease

	% C-reactive protein responder		
	Module M	Module A/M	Module A/M plus ifn-a or dex
Melanoma	42	92	–
Gastric cancer	67	65	–
CRPC	–	–	80
RCCC I	–	69	–
RCCC II	–	–	100
Angiosarcoma	–	100	–
Multivisceral LCH	–	100	–

CRPC = castration-resistent prostate cancer; RCCC = renal clear cell carcinoma; LCH = Langerhans cell histiocytosis; CRP = C-reactive protein; Module M = metronomic low-dose chemotherapy; Module A = pioglitazone plus etoricoxib or rofecoxib; Clinical response = stable disease, partial remission, and complete remission

Table 13.3 C-reactive protein follow-up in CRP responder/non-responder

	CRP responder (>30%) Mean CRP levels at baseline (mg/dl)/after 4–6 weeks	CRP non-reponder Mean CRP levels at baseline (mg/dl)/ after 4–6 weeks	P-value
Metastatic melanoma module M and A/M	21.7/11.8	20.2/69.6	0.003/0.001
Metastatic gastric cancer module M	27.5/6.47	31.2/78.3	0.02/0.004
Metastatic gastric cancer module A/M	20.4/6.7	25.6/69.1	0.07/0.02
RCCC module A/M	–	47.8/41.7	0.32
RCCC module A/M + interferon-a	40.2/11.3	–	0.0005
CRPC, module A/M + dexa	36.8/12.2	–	0.02
Melanoma/sarcoma, module A/M	34/12.3	52/98	0.02/0.009

CRPC = castration-resistent prostate cancer; RCCC = renal clear cell carcinoma; CRP = C-reactive protein; Module M = metronomic low-dose chemotherapy; Module A = pioglitazone plus etoricoxib or rofecoxib

Table 13.4 Inflammation control and clinical response (stable disease, PR and CR)

	Predictivity of CRP response for clinical response: Sensitivity/specificity (%)		
	Module M	Module A/M	Module A/M plus ifn-a or dex
Melanoma (Melanoma Res, 2007; PPAR Res, 2009)	62/91	75/100	–
Gastric cancer	91/100	85/86	–
CRPC	–	–	88/100
RCCC I	–	89/100	–
RCCC II	–	–	93/100
Angiosarcoma	–	83/100	–
Multivisceral LCH	–	100/–	–

CRPC = castration-resistent prostate cancer; RCCC = renal clear cell carcinoma; LCH = Langerhans cell histiocytosis; CRP = C-reactive protein; Module M = metronomic low-dose chemotherapy; Module A = pioglitazone plus etoricoxib or rofecoxib; Clinical response = stable disease, partial remission, and complete remission

that are of **pathophysiological importance** during tumor progression, irrespectively of tumor type and distinct systems integrations of inflammation-related processes. Broad therapeutic accessibility of inflammation-related processes for response induction in all tumor types indicates **constitutive spin-off of new systems functions** during metastatic stages (Table 13.4).

CRP response was predictive for clinical response in case of CRPC, RCCC, angiosarcoma, and LCH at a high level of sensitivity. In contrast, the relatively low sensitivity in metastatic melanoma suggests a lack of inter-systemic exchange

processes between tumor-associated inflammation and tumor-associated systems, promoting progression in about one third of patients (Table 13.4)**.** In patients with melanoma or RCCC, **clinical response rates consistently increased together with the induction of CRP response** from 26% to 69% and from 62% to 93%, respectively**.**

Degree of tumor response and CRP response: Clinical responses consecutive to CRP response showed varying degrees (SD, PR, CR, cCR) **(**Table 13.5**).** Particularly high rates of clinical and objective responses resulting in continuous complete remissions were observed in RCCC, CRPC, and angiosarcomas; improved PFS and OS rates were seen in melanoma, RCCC, and gastric cancer, particularly in CRP responders. Vice versa, the groups of CRP none-responder experienced

Table 13.5 Combined targeting of the modular tumor architecture (52% pre-treated patients): response behavior

	Response			
Tumor type/therapy arm	No. of patients	Partial remission (%)	Complete remission (%)	Continuous CR (%)
Sarcomas I	21	19	16	5
Angiosarcomas	6	17	33	17
Melanoma Arm M®	22	4	0	0
Arm A/M	26	11	3	3
Langerhans' cell histiocytosis (multivisceral)	2	–	100	100
Renal clear cell carcinoma (RCCC) I (no IFN-a)	18	0	0	0
Renal clear cell carinoma II (plus IFN-a)	33	35	13	9
Castration-resistent prostate cancer (CRPC)	36	28	6	6
Cholangiocellular carcinoma	21	24	5	5
Gastric cancer Arm M®	20	20	0	0
Arm A/M	22	14	0	0

Table 13.6 C-reactive protein response and progression-free/overall survival. Median progression-free (PFS)/over-all survival (OS) (months)

	CRP responder PFS/OS	CRP none-responder PFS/OS	P-value PFS/OS
Metastatic melanoma	2.0/18.0	1.2/5.3	0.016/0.045
RCCC A/M	–	4.7/16.2	–
RCCC A/M + ifn-a	11.5/25.6	–	–
Metastatic gastric cancer Module M plus A/M	6.52/12.34	2.46/5.10	0.01/0.005
Melanoma/sarcoma	3.5/–	1.0/–	0.004/–

Castration-resistent prostate cancer: Too less patients for Kaplan-Meier analysis, RCCC = renal clear cell carcinoma; CRP = C-reactive protein; PFS = progression-free survival, OS = overall survival; Module M = metronomic low-dose chemotherapy; Module A = pioglitazone and etoricoxib or rofecoxib

significant increases in mean CRP levels within 4–6 weeks on treatment and at best a retardation of progression (Tables 13.3 and 13.6).

Time intervals from CRP response to objective response were extremely variable. In most cases, objective response was preceded by CRP response: The majority (91%) of patients with objective response responded with delay (range 3.1–16 months). Only two patients with CRP response were continuously progressing. In case of rapid CRP (within 14 days) and tumor (up to 3 months) responses (angiosarcoma, CRPC), biomodulatory therapies may hit a tumor's **Achilles' heel** (tumor-associated inconsistencies), whereas delayed objective response may be due to the **inherent robustness** of tumor systems. However, the robustness of tumor systems can be eventually overcome by the therapeutic sustainability of modules over time, as shown particularly in patients with much delayed objective tumor responses. CRP response was directly paralleled by PSA response in CRPC, but only a few patients with measurable disease also showed delayed objective response.

Interestingly, **module M** may act via rather different **systems-related activities** in a tumor type-dependent manner, for instance without accompanying anti-inflammatory activity in tumors such as metastatic melanoma (objective response rate corresponded to DTIC first-line treatment) or with significant anti-inflammatory activity as in gastric cancer. Drug related differences used in metronomic chemotherapy modules seem to play an inferior role for the observed dichotomy in the mechanisms of action. Capecitabine is inefficacious as a monotherapy for CRPC [2] but highly effective in combination with an anti-inflammatory therapy approach.

Finally, we may **map** tumor-associated inflammation to show that inflammation is rather differentially integrated into the evolutionary context of tumor systems and, in fact, modular. Three types of interactions between modules may be distinguished by clinical response, PFS, and OS (Fig. 13.4):

1. **No additive activity** was found in gastric cancer but simultaneous no response-compromising activity between module M and A.
2. **Additive or synergistic activity** of both treatment modules in melanoma, sarcoma, and LCH.
3. **Intensified (specified) and concerted activity** by adding a further transcriptional modulator such as interferon-alpha in RCCC or dexamethasone in CRPC.

13.4 Discussion

The three mainstays of acquiring new insights into novel therapy approaches implementing modularity are (1) the change from the classic conclusion logic (indicating a pathway responsible for cell death) to that of **normative statements** (how to control systems-associated processes with therapy modules to achieve response); (2) the change from object-associated to **situation-associated systems interpretations** (biomodulatory therapies in metastatic tumors); and (3) the change from an intentional (reductionist) to a evolution-based **systems explanation** (systems

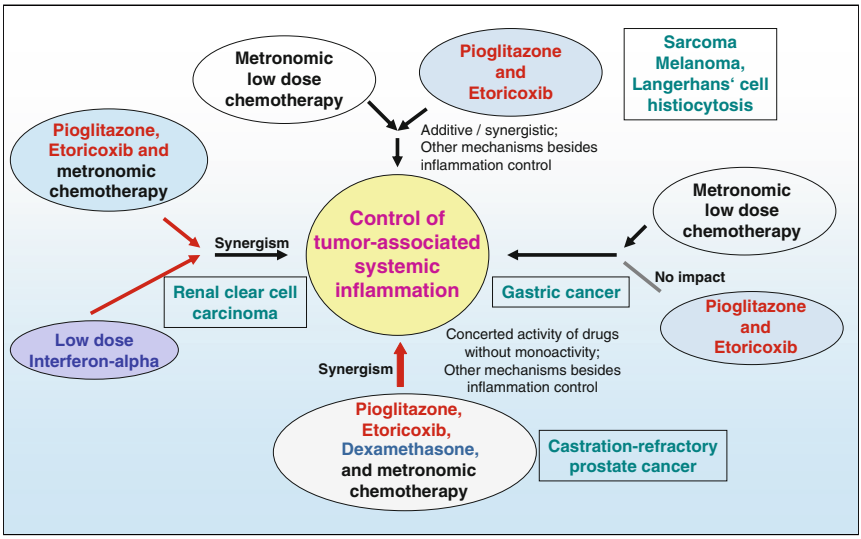

Fig. 13.4 Control of tumor-associated inflammation may be achieved by differentially interacting modular therapy approaches. Metronomic low-dose chemotherapy targets at least three systems related to inflammation, and the modular response reflects the heterogeneity in tumor-associated inflammation-related systems as well as in the acquired functional impact of inflammation-related systems

behavior and response) [1;2,5]. For situation-associated systems interpretations and systems explanations, we may now use terms derived from theoretical considerations on a tumor's modular systems behavior and intercellular rationalization processes [1;2,5].

13.4.1 Systems Rationalization and Inter-systemic Exchange Processes

The comparative interpretation of tumor systems presented, which is based on modular therapy approaches, shows that:

1. Completely differently acting biomodulatory treatment modules, such as metronomic low-dose chemotherapy or pioglitazone plus coxib, initiate suppression or reversion of tumor-associated inflammation in a **tumor- and stage-specific manner**.
2. CRP responses may further translate into clinical responses including the achievement of CR (**Table** 13.5).
3. Inflammation as a tumor-associated sub-system is differentially integrated into the context-dependent 'living world' of a tumor compartment, which is featured by tumor-specific, even tumor subtype-specific **rationalization processes**: In about one third of patients with gastric cancer or metastatic melanoma,

inflammation is not accessible to the biomodulatory therapies used in these studies [6;12] (Table 13.2).

4. Pioglitazone plus etoricoxib as well as metronomic chemotherapy **site-specifically mediate and focus on** diverse tumor-associated topologies of aggregated action effects: Prerequisite for the realization of diverse aggregated action effects are **inter-systemic exchange** and communication processes. These processes seem to be altered in metastatic melanoma, as about one third of patients with melanoma showed CRP decline to module A, but did not show any clinical response. A comparable percentage of patients with gastric cancer and elevated serum CRP levels did neither respond to inflammation nor clinically.
5. The chosen biomodulatory therapy elements act as single modules with identical modules showing differential mechanisms of action (e.g. low-dose metronomic chemotherapy in metastatic melanoma and gastric cancer or pioglitazone and coxib in RCCC, metastatic melanoma, and gastric cancer).
6. Empirically, differences may be detected in modalities of evolutionary systems development. Both tumors systems stage and evolutionary divergence of inflammation-associated systems within a particular tumor type may explain a tumor's selective sensitivity to different therapy modules in the metastatic stage (Fig. 13.4).

Systems integration of multifold interwoven inflammatory processes: The administered therapy modules may either induce clinical response in tumor types without predominant systemic inflammation in the metastatic stage, i.e. in CRPC, or without altering systemic tumor-associated inflammatory processes (efficacy of metronomic chemotherapy in melanoma). These empirical observations indicate that systems-directed activities of the respective modules may go far beyond those systems, which are directly involved in mediating tumor-associated inflammation (site-specific activity, attenuation of metastatic spread, attenuation of tumor-associated autoimmune phenomena) [1;2;18]. In this comparative analysis, we could clearly show that systems processes are multifold interwoven with one another by inter-systemic exchange processes. Simultaneous modeling of additional tumor characteristics, such as metastatic behavior, organ site-specific activity, and localized or systemic inflammation are shown to be implicit features for therapies including biomodulatory acting modules that aim at focusing on biological systems processes.

Diversity of systems processes in the metastatic stage: The present study evaluation shows that tumors can be comparatively characterized by their distinct systems biology, which may be uncovered via biomodulatory therapy approaches and respective study designs: This approach shows a **broad heterogeneity of systems processes conveying tumor-associated inflammation** (Fig. 13.5).

Tumor-associated systems processes are not uniformly integrated into a tumor systems context, neither within morphologically defined tumor stages (metastatic stage) nor within a distinct tumor type. In gastric cancer and melanoma, tumors developed either stage-specific or subtype-associated diversity of (sub)-systems by differentially developing systems-integrative processes, i.e. **rationalization,** that mediate tumor-associated inflammation. These rationalization processes are not related to histological subtypes (for instance intestinal versus diffuse type in gastric cancer). The capacity to

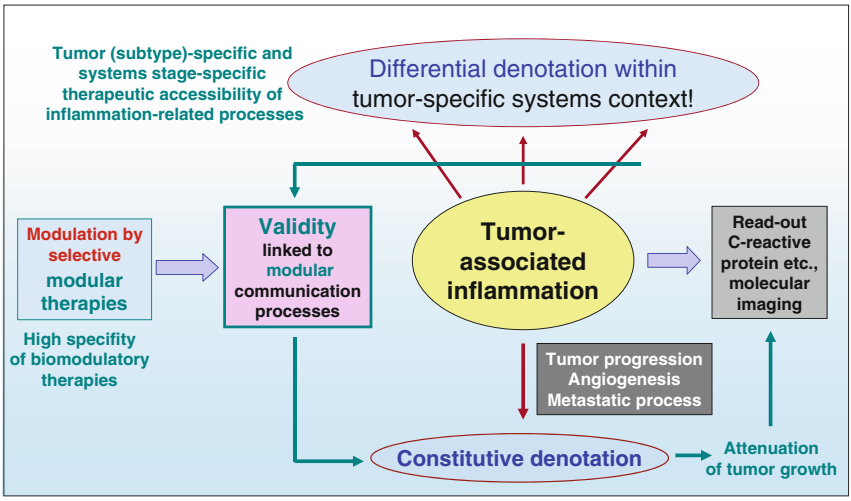

Fig. 13.5 Maps of network components and pathways cannot provide definitive functional systems interpretations, as inflammation is rather differentially integrated into the evolutionary context of tumor systems. However, three types of interactions between therapy modules may be separated by clinical response, PFS, and OS or by using markers with intrinsic functional significance (e.g. CRP): (1) No additive activity; (2) Additive or synergistic activity; and (3) Intensified (specified) and concerted activity. Tumor-specific and stage-specific modular therapeutic accessibility of inflammation-related processes indicate a constitutive spin-off of new systems functions during the metastatic process and the differential integration of inflammation into the context-dependent 'living world' of a tumor compartment. This development is featured by tumor-specific and subtype-specific rationalization processes: Inflammation-related activities are communicatively promoted and differentially adapted during tumor evolution. Systems are characterized by differential integration of inflammation (rationalization) and a distinct decoupling of functional and systems 'world'. Context rearrangement can be achieved by anti-inflammatory modular therapy approaches involving coxibs, interferons, glucocorticosteroids, and PPARalpha/gamma agonists

develop systems diversity indicates a **dissociation of the structures and functions** of **tumor systems** (e.g. inflammation) on the basis of rationalization processes. This development may impede biomodulatory accessibility for distinct therapy modules and seems to determine **systems-specific activity of the administered modules**. Therefore, biomodulatory therapies, administered as fixed modules, may contribute to discover and understand novel regulatory systems in tumor biology [19].

13.4.2 The Systems Biology of a Tumor: An Independent Feature at a Distinct Stage?

The basic idea of this series of studies was to primarily select patients with angiogenesis- or inflammation-driven tumors or both for combined anti-inflammatory and angiostatic therapy. The three suggested treatment groups with distinct biological behavior, angiogenesis-driven tumors, generally pro-inflammatory tumors, and

tumors with inflammatory characteristics in the metastatic stage are not mirrored in the detected differential systems stages, which are involved in mediating tumor-associated inflammation. As biological tumor features are not correlated to identical biologic behavior in response to biomodulatory therapy, these data indicate that tumor-associated inflammation is promoted by differentially developing tumor-associated subsystems, which characterize tumor type and stage in a similar way as the histological subtype.

Particularly from an exclusively therapeutic point of view the analysis of comparable biomodulatory therapy approaches administered in patients with histologically different metastatic tumor diseases may show that tumor-associated inflammation has a **constitutive denotation** for tumor progression and the metastatic process (Fig. 13.5), which is specifically accessible via biomodulatory therapies. Implementation of modular 'knowledge' in form of biomodulary therapies alters the validity of communicative processes: Tumor-associated inflammatory processes may evolve from their original denotation, namely promotion of tumor progression, up to the point of attenuation of tumor growth, which is indicated by the stage-specific systems marker CRP (Fig. 13.5).

The descriptive allocation of 'tumor-inherent' functions to characterize a tumor's disastrous features remains consistent with reductionist or contextualist requirements to create hierarchical levels responsible for promoting tumor growth, such as tissue invasion (matrix remodeling), establishing an inflammatory microenvironment, the insensitivity to growth inhibition, evasion of apoptosis, sustained angiogenesis, limitless replication potency, and self-sufficiency in growth signals [20]. In the reductionist picture, tumor-associated pathosphysiological features are equated with the causation of a tumor. The usefulness of this description is the integration of the tumor cell in a larger environmental context, but it reduces environmental tumor-associated activities as compliable unidirectional functions mediated by the tumor cell.

The present evaluation of clinical trials on metastatic tumors highlights the imperative and context-disrupting claim for validity of controlling therapeutic inflammation as an important prerequisite for tumor control. Inflammation control with modularly designed therapies allows the deduction of action-relevant yes or no statements that generate facts on-site in the tumor via biomodulatory therapy modules. A comparative analysis to uncover tumor systems biology may foster the transition from a context-dependent scientific and medical landscape of knowledge (the 'magic bullet' of Paul Ehrlich) to that of normative statements that interpret tumor systems behavior in a situation-associated manner (modular therapy). This change provides a promising basis for novel therapy strategies, which are needed to translate fundamental analytically-derived discoveries into personalized, i.e. systems-adapted, and thus situation-adapted therapeutic tumor strategies: This way, therapies may 'come' to the patient.

Acknowledgments This work was greatly facilitated by the use of previously published and publicly accessible research data. We would like to thank all colleagues contributing to the multicenter trials.

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Chapter 14
Searching for the 'Metabolism' of Evolution

Albrecht Reichle and Gerhard C. Hildebrand

Abstract On the background of a formal communication theory (Chapter 3) it is possible to phrase pragmatically, what is driving evolutionary processes: Communicatively linked biological systems are interweaving the nude identity of their systems objects or the arrangement of compartmentalized knowledge (*on the observer's site*) with situative biological stages or with the communicative arrangement of systems objects' validity and denotation (*on the participator's site*) by allowing the implementation of internally-derived or externally-derived modular knowledge. This knowledge is based on rules that are present in modularly arranged and rationalized systems textures, which are equitable with the 'metabolism' of evolutionary systems and purport the frame for evolutionary multiplicity.

Keywords Evolution • Communication theory • Modularity • Rationalization • Metastatic tumor

14.1 Letter

To Dr. Greaves article with the title: 'Darwin and evolutionary tales in leukemia'. Hematology Am Soc Hematol Educ Program, 2009: 1–12.

Unlike laws of nature, causal relations between initiating processes of tumor development are not anchored in an invariance of nature. Therefore, molecular and cytogenetic aberrations at initial diagnosis are generally heterogeneous [1]. However, distinct acquired genetic lesions are not distributed at random in tumor

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cells, despite the high variability of cancer causes, the heterogeneity of observed genetic aberrations, and the divergence of morphologic characteristics of diverse tumor types.

Invariance within leukemogenesis or tumorgenesis may be observed during leukemia or tumor progression. In interaction with the tissue, leukaemia and tumor (stem) cells use processes according to laws of nature to build up favorable infrastructures (systems) for proliferation [2].

A lead back to a final first principle, the 'founder phenotype or genotype' according to Darwin's reductionist considerations [1] that may be therapeutically targeted to eradicate leukemia or tumor diseases is – with exceptions – not sufficient for therapeutic purposes: Therapeutic targeting of the molecular-genetic heterogeneity of malignant (stem) cells includes multi-level difficulties.

Instead of such a lead back on a 'founder genotype', we have to deal with multiple and various constellations of functionally defined leukemia- or tumor-associated systems (i.e. inflammation, neoangiogenesis, Warburg effect, immune response, extracellular matrix remodelling, cell proliferation rate, apoptosis, coagulation effects, stem cell niches). These constellations of systems, have to be broken down to their single moments, e.g. in a reductionist sense – to evolving novel aberrant leukemia or tumor genotypes, indicating 'branching' of systems (Darwinian 'selection'), but, simultaneously, we have to understand the communicative relationship between one another rather than separately adding one system to another and thereby neglecting the presence of constitutive holistic communication architectures in biological systems [3]. These to some degree self-content-systems are simultaneously involving all systems objects, leukaemia and tumor cells as well as tumor-adjacent stroma cells [4,5]. The principle therapeutic problem of neoplasias lies in this point [2].

The reductionist Darwinian comprehension of evolution may be now advanced on the basis of observations derived from biomodulatory therapy approaches in metastatic tumors. The metastatic process may be considered as 'rapidly' evolving biologic system [2,3,6]:

Modularity of cell systems and proteins enables to constituting a 'big functional world' inside small biological networks [3,7,8]. Modularly constituted molecular or cellular architectures allow implementing modular knowledge with respective biomodulatory therapy approaches by redeeming novel validity of systems objects, the cells, pathways, molecules [2,3]. As biomodulatory therapies are sufficient to induce objective tumor response, these therapy approaches represent a methodological tool to comparatively uncover leukemia's or tumors' modular systems architectures. Therapeutically induced evolutionary steps may specify the definition of evolvability: Modularity allows to retrospectively establishing spaces for primarily non-heritable evolutionary developments, if modular events are implemented, e.g. with biomodulatory therapy [3].

Rationalization processes within tumor compartments may be separated under the view of purposes. Purposes are enmeshed in rationalized 'life-forms' of communication-driven cell systems, in such a way that we cannot oppose or circumvent them: The functional spectrum of distinct cell types within the tumor compartment

is limited despite of commonly observed huge cellular plasticity and is challenged by the required systems-associated functions directed at the systems objects [6]. These profiles of requirements lead to constitutive systems' features, which contribute to the robustness of systems. Systems-associated rationalization processes and modular architectures implicitly include discrepancies, i.e. inconsistencies, Achilles' heels, deformations or missing inter-systemic exchange processes. The proof of discrepancies is suitable to identify communication-derived rules [6]. Without these rules, evolutionary processes would not function.

Modular therapies exemplarily give indications of the 'metabolism' of evolutionary processes [3]: All hierarchies, developed by intentionally acquired knowledge, i.e. Darwin's evolving branching systems (Fig. 14.1), are leveled by modular systems considerations and by considering rationalization processes, to be finally discharged in a continuum of contingency programming and continuous inter-systemic exchange processes, respectively. Incommensurable 'worlds', the heterogeneous external physical or biochemical 'worlds' may be linked with the modularly arranged non-DNA-based heritage of the cellular 'living world' [9] and the DNA-based via the possibility for implementing modular cellular 'knowledge'. This process may result in substantial alterations of the cellular 'living world' and

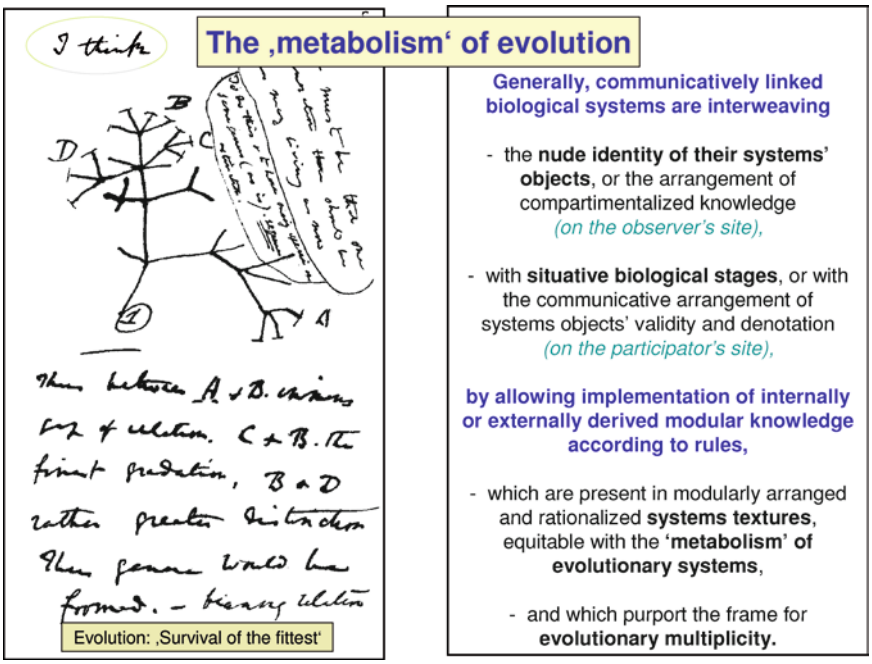

Fig. 14.1 Charles Darwin's 1837 sketch, his first diagram of an evolutionary tree from his First Notebook on Transmutation of Species (1837). Within reductionist considerations selection processes are indispensable. Modularity and rationalization processes, as discussed in a formal pragmatic communication theory, are sufficient to operationally define evolvability, which includes failure, fallacies, inconsistencies and rationalization processes

finally in molecular-genetic aberrations in tumor and stroma cells, even in transplantable stroma characteristics (e.g. fibroblasts) [10–12]. Vice versa, the (molecular-genetically altered) microenvironment facilitates clonal evolution of tumor cells [12].

Darwin has detected evolvability as an inherent feature of biological systems ('On the origin of species by means of natural selection, or the preservation of favored races in the struggle for life'; 1859). The presence of evolvability in biological systems simultaneously implicates the susceptibility towards events implementing external or internal modular 'knowledge' within holistic communicatively linked cellular systems [3,10]. The 'metabolism' of evolution, allowing implementation of internally and externally derived knowledge according to communication-associated rules may establish huge systems' diversity and context-dependent multi-functionality of proteins for creating modular cellular architectures (Fig. 14.1) [2, 3, 6, 8, 10]. 'Selection' in a non-Darwinian sense may be attributed to mechanisms covered by a pragmatic communication theory [3]. The novel 'selection' rules, based on modularity and rationalization processes may be uncovered by retrospectively establishing spaces for primarily non-heritable evolutionary developments, if modular events are implemented. As rationalization processes are inherent in biological systems, inconsistencies, Achilles' heels, deformations or missing inter-systemic exchange processes are implicitly emerging features of such systems architectures: On this background, the claim for 'survival of the fittest' should be revised. 'Selection' in the Darwinian sense relies on reductionist based observations, which do not account for the 'metabolism' of evolution as the original texture. The Darwinian notion has originally established the fundamental biological feature, namely evolvability of communicatively linked cell systems. The assumption of modularity and rationalization processes is sufficient to explain that distinct evolving tumor-associated genotypes may become clinically irrelevant, e.g. during the course of tumor diseases [6].

The 'metabolism' of evolution is generating distinct biological features of systems, i.e. survival, evolvability and finally reproducibility by redeeming validity of modular cellular features and rationalization processes. The symbolic modular architectures of the 'living world' of cell systems are reproducing themselves in form of rationalization processes, the variable integration of cells within a distinct evolving cellular 'systems world'. These processes take place within holistic communicative systems, which have been uncovered as experimentally and therapeutically accessible entity. Modularly constituted biologic systems implicitly include evolvability, i.e. the spin-off of systems functions, and rationalization processes, which are oriented on success. Coordination of actions, and strategic interventions, i.e. attenuation of tumor growth, may be (therapeutically) established by implementation of internally or externally derived modular knowledge. The possibility to choose between communication and strategic interventions is arbitrary and abstract, because it is only based on intentional perspectives of system's participators, once cell systems, at another time external systems implementing modular 'knowledge', or therapeutic operators of systems, e.g. physicians in case of biomodulatory therapies.

The overwhelming multiplicity of fossil and living species exemplifies the options of modular biological architectures and rationalization processes in the classic reductionist sense.

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Part V
Biomodulatory Therapy Approaches in Metastatic Cancer

Chapter 15
The Impact of Inflammation Control and Active Cancer Palliation on Metabolic Pathways Determining Tumor Progression and Patient Survival*

Ulrika Smedh, Annika Gustafsson, Hans Axelsson, Christian Cahlin, Christina Lönnroth, and Kent Lundholm

Abstract Strong associations are assumed between inflammation, cancer initiation, and tumor progression. Weight loss and cachexia predispose for early death in cancer disease. Usually, such cachexia conditions are characterized by systemic inflammation, which is easily monitored by increased blood levels of C-reactive protein and an elevated erythrocyte sedimentation rate. Hypothetically, eicosanoids or, more specifically, prostaglandins could be common mediators in the promotion of cancer cachexia and the fatigue syndrome. Consequently, prostaglandins, particularly prostaglandin E_2, have been reported to involve the development of anorexia, altered resting energy expenditure, tumor neoangiogenesis, elevated whole-body fat and cell metabolism, as well as blood and circulatory homeostasis in progressive cancer disease.

Thus, primary and secondary interventions with cyclooxygenase inhibitors (COX-1, COX-2) should significantly influence the appearance of overt malignancy and attenuate local tumor growth with improved survival in experimental and clinical cancer. Providing nutritional support, either by oral ingestion or parenteral nutrition, may help to prolong survival and increase wellbeing and quality of life in such patients. In our study, this treatment was combined with anti-inflammatory therapy to conceptually increase the effectiveness of supportive care.

Keywords Tumor-associated inflammation • Cachexia • Mal-nutrition • COX 2 inhibitors • Colorectal cancer • Cancer palliation • Tumor-host-interactions

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The Tumor Microenvironment 3, DOI 10.1007/978-90-481-9531-2_15,

15.1 Introduction

15.1.1 Tumor–Host Interaction

Strong associations are assumed between inflammation, cancer initiation, and tumor progression. Thus, a direct causal link exists between different malignancies and chronic inflammation. This link is sometimes related to infections and chronic exposure to toxic agents, which may interfere with genetic and epigenetic alterations that compromise gene transcription, cell reproduction, as well as tissue structures and microenvironments. Common examples are found in various solid human malignancies, such as gastric carcinoma, pancreatic carcinoma, hepatocellular carcinoma, cervical carcinoma, prostatic carcinoma, and colorectal cancer. Also, a number of classic studies have focused on metabolic and cellular alterations in tumor tissue. These studies aimed at showing significant and unique metabolisms and cellular reactions, either to explain the continuous and uncontrolled proliferation of tumor cells or to define possibilities to attenuate and interrupt tumor progression. However, most of these studies seem to essentially describe metabolic alterations similar to changes observed in normal, untransformed proliferating cells [1], particularly in the presence of attenuated oxygenation and overt hypoxia including tumor stroma interactions and neo-vascularization [2–4]. Thus, most tumor-like or tumor-specific alterations are likely to reflect rather normal cellular responses, which are usually found in healing wounds and tissue compartments during regeneration [5]. Such metabolic alterations are triggered by the local release of growth factors and cytokines from a variety of macrophages and host endothelial and immune cells stimulated by chemokines, TNF-α, histamine, proteases, various peptidergic growth factors, as well as by mediators including heparin, matrix metalloproteinases (MMPs), and serine proteases [4,6]. However, the major difference between inflammation in normal tissue and inflammation in solid tumor tissue is the continuation of inflammatory reactions during neoplastic circumstances. These reactions are caused by a lack of complex negative feedback mechanisms, perhaps due to reprogrammed cellular conditions involving epithelial mesenchymal transitions [7]. In this way, a malignant tumor initiates and orchestrates a microenvironment that escapes normal control, allows promotion of its own progression, and develops the prerequisites for a subsequent spread of tumor cells in its host [8]. The inflammatory response caused by the interaction between tumor and host cells does not only create local tissue reactions, but will also result in adaptational changes in host macroenvironments, which are apparent as systemic metabolic and immunological alterations [9,10]. Ultimately, the macrophysiological changes induced by local malignant interactions between invasive tumor cells and surrounding host cells lead to the physiological state known as cachexia, which is characterized by the progressive wasting of host tissues and systemic inflammation [11]. This condition may not be entirely related to the size of a tumor but rather correlates to a tumor's biological behavior, biochemical characteristics, and degree of

invasiveness with or without overt metastases [12]. The present study will discuss such alterations focusing on prostanoids.

15.1.2 Cancer Cachexia

Cancer cachexia involves all host tissues and organs characterized by a negative energy balance due to reduced appetite and increased resting energy expenditure [13–15]. A negative energy balance explains the initial loss of whole-body fat and the subsequent attenuation of skeletal muscle mass [16,17]. Body composition changes can be monitored in most organs [11], although cardiovascular and central nervous systems were initially believed to be functionally protected [18–21]. However, progressive and severe cancer cachexia seems to be universally detrimental for host tissues and cellular functions. Metabolic and functional adaptations are probably meant to attenuate deteriorations and extend survival as long as possible. Counter-regulatory mechanisms for such adaptations are communicated by cytokines, growth factors, prostanoids, leucotriens, and other messengers, such as classical hormones. These changes are apparently well-recognized in stressed organisms but were originally thought to partly reflect unique overall metabolic reactions in tumor hosts, particularly when combined with under-nourishment (Fig. 15.1). Usually, such cachexia conditions are characterized by systemic inflammation, which is easily determined and monitored by increased blood levels of C-reactive protein and an elevated erythrocyte sedimentation rate. Transectional multivariate analyses of large groups of unselected cancer patients suffering weight loss have confirmed that stress-related and tumor-related systemic inflammation predict survival, particularly in patients with solid gastrointestinal cancer [22]. Hypothetically, eicosanoids or, more specifically, prostaglandins could be common mediators in the promotion of cancer cachexia and the fatigue syndrome. Consequently, prostaglandins, particularly prostaglandin E_2 (PGE_2), have been reported to involve the development of anorexia, altered resting energy expenditure, tumor neoangiogenesis, elevated whole-body fat and cell metabolism, as well as blood and circulatory homeostasis in progressive cancer disease. Thus, primary and secondary interventions with cyclooxygenase inhibitors (COX-1, COX-2) should significantly influence the appearance of overt malignancy and attenuate local tumor growth with improved survival in experimental and clinical cancer [23,24].

15.1.2.1 Prostaglandin Biosynthesis

Prostaglandins are 20-carbon fatty acid derivatives found in almost every tissue and organ, mediating a number of physiological and pathological functions. These derivatives are synthesized from different essential fatty acid precursors. Prostaglandins derived from arachidonic acid are termed series-2 prostaglandins or prostanoids and include prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostaglandin I_2 (PGI_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and tromboxane A_2 (TXA_2) [25]. These prostaglandins share a common initial biosynthetic pathway, which begins with the hydrolysis of

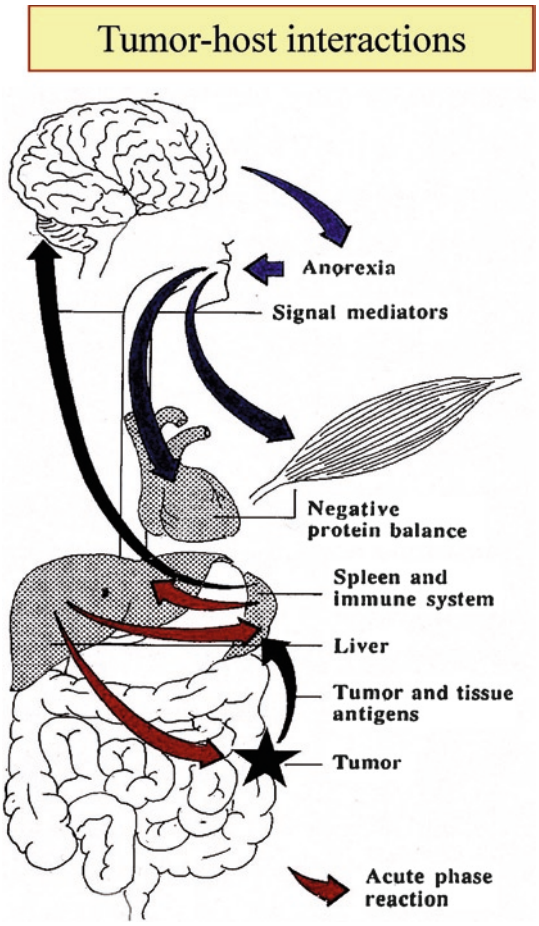

Fig. 15.1 Invasive malignant tumor growth activates the local tissue production of cytokines, growth factors, and prostanoids. These mediators are translated into a classic systemic acute phase response (*red arrows*), which is part of more specific immune reactions, such as Th_1 and Th_2 responses. Systemic cascades for signaling are also transferred to CNS centra, both by circulating mediators and afferent nerve transmission. This transfer leads to anorexia, which elicits whole-body adaptive stress responses including negative energy and protein balance, particularly in adipose tissue and skeletal muscles

cell-membrane phospholipids liberating arachidonic acid into the cytoplasm [26]. This step is mediated by membrane-bound phospholipase A_2 and activated by diverse physiological and pathological stimuli [27]. Arachidonic acid is converted by cyclooxygenase into unstable endoperoxide intermediate prostaglandin G_2 (PGG_2), which in turn is converted into oxygenated intermediate prostaglandin H_2 (PGH_2) [28]. Phospholipase A_2 and cyclooxygenase are rate-limiting steps in prostaglandin biosynthesis. Three isoforms of cyclooxygenase have been identified: COX-1, COX-2, and COX-3. COX-1 is constitutively expressed, and COX-2 is inducible by pathological stimuli [29,30]. COX-3 is an isoform of COX-1 that is preferentially expressed in the

heart and brain [31]. PGH_2 is in turn metabolized by cell-specific synthases (PGE-synthase, PGD-synthase, PGI-synthase, PGF-synthase, and Tx-synthase) into series-2 prostaglandins [32]. Prostaglandins are released from cells immediately after synthesis and act on specific cell-surface prostanoid receptors in an autocrine and paracrine fashion [33]. Alternatively, prostaglandins may also be transported by PG-transporters across cell membranes into cytoplasmatic compartments, in which effects are terminated by oxidizing and reducing enzymes [34,35].

The biological action of the prostaglandins is mediated by specific prostanoid receptors located in cell membranes. These receptors belong to the Rhodopsin-type receptor family. The receptor family is characterized by seven transmembrane domains coupled with different intracellular subunits of G proteins [36]. There are five major types of prostanoid receptors; E-prostanoid receptor (EP receptor), D-prostanoid receptor (DP receptor), I-prostanoid receptor (IP receptor), F-prostanoid receptor (FP receptor), and T-prostanoid receptor (TP receptor). Each one of these major types consists of one or several subtypes with a different structure and biological function [33], which vary according to the type of tissue and physiological condition. Functions and distributions of the receptors may also vary among species [37]. PGE_2 is considered to be involved in normal physiological functions as well as in malignant and non-malignant conditions among serie-2 prostaglandins. There are four different subtypes of EP receptors: EP_1, EP_2, EP_3, and EP_4. These receptors show an overall sequence identity of about 40%, and the putative transmembrane domains are the most conserved [38]. Biological signals are propagated by an alteration in intracellular calcium (Ca^{2+}) and cyclic adenosine monophosphate (cAMP) levels. Effects of PGE_2 are determined by the type and presence of EP receptors, which differ among cell types and organs.

PGE_2 has low affinity for the EP_1 receptor that mediates signaling by activation of phospholipase C and elevation of cytosolic Ca^{2+} concentration by activating Ca^{2+} channels. This process results in the direct activation of downstream kinases and transactivation of the HER´s-2/Neu tyrosine kinase receptor and up-regulation of the endothelial growth factor-C [39]. The EP_1 receptor also transactivates the epidermal growth factor receptor, which may promote cell proliferation and invasion [40]. The EP_2 receptor increases levels of cAMP and stimulates cellular growth by stimulating PKA and PI3K pathways [41]. The EP_3 receptor is expressed in a wide range of tissues that mediate biological signals by inhibiting adenylate cyclase and thereby decreases intracellular levels of cAMP. The EP_3 receptor is involved in acid-induced duodenal bicarbonate secretion and maintenance of mucosal integrity [42] and also participates in the regulation of tumor-associated angiogenesis and tumor growth; furthermore, the receptor has been shown to activate the Ras signaling pathway [43,44]. The generation of fever also appears to be regulated by EP_3 receptors [45]. Three receptor isoforms of EP_3 exist in mice and eight in humans, which are generated by alternative splicing and differ in their C-terminal domain [46]. The expression pattern of these receptor isoforms varies between different cell types. EP_3 receptor isoforms have been reported to differ in their ability to down-regulate adenylate cyclase, but the biological significance of this finding is not clear [47]. The EP_4 receptor has a very high affinity for PGE_2, raising intracellular levels of cAMP upon activation and stimulating cell growth and cell proliferation similar to the EP_2 receptor [41].

15.1.3 Prostanoid Related Effects in Tumor Bearers

15.1.3.1 Inflammation and Tumor Growth

The link between inflammation and the appearance and progression of cancer was first recognized in 1863, when Rudolf Virchow discovered leukocytes in neoplastic tissues [48]. The inflammatory process mediates several fundamental tumor properties, although the mechanisms involved are not yet fully understood [49–53]. Epidemiological studies imply that chronic inflammation is the origin of various types of cancer triggered by conditions, such as microbial infections (Helicobacter pylori and gastric cancer and gastric lymphoma), autoimmune disease (inflammatory bowel disease and colon cancer), and inflammation of unknown origin (chronic pancreatitis and pancreatic cancer; prostatitis and prostatic cancer). Inflammatory mediators, such as prostaglandins, chemokines, and cytokines, are present in tumor microenvironments and may create both genetic and epigenetic events for the activation of oncogenes, chromosomal rearrangement, and gene amplification as well as for the inactivation of tumor-suppressor genes. Cells transformed in this way usually show activated transcription factors (NF-kB, STAT3, and HIF1α), which may further stimulate the production of inflammatory mediators (chemokines, cytokines, and prostaglandins) and the recruitment of inflammatory cells (eosinophils, mast cells, neutrophils, macrophages, and myeloid-derived suppressor cells) leading to cascades of signaling [54–56]. Recent observations have also implied that embryonic stem cells depend on prostaglandins for control of growth, apoptosis, and perhaps differentiation [57].

15.1.3.2 Prostanoids and Metabolic Alterations

Genes for controlling fatty acid and protein metabolisms were highly down-regulated by COX-inhibition in tumor tissue, whereas genes directing carbohydrate metabolism were both up-regulated and down-regulated [58]. Such observations may contribute to overall host-metabolic effects by indomethacin attenuating catabolism caused by a growing tumor [22,59]. However, the entire host metabolism also appears to be influenced by prostanoids [22]. Distant metastases are a major cause of death in cancer with overexpression of COX-2 and increased production of PGE_2. In contrast, treatment with NSAIDs may reduce this imbalance in favor of apoptosis [23,60,61] across the PI3K-Akt-mTOR signaling in tumor cell metabolism [62]. Thus, the gene coding for Akt was drastically down-regulated, and genes coding for proteins behind cell adhesion were also down-regulated by COX-inhibition (indomethacin) [58,63]. Results derived in vivo by the application of microarray analyses show an overwhelming number of genes affected in transcription by indomethacin treatment, in which down-regulations appear to be most common. Cancer cell-intrinsic metabolism is also likely to favor growth progression as a consequence or a cause of local tumor-host cell interactions [64].

Normally, energy balance is finely tuned by the central nervous controls of appetite, digestion, ingestive behavior, energy expenditure, and heat dissipation. However,

signal alterations are only partly known in clinical aberrations, such as obesity, infections, trauma, cancer, stress, and other conditions. The CNS may respond to peripheral signals directly through messengers that cross the blood-brain barrier by diffusion or by active transport. The CNS may also respond to peripheral signals of inflammatory molecules, such as prostaglandins and interleukins, through specific receptors located on afferent autonomic nerve endings. Thus, splanchnic afferent vagal and non-vagal nerve endings seem to be important pathways for disease-induced and tumor-induced inflammatory signaling from the abdominal area to the brain. The vagus nerve is widely distributed and also innervates skin areas, mammary glands, the heart, and the lungs. Around 90% of vagal fibers below the diaphragm are sensory and project to the solitary tract nucleus in the brainstem via the sensory Nodose ganglia. Spinal afferents arise in the gut and project to NTS through the spinosolitary tract (SST) via the superior cervical ganglion (SCG). From NTS, afferent neurons project to relevant centers of food intake control in the hindbrain, hypothalamus, and forebrain. In this way, information on peripheral physiological reactions are conveyed to neural networks within the brain for integration at appropriate response levels. Such signaling may result in changes of the core temperature, metabolic rate, appetite, and ingestive behavior. Thus, the hypothalamus is the key brain region for the control mechanism in basic physiology of ingestive behavior and digestion. Here, such functions are closely related through a number of nuclei, such as the venteromedial hypothalamic (VMH), the lateral hypothalamic (LH), the paraventricular (PVN), and the arcuate (AN) nuclei, which are all involved in the control of food intake. Hypothalamic nuclei harbor neuropeptide-containing neurons that release orexigenic signals, such as neuropeptide Y (NPY), agouti-related peptide (Agrp), ghrelin, as well as anorexigenic signals, such as cocaine- and amphetamine-regulated transcript peptide (CARTp), alpha-MSH, and the corticotropin-releasing factor (CRF).

Vagus afferents respond to mechanical, chemical, and endocrine peripheral signals that may arise from adipose tissue, liver, intestine, mammary glands, pancreas, and stomach compartments (Fig. 15.2). Thus, the intravenous injection of interleukin-1β activates vagal afferents [65–67]. IL-1 as well as IL receptors have been reported to be present in the nodose ganglion, the NTS, the area postrema, as well as in the hypothalamic centers for feeding control including AN and PVN nuclei [68–70]. Evidence suggests that prostaglandins are directly involved in the activation of vagal afferents caused by IL-1 driven inflammatory reactions and vagal sensory neurons in the nodose ganglion express mRNA for the EP3 receptor [67]; consequently, indomethacin pretreatment blocks the interleukin-1β activation of vagal afferents [67].

Peripheral administration of endotoxin lipopolysaccharide (LPS) is frequently used to generate experimental inflammation leading to increased levels of proinflammatory cytokines in blood, abdominal organs, and abdominal vagal fibers. In the brain, cocaine- and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC) mRNA are up-regulated in the AN in response to peripheral LPS; CART and melanin-concentrating hormone (a product of POMC) are likewise up-regulated in the lateral hypothalamus [71]. Neuropeptides are also potent to alter food intake after central administration [72–75]. It is not yet clear whether peripheral LPS or centrally produced IL-1β affects peptide expression and release in hypothalamic neurons after LPS injections.

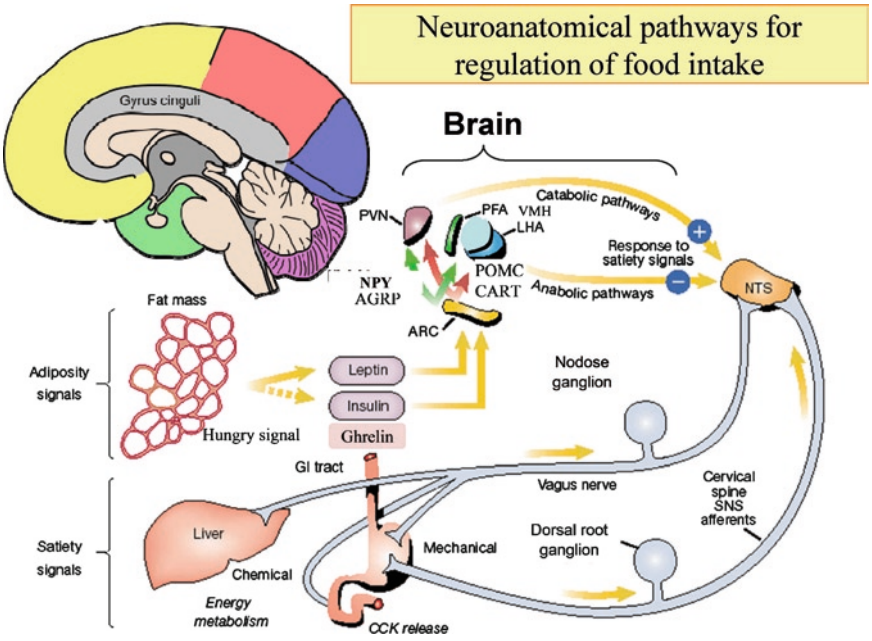

Fig. 15.2 The graph illustrates important relationships between adipose tissue and the gastrointestinal tract in communications of orexigenic and anorectic signals to different CNS levels, regulating appetite and energy homeostasis located in the brainstem and the hypothalamus. Important mediators in such communications are, for instance, leptin, ghrelin, insulin, cytokines, and prostaglandins, as described in the text

However, evidence suggests that the hypothalamic melanocortin system is involved in CNS response to tumor-induced development of anorexia-cachexia. Here, genetic or pharmacological blocking of the melanocortin-4 receptor attenuates the development of cachexia in tumor-bearing mice [76], besides observations that the central administration of a MC-4 antagonist inhibits metabolic and locomotor responses to the peripheral appearance of IL-1β [77]. Such observations imply the possibility of future pharmacological therapy of cancer-induced cachexia by targeting central nervous neuropeptide receptors. Small molecule melanocortin antagonists that readily pass the blood-brain barrier after peripheral administration have already been developed [78], but the role of such treatment strategies needs to be clinically evaluated.

15.1.3.3 Tumor Angiogenesis

Malignant tumors do not change from minimal residues into expanding overt solid tumors without angiogenesis. The net balance of positive and negative regulators promotes stimulators, such as the vascular endothelial growth factor (VEGF) that is produced and secreted from tumor cells [79,80] prior to the mediators of vascular remodeling that coopted for subsequent steps [81]. Oncogene derived proteins as well as a number of cellular stress factors including hypoxia, low pH, and nutrient deprivation

are important stimulators of angiogenesis [82]. Pro-angiogenic factors, produced by tumor cells, bind to endothelial cell receptors for induction of angiogenesis. Angiogenic stimuli cause major changes in the phenotype of "tip-cells" inside tumor cells located next to a capillary with properties of invasiveness and the ability to migrate. Tip-cells activate secreted and cell surface proteases for the partial destruction of adjacent basement membranes and extracellular matrix. Tip-cells start to migrate in directions paced by VEGF gradients. Subsequently, dissolution of the extracellular matrix allows further release of proangiogenic factors together with those produced by tumor cells. Endothelial cells proliferate and assemble in tubular structures behind migrating tip-cells, in which COX-derivates are recognized [83,84]. Newly formed blood vessels mature after the formation of a sufficient amount of vascular tubes. The initial step is the fusion of newly formed capillaries, in which tip-cells stop migrating and make contact with other tip-cells or existing capillaries. A vessel lumen is formed upon contact, and the emerging blood flow contributes to the stabilization of newly formed vessels by reducing hypoxia, thereby lowering VEGF levels. Capillaries are fused into larger vessels including arteries and veins with junctional complexes. Tumor vessels differ from normal vessels in several aspects, for instance, they spread without organization and change diameters with loss of differentiation in arterioles, capillaries, and venules.

Thus, angiogenesis is an essential factor in cancer progression propagated by proangiogenic factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor 1 and 2 (acidFGF and basicFGF) [85]. COX-2 plays an important role in tumor-associated angiogenesis [86,87] by modulating proangiogenic factors with correlations between COX-2 and VEGF expression in tumor tissue [88]. PGE_2 is regarded as a mediator of COX-2 activities in tumor angiogenesis [89]. Thus, both selective and nonselective COX-inhibitors may reduce tumor angiogenesis by inhibiting production of proangiogenic factors and subsequently the proliferation, migration, and tube formation of endothelial cells [83,84,90–95]. The gene coding for one of the three forms of VEGF (VEGF-A) was down-regulated by indomethacin, whereas others (VEGF-B and C) remained unaffected [90]. AcidFGF showed a trend towards down-regulation, whereas basicFGF showed a trend to up-regulation. Other genes in angiogenesis were mainly down-regulated. Overall, our results support the assumption that indomethacin affects tumor angiogenesis in addition to other processes related to tumor cell proliferation directed by subtypes EP_1 and EP_3 receptors [90].

15.1.4 Inflammatory Mediators in Colon Cancer

Adenocarcinoma of the colon is a common type of cancer in the Western world. A range of studies have investigated the role of various inflammatory mediators in colon cancer inhibition, development, and progression. PGE_2 is a second messenger in cell-to-cell communication, involving intracellular reactions related to G-protein coupled receptors. Systemic reactions, such as progressive weight loss, anorexia, and systemic inflammation, relate to prostaglandin activities in various organs as well as in tumor tissue [22]. Therefore attenuation of local and systemic progressive

disease would become possible by understanding ligand receptor activities in prostanoid-related metabolism and signaling pathways [58,96,97], in which COX-2 is regarded as the key enzyme for the local cellular production of PGE_2. In view of this fact, up-regulation of COX-2 may represent a global phenomenon in malignancy. However, our own studies have indicated that the tumor content of COX-2 transcript and protein are not necessarily overall increased in colorectal tumor tissue, in which local high concentrations are usually recognized as "hot spots" in contrast to the findings in the cell cultures of colon cancer [98]. This finding is not unique for COX-2 since most growth factors stain with uneven distribution among tumor cells in malignant tissue, which is a composite compartment of different clones of tumor cells, stroma, and endothelial and inflammatory cells. Up-regulation of COX-2 in tumor cells is likely to explain the majority of increased PGE_2 content in tumor tissue besides the decreased degradation of PGE_2. However, tumor stroma in colorectal tumor tissue also expresses considerable amounts of COX-2 for PGE_2 synthesis [99]. Accordingly, we reported that both COX-1 and COX-2 protein correlates to the PGE_2 content in colon cancer tissue [99], in which COX-1 tissue expression is proportionally increased to COX-2 tissue expression. This fact may explain why unspecific cyclooxygenase inhibitors effectively attenuate tumor progression [23,100,101]. mPGES-1 has been reported to be over-expressed in colorectal cancer, which is responsible for PGE_2 production [102], although increased PGE_2 levels in tumor tissue may also depend on decreased PGE_2 breakdown by HPGD. Accordingly, HPGD expression is low in tumor tissue when compared to overall levels in normal colon tissue [103]. Colorectal cancer appears to be particularly dependent on cyclooxygenase metabolites for progression [104,105]. Consequently, aspirin and conventional NSAIDs have been reported to attenuate several steps in disease progression of polyps and the subsequent invasive growth of tumor cells [106–109]. However, a recent analysis has suggested that this protection may only relate to a defined group of tumors [110].

Dukes' stages of colorectal cancer represent predictors for outcome independently of race, gender, and age with comparable results across countries [111]. Based on Dukes' staging parameters, we collected tumor material from unselected patients at primary surgery for the curative resection of newly diagnosed colorectal cancer. Immunohistochemical staining was related to tissue and blood concentration of PGE_2 as a hallmark of COX activity. Our results showed significant relationships among several key-proteins within tumor cells and stroma as well as among factors in tumor cells and stroma, indicating a "cross-talk" [99]. We could also show significant relationships between host systemic inflammation, survival, and protein staining of growth-related proteins in tumor cells and stroma [99]. Interesting and unexpected findings were that Bcl-2 expression in tumor cells and vWF in stroma were associated with prolonged survival, whereas staining of p53 and vWF in tumor cells was related to reduced survival. Bcl-2 is regarded as an inhibitor of apoptosis [112], although Bcl-2 protein has been recently reported to be related to improve prognosis of colorectal cancer [113]. In contrast, dual function of Bcl-2 was explained by interaction with the orphan nuclear receptor Nur77 bound to Bcl-2 and induced conformational changes that may convert Bcl-2 from an inhibitor to a promotor of apoptosis [112].

Our results confirmed a statistically significant relationship between tumor tissue COX-1/COX-2 staining and the overall tumor tissue content of PGE_2 in vivo [99]. A high COX-2 content suggests elevated tumor PGE_2, whereas a high COX-1 content rather predicts the opposite in tumor tissue. This divergence may be a question of less maintained physiology in tumor tissue with little retained intestinal morphology and function from its original normal mucosa, which is the main reservoir for COX-1 protein. Furthermore, a direct relationship between tumor cell proliferation and elevated host systemic inflammation was indicated in colon cancer patients. Local and systemic inflammation are known to relate to poor prognosis in colorectal cancer [23,114]. Accordingly, the correlation between COX-2 expression, PGE_2 content, and patient survival indicate a different relationship in tumors with high and low PGE_2. vWF in tumor tissue appears to be a risk factor for reduced survival, suggesting increased angiogenesis as a poor prognostic sign. Previous and present results link PGE_2 as a mediator to this pathway [90,98], although vWF may simultaneously activate different pathways in epithelial and endothelial tumor cells within a tumor [101,115] (Fig. 15.3).

Beneficial effects of NSAIDs in colorectal cancer patients have been published years ago [116,117]. Still, the molecular basis of how NSAIDs inhibit tumor progression remains unclear. Most reports have focused on PGE_2 as a major product of COX-

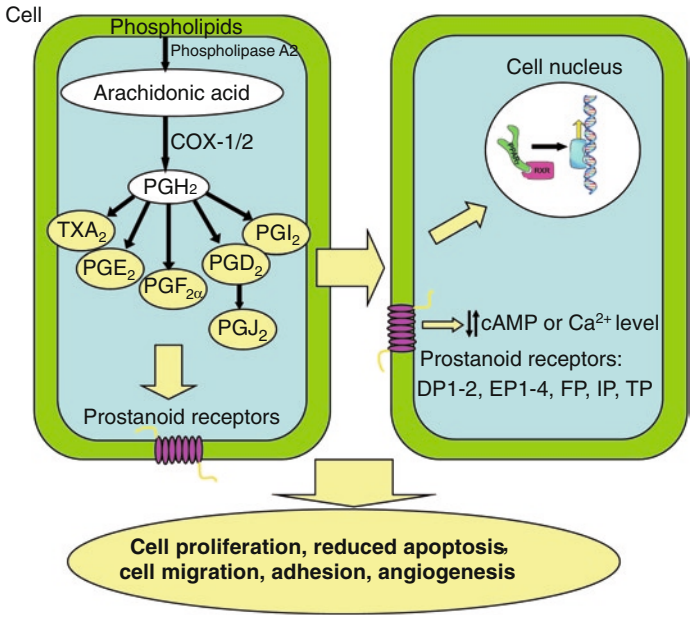

Fig. 15.3 Schematic illustration of the formation of prostanoids by COX-1 and COX-2 activities in cells with subsequent autocrine and paracrine activation of prostanoid receptors to primarily change intracellular concentrations of cAMP and Ca^{2+} levels for downstream signal transductions. These are principal biochemical alterations in most cells to control cell proliferation, cell migration, adhesion, angiogenesis, and apoptosis

2, leaving the remaining products of COX unconsidered. Therefore, we quantified PGE_2 receptor expression in human colorectal tumor tissue in comparison to expression in adjacent normal colon tissue. Expression of the EP_2 receptor subtype predicted reduced disease-specific survival [98], but overall changes in expression of any other EP subtype receptor did neither explain tumor progression nor tumor differentiation [98]. Therefore, for a more complete evaluation, we analyzed additional receptors (DP1, DP2, FP, IP, TP) for prostanoids (PGD_2, TXA_2, $PGF_{2\alpha}$, PGI_2) produced by cyclooxygenases (COX). Results showed reduced expression in four out of five prostanoid subtype receptors in Dukes A-D tumors when compared to normal colon tissue. This finding was most consistent for DPI and IP expression, whereas TP receptor expression was increased in tumor tissue. Such observations are signs of imbalanced eicosanoid receptor expression in colorectal cancer tissue. Therefore, complex relationships of prostanoids may be assumed in tumor carcinogenesis and progression. However, altered eicosanoid homeostasis in tumor tissue is well-recognized and appears to be a global tumor phenomenon [118–120], which may affect metastatic spread [121], tumor angiogenesis, cell proliferation, apoptosis, and immune reactions [101]. An obvious limitation to the information on overall tissue measurements is the risk to overlook specific alterations within or between defined cell types. However, prostanoids clearly are important factors for colorectal cancer progression, although the presentation of a simplistic model is not yet possible [63].

EP subtype receptors may be ideal targets for growth interactions among tumor tissue cells [122]. The functional response to each ligated EP receptor depends on the associated signaling pathway. A suggested role of PPARγ in colon carcinogenesis is the inhibition of cell growth and induction of apoptosis [123]. Several studies in animals have indicated that only EP_2 homozygous deletion decreases the number and size of intestinal polyps in $Apc^{\Delta 716}$ mice. Also, EP_2 receptors boost COX-2 expression by a positive feedback loop [124]. EP_1 and EP_4 knockout mice show significantly suppressed colonic aberrant crypt foci (ACF) and cell proliferation, which agrees with findings during treatment with a specific EP_1/EP_4 antagonist [125–127]. Treatment of EP_1 receptor knockout mice with the colon carcinogen azoxymethane decreases ACF formation without the effects found in EP_3 knockout mice [128]. Tissue distribution of EP receptors in normal human colon tissue shows that EP_2 is expressed on the apex of the crypt, whereas EP_1 is not expressed at all in epithelial cells [129]. Strong EP_3 expression is seen in the apex of crypts with less expression at the lateral epithelium and little or no expression at the base of crypts. Epithelial colon cells express EP_4 in a universal manner similar to mononuclear cells in the lamina propria [129]. Here, our own results indicated that EP_1 and EP_2 receptor protein were highly present in tumor cells; EP_3 occurred only occasionally, and EP_4 was not detected at all in tumor cells.

Epidemiological studies have confirmed that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with significantly decreased occurrence of colorectal tumors and decreased disease-specific mortality [116,130,131], although mechanisms unrelated to COX may also occur [122,132]. Thus, PGE_2 may promote tumor progression via its action on cell surface $EP_{1–4}$ receptors in both tumors and tumor surrounding normal cells [122]. However, reports with regard to

the question which EP receptor(s) mediate(s) the effects by $PGE_{2\,a}$ are contradictory. Some reports claim that the main effects are mainly mediated through nuclear receptors (Peroxisome Proliferator-Activated Receptors (PPARs)), although some evidence suggests that PPAR activation does not explain antiproliferative effects by NSAIDs [133–135]. The transcription factor PPARγ seems to be involved by inhibiting tumor cell proliferation in vitro as well as by suppressing tumor growth and induction of apoptosis both in vivo and in vitro [136–140]. Several studies have reported PPARγ expression in colorectal cancer [141–143]. However, the role of PPARγ remains unclear because of the down-regulation in tumor tissues with possible effects as a tumor suppressor gene, although overexpression occurs in some tumors [134,135,144,145]. PPARγ is a ligand-activated transcription factor that is only functional when heterodimerized to 9-*cis* retinoic acid receptor (RXR). PPARγ ligands have been suggested for use in chemoprevention and chemotherapy [135]. Results have shown that down-regulation of PPARγ expression in colon cancer tissue agrees with decreased apoptosis of tumor cells and increased disease progression, although multivariate analyses on a variety of prostanoids have not identified PPARγ as a predictor of tumor-specific mortality [98]. Thus, available reports do not provide a unified model of prostanoid receptor expression ($EP_{1–4}$, PPARγ) in colon cancer tissue, tumor stage, and survival; although overall COX and EP subtype receptor expression in tumor tissue has predicted disease-specific mortality in multivariate analysis. Expression of EP_2 and COX-2 have been identified to be particularly important. Thus, both the production side (COX-2) of prostaglandins (PGE_2) and the receptor signaling (EP_2) in tumor tissue are critical for the progression of colorectal cancer.

Prostanoid receptor expression in colon cancer tissue is, to some extent, affected by indomethacin treatment with reduced IP receptor expression in both tumor and normal colon tissue. IP is activated by prostacyclin (PGI_2) and has been reported to inhibit apoptosis in colonic epithelial cells [146,147]. Receptors for PGD_2 (DP1 and DP2) show increased expression in normal colon tissue during indomethacin treatment. Some evidence suggests that indomethacin may have a direct agonistic effect on DP2 receptor [148]. PGD_2 may also have several effects in tumor tissue, such as decreased proliferation including pro- and anti-inflammatory actions with significant effects on immune reactions [149–152]. Thus, different effects within a tumor compartment are likely to depend on the type of PGD_2 receptor activation (DP1, DP2 and PPARγ), in which PPARγ is usually recognized as a tumor suppressor [123]. However, indomethacin decreases its expression in both normal and tumor tissue [98].

Also, preoperative treatment with indomethacin for 3 days has caused altered expression of numerous genes of different functions, assessed on pooled RNA from Dukes A-C tumors. Gene profiling maps appearance or disappearance of gene transcripts in relation to high and low PGE_2 content in tumor tissue despite tumor stage, as reported for normal colon tissue after long-term treatment with celecoxib [153]. We have provided information on alterations in gene expression and net PGE_2 production in colon cancer tissue affecting apoptosis, differentiation, and regulation of energy metabolism in agreement with similar findings in animal tumor models [154]. Our gene algorithm analysis suggested apoptosis to be the overall, most affected pathway in

human colon cancer tissue. Extrinsic "survival factors" were particularly down-regulated to promote net apoptosis, together with decreased external growth factor exposure for the stimulation of cell cycling [23]. A speculative guess is that stroma cells are influenced to decrease the external stimulation of tumor cells, which promotes apoptosis during cyclooxygenase inhibition [99]. Thus, present clinical findings certainly emphasize that prostanoid metabolism is a complex issue in colon cancer tissue. Several hundred genes are involved, which appear to control local growth and net immune response [101,155], cell proliferation, differentiation, energy metabolism and apoptosis as also reported for normal colon tissue [153].

15.1.5 Prostanoids and Immunological Tumor Alterations

Malignant disease is characterized by the attenuation of cells mediating anti-tumor immune response, probably directed in part by PGE_2 based on reduced production of anti-tumor Th1 cytokines (TNFα, IFNγ and IL-2) [156] and increased production of Th2 cytokines (IL-4, IL-10 and IL-6) [157–159]. Many studies report that indomethacin treatment of patients with different types of solid cancer may prolong survival and improve physical functioning and quality of life [23]. However, local effects on tumor growth are certainly involved, and a similar number of studies show evidence for the attenuation of angiogenesis, decreased tumor cell proliferation, and increased tumor apoptosis [59,90,132,154]. The metabolic basis for these observations may be that COX-2 and 15-hydroxy-prostaglandin dehydrogenase expression in cancer tissue predicts tumor tissue variation of PGE_2 signaling on prostaglandin subtype receptor $E_{1–4}$ [98,160]. However, prostanoids are also known as major factors behind immune responses, which result in complex interactions that may determine disease progression and metastasis. Therefore, we consider local immune reactions as significant factors behind tumor progression, since NSAID is known to convert states of anergy into immune competence in malnourished and stressed patients [161,162].

A major observation in our studies was that many genes belonging to MHC locus on chromosome 6p21 were up-regulated in human colon cancer during short preoperative treatment with conventional NSAIDs. MHC genes control the synthesis of molecules that are essential for immune functions mediated by T-lymphocytes, macrophages, APC, and NK cells [163]. Antigen recognition by T-cells depends on the expression of HLA molecules by target cells. HLA molecules bind small antigenic peptides of enzymatically degraded proteins presented on the cell surface, which are subsequently screened and recognized by the T-cell receptor. Normally, HLA class I molecules are expressed on all cells, except RBCs and cells of the testis, presenting intracellularly derived peptide fragments to CD8+ cytotoxic T-lymphocytes. In contrast, HLA class II molecules, which are usually expressed on professional antigen presenting cells (APCs), present extracellularly derived peptide fragments to CD4+ T-helper lymphocytes [164–169].

Colon epithelial cells may express low levels of HLA-class II, although this expression is normally restricted to APCs, such as B-lymphocytes, macrophages,

and dendritic cells. The up-regulation of these molecules is associated with inflammation [170]. Attempts have been made to turn tumor cells into antigen-presenting cells by inducing HLA signaling, since human tumors often lose expression of HLA-molecules, which may leave the immune system inactivated towards tumor cells [171–174]. Thereby, increased levels of PGE_2 in colon cancer may negatively influence immunity. Arvind et al. [172] reported that SW1116 colon cancer cells express HLA class II antigens, particularly HLA-DR. PGE_2 constitutively reduced the expression of HLA-DR and removal of PGE_2 restored the levels of HLA-DR, whereas $PGF_{2\alpha}$ and LTB_4 did not affect the expression of HLA-DR. In addition, a colon cancer cell line (HT 29), which did not constitutively express HLA-DR, initiated HLA-DR expression, when cells were treated with prostaglandin inhibitors, such as aspirin, indomethacin, and sulindac. In contrast, HLA class I expression was not influenced by PGE_2. These observations agree with our results that NSAIDs (indomethacin, celebrex) up-regulate HLA class II expression in colon cancer tissue and MHC II protein in tumor epithelial cells after short-term preoperative treatment [101]. Tumors showed enough HLA class I protein for peptide presentation and CTL activation. PGE_2 suppressed immune response by EP receptor signaling, which inhibits the production of downstream targets, such as chemokines and their receptors associated with dendritic cells, macrophages, and lymphocyte function [48,175–179]. PGE_2 also down-regulated cytokines, such as TNF_α, IFN_γ, and IL-2, with T-helper cell-stimulatory function (Th1) and up-regulated T-helper cell (Th2) characterized by immunosuppressive cytokines, such as IL-4, IL-6, and IL-10 [83,156,158,175,180–182]. These suggestions agree with our results that NSAID treatment increases infiltration of B-cells, macrophages, CD4+ T-helper cells, as well as CD8+ cytotoxic T-cells in colorectal tumor tissue [101]. We found increased RNA expression of granzyme H and perforin and a trend to increased granzyme B-levels capable of activating intracellular caspases that initiate apoptosis in target cells. Granzymes are released together with perforin, which is a pore-forming protein from cytoplasmic granules of CTLs and NK cells [183–190]. Therefore, CTLs appear ready for killing target cells based on perforin protein in CD8+ cytotoxic T-lymphocytes, shown by "halos" surrounding condensed apoptotic tumor cells or disruption of tumor cell patterns after indomethacin treatment [101].

Several reports support the importance of activated tumor-specific CD8+ cytotoxicT-lymphocytes [191–194]. Accordingly, Pagés et al. [195] reported that patients suffering from colorectal cancer without any signs of metastatic spread (vascular emboli, lymphatic invasion, or perineural invasion) had increased infiltration of immune cells (CTLs) and increased content of cytotoxins. The mobilization of granulocytes, lymphocytes, and macrophages at the invasive border of gastrointestinal cancer has been recently associated with improved survival [196,197]. Monocytes and macrophages may be responsible for T-lymphocyte impairment by increased PGE_2 production [198–201]. Based on vaccine trials, consensus is growing that the cooperation of CD4+ Th1 cells and activated CD8+ cytotoxic T-lymphocytes are necessary for adequate anti-tumor immune responses. The appearance of $CD4^+$ $CD25^+$/ $CD8^+$ $CD25^+$ T-regulatory cells (Tregs) or associated molecules (immunosuppressive FOXP3 and IL-10) may thus be influenced by indomethacin exposure [202,203].

Unspecific and specific COX-inhibition may exert different effects in complex immune reactions, which involve eicosanoids. However, we believe that such differences are rather quantitatively-based on the systematic analysis of several unspecific, intermediate specific, and specific cyclooxygenase inhibitors in experimental models [100]. Besides, indomethacin is the NSAID that implied survival differences in the treatment of malignancies [23], although treatment with acetylsalicylic acid (ASA) seems to have similar effects [204–207]. Future research will eventually show fundamental differences between specific and unspecific COX-inhibition. Analysis of normal colon mucosa from patients treated with indomethacin confirmed that MHC class II genes are not up-regulated in normal mucosa. Thus, our studies showed that NSAID administration for 3 days preoperatively is enough to turn tumor microenvironments into conceptually more favorable conditions for patient outcomes. Additionally, NSAID administration is accompanied with the appearance of tumor infiltration by immune cells showing potential capacity to kill tumor cells. This finding agrees with our observations that COX activities, high tumor content of $PGE_{2,}$ and tumor expression of EP_2 increase the risk of reduced survival [98,99,101]. Thus, prostaglandins are emerging modulators of tumor-related immunity [156]. In this respect, malignant tumors may be guarded by the down-regulation of immune response through the appearance of Treg lymphocyte, as seen in wound healing. Growing tumors and healing wounds may signal growth by the same or similar mechanisms, although the initial triggers may be either a genomic alteration or a tissue matrix dysfunction.

15.1.6 Anti-Inflammatory Therapy

Several studies indicate favorable effects of the anti-inflammatory treatment of cancer development in animal tumor models, but only a few conclusive interventional studies are available in human cancer [23,84]. The study by Lönnroth et al. highlighted the possibilities to introduce NSAIDs before surgical trauma-induced inflammation with positive effects on the immune response in tumors [101]. In addition, the COX-2 inhibitor celecoxib appears to slow down growth of colorectal adenomatous polyps, which are regarded as a pre-cancerous stage [208]. Similar effects have been found after tiracoxib treatment [209]. In localized prostatic cancer, treatment with celecoxib 4 weeks prior to surgery induced cellular changes in tumors including reduced cell proliferation, angiogenesis, and enhanced apoptosis [210]. In gastric cancer, celecoxib and octreotide pretreatment prior to surgery induced apoptosis and reduced angiogenesis [211]. Moreover, beneficial effects of long-term celecoxib treatment after H. pylori eradication on regression of pre-cancerous dysplasia of the stomach have also been reported. Tumor cellular changes included increased apoptosis, reduced angiogenesis, and cell proliferation [212]. However, these interesting aspects with regard to the primary prevention of malignant transformation and appearing invasiveness have been impeded by other risk-factors, since some COX-2 inhibitors showed unwanted cardiovascular effects [213–215] that were related to specific compounds rather than to COX-2 inhibition

as such. In contrast to celecoxib, rofecoxib and diclofenak appeared to impose increased risks of cardiovascular events in a meta-analysis [215,216].

The role of prostaglandins for cancer development is well-established in animal models. However, such findings cannot be directly transferred to clinical settings. Tumors used in animal studies are usually defined by clones that hold little variation within groups, unlike the variable biochemical conditions shown in human cancers. Therefore, findings that indomethacin administration to cancer patients improves function and perhaps survival are encouraging [23]. Also, Fenwick et al. reported reduced angiogenesis in colorectal liver metastases after treatment with rofecoxib for 14 days before liver resection, but did not examine any possible effects on outcome [84]. In advanced non-small-cell lung cancer, celecoxib seems to have a beneficial effect on survival when given in combination with chemotherapy, but only in patients with tumors showing moderate to high COX-2 expression [217]. These results underline the importance of stratification of patient groups in future investigations and analyses, in which specific antagonists for the prostaglandin subtype receptors EP_{1-4} may offer new exiting possibilities [121].

Endogenous IL-1 antagonist was reported to reduce clonogenicity of leukemia cells [218], although its value in the treatment of solid cancer appears to be limited. Some TNF-α inhibitors are available for clinical use, mostly for the treatment of inflammatory bowel disease. In a phase II study on patients with pancreatic cancer (stage 2–4), no effect on survival was found, although the lean body mass was slightly increased in response to the TNF-α inhibitor infliximab [219].

Weight loss and cachexia predispose for early death in cancer disease. The typical loss of muscle and adipose tissue accompanied by increased energy expenditure in combination with reduced food intake, nausea, and anemia imply the need of metabolic and nutritional support. Providing nutritional support, either by oral ingestion or parenteral nutrition, may help to prolong survival and increase well-being and quality of life in such patients [220]. In our study, this treatment was combined with anti-inflammatory therapy to conceptually increase the effectiveness of supportive care. Thus, evidence suggests that cachexia can be delayed by providing anabolic support to counteract catabolism. Insulin treatment has been confirmed to protect adipose tissue content and thus to counteract cachexia and prolong survival [221]. Ghrelin, an endogenous orexin considered to initiate hunger, improved food intake in short-term supply to cancer patients [222]. In long-term care, the daily administration of ghrelin to patients with progressive diseases improved appetite and glucose intake, also maintaining the entire body metabolic balance [223]. Another typical feature of progressive cancer disease is anemia, which is not always related to bleeding. Treatment with human recombinant erythropoietin (EPO) prevents the development of anemia and has beneficial effects on physical functioning and quality of life without any negative effects on survival [224,225]. As yet, no evidence exists that the provision of anabolic support, either by insulin, ghrelin or EPO and by securing metabolic needs through extra nutrition, would lead to inappropriate disease progression [114]. Net effects of active palliative support to counteract cachexia improve quality of life and sometimes prolong survival [23,220,221,223–225]. Therefore, this support should be offered to patients before cachexia is fully developed.

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Chapter 16
Pioglitazone and Rofecoxib Combined with Angiostatically Scheduled Capecitabine in Far-Advanced Hepatobiliary Carcinoma

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Abstract Peroxisome proliferator-activated receptor-gamma and cyclooxygenase-2 are frequently overexpressed on cholangiocarcinoma (CC) cells and adjacent stroma cells, and might be potential therapeutic targets. A pilot phase II trial was started to analyze the activity of angiostatically scheduled chemotherapy, capecitabine 2 × 1 g/m^2 from day 15 to 28 every 3 weeks combined with an antiinflammatory/angiostatic therapy, daily 45 mg oral pioglitazone and 25 mg oral rofecoxib day 1+ in advanced CC. All 21 consecutively included patients (mean age 64 years) suffered from non-resectable far-advanced CC, 62% were pretreated. The median dose of capecitabine per cycle was 76% of that planned; the median duration of treatment was 6.8 months (range 2 to 30+). Only three patients suffered from grade 3 toxicity (hand-foot syndrome n = 2, edema n = 1). Therapy continuation was refused in one patient with HFS grade 3. Objective response was achieved in 29% of the cases including one cCR, 29% achieved SD >6 months. Median overall survival was 8 months. The median overall survival in this unselected, partially pretreated patient population compares to that observed in selected patient populations receiving second generation combination chemotherapies which were shown to be accompanied with considerable hematotoxicity. The present completely oral therapy approach combines convenience, low toxicity and efficacy, and fits to the general patients characteristics: elderly patients with tumor-associated comorbidity. Randomized trials will definitely clarify the impact of antiinflammatory treatment strategies on survival.

Keywords Hepatobiliary carcinoma • Metronomic chemotherapy • Pioglitazone • Coxib

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16.1 Introduction

Cholangiocarcinoma (CC) occurs mainly in elderly patients [1]. Carcinogenesis is at least partially based on chronic inflammatory processes in the bile ducts or liver parenchyma [2]. Fifty to ninety percent of the patients are no candidates for curative resection due to advanced disease at diagnosis [3]. Whereas tumor-associated symptoms may be resolved by supportive interventions [4, 5] tumor control by systemic therapy remains a challenge [1]. We combined two therapeutic strategies, biomodulation with rofecoxib plus pioglitazone and long-term repetitively administered chemotherapy with low-dose capecitabine. Both treatment strategies are directed on tumor stroma as well as tumor cells [6, 7].

Clinical endpoint of the present study was objective response in advanced CC. A pretreatment interval during which only the two biomodulators were administered was included to evaluate their ability to induce clinical improvement. The completely oral therapy approach shows, that progression-free survival (PFS) rates in a patient population with 62% pretreatment were similar to those described for selected patients receiving second generation pulsatile combination chemotherapy [4].

16.2 Patients and Methods

16.2.1 Patients' Characteristics

The local ethics committee approved the protocol and the patients were required to provide their written informed consent before being enrolled into the study. The present series of patients considered patients recruited between July 2001 and August 2003.

Patients with advanced or non-resectable, progressive (>25% increase in the sum of all measurable lesions at begin of study medication in comparison to last follow-up), histologically proven intra- or extrahepatic cholangiocarcinoma or gallbladder carcinoma, bidimensionally measurable disease, and life expectancy >3 months were eligible. Patients who had previously received capecitabine were ineligible. Further criteria for eligibility were as recently published [4].

16.2.2 Treatment

Pretreatment was performed with pioglitazone 45 mg once daily p.o. and rofecoxib 25 mg once daily p.o. for 14 days before starting chemotherapy. This pretreatment period was included to investigate whether biomodulation alone provides clinical

benefit. Combination treatment comprised pioglitazone and rofecoxib, administered continuously at the same doses as above, and capecitabine 1.0 g/m^2 twice daily p.o. (equivalent of a total dose of 2.0 g/m^2/day) continuously throughout the study with breaks every 2 weeks for 1 week. Treatment was continued until disease progression or for a maximum of 6 weeks after confirmation of a complete response (CR). Treatment with capecitabine was interrupted in cases of grade 2 toxicity or worse and was not resumed until toxicity resolved or improved to grade 1. When treatment was resumed, capecitabine doses were reduced as follows: (1) to 0.75 g/m^2 daily for patients who experienced the first occurrence of a grade 2 toxicity or any occurrence of a grade 3 toxicity or (2) to 1 g absolute twice daily for patients who experienced a second occurrence of a grade 2 or 3 toxicity, or any occurrence of grade 3 toxicity. Treatment was discontinued if a given toxicity occurred, despite dose reduction, for a third time at grade 2 or higher grade. Rofecoxib was reduced to 12.5 mg daily in patients developing edema >grade 1 or elevated creatinine level (>115 μmol//L).

16.2.3 Evaluation of Efficacy and Safety

Response and toxicity were evaluated in patients with minimum follow-up of at least 1 month. Objective tumor response was evaluated according to WHO criteria. CR, PR and/or stable disease lasting >6 months, were reported separately as composite parameter (clinical response).

16.2.4 Pre-treatment Evaluation and Follow-Up

Baseline evaluation included medical history, physical examination and ECOG status, complete blood cell count (CBCC), serum chemistry including electrolytes, coagulation tests, tumor markers, chest x-ray, abdominal ultra- sound, computed tomographic (CT) scans of thorax and abdomen, if required for follow-up, and facultative bone scan or CT scans of brain.

During the treatment period the patients were monitored before the start of chemotherapy (after the 14 days treatment with the biomodulators), then every 3 weeks, which included the assessment of toxicities, serum chemistry including C-reactive protein (CRP) and physical examination. Assessment of the target lesions (abdominal ultrasound, chest x-ray) was performed before a chemotherapy cycle of 3 weeks. If CT scans were necessary to evaluate response these were performed in 6–12 week intervals. In long-term responders (>6 months) the assessment intervals of toxicity and response were prolonged to 2 months. The tumor marker CA19–9 was not routinely measured, because in the presence of cholestasis it does not reflect the tumor load.

16.2.5 Statistics and Data Analysis

The endpoints of the study were objective response, secondary endpoints progression-free survival, CR, PR and/or SD as composite marker and survival, as well as safety of the study medication. The safety and response analysis was restricted to patients receiving at least one cycle of chemotherapy, lasting 3 weeks. The time to event points were estimated using the method of Kaplan and Meier. Duration of response was defined as the time interval between time of objective response and the date of disease progression. Time to progression was defined as the time interval between the start of pioglitazone/rofecoxib therapy and the date of disease progression. If the event was not yet observed at the time of last record, the patient was censored at that time point. Survival time was defined as the time from initiation of treatment (intent-to treat analysis) to the date of death, or March 15, 2004, depending on which came first. To determine, whether the achievement of CR, PR and/or SD was associated with improved survival, a landmark analysis of the 21 patients evaluable for response was performed using the definition of survival time given above. Patients who have gone off study due to drug-associated side effects were estimated as treatment failure. Relative risk of progression or death was calculated by univariate analysis using Cox-regression. Fischer's exact test and t-test were used to identify significant associations between clinical and biological variables.

16.3 Results

16.3.1 Patients

The present trial included consecutive patients with advanced non-resectable intrahepatic (n = 11), extrahepatic cholangiocarcinoma (n = 7), and gallbladder carcinoma (n = 3) (Tables 16.1–16.3). Fourteen patients had non-resectable cholangiocarcinoma (n = 14) at initial diagnosis, seven patients tumor progression of hepatobiliary tract cancer following surgery, one following radiation and two following systemic chemotherapy for CC (Table 16.1). In 19 of 21 patients (90%) multiple liver metastasis were detected. Altogether 13 of 21 patients were pretreated (62%), and 18 of 21 (86%) had a non organ-confined disease. Some patients had to be treated concomitantly prior or parallel to the study medication due to accompanying cholangitis (n = 5), liver abscesses (n = 2) and/ or bile duct obstruction (n = 11). Eleven patients received stents, two an external drainage and two photodynamic therapy of the bile duct. All patients included were evaluated for response and safety of study medication.

16.3.2 Antitumor Activity

Patients enrolled on the study protocol were characterized by far advanced disease as indicated by UICC stage, ECOG status, tumor-associated symptoms and

Table 16.1 Patient baseline characteristics

	No. of patients
Age (years)	
Mean (range)	64 (48–80)
Male/female (No. of patients)	9/12
ECOG performance status (No. of patients)	
0/1	11
2	6
3	4
Prior local therapy (No. of patients)	
Gallbladder resection	2
Hemihepatectomy	2
Segment resection	3
Lymph node resection	3
Radiation	1
Stent implantation	11
Prior systemic chemotherapy (No. of patients)	2
Infections at diagnosis (No. of patients)	
Liver abscess	2
Cholangitis	3
Portal vein occlusion (No. of patients)	1
Congenital biliary cysts	1

Table 16.2 Tumor-associated symptoms at begin of study medication

Symptoms	No. of patients
Jaundice (bilirubin >3 mg/dL)	7
Pruritus	3
Abdominal pain	9
Weight loss	5
Night sweets	2
Fever	5
Hepatomegaly	11
Right upper quadrant mass	4

complications and multimode pretreatment (62%) (Tables 16.1–16.3). The tumor characteristics are listed in Table 16.3. Most patients had comorbidity due to compensated chronic organ failure (52%: liver, lung, heart). Chronic viral hepatitis was not observed, however, liver cirrhosis (Child A) from chronic alcohol abuse was present in three patients.

Despite of these unfavorable prognostic factors in an unselected patient population objective response was achieved in 29% of the cases including one cCR, stable disease in 29% (SD >6 months) (Table 16.4). The six patients with objective response were characterized by locally advanced disease. In the patient achieving CR, a histologically proven local peritoneal carcinomatosis could be confirmed prior to treatment. Besides induction of cCR (8 months+) long-term disease stabilization in PR >12 months (n = 3: 26 months +, 18 months+, 13 months+) were surprising as well as a substantial number of disease stabilisations for more than

Table 16.3 Tumor characteristics

	No. of patients
Histological subtype (No. of patients)	
Tubular adenocarcinoma	19
Papillo-tubular adenocarcinoma	2
Grading (No. of patients)	
G1	3
G2	10
G3	8
No of liver tumors	
Solitary	2
Multiple	19
UICC staging (No. of patients)	
Stage III B	2
Stage IV A	10
Stage IV B	9
Tumor size (No. of patients)	
Maximal tumor diameter	
1–4.9 cm	15
5–9.9 cm	4
>10 cm	2
Localisation of the primary tumor (No. of patients)	
Liver lobe	4
Intrahepatic	7
Hilar	5
Bile duct	2
Gallbladder	3
Metastatic sites (No. of patients)	
Bone	1
Lung	2
Peritoneal carcinomatosis	3
Lymph node involvement	12

6 months (n = 6) even in pretreated patients (n = 4). Objective responses were seen in tumors with intermediate or poor differentiation.

Response was independent of the primary tumor localization (intra- vs extrahepatic primary), P = 0.61.

16.3.3 Progression-Free Survival (PFS)

Median PFS was 6 months (95% CI, 5–7.3 months) on an intent-to-treat analysis. Retrospective analyses showed that PFS was not significantly different in pretreated patients vs those without pretreatment (surgery, chemotherapy, photodynamic therapy), P = 0.52.

Table 16.4 Histology, stage, and grading in 12 responding patients with cholangiocarcinoma

Hist. type	Loc. of tumor	Grading	Tumor size (mm)	Metast. sites	UICC Stage	Pre-treatment	Resp.	Resp. duration (months)
T	Liver	G2	80	No	IVA	–	SD	6.0
T	Duct. cysticus	G3	21	Liver	IVA	pT3, G3, R1	SD	6.2
T	Liver	G2	75	Liver	IVA	Liver abscess drainage	SD	9.0+
T	Liver	G3	76	Loc. perit.c.	IVB	–	PR	26+
T	Duct. cysticus	G3	56	Liver	IVA	Photodyn. th.	PR	13+
T	Gallbladder	G1	61	Liver	IVA	Photodyn. th.	SD	7
T	Duct. cysticus	G2	17	Liver	IVA	Liver abscess drainage pT1a, G2, R0	SD	7
T	Liver	G2	95	Liver	IVA	–	PR	4
T	Gallbladder	G2	26	Liver	IVA	Cholecystectomy, pT3, pN1	CR	8+
T	Liver	G3	92	Liver	IVA	–	PR	18+
T	Liver	G3	102	Liver	IVA	–	PR	8
T	Liver	G2	31	Liver	IVA	Hemihepatectomy resection segment I	SD	6

T = tubular adenocarcinoma, duct. cysticus = ductus cysticus of the gallbladder, Loc. perit.c. = local peritoneal carcinomatosis, photodyn. th. = photodynamic therapy, Resp. = response, Hist. type = histologic type

16.3.4 Pre-treatment with Pioglitazone and Rofecoxib

In two patients with B-symptoms (night sweats) an attenuation of these symptoms was observed. ECOG status improved in five patients. Pain release cannot be exclusively attributed to systemic antiinflammatory therapy but also to palliative drainage of the bile ducts or control of accompanying infectious complications. The same is true for the resolution of jaundice.

16.3.5 Response Characteristics

In comparison to pulsatile chemotherapy regimens objective response was delayed, mean 4.8 months (range 3–8 months) [8]. Interestingly four patients achieving PR remained stable for more than half a year (8 months, 13 months, 18 months+, 26 months +). Four of six patients with objective response had intrahepatic primaries, two extrahepatic. All patients suffered from multiple metastatic sites in the liver (Table 16.4).

16.3.6 Survival

To date 5 of 21 patients (24%) are still alive (9+, 18+, 16+, 21+, 30 months). Overall median survival was 8 months (CI 95%, 7.1–9 months) (Fig. 16.1) on an intent-to-treat analysis. Median survival for patients with CR, PR and SD >6 months was 12 months (CI 95% 2–22.2 months), and with SD <6 months or PD, 3 months (CI 95% 2–4 months), P = 0.0001, in a landmark analysis. All deaths were tumor-associated.

16.3.7 Tolerability and Safety

Of the 21 patients enrolled, nine non-responding patients (PD, SD <6 months) received 3.5 cycles (mean, range 2–5 cycles), the 12 responding patients 16.2 cycles (mean, range 8–38 cycles). The most frequent reason for treatment discontinuation was progressive disease, occurred (5%). The median dose per cycle was 76% of that planned. The median duration of capecitabine treatment was 6.8 months. Hospitalization due to grade 3 toxicity was necessary in two patients. Seven patients required symptomatic treatment. Diarrhoea was observed in two cases, stomatitis in one, HFS grade 2 in 48%, grade 3 in 9%.

Duodenal or gastric ulcers were not observed during treatment. Grade 3 or 4 abnormalities in laboratory parameters were observed in six cases. These abnormalities were not attributable to treatment but to pre-existing liver disease or tumor progression, e.g. cholestasis. Hematotoxicity was mild in one patient (grade 1).

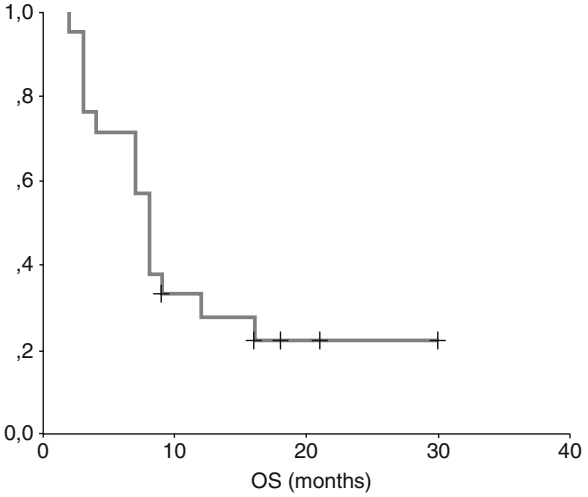

Fig. 16.1 Overall survival (OS) in 21 patients with far-advanced hepatobiliary carcinoma (intent-to-treat analysis): Median survival was 8 months (CI 95%, 7.1–9.0 months)

Table 16.5 Therapy- and tumor associated complications (WHO Grade I–IV) and response to treatment

	Hand-foot syndrome			
Patient No.	Grade 1/2	Grade 3	Tumor-/therapy-related complications[a]	Resp.
2	Cycle 3		–	SD
5	Cycle 1	–	Leukopenia, thrombopenia, grade I, cholangitits	SD
7	Cycle 4	–	–	PR
8	Cycle 5	–	Cholangitits	PR
9	Cycle 3	Cycle1	Cholangitits	SD
12	–	–	Cholangitits	SD
13	Cycle 2	–	Cholangitits	SD
14	–	Cycle 2	–	PD
15	Cycle 5	–	Edema grade 3, weight gain (3 kg)	PR
16	Cycle 3	–	Edema grade 2	CR
18	Cycle 8/9	–	–	PR
19	–	–	Cholangitis	SD
20	Cycle 9/13	–	–	PR

[a]Three further patients with edema grade I WHO with progressive disease (PD), two patients with non-cancerous ascites and PD

Other capecitabine-associated side effects, such as cardiotoxicity, were not observed. One dose reduction due to HFS was planned in the group of non-responders (PD, SD <6 months, n = 9 patients) but the patient refused to go on with a lower dose (Table 16.5). In 10 of 12 responders (CR, PR, SD >6 months, 83%) a dose

reduction of capecitabine to a final dose of twice 0.75g/m^2 per day (n = 8 pts) or twice daily 1 g absolute (n = 4 patients) was necessary between cycle 1 to 13 (mean 4.9 cycles). In 10 of 12 responding patients dose reduction due to HFS was performed before objective response has been achieved. Due to edema grade 2/3 a dose reduction of rofecoxib to 12.5 mg daily was necessary as well as accompanying therapy with diuretics in two patients. The dose of pioglitazone had not to be reduced. Cholangitis seemed not to be a therapy-related complication: The reasons were stent obstructions and in two cases tumor progression. Two patients developed non-malignant ascites due to alcoholic liver cirrhosis Child A (Table 16.5). Other side effects, such as gastrointestinal bleedings have not been observed.

16.4 Discussion

The present treatment approach combines convenience for the patient by a completely oral drug combination, tolerability of the study medication also in patients, who would have been unable to tolerate more toxic regimens, and considerable clinical benefit in the palliative care of non-selected patients with advanced CC. Hematologic toxicity was negligible and hand-foot syndrome was attenuated in comparison to the expected and reported incidence and severity during maximal tolerable dose (MTD)-guided therapy with capecitabine [8]. The median duration of capecitabine treatment in the present study was longer than reported for colon cancer [8] and the longest time of ongoing administration was 2.8 years. Comparably reduced toxicity of capecitabine may be due to the low doses being administered and to the accompanying antiinflammatory therapy with rofecoxib and pioglitazone. Thus, the favorable response and toxicity profile of the new treatment approach fits to the general patients characteristics: elderly patients with tumor-associated comorbidity.

The current study demonstrates for the first time that complete remission may be achieved with long-term intermittent low-dose chemotherapy combined with an additional angiostatic therapy approach, that PFS rates in a patient population with 62% pretreatment, poor performance status (ECOG >2, 48%) and a high rate of primary intrahepatic CCs (33%) are similar to those described for selected patients receiving second generation combination chemotherapy [9], and that multiple pretreated patients may achieve SD over long time periods.

Response rates of schedules including 5-Fluorouracil (5-FU) are ranging between 7 and 32%, those of second generation combination therapies between 22% and 35%, depending on the patients performance status and patient selection [4, 10, 11] Median PFS for combination chemotherapies are reported between 6 and 10 months [4]. Hematotoxicity of 40% seems to be considerable in docetaxel, oxaliplatin or cisplatin containing regimens [4.8].

The long-term responses to the current low-dose capecitabine schedule indicate that treatment response is not necessarily a function of the MTD but may be also achieved by a long-term administration of low doses of capecitabine including short

breaks [12]. In 83% of the patients with objective response a dose reduction to 1g or 1.5 g absolute twice daily was necessary before the achievement of objective response. Therefore, the administration of capecitabine with weekly breaks following 14 days on treatment could be equivalent to a continuous administration of low doses.

Two recently published studies identified antitumor activity of rofecoxib and pioglitazone. In angiosarcoma patients the combined treatment with the two biomodulators could induce objective response including complete remission [6]. In sarcoma and melanoma patients rofecoxib and pioglitazone were shown to modulate tumor-associated serum C-reactive protein (CRP) levels [7].

The present study cannot estimate the impact of rofecoxib and pioglitazone on outcome of CCs. In CCs serum levels of CRP are both, tumor-associated and caused by accompanying bile duct infections [13]. Therefore, CRP levels were not suitable as tumor markers for follow-up. In a retrospective analysis single-agent therapy with capecitabine has shown a poor response rate (6%). In contrast the response rate for the combined modality treatment, low-dose capecitabine plus biomodulation with a COX-2 inhibitor and a glitazone was 29% [14]. The improved response rate in a patient population with unfavourable prognostic characteristics may indicate additional activity of a presumably anti-inflammatory and angiostatic therapy. Response rates in the present study were even comparable to those in selected patient populations receiving second generation combination treatments guided by MTD [4, 9–11] Randomized trials will definitely clarify the impact of rofecoxib and pioglitazone in the treatment of advanced CCs.

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Chapter 17
C-Reactive Protein As a Secretome-Derived Biomarker for Predicting Response to Biomodulatory Therapy in Metastatic Renal Clear Cell Carcinoma

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Abstract The interaction among signaling networks of tumor and neighboring stroma cells in complex disease traits is poorly understood, and read-out parameters reflecting tumor-associated functional stages are scarce. A multi-centre phase II trial was designed to prove the hypothesis whether activation of presumably complementary receptor-triggered transcriptional cascades (via pioglitazone and interferon-a) could result in synergistic clinical effects. Therapy consisted of low-dose capecitabine 1 g/m^2 twice daily po for 14 days, every 3 weeks, day 1+, and etoricoxib 60 mg daily plus pioglitazone 60 mg daily, day 1+, and low-dose interferon-a 4.5 MU sc three times a week, week 1+, until disease progression. Fourty-five patients with renal clear cell carcinoma at a progressive disease stage and ECOG 0–2 were enrolled between March 2003 and April 2008. Forty-two percent of the patients had been systemically pretreated. Objective response was observed in 35% of the patients (PR 27%, CR 9%), which was paralleled by strong CRP decline after

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4–6 weeks' treatment. CRP values decreased from mean 42.3 mg/L, range 9.1–236, to 11.1 mg/L, range 1.1–35.6, P = 0.006. Stable disease >3 months occurred in 40%. Median overall survival and progression-free survival for the total cohort were 26.9 and 7.2 months, for CRP non-responder 13.8 and 2.6 months (95% CI, 6.5–21.1 / 0.4–4.8), and 24.4 and 11.3 months (95% CI, 22.8–31.0 / 5.7–16.9) for CRP responder, P = 0.082 / 0.017 (median observation time 26.1 months). Overall survival at 5 years was 18%. Toxicity >WHO grade 3 was reported: Hand-foot syndrome in 16 patients (36%), diarrhea in 4 patients (9%), depression in 1 patient, and pneumonia in 2 patients. (1) Clinical results of combined anti-inflammatory and angiostatic therapy were comparable with available standard therapies, although 50% of the patients had been systemically pretreated. (2) Control of tumor-associated inflammation is an important therapeutic principle in metastatic renal clear cell carcinoma.

Keywords Renal clear cell carcinoma • C-reactive protein • Secretome • Metronomic chemotherapy • Pioglitazone • Coxib • Metastatic renal cell carcinoma • Modular therapy • Systems biology antiinflammatoric agents

17.1 Introduction

Up to now, interleukin-2 has been the most active and, simultaneously, the most problematic first-line drug in inducing durable complete remission (CR) in non-resectable metastatic renal clear cell carcinoma (RCCC): Many patients are not eligible for this treatment because of expected therapy-related adverse events. For the majority of patients, the multimode targeted therapies available for RCCC are associated with a survival benefit over placebo or interferon-alpha monotherapy. The main benefit of such therapies is inducing stable disease. The drugs tie in multiple pathomechanisms, either tumor cell- or stroma cell-derived: Selected targets in RCCC are FMS-like tyrosine kinase 3 (Flt-3), mammalian target of rapamycin (mTOR), platelet-derived growth factor receptor b (PDGFRb), phosphatidylinositol 3 kinase (PI3 K), tyrosine kinase, vascular endothelial growth factor (VEGF), and vascular endothelial growth factor receptor (VEGFR) [1].

Multiple combination therapies have already been evaluated, either theme-dependently (immunomodulation, antiangiogenesis, etc.) or guided by 'historically' available 'standard' therapies. Bevacizumab showed efficacy in the treatment of RCCC when added to IFN-a [1].

Similar to these methodological approaches, future treatment strategies for advanced RCCC will probably incorporate a combination of molecular approaches, using multi-drug regimens consisting of small-molecule kinase inhibitors with biologic therapies or immunomodulatory therapies, or both.

We advanced theme-dependent therapy approaches in RCCC to biomodulatory therapies, which are adjusted to evolutionary evolving systems stages, i.e. to the spin-off of tumor-associated inflammation in the metastatic stage of RCCC. Thus,

RCCC-associated inflammation represents a therapy-relevant target for biomodulatory therapy approaches aimed at achieving objective tumor response in the range of modest toxicity [2].

Biomodulatory therapies are characterized by poor or no monoactivity of single combined drugs. However, concerted single drugs may finally alter the denotation of tumor-associated inflammatory processes by therapeutically focusing on the validity of systems features promoting tumor growth. Attenuation of tumor-associated inflammation in RCCC, as indicated by declining C-reactive protein (CRP) levels (>30% from baseline), is linked with objective tumor response – as shown – even with high sensitivity and specificity [2].

In a historical comparison, the addition of interferon-α to low-dose capecitabine, pioglitazone, rofecoxib, or etoricoxib highlighted the impact of distinct biomodulary acting combination therapies on inflammation control for improving survival: The regimen without interferon may attenuate inflammation but did not have the capacity to induce objective tumor response [2].

In an amendment approved by the local ethic committee, the study on capecitabine, pioglitazone, and etoricoxib plus low-dose interferon-α was extended because of the fact that long-term complete remissions had been observed in non-resectable metastatic RCCCs. Here, we report on 45 patients with metastatic, non-resectable, and partially systemically pre-treated RCCC.

17.2 Patients and Methods

Centers participating in the trial were the Department of Hematology and Oncology and the Department of Urology at the University Hospital Regensburg and the Departments of Hematology and Oncology at the Hospitals Fuerth and Passau.

17.3 Eligibility

The local ethics committee approved the study protocol, and patients needed to provide written informed consent before enrolment. Eligible patients were required to have progressive metastatic (according to **R**esponse **E**valuation **C**riteria in **S**olid **T**umors (RECIST) requirements) and locally recurrent or contra-lateral non-resectable RCCC. If nephrectomy was not indicated because of non-operability, clear cell histology was confirmed at a metastatic site. Patients with primarily metastatic disease underwent nephrectomy at least 21 days before initiation of treatment according to protocol. In these patients, disease progression was not a prerequisite for the start of therapy. Brain metastases were no exclusion criteria if controlled by surgery or radiotherapy prior to the start of study medication. Patients were allowed to have received an unlimited number of previous systemic therapies including chemotherapy and immunotherapy or antiangiogenic agents such as thalidomide and IFN-α,

or both (IFN-α pretreatment was no exclusion criterion because we suggested synergistic anti-inflammatory activity of pioglitazone/COX-2 inhibitor/IFN-α). Previous treatment with pioglitazone or capecitabine presented an exclusion criterion. The remaining inclusion criteria included those of the Eastern Cooperation Oncology Group (ECOG) (with the exception of serum creatinine <1.5 mg/dL).

17.4 Pre-treatment Evaluation

Apart from acquiring a medical history, baseline evaluation included a physical examination, the assessment of ECOG performance status, a complete blood cell count, serum chemistry assays, coagulation tests, a chest X-ray, abdominal ultrasound scanning, and computed tomography (CT) (scanning of the thorax and abdomen and facultative bone scanning or CT scanning of the brain if metastasis was clinically suspected). Patients were subsequently monitored before the start of chemotherapy and every 3 weeks thereafter (assessment of toxicity, serum chemistry assays, one of which measured CRP levels, and a physical examination). For patients continuing study medication, target lesions were assessed (via abdominal ultrasound or chest X-ray) before each 3-week therapy cycle. If these techniques suggested response to treatment or progressive disease, CT scans were carried out before the routinely scheduled response evaluations by CT scans in 12-week intervals.

17.5 Treatment

Patients received 1 g/m^2 oral capecitabine (Roche) administered twice daily for 14 days, every 3 weeks, from day 1+, 60 mg oral pioglitazone (Takeda), 4.5 MU IFN-α sc. (Roche) 3 times per week, from day 1+, and 60 mg oral etoricoxib or 25 mg rofecoxib (withdrawn from the market) (MSD) daily starting with day 1+. Treatment was continued until disease progression was documented or for a maximum of 6 weeks after confirmation of complete remission.

17.6 Efficacy Assessment

Response was evaluated in patients who had a follow-up duration of ≥3 weeks by the treating physicians and centrally (blinded) by the imaging unit of the University Hospital Regensburg. Response categories were assigned by means of the RECIST criteria [3]. All major responses were reconfirmed in 4–6 week intervals. Stable disease was suggested if no tumor progression occurred within 6 months of treatment. Clinical response was defined as stable disease (SD) >6 months, partial

response (PR), and complete remission (CR). Data reported represent the best response obtained during treatment according to study protocol.

17.7 Dosage Modification

Drug administration was paused for grade 2 or 3 toxicity and resumed at a reduced dosage on resolution to less than grade 2. In case of reoccurrence of dosage-limiting grade 3 or 4 toxicity, the corresponding drug was discontinued. Capecitabine therapy was continued with a 75% starting dosage for the first and 50% for the second occurrence. IFN-α administration was continued at a dose of 3 MU three times a week; COX-2 inhibitor administration at a dose of 30 mg etoricoxib every day; and pioglitazone at a reduced dose of 45 mg. According to experiences in previous phase II studies, the dosage of pioglitazone was not modified as long as a dosage reduction or discontinuation of the COX-2 inhibitor was sufficient to resolve edema or renal insufficiency to <grade 2.

17.8 Statistical Considerations

The current multicenter non-randomized phase II trial was designed to assess (1) response, (2) the qualitative and quantitative toxicity of the treatment schedules, and (3) CRP response.

The Kaplan–Meier methodology served to analyze time to progression and overall survival (OS). Overall survival and progression-free survival (PFS) was calculated from the initiation of treatment until death or until November 2009 (date of final data analysis), which ever came first. Survival analyses were done on the intent-to-treat population. Patients who died as a result of unrelated causes during therapy or who were lost to follow-up were censored.

Survival for subsets (CRP responder >30% during 4–6 weeks on treatment vs. CRP non-responder and patients with normal CRP levels at base-line) of patients was compared by means of two-sided log-rank analysis. In addition, the 'Fischer' exact and the 'Student t'-test were used to identify significant associations between chemical and biologic variables. Sensitivity and specificity of the predictivity of CRP response for clinical response were determined.

17.9 Results

17.9.1 Patients' Characteristics

In total, 45 patients (of four centers) with non-resectable metastatic RCCC were enrolled into the study between February 2003 and April 2008. Detailed patient

Table 17.1 Patients' characteristics

Parameter	Absolute	%
Age at study inclusion		
Median	63	
Range	45–76	
Sex		
Male	30	67
Female	15	33
ECOG performance status at study inclusion		
0	22	49
1	20	44
2	3	7
Nephrectomy	42	93
Surgery of metastasis	24	53
Metastatic tumors		
Lung	41	91
Lymph nodes	19	42
Bone	18	40
Liver	10	22
Adrenal gland	8	18
Contralateral kidney	5	11
Pancreas	5	11
Skin	4	9
Local relapse	3	7
Brain	2	4
Muscles	1	2
Breast	1	2
Thyreoid gland	1	2
Spleen	1	2
Peritoneal carcinosis	1	2
Histology		
Clear cell carcinoma	45	88
Histological grading		
0–3 (G0: 0; G1: 4; G2: 19; G3: 13)	36	80
Not specified	9	20
Motzer risk score		
Low (0)	15	33
Intermediate (1–2)	19	42
High (3–5)	11	24
First-line therapy	26	58
Second-line therapy	19	42

characteristics are listed in Tables 17.1 and 17.2. The age distribution corresponded to the age-related incidence of renal cell carcinoma, and a typical metastatic pattern was documented. Only a small proportion of patients had not undergone radical nephrectomy. Forty-two percent of the patients had been systemically pretreated.

Table 17.2 Patients' characteristics

Prior systemic treatment		
No	26	58
Interferon/Interleukin	3	7
Simultaneously 5-Fluorouracil and radiation	3	7
Interferon	1	2
Velbe/Interferon	1	2
Vinblastin	1	2
Vinblastin/Interferon	1	2
Sorafenib	3	2
Vindesin	1	2
Tamoxifen	1	2
Thalidomid	1	2
Sutent	2	2
Temsirolimus	1	2
Radiation prior to study	14	7
Therapy with bisphosphonates	8	18
Chemoembolisation	2	4
Radiofrequence-Thermoablation	1	2
Vaccination	1	2
Pleurodesis (Novantron)	1	2

Table 17.3 Therapy response

	Patients with RCCC (n = 45)	
Therapy response	No.	%
Complete remission (CR)	4	9
partial remission (PR)	12	27
Stable disease (SD)	18	40
Progressive disease (PD)	11	24
Therapy response (SD + PR + CR)	34	76

17.10 Treatment

All patients received at least three 3-week cycles of study medication. The median duration of study treatment was 10.5 months (95% CI, 7.2–14.7 months).

17.11 Treatment Efficacy

All 45 patients were assessable for response. At present, 11 patients are alive (24%), 3 of 4 CR patients with histologically confirmed CR, 5 patients in PR (11%) are still on treatment for 22.0+ to 58.0+, 2 patients with progressive disease are alive with alternative therapy approaches. Five patients achieving partial remissions

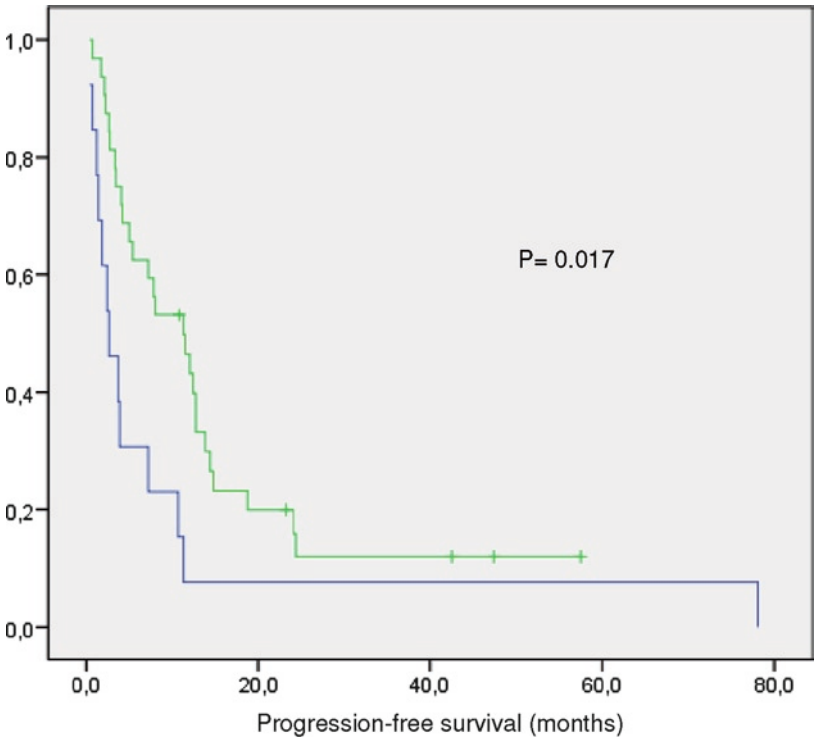

Fig. 17.1 Progression-free survival of patients with C-reactive protein (CRP) response vs. patients without elevated CRP levels or non-response (<30% CRP response during 4–6 weeks' treatment)

with only residual measurable metastatic disease in CT scans had negative positron emission tomography results, probably indicating complete remissions.

Overall clinical response (SD, PR, and CR) was 76% as detailed in Table 17.3. Objective responses were diagnosed after a median time of 4.5 months (range 2.8–8.7 months). Responses were seen at all major tumor localizations (lung, pancreas, lymph-nodes, liver, bone, and contra-lateral kidney). Metastases of patients with complete response were localized in the lung (n = 3), liver (n = 1), bone (n = 1), and in the lymph nodes (n = 4). All these patients had undergone prior tumor nephrectomy and two prior localized therapies for control of metastatic disease (chemoembolization of metastasis or surgical stabilization of a vertebra-body fracture, and radiation of further bone metastasis prior to study inclusion).

The clinical response rate of patients who had or had not received previous systemic therapy (n = 19; n = 26) was 53% and 92% respectively. Two responders received previously IFN-α.

All patients died of tumor progression (75%). After a median follow-up of 26.1 months, 12- and 24-month progression-free survival rates were 36% and 16%. 12-, 24-, and 36-month survival rates were 82%, 62%, and 36%, respectively. The

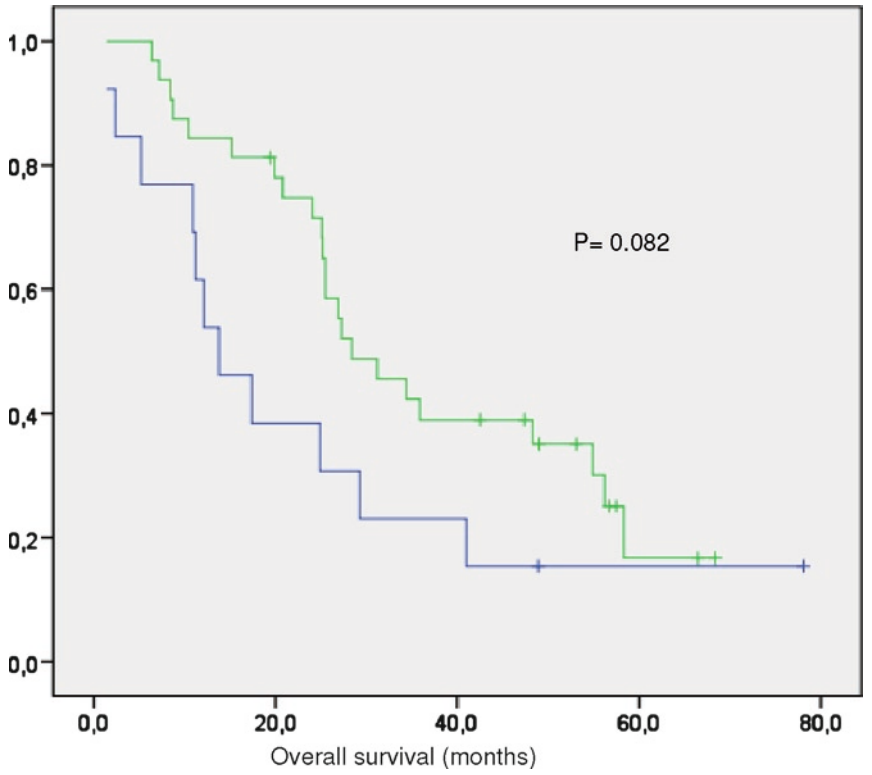

Fig. 17.2 Overall survival of patients with C-reactive protein (CRP) response vs. patients without elevated CRP levels or non-response (<30% CRP response during 4–6 weeks' treatment)

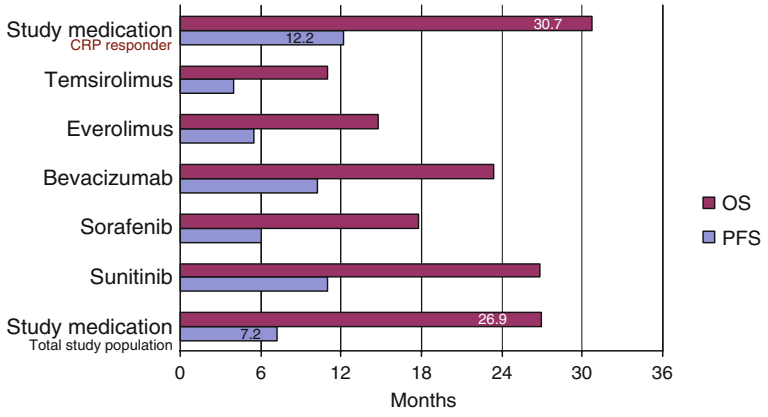

Fig. 17.3 Overall survival (OS) and progression-free survival (PFS) with targeted agents in phase III trials of advanced renal cell carcinomas in comparison with results derived from the presented biomodulatory therapy approach (Pioglitazone (Actos®), rofecoxib (Vioxx®) or etoricoxib (Arcoxia®), and Interferon-α (Roferon®) combined with low-dose metronomic Capecitabine (Xeloda®)

Table 17.4 C-reactive protein and tumor response

Number of patients (%)				
		CRP responder		
With elevated CRP levels	CRP response	Objective response (PR + CR)	Stable disease (SD)	Progressive disease (PD)
32/45 (67)	32 (100)	12 (37)	14 (44)	6 (19)

CRP response: CRP decrease >30% during 4–6 weeks on therapy

median PFS and OS rate was 7.2 months (95% CI: 3.2–11.1 months) and 26.9 months (95% CI: 22.7–31.0 months) (Figs. 17.1 and 17.2). Objective response to treatment was observed in all Motzer risk categories.

17.12 CRP Response

CRP levels were available for follow-up in all 45 patients, 32 patients (67%) had elevated CRP levels (Fig. 17.3): During therapy, CRP levels significantly decreased (>30%) in all patients with initially elevated CRP levels from mean 42.3 mg/L, range 9.1–236, to 11.1 mg/L, range 1.1–35.6 mg/L (P = 0.006). The association of CRP decline and tumor response is shown in Table 17.4. ECOG status improved in 45% of the patients with CRP response.

Explorative evaluation of CRP responder and non-responder showed significantly improved PFS (P = 0.017) and a tendency to improved overall survival (P = 0.082) for the responder group (Figs. 17.1 and 17.2). Sensitivity and specificity of CRP to predict clinical response was high at 81% and 100%.

17.13 Tolerability and Safety

The treatment regimen aimed at facilitating long-term administration of the entire study medication by a scheduled early dosage reduction in case of toxicity >grade 1. Treatment-related toxicities (>grade 2 WHO) are specified in Tables 17.5 and 17.6. Overall, the therapy regimen was well tolerated as indicated by the low number of grade 3 and 4 toxicities. Hematologic toxicity in particular was very modest.

The main toxicity was capecitabine-associated hand-foot-syndrome, which led to a dosage reduction as indicated in Table 17.6. Secondly, interferon-α dosage had to be reduced. Mild fever reactions and depression were specifically related to the additional administration of low-dose IFN-α. Fatigue after the initiation of interferon-alpha was also observed, albeit less frequently.

Table 17.5 Therapy-related toxicities > WHO grade 2

	WHO grade 3		WHO grade 4	
Therapy-associated toxicity	No.	(%)	No.	(%)
Leukopenia	2	(4)	–	–
Anemia	2	(4)	–	–
Hand-Foot-Syndrome	16	(36)	–	–
Nausea/Vometing	3	(7)	–	–
Fatigue	2	(4)	–	–
Creatinine	1	(2)	–	–
Diarrhoea	2	(4)	2	(4)
Edema	2	(4)	–	–
Infection	1	(2)	1	(2)
Pneumonia	2	(4)	–	–
Mucositis	2	(4)	1	(2)
Stomatitis	–	–	1	(2)
Heart failure	1	(2)	–	–

Table 17.6 Dose modification

	Number of patients			
Dose modification	Capecitabine	Interferon-alpha	Etoricoxib/ Rofecoxib	Pioglitazone
Twice daily 1 g absolute	29	–	–	–
Twice 4.5 Mio IE per week	–	20	–	–
30 mg or 12.5 mg/d daily	–	–	9 / 6	–
30 mg daily	–	–	–	2
Therapy breaks (<2 Wochen)	3	3	0/1	–

Edema and elevation of creatinine levels led to a dosage reduction of etoricoxib. Six patients transiently received mild diuretic therapy (weight gain, edema). Because of renal insufficiency (4/4 patients) and hypertension (1/1 patient), COX-2 inhibitors were discontinued after 3–5 treatment cycles. Dosage reduction of pioglitazone became necessary in only a few patients due to edema.

One patient with known angina pectoris experienced symptoms during study medication, which were resolved after coronary stent implantation. The dosage of one or more drugs was reduced in 64% of the patients (Tables 17.5 and 17.6). Only two patients discontinued therapy because of drug-related toxicities after 2.5 months (depression grade 3) and 6 months (hand-foot-syndrome grade 3).

17.14 Discussion

We can now provide the long-term follow-up of an extended study population treated with a biomodulatory therapy approach for non-resectable, partially systemically pretreated (42%) metastatic RCCC. The therapy regimen is characterized by the inclusion of biomodulatory acting drugs, particularly by the introduction of a combined transcriptional stimulation with interferon-alpha and pioglitazone.

Recent study results are confirmatory in every aspect:

- Combined biomodulatory treatment has the capacity to induce durable, even pathologically confirmed complete remission in metastatic RCCC.
- PFS and OS rates compare to those established in first-line treatments, although 42% of the study population had been systemically pretreated, 51% of the patients had an ECOG performance status >0, and 67% of the included patients had elevated CRP levels at base-line as a poor prognostic parameter [4–12] (Fig. 17.3).
- CRP response >30% or normalization had high sensitivity (82%) and specificity (100%) to predict clinical response (SD, PR and CR).
- Clinical responses occurred in a range of comparably low toxicity rates [1].

The origin of frequently increased serum CRP levels in RCCC is complex: CRP belongs to the secretome of malignant cells in RCCC and hepatocytes, which respond to systemic tumor-associated pro-inflammatory processes [13]. Elevated CRP levels have a negative impact on the overall survival rate in patient populations receiving surgery for primary or metastatic RCCC [4]. The present study results also show that the resolution or even the attenuation of tumor-associated inflammatory processes with non-cytotoxic biomodulatory therapies may improve PFS and, as a tendency, OS in non-resectable metastatic disease.

Besides metronomic low-dose capecitabine, the transcriptional modulators interferon-alpha and pioglitazone may be the main team players of the presented schedule. Both drugs have – similar to low-dose capecitabine – poor monoactivity at the respective dosage levels. Interferon-alpha decisively attenuates inflammation in normal volunteers, adding a decisive clinical benefit in RCCC patients. This benefit was missing in a historical control group that had not received interferon-alpha in addition to metronomic low-dose capecitabine, etoricoxib, and pioglitazone**,** although CRP response could be frequently observed in this regimen [2, 14–17]. At respective cytotoxic dose levels, the combination capecitabine (twice daily 2 g/m^2) and pegylated interferon-alpha (180 mug per week) had shown clinical activity [18].

The second point of interest is the presented therapy schedule itself. This schedule was not designed to theme-dependently interfere with more or less ‘tumor-specific’ targets, which turned out to be therapeutically relevant in the ‘general model patient’ with RCCC [1].

The activity profile of the administered drugs builds upon their ability to regulate systems functions both in tumor and adjacent stroma cells [19]. The biomodulatory activity keeps the range of toxicities modest.

The respective targets for the drugs are ubiquitously available in the tumor compartment. Concerted alteration of the holistic communicative infrastructure may be now an explanation for the observed attenuation of tumor growth or induction of complete remission. Response cannot be pinned down to suggested stereotypically available tumor-specific pathways, which is a typical explanation of the activity of small molecules or antibodies in combination therapies of contextualist design. The therapeutic handicap of these theme-dependent therapy approaches is that we presuppose distinct (pathologic) pathways as exemplarily relevant for the 'general patient' with RCCC.

In contrast to the classic multitargeted theme-dependent therapies of contextualist design, the novel generation of biomodulatory therapies may be oriented at the tumor-specific, the stage-specific, and the evolving situation-specific spin-off of systems functions, in our case tumor-associated inflammation. Translated into communicative systems terms, the validity of tumor-associated inflammation may be therapeutically 'indirectly' altered by biomodulation to change its original denotation, namely tumor promotion [20, 21]. In 18% of the patients, alterations in the intersystemic exchange processes have to be suggested, as inflammation but not tumor progression may be controlled by the study medication.

The 'indirect' attenuation of tumor growth necessitates the assumption that specific evolutionary linked functions of systems objects (proteins, pathways, cells, etc.), which are commonly featured in form of their nude identity beyond a systems context, may be redeemed in an evolutionary context by the holistic communicative tumor system.

Proteins from the tumor-associated secretome, indicating a functional tumor-associated systems status, are precious systems markers for the successful and clinically relevant modulation of particular tumor-associated systems as long as intersystemic exchange processes remain undisturbed.

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Chapter 18
Modular Therapy Approach in Metastatic Castration-Resistent Prostate Cancer

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Abstract The present multi-center phase II study was designed to support the hypothesis that networking agents which bind to ubiquitous accessible targets in metastatic castration-resistent prostate cancer (CRPC) may counteract neoplasia-specific aberrant cellular functions, thereby mediating PSA response. Patients with metastatic CRPC received low-dose chemotherapy with capecitabine 1 g twice daily plus dexamethasone 1 mg daily for 14 days every 3 weeks, COX-2 blockade with rofecoxib 25 mg (or etoricoxib 60 mg) daily combined with pioglitazone 60 mg daily, starting with day 1 + until disease progression. Thirty six patients with metastatic CRPC were enrolled; n = 18 (50%) had been extensively pretreated with radio- or radionuclide therapy, n = 16 (44%) with chemotherapies; and n = 8 patients (22%) were medically non-fit, having an ECOG-score of 0–2. Nine out of fifteen patients with PSA response >50% showed objective response. Median time to PSA response was 2.4 months (range 1.0–7.3 months). Two out of nine patients responding with PSA <4 ng/mL showed complete resolution of skeletal lesions; thirteen patients had a stable course of disease, and five patients experienced progressive disease. Median progression-free survival (PFS) was 4.0 months (2.8–5.1 months) and median overall survival (OS) 14.4 months (10.7–17.2 months). Toxicities according to WHO grade III were: Hand-foot syndrome (n = 1), hematologic toxicity (n = 7), edema (n = 1),

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Cushing syndrome (n = 1). This is the first study reporting complete resolution of skeletal lesions in CRPC by means of a biomodulatory therapy approach. The study may clinically support the above-mentioned hypothesis [27].

Keywords Castration-resistent prostate cancer • Metronomic chemotherapy • Modular therapy • Pioglitazone • Coxib

18.1 Introduction

The standard treatment for metastatic prostate cancer is androgen deprivation therapy [1]. Unfortunately, most men become resistant to hormonal manipulation. This disease stage is defined as castration-resistent prostate cancer (CRPC). Approximately 12% of patients with newly diagnosed prostate cancer (218,890 in the US in 2007) will die of metastatic CRPC [2].

Two pivotal trials of docetaxel-based chemotherapy were reported in 2004. For the first time, a survival benefit was observed for chemotherapy in CRPC [3, 4]. Thus, the results from these two studies have changed patients' expectations of treatment outcome from pure palliation to improved survival.

However, after the hormonal management of metastatic castration-sensitive PC, docetaxel-based chemotherapy represents a change to cytotoxic therapy, which may be less well-tolerated, especially by elderly co-morbid patients with limited bone marrow reserve due to preceding radiotherapy. Comprehensive recommendations for elderly patients with CRPC are still lacking [5]. Although taxanes represent the most active agents for the first-line treatment of metastatic CRPC, most patients subsequently show disease progression during taxane-based treatments [1].

Many trials now focus on improving the efficacy of docetaxel by combining it with novel agents. Several studies investigate new cytotoxic agents to define their role for the second-line treatment of CRPC [6–8]. So far, no agents have been approved for second-line therapy in CRPC. However, common practice of oncologists is to continue treatment after docetaxel failure.

Therefore, efficacious therapy approaches are required, meeting the specific clinical requirements in the therapy of CRPC: Therapies have to cope with elderly and often medically none-fit patients and with patients suffering from limited bone marrow reserve due to extended radio- or radionuclide therapy.

Angiostatic therapy approaches are now being established: Limited data are available on metronomic low-dose chemotherapy [9]. Bevacizumab (Avastin), a recombinant humanized antivascular endothelial growth factor (anti-VEGF) antibody that specifically inhibits VEGF, has shown activity in CRPC as add-on to chemotherapy [10, 11].

First promising data are now available for the combination of both angiostatic approaches in metastatic breast cancer [12]. Besides angiostatic approaches, anti-inflammatory therapy in CRPC seems to be promising, based on findings that

inflammation represents an important systems feature during tumor evolution in CRPC [13–15].

These recent observations further provide the rationale to combine angiostatic and anti-inflammatory therapy (coxib, dexamethasone, and pioglitazone) in patients with CRPC, who are either highly pre-treated or medically none-fit and are motivated to receive treatment, considering that no therapy has been approved in this setting.

Cytotoxic drugs typically produce a decline in PSA and a regression of target lesions. In contrast, agents that act to slow tumor growth, i.e. combined angiostatic and anti-inflammatory therapies, may not [16]. Instead, these agents may inhibit osteoblastic bone destruction or tumor-related angiogenesis and inflammatory processes, thereby slowing tumor progression [17, 18]. For example, a bone-directed therapy may prevent disease-related complications in the skeleton without influencing the growth of soft-tissue disease: The nuclear receptor agonist pioglitazone used in the present study may prevent the differentiation of bone marrow stem cells to osteoblasts (chapter 19) [19]. Therefore, besides the primary standard endpoint PSA response, an important secondary endpoint for non-cytotoxic drug combinations is survival and the question whether such a therapy may be efficaciously administered at modest toxicity rates for a long period of time.

18.2 Patients and Methods

The main eligibility criteria included patients with CRPC who (1) had been pre-treated either with chemotherapy (docetaxel, mitoxantrone, etoposide, or other cytotoxic drugs) or radiotherapy, (2) were none-eligible for standard chemotherapy because of co-morbidity, (3) suffered from disease progression (PSA or nodal or visceral site progression) and had a treatment-free interval from the last CT of ≥6 weeks. Bisphosphonates were permitted. Patients needed to have adequate major organ function. Written informed consent was required from all patients before enrolment into the trial (Table 18.1). The institutional ethic committee approved the protocol.

Androgen blockade had to be interrupted for at least 4 weeks with flutamide and for 6 weeks with bicalutamide, respectively. The number of prior hormonal therapies was not limited. Luteinizing hormone-releasing hormone agonist (LHRHa) treatment was continued during the study. Testosterone levels were not measured before starting study medication.

Biochemical progression was defined as >50% prostate-specific antigen (PSA) increase between two independent measurements at 2-week intervals.

Patients received capecitabine 1 g twice daily plus dexamethasone 1 mg daily for 14 days, every 3 weeks; pioglitazone 60 mg daily, rofecoxib 25 mg (or etoricoxib 60 mg) daily from day 1 until disease progression.

Early dose reductions were permitted (WHO toxicities grade I–II) to primarily facilitate long-term drug administration: Capecitabine doses were reduced to 1 g absolute twice daily in patients developing hematotoxicity grade I–II, hand-foot

Table 18.1 Main eligibility criteria

Age >18 years
ECOG 0–2
Life expectancy ≥3 months
Histological confirmed adenocarcinoma of the prostate
Castration-resistent disease
Previous chemotherapy or extensive radiotherapy (one third of hematopoietic bone marrow)
or not eligible for standard chemotherapy (medically none-fit patients)
Progressive disease after previous chemotherapy or radiotherapy
PSA increase >50% on hormonal therapy measured on two consecutive occasions, 5.0 ng/mL minimum level for entry
or objective evidence of progression on CT scan or bone scan, or both
New symptomatic bone metastases
Written informed consent signed by the patient
Absolute neutrophil count ≥1.5/nL
Hemoglobin ≥9 g/dL
Platelets ≥1,00,000
Creatinine ≤1.5 mg/dL
Transaminases <2 ULN

ECOG (Eastern Cooperative Oncology Group); PSA, prostate-specific antigen; CT, computed tomography; ULN, upper limit of normal

syndrome, or diarrhea grade I–II WHO. In case of edema or renal insufficiency (creatinine >1.5 mg/dL), rofecoxib was reduced to 12.5 mg and pioglitazone to 30 mg daily, if symptoms improved after a break of <2 weeks.

In contrast to heart failure NYHA >1, controlled hypertension and diabetes mellitus were no exclusion criteria. In case of diabetes mellitus, the pre-study medication had to be adapted to prevent hypoglycemia.

Baseline evaluation included the complete medical history and physical examination, assessment of the ECOG status, the PSA value, bone scans, and total-body computed tomography scans (CT).

Treatment was administered until disease progression (PSA or objective progression).

The primary end point was the assessment of the response rate (PSA and the objective response of every cycle and all three cycles). Secondary endpoints included toxicity, progression-free survival (PFS), and overall survival (OS).

PSA response after three cycles was critical for the continuation of the study therapy to anticipate early break-up of study medication because of flare phenomena. Major PSA response was defined as a reduction from baseline of ≥50% on two consecutive measurements taken at least 2 weeks apart. Minor response was defined as ≥25–49% PSA decrease; <25% PSA decrease up to <25% PSA increase was considered stable disease. Decline from baseline progression was defined as ≥25% increase from nadir and an increase of at least 5 ng/mL, or back to baseline, whichever was lowest, taken in two consecutive measurements at least 2 weeks apart.

Patients with measurable disease were assessed for response to therapy according to the standard Response Evaluation Criteria in Solid Tumors (RECIST) criteria, computed tomography (CT) or magnetic resonance imaging (MRI), or bone scan

every 12 weeks. In case of bone scans, outcome is reported as new lesion or no new lesion. A further new lesion in a confirmatory scan two cycles apart was estimated as progression and resolution of all lesions only in cases with skeletal involvement and PSA levels <1 ng/mL as complete elimination of disease.

We did not calculate a sample size before starting this prospective study because our patients were highly pretreated. Furthermore, we did not expect a hypothetical PSA response rate in this subset of patients.

Time to progression and overall survival were analyzed by the product-limit method (Kaplan-Meier).

18.3 Results

Between January 2003 and May 2006, 36 patients from three different institutions were enrolled. All patients were assessable for PSA response and toxicity data. Patient characteristics are shown in Table 18.2. All patients had bone disease with

Table 18.2 Patient characteristics

No. of patients	36
Age (years)	71 (66–86)
ECOG	1 (0–2)
Gleason score	7 (4–9)
≤7	11
>7	25
PSA, ng/mL (range)	308 (14–2313)
Previous local therapy	
Surgery	23
Radiotherapy (prostate/bone)	13/18
Previous hormonal treatment	
LHRH analoga	36
Anti-androgens	
Bicalutamide	36
Flutamide	22
Cyproterone acetate	12
Estrogen	3
Radionuclide therapy	3
Previous chemotherapy	
Docetaxel	16
Mitoxantrone	5
Etoposide	2
Cyclophosphamide	1
Estramustine	4
Platinum compounds	1
Previous bisphosphonates	36

ECOG (Eastern Cooperative Oncology Group); PSA, prostate-specific antigen; LHRHa, Luteinizing hormone-releasing hormone agonist. Data are expressed as median (range)

or without nodal disease or evidence for visceral spread, or both. Distributions of metastatic sites in the total patient cohort were similar to those observed in large phase III trials [3, 4].

Docetaxel: 70 mg/ m^2 every 3 weeks plus prednisone (5 mg twice daily); mitoxantrone: 12 mg/ m^2 every 3 weeks plus prednisone (5 mg twice daily); etoposide: orally 50 mg daily 1; cyclophosphamide: orally 50 mg daily; cisplatin: 50 mg/ m^2 every 3 weeks.

Extended radiotherapy = at least one third of blood-generating bone marrow in 13 patients.

Each patient had received several hormonal therapies for metastatic disease. Sixteen patients with preceding chemotherapy (n = 16) had already received at mean 2.1 (range 1–5) chemotherapy regimens for CRPC. The median number of previous chemotherapy cycles was 9 (range 2–17 cycles). 29 patients (81%) had previously received extensive radiotherapy or chemotherapy, or both.

All patients showed increasing PSA levels, and 13 patients (36%) measurable progression. Fourteen out of sixteen patients who had received docetaxel (70 mg/ m^2 every 3 weeks) plus prednisone as first-line therapy were treated with second- to fourth-line regimens before study inclusion. Two patients were medically non-fit and therefore not eligible for standard first-line therapy with docetaxel. Six other patients were medically non-fit and had previously received extensive radiotherapy. Twelve patients did not qualify for docetaxel treatment because of preceding extensive radio- or radionuclide therapy, or both. Metastatic sites were bones (multiple bone lesions), liver, lung, and lymph nodes (Table 18.3). All patients stopped previous chemotherapy for 6 weeks and showed a PSA increase of >50%.

Eighty-six percent of patients had a good ECOG performance status (0–1), 14% had ECOG 2. Impaired bone marrow function (chemotherapy, extended radiotherapy) associated with bicytopenia was frequently observed (47%) at study inclusion. 26% of patients had a previous history of controlled hypertension (28%) and diabetes mellitus (19%). 2 patients (6%) suffered from cancer-related disseminated intravascular coagulation (DIC).

Two hundred and sixty- seven cycles of capecitabine, pioglitazone, rofecoxib, and dexamethasone were administered (mean 7.4, range 2–57). Dose reductions, all

Table 18.3 Clinical manifestations of progressive castration-resistent prostate cancer

Manifestation	N = 36 (%)
Rising PSA	100
Bone	100
Substantive pain	28
Soft-tissue lesions	8
Lung/liver	16
Lymph nodes	24
Prostate/prostate bed	2
Meningeal involvement	2

PSA, prostate-specific antigen

drugs included, were necessary in 89% of patients: Capecitabine to daily 1.5 g absolute (86%), rofecoxib to 12.5 mg (39%), pioglitazone to 45 mg (28%), and dexamethasone to 0.5 mg (53%). The schedule of capecitabine resulted in a mean dose intensity of 0.5 g/m^2 twice daily or 55% of the planned dose intensity.

Capecitabine was reduced because of hand-foot syndrome in 36% of 287 cycles and because of hematotoxicity grade I–II in 51%. Treatment was interrupted 21 times for less than 2 weeks (10%). Reasons for delay were hematologic toxicity (6 cycles), non-hematologic toxicity (14 cycles), and patient's request (1 cycle). Of 36 patients, 34 patients (94%) completed 2 cycles, 16 patients (44%) 4 cycles, 6 patients (17%) 6 cycles, 6 patients (17%) >10 cycles, and 2 patients (6%) >24 cycles.

18.4 Biochemical and Objective Responses

Major and minor PSA responses were observed in 15 (42%) and 3 (8%) patients, whereas stable disease and disease progression were seen in 13 (36%) and 5 patients (14%) (Table 18.4). Overall PSA decline of >25% (including major and minor responses) occurred in 50% of patients. Median time to PSA response was 2.4 months (range 1.0–7.0 months). Two patients showed complete resolution of bone lesions in confirmatory bone scans and declining PSA levels of <1 ng/mL. Flare-up phenomena with up to 1.8 fold PSA increase occurred in 47% of PSA responders within the first two cycles.

Fifteen major PSA responses were observed in patients without previous response to docetaxel and consecutive chemotherapy (n = 4) or extensive radiotherapy (n = 7), and in medically none-fit patients (n = 4). Two patients who had previously received extended radiotherapy showed resolution of skeletal involve-

Table 18.4 Responses and survival rates according to follow-up

Response	No. of patients (%)
Biochemical (36 evaluable patients)	
Major response	15 (42)
Minor response	3 (8)
Stable disease	13 (36)
Progression	5 (14)
Objective responses in 13 evaluable patients	
Resolution of bone lesions	2 (15)
Partial response	7 (54)
Stable disease	3 (23)
Progressive disease	3 (17)
Progression-free survival (95%CI)	4.0 months (2.8–5.1 months)
Median overall survival (95%CI)	14.4 months (10.7–17.2 months)

95% CI: 95% confidence interval. Response criteria as reported in patients and methods

ments. 1 patient with complete response relapsed after 27 months; the other has been relapse-free since 43 months. Major responses were independent of PSA level at study inclusion.

Objective responses (lymph nodes n = 6, lung n = 1, bone n = 2) were observed in 9 out of 15 patients with major PSA response (60%). Objective responses occurred between cycle 3 and 9, mean 4.6 months (Table 18.3). Resolution of single skeletal lesions and significant regression of activity as indicated by bone scans occurred in further 6 patients with major PSA response. As indicated, only one partial remission was observed in patients with visceral lesions. Patients with DIC were multiply pre-treated and showed early progression within the first three cycles.

18.5 PFS and Overall Survival

Median PFS was 4.0 months (95% confidence interval [95% CI], 2.8–5.1 months) with a median overall survival of 14.1 months (95%CI, 10.7–17.2). No further study medication was administered to patients after disease progression. The two patients with complete elimination of disease received the study medication beyond complete remission because of disseminated bone involvement at inclusion and the good tolerability of the study medication.

18.6 Toxicity

In general, treatment was well tolerated. No toxic deaths occurred. The most important grade III toxicities are listed in Table 18.5. Toxicity WHO grade IV did not occur because of early dose reduction according to protocol (Table 18.4).

Table 18.5 Toxicity data experienced per patient ($n = 36$)

Toxicity	Grade I–II	Grade III	Grade IV
Neutropenia	8 (22%)	0%	0%
Anemia	14 (39%)	6 (8%)	0%
Thrombocytopenia	9 (25%)	1 (3%)	0%
Edema	18 (50%)	1 (3%)	0%
Fatigue	2 (6%)	0%	0%
Nausea/vomiting	3 (8%)	0%	0%
Diarrhea	6 (17%)	0%	0%
Hand-foot syndrome	12 (33%)	1 (3%)	0%
Cushing syndrome	1 (3%)	1 (3%)	0%
Dyspnea	1 (3%)	0%	0%

18.7 Discussion

Treatment of most patients with secondary progression of CRPC, of medically non-fit patients, or of those with limited bone marrow reserve remains difficult. Adequate systemic second-line therapies meeting their clinical requirements are needed. Several regimens have been tested in this setting. Nevertheless, no second-line treatment has been approved so far [6–8].

We assessed the efficacy and safety of capecitabine, pioglitazone, rofecoxib (etoricoxib), and dexamethasone (in metronomic treatment schedules) in a group of highly pretreated (chemotherapy or radiotherapy, 81%) or medically non-fit patients with CRPC. The most interesting finding is that responses, namely resolution of metastatic skeleton lesions for more than 2 years, may occur in a pretreated group of patients with CRCP. Additionally, responses were accompanied by a modest rate of side effects. The addition of a cyclooxygenase-2 inhibitor (celecoxib) could attenuate capecitabine-related toxicity in metastatic breast cancer [20].

Our study represents the first investigation with an exclusively combined biomodulatory therapy approach in patients with CRPC, i.e. each therapy component has modest or no monoactivity [21–23]. Long-term tumor control could only be achieved by concerted biomodulatory mechanisms of action [24]. Schedules including metronomic drug administration for treatment of CRPC (alkylating agents or dexamethasone) have shown to be efficacious in retrospective analyses of patient cohorts [9, 25].

Single stimulatory or inhibitingly acting drugs (i.e. modulators of transcription factors) do neither exert monoactivity in the respective metastatic tumor type (capecitabine, pioglitazone, rofecoxib) nor are they directed at potentially 'tumor-specific' targets [24]. Reductionist considerations may therefore not explain how multimodal, less toxic systems-directed therapies are able to induce frequently delayed objective responses and even continuous complete remission. Communication-technical considerations will be helpful to uncover mechanisms of action of modularly designed therapy approaches and to conceptualize how this novel way of treatment modulates sub-cellular and cellular communication [24, 26].

The most impressing activity of the presented schedule was found in skeletal lesions: Pioglitazone may decisively impact stromal tumor components, for example by inhibiting osteoblast differentiation besides direct activity on tumor cells (chapter 19) [19]. Alkaline phosphates (AP) levels in serum were not systematically evaluated. However, rapidly declining AP levels have been observed in single patients prior to PSA response. Alkaline phosphates of the bone are produced by osteoblasts.

The modular designed therapy approach may still be efficacious in unfavorable clinical situations: Major PSA responses were observed in patients without previous response to docetaxel and consecutive chemotherapy or extensive radiotherapy, and medically non-fit patients as well.

In light of the lack of approved second-line therapies, the retreatment of patients with docetaxel after a variable period of time is widely accepted in current clinical practice. Therefore, the fact that the presented combined biomodulatory therapy approach may induce major responses in docetaxel none-responders is of high interest.

The present report highlights important key points: (1) A completely new combined modular therapy approach may induce major responses and complete resolution of skeletal lesions in CRPC. (2) Predominant responses in skeletal lesions point to a site-specific activity of the regimen: Skeletal activity is of major importance for the treatment of patients with CRPC (Table 18.3). (3) Responses may occur in patients who were compromised by previous treatments for CRPC. (4) As major responses occurred in heavily pretreated or medically non-fit patients, the observed PSA decline is encouraging, especially against the background of a modest toxicity profile even during long-term administration of study medication.

The findings of the present paper are noteworthy because they clearly demonstrate that the combination of capecitabine, pioglitazone, rofecoxib, and dexamethasone deserves further assessment. In this respect, a randomized phase II study comparing docetaxel alone versus the combination in this set of patients is warranted.

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Chapter 19
Systems-Directed Therapy in Metastatic Castration-Resistent Prostate Cancer (CRCP)

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Prostate cancer, the most frequently diagnosed neoplasia in men, represents a therapeutic challenge throughout all disease stages. Initial therapy of metastatic prostate cancer consists of androgen ablation, either by drugs (LHRH agonists) or by surgery (bilateral orchiectomy), and responses can be observed in up to 85% of patients.

However, androgen ablation is not curative, and the disease tends to recur in many patients. At this stage, further hormonal manipulation with anti-androgens and consecutive androgen withdrawal may result in response, but mostly only for a short period of time and without prolongation of survival. Novel approaches for more efficacious castration by drugs are currently investigated and hopefully available in the near future.

Therapeutic options for castration-refractory prostate cancer are limited [1]. Tannock et al. could demonstrate a survival benefit of taxotere administered three times per week. Median survival for this standard therapy in a long-term follow-up is 19.2 months [1].

Prostate cancer is a molecular-genetically and cytogenetically heterogeneous disease. During tumor progression, more and more chromosomal or molecular-genetic aberrations are acquired. The mechanisms leading to androgen resistance are still unclear. In future, molecular-genetic, frequently recurrent aberrations may serve as novel therapeutic targets. The occurrence of advanced stage osteoplastic bone disease (chapter 5) is even more frequent, and pro-inflammatory and pro-angiogenetic processes are always present during metastatic tumor progression.

Therefore, novel therapy concepts including frequently recurrent tumor-associated pathomechanisms should be implemented into the therapeutic calculus.

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In a reductionist design, multiple modes of therapy are basically available to target tumor- and stroma-associated processes contributing to tumor progression. However, a newly developed therapy approach – as pursued in our concept – is based upon altering tumor-promoting functions in such a way that these functions cease to sufficiently support tumor growth and finally break down the tumor system as a holistic functional unit.

Cellular functions of the tumor compartment may then be modified with biomodulatory therapy approaches [2–4], which means that these therapies are active even in the range of modest toxicities (no maximum tolerable doses).

Modularity – as an intrinsic feature of proteins – may be targeted to therapeutically modify tumor growth-promoting functions [3]. Thereby, cellular proteins serving as carriers and mediators of tumor-associated functions are transferred to novel functions by altering their molecular and cellular context [2,5]. Proteins get novel denotations by therapy-induced modifications of general conditions ('background knowledge'). These variable but calculable conditions may determine the specific functions of proteins in the first place. Transcription factors in particular may capture opposing functions by context-dependently redeeming novel validity and denotation (Fig. 19.1).

A further important but frequently disregarded aim is targeting tumor-inherent rationalization processes [3]: Tumor growth-promoting functions are constituted in a tumor- and stage-specific way. This phenomenon leads to inconsistencies between the functional world of single cell compartments within a tumor. The inevitably developing functional demands, which are made up by the evolving tumor system, are directed to single tumor-associated cell compartments. The development of inconsistencies constitutes the Achilles' heel of a tumor.

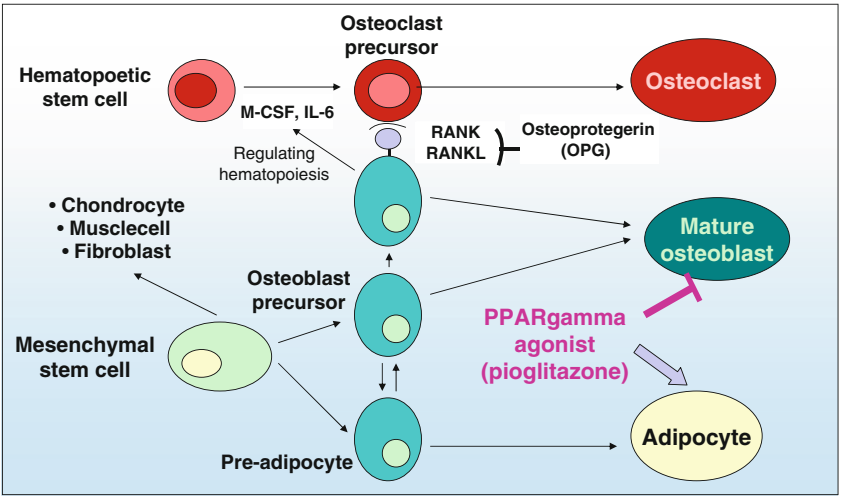

Fig. 19.1 The peroxisome proliferator-activated receptor-gamma (PPARgamma) agonist pioglitazone may significantly suppress differentiation to mature osteoblasts. Osteoblasts as mesenchymal-derived cells generally play a decisive role for promoting malignant behavior

Here, we present a novel all-oral biomodulatory therapy, which is characterized by the limited mono-activity of the single components in CRPC. Primary aim of this phase II trial was the rate of PSA response in CRPC.

Patients with confirmed CRPC and confirmed tumor progression after androgen withdrawal were included in the present phase II trial. Patients had to fulfill the criteria for CRPC according to the EAU guidelines and must not have received prior chemotherapy. Patients had daily administrations of imatinib (400 mg once daily), pioglitazone (60 mg once daily), etoricoxib (60 mg once daily), treosulfan (250 mg twice daily) and dexamethasone (1 mg once daily). Patients were treated for 6 months or until tumor progression. PSA values, ECOG status, and quality of life were continuously monitored during the study.

This interims report from two study centers (meanwhile closed phase II study) focuses on response behavior (PSA response) and the response of bone lesions in bone scans.

PSA levels decreased to <1 ng/mL (five patients) and <4 ng/mL (one patient) respectively, independent of initial PSA levels and the velocity of PSA response. PSA response was associated with complete resolution of bone lesions in two patients. Significant abatement of skeletal lesions was observed in four patients (Figs. 19.2 and 19.3). Interestingly, responses may continue for over 1 year without medication. Five patients were treated for over 21 months. Remarkably, PSA doubling time of <2.4 months before enrolment into the study protocol was very short.

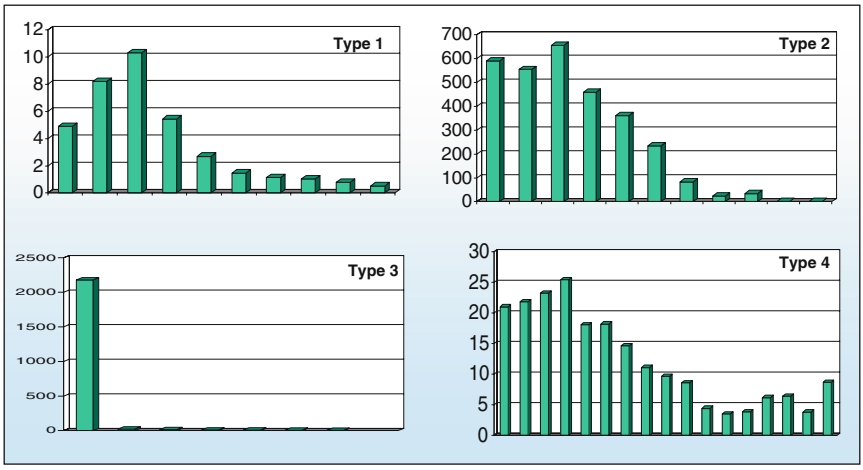

Fig. 19.2 **Type 1**: Increase of PSA levels during the first two cycles of study medication, consecutively steadily decreasing PSA levels (two patients). **Type 2**: Stable PSA levels during the first two cycles of study medication, followed by continuously decreasing PSA levels (two patients). **Type 3**: Dramatic PSA decrease within the first 2 months of study medication with PSA nadir of 0.7 ng/mL after 12 months. **Type 4**: Slightly increasing PSA levels during the first three cycles on study medication followed by a slow but continuous PSA decrease to 3 ng/mL: PSA level doubled after 12 months

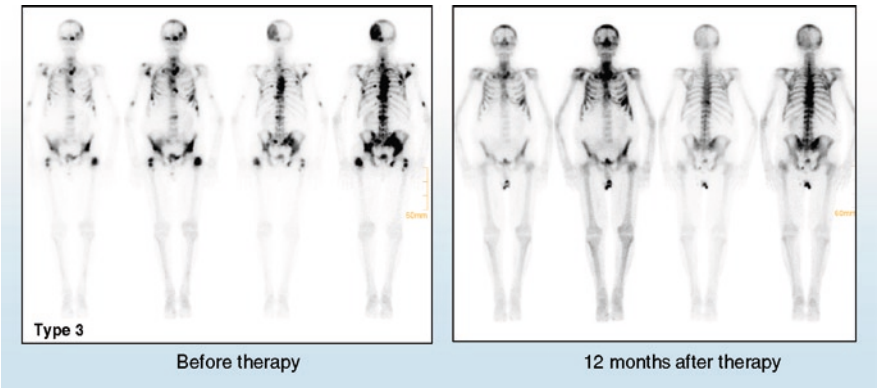

Fig. 19.3 Nearly complete resolution of skeletal lesions and meningeal involvement in an 80-year-old man with CRPC

Even with the low number of patients evaluated, the study results show that biomodulatory therapy may induce responses, very rapid as well as independently of the initial tumor spread (tumor mass). These findings indicate that biomodulatory therapies may really target the Achilles' heel of CRPC or, alternatively, may stably modulate tumor growth-supporting functions over longer time periods in such a way that finally objective tumor response can be achieved, even if delayed. An already completed study, also based on a biomodulatory therapy approach, seem to be confirmed by the present study [2]. The question whether the quality of response in the present study is prognostically relevant (PSA response versus objective tumor response) needs to be investigated.

A randomized phase II trial versus taxotere and prednisone shall start soon.

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Part VI
Criteria for Checking Systems Behavior and Creating Predictions: Systems-Associated Biomarkers and Molecular Imaging

Chapter 20
Early Detection of Systems Response: Molecular and Functional Imaging of Angiogenesis

Fabian Kiessling and Wiltrud Lederle

Abstract Non invasive imaging plays a crucial role in monitoring the efficacy of tumor therapy in the clinics. In addition, it has also been established in preclinical research and can favorably bridge from preclinical research to the clinics. However, up to now clinical imaging is mostly morphologic and does not meet the demands for innovative molecular and personalized therapy concepts. In order to become more disease and therapy specific, functional and molecular imaging strategies are of general interest. In this context, imaging of tumor angiogenesis as a general phenomenon of most tumors and as an important target for tumor therapy is an attractive approach.

This chapter reports on current strategies to assess functional parameters of vascularization (e.g. relative blood volume, perfusion, vessel permeability) as well as molecular vascular profiles by non invasive imaging. Hereby, CT, MRI, PET, optical imaging and ultrasound are covered. It is also reported how these tools can be used to assess tumor response to therapy and which role they may play in preclinical research and clinical use.

Keywords Molecular imaging • Functional imaging • Perfusion • Therapy monitoring • Angiogenesis • Personalized medicine

Abbreviations

A	Amplitude
BOLD	Blood oxygenation level dependent
CT	Computed tomography
CLIO	Cross linked iron oxide particle
[64]Cu-ATMS	[64]Cu-allyltrimethylsilane

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DCE CT	Dynamic contrast enhanced computed tomography
DCE MRI	Dynamic contrast enhanced magnetic resonance imaging
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
[18F]FAZA	[18F]-fluoroazomycin arabinoside
[18F]FDG	[^{18}F]fluoro-desoxy-glucose
FGF-2	Fibroblast growth factor-2
[18F]FLT	3′Deoxy-3′-[18F]fluorothymidine
[18]F-MISO	[18F]-Fluoromisonidazole
Gd-DTPA	Gadolinium-Diethylenetriaminepentaacetate
ICAM-1	Inter-cellular adhesion molecule 1
k_{ep}	Uptake rate constant (extravascular space per unit volume)
K_{trans}	Volume transfer constant
MION	Monocristalline iron oxide nanoparticle
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MT1-MMP	Membrane type-1 matrix metalloproteinase
NIRF	Near-infrared fluorescence
OI	Optical imaging
PFC	Perfluorocarbon emulsion
PET	Positron emission tomography
QD	Quantum dot
SCC	Squamous cell carcinoma
SPECT	Single photon emission computed tomography
SPIO	Superparamagnetic iron oxide nanoparticle
SU11248	Sunitinib malate
TGF-ß	Transforming growth factor beta
T1w	T1 weighted
USPIO	Ultrasmall superparamagnetic iron oxide nanoparticle
US	Ultrasound
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2
v_{ep}	Extracellular volume fraction

20.1 Introduction

Up to now monitoring of tumor size and morphology is standard for staging and therapy monitoring of cancer. However during recent years it has become clear that tumor load does not always correlate with patient survival and that the change in tumor size can appear too late to allow for adapting the therapy. As a consequence morphologic imaging with computed tomography (CT) and magnetic resonance imaging (MRI) is more and more supplemented by positron emission tomography (PET) providing deeper insight into the tissue pathophysiology such as metabolism ([18F]FDG-PET) or proliferation ([18F]FLT-PET). Novel tracers capable of

detecting apoptosis or tumor hypoxia ([18F]FAZA, [18]F-MISO, [64]Cu-ATMS) are currently in evaluation in preclinical and clinical studies. However, their value for monitoring therapy efficacy is not finally clear. Also tracers targeting markers of tumor angiogenesis are currently being investigated, which does not only hold true for PET imaging but also for other nuclear medicine imaging modalities as well as for MRI, ultrasound (US) and optical imaging (OI). Beside these strategies for molecular imaging, the vascularization and angiogenic activity of tumors can also be described by indirect measures, such as relative blood volume, perfusion, vessel permeability and vessel maturation. These parameters have been investigated in many preclinical and clinical studies and their capability of indicating early tumor therapy response is well proven. All of the upper mentioned imaging modalities can be used for functional imaging of tumor vascularization and even CT as one of the most important workhorses in clinical routine has shown promising results.

Nevertheless, when looking to the recent 10–15 years of research only few functional and molecular imaging methods for the assessment of angiogenesis have been established in the clinics (e.g. for breast and prostate tumor detection and characterization). Clinical indications are mostly the detection of tumors and the increase in the accuracy of the diagnosis but not the assessment of tumor response to therapy. This is contrasted by the increasing use of these techniques in academic and industrial preclinical research, where leading pharmaceutical companies have built up large small animal imaging units.

There are several reasons that may explain the retarded clinical establishment: First, in order to see changes in tumor angiogenesis patients must be imaged before starting the therapy because vascularization of most tumors is too variable to set up a predefined baseline. In addition, repeated imaging in fixed time intervals requires a well organized, time consuming and cost intensive patient management. Only specialized comprehensive cancer centers are usually capable of initiating and performing such complex interdisciplinary clinical trials. Second, personalized therapy schedules with the option of an early change in the therapeutic conduct are not broadly established and thus the knowledge about early response to therapy may often remain without therapeutic consequences. A diagnostic marker, however, without direct influence on the therapeutic conduct will not be used. Nevertheless, it can be expected that this will change with the increasing use of cost intensive molecular therapeutics (e.g. antibodies against VEGF or growth factors). Third, there is a high heterogeneity between the institutions regarding the imaging and postprocessing protocols and thus, study results are often not comparable. Fourth, the market for new and in particular for targeted diagnostic drugs is significantly smaller than for therapeutics and the demands on safety and effectiveness are comparable or even higher. Thus high development costs are opposed by a considerably small outlet. This makes pharmaceutical companies hesitate to bring new diagnostic probes to the clinics.

After all these arguments the question rises if in future the assessment of system's response to therapy will be performed using surrogate markers of tumor angiogenesis. The authors believe "yes". However, there is much need for research on finding the most reliable and cost effective biomarkers and imaging modalities. For this purpose multicentre studies and a more standardized evaluation of these markers are required. Furthermore, there should be a co-development of new diagnostic

biomarkers and therapeutics in preclinical research with the aim of subsequently translating integrated therapy and monitoring concepts into the clinics. Hereby, imaging of angiogenesis will become a powerful tool to personalize treatment and to improve the efficacy of new therapeutic concepts.

In the following it will be explained how angiogenesis can be assessed non invasively and how anti-angiogenic treatments can be monitored. Also the advantages and the limitations of the imaging modalities and applications will be addressed which may help the reader to identify the most optimal imaging strategy for his particular demand.

20.2 Vascular Volume Fraction, Tumor Perfusion, Vessel Permeability and Vessel Maturation

In comparison to vessels in physiological tissues tumor vessels are more immature, disorganized, leaky, and characterized by significant shunt perfusion. The immature nature of the vessels is reflected by a loose association of pericytes and smooth muscle cells with the endothelium. These characteristics lead to altered physiological parameters like blood volume, blood flow and vessel permeability that can be measured by various non-invasive imaging modalities. In the following, different imaging modalities will be introduced with respect to their sensitivity and specificity for the visualization of different parameters of the tumor vasculature.

20.2.1 PET and SPECT

Tissue blood volume can be routinely determined by [^{15}O]carbon monoxide PET. Since this radiotracer irreversibly binds to hemoglobin it can be used as a blood pool tracer [1]. Alternatively radio-labeled macromolecules such as polymers or proteins are often used as intravascular tracers to determine the relative blood volume and perfusion [1]. Besides macromolecules, tumor perfusion can be assessed by [^{15}O]water PET [1]. To this end, the uptake of [^{15}O]water in the tumor and in a tissue-feeding artery (arterial input function) is measured and analyzed using a one-compartment model. In clinical trials on primary tumors and metastases cytostatic therapy effects could reliably be detected by PET using [^{15}O]water and [^{15}O]carbon monoxide [1]. While the managing effort and the costs for these kinds of examinations are considerably high the excellent sensitivity for radiotracers and the possibility of absolute quantification (at least given for PET) are its major strengths.

20.2.2 Computed Tomography

A simple method to estimate tissue blood flow by dynamic contrast enhanced (DCE) CT during the first passage of a contrast agent has been proposed by Miles [2].

This model has been applied for characterizing blood flow in liver metastases and other tumors. In lung nodules, perfusion data obtained by perfusion CT strongly correlated with the [^{18}F]-desoxy-glucose (FDG) uptake in PET. In liver cancer, it has been shown that increased perfusion of the metastases and the adjacent liver tissue correlates with increased patient survival.

Nowadays, multislice CT scanners can acquire scans from more than 128 sections simultaneously with a high temporal resolution, thus strongly improving the quality of the measurements. However, the high x-ray doses required for high resolution DCE CT scans are still considered to be problematic.

Additional physiological tissue parameters can be obtained using more complex pharmacokinetic models for the analysis of density-time curves. In oncology, the models of Tofts [3] and Brix, [4] and modifications thereof are most frequently applied for the analysis of DCE CT and DCE MRI data. Both models are two compartment models and base on the assumption that there is a central blood compartment and an extravascular, extracellular compartment (interstitial space) with free bi-directional exchange of contrast material between both compartments. By using a measured arterial input function the quantitative determination of the relative blood volume, the blood flow (perfusion) and the surface-area permeability product can be obtained.

Simplifications of these models have been made by estimating the course of the arterial input function. Doing so, the need for imaging with very high temporal resolution (<2 s/image) decreases and imaging of more slices per time point or imaging with a better contrast to noise ratio becomes possible. However, this simplification should always be considered as a compromise since it goes along with a significantly reduced assignment of the outcome parameters to physiological measures Nevertheless, most groups still interpret the extracellular volume fraction, v_{ep} (Tofts model) and the amplitude, A (Brix model) as measures of the distribution volume (respectively correlatives of the relative blood volume) and K_{trans} (Toflts model) and k_{ep} as indicators of tissue perfusion and the surface-area permeability product.

20.2.3 Magnetic Resonance Imaging

Although MRI is less quantitative than PET, SPECT or CT, it offers several attractive applications to study tumor vacularization and vessel function in vivo with a high spatial resolution and an excellent tissue contrast. These include dynamic contrast-enhanced (DCE) MRI but also MR applications without the use of contrast agents based on the blood oxygenation level dependent (BOLD) contrast.

In T2*-weighted DCE MRI, the transient local magnetic field inhomogeneities (susceptibility effect) that arise from the passage of a short contrast media bolus through the capillary network are monitored in the tumor and the feeding artery [5]. Postprocessing of these DCE MRI scans is usually based on the indicator-dilution model. Not only the blood flow and volume can be determined by deconvolution, but also the mean transit time. However, it has to be considered that the model only provides reliable data of the relative blood volume if the contrast agent does not extravasate

during the first pass. This precondition is not given in tumors due to the high vessel permeability, resulting in an extravasation rate of up to 45% during the first pass. Nevertheless the above mentioned parameters may be used for the descriptive characterization of tumor angiogenesis and might provide a valuable basis for the classification of lesions, e.g. in liver, breast and brain [5,6]. For example in patients, low grade astrocytomas with high risk of early recurrence could be pre-selected based on their high relative blood volume and response of highly malignant brain tumors to anti-angiogenic drugs was reflected by a decrease in tissue "perfusion" and "relative blood volume".

T1w DCE MRI is the most frequently chosen approach to characterize tumor vascularization and has become part of the clinical routine to classify suspect breast lesions [5]. Direct inspection of the contrast enhancement curves is clinically used in order to characterize suspect lesions. Key criteria for the assessment are the maximum enhancement after contrast agent injection and the slope of the wash out. Usually the upslope and the downslope of the tumor signal intensity time curve are steeper and the maximum enhancement is higher. As an alternative to these descriptive parameters, signal-time courses can be analyzed quantitatively by pharmacokinetic modeling as already described for CT [3,4]. Pharmacokinetic analysis of DCE MRI data allowed an improved characterization of the tumors e.g. in breast, prostate, uterine cervix, and other organs [5]. This was also true for systemic disorders like multiple myelomas, where DCE MRI was capable of depicting and classifying the bone marrow infiltrate. T1w DCE MRI is also an excellent tool to study early response of tumors to anti-angiogenic therapy [5,6]. For example in squamous cell carcinoma xenografts measures of the contrast agent distribution volume were shown to decrease significantly earlier after start of treatment than tumor volumes do (Fig. 20.1). However, it was also shown that these parameters may re-increase as soon as the tumor starts to shrink and the remaining vessels draw closer.

K^{trans} and k_{ep} were often shown to indicate tumor response to therapy and mostly decrease during therapy. However, there are controversial results where no change or even an increase was found. Most probably this is due to differences between tumor models and treatments and due to the fact that these parameters are influenced by many factors including perfusion, vessel permeability and size of the interstitial space. Perfusion may mostly decrease but can also increase due to vessel normalization and more laminar flow conditions. The influence of vessel permeability on these parameters depends on tumor leakiness and the contrast agent used. With clinical contrast agents and without considering measured arterial input function vessel permeability can hardly be assessed. In this context the use of contrast media of higher molecular weight is recommended. Unfortunately, up to now such contrast media are only available for the preclinical use.

Prediction of anti-angiogenic treatment efficacy and effects of anti-angiogenic treatments were also sensitively imaged by a decrease in the vascular volume fraction measured by USPIO (Ultrasmall Superparamagnetic Iron Oxide Nanoparticle)-enhanced steady state MRI [7,8].

Alternatively to contrast enhanced MR methods, "Blood Oxygenation Level Dependent" (BOLD) imaging may be applied. BOLD imaging bases on MR-sequences

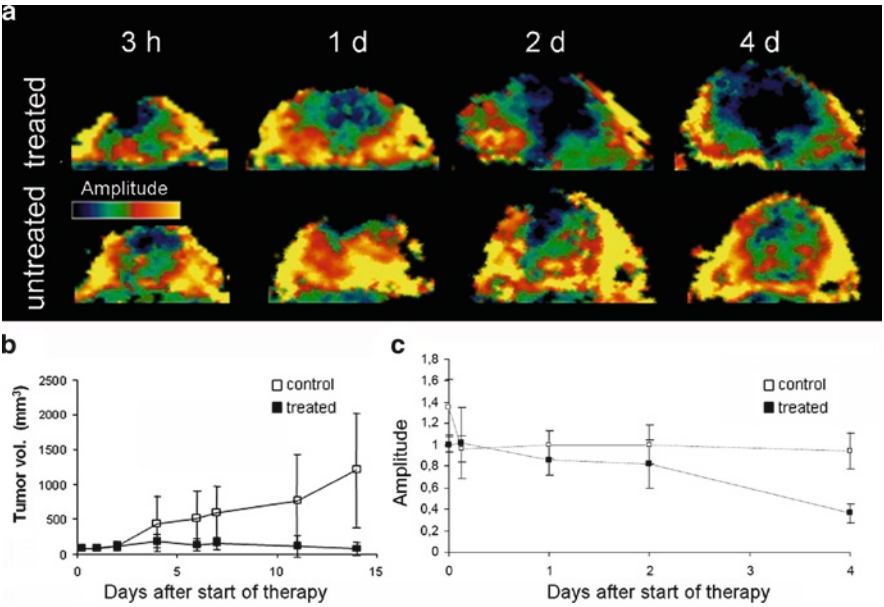

Fig. 20.1 Color coded parameter maps of the contrast agent distribution volume (Amplitude, Brix two compartment model) of an untreated (A, *upper row*) and an anti VEGFR2-antibody treated (**a**, *bottom row*) squamous cell carcinoma xenograft. While during the first 4 days of treatment sizes of the untreated and treated tumor are not significantly different, there is a significant decrease in the central vascularization in the treated tumor. The quantitative data on tumor volume changes and the change of "Amplitude" in untreated and treated mice are shown in (**b**) and (**c**) (Figure modified from [34]

that are sensitive to changes of the tissue T2* contrast. Since the oxygenation of hemoglobin reduces T2*, the signal in the tissue increases by increasing the oxygen amount in the breathed air. Using this endogenous contrast, changes in blood flow, vasodilatation and in the level of hemoglobin oxygenation can be detected. Studying the reactivity of vessels to hyperoxia and hypercapnia mature and immature vessels in tumors could be differentiated [9]. Furthermore, the effects of anti-angiogenic therapies in preclinical and clinical trials as well as early effects of a photodynamic therapy on melanoma xenografts in mice [10] were successfully monitored by BOLD.

20.2.4 Vessel Size Imaging

In 2001 Tropres developed a MRI technique capable of imaging the mean microvascular diameter in tumors [11]. Here, T2- and T2*-relaxation times of the tissue are determined before and after administration of a paramagnetic contrast agent. Since the change of T2* is static and thus mostly dependent on the relative

blood volume while the change of T2 also is influenced by the mean vessel size and number, the mean vascular diameter can be determined. Zwick and coworkers showed that in squamous cell carcinomas the degradation of small immature vessels occurring after administration of a multispecific tyrosine kinase inhibitor leads to an increase in the mean vessel size [12]. However, this trend does not seem to hold true for every tumor model and other groups recently reported on increasing mean vessel sizes after blocking VEGF signaling. Most probably significant differences in the vascular composition are responsible for these controversial results.

20.2.5 Ultrasound Imaging

Doppler ultrasound imaging allows the visualization of vessels and the estimation of blood velocity and relative blood volume without injection of contrast material. It bases on the frequency shift of an acoustic wave occurring by its reflection and scattering from a moving blood cell. If the object is moving towards the ultrasound transducer, the frequency increases and if it moves away the frequency decreases. The frequency shifts can be colour coded pixelwise and overlayed on the morphologic B-mode images to visualize the vessels and its flow direction and speed. It has been reported that Doppler ultrasound is capable of assessing changes in tumor blood flow in larger vessels after anti-angiogenic and gene therapy using clinical ultrasound systems operating between 3 and 15 MHz. However, with these frequencies the majority of tumor vessels are not captured since particularly small immature vessels being most sensitive to anti-angiogenic drugs have too slow blood velocities to be assessed. The sensitivity can be increased by increasing the ultrasound frequency and Jugold and coworkers showed the capability of high frequency ultrasound (40 MHz) to display a decrease in relative tumor blood volume after administration of the VEGFR-2 blocking antibody DC101. Unfortunately, by increasing the ultrasound frequency its tissue penetration capability decreases. Therefore, with 40 MHz transducers only superficial structures or small animals can be investigated. Alternatively, ultrasound contrast agents consisting of 1–3 μm large stabilized air bubbles may be used [13]. These can be destroyed in vivo by high energy ultrasound hereby emitting a strong non linear signal that can be measured by Doppler. Also non destructive imaging techniques may be applied which mostly catch the non linear reflections of the microbubbles.

By generating maximum intensity over time courses the relative blood volume can be determined easily. Alternatively, during a steady state microbubble concentration in the blood, a destructive pulse can be applied and the replenishment recorded. This so called intermittent imaging was initially described by Wei and colleagues and enables the quantification of perfusion and relative blood volume [14]. Meanwhile there are many papers reporting on the successful use of these techniques to monitor chemo- and radiotherapy response. In context with anti-angiogenic therapies Palmowski and coworkers observed significant differences between untreated and treated tumors in mice as early as 1 day after start of

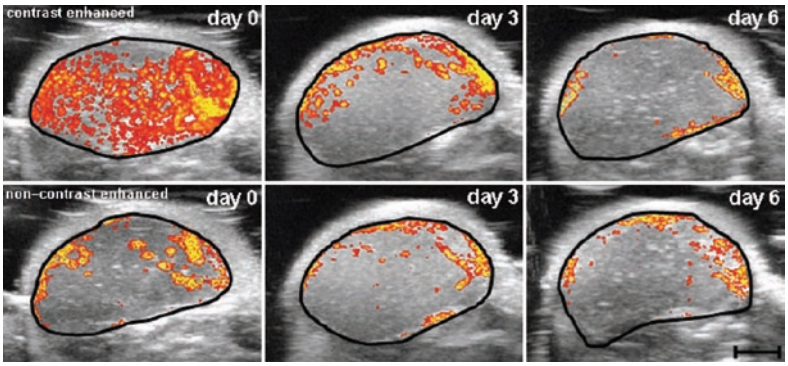

Fig. 20.2 Contrast-enhanced and non-contrast-enhanced most intensity projections of A431 squamous cell carcinoma xenografts during treatment with a multispecific tyrosine kinase inhibitor. Already after 3 days, a strong collapse of vascularization in the tumor center can be observed by contrast-enhanced imaging, indicating strong and early degradation of small immature vessels. As expectable larger and more mature vessels predominantly located at the tumor periphery and displayed by non–contrast-enhanced Doppler imaging, are less responsive. Particularly between day 3 and 6, further decrease in vascularization is only visible on the contrast enhanced image. Bar, 1 mm (Figure taken from [15])

SU11248-treatment. Nevertheless, xenograft-tumors are known to be different from human tumors and so it is not surprising that initial clinical studies on monitoring cytostatic tumor therapies with angiostatin, thalidomide and tamoxifen in patients with contrast-enhanced US revealed mixed results of decreased, unchanged or even increased vascularization in response to therapy [13].

As already mentioned non contrast enhanced Doppler ultrasound mostly catches vessels with a considerably high blood flow usually being more mature, larger and of a more linear course. In contrast, contrast enhanced ultrasound imaging catches all vessels. Thus, the more mature vessels fraction can be distinguished from the total vascularization (Fig. 20.2). By combining both ways of scanning, it could be shown that during tumor treatment with a multispecific tyrosine kinase inhibitor the total vascularization decreased over 9 days, while there was a re-increase in the “mature” vessel fraction from day 6 on indicating normalization of the vasculature [15].

20.3 Molecular Imaging

A more specific characterization of the tumor tissue can be achieved by using diagnostic probes that target specific molecules, which is determined as molecular imaging. The aim of molecular imaging is to obtain specific information for a better tumor diagnosis and for the assessment of specific therapy effects at early treatment stages. In the following, several approaches to specifically target angiogenic marker molecules by different imaging modalities are discussed.

20.3.1 (Bimodal) Molecular MRI Probes

Due to its low sensitivity for marker molecules, specific targeting of angiogenic vessels by MRI requires an intense accumulation of contrast agents at the target. However, MRI provides an excellent soft tissue contrast combined with a high spatial resolution as compared with other non invasive imaging modalities. Superparamagnetic iron oxide particles such as MION, SPIO, USPIO and CLIO coated with dextrane and its derivates or with other coating materials such as citrate and silica generate a strong negative effect on T2-weighted and T2*-weighted MR images. In this context, a non-invasive imaging approach on angiogenesis was performed by covalently coupling cross linked iron oxide particles (CLIO) to the anti-human E-selectin antibody fragment H18/7 F(ab′)2. In this study, human vascular endothelial tubules in matrigel were implanted in athymic mice and could be visualized due to upregulation of the E-selectin in response to stimulation with interleukin-1 [16].

One frequently addressed angiogenic marker is the αvβ3 integrin receptor, which is expressed on the surface of endothelial cells. It plays a crucial role for cell-cell and cell-matrix interactions and is involved in cell migration by interacting with specific signal molecules like VEGF. For targeting the αvβ3 integrin receptor, several ligands were developed including monoclonal antibodies and small peptide sequences. RGD is a small peptide that has a strong affinity for αvβ3 integrin. RGD-conjugated USPIOs have been successfully applied for the imaging of the angiogenic tumor endothelium in SCCs [5]. However, one has to consider that high amounts of the RGD-containing diagnostic probe have to accumulate in the target tissue to overcome the limited sensitivity of MRI to contrast agents. In a recent study it was shown that a diagnostically relevant dose of RGD-USPIO can induce unwanted biological side-effects in tumor cells themselves [17].

Liposomes are frequently used carriers for biologically active compounds and consist of spherical lipid bilayers with 50–1,000 nm diameter. These nanoparticles can be generated with varying size, phospholipid composition and surface characteristics. Liposomes can either be used as carrier of genes and therapeutics or can be loaded with contrast agents. Gd-DTPA loaded liposomes were coupled either with RGD or antibodies against αvβ3 integrin [5,18] for angiogenesis imaging by MRI. Using these specific probes, a heterogeneous expression of αvβ3 -integrins at the margin of experimental tumors was detected. Mulder and colleagues used αvβ3 integrin targeted bimodal liposomes to quantitate angiogenesis in a tumor mouse model with magnetic resonance imaging (MRI) and evaluated the therapeutic efficacy of the angiogenesis inhibitors anginex and endostatin. Validation of the MRI by fluorescence microscopy revealed a high correlation of the measured MRI signals with the microvessel density. Thus, this study provides evidence that molecular MRI can be used for the non-invasive assessment of anti-angiogenic therapy effects [19].

Besides αvβ3-integrins aminopeptidases are often overexpressed on the tumor endothelium and can be targeted by the cyclic tri-peptide cNGR. Imaging of aminopeptidases by MRI has been successfully applied using paramagnetic quantum

dots labeled with cNGR. The use of quantum dots also allowed the localization of the particles on tumor sections by immunofluorescence microscopy [20].

Perfluorocarbon emulsions (PFC) can be used for both, US and MRI. These emulsions consist of PFC droplets with a mean diameter of approximately 250 nm in suspension and can be labeled with different ligands at the outer surface. Like microbubbles used for US (see below), PFC emulsion droplets remain intravascular. Site directed emulsion droplets used for US imaging attach to target molecules on the cells, thereby forming a thin acoustically reflective layer between the targeted surface and the surrounding medium. Including Gd-chelates in the emulsion, these droplets can be additionally used as MRI contrast agent by generating a positive contrast. Flacke and colleagues generated emulsion droplets tagged with an anti-fibrin monoclonal antibody and used them to visualize angiogenic vessels in vulnerable plaques in vivo by MRI [21].

20.3.2 Ultrasound (US)

Contrast-enhanced ultrasound uses small gas filled microbubbles which remain strictly intravascular due to their diameter of about 0.7–10 μm. In contrast to the clinically used ultrasound contrast agents, target-specific ultrasound requires the coupling of specific ligands to the microbubble shell. Target-specific ultrasound contrast agents exist as soft- (e.g. phospholipid) and hard-shelled (e.g. polymer based) microbubbles [13]. The coupling of streptavidin to the membrane of microbubbles allows a flexible and easy labeling with biotinylated ligands. Using modern US-techniques that utilize harmonic effects and conversion pulse imaging even single microbubbles can be detected in the tissue, thus highlighting the power of molecular ultrasound.

Using target-specific microbubbles, US is capable of depicting early tumor angiogenesis [13]. Since the microbubbles strictly remain intravascular, specific targets are generally molecules that are either induced in activated endothelial cells or markedly up-regulated compared with quiescent endothelial cells. Besides normal endothelium, lymphatic endothelium can be successfully targeted using L-selectin specific microbubbles [13].

One prominent angiogenic target in ultrasound is the $\alpha_v\beta_3$ integrin. It is highly expressed on activated endothelial cells and almost absent on quiescent endothelial cells in the stable vasculature. $\alpha_v\beta_3$ integrin specific microbubbles have been either conjugated to cyclic RGD peptides or to specific antibodies and have demonstrated significant binding capacities to angiogenic endothelial cells in vitro and in vivo. Microbubbles conjugated to a cyclic RRL peptide also showed a significant accumulation in s.c. human prostate carcinoma xenografts in mice. Echistatin, a viper venom disintegrin with an RGD sequence was conjugated to microbubbles and demonstrated its potential in imaging angiogenic vessels in FGF-2 enriched matrigel plugs in mice. Additionally, echistatin conjugated microbubbles were successfully applied for $\alpha_v\beta_3$ integrin based angiogenesis imaging in a rat intracerebral glioma model.

Accumulation of the $\alpha_v\beta_3$ integrin targeted microbubbles was greatest at the periphery of tumors with the highest $\alpha_v\beta_3$ integrin expression and correlated well with tumor microvascular blood volume. These results highlighted the advantage of combining different parameters for the analysis of angiogenesis, e.g. microbubble retention with relative blood volume [13].

The most prominent angiogenic marker that is frequently used in ultrasound based angiogenesis imaging is the vascular endothelial growth factor receptor 2 (VEGFR-2) [13]. VEGFR-2 antibody coupled microbubbles showed a significantly higher accumulation in subcutaneously implanted tumors than unspecific control microbubbles. In addition, the retention of VEGFR-2 specific microbubbles was much stronger in "highly invasive metastatic" than in "non-metastatic" breast tumours, thus demonstrating the capacity of targeted ultrasound of assessing even the angiogenic activity.

Besides characterizing tumor angiogenesis and vascularization, molecular ultrasound has been identified as powerful modality for the assessment of anti-angiogenic treatment effects. For the imaging and analysis of anti-angiogenic therapy, antibodies either against the VEGF/VEGF-receptor complex were used in an orthotopic model of pancreatic cancer or antibodies against VEGFR-2 and/or antibodies against CD105 (endoglin) were applied in two subcutaneous models of pancreatic cancer [22]. Targeted microbubbles showed a significantly higher enhancement in the tumor vasculature than untargeted or control IgG–targeted microbubbles. The video intensity from targeted microbubbles correlated with the expression level of the marker molecules (CD105, VEGFR-2, or the VEGF-VEGFR complex). The decrease in video intensity correlated with a decreased microvessel density in tumors after anti-angiogenic or cytotoxic therapy.

The effects of MMP inhibition (Prinomastat) were also assessed with microbubbles against VEGFR-2 and cyclic RGD (ligand for $\alpha_v\beta_3$ integrin) [23] (Fig. 20.3). A significantly lower accumulation of target specific microbubbles was observed in treated tumors as compared with untreated ones. Histologic analysis revealed that the lower VEGFR-2 and $\alpha_v\beta_3$ integrin concentrations in treated tumors were due to a general decrease in relative vessel density. Thus, this study clearly demonstrated that only a combined analysis of relative blood volume and of molecular marker expression clarifies whether alterations in microbubble retention are based on a general change in the endothelial surface (e.g. relative blood volume) or on a marker expression change on the endothelial cells.

An alternative endothelial marker used for molecular ultrasound is CD105 (endoglin), a TGF-ß co-receptor that is over-expressed by activated endothelial cells. As already described, the potential utility of CD105 in imaging tumor development and anti-angiogenic therapy has been well-documented in s.c. and orthotopic pancreatic tumors in mice [22].

In addition, microbubbles targeted against P/E-selectin and VCAM-1 were successfully applied in subcutaneously implanted tumors [13] In a recent study, molecular ultrasound imaging was used to intraindividually track changes in the expression of the angiogenic markers $\alpha_v\beta_3$ integrin and ICAM-1 in response to carbon ion irradiation in a rat prostate cancer xenograft [24]. A higher binding of

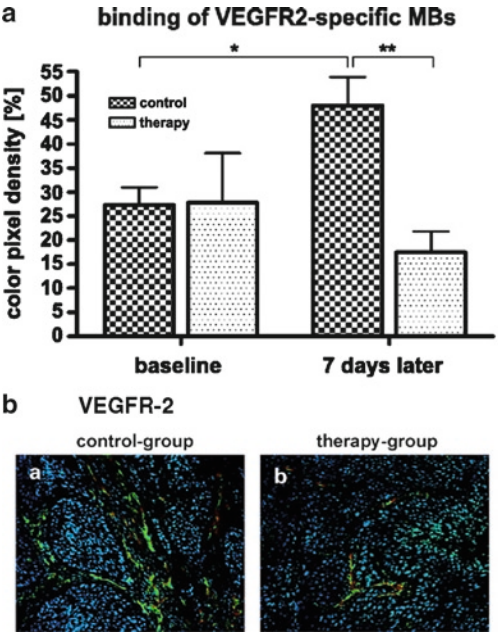

Fig. 20.3 Accumulation of VEGFR-2 specific microbubbles in tumors before and after therapy. A, before therapy, the amount of stationary VEGFR-2 specific microbubbles is similar in the control and therapy group. Note the increased accumulation of VEGFR-2 specific microbubbles in the controls after 7 days of tumor growth. * P <0.05; ** P <0.01 (n = 5 animals). B, Immunostaining of tumor sections for VEGFR-2 (green), CD31 (red), cell nuclei (Hoechst, blue). Note that VEGFR-2 and CD31 are both reduced in the therapy group (b) (Figure adapted from [23])

$\alpha_v\beta_3$ integrin and ICAM-1 specific microbubbles was observed in irradiated tumors compared to the controls. After normalization of the amount of accumulated microbubbles to the relative blood volume, differences between irradiated and control tumors became more prominent, thus indicating that carbon ion irradiation upregulated ICAM-1 and $\alpha_v\beta_3$ integrin expression in the tumor neovasculature.

20.3.3 PET and SPECT

[^{18}F]Galacto-RGD PET was applied for visualizing αvβ3 integrin on the angiogenic endothelium in mouse skin cancer xenografts and in patients with melanoma and sarcoma. Targeting $\alpha_v\beta_3$ integrin with [^{18}F]Galacto-RGD provided in most cases a higher spatial signal intensity and resolution as the analysis of the tumor metabolism by [^{18}F]FDG [25]. However, since the small [18F]Galacto-RGD can extravasate and since melanoma and sarcoma cells also express αvβ3 integrin themselves, enhancement of the contrast agent can derive from both, activated endothelial cells and tumor cells.

Additionally, membrane type-1 matrix metalloproteinase (MT1-MMP) was targeted on activated endothelial cells using liposomes linked to stearoyl-Gly-Pro-Leu-Pro-Leu-Arg (GPLPLR-Lip) [26]. An about 4-fold higher accumulation of these targeted liposomes was observed in tumor bearing mice compared to control animals.

As compared to PET, the sensitivity of SPECT for radiopharmaceuticals is about one order of magnitude lower and the quantification of the acquired emission data is more complex. On the other hand, handling SPECT tracers is less problematic due to the longer half-life of the radionuclides and it allows the use of radiotracers with different photon energy at the same time. Site directed SPECT tracers like an RGD labeled peptide with Technetium-99 m [27] or an Indium-111 labeled αvβ3 integrin targeted agent, have been generated and were used for angiogenesis imaging in preclinical and clinical trials.

Because of the wider availability of γ-cameras and SPECT scanners in the past, VEGFR imaging was achieved with SPECT earlier than with PET. Several radioisotopes, such as ^{123}I, ^{111}In, ^{99m}Tc, ^{64}Cu, and ^{89}Zr, have been used for either SPECT or PET applications [28]. ^{123}I-VEGF$_{165}$ and ^{123}I-VEGF$_{121}$ were used for VEGFR scintigraphy of primary tumors and their metastases [28]. In a clinical study on nine patients the majority of primary pancreatic carcinomas and their metastases could be visualized on ^{123}I-VEGF$_{165}$ scans.

In a recent study, bevacizumab was labeled with ^{111}In and ^{89}Zr for SPECT and PET, respectively. Nude mice with human ovarian xenograft tumors were injected with ^{89}Zr-bevacizumab, ^{111}In-bevacizumab, or ^{89}Zr-IgG. PET revealed tracer uptake in well-perfused organs up to 24 h after injection and clear tumor localization at 72 h after injection and beyond. Although the tumor uptake of ^{89}Zr-bevacizumab was higher than that of ^{89}Zr-IgG, the absolute tumor uptake (<8 %ID/g) was much lower than that of other radiolabeled antibodies reported in the literature. The higher uptake of ^{89}Zr-bevacizumab than ^{89}Zr-IgG may have been attributable to the different levels of passive targeting of individual antibodies, even though they were isotype-matched IgG. Whether the levels of VEGF expression are significantly different during different stages of tumor development, in turn leading to different levels of tumor uptake of tracers, needs to be studied [28].

Recently, VEGF$_{121}$ was labeled with ^{64}Cu for PET of VEGFR expression. Small-animal PET imaging revealed rapid, specific, and prominent uptake of ^{64}Cu-DOTA-VEGF$_{121}$ in highly vascularized small U87MG tumors with a high VEGFR-2 expression but a significantly lower and sporadic uptake in large U87MG tumors with low VEGFR-2 levels. The study demonstrated the dynamic nature of VEGFR expression during tumor progression, in that even in the same tumor model, levels of VEGFR expression were dramatically different at different sizes and stages [28].

In a follow-up study, a VEGFR-2–specific fusion protein, VEGF$_{121}$/rGel (VEGF$_{121}$ linked to recombinant plant toxin gelonin) was used to treat orthotopic glioblastoma in a mouse model. ^{64}Cu-VEGF$_{121}$/rGel PET imaging was successfully used to estimate the tumor targeting efficacy of the therapeutic substance and to define the dose intervals. That study suggested that clinical multimodality imaging and therapy with VEGF$_{121}$/rGel may provide an effective means of prospectively

identifying patients who will benefit from $VEGF_{121}$/rGel therapy and then stratify, personalize, and monitor treatment to obtain optimal survival outcomes [29].

To date, there has been only one report on SPECT of integrin $\alpha_v\beta_3$ with a nanoparticle-based tracer. ^{111}In-Labeled perfluorocarbon nanoparticles were tested for the detection of tumor angiogenesis in rabbits implanted with Vx-2 lung carcinoma tumors. At 18 h after injection, the mean tumor activity in rabbits receiving integrin $\alpha_v\beta_3$–targeted nanoparticles was about fourfold higher than that obtained with control nanoparticles [28].

20.3.4 Optical Imaging (OI)

Optical imaging technologies have the advantage of a high sensitivity for contrast agents with a high resolution. However, scattering limits the penetration of light in tissues. To display molecular targets that are located deeper in the tissue of small animals, near-infrared fluorescence (NIRF) optical imaging can be used. In the near-infrared range (650–900 nm), water and biological tissues have minimal absorbance, scattering and auto-fluorescence, allowing efficient penetration and emission of photons with a low scattering within the tissue. Analogous to other non invasive imaging modalities, in vivo NIRF imaging of angiogenesis and lymphangiogenesis can be performed using specific near infrared fluorochrome labeled contrast agents against $\alpha_v\beta_3$ integrins [30], L-selectin [31] and heparan sulfates [32]. Cheng and colleagues [30] generated Cy5.5-conjugated mono-, di-, and tetrameric RGD peptides and compared their effects on integrin avidity and tumor targeting efficacy in a subcutaneous U87MG glioblastoma xenograft model. High receptor binding affinity and receptor-mediated endocytosis was observed for all fluorescent probes. However, the tetrameric RGD peptide Cy5.5 conjugate showed the highest tumor uptake and tumor to normal tissue contrast.

The availability of activatible contrast agents makes OI a unique tool for imaging enzyme activity in vivo, including the analysis of the molecular mechanisms of angiogenesis and the non invasive assessment of therapeutic effects. Bremer and coworkers performed in-vivo imaging of MMPs activated in tumors using activatable Cy5.5 fluorescent probes. The fluorochromes were linked to an MMP substrate and fixed on a polymeric backbone, hereby quenching each other due to the close local assembly. After cleavage of the substrate by active MMPs, the fluorochromes become de-quenched, resulting in photon emission. This approach was not only very well suited for visualizing and assessing the MMP activity in fibrosarcoma and breast cancer models but also for monitoring the effects of MMP-inhibition [33] and of cytostatic drugs (Fig. 20.4).

Besides fluorochromes like Cy5.5, QDs are frequently used for optical imaging. These are inorganic fluorescent semiconductor nanoparticles that have several advantages like high quantum yields, high molar extinction coefficients, strong resistance to photobleaching and chemical degradation, continuous absorption spectra spanning the range from UV to near-infrared, narrow emission spectra

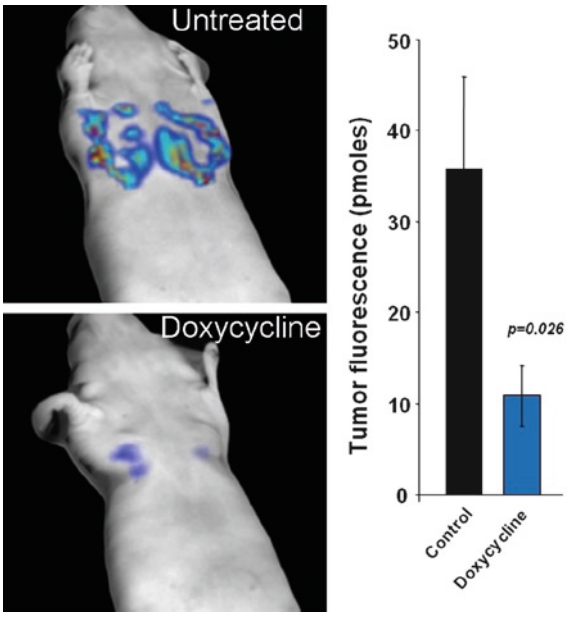

Fig. 20.4 Selective MMP detection in HT-29 colon adenocarcinoma xenografts. NU/NU mice were injected subcutaneously with HT-29 tumor cells bilaterally in the both mammary fat pads. After 1 week, mice either remained untreated or were treated with 100 μL of 10 mg/mL (1 mg/mouse) doxycyline 1× per day subcutaneously. Mice were injected with an activatable MMP probe (MMPSense750 FAST, Visen Medical) 24 h after starting the treatment and imaged 6 h later. Whereas the treatment did not reduce tumor volumes yet, MMP activity is already reduced significantly (Image kindly provided by Jeffrey D. Peterson, Visen Medical, Boston)

(typically 20–30 nm full width at half maximum), and large effective Stokes shifts Specific targeting can be achieved by attaching targeting ligands to the QD surface. NIRF imaging of integrin $\alpha_v\beta_3$ on tumor vasculature with RGD peptide–conjugated QD705 was successfully performed on the tumor vasculature in s.c. U87MG tumors. The large size of the QD705–RGD conjugates (~20 nm in diameter) prevented efficient extravasation; therefore, the QD705–RGD conjugates mainly targeted integrin $\alpha_v\beta_3$ on the tumor vasculature.

Nevertheless, the translation of QDs to clinical applications remains critical due to inefficient delivery, potential toxicity, and the lack of quantitative detectability [28].

20.4 Outlook

Already today a concise characterization of tumor vascularization can be achieved by non invasive imaging that can potentially be applied in preclinical and clinical research as well as in clinical routine. In this context, multimodal and hybrid, respectively fusion imaging is required to cover all relevant pathophysiological aspects.

Whether a combination of different surrogate markers is finally required in the clinics or whether single parameters have sufficient power to characterize cancer and to monitor therapy response is still an open question and will also depend on the chosen therapy. In particular, personalization of combination treatments of chemotherapeutic and anti-angiogenic drugs will most probably require a more complex imaging strategy to evaluate the efficacy of the individual components. In this context, it is so far unclear how a differentiation between anti-angiogenic and chemotherapy effects can be achieved since both are known to decrease functional and molecular characteristics of the vascularization, e.g. the relative blood volume.

In summary, non invasive imaging of angiogenesis is highly valuable to assess system's response to therapy early, reliably and sensitively. With increasing standardization of the measures and with making the outcome parameters more quantitative, non invasive imaging of angiogenesis can be expected to play an essential role in cancer management. Nevertheless, it must also be taken into account that particularly for the clinics such imaging strategies are cost intensive and that economic considerations may hamper its acceptance. However, in personalized combination therapies cost effectiveness can be achieved if imaging of angiogenesis helps to early identify the non response to cost intensive components (e.g. an anti-angiogenic drug), to optimize dosing and to re-arrange the treatment.

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Chapter 21
Secretome Proteomics, a Novel Tool for Biomarkers Discovery and for Guiding Biomodulatory Therapy Approaches

Verena Paulitschke, Rainer Kunstfeld, and Christopher Gerner

Abstract Secretome analysis represents a novel technology for biomarker discovery based on proteome profiling of proteins secreted by both primary tumor cells and tumor associated cells. Tumor cells are able to establish a permissive and supportive environment for survival and cell growth and to facilitate invasion and metastasis by modulating the stromal host compartment. The onset of these characteristic events seems to precede tumor progression. Due to the leaky nature of newly formed blood vessels and the increased hydrostatic pressure within tumors, secreted proteins are most plausibly shed into the blood. Thus, proteins specifically secreted by these cells may serve as early disease biomarkers. Biomarker candidates identified by secretome proteomics combined with the application of appropriate bioinformatic tools can then be validated in human plasma/sera. Besides biomarker discovery secretome analysis will also shed light on mechanisms of tumor progression offering novel targets for therapeutic intervention. The tumor-stroma cell cooperativity is reversible and may thus be directly accessible to therapeutic intervention. In conclusion, secretome proteomics offers new insights into the pathophysiology of tumor progression, and allows the identification of novel biomarkers and of new drug targets.

Keywords Tumor-associated stroma cells • Secretome profiling • Biomodulatory therapy • Mass spectrometry • Primary human cells • Bioinformatics

Abbreviations

AFP	Alpha-fetoprotein
CAF	Cancer associated fibroblast
CEA	Carcinoembryonic antigen

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CPL/MUW – database	Database of the Clinical Proteomics Laboratories at the Medical University of Vienna
DIGE	Differential in-gel electrophoresis
Gpm	Global proteome machine organisation
ICAT	Isotope coded affinity tag
LMW	Low-molecular-weight
MIAPE	Minimum information about a proteomics experiment
PRIDE	PRoteomics IDEntifications database
PSA	Prostate specific antigen
ROC	Receiver operating characteristic
SILAC	Stable isotope labeling by amino acids in cell culture
SOP	Standard operating procedure
SVM	Support vector machines
TIF	Tissue interstitial fluid

21.1 Introduction

21.1.1 The Proteome

The proteome, first defined by Williams in 1996 [1], is the protein complement of genomic functionality and is defined as the set of proteins which are present in a cell, tissue or organism. The proteome is highly dynamic and may respond to almost any kind of environmental stimuli, most obviously it varies according to cell type and functional state of cells. The proteome in a body fluid, cell, tissue, or organism represents only a subset of all possible gene products at a certain point of time and cannot be directly predicted from gene expression. Proteins may exist in multiple varieties due to posttranslational modifications which affect protein structure, localization, function and turnover. These specific changes may reflect immediate and characteristic changes in response to disease processes. Especially the low-molecular-weight (LMW) range proteome is believed to be very useful for analysis of disease progression and response to treatment [2].

21.1.2 Clinical Proteomics

The goal of clinical proteomics is to obtain the most comprehensive insight into pathophysiological conditions derived from protein expression profiles as they occur in vivo. Proteins play a fundamental role in controlling multiple functions within a cell's organization. They serve as building materials, enzymes and biological transport machines, as well as sensors processing and transferring information. Cells consist of thousands of proteins executing diverse operations, not only highly

coordinated, but also dependent upon each other. Cells may newly produce specific proteins when they encounter challenges for specific functions. When cells encounter unusual situations, they try to adjust to it by expressing proteins which may help to deal with the new situation. Such proteins, specifically synthesized on demand, may indicate characteristic disease states and may thus serve as diagnostic markers. Detection of such aberrations in protein expression in diseased tissues may lead to a better understanding of the cellular pathology and thereby support the development of new therapeutic strategies. Therefore, proteins have attracted attention to biomarker discovery: One of the central applications of proteomics has become the classic protein biomarker discovery and the uncovering of functional tumor-associated systems stages, e.g. inflammation, neoangiogenesis, proliferation behaviour and others.

Clinical proteomics focuses on the analytical and clinical implementation and validation of novel biomarkers and aims to gain a better understanding of disease processes which may support the implementation of novel treatment options. Therefore it is critically dependent on high-throughput analysis platforms which have to provide reproducible and reliable protein patterns, bioinformatics tools for data comprehension and interpretation. Furthermore it has to refer to a well-defined patient cohort including all necessary anamnestic and physiologic parameters for instance age, sex, hormonal status and treatment. Sample collection and biobank organization have to be SOP-driven. The samples should be rapidly analyzed since transportation and storage may lead to artifacts like selective damage or aggregation of specific cell subpopulations or shedding of cell surface markers. To collect comprehensive information about sample technical analyses such as genomics, metabolomics, lipidomics, glycomics, transcriptomics, flow cytometry with definition of specific cell populations may be combined [2].

As a matter of fact, despite of intensive efforts in proteomics in the recent years, few novel disease biomarkers have been discovered. Since 1998 the rate of introducing newly approved protein targets has been declining to an average of one per year in the USA [3,4]. Therefore, novel analysis models and procedures have to be defined for biomarker discovery, which are highlighted in this review.

21.1.3 Metastasis and Tumor Microenvironment

Especially in oncology novel biomarkers are urgently needed. Due to metastasis cancer is a major cause of mortality worldwide with ten million new cases and more than six million deaths per year [5]. Early detection of incipient remodeling processes indicating metastatic progression and the development of appropriate therapeutic approaches may substantially improve patient survival.

The tumor microenvironment consists of a multi-facetted spectrum of highly specialized cell types, e.g. mesenchymal cells, myelomonocytic cells, endothelial cells and immune cells. The metastatic process is decisively driven by stromal processes, particularly facilitated by neoangiogenesis, lymphangiogenesis and accompanying

inflammatory processes. Growth factors secreted by the stromal cells may serve as survival factors for cancer cells [6]. The tumor microenvironment, through the process of aberrant cell growth, cellular invasion and altered immune system function, contributes a unique sum of proteins secreted, with cytokine and chemokine or enzymatic activity (for example, matrix metalloproteinases) [7,8]. This generates an unbalanced or altered stoichiometry of agonists and antagonists within the tumor profile compared to the 'normal' milieu and can provide characteristic fingerprints applicable as specific and sensitive biomarkers for various purposes [9].

21.2 Biomarker

21.2.1 Definition

A biomarker is objectively measurable indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

Different types of biomarker can be evaluated: prognostic, which characterize the course of disease, predictive to monitor the response to treatment, diagnostic which demonstrate the evidence of disease and pharmacodynamic for the purpose to show efficacy of treatment.

A surrogate endpoint is a biomarker that is intended to substitute for a clinical endpoint, a characteristic or variable that reflects how a patient feels, functions, or whether he is going to survive.

A surrogate endpoint is expected to predict clinical benefit such as decreased pain, quality of life, DFS (disease free survival), OS (overall survival) and cure.

Cancer biomarkers have to enhance the potential to screen, diagnose, prognosticate, localize and stage tumors, or predict and monitor the therapeutic responses to various cancers. Therefore cancer biomarkers have to be correlated with the clinical situation and can be classified into four broad categories related to tumor burden, cancer risk, tumor-host interaction and function.

21.2.2 Biomarker in Cancer

Metastatic cancer presents a substantial clinical challenge since there is a lack of adequate approaches to properly define disease subgroups for rational treatment design and selection. In addition the majority of cancers are initially diagnosed in advanced stages. Some important markers commonly employed in clinical diagnosis include CEA (carcinoembryonic antigen), PSA (prostate specific antigen), AFP (alpha-fetoprotein), CA 125, CA 15–3, and CA 19–9. Current diagnostic methods are limited in their ability to diagnose early disease and accurately predict individual

risk of disease progression and outcome. None of these markers is known to have high specificity and sensitivity or to exhibit prognostic value for neoplasms [10]. This may be attributed to the high heterogeneity in cancer patients with a lot of varying parameters such as tumor size, location, histology, depth, stage, grade, ulceration, age, sex etc. The emerging pattern of molecular complexity in tumors mirrors the clinical diversity of the disease. This highlights that cancer is not a single disease but a heterogeneous group of disorders that arise from complex molecular changes [11]. Thus, there is a growing consensus that marker panels, which are more sensitive and specific than any individual marker, will increase the accuracy of early-stage cancer detection.

21.2.3 Stages of Biomarker Development

The **discovery phase** represent an 'unbiased' experimental setup, here high-throughput methods are of outstanding relevance. The next phase, '**qualification**,' serves for the confirmation that the differential expression of candidate proteins observed in the discovery phase can be verified using alternative, targeted methods. In addition the differential expression of candidate biomarkers has to be verified human plasma/serum samples. During the discovery and qualification phase the consistency of association between marker and disease and the marker sensitivity and specificity has to be demonstrated. In the **'verification'** phase the analysis has to be extended to a larger number of human plasma samples, incorporating a broader range of cases and controls. Here the environmental, genetic, biological and stochastic variation in the population has to be considered. In the verification phase the sensitivity of biomarker candidates is affirmed and specificity has to be assessed [3].

21.2.4 Proteomic Technology in Biomarker Discovery

Important sources for biomarkers should be represented by proteins in the blood. The exact number of proteins in blood is not known. Efforts by different laboratories of the Plasma Proteome Project led to the identification 889 proteins identified with a confidence level of at least 95%. It is estimated that the plasma proteome may contain up to 10,000 proteins [12]. Proteome analysis is a promising tool for the discovery of novel and innovative cancer biomarkers [13]. Over the past decade, serum and plasma proteomics aimed to identify potential cancer biomarkers [14]. Since these markers are present in low amounts in blood samples, the direct isolation requires a labor-intensive process involving the depletion of abundant proteins and extensive protein fractionation.

This classical approach comparing the plasma protein profiles of the healthy donor to the patient largely failed during the discovery phase. An inherent problem

of blood proteomics is the **complexity** of the protein composition, comprising an enormous diversity of proteins and protein isoforms, the dynamic range of plasma and other biofluids and the tremendous extend of human and disease variation. In addition the anticipated **low relative abundance** of many disease-specific biomarkers represents a pitfall: the concentration range in human plasma covers ten orders of magnitude, which means that certain biomarkers may be ten billion fold less abundant than serum albumin. Due to these pitfalls of blood proteomics it has been proposed to rather analyze diseased tissue or biological fluids close to diseased sites (for example tissue interstitial fluid (TIF)). Here the relevant proteins are expected to occur at higher concentrations which facilitates biomarker discovery.

Alternatively, the secretome of cancer cells [15] and tumor associated cells can be analyzed and verified subsequently in human blood by ELISA analyses. Following completion of the Human Genome Project, scientists postulated that important cancer biomarkers will be secreted proteins, as about 20–25% of all cell proteins are secreted [16]. Actually some classical cancer biomarkers (e.g., CEA, Her2-neu) are cell-membrane bound, with their extracellular domains eventually shed into the circulation [14].

21.3 Secretome as Reservoir for Biomarker Discovery

21.3.1 Definition

The secretome is defined as the set of secreted proteins [17,18]. The term "secretome" was first referred by Tjalsma et al. [17] to secreted proteins of *Bacillus subtilis* in a genome-based global survey. The secretome is composed of proteins that are actively secreted, shed from the cell surface and intracellular proteins, which are accidentally released into the supernatant. Cell lysis resulting from necrosis releases relatively large amounts of protein when compared to secretion. The secretome harbors proteins released by a cell, tissue or organism through various mechanisms including classical and nonclassical secretion as well as secretion via exosomes [19]. Secretion may occur either constitutively (continuously) or be regulated and triggered on demand resulting from different functional cell states.

21.3.2 The Cancer Secretome

The cancer secretome, the totality of proteins released by cancer cells, has been attracting wide attention as it is a potential reservoir of cancer biomarkers. Secreted proteins may determine, control and coordinate many biological processes such as growth, cell division and differentiation, invasion, metastasis, angiogenesis and lymphangiogenesis via an endocrine, paracrine or autocrine way. In addition it is known that the tumor microenvironment contributes to tumor development and

progression via communicative processes, mediated by cytokines, chemokines, hormones and specifically secured communication structures (e.g. gap junctions) [8]. Therefore also secreted proteins shed by tumor associated cells need to be considered [9]. Protein secretion exerts autocrine and paracrine biological functions rather than maintenance of basic metabolism. Therefore, specifically secreted proteins may much better be related to the exertion of biological functions compared to cytoplasmic proteins. These proteins eventually end up in the bloodstream, and thereby may have a potential as non-invasive biomarkers [9]. Their biological key roles make them good targets and sources for therapeutical and drug-based intervention as well as tools for diagnosis and prognosis. Thus, great interest is currently focused on the characterization of secreted proteins in order to identify novel biomarkers. The leaky nature of newly formed blood vessels and the increased hydrostatic pressure within tumors increase the chance to find secreted proteins in the blood stream [9]. A pathological situation thus tends to push molecules from the tumor interstitium into the circulation. Therefore it seems to be plausible that proteins produced by the microenvironment will be shed into the blood, making ongoing processes of tumor development detectable [9]. Combinations of markers that are indicative for the specific interactions of the tumor tissue microenvironment will achieve higher specificity and higher sensitivity than the application of any single marker. Candidate biomarkers are expected to exist at very low concentrations diluted in blood plasma with highly abundant proteins such as albumin, which exist in billion-fold excess. At early stages of disease, cancer-specific proteins will always constitute an evanescent subfraction of the proteome representing a true analytical challenge. Noteworthy, early-stage disease lesions such as carcinoma in situ represent tumor cell numbers hardly exceeding several thousand cells. However, the affected microenvironment comprises many more cells compared to the number of tumor cells. Thus proteins derived from tumor associated stroma cells will be produced by more cells and may accumulate to higher amounts. Consequently it can be expected that such proteins will be better accessible for diagnostic purposes than proteins derived from cancer cells themselves. Secretome analysis is applicable to cultured cells as well as tissue specimens [9]. The most comprehensive analysis results, however, are obtained in case of isolated and cultured cells.

In contrast to secreted proteins as new candidates for blood biomarkers, specific proteins identified in the cytoplasm rather represent biomarker candidates accessible to immunohistochemical analysis. Cytoplasmic proteins also comprise specific indicators of functional cell states and cell activities. Combining the information of both secreted and cytoplasmic proteins further supports the detailed understanding of complex patho-physiological processes.

21.3.3 Development of Rational Therapy Design by Secretome Analysis

For many years, the main principle in the treatment of metastatic cancer has been the cyclic administration of high-dose chemotherapy, which is a unselective

strategy based on cytotoxic effects [20]. Chemotherapy uses the small window between killing of rapidly dividing cancer cells and spearing healthy tissues. All tissues with a high proliferation rate are affected by chemotherapy leading to severe and dose limiting side effects such as myelosuppression, damage of the intestinal mucosa and severe skin reactions. Due to this issue, cycles of therapy have to be interrupted by drug-free periods to allow normal tissue to recover. Although the initial effects of chemotherapy are often quite impressive in terms of depleting tumor mass, the duration of remission is often short and resistance may be induced. This risk of selecting chemoresistant cell clones can be linked to the genetic instability and the high mutational rates and heterogeneity of tumor cells. In order to overcome this drug resistance, doses of chemotherapy can either be increased; intervals shortened or chemotherapeutic combination strategies can be chosen. All these options are subsequently potentiating side effects [9].

For an accurate, individualized assessment of risk of disease progression it was suggested to classify disease subgroups and rationally select treatments to substantially affect the outcome of advanced disease. Sekulic et al. [11] discuss that the low overall response rates observed in clinical trials that rely on clinical disease features for patient selection might simply reflect a relatively low percentage of patients with the disease susceptible to a given therapeutic agent or combination. As a consequence, patient selection for clinical trials and selection of therapy on the basis of individual molecular attributes might be necessary to improve response rates to any kind of therapy. Sekulic et al. propose that the detailed consideration of each single patient will overcome the problems of heterogeneity and may lead to a new classification by genomic techniques [11]. Newer individual sequencing data, however, suggest that the heterogeneity of genetic aberrations even within a single patient is by far too large to enable patient stratification. Another stratification option may be derived from the specificity of protein expression profiles which are largely dependent on functional states of cells. Cells make proteins in order to fulfil specific tasks. Functional activation, therefore, inevitably results in the expression of a protein cluster dedicated to fulfil the newly requested functions. Specific pathologic processes may, therefore, be characterized by functional protein signatures. These proteins, here designated as functional protein signatures, may thus enable the identification of relevant functional cell states. In contrast to the genomic techniques focusing on hereditary predisposition, proteome analysis is able to detect when and to what extend the risks have become manifest. For characterisation of diseases, functional aberrations causative for the disease have to be distinguished from aberrations resulting from these primary functional aberrations. To give an example, uncontrolled proliferation is a common process characteristic for neoplasia. The detection of a common process will not support disease sub-classification. Different kinds but characteristic stressors such as inflammatory activation, oxidative stress, DNA damage or ER stress, however, may be causative for disease states such as uncontrolled proliferation. Each kind of stressor is specifically detectable by a defined protein signature providing the basis for functional disease classification. Understanding and detecting the variety of mechanisms leading to a common pathology may serve patient stratification aiding rational therapeutic

concepts better than the consideration of downstream consequences of pathological processes. As a consequence, protein clusters rather than single proteins will serve as biomarkers. Such application may be more feasible than individual genetic profiling to support optimal therapeutic decisions.

In search for alternative strategies for the treatment of advanced cancer, targeting the tumor stroma seems to be a promising tool since this approach is not cytotoxic but interferes with the cooperativity of tumor and tumor stroma cells. This concept is based on the improving understanding that tumor development is associated with the transformation of normal stroma into an "activated" stroma phenotype. Tumor cells are able to establish a permissive and supportive environment for survival and cell growth and to facilitate invasion and metastasis by modulating the stromal host compartment. Targeting this interference between tumor and tumor stroma may consistently lead to a reduction of tumor growth and metastasis. The targets in this approach are genetically normal activated cells which will not be able to escape therapy due to genetic instability and clonal selection. Therefore, targeting these cells should lead to a reduction of development of resistance. This strategy is also considered to be less toxic and thus allows sustaining the therapeutic pressure continuously over longer time periods [9]. Considering that the stroma provides proteins supporting tumor survival, a blockage of this process might chemosensitise the tumor. Therefore, this approach might serve as an efficient combination therapy with chemotherapeutic agents. The enhanced knowledge generated by secretome analysis of molecular aberrations involving important cellular processes, such as cellular signaling networks, regulation of cell cycle and cell death, will contribute to better diagnosis, accurate assessment of prognosis, patient stratification and rational design of effective therapeutics.

21.3.4 Clinical Application

Secretome analysis aims to address three important features of clinical proteomics [9]:

1. Tumor cells may recruit stromal cells for the secretion of growth factors which serve as powerful survival factors. The onset of these characteristic events seems to precede tumor progression. These secreted proteins may have a good chance entering the bloodstream, due to the leaky nature of newly formed blood vessels and the increased hydrostatic pressure within the tumors. Stroma cell secretion of bioactive molecules, which may serve as **diagnostic biomarkers**, are early events in carcinogenesis and may thus enable the early detection of cancer progression.
2. Proteome profiling may identify molecular signatures of processes which promote metastasis. Secretome analysis of defined cell populations offers the opportunity to identify the contribution of the involved cell types and thus the underlying **pathomechanisms**. These pathways rather than single proteins should be monitored and targeted.

3. Transformation of cancer cells is an irreversible process which may be corrected only by apoptotic cell death. Tumor therapy usually targets cancer cells; modern therapy concepts include targeting the stroma in an anti-angiogenic and anti-inflammatory fashion. Cooperativity contributed by stromal cells is reversible and thus directly accessible to **therapeutic intervention**. Most importantly, stroma derived survival factors shall be decreased resulting in a higher chemosensitivity of the tumor cells. Detailed understanding of the responsible processes may thus enable the design of completely new therapeutic strategies.

21.4 Methods

To gain reliable insights into the cancer secretome it is obligatory to prepare samples which are clearly defined and as pure as possible. Secreted proteins occur in body fluids, the direct analysis of potential marker proteins from such samples is hindered by the high complexity and dynamic range of resident plasma proteins. A cell is the smallest independent protein synthesis unit, therefore a reduction of sample complexity to single cell types greatly improves the chances to identify low abundant proteins. It has been observed that proteins secreted by tumor cells in vitro may very well reflect the proteins secreted by tumors in vivo [21]. Therefore, the routine method used is to analyze the secreted of tumor cells or tumor stroma cells in vitro [21]. Mbeunkui et al. [22] performed a comprehensive study of the secretome of three metastatic cancer cell lines and demonstrated that an incubation time of 24 h and 60–70% cell confluence were considered as optimal cell incubation conditions (Fig. 21.1). Due to the low abundance of secreted proteins, the contamination by non-secreted proteins may mask the proteins of interest. The discrimination of genuine secreted proteins from non-secreted proteins is a major issue that needs to be answered in every single experiment [21].

In addition, secreted proteins present in the culture media usually occur at low concentrations, which is often below the ng/mL range. These proteins should be concentrated before proteomics analysis [21]. Ultrafiltration can be used for the concentration of the secretome [21]. Alternatively, precipitation can be performed with acetone or ethanol.

21.4.1 2D-gel Electrophoresis

Zwickl et al. [23] have established a metabolic labeling-based technology with [^{35}S]-labelled methionine and cysteine which allows for the sensitive and selective detection of secreted proteins. They demonstrated the applicability of this method by a study on secretome profiles of a hepatocellular carcinoma-derived cell line. These cells were incubated in the presence of [^{35}S]-labelled methionine and cysteine. Subsequently, the cell supernatant was filtered, precipitated and subjected to

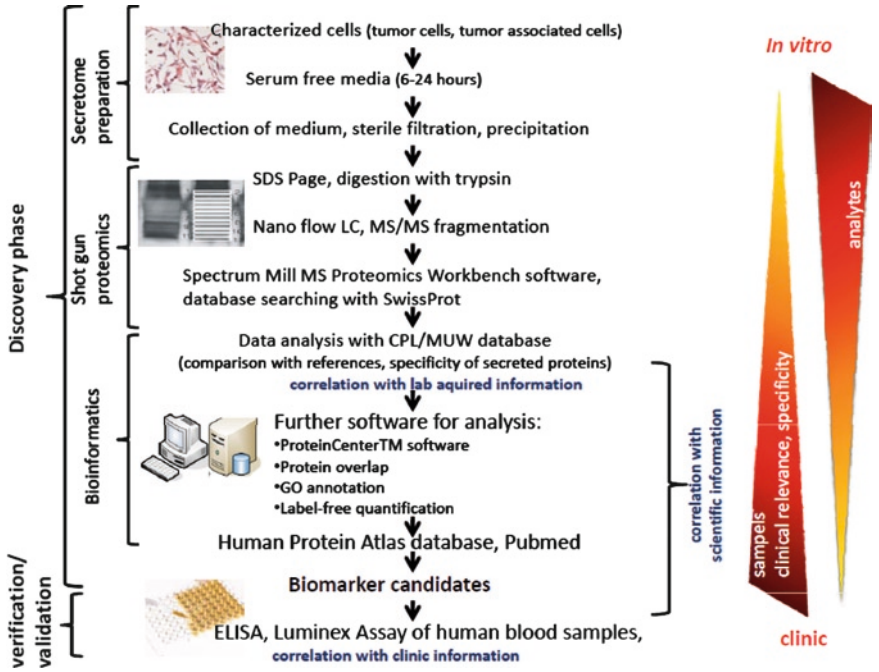

Fig. 21.1 Workflow of secretome proteomics. Secretome preparation is performed with well-characterized tumor or tumor associated cells. Supernatant collection, sterile filtration and precipitation is performed after 6–24 h incubation of the cells in special formulated serum free media. For shot gun proteomics the protein samples are separated by SDS-gel electrophoresis followed by tryptic in-gel digestion and peptide separation by nano-flow LC. Peptide identification is accomplished by MS/MS fragmentation analysis and the MS/MS data are interpreted by the Spectrum Mill MS Proteomics Workbench software and searched using the UniProt Database. Biomarker candidates are selected considering own laboratory and public available expert information. In the verification and validation phase performing ELISA studies in human blood samples these candidates are correlated with clinic information. Specificity and clinical relevance is increased starting from in vitro to clinic while the number of analytes is decreased

two-dimensional gel electrophoresis. After staining proteins were detected by fluorescence staining and autoradiography. Fluorescence staining detects all proteins, in contrast autoradiography detected only those proteins synthesized and secreted by living cells during the metabolic labeling period. All identified 16 protein spots in autoradiography were found to be authentic secreted proteins.

The disadvantages of 2-DE are the low sensitivity in the detection of proteins in low concentrations, the poor representation of hydrophobic membrane proteins in 2D-gels, furthermore the technique is time-consuming, labor-intensive and has a relatively low efficiency in protein detection due to limited amenability to automation [21]. To circumvent some of these inherent problems of the standard 2-DE procedure, a modified method, differential in-gel electrophoresis (DIGE) has been developed by GE Healthcare [24], where three charge and mass-matched fluorescent dyes (Cy2, Cy3 and Cy5), are utilized. These dyes can primarily combine covalently with lysine.

Different protein samples are differently labeled by these fluorescent dyes, then mixed and visualized in one gel. DIGE reduces the experimental variations using one gel for three samples [19]. Instead this method is not applicable to those proteins without lysine (in case of minimal dyes) or cysteine (in case of saturation dyes).

21.4.2 Mass Spectrometry

A mass spectrometer consists of three components: (a) an ion-producing source, (b) a mass analyzer to measure the mass-to-charge ratio (m/z) of the ionized molecule, and (c) a detector that registers the number of ions. A typical shotgun proteomic experiment generally consists of five stages: (1) proteins present in cell lysates, tissue or body fluids are separated by fractionation or affinity selection to define the subproteome, (2) enzymatic degradation of proteins to peptides by trypsin, (3) peptides are separated by reversed phase nano-flow HPLC and eluted into an electrospray ion source where they become charged single molecules in the gas phase which may enter the MS. Isotope-labeling methods, such as isotope coded affinity tag (ICAT) and stable isotope labeling by amino acids in cell culture (SILAC), can be used to introduce quantitative aspects in cancer secretome analysis [25]. These label based approaches are expensive, time-consuming and not always feasible due to the limitation of available tags for primary human materials [25]. We have started to systematically analyze secretomes of various primary and cultured human cells [9,26]. Therefore we have standardized a procedure to bioinformatically filter the truly secreted proteins from contaminant proteins regarding the known main contaminants, i.e. cytoplasmic proteins and serum proteins and as well regarding signal peptides characteristic for secreted proteins . Secreted proteins are then classified with respect to cell type specificity and their relation to functional cell states which are investigated in vitro by functional activation. The relation of identified proteins to the most plausible cells of origin as supported by the CPL/MUW database [27] greatly facilitates the interpretation of complex proteome profiles as derived from human serum samples (Figs. 21.1 and 21.2).

The applied standard procedure to analyse secretomes is detailed in the following (Fig. 21.1). For the accumulation of secreted proteins cells are incubated in serum-free specialized media formulations for 6–24 h at 37°C. For isolation of the secreted protein fraction, the cell supernatant is collected, sterile filtrated to remove cellular debris and precipitated by the addition of ethanol. For the isolation of the corresponding cytoplasmic proteins, all buffers are supplemented with protease inhibitors. Cells are lysed in hypotonic lysis buffer and pressed through a 26 g syringe in order to open the cells by rupture. The cytoplasmic fraction is separated from the nuclei by centrifugation and precipitated by the addition of ethanol. All protein samples are dissolved in sample buffer (7.5 M urea, 1.5 M thiourea, 4% CHAPS, 0.05% SDS, 100 mM DDT) and separated by SDS-gel electrophoresis followed by tryptic in-gel digestion. For shotgun analysis, peptides are separated by nano-flow

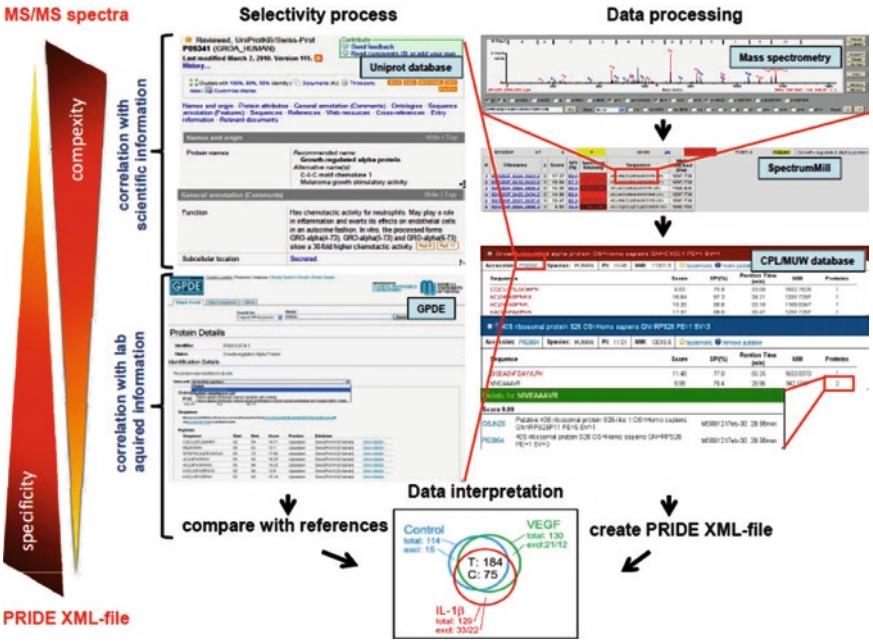

Fig. 21.2 All proteome identification data are based on peptide fragmentation spectra. Blast search of each peptide reveal the corresponding proteins. All peptides related to a single protein become sorted accordingly. Ambiguity may arise due to partial sequence similarities of different proteins, which may not allow to assign a peptide to a single protein only. Uniprot and the CPL/MUW database assist in the selection of the most plausible candidate. Data of various experiments are combined to obtain reference maps of single cell types at specific states. The specificity of any single protein expression with respect to cell types may be retrieved using the GPDE. Overlap and specificity of proteome maps can be visualized by accurate Venn diagrams. During this process specificity is increased while complexity is decreased

LC (1100 Series LC system, Agilent, Palo Alto, CA) using the HPLC-Chip technology (Agilent) equipped with a 40 nl Zorbax 300SB-C18 trapping column and a 75 µm × 150 mm Zorbax 300SB-C18 separation column at a flow rate of 400 nl/min, using a gradient from 0.2% formic acid and 3% ACN to 0.2% formic acid and 50% ACN over 60 min. Peptide identification is accomplished by MS/MS fragmentation analysis with an ion trap mass spectrometer (XCT-Ultra, Agilent) equipped with an orthogonal nanospray ion source. The MS/MS data are interpreted by the Spectrum Mill MS Proteomics Workbench software (Version A.03.03, Agilent) and searched against the SwissProt Database (UniProt Version 15.4 containing 20,328 protein entries) (Figs. 21.1 and 21.2) allowing for precursor mass deviation of 1.5 Da, a product mass tolerance of 0.7 Da and a minimum matched peak intensity (%SPI) of 70%. Due to previous chemical modification, carbamidomethylation of cysteines is set as fixed modification. The reliability of peptide identifications from MS/MS spectra relates to spectral quality indicated with specific scores. The scores are essentially calculated from sequence tag lengths, but also mass deviations are

considered. To assess the reliability of the peptide identifications, searches are performed against the corresponding reversed database. Further details are accessible via www.meduniwien.ac.at/ proteomics.

A protein fraction may be contaminated with keratins derived from dust and comprise identifications with questionable identification quality. To make appropriate decisions, we make use of lists of common contaminants as well as reference lists dependent on the kind of sample comprising "expected" proteins. Only those putative identifications are included, which are present in the according reference list, while all other are discarded. The resulting protein profile is classified using the CPL/MUW database to support subsequent data interpretation (Fig. 21.1). Classification considers common housekeeping proteins, cell type-specific proteins and proteins related to the exertion of specific functions. Furthermore, other public available data as the gene ontology (GO) can be included. Protein expression data derived from methods other than mass spectrometry such as Protein Atlas and gene expression data may support the final decision for expression specificity and thus choice of biomarker candidates. Such biomarker candidates have to be verified and validated performing ELISA studies with human blood samples and by correlation with clinic data. Specificity and clinical relevance is increased starting from in vitro to clinic while sample size is decreased (Fig. 21.1).

21.5 Bioinformatics

Proteomes of biological samples typically consist of thousands of different proteins with a concentration range spanning nine or more orders of magnitude [28]. Only technically demanding high-throughput technologies such as mass spectrometry may actually cope with such an analytical challenge [29]. Modern machines produce more than 10,000 peptide fragmentation spectra per hour, piling up to huge amounts of data for each experiment. As a consequence, there is no proteome profiling without the assistance of well-performing computers and sophisticated bioinformatics tools.

A typical workflow to analyse proteomics data would consist of several independent but interrelated steps. These include interpretation of spectra, subsequent protein identifications and quantifications as well as the assignment of specifically expressed proteins based on comparative analysis. While several different and powerful software packages exist to support these steps such as Mascot [30], SEQUEST [31] and Spectrum Mill [32], there is still urgent demand for further improvements. In the following, the implications of each step will be presented in more detail.

To begin with more technical aspects, there is still a broad variety of data formats and protein sequence databases which complicate the exchange and comparison of data generated by different laboratories. It was the initiative of the European Bioinformatics Institute to establish a common data format, PRIDE-XML, which can be realized starting from almost any kind of existing data format. To support the dissemination of complex proteome data, the public data repository

PRIDE (PRoteomics IDEntifications database, http://www.ebi.ac.uk/pride) was installed [33]. The Global Proteome Machine Organisation (gpm) at gpmdb. rockefeller.edu was established to improve the quality of proteome analysis data relying on tandem mass spectrometry, to make results portable and to provide a common platform for testing and validating proteomics results [34]. These important tools provide access to thousands of proteome analysis experiments and supports documentation of published data.

To summarize, clinical proteomics needs standard operating procedures and guidelines for data generation, data analysis and validation of datasets [35] since the biomarker discovery has suffered in the past from inconsistent data acquisition, statistical interpretation and validation [36]. These standards are represented by (1) the use of standards in the data format and storage (mzXM/mzData), (2) by public data repositories (Peptide Atlas, PRIDE, SwissProt/Uniprot and (3) the integration of a complex database including biological information and different bioinformatic programs using to link different protein lists for instance to specific pathways [2].

Data mining strategies fall into two categories: unsupervised (analogous to clustering) and supervised (analogous to classification) such as classification and regression trees and support vector machines (SVM) [36]. Each algorithm has inherent strengths and weaknesses, which must be matched to the different statistical problems [36]. Some of these softwares are (Fig. 21.1):

1. ProteinCenter software, a proteomics data mining and management software, can be used to predict the function of the identified proteins based on universal GO annotation terms. Here a comparison of cell line secretomes with each other and a functionally categorization can be performed [36,37].
2. The SignalP program can be used to determine the presence of secretory signal peptide sequences and thus predict potential secretion.
3. The SecretomeP program offers the possibility to predict non-signal peptide-triggered protein secretion and to distinguish between protein secretion pathways-the classical and non classical pathway [37].
4. MetaCore (GeneGo, St. Joseph, MI) is used for biological network building and describe millions of relationships between proteins, according to publications on proteins and small molecules including direct protein interactions, transcriptional regulation, binding or enzyme-substrate interactions [37].

In the process of biomarker discovery, a single biomarker may hardly provide sufficient specificity; often several biomarkers have to be combined. Here a two-step process is required:

1. Biomarkers have to be identified employing statistics for multiple testing.
2. They are combined in a predictive model using some of the algorithms [36].

Support Vector Machines (SVMs) offer a cross-validated predictive statement, which is an important issue in biomarker combination. In the case of making a predictive diagnosis through the combination of biomarker, it is possible to calculate the level of confidence with a classification algorithm. Two basic considerations

have to be applied: (1) the number of independent variables should be kept minimal and (2) a blinded validation set should be included [2]. Diagnostic accuracy establishes how accurately the test discriminates between those with and without the disease and is determined by calculating the test's sensitivity, specificity, likelihood ratio and receiver operating characteristic (ROC) curve [36].

One inherent problem of the high throughput technology mass spectrometry becomes evident upon consideration of statistical aspects [38]. A confidence level of 99.5% for the assignment of peptide sequences to fragmentation spectra suggests very high validity of data which is currently hardly realised. Modern equipment may allow the researcher to identify thousand different peptide sequences per hour. A confidence interval of 99% implies that five out of the thousand peptides are not correct. A typical experiment consists of around ten injections, summing up to 50 or more false peptide assignments. Comparative analysis of two groups of experiments summarizing five independent experiments would already sum up to 500 false peptide assignments. Complex analyses may require the consideration of hundreds of experiments. In such a case, a confidence rate of 99.5% per peptide identification may result in a chance to receive false results from a database query higher than 50%.

The only way out of this dilemma will be the consideration of expert knowledge in data analysis [27]. Currently, only quality features of individual spectra are considered for the assignment of amino acid sequences. Each decision is made independent of any other data. Actually, there are chances to make use of other data. We know that a given peptide has characteristic and reproducible chromatographic mobility as well as ionization and fragmentation characteristics. Therefore, the accessible knowledge of successfully identified peptides may facilitate the decision of peptide assignments in case of uncertainty. Furthermore, consideration of knowledge of the origin of the sample may greatly improve data consistency. To give an example: analysis of a mitochondrial fraction may allow some contaminating proteins derived from the endoplasmatic reticulum, but hardly from the cell nucleus. The analysis of a liver sample may include proteins from e.g. immune cells but hardly proteins specific for the heart. Although these implications seem trivial, they require complex expert system programming in order to be automatically implemented in the high throughput analysis of data. The systematic assessment of ontologies may, however, enable the implementation of such strategies.

The processing of data as realized in case of the CPL/MUW-database is outlined in the following. Actually, all protein identifications are based on peptide fragmentation spectra (mass spectrometry) (Fig. 21.2). Amino acid sequences are derived from the spectra and all related peptides identified during a LC-MS/MS run are sorted according to proteins they are derived from (SpectrumMill software). Actually, there are peptides which may be allocated to more than one protein, which need to be nominated in an easily accessible fashion (Fig. 21.2). In such a case, several considerations have to take place. The ambiguity may be solved by consideration of gene expression data and previously determined protein expression data. Consequently, established knowledge made available via the SwissProt-database needs to be accessed, while laboratory-owned data may as well aid the

decision process (Fig. 21.2). On the other hand, known potential contaminants such as keratins should be known to avoid misassignments. After the decision process resulting in protein lists comprising all relevant experimental and peptide identification data as realized via PRIDE XML-files, interpretation of data may be enabled by comparative analysis (Fig. 21.2). To provide an example: we have analysed secretomes of primary human endothelial cells at normal, angiogenic and inflammatory cell states. Accurate Venn diagrams displays the relation between these protein fractions (Fig. 21.2). Out of a total of 184 different proteins identified, 75 were found in all three kinds of cells. 114 proteins were secreted by untreated cells, 14 of which were not identified at the other two functional states. One twenty-nine proteins were identified in IL-1β-treated cells, 33 of those were not identified at the other two functional states. Actually, some of them were found as well secreted by e.g. inflammatory activated macrophages, leaving 22 proteins apparently specific for inflammatory activated endothelial cells. This kind of comprehensive comparative analysis may strongly support the interpretation of complex data.

While data acquisition and protein identification may be considered as relatively simple tasks, there is still obvious demand for tools supporting data interpretation. These processes organize the data with respect to experiments and cell types, but not to functional aspects. Currently there is still obvious demand for further tools supporting data interpretation. The application of -omics techniques often leave the researcher with very long lists of identified genes and proteins which are impossible to comprehend. Current strategies try to relate expression data to signaling pathways in order to support biological interpretation [39–41]. There are still major limitations to these approaches. In many cases, the known involvement of a gene or a protein in a specific signaling or metabolic pathway would highlight the protein as such. Comparative analyses, however, record up- or down-regulation of proteins. Switching on a specific pathway does not necessarily mean that relative amounts of proteins involved in the pathway would be regulated. In many cases, however, the activation of a specific pathway would result in the up-regulation of proteins which are not at all involved in the exertion of the signaling or metabolic event. For the identification of the involvement of pathways, which is evidently desirable, databases would be required which exhibit consequences of pathway activation rather than involvement in pathways. There is still a demand for such databases.

Another shortcoming of current analysis strategies is the preferential assignment of tissue-specific expression patterns rather than cell type-specific expression patterns. Actually it is obvious that tissues are made of different kind of cell types. Some cell types such as immune cells occur in all tissue types, other cell types specifically occur in a single organ. It is the specific functional characteristics of hepatocytes which give raise to liver-specific specific proteins, liver cells other than hepatocytes do not express liver-specific proteins. Therefore, it would be more accurate to talk about hepatocyte-specific proteins rather than liver-specific proteins. There are databases listing organ-specific protein expression but no databases listing cell type-specific protein expression.

For this reason we established the following data analysis strategy. First of all the proteome profiles of isolated organelles which commonly occur in cells, such

as nuclei, mitochondria , ribosomes and proteasomes were determined. Such analyses obviously allow for the fact that cell type-specific proteins may as well occur in organelles such as nuclei but very much account for the fact that the basic protein composition of these organelles is highly similar. A proteome profile of a cell may thus already be structurally sorted according to the belonging to an organelle. As a consequence, a long protein list may already become much easier to be interpreted as related groups of proteins are identified.

The next step of systematic analyses focuses on cell types. We have already determined proteome profiles of lymphocytes, monocytes, dendritic cells, neutrophils, fibroblasts, endothelial cells, various epithelial cells and many others and classified both commonly expressed proteins as well as cell type-specific proteins. Some of these data have been made available to the public via the CPL/MUW database at www.meduniwien.ac.at/proteomics/database [27]. The expression specificity of several thousand proteins with respect to cell types can thus be immediately determined.

The SQL database (CPL/MUW – database of the Clinical Proteomics Laboratories at the Medical University of Vienna) facilitates (i) quality management of protein identification data, which are based on MS, (ii) the detection of cell type-specific proteins and (iii) of molecular signatures of specific functional cell states [27].

Proteome analyses of clinical materials constitute a big challenge for investigators due to its great complexity. Exact planning and documentation of each analysis step is crucial to enable meaningful data interpretation. This is why we strictly follow the established rules of the “minimum information about a proteomics experiment” (MIAPE) [35]. According to highest international standards, submit all relevant proteome analysis data to the international repository for proteome analysis data, the PRIDE database. We have already successfully implemented a program which automatically translates experimental data out of our database to a standardized PRIDE-XML format using international standardized ontology-terms to describe all experimental details (http://www.ebi.ac.uk/ontology-lookup/) [41]. Furthermore, we have programmed a proteome analysis database referring to the investigation of cross-cell type and cross-species comparisons of proteome analysis data derived from both, 2D-PAGE and shotgun analysis [27].

Proteins fulfil biological functions. If a cell enters a characteristic functional state it may need proteins not expressed under normal conditions. Such proteins may be specifically expressed only when the cells enter the functional state. As a consequence, the identification of such specifically expressed proteins may identify the corresponding cell state. Any disease-related symptom is a consequence of aberrant cell activities associated with the disease. Identification of aberrant cell activities may thus identify diseases. When investigating disease biomarkers we should consider the fact that proteins were designed by evolution to exert functions rather than to indicate diseases to medical doctors. Therefore, there are no protein biomarkers specific for a disease; there are only, actually plenty of, biomarkers specific for biological functions. If such an aberrant function is specifically associated with a certain disease the corresponding protein may be considered as a disease biomarker.

We have started to systematically assess protein expression profiles of cells at characteristic functional states. As expected, we were able to identify several specifically expressed proteins. These include proteins specifically related to functional states such as cell proliferation or inflammatory activation which may be entered by different kinds of cells. Actually, there are proteins which we found to be exclusively expressed by a single cell type at a specific cell state but not by any other cell. Therefore, these proteins are classified into organelle-derived, cell type-specific, cell state-related and cell type cell state-specific proteins. Comparisons of normal and diseased tissue proteome sample therefore result in the consideration of alterations in the abundance of organelles (indicative for, e.g. rate of mitochondrial respiration compared to glycolysis), the consideration of alterations of the occurrence of cell types (indicating e.g. invasion of immune cells or increase in the number of fibroblasts), the consideration of cell states (assessment of cell proliferation, cell stress, apoptosis, inflammatory activation of myofibroblast formation) and finally the occurrence of specific cell entities (e.g. type II macrophages). The knowledge of disease-associated aberrations in one or several of these aspects may thus allow us to design highly specific marker panels.

21.6 Identification of Biomarker Candidates by Secretome Analysis

Secretome analysis is an upcoming field of cancer research. This chapter gives a brief overview of the latest key secretome studies:

Recently, secretome analysis based on a LC-MS/MS label-free quantitative proteomics approach was used to compare the secretome of a primary cell line SW480 with its lymph node metastatic cell line SW620 from the same colorectal cancer patient [25]. They identified a total of 910 proteins from the conditioned media and 145 differential proteins between SW480 and SW620 (>1.5-fold change). Among them, trefoil factor 3 and growth/differentiation factor 15, two proteins upregulated in the metastatic cell line SW620, were analyzed in a large cohort of clinical tissue and serum samples and confirmed as biomarker candidates for the prediction of colorectal cancer metastasis [25]. Here secretome analysis allowed new insights into the pathophysiology of tumor progression.

An important study for a systematic identification of unique markers for colorectal cancer was performed by Wu et al. [42]. Secretomes of 21 cancer cell lines derived from 12 cancer types (colon cancer, leukemia, bladder cancer, lung cancer, NPC, hepatocellular carcinoma, cervical carcinoma, epidermoid carcinoma, ovary adenocarcinoma, uterus carcinoma, pancreatic carcinoma and breast cancer) were compared. Collapsin response mediator protein-2 (CRMP-2) was only secreted by the colorectal cell lines (Colo205 and SW480) but not any other cell lines tested and was therefore selected for further evaluation. Initially CRMP-2 was identified as a mediator required for semaphoring triggered growth cone collapse and was associated with carcinogenesis by p53 regulation. ELISA analyses of plasma

samples from colorectal patients and healthy controls were performed to examine the levels of CRMP-2 and CEA revealing that the sensitivities of plasma CRMP-2 and CEA were found to be 60.5% and 42.9%, respectively. This secretome analysis led to a novel marker, CRMP-2, which may be a colorectal marker superior to CEA. However, a large cohort study is required to validate the utility of plasma CRMP-2 levels for CRC screening and diagnosis.

In addition these authors analyzed proteins released by most cancer cell lines (pan-cancer marker candidates) and assigned these to specific secretion mechanisms. In the conditioned media of cancer cells proteins may be released via various cellular mechanisms, including classical secretion and nonclassical secretion pathways, as well as secretion via exosomes. The exocytosis of membranous vesicles called exosomes was initially described in antigen-presenting cells such as B-lymphocytes and dendritic cells, and was later found to also occur in tumor cell lines. The authors assigned some identified proteins to characteristic constituents of exosomes including ubiquitously expressed molecules such as intracellular metabolic enzymes (pyruvate kinase and alpha enolase), cytoskeletal proteins (actin, cofilin, tubulin, and moesin), and chaperones (HSP90 and HSP70). To determine whether some proteins may have been released into the medium by cell death, cell viability has to be measured.

To get panels of serum biomarkers for lung cancer, Xiao et al. [43] compared the secretome of primary cultures of lung cancer cells and the adjacent normal bronchial epithelial cells of six lung cancer patients using one-dimensional PAGE and nano-ESI MS/MS . They demonstrated that a panel of four proteins, CD98, fascin, polymeric immunoglobulin receptor/secretory component and 14- 3-3 η had a higher sensitivity and specificity than any single marker.

To characterize extracellular events such as cell-to-cell interactions and cell-to-extracellular matrix interactions associated with breast cancer progression on the genomic level, gene profiles of secreted proteins were investigated in a cell line of human proliferative breast disease. Differentially expressed genes were searched for genes encoding secreted proteins in three public databases. The analysis displayed two clusters of secretome genes with expression changes correlating with proliferative potential [44].

Celis et al. [45] employed 2-DE and MALDI-TOF-MS to analyze the tumor interstitial fluid (TIF), which was collected of freshly dissected invasive breast carcinomas. From TIF, which perfuses the breast tumor microenvironment, they identified 267 primary translation products, involved in cell proliferation, invasion, angiogenesis, metastasis and inflammation.

A novel technology for investigating in vivo cancer secretome was recently developed by Huang and colleagues [46]. They collected the samples for further secretome analysis by implanting capillary ultrafiltration (CUF) probes into tumor masses of a live mouse at the progressive and regressive stages. Five of the detected proteins, including cyclophilin-A, S100A4, profilin-1, thymosin beta 4 and 10, which previously correlated to tumor progression, were identified at the progressive stage. They also identified specifically secreted proteins at the regressive stage called fetuin-A, alpha-1-antitrypsin 1–6, and contrapsin.

Very recently, a secretome analysis of 23 human cancer cell lines derived from 11 cancer types using one-dimensional SDS-PAGE and nano LC-MS/MS (GeLC-MS/MS) was performed on LTQ-Orbitrap MS to generate a comprehensive cancer cell secretome [37]. The identified proteins were selected as potential marker candidates according to three categories: (i) proteins apparently secreted by one cancer type but not by others (cancer-type–specific marker candidates), (ii) proteins released by most cancer cell lines (pan-cancer marker candidates), and (iii) proteins putatively linked to cancer-relevant pathways [37]. This analysis yielded 6–137 marker candidates selective for each tumor type and 94 potential pan-cancer markers. Among these, the monocyte differentiation antigen CD14 (for liver cancer), stromal cell-derived factor 1 (for lung cancer), cathepsin L1 and interferon-induced 17 kDa protein (for NPC) were selected for validation as potential serological cancer markers.

Immunohistochemistry revealed that bile salt sulfotransferase, ornithine carbamoyltransferase, monocyte differentiation antigen CD14**,** and isoform 1 of asialoglycoprotein receptor 2 were less immunoreactive in tissues of other cancer types, while multidrug resistance protein 1 and vitamin K-dependent protein C were overexpressed in hepatocellular carcinoma versus other cancers. Bladder cancer tissues reacted more strongly with proteins such as cadherin-6, squalene synthetase, ribophorin II, and 15-hydroxyprostaglandin dehydrogenase while the levels of neurogenic locus notch homolog protein 3 and trefoil factor 1 were higher in breast cancer tissues versus tissues of other cancers [37]. The stromal cell-derived factor 1 (CXCL12) reacted more strongly with lung cancer tissues. In addition, Wu et al. confirmed the significantly elevated plasma levels of two candidates (CD14 and SDF-1/CXCL12) in hepatocellular carcinoma and lung cancer patients [37].

In our recent study, we analyzed the secretomes of primary melanocytes, cultured melanoma cells and representatives of the most prominent stroma cells including fibroblasts, endothelial cells and dendritic cells by shotgun proteomics [9]. We consider the assessment of cell type-specific secretion characteristics as a prerequisite before potential relevant alterations of tumor-associated stroma cells can be recognized. In case a tumor-associated fibroblast secretes a protein not secreted by normal fibroblasts, but secreted e.g. by normal endothelial cells, such a protein would hardly be useful as biomarker. This is why we systematically analyzed the most important representatives of tumor-associated stroma cells. This strategy enables us to identify proteins which are aberrantly expressed by tumor-associated fibroblasts but not in any normal counterparts isolated from healthy background [9]. We performed secretome and proteome profiles generated from normal human skin fibroblasts in comparison to melanoma-associated fibroblasts isolated from mouse xenografts and fibroblasts from bone marrow of multiple myeloma patients. Further mutual comparisons were enabled including proteome profiles of melanocytes and M24met melanoma cells. All shotgun proteomics data have been made accessible via the PRIDE database. Amongst others, the candidate biomarkers GPX5, secreted by melanoma cells, in addition to periostin and stanniocalcin-1, which are expressed by melanoma-associated fibroblasts, were identified. Due to this data we started to investigate tumor associated fibroblasts of

primary melanoma and primary melanoma cells in a more systematic fashion by rtPCR, comparative genomic hybridization and cytoplasmic proteome and secretome analysis. This information will enable us to better understand cellular processes of the tumor and tumor associated cells in order to define new therapeutic agents and rational concepts for melanoma treatment and to detect biomarkers.

Secretome analysis is a novel research area offering new opportunities for biomarker discovery and drug development. However, despite promising results highlighted in this chapter, more systematic and hypothesis driven studies are needed. As primary cells are highly sensitive living units, any alteration in culture condition may result in aberrant protein secretion. Therefore, for clinical proteomics supporting biomarker discovery it is inevitable to refer to a SOP driven data resource of secretomes to enable an appropriate correlation of scientific with patient-derived information.

21.7 Conclusion

The identification of potential marker proteins is not trivial. Comparative analysis of serum samples and tissue specimen is hindered by the natural complexity of protein expression. Diseases like cancer mean a variety of de-regulated cell processes all of which eventually causing characteristic aberrant protein expression. Different kinds of patho-physiological processes may be associated with tumor development, such as involvement of the immune system, alterations of the microenvironment and characteristic processes in the cancer cells themselves. This complexity is further enhanced by the individual heterogeneity in disease in addition to heterogeneities introduced by the involved experimental procedures. Low abundant proteins may be hard to identify as long as they are present in a complex protein mixture together with other proteins, several at million fold higher concentrations. Dependent on the protein mixture, positive identification of actually present, but low abundant proteins may thus fail. Statistical evaluation of comparative proteome analysis data may thus not be able to identify the truly relevant proteins. One possible concept to overcome this inherent heterogeneity is based on the functional analysis of cell types in advance. It is predicated on the characterization of smallest independent units and tries to find a combination of independent units to match the molecular profile of an individual sample. This smallest unit capable of protein synthesis, the cell, decides whether or not to produce proteins with specific activity which may become related to a disease.

In mathematics the strategy to refer to independent functions is called Fourier transform which makes a complex function amenable for further analysis. The smallest independent and potentially predictable protein synthesis machinery unit is a cell. Since every functional cell aberration is associated with aberrations of protein expression when compared to normal, the cell is an optimal starting point for biomarker discovery. Like Fourier transform in physics, the establishment of profiles of the smallest autonomous protein production units in the body, i.e. cells,

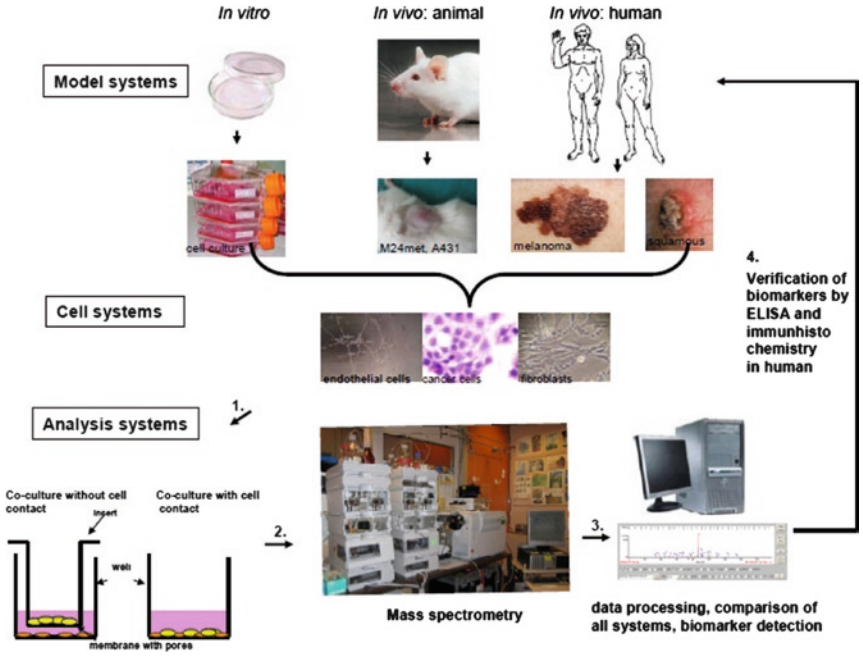

Fig. 21.3 The novel approach detecting biomarkers and defining potential therapeutic targets. The basic strategy for biomarker discovery is visualized. As model systems cultured cell lines, animal models for melanoma and squamous skin cancer and biopsy specimens of human skin cancer are presented. In all cases the secretome of the same isolated cell types (i.e. cancer cells, endothelial cells and fibroblasts) is analyzed. In further steps it is envisaged to analyze for specific cell-cell interactions mimicking characteristic tissue states for example by applying different co-cultures starting from in vitro to in vivo models. In a last step these results shall then be evaluated in the human background in the tissue and blood profile [9]

may greatly facilitate the interpretation of complex proteome profiles as derived from human serum or tissue samples (Figs. 21.2 and 21.3). All proteomes, i.e. protein mixtures, should it be from tissues, blood, plasma or other body fluids can be expressed as a function of cellular proteomes. The assignment to cellular proteome reference maps will lead to a massive reduction of apparent complexity (Fig. 21.2). Therefore possible candidates can be extracted by defining the involved cell systems such as cancer cells and distinguished cell of the environment including fibroblasts and endothelial cells in a first step. With the aid of specialized databases, for instance the CPL/MUW-database [27], specificities and commonalities of protein expression profiles of such different cells can be quickly assessed. Therefore, early teamwork between the clinical level, bioinformatics, medical informatics, and proteomic scientists is needed to overcome the current limitations.

One key question relates to our ability to draw appropriate conclusions for (short-, mid-, or long-term) therapeutic approaches and consequences from the highly dynamic proteome profiles. Specific cellular systems and subsystems and

functional components have to be defined prior to the analyses of a complex organism influenced by various states of disease. Integration of proteomics and cell-based technologies will allow the description of the molecular setup of normal and abnormal cell systems leading to the standardized discrimination of abnormal cell states in disease permitting for instance the design of individualized therapies, the prediction of further disease course in patients, the identification of new pharmaceutical targets, and establishment of a standardized framework of relevant molecular alterations in disease [2].

We make use of three different model systems (cell culture, tissue in vivo and human being), all have their strength and weakness starting from in vitro to human. The complexity but also relevance is increased from in vitro to human being. Therefore we combine all these systems (Fig. 21.3).

Our strategy is composed of seven independent steps (Fig. 21.3) [9]:

1. Establishment of relevant model systems mimicking various functional cell states including characteristic in vitro cell activation experiments and (non-) contact co-cultures
2. Standardization of protein isolation
3. Standardization of MS-procedures
4. Generation of proteome reference maps for human primary cells
5. Data organization via database
6. Interpretation of data from diseased tissues by the use of multiple reference maps
7. Verification of biomarkers or possible therapeutic targets by i.e. ELISA, immunhistochemistry, Western blot

In a last step these results shall then be evaluated in the human background in the tissue and blood profile (Fig. 21.3). ELISAs for instance the Luminex system [47] are to be established for the most promising candidates (including the specifically expressed proteins mentioned above). These assays will then be used to assess protein levels of candidate biomarkers in serum samples of patients. For validation we begin with assaying patients whose fibroblasts were found in vitro to secrete large amounts of candidate biomarker proteins. These data are then compared to serum samples derived from patients whose fibroblasts were found not to secrete these factors. This step of analysis will allow us to assess whether serum protein levels of these marker proteins are indeed related to the in vitro fibroblast expression levels as anticipated. The secretion specificity of the cancer associated fibroblasts has to be assessed by comparison to the secretomes of fibroblasts, endothelial cells, tumor cells and macrophages, which contribute to tissue remodeling and repair [9,26,48]. Here, we present a novel technical approach to better understand the mechanisms of tumor progression and metastasis by involving the microenvironment. The approach is of tremendous importance since it will allow us new insights in the pathophysiology of tumor progression, leading to the identification of novel biomarkers for early detection and prognosis and may lead to the identification of new therapeutic targets. The plethora of data will offer new opportunities to develop biomarker sets for ELISA analysis for the clinical routine [9]. The combination of a set of relevant

markers will yield an improvement of sensitivity and specificity of the screenings. By focusing on secreted proteins which are early shed by the microenvironment into the blood, specific information about the actual status of the patient and define a fingerprint of the tumor status in the patient can be gained. This strategy may enable early diagnosis of metastatic processes and offers an opportunity for a rational therapy selection. Candidate biomarkers shall be evaluated in clinical studies by correlation with the progression free and overall survival. This concept may be able to establish novel classifications, to define patient subgroups and to consequently allow us to enhance the often low overall response rates observed in clinical trials.

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Chapter 22
Cyclooxygenase 2 (COX2) and Peroxisome Proliferator-Activated Receptor Gamma (PPARG) Are Stage-Dependent Prognostic Markers of Malignant Melanoma

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Abstract COX2 and PPARG are differentially expressed in many human tumors and have emerged as potential targets of biomodulatory cancer therapy. Using three tissue microarrays (TMA) we studied the correlation of COX2/PPARG immunoreactivity in a broad spectrum of tumors focussing on the correlation between clinicopathologic features and outcome of patients with malignant melanoma (MM).

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TMA-1 consisted of normal and tumor tissues (n = 3,448) from 47 organs and tissue entities including skin neoplasms (n = 323) of melanocytic (MM, benign nevi) and non-melanocytic origin (squamous cell carcinomas, basal cell carcinomas, Kaposi sarcomas, histiocytomas, capillary hemangiomas, sebaceous adenomas). TMA-2 consisted of 88 MM with follow-up data, 101 MM metastases and 161 benign nevi. TMA-3 (n = 194) consisted of MM metastases from 36 patients with metastatic stage IV melanoma who had participated in a randomized phase II trial using a stroma-directed biomodulatory approach combining COX/PPAR-targeting with metronomic low-dose chemotherapy.

COX2 immunoreactivity significantly increased from benign nevi (51%) to primary MM (86%) and MM metastases (91%; $P < 0.001$, TMA-2). In case of primary MM, positive COX2 staining was associated with advanced Clark levels ($P = 0.004$) and shorter recurrence free survival ($P = 0.03$). Similarly, PPARG immunoreactivity was significantly increasing from benign nevi (0%) to MM (22%) and MM metastases (33%; $P < 0.001$). However, PPARG expression in primary MM was not associated with any of the clinico-pathologic characteristics or tumor progression and overall survival. On the other hand, patients with PPARG-positive MM metastases who had been treated either with biomodulatory metronomic chemotherapy (trofosfamide) alone or combined with COX2/PPARG-targeting drugs, i.e. rofecoxib and pioglitazone, showed a significant advantage concerning progression-free survival ($P = 0.044$).

We conclude that the expression of COX2 and PPARG is a frequent finding in the progression of MM. Regarding primary MM, the expression of COX2 indicates an increased risk of tumor recurrence, i.e. melanoma progression. In metastatic MM the expression of PPARG may serve as positive predictive marker of potential responsiveness to biomodulatory stroma-targeted therapy (Meyer S, Vogt T, Landthaler M, et al (2009). Cyclooxygenase 2 (COX2) and Peroxisome Proliferator-Activated Receptor Gamma (PPARG) Are Stage-Dependent Prognostic Markers of Malignant Melanoma. PPAR Res 2009: 848645).

Keywords Tissue microarray • PPARgamma • COX2 expression in tumor tissue • Metastatic melanoma • Castrate-resistent prostate cancer • Biomarker analytics • Biomodulatory therapy

Abbreviations

MM	Malignant melanoma
TMA	Tissue microarray
IHC	Immunohistochemistry
COX2	Cyclooxygenase 2
PPARG	Peroxisome proliferator-activated receptor gamma

22.1 Introduction

Cyclooxygenases (COXs) catalyze the first rate-limiting step in the conversion of arachidonic acid to prostaglandins. Two COX isoenzymes have been identified: COX1 is constitutively expressed in most tissues and mediates the synthesis of prostaglandins in normal physiological processes, whereas COX2 is not detectable in most normal tissues but is rapidly induced by various stimuli such as inflammatory reactions [1]. COX2 is also expressed in various tumor types [2], and levels of expression have been shown to correlate with invasiveness and prognosis in some tumor entities, suggesting an important role of COX2 in tumor development and progression. Epidemiological studies show that prolonged COX2 inhibition through acetylsalicylic acid or other nonsteroidal anti-inflammatory drugs (NSAIDs) might offer some protection against colon cancer and some other malignancies [3,4]. Accordingly, in animal experiments COX2 inhibitors can reduce the incidence of colon carcinoma in APC knockout mice treated with chemical carcinogens [5]. The mechanism by which COX2 expression accelerates tumorigenesis is poorly understood. However, a potential role of COX2 in epithelial and melanocytic skin cancer development is also not unlikely, since COX2 is frequently expressed in malignant melanomas (MM) [6,7] and squamous cell carcinomas of the skin [8,9].

The peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear hormone receptor subfamily of ligand-activated transcription factors. There are three known subtypes of peroxisome proliferator-activated receptors; PPARA, PPARD, and PPARG. The latter is involved in physiological adipocyte differentiation and differentially expressed in several types of human cancers [10], e.g. in prostate cancer [11,12], breast adenocarcinomas [13], overian cancer [14,15], lung cancer [16], and colon cancer [17]. Accordingly, PPAR ligands were shown to inhibit the growth of cells from different cancer lineages in vitro [18]. In human melanoma cell lines the anti-proliferative and apoptosis-inducing effect of PPARG ligands was demonstrated, too [19,20].

Current research data and clinical experience suggest that PPARA/G can mediate both direct antitumoral and immunomodulatory effects and a broad spectrum of stroma modulating activity including anti-angiogenic, anti-inflammatory and immuno-augmentative effects [21,22]. Examples of super-additive complementation of PPARG agonists by COX2 inhibitors and metronomic chemotherapy are well documented experimentally and in clinical trials, respectively [10,16,23].

We had studied such combined tumor-stroma-targeted cancer therapy using PPARG agonists and COX2 inhibitors in the second-line treatment of advanced metastatic melanoma disease [22,23]. In a randomized multi-institutional phase II trial including 76 mostly chemorefractory patients with progression of metastatic melanoma (stage IV melanoma according to AJCC criteria), we had observed a significantly prolonged progression-free survival in the group of patients that received angiostatically scheduled low-dose metronomic chemotherapy (trofosfamide) in combination with a PPARG agonist (pioglitazone) and a COX2 inhibitor

(rofecoxib) compared to the group of patients who received metronomic chemotherapy alone [22]. Accordingly, tumor associated inflammatory and angiogenic processes mediated by COX2 overexpression or PPARG deficiency were suggested to play a pivotal role in the biology of melanoma progression [22]. However, there is insufficient data on the expression of both target molecules; therefore, their prognostic and therapeutic relevance in MM is still unclear.

The study presented herein is based on a high-throughput tissue microarray (TMA) analysis, a highly efficient technology for investigating large numbers of tumors. To the best of our knowledge this is the largest study of this topic which can link expression data with extensive follow-up data of melanoma patients, respectively. In addition, as we gather extensive data on various other cancers and normal tissues (47 organs and tissue entities) we can put the specifities of the melanoma data into a broader oncologic context.

22.2 Materials and Methods

Tissue Microarrays (TMAs). TMA construction was performed as described previously [24]. The local Institutional Review Boards of the Universities of Regensburg and Basel granted approval for this project.

The first TMA (**TMA-1**) contained formalin-fixed, paraffin-embedded tissue punches from the archives of the Institute of Pathology, University of Basel, Switzerland. A comprehensive TMA was created by transferring representative tissue cylinders with a diameter of 0.6 mm to seven new paraffin blocks as described by Bubendorf et al. [25]. Representative areas of different subtypes for the most frequent tumor entities and their corresponding non-tumorous tissue were selected for analysis. Four μm sections of the resulting TMA block were cut and mounted to an adhesive-coated slide system (Instrumedics Inc. Hackensack, New Jersey, USA). The constructed multi-tumor TMA-1 consisted of 3,448 primary tumors from 132 different tumor subtypes and 26 different normal tissues and allowed us to determine the prevalence of COX2 and PPARG expression in non-tumorous tissues and corresponding malignant tumors. Samples from skin (n = 330), lung (n = 217), brain (n = 228), breast (n = 218), colon (n = 204), soft tissue (n = 150), salivary gland (n = 152), testis (n = 126), ovary (n = 140) and kidney (n = 144) were the major tissues assembled on this TMA. The evaluation of tissue and clinical data was performed on the basis of anonymized patient data according to the regulations of the University of Basel Institutional Review Board. Detailed tumor and tissue characteristics can be found in Table 22.6, 22.7 and Figure 22.5, 22.6. The skin-related datasets were extracted and are summarized in Table 22.1, the other data sets in Figure 22.1 to 22.4 and Table 22.6 and 22.7.

The second TMA (**TMA-2**) was constructed as described by Wild et al. [26] and contained a total of 350 formalin-fixed, paraffin-embedded human tissues: 88 (25.1%) primary malignant melanomas, 101 (28.9%) metastases, and 161 (46.0%) benign nevi. H&E-stained slides of all tumors were evaluated by two surgical pathologists

Table 22.1 COX2 and PPARG expression analysis of skin tumors using TMA-1

	Cytoplasmic COX2 immunoreactivity						Nuclear PPARG immunoreactivity						
Tumor entity	n analyzable	0 (n)	1 + (n)	2 + (n)	3 + (n)	P^a	n analyzable	0 (n)	1 + (n)	2 + (n)	3 + (n)	P^a	P^b
TMA-1: total (n = 323)	186	34	86	54	12		212	143	50	19	0		**0.0003**
Melanocytic lesions													
Malignant melanoma	38	0	16	17	5	**0.02**	41	21	8	12	0	**0.001**	**0.01**
Benign nevus	19	4	7	8	0		24	22	2	0	0		1.00
Epithelial tumors													
Squamous cell carcinoma	30	3	10	11	6	0.07	33	23	7	3	0	**0.001**	0.62
Basal cell carcinoma	31	7	16	7	1		33	11	21	1	0		0.57
Connective tissue tumors													
Kaposi sarcoma	15	6	8	1	0	0.61	18	13	5	0	0	0.13	1.00
Benign histiocytoma	16	8	7	1	0		22	19	1	2	0		0.47
Capillary hemangioma	14	3	9	2	0		18	16	2	0	0		1.00
Adnexal tumors													
Benign sebaceous adenoma	23	3	13	7	0		23	18	4	1	0		1.00

[a]Fisher's exact test (2-sided); bold face representing significant data;
[b]Fisher's exact test (2-sided); association of COX2 and PPARGIHC within single tumor entities

(T.V., P.J.W.). Clinical follow-up data, provided by the Central Tumor Registry of the University of Regensburg, were available for all patients with primary malignant melanomas (n = 88). The median follow-up for all patients was 54 months (range 0–135 months), whereas the median follow-up for censored patients (n = 74) was 63.5 months. Characteristic parameters of TMA-2 are summarized in Table 22.2.

The third TMA (**TMA-3**) was constructed on the basis of a randomized multi-institutional phase II trial using an angiostatic biomodiulatory approach to assess the impact of COX2- and PPAR-targeted therapy in combination with metronomic low-dose chemotherapy in patients with advanced metastatic stage IV melanoma [22]. The clinical trial was designed to select metronomic chemotherapy alone (arm A: trofosfamide 50 mg orally three times daily, day 1+) or combined anti-inflammatory/angiostatic treatment (arm B: trofosfamide as above mentioned plus rofecoxib 25 mg orally, day 1+, and pioglitazone 60 mg orally, day 1+) for further evaluation. A total of 76 patients, mostly (>60%) refractory to at least one previous chemotherapy with maximum tolerated doses, and progression of metastatic melanoma were included; from the Institute of Pathology and the Department of Dermatology (University of Regensburg, Germany) 194 formalin-fixed paraffin-embedded metastatic tissues of 36 patients (47%) were available for further immunohistochemical analysis. The local ethic committee had approved the study (Table 22.3).

Prior to TMA-construction, H&E-stained slides of all specimens were evaluated by two dermatopathologists (T.V., S.M.) to identify representative metastatic areas. Clinical follow-up data with a median follow-up period of 9 months (range 1–43 months) were available for 35 melanoma patients (97%), i.e. 12 patients (33%) who received metronomic chemotherapy alone (arm A) and 23 patients (64%) with combined anti-inflammatory/angiostatic treatment (arm B). Median follow-up of censored patients was 7 months (range 2–43 months). Characteristic parameters of TMA-3 are given in Table 22.4.

Immunohistochemistry (IHC). Immunohistochemical studies utilized an avidin-biotin peroxidase method with a 3-amino-9-ethylcarbazole (AEC) chromatogen. After antigen retrieval (steam boiler with citrate-buffer, pH 6.0 for 20 min) immunohistochemistry was carried out applying the ZytoChemPlus HRP Broad Spectrum Kit (Zytomed Systems, Berlin, Germany) according to the manufacturer's instructions. The following primary antibodies were used: anti-COX2 (mouse monoclonal, Cayman Chemical, Ann Arbor, MI, USA; dilution 1:200, final concentration 2.5 μg/ml), anti-PPARG (rabbit monoclonal, Cell Signalling, New England Biolabs GmbH, Frankfurt am Main, Germany; dilution 1:400), anti-TP53 (mouse monoclonal IgG, clone Bp53–12 (sc-263), Santa Cruz Biotechnology Santa Cruz, CA; dilution 1:1,000), and anti-Ki-67 (rabbit monoclonal, clone MIB1; DakoCytomation GmbH, Hamburg, Germany; dilution 1:10, final concentration 5 μg/ml). As a positive control for COX2 and PPARG IHC, a colon carcinoma with known COX2 and PPARG expression was chosen. Normal tissue samples of ten different organs were considered as negative controls. Two pathologists (F.B., S.M.) performed a blinded evaluation of the stained slides. Cytoplasmic COX2 and nuclear PPARG immunoreactivity was estimated using an arbitrary semi-quantitative four-step scoring system (0–3+), based on the intensity of cytoplasmic COX2 staining [6] and the percentage of PPARG positive

Table 22.2 Clinico-pathologic parameters in relation to COX2 immunohistochemistry using TMA-2

		Cytoplasmic COX2 immunoreactivity						Nuclear PPARG immunoreactivity					
Variable	Categorization	n analyzable	0 (n)	1 + (n)	2 + (n)	3 + (n)	P^b	n analyzable	0 (n)	1 + (n)	2 + (n)	3 + (n)	P^b
Primary malignant melanomas													
Clark level	II	4	3	1	0	0	**0.004**	2	2	0	0	0	0,793
	III	14	3	6	3	2		12	11	1	0	0	
	IV	52	2	27	15	8		52	39	10	2	1	
	V	13	4	2	6	1		11	9	1	0	1	
Tumor thickness	≤2.0 mm	35	8	17	6	4	0.104	31	24	6	1	0	0,762
	>2.0 mm	49	4	20	18	7		47	37	7	1	2	
Growth pattern[a]	SSM	37	6	15	11	5	0.748	8	5	3	0	0	0,685
	LMM	3	2	0	1	0		36	29	6	1	0	
	NM	29	2	14	9	4		5	4	1	0	0	
	ALM	6	1	3	1	1		27	21	3	1	2	
	ONA	9	1	5	2	1		2	2	0	0	0	
TP53 immunoreactivity	<5%	67	11	28	20	8	0.308	63	49	10	2	2	0,883
	≥5%	15	0	8	4	3		15	12	3	0	0	
Ki-67 labeling index	<5%	68	11	29	18	10	0.295	64	53	9	1	1	0,101
	≥5%	14	0	7	6	1		14	8	4	1	1	
Melanoma metastases													
Lymph node		42	3	9	4	26	**0.013**	42	32	6	2	2	0,136
Skin		56	6	27	6	17		53	32	18	1	2	
Benign nevi													
Compound & junctional		47	39	7	1	0	**<0.001**	53	53	0	0	0	–
Dermal		21	15	6	0	0		45	45	0	0	0	
Congenital		51	4	45	2	0		50	50	0	0	0	

[a]SSM, superfical spreading melanoma; LMM = lentigo maligna melanoma; NM = nodular melanoma; ALM = akro-lentiginous melanoma; NOS, not otherwise specified

[b]Fisher's exact test (two-sided), bold face representing significant data

Table 22.3 Univariate analysis of factors regarding tumor recurrence and death

		Tumor recurrence (RFS)			Death (OS)		
Variable	Categorization	n[a]	Events	*P*[b]	n[a]	Events	*P*[b]
Age at diagnosis	≤60 years	48	25	0.7	48	7	0.6
	>60 years	40	18		40	7	
Gender	Female	39	15	0.06	39	5	0.4
	Male	49	28		49	9	
Clark level[c]	II	5	0	0.4	5	0	0.3
	III	15	8		15	2	
	IV	54	28		54	8	
	V	13	7		13	4	
Tumor thickness	≤2.0 mm	38	14	**0.03**	38	4	0.2
	>2.0 mm	50	29		50	10	
Ki67 labeling index	<5%	33	17	0.7	33	7	0.9
	≥5%	36	16		36	7	
Cytoplasmic COX2 IHC	score 0	12	2	**0.03**	12	0	0.1
	score 1+–3+	72	39		72	14	
Nuclear PPARG IHC	score 0	61	28	0.2	61	11	0.6
	score 1+–3+	17	10		17	2	

[a]Only initial and unifocal malignant melanomas were included;
[b]log rank test (two-sided), bold face representing significant data;
[c]according to UICC: TNM Classification of Malignant Tumours. 6th edn (2002) Sobin LH, Wittekind CH (eds.) Wiley, New York

cell nuclei [7]: 0 (negative): no cytoplasmic COX2 staining/PPARG staining 0% of cell nuclei; 1+: weak COX2 staining/PPARG staining 1–9%; 2+: moderate COX2 staining/ PPARG staining 10–50%; 3+: strong COX2 staining/ PPARG staining greater than 50%. Causes of non-interpretable results included lack of tumor tissue and presence of necrosis or crush artifact. The percentage of tumor cells with nuclear Ki-67 and TP53 staining was determined as described previously [27]. Ki-67/TP53 labeling was considered high if at least 5% of the tumor cells were positive.

Statistical analysis. Specimens on TMA-1 and TMA-2 were considered independently. Concerning TMA-3, COX2 and PPARG immunoreactivity were examined for a mean of 5 metastatic samples per patient (range 1–15); the median level of COX2 and PPARG immunoreactivity was chosen for further analyses using the SPSS version 16.0 (SPSS, Chicago, IL, USA). P-values <0.05 were considered significant. Contingency table analysis and two-sided Fisher's exact tests or X^2-tests were used to study statistical associations between clinico-pathological and immunohistochemical data. Retrospective overall and progression-free survival curves comparing patients with and without any of the variables were calculated using the Kaplan-Meier method, with significance evaluated by two-sided log rank statistics. For the analysis of progression-free survival, patients were censored at the time of their last progression-free clinical follow-up appointment. For the analysis of overall survival, patients were censored at the time of their last clinical follow-up appointment or at their date of death not related to the tumor. For multiple testing, the closed test principle was used (Table 22.5).

Table 22.4 Clinico-pathologic parameters in relation to COX2 and PPAR immunohistochemistry and results of univariate survival analysis using TMA-3

		Median COX2 immunoreactivity						Median PPARG immunoreactivity						Death (OS)			Tumor progression (PFS)		
Variable	Categorization	n analyzable	0(n)	1 + (n)	2 + (n)	3 + (n)	P^a	n analyzable	0(n)	1 + (n)	2 + (n)	3 + (n)	P^a	n	Events	P^b	n	Events	P^b
Advance melanoma patients																			
Age																			
	<60 years	12	0	3	8	1	0.146	12	8	4	0	0	1.000	12	7	0.152	12	11	0.163
	≥60 years	22	1	9	6	6		22	13	7	1	1		22	14		22	18	
Initial tumor stage																			
	pT1	3	0	0	1	2	**0.040**	3	1	1	0	1	0.588	2	1	0.690	2	2	**0.016**
	pT2	1	0	0	1	0		1	1	0	0	0		1	1		1	1	
	pT3	13	0	6	7	0		13	9	3	1	0		13	9		13	11	
	pT4	9	1	2	2	4		9	4	4	0	1		9	6		9	6	
	Melanoma in situ	1	0	0	1	0		1	1	0	0	0		1	0		1	1	
Initial regional lymph node status																			
	pN0	11	0	3	6	2	0.470	11	6	4	1	0	0.472	11	6	0.980	11	9	0.894
	pN1	10	0	4	4	2		10	7	2	0	1		9	8		9	8	
	pN2	6	0	2	2	2		6	3	3	0	0		6	4		6	5	
	pN3	2	1	0	0	1		2	1	0	0	1		2	1		2	1	
Study therapy																			
	A: trofosfamide	12	1	5	5	1	0.342	12	9	1	1	1	0.074	12	10	0.570	12	10	0.898
	B: trofosfamide + rofecoxib + pioglitazone	24	0	8	9	7		24	13	10	0	1		23	12		23	20	
CRP																			
	0	14	1	1	7	5	**0.004**	14	8	5	0	1	0.617	14	9	0.115	14	11	0.128
	1	10	0	7	3	0		10	7	2	1	0		10	10		10	10	

[a]Fisher's exact test (two-sided), bold face representing significant data;
[b]log-rank test (two-sided)

Table 22.5 Univariate analysis of factors regarding tumor progression and death using TMA

		Death			Tumor progression		
Variable	Categorization	n	Events	P^a	n	Events	P^a
Advance melanoma patients							
Age							
	<60 years	12	7	0.152	12	11	0.163
	≥60 years	22	14		22	18	
Initial tumor stage							
	pT1	2	1	0.690	2	2	**0.016**
	pT2	1	1		1	1	
	pT3	13	9		13	11	
	pT4	9	6		9	6	
	Melanoma in situ	1	0		1	1	
Initial regional lymph node status							
	pN0	11	6	0.980	11	9	0.894
	pN1	9	8		9	8	
	pN2	6	4		6	5	
	pN3	2	1		2	1	
Study therapy							
	A: trofosfamide	12	10	0.570	12	10	0.898
	B: trofosfamide + Rofecoxib + Pioglitazone	23	12		23	20	
Cytoplasmic COX2 IHC							
	Score 0 to 1+	14	10	0.505	14	13	0.338
	Score 2+ to 3+	21	12		21	17	
Nuclear PPARG IHC							
	Score 0	22	15	0.179	22	21	**0.044**
	Score 1+ to 3+	13	7		13	9	

[a]Log-rank test (two-sided)

22.3 Results

TMA-1. Investigation of COX2 and PPARG protein expression in 323 benign and malignant skin tumors using a comprehensive multi-tumor TMA (TMA-1) was informative in 57.6% (186/323) and 65.6% (212/323) of cases. COX2 and PPARG expression of any intensity (score 1+–3+) was detected in 81.7% (152/186) and 32.5% (69/212) of informative cases, respectively. Table 22.1 summarizes the expression data and statistical analysis of COX2 and PPARG immunoreactivity of each skin tumor entity on TMA-1. For connective tissue tumors (Kaposi sarcoma, capillary hemangioma, benign histiocytoma) no significant differences could be found in benign versus malignant tumors ($P = 0.61$ and $P = 0.13$). Regarding epithelial tumors (squamous cell carcinomas, basal cell carcinomas) positive PPARG staining was detected significantly more often in basal cell carcinomas than in squamous cell carcinomas ($P = 0.001$). Surprisingly, 86.9% of benign skin adnexal tumors (sebaceous adenomas) were positive for COX2; 21.7% positive for PPARG. Regarding melanocytic lesions, 100% (38/38) of primary melanomas and 78.9% (15/19) of benign nevi revealed at least weak COX2 immunoreactivity (score 1+–3+); 48.7% (20/41) of primary melanomas and 8.3% (2/24) of benign nevi demonstrated PPARG positivity (1+–2+). Accordingly, compared to benign nevi, expression of both COX2 and PPARG was significantly increased in primary melanomas ($P = 0.02$ and $P = 0.001$).

Besides skin tumors, COX2 and PPARG expression was analyzed in many other benign and malignant tissue types from 46 different organs using a comprehensive multi-tumor TMA-1. As shown in Tables 22.6 and 22.7, differential COX2 and PPARG expression between normal and neoplastic tissue could be observed for almost every tissue type investigated. In prostate cancer, for example, COX2 expression continuously increased from prostatic hyperplasia to prostatic intraepithelial neoplasia (PIN) to organ-confined prostate cancer to hormone-refractory prostate cancer to metastatic disease (supplemental Fig. 22.5 and 22.6).

TMA-2. Based on the results of TMA-1, a second TMA (TMA-2) with clinical follow-up data sampling primary malignant melanomas and melanoma metastases as well as benign nevi was constructed. COX2 and PPARG immunoreactivity was informative in 86.0% (301/350) and 91.7% (321/350) of cases, respectively. Expression of COX2 and PPARG of any intensity was detected in 73.8% (222/301) and in 15.0% (48/321) of informative cases. Representative negative and positive COX2 and PPARG immunostaining patterns in malignant melanoma are shown in Fig. 22.1a–d. Figure 22.2a and b summarize the results of COX2 and PPARG IHC for primary melanomas, metastases and nevi on TMA-2. The percentage of COX2 positive cases significantly increased from benign nevi (51%) to primary melanomas (86%) and melanoma metastases (91%; $P < 0.001$; Fig. 22.2a). Likewise, PPARG immunoreactivity significantly increased from benign nevi (0%) to malignant melanomas (22%) and melanoma metastases (33%; $P < 0.001$; Fig. 22.2b). Clinico-pathologic variables of melanoma patients were correlated with COX2 and

Table 22.6 Frequency of COX2 protein expression in 132 human tumor types

Tumor type	No. of tumors analyzed	COX2 protein expression			
		Negative (%)	Weak (%)	Moderate (%)	Strong (%)
Adrenal gland					
Adrenal gland adenoma	13	0	7.7	7.7	84.6
Adrenal gland carcinoma	6	0	0	0	100.0
Pheochromocytoma	27	0	33.3	48.1	18.5
Anus					
Anus, squamous cell cancer	3	33.3	33.3	33.3	0
Brain					
Cerebrum, grey substance, normal	5	40.0	20.0	40.0	0
Cerebrum, white substance, normal	5	100.0	0	0	0
Meningeoma	42	71.4	26.2	2.4	0
Ependymoma	9	11.1	11.1	66.7	11.1
Astrocytoma	30	10.0	46.7	30.0	13.3
Glioblastoma multiforme	34	23.5	47.1	20.6	8.8
Oligodendroglioma	17	17.6	17.6	58.8	5.9
Medulloblastoma	4	0	25.0	75.0	0
Esthesioneuroblastoma	2	0	0	100.0	0
Breast					
Breast, normal	3	0	0	0	100.0
Breast, ductal cancer	43	11.6	58.1	23.3	7.0
Breast, lobular cancer	30	16.7	53.3	26.7	3.3
Breast, medullary cancer	25	4.0	36.0	48.0	12.0
Breast, tubular cancer	16	37.5	56.3	6.3	0
Breast, mucinous cancer	23	47.8	26.1	13.0	13.0
Breast, apocrine cancer	3	33.3	0	66.7	0
Breast, cribirform cancer	5	40.0	40.0	20	0
Breast, Phylloides tumor	12	16.7	83.3	0	0
Colon					
Colon, mucosa, normal	2	50.0	50.0	0	0
Colon adenoma, mild dysplasia	31	16.1	58.1	22.6	3.2
Colon adenoma, moderate dysplasia	33	18.2	54.5	18.2	9.1
Colon adenoma, severe dysplasia	23	26.1	43.5	30.4	0
Colon, adenocarcinoma	40	10.0	55.0	32.5	2.5
Endometrium					
Endometrium, normal	6	0	33.3	33.3	33.3
Endometrium, endometroid carcinoma	39	2.6	33.3	53.8	10.3
Endometrium, serous carcinoma	13	7.7	30.8	46.2	15.4
Esophagus					
Esophagus, normal tissue	6	83.3	16.7	0	0
Esophagus, adenocarcinoma	6	0	66.7	16.7	16.7

(continued)

Table 22.6 (continued)

Tumor type	COX2 protein expression				
	No. of tumors analyzed	Negative (%)	Weak (%)	Moderate (%)	Strong (%)
Esophagus, squamous cell carcinoma	26	3.8	42.3	26.9	26.9
Esophagus, small cell carcinoma	1	0	0	0	100.0
Fat tissue					
Liposarcoma	26	26.9	50.0	23.1	0
Gall bladder					
Gall bladder, normal	4	33.3	66.7	0	0
Gall bladder, adenocarcinoma	18	5.6	22.2	55.6	16.7
GIT					
GIST	28	17.9	57.1	10.7	14.3
Hematologic (n = 6)					
AML	1	0	0	100	0
CML	4	0	25	25	50
Kidney					
Kidney, cortex, normal	5	0	0	20.0	80.0
Kidney, clear cell cancer	46	2.2	32.6	54.3	10.9
Kidney, papillary cancer	34	8.1	16.2	51.4	24.3
Kidney, chromophobe cancer	13	7.7	30.8	30.8	30.8
Kidney, oncocytoma	7	0	0	14.3	85.7
Larynx					
Larynx, squamous cell carcinoma	32	37.5	40.6	15.6	6.3
Liver					
Liver, normal	2	0	0	0	100.0
Hepatocellular carcinoma	29	0	6.9	6.9	86.2
Lung					
Lung, normal	4	0	0	0	100.0
Lung, squamous cell carcinoma	43	2.3	55.8	27.9	14.0
Lung, adenocarcinoma	47	6.4	59.6	27.7	6.4
Lung, large cell cancer	43	9.3	32.6	37.2	20.9
Lung, small cell cancer	39	17.9	53.8	25.6	2.6
Lymphatic tissue					
NHL, diffuse large B	16	0	25.0	68.8	6.3
MALT lymphoma	22	0	27.3	68.2	4.5
Hodgkin lymphoma, mixed cell	13	15.4	23.1	23.1	38.5
Hodgkin lymphoma, nodular sclerosis	23	13.0	34.8	26.1	26.1
Lymph node					
Lymph node, normal	3	0	33.3	66.7	0
NHL, others	15	0	53.3	33.3	13.3
Mouth					
Mouth, normal	8	0	0	0	8
Oral cavity, squamous cell carcinoma	36	33.3	36.1	25.0	5.6

(continued)

Table 22.6 (continued)

Tumor type	COX2 protein expression				
	No. of tumors analyzed	Negative (%)	Weak (%)	Moderate (%)	Strong (%)
Myometrium					
Leiomyoma	52	59.6	40.4	0	0
Nerve tissue					
Neurofibroma	26	69.2	30.8	0	0
Ovary					
Ovary, normal	4	75.0	0	0	25.0
Ovary, serous cancer	41	0	56.1	39.0	4.9
Ovary, mucinous cancer	14	14.3	35.7	35.7	14.3
Ovary, endometroid cancer	41	4.9	80.5	12.2	2.4
Ovary, dysgerminoma	2	0	0	50.0	50.0
Ovary, yolk sack tumor	1	0	0	0	100.0
Ovary, undifferentiated carcinoma	1	0	0	100.0	0
Ovary, Brenner tumor	9	44.4	44.4	11.1	0
Pancreas					
Pancreas, normal tissue	9	0	11.1	55.6	33.3
Pancreas, adenocarcinoma	39	2.6	23.1	46.2	28.2
Parathyroid					
Parathyroid, normal	3	0	33.3	33.3	33.3
Parathyroid, adenoma	15	0	26.7	26.7	46.7
Parotis					
Parotis, normal	5	0	0	0	100.0
Salivary gland, small cell cancer	1	0	0	100.0	0
Salivary gland, squamous cell cancer	2	0	100.0	0	0
Salivary gland, unclassified carcinoma	1	0	0	100.0	0
Salivary gland, undifferentiated carcinoma	6	33.3	33.3	16.7	16.7
Penis					
Skin, penis normal	3	100	0	0	0
Penile carcinoma	33	0	54.5	33.3	12.1
Pharynx					
Pharynx, lymphoepithelial carcinoma	4	0	0	50.0	50.0
Pituitary					
Craniopharyngeoma	4	25.0	75.0	0	0
Pleura (n = 28)					
Malignant mesothelioma	14	7.1	35.7	21.4	35.7
Prostate (n = 134)					
Prostate cancer, untreated	45	6.7	37.8	44.4	11.1
Prostate cancer, hormone refractory	30	0	23.3	26.7	50.0
Salivary gland (n = 153)					
Salivary gland, adenolymphoma	29	0	17.2	44.8	37.9

(continued)

Table 22.6 (continued)

Tumor type	COX2 protein expression				
	No. of tumors analyzed	Negative (%)	Weak (%)	Moderate (%)	Strong (%)
Salivary gland, pleomorphic adenoma	43	7.0	65.1	27.9	0
Salivary gland, cylindroma	41	7.3	36.6	53.7	2.4
Salivary gland, mucoepidermoid cancer	5	60.0	40.0	0	0
Salivary gland, adenocarcinoma	1	0	0	100.0	0
Salivary gland, acinus cell cancer	5	40.0	40.0	20.0	0
Skeletal muscle (n = 26)					
Rhabdomyosarcoma	10	0	20.0	40.0	40.0
Skin (n = 359)					
Skin, normal	3	100.0	0	0	0
Skin, basalioma	31	22.6	51.6	22.6	3.2
Skin, squamous cell cancer	31	10.0	33.3	36.7	20.0
Skin, Merkel cell cancer	3	0	0	33.3	66.7
Skin, malignant melanoma	38	0	42.1	44.7	13.2
Skin, benign nevus	19	21.1	36.8	42.1	0
Benign histiocytoma	16	50.0	43.8	6.3	0
Dermatofibroma protuberans	1	0	0	0	1
Kapillary hemangioma	14	21.4	64.3	14.3	0
Kaposi Sarcoma	15	40.0	53.3	6.7	0
Skin appendix (n = 32)					
Skin, benign appendix tumor	23	13.0	56.5	30.4	0
Small intestine (n = 20)					
Small intestine, normal	3	33.3	33.3	33.3	0
Small intestine, adenocarcinoma	9	11.1	44.4	44.4	0
Smooth muscle (n = 40)					
Leiomyosarcoma	31	9.7	87.1	3.2	0
Soft tissue (n = 156)					
Paraganglioma	7	0	28.6	42.9	28.6
Lipoma	18	72.2	22.2	5.6	0
Malignant fibrous histiocytoma	23	34.8	47.8	17.4	0
Fibrosarcoma	8	0	50.0	50.0	0
Synovial sarcoma	2	50.0	50.0	0	0
Alveolar sarcoma	1	0	0	100.0	0
Epitheloid hemangioma	1	0	0	0	100.0
Epitheloid Sarcoma	2	0	50.0	50.0	0
Hemangiopericytoma	5	0	40.0	60.0	0
Glomus tumor	5	20.0	20.0	60.0	0
Angiosarcoma	3	33.3	33.3	0	33.3
Ganglioneuroma	2	0	50.0	50.0	0
Granular cell tumor	5	0	60.0	40.0	0
PNET	15	0	26.7	40.0	33.3
Angiomyolipoma	1	0	100.0	0	0

(continued)

Table 22.6 (continued)

Tumor type	COX2 protein expression				
	No. of tumors analyzed	Negative (%)	Weak (%)	Moderate (%)	Strong (%)
Stomach					
Stomach, normal	3	0	0	66.7	33.3
Stomach, diffuse adenocarcinoma	22	4.5	59.1	27.3	9.1
Stomach, intestinal adenocarcinoma	39	5.1	25.6	64.1	5.1
Tendon sheet					
Tendon sheet, giant cell tumor	23	13.0	60.9	21.7	4.3
Testis					
Testis, normal	5	0	0	100.0	0
Testis, seminoma	48	0	22.9	33.3	43.8
Testis, non-seminomatous cancer	52	7.7	28.8	51.9	11.5
Testis, mixed cancer	2	0	0	100.0	0
Testis, teratoma	5	0	0	100.0	0
Thymus					
Thymus, normal	2	50.0	0	50.0	0
Thymoma	18	0	66.7	33.3	0
Thyroid					
Thyroid, normal	3	33.3	33.3	33.3	0
Thyroid, adenoma	38	2.6	13.2	42.1	42.1
Thyroid, follicular cancer	46	4.3	15.2	17.4	63.0
Thyroid, papillary cancer	32	0	15.6	43.8	40.6
Thyroid, anaplastic cancer	5	0	20.0	40.0	40.0
Thyroid, medullary cancer	9	22.2	22.2	11.1	44.4
Urinary bladder					
Urinary bladder, normal	1	0	100.0	0	0
Urinary bladder cancer, non-invasive urothelial cancer	29	13.8	65.5	20.7	0
Urinary bladder cancer, invasive urothelial cancer	30	0	30.0	36.7	33.3
Urinary bladder, squamous cell cancer	5	20.0	0	40.0	40.0
Urinary bladder, small cell cancer	3	33.3	0	0	66.7
Urinary bladder, sarcomatoid cancer	8	25.0	25.0	25.0	25.0
Urinary bladder, adenocarcinoma	3	0	66.7	33.3	0
Uterus					
Uterus, carcinosarcoma	6	0	33.3	66.7	0
Endometrioid stroma sarcoma	3	0	100.0	0	0
Uterus, cervix					
Cervix, normal	1	100.0	0	0	0
Uterus, cervix, cervical intraepithelial neoplasia, grade 3	9	77.8	22.2	0	0
Uterus, cervix, squamous cell carcinoma	17	17.6	29.4	41.2	11.8

(continued)

Table 22.6 (continued)

Tumor type	COX2 protein expression				
	No. of tumors analyzed	Negative (%)	Weak (%)	Moderate (%)	Strong (%)
Uterus, cervix, adenocarcinoma	2	50.0	0	0	50.0
Vagina					
Vagina, squamous cell carcinoma	3	0	100.0	0	0
Vulva					
Vulva, squamous cell cancer	32	12.5	37.5	31.3	18.8
ZNS					
Malignant Schwannoma	7	14.3	42.9	28.6	14.3
Schwannoma	37	45.9	35.1	18.9	0

PPARG expression (Table 22.2). In primary melanomas, positive COX2 immunoreactivity was significantly related to advanced Clark levels ($P = 0.004$), but no other clinico-pathologic variables such as tumor growth pattern, p53 immunoreactivity and Ki-67 labeling index. Skin metastases demonstrated a gradually weaker COX2 immunoreactivity compared with lymph node metastases ($P = 0.013$). Among the various types of benign nevi on TMA-2, COX2 expression was significantly increased in congential nevi compared to compound, junctional and dermal melanocytic nevi ($P < 0.001$).

According to a univariate analysis, tumor progression was significantly related to both melanoma thickness and COX2 immunoreactivity, respectively ($P = 0.03$; Table 22.3); i.e. expression of COX2 was associated with shorter progression-free survival ($P = 0.03$; Fig. 22.3). In contrast, PPARG expression of primary melanomas was not associated with any of the variables neither the clinico-pathologic ones nor progression-free and overall survival (Tables 22.2 and 22.3).

TMA-3. Using TMA-3, the prognostic and therapeutic meaning of COX2 and PPARG expression was analyzed in patients with advanced metastatic melanoma disease (n = 36). All patients received angiostatic biomodulatory treatment with trofosfamide alone (arm A, n = 12) or in combination with rofecoxib and pioglitazone (arm B, n = 24). COX2 and PPARG protein expression of metastatic tissues was informative in all 36 cases. Expression of COX2 and PPARG of any intensity was detected in 97.2% (35/36) and in 38.9% (14/36) of patients, respectively. Clinico-pathologic variables of this cohort of patients with advanced metastatic melanoma disease were compared relative to COX2 and PPARG expression (Table 22.5).

Considering all 36 patients receiving biomodulatory therapy expression of PPARG (score 1+–3+) in the metastases was significantly associated with longer progression-free survival ($P = 0.044$) but not with overall survival ($P = 0.179$; Fig. 22.4a and b). Expression of COX2 (score 2+–3+) in the metastases, however, was not associated with overall and progression-free survival, respectively (Fig. 22.4c and d).

Table 22.7 Frequency of PPARG protein expression in 132 human tumor types

	PPARG protein expression				
Tumor type	No. of tumors analyzed	Negative (%)	Weak (%)	Moderate (%)	Strong (%)
Adrenal gland					
Adrenal gland adenoma	13	23.1	46.2	30.8	0
Adrenal gland carcinoma	6	33.3	50.0	16.7	0
Pheochromocytoma	27	22.2	37.0	14.8	25.9
Anus					
Anus, squamous cell cancer	3	66.7	0	0	33.3
Brain					
Cerebrum, grey substance, normal	12	100.0	0	0	0
Cerebrum, white substance, normal	4	100.0	0	0	0
Meningeoma	40	82.5	15.0	2.5	0
Ependymoma	10	90.0	10.0	0	0
Astrocytoma	28	92.9	3.6	3.6	0
Glioblastoma multiforme	34	50.0	32.4	14.7	2.9
Oligodendroglioma	15	60.0	40.0	0	0
Medulloblastoma	4	100.0	0	0	0
Esthesioneuroblastoma	2	100.0	0	0	0
Breast					
Breast, normal	4	25.0	75.0	0	0
Breast, ductal cancer	46	52.2	34.8	6.5	6.5
Breast, lobular cancer	32	92.9	4.8	2.4	0
Breast, medullary cancer	26	46.2	46.2	7.7	0
Breast, tubular cancer	21	61.9	14.3	19.0	4.8
Breast, mucinous cancer	26	65.4	30.8	3.8	0
Breast, apocrine cancer	3	66.7	33.3	0	0
Breast, cribirform cancer	6	50.0	33.3	16.7	0
Breast, Phylloides tumor	12	91.7	8.3	0	0

Colon					
Colon, mucosa, normal	2	50.0	50.0	0	0
Colon adenoma, mild dysplasia	33	63.6	36.4	0	0
Colon adenoma, moderate dysplasia	40	67.5	27.5	0	5.0
Colon adenoma, severe dysplasia	27	44.4	55.6	0	0
Colon, adenocarcinoma	42	28.6	23.8	45.2	2.4
Endometrium					
Endometrium, normal	7	57.1	42.9	0	0
Endometrium, endometroid carcinoma	42	31.0	59.5	9.5	0
Endometrium, serous carcinoma	17	35.3	52.9	5.9	5.9
Esophagus					
Esophagus, normal tissue	9	88.9	0	0	11.1
Esophagus, adenocarcinoma	7	57.1	28.6	0	14.3
Esophagus, squamous cell carcinoma	30	63.3	13.3	6.7	16.7
Esophagus, small cell carcinoma	1	100.0	0	0	0
Fat tissue					
Liposarcoma	26	84.6	33.8	0	11.5
Gall bladder					
Gall bladder, normal	6	100.0	0	0	0
Gall bladder, adenocarcinoma	23	69.6	26.1	0	4.3
GIT					
GIST	28	64.3	0	14.3	21.4
Hematologic					
AML	1	100.0	0	0	0
CML	3	33.3	33.3	0	33.3

(continued)

Table 22.7 (continued)

Tumor type	PPARG protein expression				
	No. of tumors analyzed	Negative (%)	Weak (%)	Moderate (%)	Strong (%)
Kidney					
Kidney, cortex, normal	7	71.4	28.6	0	0
Kidney, clear cell cancer	48	27.1	31.3	27.1	14.6
Kidney, papillary cancer	44	18.2	25.0	34.1	22.7
Kidney, chromophobe cancer	13	23.1	23.1	46.2	7.7
Kidney, oncocytoma	7	14.3	14.3	0	71.4
Larynx					
Larynx, squamous cell carcinoma	38	92.1	7.9	0	0
Liver					
Liver, normal	13	15.4	38.5	38.5	7.7
Hepatocellular carcinoma	31	25.8	25.8	41.9	6.5
Lung					
Lung, normal	12	33.3	41.7	8.3	16.7
Lung, squamous cell carcinoma	50	42.0	24.0	30.0	4.0
Lung, adenocarcinoma	48	39.6	25.0	22.9	12.5
Lung, large cell cancer	45	44.4	17.8	26.7	11.1
Lung, small cell cancer	39	84.6	12.8	2.6	0
Lymphatic tissue					
NHL, diffuse large B	17	52.9	29.4	5.9	11.8
MALT lymphoma	23	47.8	21.7	17.4	13.0
Hodgkin lymphoma, mixed cell	15	60.0	20.0	13.3	6.7
Hodgkin lymphoma, nodular sclerosis	30	66.7	13.3	3.3	16.7
Lymph node					
Lymph node, normal	13	100.0	0	0	0
NHL, others	18	66.7	11.1	16.7	5.6

Mouth					
Mouth, normal	9	100.0	0	0	0
Oral cavity, squamous cell carcinoma	40	90.0	5.0	2.5	2.5
Myometrium					
Leiomyoma	58	89.7	5.2	3.4	1.7
Nerve tissue					
Neurofibroma	25	100	0	0	0
Ovary					
Ovary, normal	4	100	0	0	0
Ovary, serous cancer	45	64.4	15.6	8.9	11.1
Ovary, mucinous cancer	21	19.0	52.4	9.5	19.0
Ovary, endometroid cancer	46	67.4	17.4	6.5	8.7
Ovary, dysgerminoma	2	0	50.0	50.0	0
Ovary, yolk sack tumor	1	0	100.0	0	0
Ovary, undifferentiated carcinoma	1	0	0	100.0	0
Ovary, Brenner tumor	9	55.6	22.2	22.2	0
Pancreas					
Pancreas, normal tissue	9	88.9	0	11.1	0
Pancreas, adenocarcinoma	45	51.1	24.4	13.3	11.1
Parathyroid					
Parathyroid, normal	4	75.0	25.0	0	0
Parathyroid, adenoma	17	20.0	53.3	13.3	13.3
Parotis					
Parotis, normal	5	100.0	0	0	0
Salivary gland, small cell cancer	1	100.0	0	0	0
Salivary gland, squamous cell cancer	2	100.0	0	0	0
Salivary gland, unclassified carcinoma	1	0	0	0	100.0
Salivary gland, undifferentiated carcinoma	4	100.0	0	0	0

(continued)

Table 22.7 (continued)

Tumor type	PPARG protein expression				
	No. of tumors analyzed	Negative (%)	Weak (%)	Moderate (%)	Strong (%)
Penis					
Skin, penis normal	4	75.0	25.0	0	0
Penile carcinoma	36	50.0	25.0	22.2	2.8
Pharynx					
Pharynx, lymphoepithelial carcinoma	4	75.0	25.0	0	0
Pituitary					
Craniopharyngeoma	4	75.0	25.0	0	0
Pleura					
Malignant mesothelioma	18	66.7	33.3	0	0
Prostate					
Prostate cancer, untreated	47	59.6	25.5	2.1	12.8
Prostate cancer, hormone refractory	34	67.6	23.5	0	8.8
Salivary gland					
Salivary gland, adenolymphoma	29	13.8	86.2	0	0
Salivary gland, pleomorphic adenoma	49	69.4	22.4	6.1	2.0
Salivary gland, cylindroma	44	88.6	4.5	6.8	0
Salivary gland, mucoepidermoid cancer	5	40.0	20.0	20.0	20.0
Salivary gland, adenocarcinoma	1	100.0	0	0	0
Salivary gland, acinus cell cancer	4	100.0	0	0	0
Skeletal muscle					
Rhabdomyosarcoma	10	50.0	50.0	0	0
Skin					
Skin, normal	10	90.0	0	0	10.0
Skin, basalioma	33	33.3	63.6	3.0	0
Skin, squamous cell cancer	33	69.7	21.2	9.1	0
Skin, Merkel cell cancer	4	100.0	0	0	0

Skin, malignant melanoma	41	51.2	19.5	29.3	0
Skin, benign nevus	24	91.7	8.3	0	0
Benign histiocytoma	22	86.4	4.5	9.1	0
Dermatofibroma protuberans	1	100.0	0	0	0
Kapillary hemangioma	18	88.9	11.1	0	0
Kaposi sarcoma	18	72.2	27.8	0	0
Skin appendix					
Skin, benign appendix tumor	23	78.3	17.4	4.3	0
Small intestine					
Small intestine, normal	3	100.0	0	0	0
Small intestine, adenocarcinoma	11	36.4	18.2	18.2	27.3
Smooth muscle					
Leiomyosarcoma	35	60.0	22.9	5.7	11.4
Soft tissue					
Paraganglioma	7	14.3	85.7	0	0
Lipoma	22	100.0	0	0	0
Malignant fibrous histiocytoma	22	81.8	9.1	0	9.1
Fibrosarcoma	8	88.9	11.1	0	0
Synovial sarcoma	2	50.0	50.0	0	0
Alveolar sarcoma	1	0	0	100.0	0
Epitheloid hemangioma	1	100.0	0	0	0
Epitheloid sarcoma	2	100.0	0	0	0
Hemangiopericytoma	4	75.0	0	0	25.0
Glomus tumor	5	80.0	20.0	0	0
Angiosarcoma	3	100.0	0	0	0
Ganglioneuroma	7	100.0	0	0	0

(continued)

Table 22.7 (continued)

Tumor type	PPARG protein expression				
	No. of tumors analyzed	Negative (%)	Weak (%)	Moderate (%)	Strong (%)
Granular cell tumor	6	83.3	0	0	16.7
PNET	14	85.7	0	7.1	7.1
Angiomyolipoma	1	0	0	0	100.0
Stomach					
Stomach, normal	6	66.7	33.3	0	0
Stomach, diffuse adenocarcinoma	22	54.5	13.6	27.3	4.5
Stomach, intestinal adenocarcinoma	40	25.0	22.5	30.0	22.5
Tendon sheet					
Tendon sheet, giant cell tumor	24	54.2	29.2	8.3	8.3
Testis					
Testis, normal	11	27.3	72.7	0	0
Testis, seminoma	49	26.5	57.1	16.3	0
Testis, non-seminomatous cancer	55	47.3	34.5	16.4	1.8
Testis, mixed cancer	2	0	0	0	100.0
Testis, teratoma	4	66.7	16.7	0	16.7
Thymus					
Thymus, normal	5	100.0	0	0	0
Thymoma	19	42.1	36.8	5.3	15.8
Thyroid					
Thyroid, normal	3				
Thyroid, adenoma	42	85.7	7.1	4.8	2.4
Thyroid, follicular cancer	48	45.8	37.5	14.6	2.1
Thyroid, papillary cancer	35	77.1	17.1	2.9	2.9
Thyroid, anaplastic cancer	5	80.0	0	0	20.0
Thyroid, medullary cancer	9	55.6	33.3	11.1	0

Urinary bladder					
Urinary bladder, normal	2	0	100.0	0	0
Urinary bladder cancer, non-invasive urothelial cancer	31	67,7	22.6	9,7	0
Urinary bladder cancer, invasive urothelial cancer	36	25,0	33.3	27.8	13.9
Urinary bladder, squamous cell cancer	5	60.0	40.0	0	0
Urinary bladder, small cell cancer	5	100.0	0	0	0
Urinary bladder, sarcomatoid cancer	8	62.5	25.0	12.5	0
Urinary bladder, adenocarcinoma	3	66.7	0	0	33.3
Uterus					
Uterus, carcinosarcoma	6	33.3	50.0	16.7	0
Endometrioid stroma sarcoma	3	100.0	0	0	0
Uterus, cervix					
Cervix, normal	4	75.0	25.0	0	0
Uterus, cervix, cervical intraepithelial neoplasia, grade 3	13	100.0	0	0	0
Uterus, cervix, squamous cell carcinoma	26	57.7	34.6	7.7	0
Uterus, cervix, adenocarcinoma	2	100.0	0	0	0
Vagina					
Vagina, squamous cell carcinoma	3	33.3	66.7	0	0
Vulva					
Vulva, squamous cell cancer	32	19.4	69.4	11.1	0
ZNS					
Malignant schwannoma	8	87.5	12.5	0	0
Schwannoma	35	80.0	17.1	2.9	0

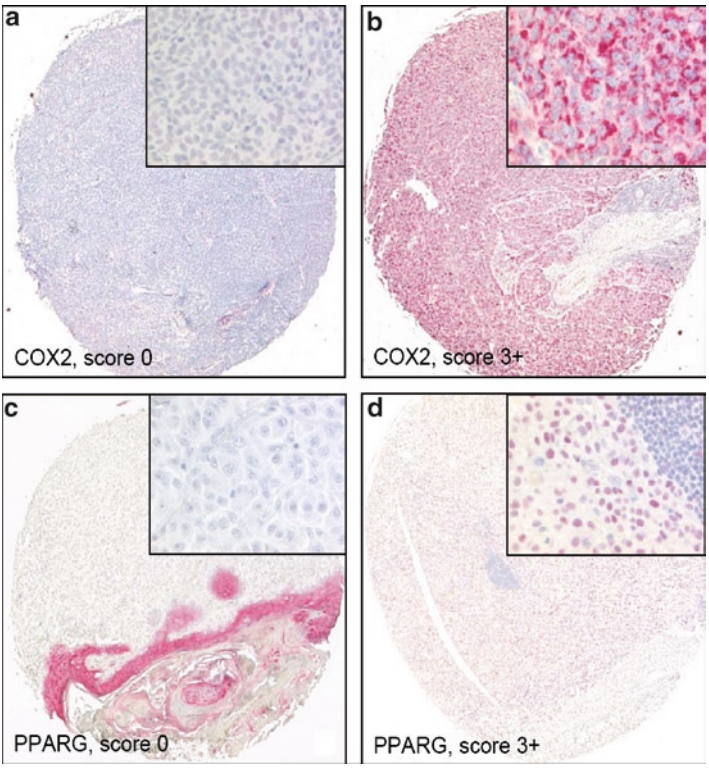


Fig. 22.1 (**a**–**d**) Immunohistochemical COX2 and PPARG staining of malignant melanomas on TMA-2. Original magnification 10x (insets 200x). Representative examples of a primary malignant melanoma with negative (**a**) and strong (**b**) immunoreactivity for COX2. Representative examples of a primary malignant melanoma with negative (**c**) and strong (**d**) immunoreactivity for PPARG

22.4 Discussion

In this study, we demonstrate by a comprehensive multi-tumor TMA that COX2 and PPARG are differentially expressed in a broad spectrum of normal and malignant tissues. Focussing on tumors of the skin we can further confirm that COX2 immunoreactivity of primary MM is significantly associated with advanced Clark levels ($P = 0.004$) and shorter recurrence-free survival ($P = 0.03$). PPARG expression of primary MM, however, does not provide significant prognostic information. Yet, by analysis of COX2 and PPARG expression in MM metastases of patients who had received biomodulatory therapy, we can show that only the expression of PPARG is significantly associated with longer progression-free survival ($P = 0.044$). Hence, our study confirms the prognostic meaning of COX2 in patients with primary MM

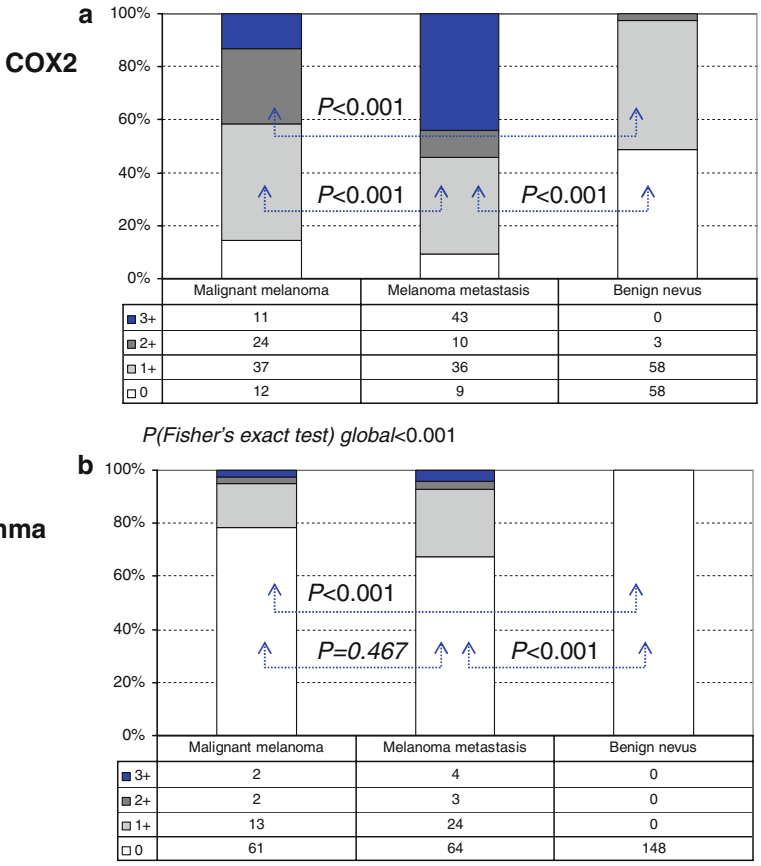
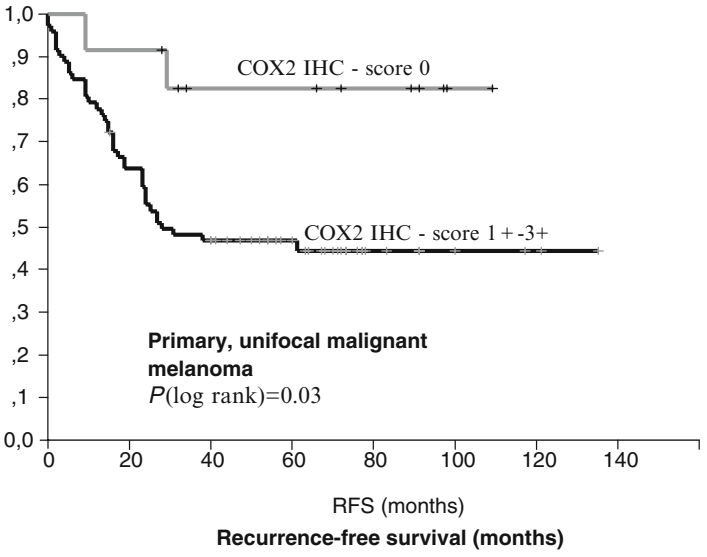


Fig. 22.2 (**a**, **b**) Cumulative bar charts of COX2 (**a**) and PPARG (**b**) immunoreactivity in melanocytic skin tumors using TMA-2

and adds a new late-stage histolpathological marker, PPARG, which may be predictive for responsiveness to biomodulatory therapy in advanced metastatic MM. To our knowledge this is the first TMA study demonstrating that PPARG protein expression may be a positive prognostic marker indicating responsiveness to stroma-targeted therapy in the late metastatic stage (IV) of MM disease, i.e. in patients refractory to conventional first-line chemotherapy, mostly with dacarbacine (Fig. 22.4).

Consistent with previously published data on melanocytic skin lesions [6, 7] our immunohistochemical analysis of benign nevi, primary MM and MM metastases show that COX2 and PPARG immunoreactivity significantly increases from benign nevi to primary MM and MM metastases. In other organs, however, e.g. in primary cancers of the lung versus normal lung tissues, decreased expression levels of PPARG were found and associated with poor prognosis [16]. At first sight, these finding are in contrast to the upregulation of PPARG in primary MM and MM metastases versus benign nevi observed with TMA-2. But, as our data also show, this upregulation



No. of melanoma patients at risk:

Time (months)	0	20	40	60	80	100	120
score 0	12	11	7	7	5	1	0
score 1 +-3+	72	45	33	22	7	4	2

Fig. 22.3 Distribution of time (months) to tumor-related death among patients with primary malignant melanomas showing negative (0) or positive (1+ to 3+) COX2 immunoreactivity as estimated by the Kaplan Meier method

does not correlate with the outcome of MM patients indicating a distinct role of PPARG in primary MM and MM metastases. Notably, in the advanced metastatic stages of MM enclosed in this study, patients with PPARG-positive metastases versus PPARG-negative metastases show a significant survival benefit concerning progression-free survival ($P = 0.044$) *not* dependent on whether angiostatically scheduled metronomic chemotherapy (trofosfamide) was administered alone or in combination with pioglitazone (PPARG agonist) and rofecoxib (COX2 inhibitor) as additional biomodulatory therapy. Considering PPARG or COX2 as candidate substrates for targeted cancer therapy, it could be assumed that only patients with PPARG- or COX2-positive metastases and additional PPARG-agonistic or COX2-inhibitory therapy would show a survival benefit compared with patients treated with metronomic chemotherapy alone. Yet, subgroup analysis with TMA-3 did not show a significant survival benefit for these patients. Thus, our study supports current concepts that targeting COX2 and PPAR is more a tumor-stroma effective approach than an approach depending on the status of target expression of the tumor itself [21,22]. Possible explanations of this paradoxon are multifaceted and complex. There may be numerous “off-target” effects of the involved drugs, e.g. modulation of COX2/PPARG-independent pathways [16,18,21]. According to the paradigm of biomodulatory stroma targeting approaches [21,28] the effects may be

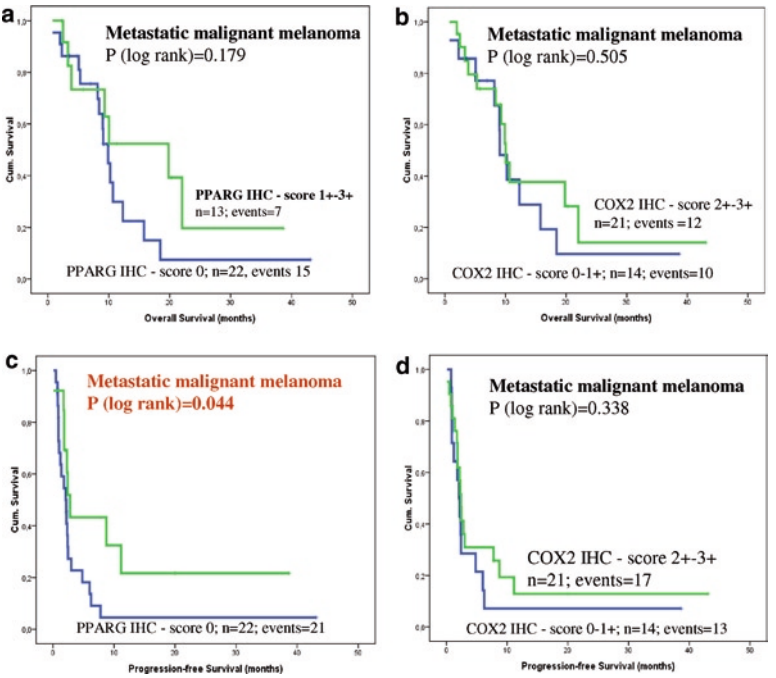


Fig. 22.4 (**a**–**d**) Distribution of time (months) to death and tumor progression among patients with advanced metastatic melanomas in correlation with immunoreactivity of PPARG (**a**, **b**) or COX2 (**c**, **d**). All patients received biomodulatory treatment. The calculation was performed according to the method of Kaplan and Meier

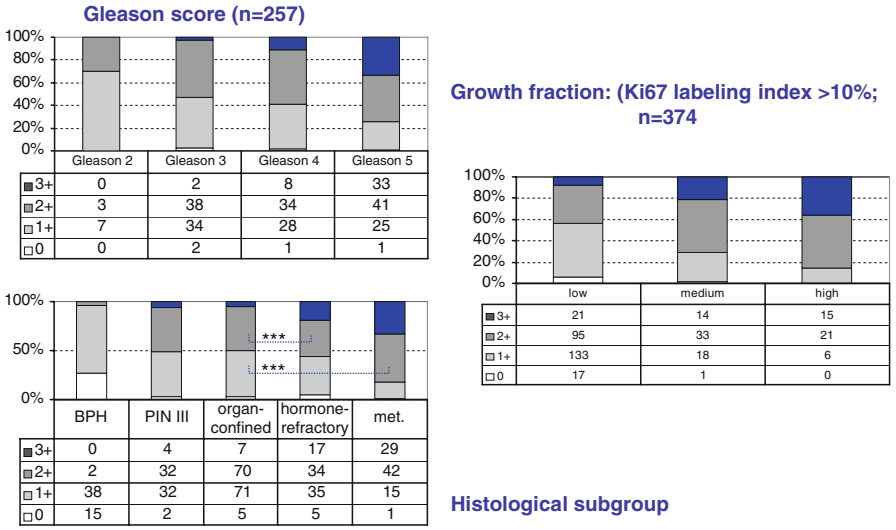


Fig. 22.5 Prostate cancer: COX-2 Expression during malignant progression using TAM-1

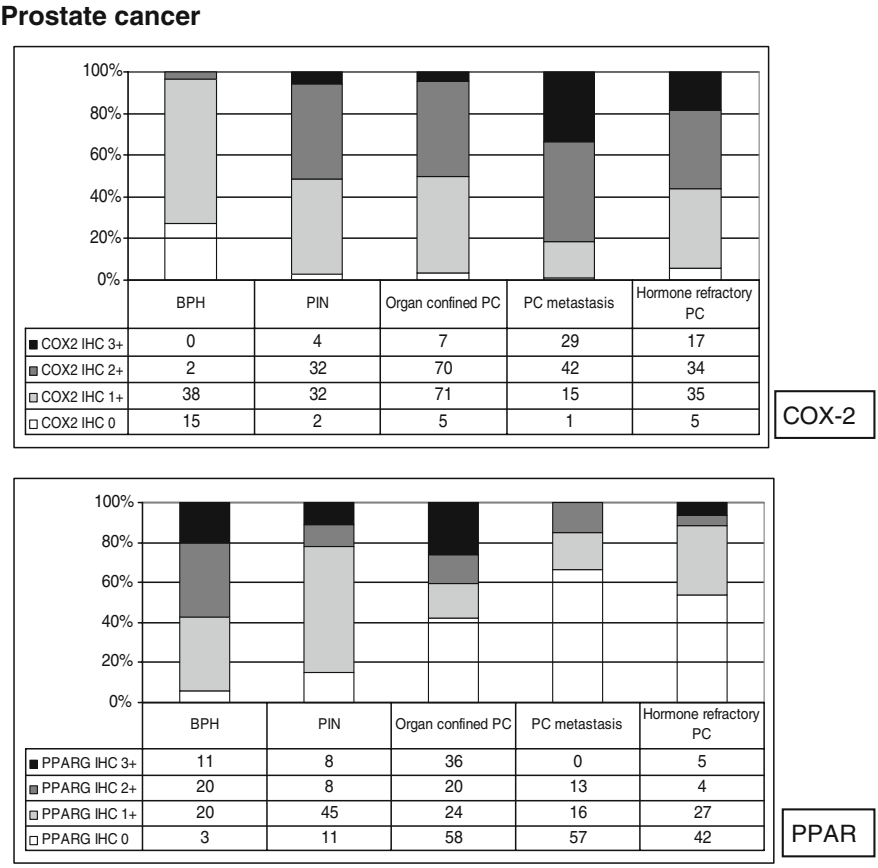


Fig. 22.6 Cumulative stage-dependent bar charts of COX2 and PPARG immunoreactivity in prostate cancer using TMA-1

indirect due to modifying the tumor stroma; i.e. the therapy mainly exploits the dependence of cancer tissues on functions of the stroma providing a permissive and supportive environment for tumor cell survival, growth, invasion and formation of metastases. A variety of soluble agents such as chemokines, growth factors, lipids, angiogenetic factors, proteinases and proteinase inhibitors are involved in a complex crosstalk between tumor and stroma. Stroma targeted approaches aim to inhibit tumor growth and invasion by disruption of this tumor-stroma interaction. Interestingly, stromal cells in the tumoral microenvironment can also differ from their normal counterparts in the expression of biologically meaningful molecules [29] including also COX2 and PPARG expression. For instance, upregulation of these effectors could be detected in stromal myofibroblasts surrounding colon adenocarcinomas [30] (Fig. 22.6).

Therefore, to fully evaluate and understand the potential of COX2 and PPAR modulation in MM further studies using TMAs punching the surrounding stroma

may be interesting future work. Based on the large comprehensive amount of data gained in this study it seems to be promising to further develop experimental protocols that employ COX2/PPAR biomodulation. The combination of both drugs is a logical consequence of experimental studies indicating that COX2 and PPARG signalling pathways are multiply intertwined: PPARG ligands suppress COX2 expression induced by lipopolysaccharide and phorbol myristate acetate in macrophages, astrocytes and epithelial cells [16]. Moreover, expression of COX2 was suggested to be regulated by a negative feedback loop involving PPARG and NF-κB [31,32]. PPARG agonists were shown to down regulate COX2, potentiate the apoptotic effects of chemotherapeutic agents, and inhibit the growth of human melanoma cell lines in vitro [19,20]. Consistently, the randomized phase II trial by Reichle et al. [22] including chemorefractory patients with progressive metastatic stage IV melanoma disease demonstrated a significantly prolonged progression-free survival if metronomic low-dose chemotherapy (trofosfamide) was combined with pioglitazone (PPARA and G agonist) and rofecoxib (COX2 inhibitor). In summary, COX inhibitors and PPAR agonists are a beneficial adjunct in biomodulatory therapy of MM rather independent of the presence of the targeted substrates in the cancer cells themselves. The expression of PPARG in the cancer, however, can indicate a higher probability to respond to stroma-targeted approaches also without drugs aiming on PPAR.

In conclusion, our study provides a late-stage prognostic marker, PPARG expression, which correlates with responsiveness to biomodulatory stroma-targeted therapy. But it should be kept in mind that the indication for such approaches cannot be solely based on selected features of the cancer cell itself but must consider the complexitiy of the stroma-tumor interaction, i.e. the microenvironment, including angiogenesis, immuno-effects and functions of the connective tissue, as well. Therfore, further prospective clinical trials are needed to validate the meaning of PPARG and COX2 targeting as a part of biomodulatory therapeutic approaches.

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Part VII
Pharmacological Considerations on Systems Biological Therapy Approaches

Chapter 23
Uncovering Tumor Systems Biology by Biomodulatory Therapy Strategies

Albrecht Reichle

Abstract How can get structured therapies in metastatic cancer a source for detecting tumor-associated systems-biological processes as adjustable sizes available for biomodulatory therapies?

A therapy-derived methodological approach to explore tumor-associated systems biology should be explicated and developed by means of analyses of recently published biomodulatory therapy approaches introducing combined anti-inflammatory; angiostatic; and immunomodulatory therapy in the treatment for advanced chemorefractory tumors of quite different origin. Biomodulatory therapy approaches in tumors intend to develop systems-terms that provide a basis for broadening therapy-relevant capacities by regulating biological systems processes for tumor control. Combined targeted therapies of tumor-associated wound healing mechanisms, namely inflammation and neoangiogenesis, have shown that – using an approach for understanding systems biology as adjustable size – we may break through the barrier of complexity of tumor-stroma-interactions in a therapeutically relevant way. Targeting the tumor systems' topology of aggregated action effects (inflammation, neoangiogenesis, Warburg effect, immune response, extracellular matrix remodeling, cell proliferation rate, apoptosis, coagulation effects) may open up the perspective of individualized tumor therapy.

Keywords Combined transcriptional modulation • Metronomic chemotherapy • Tumor-associated inflammation • Metastatic tumors

23.1 Introduction

The present systems theoretical discussion is based on a series of published clinical trials on systemically pretreated metastatic tumors with different histologies [1–7]. The therapy approaches applied are uniformly characterized by a poor or missing

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mono-activity of the respective anti-inflammatory and angiostatic acting drugs (peroxisome proliferator-activated receptor (PPAR) alpha/gamma agonists, interferon-alpha, glucocorticoides, cyclooxygenase-2 inhibitors, metronomic low-dose chemotherapy). These drugs were administered in different combinations in a wide range of tumor types. Seemingly unexpected, these therapy approaches have the capacity to induce objective and even long-term tumor response at a low rate of treatment-associated side effects. Clinically, the mechanisms of action could be followed in the resolution of tumor-associated disease traits and in corresponding laboratory parameters in the peripheral blood. This constellation of collected parameters now offers new insights about the object of interest, i.e. the tumor tissue as a networking system, which is still susceptible to concerted regulatory, and, most importantly, to clinically relevant therapeutic interventions.

Methodological discussions based on practical and emancipatory knowledge-guiding interests should (1) uncover the constraints for a systems-biological consideration of tumor-associated biological processes, (2) straighten out how systems-biological processes may be detected in tumor tissues via regulatory designed biomodulatory therapy strategies, (3) state what kind of scientific program should be discharged on the basis of systems-biological considerations, and (4) specify how new theorems may be constructed logically.

Structured therapy-derived observations are aimed at uncovering systems structures, at understanding probably still anonymous regulatory systems by regularly observable biomarkers, and at augmenting the therapy-relevant capacity for therapeutic biomodulatory interventions by a systems-biological understanding of tumor-stroma interactions.

Systems theories about the ‘inner life’ of tumors should describe rather complex interactions among tumor-associated phenomena that are neither classified causally nor randomly in such a way that they may be described statistically or generally with mathematical models. Tumors can be considered as open systems, in which phenomena such as self-organization, non-linearity, interdependence, and self-regulation (homeostasis) or phenomena mediated by attractors may be observed [8,9]. Compared to the traditional attainment of predictions about the system’s behavior by analytical-empiric analyses of its structures and functions, the obtaining of systems-biological insights by systematized biomodulatory therapies represents a new perspective. This method is completely divergent but presumably complementary to the reductionist approach that aims at targeting acquired and poorly predictable aberrations in tumor cells.

23.2 Problems with Therapy Strategies in Metastatic Tumors in a Historical Context

Advanced tumor disease is frequently associated with a reduced performance status of the patient. Therapy, even palliative approaches, may further transiently worsen the patient’s performance status. At this stage, many malignant diseases are often incurable, so that comprehensive palliative medicine represents the most important therapeutic intention.

Practical knowledge-guiding interests. Both treatment-related and disease-related comorbidity, which is characterized by multifold arising tumor-associated disease traits, may determine the quality of life for patients. Combination therapies are often used for controlling advanced tumors. These therapies are mainly characterized by a steeply increasing toxicity caused by adding one drug to another at maximal tolerable doses and, at the same time, a relatively modest improvement in overall survival, if at all. This dilemma has to be faced when choosing adequate treatment schedules for patients in palliation. Pharmacogenomic approaches may minimize toxicity in individual cases. A further individualization of therapies, however, is difficult or even impossible to achieve because of missing treatment-related biomarkers that indicate response aside from traditional tumor markers.

Further and relatively frequent adverse effects in palliative treatment are poor chemosensitivity to cytotoxic drugs available for many advanced tumor types and the circumscribed benefit of the so-called 'targeted therapies' in tumors with corresponding target over-expression. For ultimate tumor response, more information and insights are necessary on how tumor-associated disease traits (e.g. inflammation, angiogenesis) may get interactively manageable – data that necessitate therapies guided by biomarkers related to pathophysiologically relevant tumor-associated processes.

Advanced metastatic disease is often associated with a poor tolerability of the therapy regimen. Therefore, the question of the most important aim arises: achievement of tumor response or, alternatively, disease stabilization with presumably more modest side effects. Therefore, long-term administration of a less toxic biomodulatory regimen for long-term disease control is worth considering with respect to the chronification of a malignant disease. Sequentially administered pulsed therapy approaches may already improve palliation, for instance in colorectal cancer [10].

Emancipatory knowledge-guiding interests. Knowledge-guiding interests are developing and getting emancipated to the same extent as traditional treatment procedures are being customized or diverse interests established. Structures of distorted communication may be durably institutionalized: Established treatment strategies refer to the conflicting interests of medical and pharmaceutical personnel, who aim at optimizing response rates, and of patients, who are also interested in an improvement of both quality of life and long-term disease control besides disease eradication. As shown by many studies, the administration of a combination of cytotoxically acting therapy elements – which is frequently guided by the simple availability of drugs – often shows a moderately enhanced efficacy at a simultaneously enhanced toxicity profile. Conventional treatment strategies are established on the assumption that tumor cells have to be targeted directly and have to be disposed of by cytotoxic drugs or pathway inhibitors, or by immunologic, antibody-, or cellular-mediated attacks. Emancipatory aspects of knowledge-guiding interests are reflected by the fact that a drug needs to demonstrate mono-activity before its possible approval for clinical practice. A concerted regulatory activity of drugs without mono-activity of the single drug, probably at respective low dosages, is excluded as a matter of principle.

23.3 Explorative Considerations

Unlike laws of nature, causal relations between initiating processes of tumor development are not anchored in an invariance of nature. Therefore, molecular and cytogenetic aberrations at initial diagnosis are generally heterogeneous in both tumors and individual tumor types. Invariance within the tumor process may be observed during tumor progression. In interaction with normal human tissue, tumor cells use processes according to laws of nature for building up a favorable infrastructure for proliferation. Presently, two major clinical interpretations seem to be continuative: (1) Tumor development may be described embryo-genetically, and (2) tumors may be figuratively conceived as 'never healing wounds'. For the first time in 1986, Dvorak interpreted these laws of nature as tumor-associated 'wound healing' mechanisms, for instance angiogenesis, inflammation, immunology, remodeling of the extracellular matrix, specific changes in cell metabolism and coagulation, and altered behavior in proliferation [10–15]. With this interpretation, Dvorak addressed the systems biology of tumors in a contemporary context. Up to now, a tumor's systems biology has rarely presented a target for a systematic approach in cancer treatment.

Systems-immanent 'dysbalances'. In tumors, unsolved tumor-specific problems concerning the control of self-regulating systems have been observed that are based on a dysregulation of constitutive elements such as transcription factors due to acquired molecular-genetic aberrations [16,17]. The constitutive dysregulation of transcriptional activity is shown to be an important target for biomodulatory therapy approaches in metastatic cancer. The dysregulated systems biology of a tumor may commonly not be understood mono-causally or explained context-free. Systems biological considerations target on a dysbalance between interfering functional elements in the tumor in such a way that conditioning and conditioned tumor-promoting elements (e.g. wound healing mechanisms) also behave reciprocally under therapeutic aspects.

The dysregulation of wound healing mechanisms is reflected in tumor-associated disease traits (e.g. tumor-associated inflammation, ECOG performance status, coagulation disorders, tumor-associated auto-immunity, and metastases). On a molecular level, it can be observed in the dysregulation of (nuclear) transcription factors, both in tumor and neighboring stroma cells. In a concerted action, transcription factors regulate distinct gene cascades and consecutively important cell functions for survival. Their cooperative interaction is also important for the survival of tumor cells.

23.4 Uncovering Systems-Biological Processes in Tumor Tissues by Biomodulatory Therapy Strategies

Generation of biomodulatory treatment strategies. Biomodulatory therapy approaches in tumors intend to develop systems-terms that provide a basis for broadening therapy-relevant capacities by regulating biological systems processes for tumor control. Systems-biological processes may be regulated via (nuclear)

transcription factors or by specifically targeting corresponding 'wound healing mechanisms' (e.g. tumor neoangiogenesis, tumor-associated inflammation and immunology). Epigenetic or embryo-genetic processes are additional targets to modulate systems-relevant mechanisms. Drugs acting as biomodulators, should promote the therapeutic impulse for self-organization, self-stabilization, or achievement of a new homeostasis in the tumor tissue for attenuation of tumor growth. An appraisal of the functional status, for example, tumor-associated wound healing mechanisms would be helpful in future to choose the most adequate (personalized) biomodulatory therapy approach.

Generation of differentially induced tumor-stroma-organizations. The combined activity of regulatory and pleiotropic agents, such as the administered transcription modulators (dexamethasone, pioglitazone, interferon-alpha), or agents modulating tumor-associated inflammatory, and immunological processes with a close link to angiogenesis (COX-2 inhibitor, metronomic low-dose chemotherapy) may shape the tumor's organization by simultaneously attenuating multiple activities involved in tumor growth. Targeting constellations of constitutive dysregulated tumor-stroma-interactions such as inflammation and angiogenesis should result in tumor control (Fig. 23.1).

This hypothesis has been supported in recently performed trials by treatment-related characteristics (chapter 12): (1) No or poor agent activity of each administered drug (predominantly combined regulatory activity) when given alone, (2) a very moderate toxicity profile during long-term drug administration (presumably no dose-response relationship), (3) very delayed objective responses (stable shaping and focusing of the tumor systems organization), (4) improved overall survival

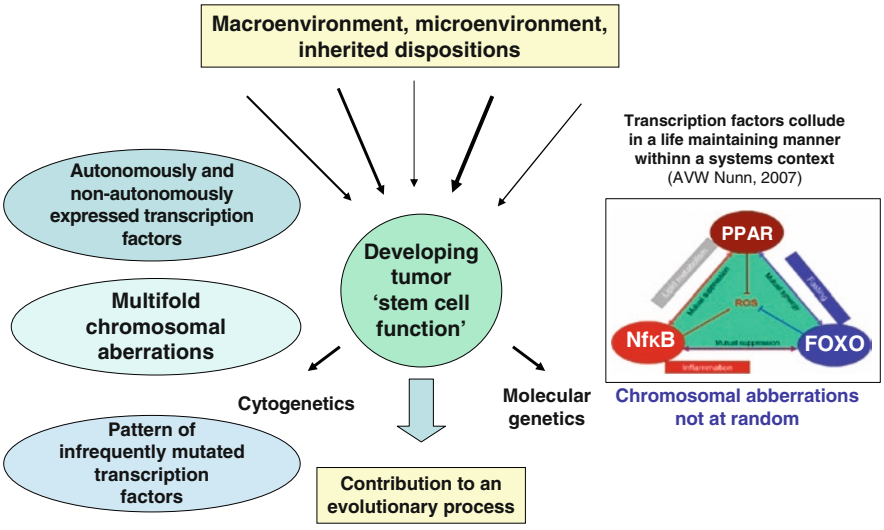

Fig. 23.1 Important transcription factors are infrequently mutated in tumor cells. Therefore, modulators of transcriptional activity in tumor and adjacent stroma cells provide biomodulatory access to alter systems functions with the aim to attenuate tumor growth

without an increase of the response rate (biomodulatory activity), (5) significant modulation of tumor-associated disease traits such as inflammation, ECOG status, and paraneoplastic syndromes (biomodulation-derived biomarkers), (6) activity depending on the metastatic organ site (tumor-stroma-specifity as expected from the known differential behavior of the various cell types within the tumor compartment, and the varying stroma cell compositions at the different metastatic sites), and (7) predominant site of progression at the original localization of the metastases (hints for impact on metastatic processes) [1–7].

The fundamental potency and specifity of co-regulatory activities of nuclear receptor modulators may be exemplarily shown by the action of PPAR ligands [18–20]: In prospectively designed clinical trials, dual PPAR-alpha and gamma agonists have the capacity to lower the incidence of cardiovascular events in patients with diabetes mellitus. On the other hand, retrospectively performed analyses of a specific PPAR-gamma agonist reveal an increased incidence of cardiovascular events in the same group of patients. This means that, in the first case, side effects based on a strong anti-inflammatory activity may accomplish the therapeutic repertoire of treating diabetes on a systems-biological level – besides the originally intended effect of lower serum glucose levels. In the other case, serum glucose levels are also lowered but disease-related inflammation is obviously not controlled. Thus, the observation of side effects – even unexpected ones – becomes highly important when co-regulatory activities of modulators of transcription factors are used therapeutically. Co-regulatory activities are generally important for treating complex disease traits: The impressive amplified anti-inflammatory activity of PPAR-alpha/gamma agonists combined with glucocorticoides has been shown preclinically. Thus, this therapy may become successfully implemented in the treatment of hormone-refractory prostate cancer as both anti-inflammatory and anti-osteoplastic treatment [21].

Targeting multiple disease traits (Fig. 23.1). As demonstrated in multiple clinical trials including angiogenesis inhibitors or anti-inflammatory drugs, the targeting of single wound healing mechanisms may result in tumor response. In recent trials, we have extended these experiences to anti-inflammatory therapy: (1) Anti-inflammatory therapy adds further benefits to angiostatic therapy, and (2) the intensity of an anti-inflammatory approach may have significant impact on outcome. Based on these systems-biological observations, we now postulate tumor-associated inflammation as both a pathophysiologically important element and as a therapeutic target, but without presupposing causal relationships between inflammation and tumor progression. The combined targeting of wound healing mechanisms may even induce objective response including complete remission and continuous complete remission. Successful combined targeting of ‘wound healing’ processes with transcriptional regulators in tumor and adjacent stroma cells reveals preserved regulatory elements in individual tumor types [1–7].

Combination of approved drugs. In contrast to ‘causal’ therapy approaches that aim at blocking aberrant tumor-associated pathways by a restricted repertoire of highly specific drugs, multiple potential modulators (activators and deactivators) of transcriptional processes or of wound healing processes are available for

biomodulatory therapy approaches (chapter 24). The introduction of approaches targeting systems-relevant processes is not exclusively dependent on the development of new drugs. Established medications may be used for unintended purposes. The main therapeutic focus is implementing single drugs in such a way that a concerted biomodulatory activity may arise in the context of a systems-biological approach. Consequently, mono-activity of a single drug is no prerequisite for inclusion in a combined therapy approach. Drugs with biomodulatory activity (e.g. lenalidomide, bevacizumab) could even be used again in second-line, and then within a systems-biological therapeutic approach (trials are being conducted) [22,23].

23.5 Program of a Scientific Theory

Conventional therapy methods frequently neglect the complexity of the tumor compartment. They mainly target the molecular-genetically highly variable tumor cell, whose variability is explained by the complexity of tumor development. By blocking a pathological signaling pathway with a small molecule or antibody, the whole tumor system should be destroyed, synonymously with the virtual assumption that tumor development could result from a single causative principle. Lessons we have learnt from reductionist therapy strategies in relatively rare tumor entities such as chronic myelocytic leukemia, gastrointestinal stroma tumors (tyrosine kinase inhibitors), promyelocytic leukemia (all-trans retinoic acid) or Flt3 positive acute myelocytic leukemias are obviously not conferrable to most of the other advanced tumor types.

Induction of complete remission is a frequent prerequisite of reductionist therapy approaches aimed to improve overall survival. If responses are not achievable with such reductionist methods, therapies have to meet criteria of systems-biological processes to gain fundamental changes in the biology of metastatic diseases aimed to improve survival via disease chronification.

When gathering the first clinical results on systems-biological treatment approaches in metastatic cancer, criticism against the exclusive preference of reductionist therapy approaches may be reworded: Successful biomodulatory therapy approaches in different metastatic tumor types contradict the paradigm that, for the most part, only drug-mediated blockades of more or less tumor-specific aberrant pathways may induce tumor response; a paradigm that is supported by an overwhelming number of clinical data.

1. A lead back to a final first principle that may be therapeutically targeted to eradicate metastatic cancer is generally not permitted, in particular in knowledge of the multi-faceted activity profile of biomodulatory agents. However, instead of such a lead back to a first principle, we have to deal with multiple and various element constellations, one of which, for example, is tumor-associated inflammation. The constellation of elements has to be broken down to its single moments; but – simultaneously – we have to understand the relationship between

one another rather than separately adding one to another and thereby neglecting their importance within the complex constellation. The principle therapeutic difficulty is based on this point. Systems-oriented therapies provide tools to cope with these basic problems.

2. Elements in motion (e.g. angiogenesis, inflammation, etc) are met in the circle of functions triggered by the biomodulatory therapeutic activities chosen. Thus, stability in systems biology is presumed to be dynamic (chapter 26). Biomodulatory therapies have the continuing ability to get adapted to interacting tumor-associated elements for achieving therapeutic response (individualized therapy). Biomarkers (e.g. secretome parameters) are indicating efficacious modulation of single disease traits. Therefore, in the immediate present and future, biomodulatory therapy approaches of metastatic tumors could be methodological tools of individualized tumor therapy: Close monitoring would further allow us to choose other modulator combinations to facilitate objective tumor response in case of weak interactivity. For example, a broad variety of drugs is currently available to control tumor-associated inflammation or neoangiogenesis. On the basis of biomarkers, success and failure of a biomodulatory approach may be calculated for individual patients.

Biomodulation in metastatic tumors provides a tool for recognizing patterns in therapy-associated events via biomodulation-derived biomarkers (chapter 20, 21). Thereby, it enables the shaping of the tumor systems organization and the uncovering of endogenous sources such as transcription factors and their cross-talks for managing growth behavior by counterbalancing tumor systems biology.

Counterbalancing these transcriptional dysregulations by biomodulatory therapies – either directly by modulators of transcription factors (e.g. NFkappa-B modulators, PPAR agonists/antagonists, glucocorticoides, interferon-alpha) or indirectly by targeting wound healing mechanisms (e.g. anti-angiogenic, anti-inflammatory approaches) or epigenetic changes – may resolve tumor-associated disease traits and thereby control tumor growth. In future, network relationships will need to be elaborated in more detail.

From a therapeutic point of view, the systems-biological model does not specify whether a wound healing mechanism has to be suppressed or stimulated to achieve tumor control: Inflammation control as well as stimulation of inflammation may control tumor growth, immuno-suppression, and immune-stimulation [1–7,24]. Probably contradictory decisions could be associated with the same capacity to achieve tumor control in a distinct tumor type. Thus, the question arising is which therapeutic approach is easier to put into practice, is probably more compatible with other therapy approaches, and is the most tolerable one with regard to side effects.

Systems theoretical considerations derived from biomodulatory therapy approaches may provide an additional platform to discuss new treatment strategies. This applies in particular to advanced tumors, for which no routinely recommendable therapies exist in the metastatic stage because of known poor chemosensitivity or significant therapy-associated toxicity. Particularly in these multi-drug resistant tumor types, systems-biological considerations may align therapeutic options to

tumor development at the respective organ site, which means that its biological history may implicate therapeutic calculations. As shown in recently published trials, inflammation as well as special stroma compositions at different organ sites (e.g. osteoplastic metastasis in prostate cancer) may be specifically targeted by combined biomodulatory approaches [1–7].

23.6 Constitution of a New Kind of Consideration About Objects of Interest

The construction of therapy-directed systems terms about malignant processes (tumor-stroma-interactions) should be developed combining different scientific empiric/analytic as well as hermeneutic approaches. Therefore, it is necessary to discuss the logic construction of developed theorems, the relation of systems theory to biomodulatory therapy approaches, criteria for checking systems behavior and creating predictions, as well as checking procedures. The constitution of the new kind of consideration about the objects of interest – a therapy-related systems theory – is different from the exclusive analytic/empiric systems term that derives from results generated by functional genomics/proteomics in tumor systems biology.

Logic construction of theorems. A tumor's systems biology should not be interpreted out of its context. The requirements of application (therapy schedule, tumor type) and the number of surrogate markers (secretome parameters derived from stroma and/or tumor cells and results from molecular imaging) define the way the interpretation is conducted. Additionally, they define the hermeneutic understanding of extremely complex cellular interactions corresponding to the chosen picture, i.e. the wound healing mechanisms, and enable insights into more abstract evolutionary processes. In the present case, this means the following: Naturally, the administered drugs – particularly the transcriptional active modulators – still have insufficiently illuminated spectrums of activities, which may be even dependent on the cell type. General interpretations of the systems biology do not obey the same categories of refutation as general theories and remain per se open for discussion. The discourse serves to provide explanatory statements of problematic scopes of opinions and norms. The logic of explaining tumor systems biology is the result of a connection between a hermeneutic understanding of tumor growth, for instance as wound healing mechanisms, and the causal explanation (e.g. co-regulatory activity of transcription factors, targeting of wound healing mechanisms). Methodologically, the reductionist approach, restricted in terms of a limited interpretation of tumor-associated phenomena, is closely integrated in systems-biological approaches that are open for the detection of new networking interactions (experimental part). Thereby, the context of discovery (modulation of tumor-associated disease traits, biomarkers) has to be consistently separated from the context of justification (rational for a biomodulatory therapy approach).

Relation systems theory of tumors to biomodulatory therapy. Statements about phenomena linked to cellular functions or regularly observed intercellular events

that constitute the systems biology of tumors may be retranslated in (1) therapies with a rational and pragmatic purpose – that means in differential biomodulatory treatment strategies – but also in (2) a new hermeneutic understanding of empirically and analytically collected results (evolutionary tumor-associated processes) or (3) may discharge into specific analytical approaches.

Published phase II trials on combined targeted therapy of tumor-associated wound healing mechanisms, for instance inflammation and neoangiogenesis, have shown that – using an approach for understanding systems biology as adjustable size – we may break through the barrier of complexity of tumor-stroma-interactions in a therapeutically relevant way. For a targeted modulation, elements such as inflammation and neoangiogenesis are available, which are dysregulated on the basis of acquired chromosomal aberrations. Biomodulation of systems-biological processes facilitate comparatively high efficacy at moderate toxicity [1–7].

Criteria for checking systems behavior and creating predictions. The focus on the systems biology of a tumor as the original target of a cancer therapy necessitates biomarkers that indicate stable response in the field of tumor-associated disease traits (secretome analytics, and molecular imaging) or tumor-associated phenomena such as inflammation, angiogenesis, coagulation, and metabolism.

Efficacious biomodulation. Rather than the primary or 'classic' markers for tumor response including tumor shrinkage or decrease of tumor markers, this new group of markers (molecular imaging, cellular secretomes) reflects efficacious biomodulation. However, we are aware of the limitation that some of these tumor-associated phenomena – which mirror tumor biomodulation – are sometimes difficult to follow on a systemic level. They can not be uniformly interpreted across tumor entities as demonstrated in our example of castration-resistent prostate cancer (CRPC) in comparison to other tumors, when inflammation seems to be differently integrated in the pathophysiology of a tumor: Prostate-specific antigen (PSA) decline was paralleled but not preceded by a C-reactive protein (CRP) decline in CRPC, whereas, in other tumor types including RCCC, decrease of CRP or ECOG performance improvement preceded tumor response [1–7].

The more pronounced the dysregulation of transcription factors in tumor and adjacent stroma cells compared to normal tissue, the more specific a biomodulatory therapy approach could be selected. An open question might be the frequency of escape phenomena of the tumor tissue during biomodulatory therapies and how to overcome these mechanisms. At least, recently published data have shown that relatively favorable, progression-free survival rates in patients responding to the new therapy concepts are not at the cost of enhanced rates of rapid progression.

Checking procedures. Traditional checking levels, tumor shrinkage (computed tomography, tumor markers), and side effects may be expanded by systems-relevant biomarkers, which may be related to objective tumor response. Biomarkers may be followed locally by metabolic or vascular imaging techniques or systemically in parameters of the peripheral blood. For example, CRP has been shown to be very useful for detecting sufficient control of tumor-associated inflammation.

Safety aspects. The therapeutic index is a measure for the safety of a drug or a drug combination and indicates the margin between therapeutic and toxic doses: the

bigger the margin, the less dangerous the drug(s). The application of the therapeutic index on biomodulatory therapy approaches is limited. As shown in multiple clinical trials, biomodulatory therapies may work on a low toxicity level, as biomodulatory dosages of single drugs are not identical with maximal tolerable doses. However, because of the concerted activity of the chosen drugs, these therapy approaches have to be checked for unexpected side effects. Reduced toxicity should be achieved by utilizing the co-regulatory activities of transcriptional modulators. Co-regulatory activities may simultaneously specify both the desired therapeutic effect and side effects (PPAR agonists!), and may even save up dose of single drugs (glucocorticoide dose in combination with glitazones) [21].

23.7 Discussion

Practical and emancipatory interests in therapies integrated in the coherence of science bring together the constitution of new objects of interest, **therapy-derived systems biology**, and their pragmatic application, here in form of biomodulatory therapy approaches. These interests led to a methodological approach aimed at uncovering systems-biological processes by differentially administered biomodulatory drugs for the control of tumor growth. Biomodulatory derived changes in the tumor may demerge individually moving processes within the tumor tissue into more easily elusive constellations, for example wound healing mechanisms. Therefore, these therapy approaches point at a way from bedside to bench: Detectable constellations in tumors may be integrated in systems-biological models to modify and specify tumor-associated constellations and phenomena by biomodulatory approaches, even to adapt therapies to individual constellations [25,26]. On the other hand, constellations may be consecutively analyzed analytically or empirically at the bench and may be retranslated into new (hermeneutic) systems interpretations. Thus, the methodology may partially reverse the traditional information flow, which is affected by the predominant transfer from analytical sciences to applied sciences.

A striking difference is visible in the pragmatic function, which generated data in different scientific areas. Here, we can combine therapeutically derived information on systems biology to establish systems-biological models. Information may be generated on three levels: Biomodulatory processes (systems-associated prognostic markers), processes indicating tumor response (traditionally tumor shrinkage, now molecular imaging, cellular secretomes), and side effects on the level of the whole organism [25–28].

The claim for objectivity on systems-biological processes studied via biomodulatory therapy approaches is based on a possible **virtualization of the engagement to get experiences or decisions**. The virtualization is enabled by a communicative evaluation of hypothetical requirements for the validity of a systems-biological model and hereby allows the generation of provable knowledge. These new methodological approaches for studying systems biology by a therapy-guided method

may be an important supplementation of the established analytical/empirical studies on functional genomics in systems biology [29].

Studies are being conducted to investigate whether the two divergent therapy approaches are compatible: systems-directed biomodulatory therapies targeting constellations of constitutive dysregulated tumor-stroma interactions to achieve self-control ('communication design') combined with reductionist approaches and pathway/signaling-blocking treatments that virtually lead back tumor development to a first causal principle.

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Chapter 24
Breathing New Life into Old Drugs: Indication Discovery by Systems Directed Therapy

Annika Bundscherer and Christian Hafner

Abstract In the treatment of chemorefractory and metastatic cancer new concepts such as stroma-targeted and antiangiogenetic strategies emerge as powerful alternatives to conventional regimes. In this context, several well established drugs such as IMiDs, COX 2 inhibitors, mTOR antagonists, and PPARγ agonists attract increasing attention. Beyond their primary field of indication, these drugs have demonstrated broad anti-tumoral activity such as induction of apoptosis and inhibition of tumor cell proliferation. In addition, by interrupting the tumor-stroma interaction, these agents also reveal antiangiogenetic and immuno-modulating effects. Compared to conventional high dose chemotherapy, stroma-targeted strategies are thought to be less susceptible to the development of drug resistance and to cause less toxicity. Taking into account that combinatorial use and repurposing of biomodulating drugs might potentiate the antineoplastic effects without causing life threatening toxicities, targeting the tumor stroma is judged to be a promising approach in tumor palliation.

Keywords IMiDs • COX 2 inhibitors • mTOR antagonists • PPARγ agonists • Indication discovery • Repurposing of drugs

Abbreviations

bFGF	Basic fibroblast growth factor
CDK	Cyclin dependent kinase
CNS	Central Nervous System

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COX	Cyclooxygenase
FKBP-12	FK 506 binding protein 12
HIF	Hypoxia inducibe factors
HUVEC	Human umbilical vein endothelial cells
IGFR	Insulin-like growth factor receptor
IL	Interleukin
IMiDs	Immunmodulatory drugs
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa B
ICAM	intercellular adhesion molecule
NK-cells	Natural killer cells
NSAID	Non steroidal anti-inflammatory drugs
NSCLC	Non small cell lung cancer
PAI-1	Plasminogen activator inhibitor
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PGE_2	Prostaglandin E2
PI3K	Phosphatidyl-inositol 3 kinase
PPAR	Peroxisome proliferator-activated receptor
PTEN	Phosphatase and tensin homologue deleted on chromosom ten
RNA	Ribonucleic acid
RXR	Retinoid X receptor
TDZ	Thiazolidinediones
TGFβ	Transforming growth factor β
TIMP	Tissue inhibitor of MMP
TNFα	Tumor necrosis factor α
TSC 1/2	Tuberous sclerosis complex 1/2
TSP	Thrombospondin
TXA	Thromboxane A
VEGF	Vascular endothelial growth factor

24.1 Introduction

The treatment of chemorefractoy and metastatic cancer remains to be a great challenge in oncology. With the aim of killing as many malignant cells as possible, high-dose chemotherapy schedules have been designed. As chemotherapeutic agents disrupt DNA replication and cause DNA damage in rapidly dividing cells, all tissues with a high proliferation rate are affected by these agents, resulting in severe and dose limiting side effects such as damage to the intestinal mucosa,

myelosuppression and hair loss. For this reason, prolonged breaks between successive cycles of therapy are required to allow the normal tissue to recover.

The initial response to chemotherapy often is rather impressive but mostly short-lived. Due to their genetic instability, tumors that initially responded to chemotherapy become drug resistant and the patients experience a relapse. Several strategies such as multidrug combination, escalating the maximal tolerated dose and impairing side effects by supportive treatment have been established to overcome this drug resistance [1]. However, progress has been modest concerning quality of life and survival in many tumor entities. Therefore, scientists and clinicians have sought for new strategies of cancer treatment to overcome these limitations.

Interestingly, in recent years several well established drugs, originally developed for the treatment of non-oncologic diseases, such as IMiDs, COX 2 inhibitors, mTOR antagonists and PPARγ agonists revealed anticancer potential. Beside direct anticancer activities like induction of apoptosis, cell cycle arrest and inhibition of tumor cell proliferation, these biomodulators also are able to modify the interaction between tumor and stroma cells. The understanding is growing that cancer cells alter the stroma host compartment in many ways to establish a favorable environment for survival and cell growth and to foster invasion and metastasis. The cross-talk between tumor and stroma is mediated by a variety of soluble agents such as cytokines, growth factors and extracellular matrix proteins as well as by direct cell-cell contact [2]. The aim of stroma-targeted therapy is to disrupt the tumor-stroma interaction. The targets of this innovative concept are not the tumor cells themselves but peritumoral stroma cells such as fibroblasts, endothelia and inflammatory cells. One major advantage of this kind of therapy is the suggested genetic stability of stroma cells. Thus, stroma targeted approaches are judged to be less susceptible to the development of drug resistance [3]. Furthermore, tumor associated stroma cells were shown to express different surface molecules than cancer cells. For this reason, a selective intervention could be feasible. Since the required drug concentrations for stroma targeted therapy are usually lower than for conventional chemotherapy, side effects are expected to be less severe and quality of life can therefore be improved. For this reason, stroma targeted therapy is a promising approach in tumor therapy. However, the primary aim of this kind of therapy is stabilisation of disease and prolongation of progression free survival rather than tumor remission.

One very important component of the tumor stroma is the endothelial cell of tumor vessels. As the size limit for sufficient oxygen diffusion is 100–200 μm, tumors cannot grow beyond this critical size or metastasize without proficient blood supply. For this reason, tumor cells alter the balance between pro- and antiangiogenetic factors and recruit their own blood vessels by angiogenesis [4]. This “angiogenetic switch” can be triggered by metabolic or mechanical stress, immune and inflammatory response as well as by genetic mutations. Due to different mechanisms involved in tumor angiogenesis, the architecture of healthy vessels differ fundamentally from that found in the tumor vessels, which often are heterogeneous, irregular and leaky. The tumor endothelial cells are disorganized and express imbalanced surface molecules [5]. These structural differences open the gate for a selective antiangiogenetic tumor therapy.

This chapter will focus on the mechanisms of the anticancer and stroma-targeting activities of thalidomide, COX 2 inhibitors, mTOR antagonists and PPARγ agonists as well as the results of clinical trials.

24.2 IMiDs

24.2.1 History of Thalidomide

The glutamic acid derivative thalidomide was first synthesized in 1954 and revealed to be an effective sedative and sleep-inducing agent. Due to its anti-emetic effects, it was also used for the treatment of morning sickness in pregnant women. In 1961 thalidomide had to be withdrawn from the market because of severe congenital limb defects which were associated with the use during pregnancy [6]. Although this agent had written an inglorious chapter in the history of medicine, thalidomide is now being re-evaluated for its antiangiogenic effect and potential use in the treatment of various diseases including AIDS and cancer [7]. Being an effective therapeutic agent in the treatment of various inflammatory and dermatologic conditions, thalidomide was recommended by the World Health Organization for the therapy of lepromatous leprosis and was approved for sale in the USA in 1998 [6]. With the intent of reducing the teratogenic risk, the use of thalidomide is restricted by the mandatory System of Thalidomide Education and prescribing Safety program, making thalidomide to the most restrictively prescribed agent ever approved. In the meantime, synthetic thalidomide analogues possessing more potent immunoregulatory properties while lacking the side effect profile of the first generation drug have been designed [8]. Substances such as CC-4047 (actimide) und Revlemid are among these new IMiDs (immunomodulatory drugs) [9].

24.2.2 IMiDs in Cancer

Several experimental and clinical studies investigating the anticancer effect of IMiDs are ongoing or already finished (Fig. 24.1). A novel sugar-substituted thalidomide derivative, STA-35, was potent in inhibiting HL-60 cell proliferation in vitro and induced apoptosis by the suppression of NF-κB activation [10]. In addition to these direct anticancer effects, the antineoplastic activity of thalidomide and its analogues is based on immunomodulating and antiangiogenetic mechanisms as well as epigenetic modelling. Thalidomide was shown to inhibit TNFα production by increased degradation of TNFα mRNA [11] and to reduce the density of cell surface molecules involved in the adhesion cascade such as ICAM-1, VCAM and E-selectin [12]. Also an enhancement of TH-1 type immune activity as well as an augmentation of NK cell cytotoxicity were detected [6]. Furthermore, IMiDs impaired the metastatic capacity of murine colorectal cancer cell lines both in vitro and in vivo [13].

The tumor associated angiogenesis can be blocked by IMiDs via different pathways. Lenalidomide had inhibitory effects on the associations between

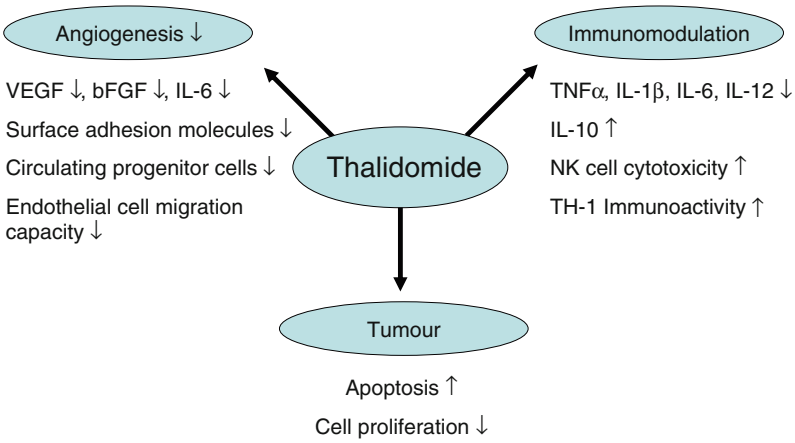

Fig. 24.1 Thalidomide, mechanisms of action: Antitumoral and stroma-targeted effects of thalidomide VEGF = vascular endothelial growth factors, bFGF = basic fibroblast growth factor, IL = interleukin, TNF = tumour necrosis factor (From [141; Fig. 12.4, p. 231]. With kind permission of Springer Science and Business Media)

cadherin 5, beta-catenin and CD31 and adherens junction proteins whose interaction is critical for endothelial cell cord formation. Furthermore, inhibition of hypoxia-induced processes and of VEGF-induced PI3K-Akt pathway signaling could be detected [14]. Thalidomide inhibited vasculogenic mimicry channel and mosaic vessel formation in melanoma through the regulation of vasculogenic factors. The reduction of VEGF, NF-κB, PCNA, MMP-2, MMP-9 protein expression and MMP-2, MMP-9 mRNA levels was described [14]. In addition, IMiDs might diminish circulating endothelial progenitor cells and affect endothelial cell migration capacity. The knowledge about antineoplastic effects of IMiDs is not limited to the results of preclinical studies. In a clinical trial, 18 men suffering from high-risk prostate cancer were given thalidomide at doses escalated to 600 mg for 12 weeks, followed by radical prostatectomy. Tissue microarray analyses indicated modulation of vascular marker expression accompanied by a reduction in microvessel density in the treated group [15].

24.2.3 IMiDs in Clinical Trials

Evidence is growing that single agent lenalinomide is effective and well tolerated in relapsed, refractory multiple myeloma [16,17]. Also the anticancer activity of dexamethasone can be improved in advanced multiple myeloma patients. With a 60.2% response rate compared to 24% in the placebo group, lenalinomide plus dexamethasone was shown to be more effective than high-dose dexamethasone alone [18]. In addition, in patients with newly diagnosed myeloma combining lenalinomide with low-dose dexamethasone was associated with lower toxicity and better overall survival compared to the combination of lenalinomide and high-dose

dexamethasone [19]. The efficiency of thalidomide and its analogues in treatment of patients suffering from other malignancies including melanoma [20–22], glioma and pancreatic cancer has already been shown in clinical trials [23–27]. After 12 month of treatment with thalidomide, two out of three patients with Kaposi sarcoma showed a complete response [28]. In another clinical trial, the effects of a therapy with oral thalidomide appeared to be comparable with those of a single agent intravenous chemotherapy in women with advanced ovarian cancer [29]. However, in a large randomized double-blind placebo-controlled trial of thalidomide in combination with gemcitabine and carboplatin in advanced non-small-cell lung cancer, no improvement in overall survival but an increased risk of thrombotic events could be detected [30]. Similar results were obtained in a study combining thalidomide and chemotherapy in patients with small cell lung cancer [31].

24.3 COX 2 Inhibitors

24.3.1 Cyclooxygenase – Isoforms and Function

The cyclooxygenase (COX) is a key enzyme in the conversion of arachidonic acid to prostaglandins. COX 1, the constitutively expressed isoform, is involved in the regulation of several housekeeping processes such as induction of platelet aggregation and gastrointestinal cytoprotection [32]. While COX 1 can be found in almost all tissues, the expression of the early response gene COX 2 can be rapidly induced by a variety of inflammatory processes including cancer. Under physiological conditions, COX 2 is constitutively expressed in selected tissues like ovarian follicles and seminal vesicles [33]. In the kidney, COX 2 plays a crucial role in the regulation of sodium balance and the maintenance of the perfusion under stress. In addition, COX 2 is important for body temperature control and establishment of pain sensation in the central nervous system. COX 2 expression was also detected in a variety of malignancies including pancreatic, gastric, cervical, breast and prostate cancer [32].

The identification of the two COX isoforms opened the gate for the development of selective COX 2 inhibitors. These new drugs were supposed to reveal similar anti-inflammatory, antipyretic and analgesic activity as unselective NSAIDs without causing gastrointestinal side effects [34]. As a long term application of Rofecoxib was associated with an increased cardiovascular risk, this substance had to be withdrawn from the market and the indications for other selective COX 2 inhibitors have been restricted.

24.3.2 COX 2 in Cancer

COX 2 is involved in carcinogenesis (Fig. 24.2), promotes tumor cell invasion, metastasis and angiogenesis via different pathways and facilitates escape from the host surveillance mechanisms. In a variety of cancers, COX 2 overexpression could be detected. In several preclinical studies, COX 2 inhibitors were potent in inhibiting

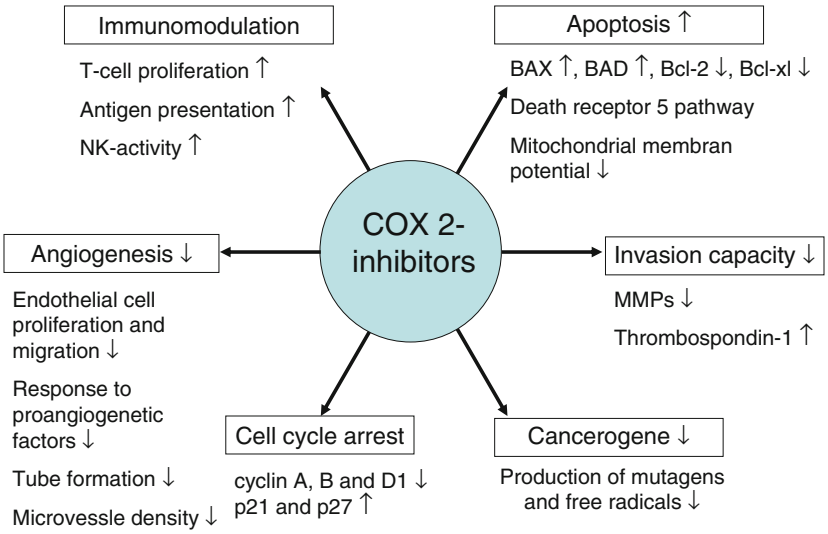

Fig. 24.2 Antitumoral and stroma-targeted effects of COX 2 inhibitors NK = natural killer cells, MMPs = matrix-metalloproteinases (From [141; Fig. 12.2, p. 225]. With kind permission of Springer Science and Business Media)

tumor cell growth in vivo and in vitro. Via increasing the level of proapoptotic BAD and decreasing Bcl-xl concentration as well as reduction of the Bcl-2/Bax ratio [35,36], activation of caspase 3, 7, 8 and 9 [36–39] and activation of the mitochondrial pathway of apoptosis by decrease in mitochondrial membrane potential [40], COX 2 inhibitors are able to trigger programmed cell death in cancer cells.

In addition, COX 2 inhibitors induced cell cycle arrest by decreased expression of cyclin A, B1 and D1 as well as CDK1 and induction of the CDK inhibitors p21 and p27 [41–43]. Interestingly, in a study using low COX 2 expressing and high COX 2 expressing gastric carcinoma cells, the growth inhibitory effect by decreasing bcl-2 expression was COX 2 dependent and the increase of p21(WAF1) and p27(KIP1) appeared to be independent of COX 2. Furthermore, the antiproliferative effects of celecoxib were comparable in cells with stable transfections of small interfering RNA (siRNA) against COX 2 and negative control vector cells [44]. As similar results were obtained in several studies using cell lines with low COX 2 baseline expression, in COX 2 deficient cell lines or after silencing COX 2 by antisense depletion, the antitumoral activity of COX 2 inhibitors was judged to follow COX 2 independent pathways [43,45–47]. Schiffmann et al. tested the effects of different COX 2 inhibitors as well as methylcelecoxib (DMC), a close structural analogue of celecoxib lacking COX 2-inhibitory activity, in COX 2 overexpressing and COX 2 deficient cell lines. Interestingly, only celecoxib and methylcelecoxib decreased cell survival by induction of apoptosis and cell cycle arrest and reduced the growth of tumor xenografts in nude mice. For this reason the researchers postulated that the anticancer efficiency of celecoxib seems to be no class effect of coxibs [48].

Promoting endothelial cell migration by increased TXA_2 levels, stimulating the production of angiogenetic factors such as VEGF, PDGF, bFGF and TGFβ, and

triggering tube formation, COX 2 plays an important role in the tumor associated angiogenesis [33]. These pathways can be blocked by application of selective COX 2 inhibitors. In an orthotopic implantation tumor model of colon cancer, celecoxib enhanced tumor cell apoptosis and inhibited tumor growth and angiogenesis by inhibiting COX 2, PGE_2 synthesis, and VEGF and MMP-2 mRNA expression in tumor tissue [49]. In several preclinical and clinical studies a reduction of microvascular density as well as decreased VEGF concentrations are described [50–52]. In a clinical trial treating 45 patients with prostate cancer with oral celecoxib for four weeks prior to radical prostatectomy, a decrease in tumor cell proliferation, microvessel density, angiogenesis and HIF-1 but an enhancement in apoptosis could be observed [49]. As in healthy endothelial cells only COX 1 can be found, while cancer associated endothelial cells often express both isoforms [53], a selective destruction of the tumor vasculature seems to be possible.

Angiogenesis is not the only target in the tumor-stroma interaction that is affected by COX 2 inhibitors. Liu et al. detected a reduction of lymphatic vessels and lymph node metastasis in lung adenocarcinoma [54]. In a Lewis lung carcinoma animal model, oral administration of high dose celecoxib significantly inhibited tumor growth as compared to a low dose treatment. In combination with radiotherapy, high dose celecoxib reduced the number of pulmonary metastases and delayed tumor growth to a greater extent than celecoxib or radiotherapy alone [55].

Blocking COX 2 function can enhance the function of immune cells in the stroma. PGE_2 impacts T-cell proliferation and antigen presentation allowing the tumor to escape host surveillance mechanisms [56]. In addition, tumor induced IL-10 production and activation of T-regulatory cells attenuate antitumor immune response [57,58]. As a consequence, the specific blockade of COX 2 triggers recognition and lysis of metastatic tumor cells by modulation of NK activity [59] and alters the balance of IL-10 and IL-12 [60].

Results of several studies implicate the importance of COX 2 inhibitors not only in treatment of malignancies but also in cancer chemoprevention. The evidence is compelling that NSAIDs as well as selective COX 2 inhibitors have strong potential for the chemoprevention of different tumor entities including colon, breast, lung and prostate cancer [61]. Also in bladder cancer a protective role of rofecoxib and celecoxib against tumor growth was detected [62,63].

In the meantime the results of several clinical studies using COX 2 inhibitors as single agent or in combination with other biomodulating or cytotoxic drugs are available [64–72]. As in a randomised phase II study 75% of the patients who received celecoxib but only 31% of the placebo patients showed clinical response, celecoxib seemed to have activity in the treatment of high grade cervical dysplasia [73]. Celecoxib as a single agent showed efficiency in the treatment of tumor cachexia [74]. Combining celecoxib with chemotherapeutic agents showed encouraging results in preclinical studies.

Celecoxib potentiated the antiproliferative effect of cisplatin on vulva cancer cells in vitro [75], and in a xenograft model of colon cancer celecoxib enhanced the antitumor effects of oxaliplatin [76].

However, in a clinical study the combination of celecoxib and doxetaxel, paclitaxel or carboplatin did not improve the response rate in patients with NSCLC [77,78].

24.4 mTOR Antagonists

24.4.1 The mTOR Receptor

The atypical serine-threonine kinase mTOR (mammalian target of rapamycin) is a master switch between anabolic and catabolic metabolism and plays a crucial role in the regulation of cell proliferation, differentiation, migration and survival [79]. Growth factor signaling is transmitted via the IGFR-PI3K-AKT-mTOR cascade. While mTOR function can be activated by growth factors, amino acids, ATP and glucose, the tumor suppressor proteins TSC1/2 and PTEN are able to inhibit mTOR activity. Because mutations of the tumor suppressor gene *PTEN* can be frequently found in malignancies, enhanced mTOR signaling can lead to uncontrolled cell proliferation. These cells are also hypersensitive to growth inhibition by blocking mTOR function. As a consequence, targeting mTOR could be a promising strategy in tumor therapy [80].

24.4.2 mTOR Antagonists

Rapamycin is a natural fungicide which is used as an immunosuppressive drug to prevent allograft rejection in organ transplant patients. After binding to its immunophilin FK 506 binding protein (FK-BP12), rapamycin inhibits mTOR function. As a result, arrest of cell cycle, inhibition of cell proliferation as well as 5–20% reduction in total protein synthesis can be induced in many cancer cells. Although the anticancer efficiency of rapamycin was described during its preclinical evaluation [81,82], this potential could not be clinically used until a series of rapamycin analogues with improved pharmacological properties were designed, including CCI-779, RAD-001 and AP-23573 [83].

24.4.3 Blocking mTOR Function in Cancer

In several preclinical studies, incubation of cancer cells with rapamycin or analogues reduced tumor cell proliferation [38,84,85] and induced arrest in G_1 phase of the cell cycle. Mechanisms like downregulating Cyclin A, D, E and survivin, as well as upregulation of CDK inhibitors p21 and p27 are involved in this process [85–87] (Fig. 24.3). One interesting question is, whether mTOR antagonists are able to induce apoptotic cell death in cancer. Rapamycin blocked cell cycle progression in renal, endometrial and lung cancer without inducing apoptosis [88–90]. In contrast, in anaplastic lymphoma an increase of apoptotic cell death was accompanied by a reduction of antiapoptotic proteins bcl-2, bcl-xl and c-Flip [86]. RAD001 significantly induced apoptosis in nasopharyngeal carcinoma. In addition, additive growth inhibitory effects could be obtained by a combinatorial treatment with RAD001 and

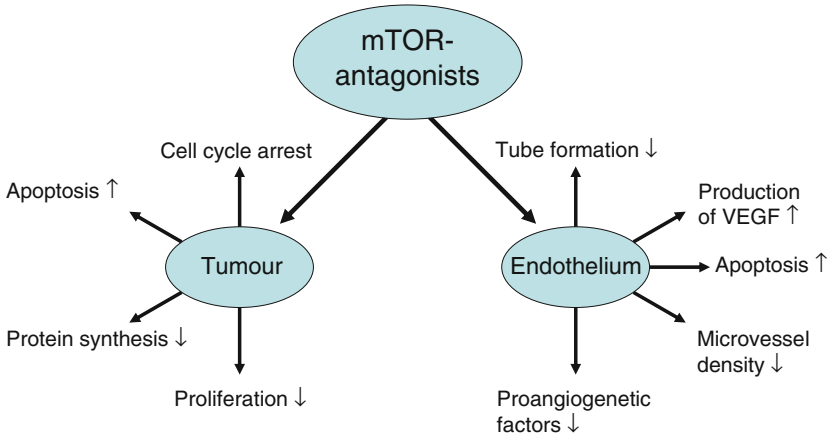

Fig. 24.3 Antitumoral and stroma-targeted effects of mTOR antagonists VEGF = vascular endothelial growth factors (From [141; Fig. 12.3, p. 229]. With kind permission of Springer Science and Business Media)

cisplatin [91]. Also in endometrial, ovarial and scirrhous gastric cancer a combination of mTOR antagonists with cytostatic agents led to a potentiation of antineoplastic effects by increasing growth inhibition and apoptosis [92–95] (Table 24.1).

Beside direct anticancer activities, mTOR antagonists also target stroma mediated mechanisms such as metastasis, invasion and angiogenesis. In a human renal cancer metastasis model, rapamycin reduced the number of pulmonary metastases and prolonged survival [89].

Cancer tissues with enhanced mTOR function are highly vascularized [96], a process which is regulated via hypoxia-inducible factor (HIF) induced transcription of proangiogenetic factors such as VEGF and PDGF. Overexpression of mTOR is able to increase the levels of HIF-α and subsequently of VEGF in tumor cells. Inhibition of mTOR signaling can interrupt this mechanism and block tumor associated neoangiogenesis. In some tumor entities, HIF α and VEGF levels as well as the response of endothelial cells to stimulation by VEGF could be reduced by mTOR antagonists [96–98]. In addition, rapamycin inhibited the proliferative, migratory, adhesive and tube formation capacity as well as differentiation of endothelial progenitor cells and decreased the level of endothelial nitric oxide synthase [99,100]. An increased susceptibility of tumor specific vessels to thrombosis was described after treatment with rapamycin [101]. Furthermore, the mTOR antagonist RAD001 reduced VEGF expression and microvascular density in a xenograft model of human hepatocellular carcinoma [102].

24.4.4 mTOR Antagonists in Clinical Trials

Clinical studies using rapamycin and its analogues in cancer therapy showed encouraging results [103–105]. Forty-one patients suffering from metastatic renal

Table 24.1

Cancer	Drugs	Clinical trail	Result	Reference
Angiosarcoma	Pioglitazone, Rofecoxib, Trofosfamide (metronomic)	Pilot study (n = 6)	2 × CR 1 × PR 3 × SD	[64]
Chronic lymphocytic leukemia	Thalidomide	Phase II (n = 28)	1 × CR 3 × PR 14 × SD	[26]
Glioblastoma multiforme	13 cis retinoic acid, Celecoxib	Phase II (n = 25)	44% SD	[65]
Glioma	Pioglitazone, Rofecoxib, Tremozolomide or Capecitabine (metronomic)	Phase II (n = 14)	29% SD	[66]
Histiocytosis	Pioglitazone, Rofecoxib, Trofosfamide (metronomic)	Case report	Tumor regression	[67]
Hodgkin's lymphoma	Thalidomide Vinblastine	Phase II (n = 11)	4 × PR 2 × SD	[23]
Kaposi-sarcoma	Pioglitazon, Rofecoxib Trofosfamid (metronomic)	Case report	SD > 1 year	[69]
Lung cancer	Celexocib, Paclitaxel, Carboplatin	Phase II (n = 29)	17%CR 48% PR	[68]
Lung cancer	Everolimus Gefitinib	Phase I (n = 10)	2× PR	[105]
Lung cancer	Thalidomide, Irinotecan, Gemcitabine	Phase II (n = 20)	10% PR 70% SD	[25]
Lung cancer	Thalidomide Carboplatin Etoposide	Phase II (n = 25)	4 × CR 13 × PR	[24]
Mantle cell lymphoma	Lenalidomide	Phase II (n = 15)	3 × CR 5 × PR	[27]
Melanoma	Thalidomide Interferon α2b	Pilot study (n = 15)	1 × PR 3 × SD	[21]
Melanoma	Pioglitazone, Rofecoxib Trofosfamid (metronomic)	Phase II (n = 19)	19% OR 14% SD	[70]
Melanoma	Thalidomide Dacarbazine	Phase II (n = 13)	1 × PR 1 × SD	[22]

(continued)

Table 24.1 (continued)

Cancer	Drugs	Clinical trail	Result	Reference
Multiple myeloma	Lenalinomide Dexamethasone	Phase III (n = 176)	60.2% response rate	[18]
Non-Hodgkin's lymphoma	Cyclophosphamide (metronomic) Celecoxib	Phase II (n = 32)	2 × CR 9 × PR	[72]
Pancreatic cancer	Gemcitabine, Celecoxib	PhaseII (n = 42)	4 × PR 26 × SD	[71]
Renal cell cancer	Everolimus	Phase II (n = 41)	14% PR 73% SD	[103]
Renal cell cancer	Temsirolimus Interferon α	Phase I/II (n = 71)	8% PR 36% SD > 24 month	[104]
Soft tissue sarcoma	Pioglitazone, Rofecoxib Trofosfamid (metronomic)	Phase II (n = 21)	11% OR 11% SD	[70]
Mantle cell lymphoma	Temsirolimus	Phase II (n = 29)	1× CR 10× PR	[108]

cell carcinoma were treated with an oral dose of 10 mg everolimus daily. With a progression free survival of at least 6 month for approximately 70% of the patients, everolimus showed an encouraging anticancer efficiency. As evidence is growing that temsirolimus benefits patients with advanced renal cell carcinoma [106] this mTOR antagonists is suggested to be the standard therapy for patients with poor prognosis [107]. In addition, improving progression free survival and objective response, single agent temsirolimus was shown to be effective in the treatment of relapsed or refractory mantle cell lymphoma [108,109]. Also in patients with myelodysplastic syndrome, everolimus was well tolerated [110]. Furthermore, biopsy proven cutaneous Kaposi sarcoma lesions disappeared in kidney-transplant recipients after 3 month of treatment with sirolimus [111]. Currently, several clinical studies are ongoing or have already been finished evaluating the anticancer potential as well as the safety, tolerability and pharmacokinetic and pharmacodynamic properties of the new mTOR antagonist deforolimus [112,113]. The available data indicate a high potential of mTOR antagonists in the treatment of advanced cancer. Further studies are warranted to use this potential in the treatment of cancer patients in the future.

24.5 PPARγ Agonists

24.5.1 The PPARγ Receptor

Thiazolidinediones like pioglitazone, ciglitazone and rosiglitazone are commonly used as insulin-sensitizer in the treatment of type 2 diabetes. They are ligands of the peroxisome proliferator-activated receptor γ (PPARγ), which is mainly expressed in adipocytes and cells of the immune system and is an important regulator of the cellular metabolism. The three identified isoforms, PPARα, PPARβ/δ and PPARγ, are members of the nuclear hormone receptor superfamily. After heterodimerisation with the 9-cis retinoacid receptor RXR, PPARs respond to ligand activation through the regulation of gene expression [114].

24.5.2 PPARγ in Cancer

Similar to thalidomide, COX 2 inhibitors and rapamycin, PPARγ agonists are able to induce growth inhibition, apoptosis and cell cycle arrest in a variety of human cancers [115–117] (Fig. 24.4). Although the underlying mechanisms are not fully understood yet, some of the antineoplastic effects seem to be independent from PPARγ signaling [118–120]. PPARγ protein or mRNA expression was detected in many human cancer tissues. In some malignancies like glioblastoma and adrenocortical carcinoma, PPARγ expression was even higher than in healthy tissue [121,122]. For this reason it is discussed if a therapy with PPARγ agonists could

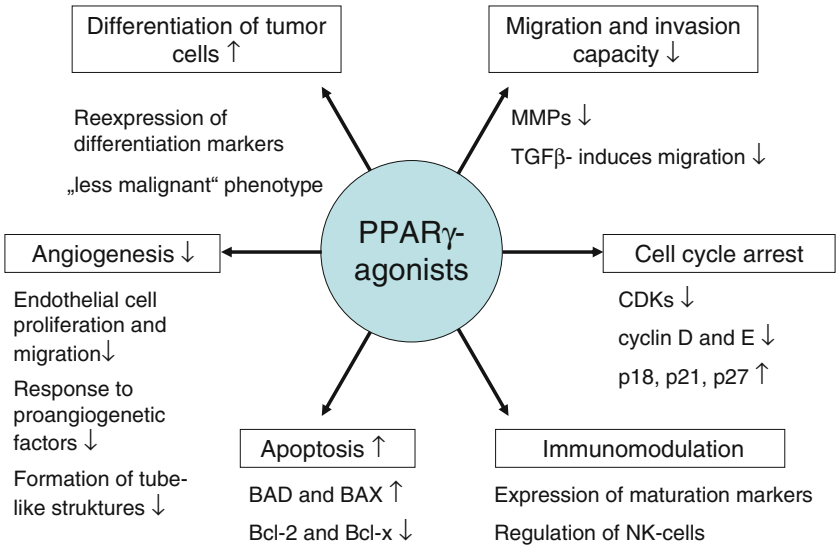

Fig. 24.4 Antitumoral and stroma-targeted effects of PPARγ agonists MMPs = matrix-metalloproteinases, TFGβ = tissue growth factor β, CDKs = cyclin dependent kinases, NK cells = Natural killer cells (From [141; Fig. 12.1, p. 223]. With kind permission of Springer Science and Business Media)

selectively target tumor tissue. Compared to normal myometrium, uterine leiomyoma was more sensitive to inhibition by ciglitazone [123] and pioglitazone had stronger growth inhibitory effects in leukemia cells than in hematopoietic stem cells obtained from healthy volunteers [124]. PPARγ activation also enhances gene expression in malignancies. In several cancer entities including melanoma [125], thyroid carcinoma [126] and in promyelocytic cell lines [127], a reexpression of differentiation markers could be induced by PPARγ agonists, which may be associated with a better clinical prognosis.

In addition to direct anticancer effects, PPARγ interact with the tumor stroma. One important mechanism in the tumor progression and invasion is the proteolytic matrix degradation by matrix-metalloproteinases (MMPs) and members of the plasminogen activating system [128]. PPARγ agonists effectively reduce tumor cell invasion and metastasis by inhibiting MMP-2, MMP-7 and MMP-9 activity as well as upregulating TIMP-1 and PAI-1 [129–132]. Furthermore, rosiglitazone reduced the number of lung metastases in a murine mammary tumor model, a process that could be associated with the decrease of MMP-9 expression level and reduced adhesion, migration and invasion of tumor cells [133]. As PPARγ heterodimerises with RXR, a combined therapy seems to be a reasonable approach in cancer treatment. In glioblastoma cell lines PPARγ and RXR ligands synergistically decreased tumor cell invasion and induced apoptotic cell death by increasing cytochrome c, caspase 3, Bad and Bax levels while decreasing Bcl-2 and p53 [132].

In addition to the reduction of tumor cell invasion and metastasis, targeting PPARγ also affects tumor associated neoangiogenesis. Treatment with PPARγ

ligands significantly impaired bFGF and VEGF-mediated proangiogenetic effects in the chick chorioallantoic membrane model [134] and inhibited leptine induced endothelial cell migration [135]. VEGF expression and microvascular density was significantly decreased in vitro and in vivo after application of rosiglitazone or pioglitazone in preclinical studies using pancreatic or ovarial tumors [136,137]. PPARγ ligands combined with daily low-dose chemotherapy, which is referred to as metronomic chemotherapy, was shown to cause synergistic antiangiogenetic effects. Metronomic chemotherapy induces endothelial cell apoptosis via enhanced expression of TSP-1 and subsequent activation of endothelial CD36 receptors. As PPARγ ligands can booster CD36 expression, endothelial cells are more susceptible for thrombospondin-1-mimetic peptides [138]. For this reason a combinatorial treatment with PPARγ agonists and metronomic chemotherapy might be a promising strategy in tumor palliation. In recent years the combination of PPARγ agonists and COX 2 inhibitors with metronomically scheduled trofosfamide turned out to be effective in the palliative therapy of several tumor entities including angiosarcoma, melanoma, soft tissue sarcoma, Langerhans' cell histiocytosis, Kaposi sarcoma and hepatocellular carcinoma [64,67,69,70,139,140].

In conclusion, several well established drugs which are in clinical use for non-oncological indications are promising new tools in tumor palliation. Especially when used in combination, these agents could enhance synergistically antitumor effects and overcome single agent induced drug resistance.

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Part VIII
Tumors' Systems Biology: Implications for Personalized Therapy

Chapter 25
A Methodological Approach to Personalized Therapies in Metastatic Cancer

Albrecht Reichle, Thomas Vogt, and Gerhard C. Hildebrandt

Abstract Personalized medicine should consist of a methodological therapy approach. Therefore, metastatic tumors have to be rendered usable for innovative action-theoretical therapy approaches to generate therapy-relevant tumor models and to uncover novel patterns of targets.

A new therapeutic level could be accomplished by introducing a pragmatic communication theory based on clinical results from less toxic combined biomodulatory therapies, altering the validity and denotation of cellular biochemical processes. A post-genomic view expands the role of proteins as an element within a network of communicative interactions. In a more abstract way, proteins and cells can be expressed as systems objects, which acquire contextual functions within circumscriptive functional modules or within the holistic communicative network of a tumor system. Biomodulatory therapies allow access to modular systems features as well as to the discrepancies between the functionality of single cell systems within a tumor compartment and the site-specific systems requirements of an organ (rationalization).

This way, modular tumor architectures, rationalization processes, deformations, and the Achilles' heels of tumor systems may be implemented into therapeutic considerations to expand therapy options by individual systems-relevant and stage-relevant features (secretome, molecular imaging). Multi-level decision-making during therapy, i.e. biomarker-guided selection of therapies for individual patients, consecutively necessitates novel trial designs.

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Selection of patients for therapy could be replaced by selecting therapies for patients, corresponding to the stage-dependent developmental status of a tumor system in an individual patient.

Keywords Adaptive trial design • Systems biology • Modularity • Metastatic tumor • Personalized tumor therapy • Molecular imaging • Secretome analytics

25.1 Personalized Medicine: Post-metaphysic Thinking

The overall aim of personalized medicine is the improvement of benefits: The risk ratio for patients needs to be decreased by specifying the diagnosis of tumor diseases and by improving the outcome and the delivery of the 'right' drug at the right dosage and appropriate time. The introduction of biomarkers, pathway signatures, tumor genomics, and pharmacogenomics involves the identification and application of markers and scores. These instruments correlate with drug response, treatment efficacy, or adverse events, and represent a prerequisite for critical drug discovery, for the development of decisions and, ultimately, for **novel clinical trial designs** to personalize tumor therapy.

This specific modern trend, which has seized the theoretical concept of personalized medicine, lies to a lesser extent in novel methodologies to approach the individualization of tumor therapies. This trend is rather based on the versatile motifs of science and scientists who aim at getting closer to the particular circumstances, i.e. the individual situation and tumor disease, of an individual patient.

The either scientifically-based or interest-guided motifs for personalizing tumor therapy compulsorily lead to paradoxes: Primarily theoretically orientated disciplines of medical science postulate the inversion of leadership by putting theory before clinical practice and by exchanging their original order, although this exchange is probably unintentional. As a result, innovation is required for novel trial designs.

The diverse motifs and emancipatory interests of modern trends on personalized tumor therapy directly lead to new paradigms of care, which need to be publicly communicated. From a scientific perspective, personalized therapy has to be based on methodology and delineated from **therapy-relevant tumor models** to deal with the vast amount of knowledge available on tumor diseases.

By focusing the view on regained novel scientific objects, namely patients and their postulated individual tumor disease, rather different scientific areas of knowledge need to be connected to meet the therapeutic requirements of patients and their disease. Large, highly diversified data sets must be fused and computeralized. New languages termed **'ontologies'** are recommended to be generated, combining seemingly incommensurable data from various scientific fields [1,2]. This computeralization requires more than sophisticated computer programs; as such programs may only partially rescue or put in order these incommensurable data fields or, at best, generate some new perspectives and hypotheses to be proven by experimental data.

25.1.1 Therapy-Relevant Phenotype

The therapeutic accessibility of therapy-relevant situational information about a patient's tumor-associated disease traits (modularity, rationalization processes, spin-off of novel systems characteristics) is equally necessary. These attempts are at an early stage: Uncovering a tumor's whole genome with its frequently multifold aberrations does not necessarily promise the prediction of a tumor's **therapy-relevant phenotype**, even if diverse aberrant signaling signatures are incorporated into therapeutic considerations. In future, therapy-relevant phenotypes may be ascertained on a broadened methodological basis and may differ from particularized biologic parameters, signaling and genome signatures, or complex computer-based scores with predictive potency for survival or metastatic behavior [5].

25.1.2 The Reductionist Therapy Approach

The multiplicity of reductionist knowledge on tumor biology derived from highly diversified scientific fields has to be focused in individual patients by demonstrating coherency and practicability-requirements that include multimode aspects.

Therapy-relevant knowledge on tumor biology is preferentially derived from acquired gene aberrations or from altered gene expression in tumor cells. Because of the suggested genetically-based causation of tumor disease, genes and their gene products become therapy-relevant targets (drug discovery), in addition to pathophysiologically relevant targets including stem cell niches [6]. The contextualist perspective is met by multi-level reductionistically designed therapy approaches, which are now specified by complex molecular signature analytics [7].

The central problems, however, that need to be resolved in individual patient care remain the complexity, the multi-level hierarchies, and the facticity of a phenotypically realized individual tumor disease.

The newly uncovered metaphysic privilege of unity in individual patient care replenishes the contextualist preference of multiplicity before unity in a conspirative sense: Incommensurable 'worlds' are merged into **'ontologies'** [8]. Even the contextualist compilation of tumors, which primarily focuses on the inherent coherency of aberrantly expressed genes, has to face the problem that genes, as aberrantly as they may be expressed, do not represent any programs themselves. The expression of digitally coded aberrant genetic information has to be considered in the context of an analogously working none-DNA-based heritage [9]. This heritage contributes to the growth 'program' of tumor cells by adding, for example, modularity, rationalization processes, and contingency programming for efficacious tumor propagation and maintenance. Non-autonomous factors of tumor cells from the microenvironment additionally configure tumor growth (chapter 23) [10].

25.1.3 The Holistic Therapy Approach

The phenotypically-based aspect of tumor-inherent **rationalization processes** is embedded in the holistic communication processes of a tumor (a tumor's 'living world'). In light of this fact and the developing **modularity** within complex tumor systems, the process of particularization of socially linked cells and sub-cellular functions may represent something different than the disposal of self-reflexively guided pathways, networks, genes, and tumor stem cells. Genes do not represent programs, and transmission of information is not limited to a one-way direction [4]!

Multi-level particularization, conceptualized by the suggested multi-level differentiation of systems with genes seen as the universal originators (genes and causation), is ambiguous for therapeutic issues. Developing a systems description seems rather more important, which does not exclusively consider levels of action (tumor genomics, pathway analytics, and niches).

The methodological development of modularly designed therapies for metastatic tumors may be one answer to the universal competition of unity in patient care and the multiplicity of reductionist (contextualist) knowledge [4]. Communicative action with metastatic tumors in terms of biomodulatory therapies may be a further step in personalizing patient care.

25.2 The Idea of Homogeneous Patient Subsets

25.2.1 Evidence-Based Therapy: Uncovering Prognostic Parameters

The choice of cytotoxic agents is empirically-based and geared to fit the average patient. However, only in a few tumor types, such as germ cell tumors [11], does the majority of patients benefit from classical therapeutic regimens. Molecular rationales currently considered for cytotoxic agents in metastatic tumor therapy are different in vitro sensitivities of tumor cells, for instance with defects in post-replication repair genes (cisplatin), an helicase sgs1 mutant (cytarabine), or defects in double strand break repair (camptothecin, idarubicin and mitoxantrone). Some agents are selective for a broader range of DNA damage repair mutants, and some agents are non-specific [12].

In most types of cancer, a high percentage of patients may receive an inefficacious combination of cytotoxic drugs. With a better understanding of the mechanisms underlying efficacy and toxicity of anti-cancer drugs, medical research is now focusing on personalizing treatment strategies. These strategies involve the combination of preferentially genetic characteristics but also incorporate pathophysiological features and micro-environmental factors together with traditional tumor characteristics (histology, tumor spread), which currently drive clinical decision-making.

Increasing knowledge about genetic and molecular-genetic changes in tumor cells offers new insights into the development and spreading of tumors, allowing a separation of patients at risk for tumor development and patients suffering from early metastatic spread [13]. Simultaneously, these genetic changes can present therapeutic targets both on a protein level or an RNA level. A steadily growing variety of drugs has been developed, tailored to inhibit specific tumor-associated molecules, such as enzymes, receptors, and pathways. These drugs are hoped to cope with the molecular-genetic-based heterogeneity of tumors: Ever decreasing patient cohorts, which are characterized by distinct patterns of molecular-genetic characteristics, are intended to receive therapies that are as specific, as tolerable, and as efficacious as possible [14].

What do we accomplish? Hope is generally focused on a patient's personal tumor genomic and the complete catalogue of acquired gene aberrations. What happens, if a tumor's genotype is really individual and even intra-individually heterogeneous, or if no tumor really matches another? Such thoughts may be particularly relevant in so far fairly untreatable types of cancer, such as pancreatic cancer, comprise up to 70 gene aberrations on average. To keep **evidence-based medicine** in our therapeutic decisions, we have again to look for homogeneous patient subpopulations, who share an intersection of aberrantly expressed genes or other prognostic parameters and who may be treated homogeneously [15].

25.2.2 Individual Tumor Disease

The limitations in the homogeneity of patient cohorts are obvious: Aberrant genes, as homogenously as they may be distributed within distinct tumor types, do not represent unidirectional programs, which definitely constitute a tumor's phenotype in a distinct host organ. We also have to take into account the non-DNA-based heritage of tumor and stroma cells [9]. Intersystemic exchange processes between the 'heritages' are minted by communication acts [4]. By introducing a pragmatic communication-theoretical approach, the intentionally uncovered structural levels are resolved in equivalent communicative structures bent on the respective systems objects. Now, the socially interwoven tumor and stroma cell community evolves as a holistic communication-driven structure, which provides internal access via modular therapy approaches, thereby disclosing its modularly designed architecture (recons **tumor tractibility** of modular structures) [3,4].

Communicative tumor (sub)-systems do not obey nominal conditions in an evolutionary process but adhere to rules to meet the validity of communication processes: Phenotypically distinguishable individual tumor disease may constitute within the predetermined range of-at least to some degree-autonomous tumor development (see chapter 26). These self-evident presumptions compromise the phenotypical homogeneity of tumors [3].

Phenotypical matchlessness of an individual tumor disease is in conflict with the search for homogeneity and common features in larger patient cohorts.

The homogeneity of cohorts is a prerequisite to keep tumor therapy evidence-based, because trials using targeted therapies do frequently not succeed without prior molecular-genetic-based tumor selection.

Additionally, the interference of drugs with a patient's organ system has to be taken into consideration. Drug interactions in combination therapies as well as pharmacogenomically based variants, which contribute to reduced drug tolerability and efficacy, have also to be considered when planning personalized therapies [16].

25.2.3 Novel Therapy-Relevant Methodological Approaches

Leveling hierarchical orders by communication-guided considerations aids the establishment of **novel therapy-relevant targets that lie in the communicative tumor system**: The holistic communicative process itself, a tumor's living world, is placed at the investigators' disposal.

A tumor's 'living world' gathers various input signals and mediates the validity and denotation of communicatively-linked biochemical pathways. This communication-driven biochemical or cellular 'background' is directly involved in tumor evolution and may be featured by novel systems-related similarities among tumors – besides established histological findings and genome signatures [3]. In interaction with normal human tissue, tumor cells use communication-linked processes according to laws of nature to build up a favorable infrastructure for their proliferation. Also leukemia cells with stem cell functions showing an unlimited capacity of self-renewal in vitro are communicatively integrated in a highly aberrant stromal environment. These tumor or stroma cell processes are accessible in a reconstructive way via biomodulatory therapies and may be classified to generate novel, presumably homogeneous tumor systems characteristics, as indicated by the uncovered differential features of tumor-associated inflammation [4,17].

Therefore, the search for homogeneous patient populations may be more successful by the additional incorporation of novel methodologically-based procedures: A second, communication-driven objectivation of tumor features is now available describing **tumor-comprehensive systems stages**. These systems stages mirror situate, phenotypically characterized dispositions of the tumor and may be used as therapy-relevant targets to further personalize tumor therapies [3,4] (Fig. 25.1).

25.3 Differential Model-Creating Determinants

25.3.1 Hierarchical Therapy-Relevant Structures

The descriptive allocation of 'tumor-inherent' functions to characterize a tumor's disastrous features remains consistent with reductionist or contextualist requirements to create hierarchical levels responsible for promoting tumor growth, such as tissue invasion (matrix remodelling), inflammatory microenvironment, insensitivity to growth inhibition, evasion of apoptosis, sustained angiogenesis, limitless replication

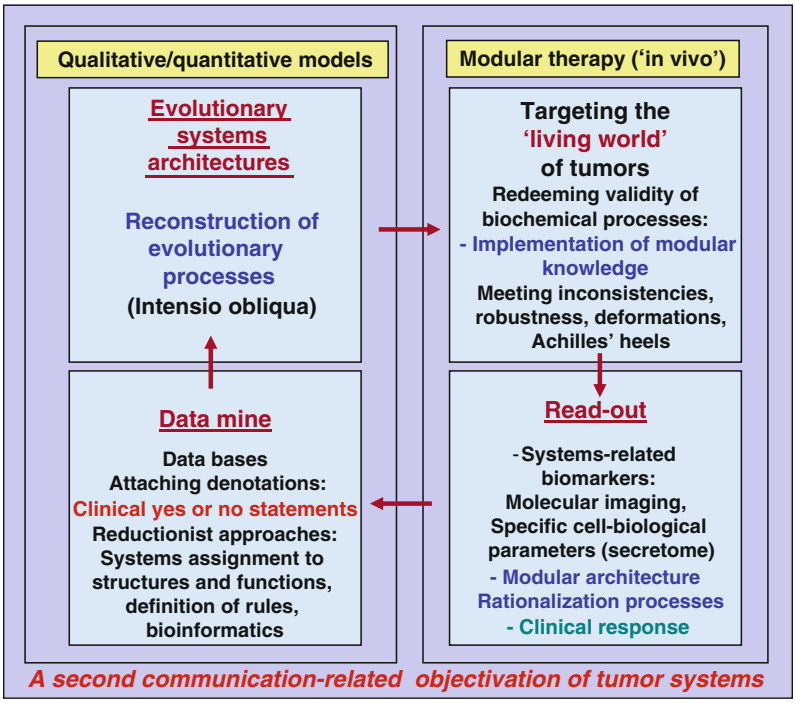

Fig. 25.1 Reconstructive analyses of tumor-specific evolutionary processes may be achieved by iterative cycles of differentially structured (combined) modular tumor therapies and evaluation of modular systems and tumor response: Modular therapies generate systems-related read-outs, consecutively leading to decision-relevant yes or no statements. Qualitative and quantitative systems analyses may be supplemented and broken down to an analytical level by complementary molecular-biological data mining. Thereby, systems-relevant functions may be assigned to specific structures within stage-specific rationalization processes resulting in systems classification

potency, and self-sufficiency in growth signals [18]. Highly specific metabolic changes in tumor cells and the impact of coagulation are frequently neglected (chapter 3 and 7) [17,18–21]. In the reductionist picture, tumor-associated pathophysiologic features are equated with the causation of tumors. The usefulness of this description is the integration of tumor cells into a larger environmental context. However, this description reduces environmental tumor-associated activities as compliable unidirectional functions mediated by tumor cells.

The newly uncovered systems perspective, which is frequently underestimated, moves its focus to the discrepancies that develop between the functional world of tumor-associated cell systems and the functional requirements imposed by rationalization processes and triggered by a tumor's systems 'world' [3,4]. Systems may coordinate the 'idea' of different cell types (Table 25.1). From the perspective of a participant within a tumor system, novel qualities of systems objects, i.e. cells and sub-cellular biochemical processes, may be described: (1) Modular tumor architecture emerges, which is accessible for biomodulatory therapy approaches. (2) In comparative analyses, tumor systems may be characterized as rationalized tumor-specific

Table 25.1 Evolution: the 'metabolism' of systems development

Reproduction of social cell communities or realization of social functions
Social integration, coordination of 'ideas' of different cell types: 'Theory of communicative action'
Socialization of cell systems and interpretation of requirements

systems features, such as inflammation. (3) Achilles' heels can be uncovered if functions may only be arduously kept up to maintain the systems context as well as (4) systems-related deformations of cellular functions. (5) New systems features, i.e. tumor-associated inflammation, may spin off [3,4].

25.3.2 May Hierarchical Structures Be Abated for Therapeutic Purposes?

Considerations involving evolutionary tumor processes have to abate hierarchical aspects to establish communicative systems architectures. Systems objects (cell types, pathways, transcription factors, etc.) acquire modular features because their function is non-randomly dependent on the multimode bundled functions of the so far unrecognized background. This background redeems the validity of systems objects in the first place to establish distinct denotation of communicatively-linked biochemical processes [4,22]. As shown, modularly-designed tumor therapies may also redeem the validity of systems objects in the tumor compartment, which represents an important novel mechanism to therapeutically achieve tumor control [4].

Hitherto existing perspectives favoring unity of patient care and contextualism are likely to consider communicative actions in terms of modularly-designed tumor therapies as too weak and presumably inefficacious. The reason for this view is that all hierarchies developed by intentionally acquired knowledge are leveled to be discharged in a continuum of contingency programming, in modularly-evolving systems features, and in continuous inter-systemic communicative exchange processes. On the other hand, the methodology of communicative therapeutic intervention (modular therapy) seems to be too potent from a contextualist perspective. This view may be caused by the fact that incommensurable 'worlds', such as non-DNA-heritage and DNA heritage or different techniques for implementing modular knowledge and various modular tumor architectures, turn out to be pervious, despite their qualitatively rather heterogeneous features.

25.3.3 Model-Creating Determinants

Competing model systems, both reductionist and holistic, show **different model-creating determinants** (Table 25.2). The genetic background used for developing tumor models is now contrasted by communicatively-derived modular architecture.

Table 25.2 Differential model- and therapy-creating determinants

Determinants	Assessment tools
Evolution (principles of communication)	• Modularity • The tumor's 'living world' as communicative holism • Denotation and validity of communication processes
Systems 'world' versus functional 'world' (functional diversification of cell systems)	• Rationalization • Intersystemic exchange processes • Achilles' heel • Deformation • Topology of aggregated action effects
Pathophysiology	• Angiogenesis, inflammation, metabolism, extracellular matrix remodelling, coagulation, proliferation, etc
Histology/biochemistry Genetics	• Structure, function, interaction • Pathway signatures • Functional genomics

Therapy-relevant tumor models have to be realized, discussed, and balanced against each other before developing or planning appropriate therapies in distinct tumor types and systems stages. Thus, differentially-applied methodological approaches to implement therapy strategies for metastatic tumors may bring appropriate therapies closer to the patient: The most efficacious therapeutic approach for an individual patient is now becoming scientifically evaluable.

Basic science is covering versatile forms of acquired knowledge that is based on differentially applied methodologies-also in the field of systems biology. The simple perception of facts without considering the methodological background leads to conclusions that may not be equated with science, for example, if aberrant genes are unidirectionally equalized with the causation of an altered phenotype**.** This equation may be appropriate for Philadelphia chromosome-positive chronic myelocytic leukemia but cannot be that easily applied in tumors with multiple chromosomal aberrations, such as pancreatic cancer [23,24].

The different methodologies for creating tumor models complement each other in the same way as the **benchmarks of communicative systems correspond to the components** of which functional sequences are composed (Table 25.3). Systems biological considerations rely on studies of basic science, which primarily try to disassemble complexity and measure the activity of isolated systems components. Such an approach is very successful in characterizing the individual parts but very limited in reconstructing how single components are communicatively integrated and rededicated within a systems context (modularity): Depending on the host, the developmental status, and the systems context, genes and their gene products may have completely different, sometimes opposing functions. Obviously, the communicatively-linked biochemical or cellular background may define the validity and denotation of distinct systems objects, for instance transcription factors. The term 'oncogene' surely does not cope with the evolutionary function of a distinct gene.

Table 25.3 Change of paradigms: The three mainstays for acquiring new insights into novel therapy approaches implementing modularity

• **Therapeutic access from inside** in a comprehensive and reconstructive way (the participator's view)	• **Observation-guided**, contra-intuitive knowledge (the observer's view)
• **Normative statements** how to control systems-associated processes with therapy modules to achieve response	• **Classic conclusion logic**, e.g. indicating a pathway responsible for cell death: **cause-effect-chain**
• **Situation-associated systems interpretations**: modular architecture, rationalization processes, evolutionary context	• **Object-associated, intentional interpretation (nude identity)**: theme-dependent context-knowledge, compartmentalized systems

25.4 Modularity and Rationalization of Tumor-Associated Functions: Therapeutic Targets for the Therapy of Metastatic Tumors

Modularity of cells and cell systems is a ubiquitous intrinsic biologic dimension, which becomes of exceptional interest during evolutionary processes, i.e. during tumor growth. In the first place, modularity may establish multi-functionality and evolvability within a holistic communicative tumor cell system. Modularity either descriptively (modular therapy approaches) or mathematically seizes the phenomenon that the various, sometimes even opposing, references of the systems objects are interwoven situational biological stages, i.e. they are embedded in the communicatively arranged validity and denotation of systems objects [4].

Cellular functions, such as signal transmission or cell cycle control, are carried out by 'modules' made up of small networks, which are composed of numerous interacting molecules. The understanding of how modules work depends on combining phenomenological analysis (uncovering of rationalization processes) with molecular-biological studies. Proteins are traditionally identified based on their individual actions as enzymes, signaling molecules, or structures constituting aggregates in cells. But the post-genomic view now expands the role of proteins as an element within a network of communicative interactions. A more abstract term for a protein-in a communicative sense – is 'systems object', which acquires contextual functions within circumscriptive functional modules or within the holistic communicative network of a tumor system (chapter 26) [4,25].

Various possibilities seem to exist to redeem novel validity and denotation during evolutionary tumor processes independent of the presence of acquired genomic aberrations. Multi-functionality has been observed as a feature of protein evolution: As an example may serve the protein p53 [22,26,27].

Single molecules acquire cell type-specific functions, and diversification of signaling pathways may occur [28]. The highly specified systems-mediated regulation of transcription factors, such as NF-kappaB, may induce even opposing biological effects within the same clonal cell population [29,30]. In response to diverse stimuli, transcription factors alter their interactions to varying degrees, thereby rewiring

networks. A few transcription factors serve as permanent hubs but most act transiently only in certain conditions [31].

Modularization is suggested to be a universal characteristic of real networks because of the advantages it adds to the multi-functionality, robustness, and evolvability of networks. Zhang stated 'modularity may constitute a big world inside small world networks' [32]. Shorter network diameters could provide higher functional efficiency: An intrinsic tradeoff between network efficiency and multi-functionality, robustness, and/or evolvability seems to exist.

The modular architecture of biologic networks allows the selective implementation of biomodulatory acting agents. The implementation of modular knowledge provides an important therapeutic instrument. Biomodulatory therapies facilitate the reconstructive analysis of tumor and stage-specific rationalization processes, for example tumor-associated inflammation.

Modular therapies constitute a novel frame work for qualitative (clinical response, tumor site-specific activity) or quantitative analysis (systems-associated biomarkers, imaging techniques) of modular tumor architectures as a prerequisite for reconstructing or redesigning functional modules or rationalization processes from their cellular or molecular constituents.

25.5 Creating a Cancer-Drug Portfolio: Interest in the Technical Disposability over Verifiable Tumor-Associated Processes

25.5.1 The Classic Approach: Cytotoxic Therapy

The availability of cytotoxic agents brought great progress to the treatment of metastatic cancer. Cytotoxic agents are still indispensable. However, a high proportion of patients has to frequently face side effects together with no or moderate activity of the administered cytotoxic drugs. Therefore, the following question has arisen: Which patients do really benefit from combined cytotoxic therapy in the light of its additive effects on subjective well-being and the impaired quality of life counterbalancing the often limited treatment efficacy. As a consequence, this dilemma has propelled the search for both prognostic markers and pharmacogenomic parameters.

The handicap of prognostic markers persists because they are closely related to the type of therapy as well as to the biology of a disease [33]. Frequently, negative prognostic markers do not open up perspectives for alternative therapy strategies, and patients depend on the development of novel agents in the long run.

The dogma that aberrant genes cause cancer disease has reinforced the conception to counteract the activity of these genes by corresponding inhibiting agents on protein or RNA level (anti-sense technology). This method aims at reducing hardly calculable side effects and at developing a more biologically oriented therapy, which is now often equalized with personalized therapy.

25.5.2 Targeted Therapy

The term ‘targeted therapy’ reflects the suggested close relation between genetic (molecular-genetic) aberrations and cancer causation. Consecutively, the idea is that – if the cause has been eradicated within a tumor cell – the tumor cell cannot and will not survive.

Trastuzumab has been one of the pioneers in a series of designer drugs selected for patients on the basis of the molecular profile of their tumor [34]. Instead of the conventional ‘one size fits all’ approach, trastuzumab provided a novel way to attack cancer specifically. The successful approval of trastuzumab for the treatment of Her2-neu positive breast cancer has furthered the idea of personalized medicine. However, only a limited number of studies highlight tumor-associated gene aberrations, showing that tumors best respond to respectively targeted therapy approaches. A special concern remains the reliability of thresholds of biomarkers and their analytical validity. Recent studies have emphasized the shortcomings of the ‘gene and causation approach’ by showing that the genetic backbone of many cancers is both complex and overlapping: A wide variety of rare genetic aberrations are implicated in many types of cancer, and mutations in distinct signaling pathways are often not tumor-specific [35].

A consistently reductionist procedure for studying systems interrelations seems to be the deconstruction of aberrant tumor cell-associated signaling pathways. Tumor-associated gene expression signatures are consecutively reconstructed as ‘modules’ [7]. Aim of this methodological procedure is to combine multiple targeted therapy approaches to finally achieve better tumor cell control [36,37].

Because of antibodies detecting cancer cell epitopes, the main symptoms of a disease may be successfully eliminated; but the introduction of novel technologies, such as biospecific antibodies (synthetic biology), may even eradicate minimal disease residuals [38].

Combinations of chemo-therapy and targeted therapy primarily arise from drug availability and from the fact that most standard therapies comprise cytotoxic agents, which often makes targeted therapies a simple “add-on” component. Recent studies have shown that the efficacy and toxicity of such combination therapies is poorly predictable [39].

The consideration of stroma components and their close link to tumor progression has led to the introduction of antiangiogenic therapy approaches. These therapies served as a proof of principle that tumor-associated stroma components are aberrantly expressed and therefore appropriate targets for tumor therapies [40].

25.5.3 A Tumor’s Holistic Communicative Structure as a Therapeutic Target

Biomodulatory therapies now establish access to rather novel patterns of targets, which predominantly lie in the communicative structure of a tumor compartment. The holistic communicative system itself is the therapeutic object, whereas physicians

therapeutically participate in the system to guide modular processes. In the course of biomodulatory therapies, **tumors may be reconstructed** from their evolutionary site. Biomodulatory therapies may implement modular knowledge by redeeming the validity of systems objects. Such therapies may communicatively specify the denotation of molecules, for instance distinct transcription factors. Their denotation is linked to the communicatively structured background, gathering and mediating the multiplicity of input signals [41].

The modular systems structures of tumors are therapeutically accessible to regulatory acting target sites that evolve during tumor progression. The repertoire of drugs abruptly expands (Table 25.4) with the introduction of systems-therapeutic (modular) concepts because:

1. Substances with unintended indication, such as drugs modulating the transcriptional networking of both tumor and stroma cells, may be introduced for therapy [41,42].
2. Contrary to the molecular-genetic heterogeneity of tumor cells, tumor growth-promoting systems show a high level of similarities (for example, angiogenesis and inflammation). Therefore, a similar repertoire of drugs may be available, which target and regulate corresponding tumor-associated subsystems mirrored by biomarkers [17].

Table 25.4 Reductionist and systems-directed therapy approaches

Systems level	Target	Therapy approaches
Tumor cells	Tumor cell-specific pathways, epitopes, etc. (reductionist)	• Cytotoxic chemotherapy • Small molecules • Antibodies, cellular therapies
Stroma cells	Stroma cell functions (reductionist)	• Education, re-education • Elimination, Trafficking • Vaccines
Modularity, evolvability	Modular tumor architecture (systems-directed)	• Biomodulatory therapies • Synthetic biology
Pathophysiology	Angiogenesis, inflammation, apoptosis metabolism, extracellular matrix remodelling, coagulation, proliferation etc.	• **Biomodulatory therapies** • Antibodies • Small molecules
(Immune-) Histology Biochemistry	• Tumor cell-specific molecules • Structures, functions, interactions • Pathway signature; functional genomics	• Combination of small molecules • Anti-sense therapy • Antibodies
Systems versus functional 'world'	Varying cellular mediators of similar cell functions: • Rationalization, Achilles' heel • Intersystemic exchange processes • Topology of aggregated action effects	• **Biomodulatory therapies** • Antibodies • Small molecules

3. Targeting functionally defined subsystems becomes of increasing interest, as subsystems may be exclusively functionally defined in a systems context but simultaneously linked to alternating structural systems [43]. Targeting functional systems structures provide a new therapeutic window favoring concerted biomodulatory strategies.
4. Beyond that, it should be possible to abstract traditionally described subsystems: Drugs with biomodulatory activity as (nuclear) transcription factors regularly have an activity profile far above the capacity of hermeneutic comprehension [17]. Transcriptional networking may have a decisive regulatory impact on tumor promotion, for instance, on the angiogenic switch or on tumor stem cell behavior [44]. Indeed, the abdication of hermeneutic comprehension constituted a prerequisite of modern science.
5. Complimentary reciprocal activity, during which subsystems may generate each other, may be analyzed as currents of intersystemic exchange. Therefore, from a therapeutic point of view, the systems-biological model does not specify whether a systems function has to be suppressed or stimulated to achieve tumor control: Inflammation control as well as stimulation of inflammation may control tumor growth, immunosuppression, and immune stimulation [17,45]. Contradictory decisions could be associated with the same capacity to achieve tumor control in a distinct tumor type. Thus, the questions arising are: What therapeutic approach would be easier to put into practice, what approach is likely to be more compatible with other therapeutic approaches, and what is the most tolerable approach with regard to side effects?
6. Based on the reductionist use of drugs for tumor therapy, a drug should have significant mono-activity and still be acceptable at maximum tolerable doses. Now, in the light of biomodulatory therapy approaches, demands are revolving. De Vita phrased: ‘If an agent modulates a target in preclinical models and the expected downstream effect induced by target interaction is observed, perhaps this provides sufficient evidence to test the agent in a clinical trial, even in the absence of demonstrated efficacy in preclinical models, provided there is enough information to determine a safe starting dose’ [46]. A large number of drugs could be integrated into modularly designed therapy approaches because their single prerequisite is biomodulatory activity in a concerted action.
7. In the near future, biomodulatory therapy approaches of metastatic tumors could be methodological tools for **personalized tumor therapy**: In contrast to ‘causal’ therapeutic approaches aiming at the blockage of aberrant tumor-associated pathways by a restricted repertoire of highly specific drugs, multiple potential modulators **(activators and deactivators)** of transcriptional processes are available for biomodulatory therapy approaches. According to our experience, mono-activity of a single transcription modulator is no prerequisite for its successful use, and the combined administration activity of all modulators could be followed by respective biomarkers. Close monitoring would further allow us to choose other modulator combinations in case of weak interactivity to facilitate objective tumor response [17].

8. The simultaneous communicative therapeutic interaction with systems entails the administration of **low-dose levels of each biomodulatory acting drug** within a combined schedule. Achieving cytotoxicity with maximum tolerable doses is not of primary concern any more.

25.5.4 Expansion of Therapeutic Options

The therapeutic capacity of biomodulatory therapy approaches to meet phenotypically featured **systems stage-specific and modular architectures** points to an asymmetry between reductionist and communicative systems-directed therapy approaches (Table 25.4). The extent of attaining therapy-relevant targets with (combined) reductionistically derived 'targeted' therapy approaches seems to be more limited compared to the provided prospects for targeting systems-relevant rationalization processes within a tumor, for instance tumor-associated inflammation: (1) Multiple systems features, the topology of aggregated action effects, robustness, inconsistencies, deformations, inter-systemic exchange, and rationalization processes may establish a broad capacity to resolve therapeutic problems. (2) A broad series of stimulatory and inhibitory drugs without mono-activity in a respective tumor type could be introduced into combined biomodulatory treatment schedules [17].

Furthermore, analytical data point to a postulated asymmetry between the therapeutic capacities of modular therapeutic options versus reductionist approaches: The extremely high frequency of un-anticipated actions of approved drugs, which are observed by screening against complex pathways, supports the model of robustness. Transcriptional modulators, which carry out tasks such as lowering the serum glucose level (PPARgamma agonists), are likely to achieve additional effects. Thus, such modulators may acquire completely novel denotations in combination with other transcriptional modulators, such as dexamethasone and interferon-alpha (indication discovery) [47].

Un-anticipated actions of approved drugs contrast a narrow selection of patients for studies. On the other hand, for some drugs, patient selection seems to be the only path to therapeutic success.

25.6 Monitoring Therapy

The incorporation of biomarkers into drug developing and drug monitoring processes will improve the understanding of how therapies or therapeutic strategies work. This incorporation will allow a more accurate identification of patients benefiting from these therapies. The aim of incorporating a patient's tumor systems-related data in treatment planning is becoming reality.

25.6.1 Integration of the Classic Reductionist Approach

Genetic testing allows selection of the best treatment. Physicians start to use detailed information about the tumor genomes of a patient to decide which treatment will be best [7,48–50].

The classic biomarker for reductionist therapy approaches is related to a drug's target. Consequently, prognostic tumor cell-associated parameters directly mirror response or futility of distinct groups of respective targeted agents.

For true effectiveness, more than one single biomarker has to be developed in separate training and test sets. The inherent problem associated with, for instance, microarray data sets is termed 'overfitting' the data. This process occurs when many elements, for instance genes, are correlated with a few clinical end points such as survival, recurrence, etc.: Only a small number of a long list of genes may be found to correlate in expression and by random chance with one of the few possible clinical end points [51].

As a next step, the integration of multi-parameter clinico-pathological variables including imaging and biomarker data (commonly termed systems pathology) may result in a highly accurate tool for predicting clinical outcome [52].

25.6.2 Are Therapeutic Approaches Developing into a Systems-Associated Marker-Guided Therapy?

Systems stage and systems architecture (for instance inflammation): The technique of communicative action, which allows the implementation of modular (therapeutic) knowledge, connects rather incommensurable scientific worlds of communicatively linked structures, i.e. digitally coded DNA and analogously operating non-DNA-based heritage [17]. Consecutively, the classification of tumor-associated structures and functions (systems stages) has novel practical and particularly therapeutic impact (Table 25.5).

The communicatively uncovered and frequently unconsidered molecular and cellular 'background' involved in tumor evolution gathers the diversity of input signals and mediates the validity and denotation of multifold communicatively-linked biochemical pathways and cellular functions. This background may be featured in **novel modular systems similarities among tumors and tumor-specific rationalization processes**. Modularity is shown to be a separate basic functional attribute of a tumor besides tumor histology and molecular tumor biology. In interaction with normal human tissue, tumor cells use communication-linked processes according to laws of nature to build up a favorable infrastructure for proliferation. These processes are accessible in a reconstructive way via biomodulatory therapies and may be classified to get novel, presumably homogeneous tumor characteristics as indicated by differential characteristics of tumor-associated inflammation.

Table 25.5 Modular versus reductionist-derived molecular therapy approaches

	Choosing alternatives	
	Modular therapies	Magic 'bullets'
Malignant systems separation	High grade of rationalization	Low grade of rationalization
Systems' robustness	High	Low
Systems' evolution	Extended systems diversification (high grade of complexity)	Low grade systems diversification (e.g. CML)
Therapeutic targets	– Inconsistencies – Deformations (Achilles' heel) – Rationalization processes – Validity of communication processes – Malignant behavior (tumor stem cell niche) – Tumor and stroma cells	– Reductionist targets – (Blockade of pathways, receptors etc.) – Denotation of communication processes – Bulk of malignant disease – Cancer stem cell
Treatment-related toxicity	Biomodulatory efficacious doses	Maximal tolerable doses

Common systems features: The most important task is to look for common systems features ('topologies', inconsistencies) within different tumor types to get action-theoretically guided classifications of distinct tumor-associated evolutionary systems processes. Furthermore, classification is essential because classification is the basic language of medicine and of systems organizations across different tumor types, which need to be clearly defined. The uncovering of common features in different tumor types is only the beginning: Lymphomas could soon be classified according to their activation of inflammatory signaling pathways [53]; common stroma gene expression sets may be detected in response to tumor invasion [54]; and neoplasias may be classified according to their responsiveness towards combined modulation of transcriptional networking [17]. Another attempt may be the formulation of stroma scores, which still seems to neglect functional system aspects [3,10,17].

25.6.3 Tumor Type-Specific and Systems Stage-Specific Therapy

Because of the increasing experience in applying diversified methodological approaches for tumor therapy, we have developed a common understanding of important elements and principles required for distinct tumor type-specific and systems stage-specific tumor therapies. These considerations will lead to novel systems-based tumor classifications and to novel risk assessment, risk management, and risk communication. Risk management will continue to be a balancing act of competing priorities and needs but will be methodologically amenable and thus more personalized. Flexibility and scientifically-based adequate judgment are the ultimate keys to appropriate, successful risk decisions.

Tumor-specific and stage-specific therapeutic accessibility of inflammation-related processes to induce response in all tumor types indicates a constitutive spin-off of new systems functions during metastatic processes. Furthermore, the multimode therapeutic accessibility shows differential integration of inflammation into the context-dependent 'living world' of tumor compartments featured by tumor-specific and subtype-specific rationalization processes: Inflammation-related activities are communicatively promoted and differentially adapted during tumor evolution. Empirically, differences may be detected in the modalities of evolutionary systems development and in the acquired functional impact of inflammation-related systems. Biomodulatory therapies, administered as fixed modules, may contribute to discover and understand novel regulatory systems in tumor biology.

The modality of response induction is decisively affected by the change from histologically derived and molecular-genetically derived object-associated therapy developments (pathways, gene expression pattern, stem cell niches) to situation-associated and **stage-related tumor systems interpretations** for establishing tumor therapy, for example 'late-stage therapy' in metastatic melanoma. The holistic approach relies on the induction of **inter-systemic exchange processes**, which may be initiated by systems-targeting therapy modules [3]. This approach contrasts with the reductionist approach, which primarily aims at blocking communication processes, such as signaling pathways, and aberrant gene expression.

As shown in a recent study, a new generation of biomarkers may now empirically predict response to systems-targeted therapies: PPARgamma expression in melanoma cells is stage-specific (late-stage) and may predict response to 'anti-angiogenic' therapy approaches, independent of the administered biomodulatory therapy [55]. Future treatment response may be better monitored by evolving molecular imaging techniques because these techniques may decisively contribute to follow the biomodulatory activity of systems-directed therapies before objective response is achieved.

25.6.4 Guiding Systems-Directed Therapies

Stage-specific and systems-related prognostic markers (PPARgamma expression as 'late-stage' marker in melanoma; COX-2 as early stage prognostic marker) and markers describing the functional status of systems, such as serum C-reactive protein (CRP) levels, may guide systems-directed approaches with high predictivity for clinical response. A broad and heterogeneous repertoire of drugs is available to modulate distinct systems behaviors, for example inflammation: As shown, inflammation-related processes remain pathophysiologically important for response induction, irrespective of the mode of tumor-specific integration of rationalization processes.

Cell type-specific proteins, detected by analyzing the **secretome of distinct tumor-associated cell types** (Chapter 21) mirroring their functional status, could become a tool for biomarkers for guiding systems therapies and could give hints on mechanisms of action in a reductionist sense [56].

Molecular imaging: Molecular imaging (chapter 20) is an emerging field that joins molecular and cell biology for non-invasive tumor imaging [57,58]. These techniques require the development of necessary assays and ways for in vivo monitoring distinct molecular changes. Molecular imaging will allow a better understanding of the biological evolution of cancer, leading to improved diagnosis and disease management. Furthermore, molecular imaging may facilitate the observation of specific molecular and biological processes influencing tumor response behavior (proliferation, apoptosis, inflammation, metabolism, and angiogenesis). Novel imaging techniques could decisively contribute to foster **therapy selection for patients (patients' situational tumor systems stage)**, which could play a critical role in cancer detection, drug development, and finally in personalized tumor treatment.

25.7 Implementation of New Therapy Models

25.7.1 Can Patient Selection for Therapy Be Improved or, Vice Versa, Can Therapy Selection Be Improved for Patients?

The gene-causation-approach serves as a methodological basis for drug development as well as for guiding patient selection according to distinct molecular-genetic criteria or genomic signatures. Therapeutic aim remains the diversification of targeted cancer therapy. The advances in genomic technologies have the potential to add substantial value to current medical practice by using both the genetic characteristics of the metastatic disease and the genotype of the patient (pharmacogenomics).

The inclusion of tumor systems biology into the therapeutic calculus, i.e. modularity, and the rationalization processes besides the whole genome's molecular genetics, allow more choices for differential tumor therapies dependent on a tumor's genetically-based and evolutionary status.

Table 25.5 outlines the selection options for systemic tumor therapies:

1. Tumor systems may be assessed according to rationalization aspects (for instance, how is inflammation implemented and rationalized?). Systems that are based to a high degree on division of functions seem to be less susceptible to reductionistically designed therapeutic perturbations. Tumor cells in such rather robust systems are characterized by multifold chromosomal aberrations.
2. A tumor's **robustness** is likely to be a further decision criterion. Failure of single agent-targeted therapies or multi-agent chemotherapies may be a measure for the resistance of these tumor systems towards external perturbances.
3. Awareness of **discrepancies between the functional features of cell compartments and the systems world** may uncover the inconsistencies and deformations of systems (Achilles' heels). Both biomodulatory and reductionist therapy approaches could be therapeutic options after precise identification of the Achilles' heel.

4. The introduction of modern technologies, such as microarray analyses, pathway analysis in cancer and stroma cells, and accompanying translational research, has led to some fundamental biological understanding of complex cell interactions associated with important therapeutic implications [59,60]. Analytically and empirically obtained data are important, including the myriad of prognostic markers: But the systems perspective offers the opportunity of **weighing constellations** as well as pathophysiologically important elements for taping new treatment strategies!
5. Pathophysiologically related pathway signatures could prompt combinations of reductionist therapy approaches.
6. Toxicity of therapy approaches and pharmacogenomic aspects may be decisive in co-morbid or medically none-fit patients for decision-making.

Combinations of targeted approaches with chemotherapy have brought significant progress in small molecularly-characterized subgroups of patients, sometimes also progress in palliative care for a respective molecularly non-specified patient cohort: Now, significant targeted effects beyond postulated tumor cell-specific response have to be suggested. Therefore, selection should only be done at a later stage in the process of evaluating targeted therapies [61,62].

25.7.2 Using and Incorporating Systems-Relevant Information in Clinical Trial Designs for Metastatic Tumors

Systems-related biomarkers represent a novel kind of markers, which offer the possibility of **new study designs:** Systems-related biomarkers could record early systems response. Biomodulatory therapies could be continued in case of favorable marker response; in case of unfavorable response (futility of therapy), biomodulatory therapies could be rapidly changed to finally achieve the target values of a distinct biomarker. These **adaptive trial designs** would be able to cope with the time sensitivity for achieving tumor control. Patients would be adaptively randomized, and treatment assignment probabilities could be altered to favor the treatment that appears best for a patient's biomarker characteristics. This process will allow new agents or combinations to enter the trial. In traditional trial designs, data obtained during the trials do not influence randomization probabilities.

Sequential administration of modular therapy approaches to adjust predictive systems-associated biomarkers focus on the adequate selection of biomodulatory therapies to meet the situational 'metabolism' of a tumor's evolutionary process, i.e. for example inflammation-related rationalization processes.

Controlled administration of rapidly alternating systems-directed therapies until adjustment of favorable target values, i.e. tumor imaging parameters (molecular imaging) or systems-related biomarkers could be uniformly controlled procedures for treating heterogeneous tumor diseases with distinct systems-related features.

For example, a clinical trial could demonstrate (1) whether the rate of CRP detection is meaningful in a particular tumor disease and stage, (2) what are reliable

therapy-relevant CRP thresholds, and (3) the clinical benefit of CRP-guided and CRP-directed therapies. (4) If therapies have to be rapidly changed to finally achieve marker thresholds, the following question has to be addressed: What is the treatment by marker interaction (quantitative and qualitative interaction)? Such study designs may not answer the question whether biomodulatory therapies could also benefit marker-negative patients, for instance with castration-refractory prostate cancer [4].

'High-throughput' therapeutic adoption of modular therapies on tumor-specific and stage-specific rationalization processes of sub-systems could enable personalized tumor therapy. Systems-directed approaches have not to deal with a diagnostic and therapeutic black box until objective response is recorded with traditional imaging techniques measuring tumor size.

Multilevel **decision-making during therapy and general moderate toxicity profiles** represent an important ethical justification for action-theoretical therapeutic approaches. Therapeutic risk may be recorded at an early stage of therapy and may revise the therapeutic procedure by an alternative therapy approach to finally adjust the respective systems-related biomarker at its target value: Increasing knowledge about tumor systems behavior and evolutionary developing systems structures (reconstruction) combined with representative target values (for instance, C-reactive protein) or prognostic parameters (for example, PPARgamma expression) may be helpful to guide modular therapy strategies [55].

25.8 Therapeutic Aims

Systems-directed therapies may meet rather new therapeutic requirements by a second objectivation of the tumor (Fig. 25.1): Rationalization processes and modularity are now uncovered as components of a tumor's 'living world', besides the common description of theme-dependent, reductionist subject-object relations (gene-causation-approach): The novel modeling of tumor systems significantly expands therapeutic options. Therefore, the discussion about **study endpoints** comes into focus again.

1. Biomodulatory therapies focus on the chronification of metastatic disease besides the induction of complete remission.
2. Biomodulatory therapies are tools for personalized tumor therapy.
3. Approaches may be specifically designed for the demand of tumor stages and corresponding systems stages for involved organ sites.
4. Weighing systems constellations is the basis for establishing new therapy approaches.
5. The combination of approved drugs (within therapy modules) installs new life into old drugs.
6. Therapy modules may cause cancer cells to behave more like normal cells, for instance, by modulating the 'stemness' of tumor cells, Oct 3/4 genes via orphan receptors (Peroxisome proliferator-activated receptors) [63–65].

25.9 Challenging Space

25.9.1 Communication Theory, Basic Science, and Therapy of Metastases

In the current understanding, information theory (such as cellular signal integration), basic science (with its advances in tumor genomics), and clinical tumor therapy (targeted therapy and cytotoxic therapy) seem to constitute incommensurable worlds given that the various scientific areas deal with rather different scientific objects. The proposed action-theoretical approach aims at both the therapy of metastatic tumors and the uncovering of modular systems structures. This approach represents a pragmatic communication-theoretical method for understanding communicatively linked systems objects, biochemical processes, and cell functions by communication-technical terms, namely the validity and denotation of systems objects. The formal-pragmatic communication theory exceeds information theoretical approaches because the modular feature of systems objects is acknowledged beyond the simple exchange of information.

25.9.2 Reverse Engineering, Reconstruction of Systems Features (Intensio Obliqua) Versus Forward Engineering (Intensio Recta) with the Gene-Causation-Approach

The introduction of biomodulatory therapy regimens for metastatic tumors allows the versatile involvement of clinical treatment in communication theory and basic science:

The implementation of therapies interfering with evolutionary tumor processes serves as

1. A detector of therapeutic structures based in modular tumor architecture. Although biomodulatory therapies can be seen as "targeted" as classic reductionistically designed therapies, now holistic communicative (modular) structures are the targets, which have the capacity to redeem the validity and denotation of single systems objects within communicative tumor processes.
2. Therapy-relevant action-theoretical approaches may uncover the interwoven modular tumor architecture. We may describe modular textures on a molecular basis (including molecular imaging techniques), on the background of altered cell functions in the course of rationalization processes, in form of therapy-guiding biomarkers (secretome analytics), and, where applicable, as systems-relevant prognostic parameters (Figs. 25.1 and 25.2).

Basic science is getting directly involved in the reconstructive process, even though an approach has been established directed from bedside to bench to implement clinical practical care (adaptive trial designs) as scientific object in patient care.

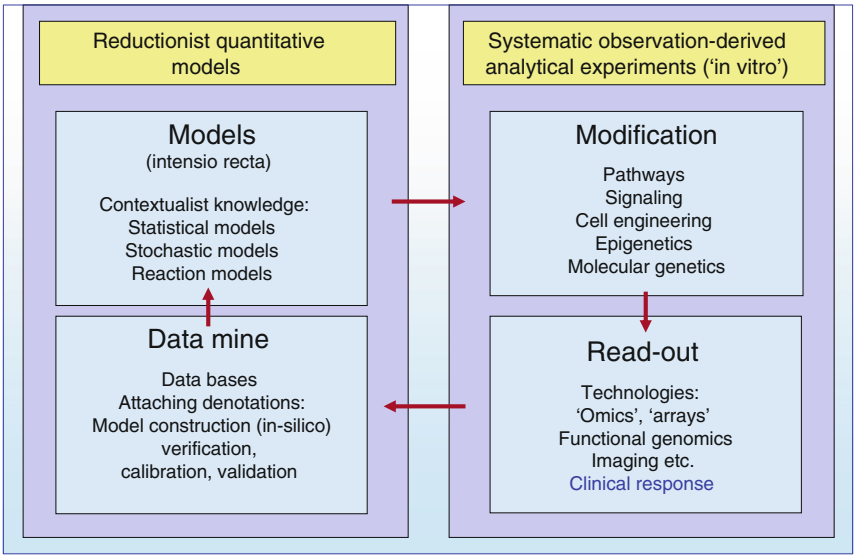

Fig. 25.2 Theme-dependent and closely interrelated areas of knowledge are the basis for reductionist approaches to uncover systems biology. According to reductionist systems interventions, scientists are observers of subject-object relations. However, if references of studied systems objects resolve during evolutionary tumor development, and systems objects are anticipating novel systems-related rationalization processes (e.g. differential integration of inflammation), then methodological considerations guided by 'intentio obliqua' (Figure 25.1) are appropriate to reconstruct evolutionary systems stages (modular approach)

25.9.3 Biomodulatory Therapy: Gene-Based and Non-DNA-Based Heritage

Prerequisite for uncovering a tumor's communicative structures, i.e. modularity and rationalization processes, is the inclusion of clinical read-out parameters because 'know that' biomodulatory therapies may achieve chronification of metastatic tumor disease, even objective and complete responses. Such therapies may induce organ-site specific activity by modulating the evolvability of metastasis and can regulate systems-relevant biomarkers.

The newly established pragmatic communication-theoretical approach **shows that causality in any particular form does not need to be a feature of every successful scientific explanation**: Primarily the 'know that', i.e. the activity of a biomodulatory therapy approach, is sufficient, whereas the 'know how' has to be further evaluated, again in a reductionist sense (Fig. 25.2).

The reductionist approach for uncovering the nature of tumor development is supplemented by an indirect, communicatively-guided biomodulatory approach **('intensio obliqua')**. Scientific knowledge about a tumor systems architecture consequently depends on the kind of implemented biomodulatory therapy and on the 'policy' of treatment.

After uncovering the architecture of rationalization processes or the identification of deformations and Achilles' heels in metastases by applying novel indirect methodology (**'intension obliqua'**), vulnerable nodal points of subsystems should be targeted by reductionist approaches. This way, approaches derived from synthetic biology could be clinically implemented.

Therefore, the therapeutic focus of reductionist approaches could be expanded beyond targeting aberrant genes or their proteins, namely by widening the targets of reductionist therapy approaches to essential functional systems features, which evolve on the background of multiple tumor-associated aberrations and rationalization processes, representing the evolutionary 'program'.

In future, we have to face the task of reconstructing a tumor's evolutionary development (reverse engineering) to the full extent (Fig. 25.1). The technique of reverse engineering is similar to methodologies for uncovering the tumor-related psychosocial development of patients on their cultural background.

As shown, tumor-associated inflammation may be rather differentially accessible for biomodulatory therapy approaches. Highly variable modular architectures for tumor-associated inflammation in various tumor types and stages have to be uncovered via systems-directed therapy approaches. This perspective allows a new comprehension of individualized tumor therapy. The time-sensitivity of a therapeutic approach in particular may be addressed.

Clinical trials have now to show how modular systems-directed therapies may be combined with tumor pathophysiology-orientated and molecular-genetically-based treatments. All these approaches have the capacity to displace classic chemotherapy in some areas. If so, personalized therapy in metastatic cancer – originally only a motif to focus therapeutic care in a single patient – may be realized with advanced methodological **access to therapy-relevant tumor models**. Selection of patients for therapy could be replenished or even displaced by selection of therapy corresponding to the stage-dependent developmental status of the tumor systems in individual patients.

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Part IX
Summary

Chapter 26
To Be an Object in a Biological System

The Necessity of a Formal-Pragmatic Communication Theory

Albrecht Reichle and Gerhard C. Hildebrandt

Abstract Based on communication-technical considerations, it has become obvious that both reductionist and holistic understandings are equitably exerted to reproduce the situational stage of a tumor disease. As required by methodology, these approaches have to virtually dissect the coherence of systems and the functional 'world' of distinct tumor systems: Differential perspectives of interaction are entangled with various levels of knowledge and consecutively with different therapy strategies.

Keywords Metastatic tumor • Holism • Reductionism • Communication theory • Modularity • Rationalization • Object • Subject

26.1 The Problematization of Established Interpretations of Evolving Tumor Systems

Therapeutically efficacious access to metastatic tumors, which is mediated by communicative interactions of biomodulatory acting drug combinations, has emerged as a trigger for the problematization of established tumor models [1,2]. Traditional models are based on reductionist or contextualist interpretations of metastatic tumors. However, these models may not explain the observed and therapeutically relevant activity of biomodulatory therapy approaches, which include drug combinations with only poor single agent monoactivity or none at all [3].

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Tumors are characteristically composed of functionally rather heterogeneous cell populations, i.e. tumor and stroma cells. Despite the ostensible morphologic heterogeneity of these cell populations, clinical trials using biomodulatory therapy approaches have shown that these heterogeneous cell communities constitute a holistic, therapeutically accessible communicative entity [3], which seems to be a contradiction. Holistic communicative processes – recently termed the tumor's 'living world' – turned out to be a novel scientifically and therapeutically accessible object offering insights into evolutionary processes: Biomodulatory therapy approaches bring transparency into holistic communicative systems by breaking into a tumor's 'living world' and by dissecting a tumor for practical purposes, such as the attenuation of tumor growth (normative notion), in comprehensible evolutionary processes.

First of all, critical scrutinizing established reductionist data interpretations of tumor evolution results in disintegration. This disintegration is based on the fact that systems objects, i.e. single cell compartments, cells, proteins, etc., of the tumor compartment, are expelled from their position as objects and are to be re-integrated into novel tumor models as situatively defined systems subjects. The observed therapy-derived phenomena, i.e. the therapeutic accessibility of the holistic communicative tumor system through biomodulatory therapies, may be adequately explained by the integration of systems-imposed activities, which are carried out by particular systems participators [3]. The routine reductionist perception of a metastatic tumor is now bereaved of its conversance and universal validity.

The recently developed formal-pragmatic communication theory basically emphasizes two perspectives of interaction with systems participators: One is based on the perception of an observer (a reductionist and contextualist point of view), the other on that of a participator (a communicative, holistic point of view). The simultaneous double-sided perspective offers the opportunity to describe systems participators as objects (in the past tense form) with the aim to formally discriminate one object from other systems objects and as situatively emerging subjects that are integrated in the evolutionary context of a biological system. Situative operational characteristics of systems participators develop by implementing modular knowledge that is either internally-derived or externally-derived, or both. Systems may be subjected to often complex configured coincidental or sequential information flows, which consecutively lead to highly specific situative changes in the function of otherwise familiar systems objects. All communicative processes adhere to rules, which lie within the holistic communicative systems texture.

Based on these communication-technical considerations, it has become obvious that both reductionist and holistic understandings are equitably exerted to reproduce the situational stage of a tumor disease. As required by methodology, these approaches have to virtually dissect the coherence of systems and the functional 'world' of distinct tumor systems: Differential perspectives of interaction are entangled with various levels of knowledge and consecutively with different therapy strategies [3].

26.2 Re-interpretation of Reductionist Considerations on Tumor Evolution

The main challenge for the formal-pragmatic communication theory is now to explain the multimode experimentally-derived results from rather different experimental positions, describing mechanisms that are involved in tumor progression. We selected the most recent important papers describing mechanisms of tumor evolution for discussing the respective reductionist interpretation of these study results.

1. Greaves impressively phrased the dilemma which arises on the basis of the reductionist interpretations of evolutionary processes during the development of acute lymphocytic leukemia [4]: On the one hand, he suggested a 'back to Darwin'-model for cancer-propagating cells that places cells with variable self-renewal potential or 'stem cells' as the units of evolutionary diversification and selection. On the other hand, he showed an only temporally limited hierarchical development of leukemia and cancer cells: 'Cancer stem cells (CSCs) could, in some circumstances, be developmentally positioned at the apex of a hierarchy'. Graves also stated – in contradiction to the postulated hierarchy – that 'there is no reason to suppose that hierarchical structures are inherently stable and maintained with cancer progression'.
2. An answer to Greaves' dilemma of the existence of probably various and alternating stages of hierarchical and non-hierarchical developments during tumor evolution is given by Raaijmakers et al. [5]. From their experimental observations, it can be delineated that 'individual microenvironment constituents can serve as regulators of tissue functions beyond that of stem cell support'. Thus, the position of the so-called 'cancer stem cell' at the apex of a hierarchy is relativized because 'the mechanism of malignancy may result from the interaction of cell autonomous and microenvironmentally determined events'. The microenvironment may be the site of the initiating event that leads to secondary genetic changes, even in heterologous cell types. These observations presuppose communicative processes between different cell types and consecutively suggest the holistic communicative systems community as the primary evolving unity. However, the question why heterogeneous neoplasias are developing upon a unique molecular-genetic aberration in a heterologous cell type remains unanswered.
3. An important observation contradicting the Darwinian selection processes (selection of the fittest) describes how analogous acting and evolution-promoting processes (genotoxic stress) are translated into digitalized reproducible genomic structures in prostate cancer cells [6]: Novel findings elucidated several unexpected general principles for non-random chromosomal translocations in tumors. 'A long-standing concept in tumor translocation has been that genotoxic stress causes direct random double strand breaks (DSBs) that lead to random translocations, with the selection of those conferring growth advantages.

> By devising and investigating a model of tumor translocations that fully mimics the frequency of in vivo events without proliferative selection', Lin et al. suggested that 'there is a site-selective immediate pattern of DSBs that ultimately dictates the pattern of tumor translocations'.

The novel communication-based tumor model may be applied to explain the mentioned findings that occur during tumor development in an evolutionary context.

26.3 The Collapsed Reductionist Interpretations of Observations on Tumor Evolution Have Now to Be Reconstructed with Novel Methodologies

Systems objects as actors within a systems-associated biological context situatively gain novel and specified assignments of identities. The novel systems-associated identity may be even contradictory and of a completely different quality to any known object-associated identity (spin-off of novel systems functions). The systems-associated identity of an actor, as the originator of a spontaneously accomplished communicatively-derived action, may be only retrospectively assigned to already established, object-associated identities. The object-associated identity only occurs as a 'historical' feature. The identity of a systems object is no inherent feature but is communicatively and situatively mediated. The more evolutionary processes involved, the more novel systems-linked identities of systems objects may be expected.

26.4 Implementation of Internally-Derived or Externally-Derived Modular Knowledge

Communicatively linked biological systems are interweaving the nude identity of their systems objects or the arrangement of compartmentalized knowledge (on the observer's site) with situative biological stages or with the communicative arrangement of systems objects' validity and denotation (on the participator's site) by allowing the implementation of internally-derived or externally-derived modular knowledge. This knowledge is based on rules that are present in modularly arranged and rationalized systems textures, which are equitable with the 'metabolism' of evolutionary systems and purport the frame for evolutionary multiplicity.

As shown by Lin et al., the implementation of modular knowledge as postulated by the formal-pragmatic communication theory may indeed initiate specific translocations within a distinctive systems context [3,6]. The liganded androgen receptors in combination with genomic stress (modular knowledge) are related to the development

of specific translocations in prostate cancer cells. This context-associated systems feature represents a pivotal example of how validity and denotation of systems objects (androgen receptor) is redeemed within a situatively characterized systems context to facilitate evolutionary processes.

Communication may be basically modular and leads to the rationalization of systems [1,3]. Implementation of modular knowledge is the configuration of the coherence between the validity and denotation of communication processes. Vice versa, modular therapies may supplement prepositional aspects of communication, i.e. the presence of a tumor's living world by normative aspects, namely by therapy-derived yes or no statements ('know that').

26.5 Objects Anticipate the Attitudes of Subjects

Context-dependent conflicting impulsions for operations mediated by distinct systems objects deprive the respective objects of the features of an object; also the objects anticipate the attitudes of a subject. The emergence of a distinct description of an object that is only available ex post is closely associated with the transition to an evolutionary novel stage of communication, also in the case of androgen receptors in prostate cancer cells when liganded in the presence of genomic stress (irradiation).

The possible 'no' by which an addressee refuses an offer for communication does neither touch the validity of a communication act nor the identity of an addressee. Both sites have to acknowledge each other as systems actors; this acknowledgement represents an important prerequisite for evolutionary progression.

26.6 The Accomplishment of the Interactive Roles of Cells Within a Tumor Tissue may Never Only Imply their Reproduction

The description that interactions of 'cell autonomous and microenvironmentally determined events' support the mechanism of malignancy during the evolution of myelodysplasia and consecutive acute myelocytic leukemia points to a communicative aspect that has been experimentally proven in a mouse model [5]. Also, this model of leukemogenesis suggests non-random molecular-genetic and genetic aberrations, even in heterologous cell types (hematopoetic cells), as a consequence of initiating molecular-genetic aberrations in mesenchymal cells.

Despite a unique initiating molecular-genetic event, variable presentations of myelodysplasia and acute myelocytic leukemia in mice underline that the accomplishment of interactive roles of cells within a tumor tissue may never only imply their reproduction, as long as interactions are communicatively, i.e. to some degree non-hierarchically, structured: Therefore, a formal-pragmatic communication theory is necessary to explain communication processes within a cellular systems context.

26.7 Homeostasis-Preserving 'Social' Subject

As actors (genes, proteins, and cells), systems participators acquire the objective relevance of both activity profiles, namely those of a (known) object as well as that of a situatively defined, evolutionary-linked systems subject. The actors simultaneously take in the perspective of another systems participator, thereby acquiring the feature of a homeostasis-preserving 'social' subject within the rules given by the 'metabolism' of evolution.

Basically, acquired molecular-genetic changes in any cell have the chance to be repaired. Yet, the repair machinery including epigenetic mechanisms may not be able to resolve the problem for several reasons: (1) because of an inability to repair or compensate; (2) because of situatively provided communicative circumstances; (3) or because genetically altered cells may not be silenced in a communicative sense by their adjacent cellular environment. If the repair machinery fails, the altered cells start to participate in a localized or more global communication process and may develop cellular systems in their function as potentially evolution-promoting novel systems objects, thereby simultaneously preserving homeostasis as systems subjects. The manifestation of evolutionary tumor processes may be multimode, dependent on the communicative rules constituting the 'metabolism' of evolution. The unlimited cellular communication community finds its support in the structure of communication lines and intersystemic exchange processes.

26.8 The Situative Identity of Systems Objects Proves the Sustained Subjectivity of Communication

The not explicitly predictable situative identity of a systems object proves the sustained subjectivity of communication as a medium, in which systems objects do not necessarily objectively acknowledge one another. Therefore, identity that backs itself is missing: (1) Identity is communicatively-derived. (2) Systems objects assume the normative expectations of the 'alter' (protein, cell etc.), the other systems participators, but they do not stereotypically redeem reductionist expectations in a distinct systems context.

The situative identity of systems objects is facilitated by the acquired systems-associated identity, which is characterized by modular and rationalized features. This identity also restrains, from an intersubjective perspective of a systems context, the capability of a systems object to redeem established object-associated identities. Therefore, the observed broad variety of hematopoetic disease traits, i.e. myelodysplasias, acute myeloid leukemias [5] is fully consistent with the formal-pragmatic communication theory, even if derivable from a single molecular-genetic aberration in a heterologous cell type (mesenchymal cells).

The object-associated identity of a systems object must not coincide with the situative identity as a systems subject. The situative identity is the originator of a spontaneous action reference, which is implemented by modular knowledge:

The liganded androgen receptor in connection with genomic stress may take on a completely novel role in prostate cancer cells. Therefore, the identity of systems objects is defined by communication rules, which lie in an evolutionary horizon. The identity of a systems object is related to the identity given by a situative evolutionary systems context. This identity is defined ex post from the perspective of an actively participating but not necessarily reified molecular or cellular systems world (noise, no specific interactions) as symbolized by the so-called 'background knowledge', which is provided by the tumor's holistic communicative world, i.e. its 'living world'.

As systems objects are getting integrated in a systems context, they are constituted as objects capable of acting, thereby developing the possibility to redeem novel denotations and validity within communicative systems by the acquisition of systems-associated requirements, i.e. normative features.

26.9 Discussion: The Privileged Access of Systems Actors

The object-associated identity serves as a descriptive distinction towards the 'alter'. The systems-associated identity as the originator of spontaneous activity aspects, i.e. spin-off of novel systems functions, represents the privileged access of a systems actor towards its own subjective microenvironment (communicative world) via expressive communication activities within a systems context. The object-associated identity of a systems object, which directly describes the communication act, is the function of arbitrary acts directed at a communicative target. These communication acts may be redeemed according to communication-derived rules and aim, for example, at preserving homeostasis; in an intentional sense, they also aim at inducing tumor control with biomodulatory therapies. Normative contexts limit the number of relations between the systems objects.

Within this communicatively defined frame, smallest multimode systems become conceivable, which implicates that multimode 'niches' supporting tumor evolution may occur on the background of evolutionary texture ('metabolism' of evolution). Consecutively, multiple clonal phenotypes may arise, fully consistent with the observations compiled by Greaves for acute lymphocytic leukemia.

The communicative systems world of a tumor has equitable systems partners, i.e. systems are not unidirectional built up by genes. Vice versa, the analogously working communicative systems have the capability to implement external or internal modular knowledge, or both, to promote the digitalization of evolutionary processes in form of reproducible genetic aberrations.

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Chapter 27
From Molecular to Modular, from Theme-Dependent to Evolution-Adjusted Tumor Therapy

Albrecht Reichle and Gerhard C. Hildebrandt

Abstract The successful implementation of biomodulatory therapies for controlling a wide variety of metastatic types of cancer has been demonstrated in multiple clinical phase II trials. These therapies have opened up new perspectives for studying **novel tumor** models, which may explain response to combined biomodulatory therapies. Hereby, insights in evolutionary systems structures have become possible.

Keywords Adaptive trial design • Modularity • Personalized medicine • Tumor evolution • Metastatic tumor

27.1 Introduction

Comprehensive interpretation of a tumor disease is a prerequisite for the successful systemic treatment of metastatic tumors. Such interpretations consider classical parameters, i.e. histology, cytogenetics, molecular-genetics, pharmacogenetics, cellular phenotypes, clinical parameters, and natural history. The course of a disease, including its therapy-related side effects, and a patient's holistic perception of the disease offer rather different perspectives for individualizing the treatment of a tumor disease.

Oncologists have to cope with **individual tumor diseases** in distinct evolutionary systems stages beyond the routine ascertainment of a widely scattered spectrum of often incommensurable tumor-associated parameters, which are finally gathered in

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a contextualist, theme-dependent compilation of tumor-associated pathomechanisms. Only this method allows consistency with the principle of medical treatment, i.e. to select therapies for patients and not – as commonly practiced – to select patients for therapies, which corresponds to the general comprehension of a **personalized tumor therapy**.

27.2 Tumors Are Communicative Networks

One of the primary tasks of oncologists is the therapeutic accomplishment of **specific functionally characterized evolutionary stages of tumor diseases**. The interpretation of a tumor's functional stage has necessarily **communicative aspects**. Tumors are now considered as communicative molecular or cellular systems, which – in contrast to the well-known reductionist theme-dependent compilation of tumor models – should be uncovered in a time-sensitive manner and discussed in an evolutionary context. Methods investigating communicative tumor processes must be detached from established exclusively reductionistically or contextualistically derived considerations about tumor-associated systems structures including the favored cause-effect-chain.

Reductionistically derived therapy approaches preferably rely on the multitude of **tumor-associated chromosomal aberrations** and the associated disturbances of protein functions or signaling pathways. Contextualistically compiled and theme-dependent treatments are characterized by the concomitant use of multiple small molecules or antibodies targeting circumscriptive tumor-associated pathomechanisms. Drugs targeting the synthetic linkage of biological processes, such as bispecific antibodies, are now increasingly used in clinical trials.

Communicative relationships primarily lie in the post-genomic world, constituting the **holistic functional world** of cells, proteins, and mediators. The main questions that need to be answered are:

- How can we get an appraisal of the **evolutionary stage** of an individual **tumor system?**
- What are the carriers and propagators of multimodal interwoven evolutionary developing tumor systems?
- Are these **systems reconstructible** and classifiable to be used as a future base for broadening therapeutic options?

A reductionistically derived answer to these questions lists multimodal specified tumor features, which are suggested to generally promote tumor growth: Tumor-associated inflammation, neoangiogenesis, Warburg effect, insufficient immunological response, extracellular matrix remodeling, cell proliferation rate, apoptosis defects, coagulation effects, cellular niches, and molecular genetics, etc. Reductionistally derived, theme-dependent knowledge does not answer the question of how these phenomena interact in the evolutionary stage of an individual tumor disease – communication, communication distortion, and communication disruption come into play.

27.3 From Molecular to Modular Tumor Biology

A prerequisite for solving the above questions is to therapeutically, and thereby communicatively, interfere with tumor systems in such a way that the following targets can be achieved:

- Objective tumor response – the crossing point of reductionistically and holistically derived therapeutic interventions
- At the same time, novel insights into how tumor systems differentially interfere with biomodulatory combination therapies

Systems-associated biomarkers derived from the cellular secretome or from molecular imaging techniques are novel indicators for differential systems response and may characterize tumor-associated systems behavior (e.g. C-reactive protein (CRP) for tumor-associated inflammation). Communicative systems architectures, their intersystemic exchange processes, and functional organizations may be compared among different tumor types to detect differential, namely modular systems activities in response to identical biomodulatory therapies.

Multimode technologies have uncovered myriads of prognostic parameters for stages of tumor diseases as well as for corresponding therapy approaches. Nevertheless, a big gap still exists for systems-derived markers, which may indicate successful biomodulation of distinct tumor-associated molecular systems or cell compartments. Ideally, systems-associated biomarkers mirror therapy-relevant changes in the behavior of tumor subsystems. These biomarkers are, to some degree, independent of the repertoire of the administered biomodulatory acting drugs.

Biomodulatory therapy approaches are marked by their ability to specifically modulate the evolvability of tumor systems with the aim of tumor control and of achieving objective response. Modular, evolutionary context-embedded activity of biomodulatory therapies is contrasted by the aspired selective theme-dependent activity (e.g. cytotoxic, anti-angiogenetic activity, etc.) of reductionist approaches for the treatment of metastatic tumors.

Biomodulatory therapies with the capacity to induce complete response proved to be, for example, **metronomic low-dose chemotherapies** plus the **combination of transcriptional modulators**. Unlike reductionist treatment approaches, biomodulation can include drug combinations with stimulatory effects. Biomodulatory therapies are often characterized by no or poor monoactivity of the single drug in the respective tumor type.

27.4 Model-Creating Capacity of Biomodulatory Therapies

The successful implementation of biomodulatory therapies for controlling a wide variety of metastatic types of cancer has been demonstrated in multiple clinical phase II trials. These therapies have opened up new perspectives for studying **novel tumor models**, which may explain response to combined biomodulatory therapies. Hereby, insights in evolutionary systems structures have become possible.

Response evaluation during the administration of biomodulatory therapies excludes reservation towards objectivity of attained therapy results. The decisive scientific turn with respect to content and methodology, of how to create objective knowledge about holistic and communicatively appreciated tumor systems, is related to the fact that available response data, including biomarkers characterizing systems behavior, allow the interpretation of

- The **individual evolutionary status** of a tumor disease in a tumor type- and stage-specific manner
- The collected data combine classic response criteria with information indicating differential systems responses, enabling **therapy-derived systems interpretations and classifications**

The model-creating capacity of biomodulatory therapies is closely linked to **novel systems-derived biomarkers** (e.g. CRP, peroxisome proliferator-activated receptor (PPAR) gamma expression), the functionally varying secretome of cells within the tumor compartment, and parameters derived from molecular imaging techniques.

The novel scientific field, **therapy-derived systems biology, covers technical and conceptual aspects**:

- How are biomodulatory therapies performed?
- What drugs may be combined?
- How may the individual **evolutionary status** of a tumor be interpreted?
- How can therapies be rapidly adapted to the tumor's situational and evolutionary status **(adaptive trial design)**.

27.5 Therapy-Derived Systems Biology: A Formal-Pragmatic Communication Theory

The two uncovered model-constituting principal determinants are the tumor systems **modular architecture** and **systems-immanent rationalization processes**. Both systems features allow the explanation of objective response to drug combinations without significant monoactivity as well as different response kinetics. The biomodulatory activity of the administered drug combinations is underlined by much delayed, but also by very rapid (striking the Achilles' heel) objective responses.

A basic assumption of the novel underlying **formal-pragmatic communication theory** is the **tumor's 'living world'** which comprises the tumor's holistic communication processes, on which we rely in every therapy. The 'living world' of morphologically defined tumor cell systems creates the term opposite to those idealizations, which originally constitute scientific (intentional) knowledge. The 'living world' is uncovered by redeeming validity of communicative tumor processes by implementing modular knowledge of the cellular and external environment (for instance for therapeutic requirements).

The tumor's 'living world' can only be divided into categories of knowledge, for example **modular systems textures**, by experimental or **therapeutic experiences** (biomodulatory therapies). Primarily the 'know that', i.e. the activity of a biomodulatory therapy approach, is sufficient, whereas the 'know how' has to be evaluated further, again in a reductionist sense. In contrast, the cause-effect-chain represents a fundamental prerequisite to justify theme-dependent therapeutic procedures, but these may be not necessarily related to the tumor's evolutionary status.

27.6 Novel Systems Determinants Constitute a 'Big World' Inside Small World Networks

Modularity in the present context is a formal-pragmatic communicative systems concept, describing the degree and specificity to which systems components (cells, pathways, molecules, etc.) may be communicatively separated in a virtual continuum, reassembled, and rededicated (e.g. co-option) to alter **validity and denotation** of communication processes. This concept refers to possible interactions between the systems objects (cells, pathways, molecules, etc.) in a tumor as well as to the degree to which the communicative rules of the systems architecture (for establishing validity and denotation) enable or prohibit the focus on the validity and denotation of systems objects. Systems objects acquire the features of symbols, which are rich in content and able to acquire novel references by rearranging validity and, consecutively, denotation. Tumors consist of modules, which become a scientific object by communicatively uncovering the **tumor's 'living world' (defined as the tumor's holistic communicative world)** with biomodulatory and therefore modularly designed events.

Biomodulatory therapies represent a novel therapeutic instrument, which supplements molecular **tumor therapies** (e.g. blockade of pathways, classic cytotoxicity) **by modular, evolution-adjusted tumor therapies**.

Rationalization processes turned out to be important targets of biomodulatory therapies: The functional spectrum of distinct cell types within the tumor compartment is limited despite the commonly observed huge plasticity and may be challenged by the required systems-associated functions directed at the systems objects. These profiles of requirements may lead to discrepancies within the systems, which may be described as **inconsistencies**, Achilles' heels, and deformations or missing intersystemic exchange processes. Additionally, we have to expect that different patterns of cell types within the tumor compartment may promote particular functions, such as tumor-associated inflammation, in a concerted action as well as in a tumor type-dependent manner.

The modularly structured and rationalized 'living world' of single cell compartments or tumor systems represents the horizon for the practice of inter- and intracellular communication and understanding, in which communicatively acting 'rationalized' subjects, i.e. cellular proteins, cellular compartments, and niches, are continuously trying to implement modular 'knowledge' by redeeming novel

validity and denotation: Modularity of cell systems and proteins enables to constitute a ‘big world’ inside ‘small world networks’.

The focus on the ‘living world’ of tumors with

- **Novel scientific instruments** (biomodulatory therapies, hermeneutic considerations)
- Novel **read-out parameters** (secretome analytics, molecular imaging)
- Scientific organization structures **(translational science)** may contribute to a contemporary diagnostic self-conception of **dynamically evolving**, stage- and tumor-specific functions. The tumor’s ‘living world’ delivers the resources for interpretation processes. With these interpretations, the operator (e.g. the oncologist) tries to cover the necessary requirements for understanding the mechanisms of action of distinct biomodulatory therapy approaches. The resulting formal-pragmatic communication theory has, presumably, broad impact on the therapeutic practice of metastatic tumors and **personalized tumor therapy**.

Reductionist systems approaches are now opposed by a **holistic communication-based model**, the tumor’s ‘living world’, which is uncovered by implementing modular knowledge into cellular and molecular environments, for instance for therapeutic requirements: The tumor’s whole communicative system is subjected to modular interventions pursuing integration of complex biochemical systems processes.

27.7 Tumors May Be Viewed and Uncovered as Communicatively Structured Holistic Systems

Biomodulatory therapies broach the issue of the tumor’s ‘living world’ as a holistic and self-contained communication process by configuring situational stage- and tumor-specific evolutionary systems features (Fig. 27.1). Therapy-derived configurations are based on rules of modular molecular architectures as well as on cellular rationalization processes. The holistic aspect allows therapy-derived situational systems interpretations in an evolutionary context as well as systems classifications **without preconceived teleological notions**.

Novel modular architectures may be configured by the **compliance** of biomodulary therapy approaches with modular tumor architectures. In case of missing redemption of validity and denotation, **noise** develops, which may produce stress that may be spontaneously silenced and repaired, or otherwise could be directly involved in redeeming novel validity and denotation of communicative processes (Fig. 27.2).

Individual tumor-associated evolutionary systems stages are **separated episodes of the tumor’s ‘living world’** with respect to distinct issues or intentions, namely the aspired growth control of respective metastatic tumors. Systematic administration of biomodulatory therapies in multiple tumor types has uncovered a novel therapeutic instrumental cascade:

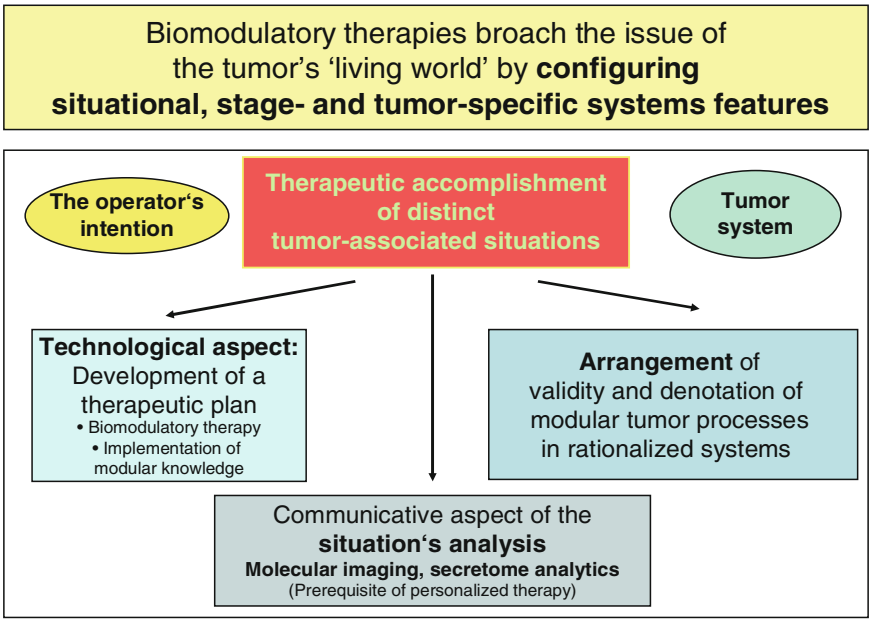

Fig. 27.1 Biomodulatory therapies broach the issue of the tumor's 'living world' as a holistic and therefore self-contained communication process by configuring situational, stage- and tumor-specific systems features. The tumor's **evolutionary-derived stages** are separated episodes of the tumor's 'living world' with respect to distinct issues or intentions, namely the aspired growth control of respective metastatic tumors

- Validity of modular communication processes may be altered by stage- and tumor-selective therapies to **refocus differential denotations of constitutive tumor processes**, e.g. inflammation.
- Thereby, modularly constituted communication processes lose their primary purpose, i.e. growth promotion, to finally induce attenuation of tumor growth.
- Conclusively, biomodulatory therapies modify the prerequisites for validity of communicative molecular or cellular processes, which are lying in the tumor's 'living world', thereby necessarily altering their denotation.

27.8 Evolutionary Systems Development

Tumor-specific and stage-specific therapeutic accessibility of, for example, inflammation-related processes to induce response in a wide variety of histological tumor types indicates:

- A **constitutive spin-off of new systems functions** during the metastatic process (tumor evolution).

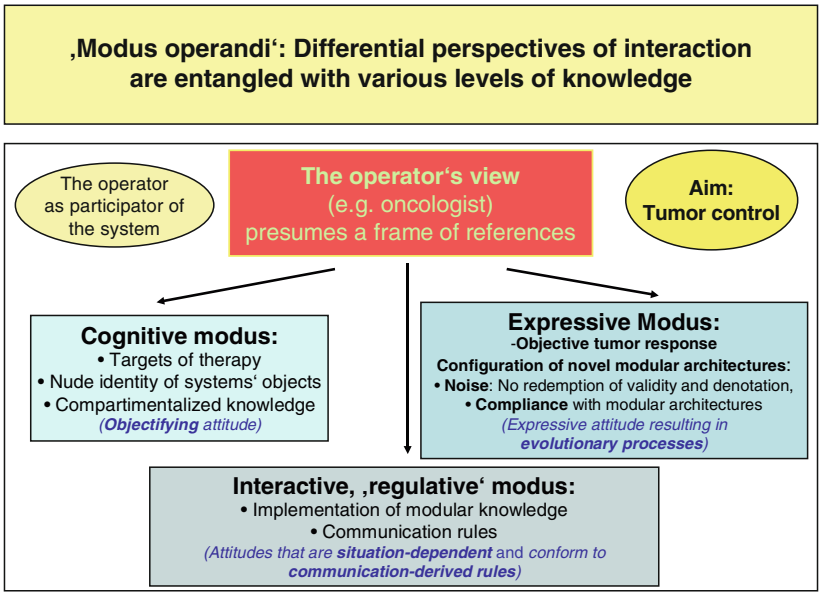

Fig. 27.2 Novel modular architectures may be configured by the **compliance** of biomodulary therapy approaches with situational architectures. In case of missing redemption of validity and denotation, **noise** develops which may produce stress that may be spontaneously silenced or repaired, or otherwise could be directly involved in redeeming novel validity and denotation of communicative processes

- Furthermore, this accessibility shows different integration of inflammation into the tumor compartments' 'living world' that is featured by tumor-specific and subtype-specific rationalization processes.
- Inflammation-related activities are communicatively promoted and differentially adapted during tumor evolution.
- Empirically, differences may be detected in modalities of **evolutionary systems development** and in the acquired functional impact of inflammation-related systems.

Biomodulatory therapies, administered as fixed modules, may contribute to the discovery and understanding of novel regulatory systems in tumor biology.

- Interestingly, identical biomodulatory therapy components (modules) induce clinical activity via differential tumor-associated systems.
- As shown, intersystemic exchange processes may be decisively disturbed (Comparative uncovering of tumor systems biology by modularly targeting tumor associated inflammation, Reichle A, Hildebrandt GC).
- In addition, biomodular therapies provide a methodological equipment to describe **evolvable tumor systems** with steadily advancing modular architectures and rationalization processes.

Modularly acting events, such as modularly designed therapies, may induce significant modular response in socially linked cell systems (prerequisite) and may **provide room for evolutionary development** by redeeming novel validity. Following modular events, molecular-genetic alterations may also occur.

The additional assumption of Darwinian selection processes is no prerequisite for explaining evolutionary processes. Selection processes are indispensable within reductionist considerations. But what is the 'vis a tergo' for selection processes? Modularity is sufficient to operationally define evolvability, which includes failure, fallacies, inconsistencies, and rationalization processes. Necessarily, evolution does not aim at selecting the fittest. Achieving compliance with modular architectures is sufficient enough, as long as reproducibility and survival remain preserved.

Biomodulatory therapies are currently being implemented in a wide variety of different metastatic tumor types. Thereby, these therapies simultaneously delineate novel tumor characteristics linked with evolutionary processes.

- **Tumor-type comprehensive anti-tumor activity of biomodulatory therapies** indicates to some degree **invariant processes of nature-promoting leukemo- and tumorgenesis**, which now have to be classified according to their modular background.
- In interaction with stromal tissue, leukemic as well as tumor (stem) cells use processes according to laws of nature to establish infrastructures **(modular systems)** favorable for proliferation.

27.9 Adaptive Trial Designs

Procedural aspects of biomodulatory therapies are closely guided by tumor-inherent rationalization processes and modular tumor architectures, which are frequently based on complex chromosomal aberrations in metastatic tumors. Modularity and rationalization as model-immanent determinants have an enormous effect on the design of biomodulatory therapy concepts, and finally necessitate **adaptive trial designs** by inclusion of systems-relevant biomarkers for follow-up. These markers may indicate early systems response as prerequisite for objective tumor response or chronification of tumor disease. On the other hand, biomodulatory therapies could be rapidly changed in case of insufficient marker response **(high 'through-put' consecutive administration of biomodulatory therapies adapted to evolutionary-derived systems stages)**.

Biomodulatory therapies pose the question about therapy-relevant study endpoints. Systems-related surrogate markers may predict disease chronification and early or delayed induction of objective tumor response. Knowledge about intersystemic exchange processes and the architectural constitution of tumor-associated modular subsystems helps to develop rapidly changing **adaptive trial designs** allowing changes of treatment modules. Implementation of adaptive trial designs requires the availability of different combinatory biomodulatory

therapies, demonstrating specific activity on stage- and tumor-specific modular systems architectures. Furthermore, intersystemic exchange processes must not be disturbed to alter validity and denotation of systems processes involved in tumor progression.

27.10 Biomodulatory Therapies Accentuate and Focus Practical Issues

Specified plans for therapeutic interventions implementing biomodulatory therapies accentuate and focus practical issues: Biomodulatory therapies and their communicative feature manage tumor-associated functional situations and stages by redeeming novel validity and denotation of the communicatively linked objects of tumor systems in a **range of modest toxicity. Thereby, such therapies may initiate organ-site specific activity**.

The formal unity of all available **competitive or supplementary systemic therapy concepts** for the growth control of metastatic tumors, derived from and directed at different communicatively designed tumor models **(reductionist, contextualist, or holistic)**, may not be sustained any more by a unique conception with regard to contents. The diversity of therapeutic conceptions decisively reflects particular **tumor models**, which we perceive as 'appropriate' in a single patient's disease.

The hermeneutic technique **(intensio obliqua)** used for the present therapy-derived systems description may now partially overcome traditional scientific procedures of discussion **(intensio recta)**. The reason for this change may be that the hermeneutic technique **remains susceptible for holistic communicative concepts**, which supply the background of all pathways, proteins, and cell functions uncovering the modular behavior of all these systems subjects in response to biomodulatory therapy approaches.

27.11 Holism and Reductionism Represent Separate, Scientifically Accessible Scopes of View

Reductionism as an alternative method to derive scientific knowledge about tumor systems shows that complex systems can be explained by theme-dependent knowledge, i.e. pathways and complex gene aberrations. Why is the emerging tumor-associated systems behavior hard to predict from a reductionist point of view? The number of interactions between components of cellular or molecular systems – which increase combinatorially with the number of components – and the interaction patterns are characteristically restricted by the respective modular evolutionary status of the tumor systems architecture (the 'living world'), thus potentially enabling the emergence of many new and subtle types of behavior. The temporally restricted appraisal of **modular**

systems arrangements in particular is the domain of holistic and therefore communication technical methodological approaches.

The successful introduction of biomodulatory therapies in metastatic tumors underlines that holistic communicative processes may be successfully studied at their own autonomous level of analysis, i.e. the tumor's 'living world', to uncover evolutionary processes as basis for therapy-relevant knowledge. In so far, socially linked, communicative tumor systems as a 'whole' are not reducible to or completely explicable in terms of reductionistically derived descriptions of tumor behavior.

To place the study of systems into manageable and simplified frameworks, the **tumor's 'living world'** is commonly conceptualized as a nested hierarchy of tumor-associated components, ranging from the DNA-based heritage to tumor and stroma cells, to tumor tissues, to the hosts' organs, and to the host. Kolch remarked that 'we try to find out the function of a system by disassembling it and measuring the activity of isolated components. This approach is very successful in characterizing the individual parts but very limited in reconstructing the evolutionary development of a system as a whole' (Kolch W (2008) Defining systems biology: through the eyes of a biochemist. IET Syst Biol 2:5–7). This systems concept as antithesis to reductionist concepts remains fully consistent with reductionist scientific approaches. This concept has to face the problem that small, circumscriptive, theme-dependent systems patterns do not necessarily explain large scale phenomena, the spin-off of novel systems features, or the evolutionary-based behavior of holistic communicative tumor systems.

The sentence '**the whole** is more **than the sum** of its **parts'** (Aristotle in The Metaphysics, 1045a10) concisely emphasizes the problem that a (tumor) system as a holistic system develops complex, often little understood stage-dependent and situate interactions. When applied to cancer, this problem may be, at least to some degree, due to the autonomous modular-based development of tumor systems (chapter 26). The therapeutically successful access to tumor systems by communicative interventions (biomodulatory therapies) may now separate the object of interest, the tumor's 'living world', which is composed as a holistic communicative system in categories of knowledge, i.e. the modular architecture and rationalization processes.

27.12 The Ambition for Personalized Tumor Therapy: Configuring Situational, Stage- and Tumor-Specific Systems Features

The ambition for personalized tumor therapy is reluctant towards any kind of functional reductionistically derived specification, trying to categorize 'tumor-inherent' functions as disastrous tumor features, i.e. tumor-associated inflammation, neoangiogenesis, Warburg effect, immunological response, extracellular matrix remodelling, cell proliferation rate, apoptosis, coagulation effects, cellular niches, or molecular genetics.

Instead, ubiquitously available therapy-relevant targets are differentially involved in distinct tumor-associated molecular or cellular subsystems, dependent on the tumor type and stage. These targets are part of a holistic view and susceptible to biomodulation. Personalized tumor therapy approaches focus on biological effects in systems involved in tumor progression by redeeming novel validity and denotation of particular modular systems and intersystemic exchange processes, which present basic mechanisms to finally attenuate tumor growth.

27.13 Outlook

The current **'colonization' of the tumor's 'living world' –** which is sometimes characterized by emancipatory interests of basic sciences, sophisticated techniques, market, capital, laws, and redtapism – has to be criticized as these colonization processes may constrain the view for principally communicatively linked tumor-associated systems processes. Instruments for merging different scientific directions for systems-theoretical considerations are missing. Basic research is predominantly technology-oriented, aligning itself with the dichotomy of structure- and function-analytical problems. Pre-clinical therapy models focusing on biomodulation necessitate closer cooperation between academic institutions, biotechnology, and pharmaceutical industries. Further advancement of various scientific resources are needed to uncover novel biomodulatory combination therapies, to study these therapies in a systems-associated context, and to develop adaptive trial designs.

Modular situation-adapted therapy approaches have to 'conquer' their position among already established theme-dependent therapy concepts compiled in a reductionist and contextualist manner. Potentially, highly chemoresistant and genetically complex tumors may become susceptible to post-genomic modular therapies. The alternative approach, experimental or therapeutic knock-down of single or multiple tumor-associated aberrations in metastatic tumors, has not yet overcome the tumor systems robustness in case of multiple or complex tumor cell-associated genetic aberrations.

The more evolutionary processes are involved in tumor progression, the more modularly designed tumor therapies could be applied, which should be of advantage in comparison to contextualistically compiled therapy concepts. Successful biomodulatory therapy approaches in castrate-refractory prostate cancer, metastatic renal clear cell carcinoma and melanoma etc. point in this direction (Systems Biology: A Therapeutic Target for Tumor Therapy, Reichle A, Vogt T, chapter 12).

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