Tumor Dormancy and Cellular Quiescence and Senescence 3

M.A. Hayat
Editor

Tumor Dormancy, Quiescence, and Senescence

Aging, Cancer, and Noncancer Pathologies

Volume 3

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Although touched by technology, surgical pathology always has been, and remains, an art. Surgical pathologists, like all artists, depict in their artwork (surgical pathology reports) their interactions with nature: emotions, observations, and knowledge are all integrated. The resulting artwork is a poor record of complex phenomena.

Richard J. Reed, MD

One Point of View

All small tumors do not always keep growing, especially small breast tumors, testicular tumors, and prostate tumors. Some small tumors may even disappear without a treatment. Indeed, because prostate tumor grows slowly, it is not unusual that a patient may die at an advanced age of some other causes, but prostate tumor is discovered in an autopsy study. In some cases of prostate tumors, the patient should be offered the option of active surveillance followed by PSA test or biopsies. Similarly, every small kidney tumor may not change or may even regress. Another example of cancer or precancer reversal is cervical cancer. Precancerous cervical cells found with Pap test may revert to normal cells. Tumor shrinkage, regression, dormancy, senescence, reversal, or stabilization is not impossible. Can prosenescence therapy be an efficient alternative strategy to standard therapies for cancer prevention and treatment?

Another known example of cancer regression is found in pediatric neuroblastoma patients. Neuroblastoma shows one of the highest rates of spontaneous regression among malignant tumors. In addition to the well-known spontaneous regression in stage 4S disease, the high incidence of neuroblastoma remnants found during autopsy of newborns suggest that localized lesions may undergo a similar regression (Guin et al. 1969). Later studies also indicate that spontaneous regression is regularly seen in infants with localized neuroblastoma and is not limited to the first year of life (Hero et al. 2008). These and other studies justify the "wait and see" strategy, avoiding chemotherapy and radiotherapy in infants with localized neuroblastoma, unless MYCN gene is amplified. Infants with nonamplified MYCN and hyperdiploidy can be effectively treated with less intensive therapy. Infants with disseminated disease without MYCN have excellent survival with minimal or no treatment. Another example of spontaneous shrinkage and loss of tumors without any treatment is an intradural lipoma (Endoh et al. 1998).

Although cancers grow progressively, various lesions such as cysts and thyroid adenomas show self-limiting growth. Probably, cellular senescence occurs in many organ types following initial mutations. Cellular senescence, the growth arrest seen in normal mammalian cells after a limited number of divisions, is controlled by tumor suppressors, including p53 and p16, and so this phenomenon is believed to be a crucial barrier to tumor development. It is well-established that cell proliferation and transformation induced by oncogene activation are restrained by cellular senescence.

Metastasis is the main cause of death from cancer. Fortunately, metastasis is an inefficient process. Only a few of the many cancer cells detached from the primary tumor succeed in forming secondary tumors. Metastatic inefficiency varies depending on the location within an organ, but the malignancy may continue to grow preferentially in a specific tissue environment. Some of the cancer cells shed from the primary tumor are lost in the circulation due to hemodynamic forces or the immune system, macrophages, and natural killer cells.

Periodic rejection of a drug by FDA, which was previously approved by the FDA, is not uncommon. Most recently, the FDA ruled that Avastin should not be used to treat advanced breast cancer, although it remains on the market to treat other cancers, including colon and lung malignancies. Side-effects of Avastin include high blood pressure, massive bleeding, heart attack, and damage to the stomach and intestines.

Unwanted side effects of some drug excipients (e.g., propylene glycol, menthol) may also pose safety concerns in some patients. Excipients are defined as the constituents of the pharmaceutical formulation used to guarantee stability, and physicochemical, organoleptic and biopharmaceutical properties. Excipients frequently make up the majority of the volume of oral and parenteral drugs. Not all excipients are inert from the biological point of view. Although adverse drug reactions caused by the excipients are a minority of all adverse effects of medicinal products, the lack of awareness of the possible risk from excipients should be a concern for regulatory agencies, physicians, and patients (Ursino et al. 2011). Knowledge of the potential side effects of excipients is important in clinical practice.

It is known that chemotherapy can cause very serious side-effects. One most recent example of such side-effects was reported by Rubsam et al. (2011). Advanced hepatocellular carcinoma (HCC) induced by hepatitis C virus was treated with Sorafenib. It is an oral multikinase inhibitor that interferes with the serine/threonine kinases RAF-1 and B-Raf and the receptor tyrosine kinases of the vascular endothelial growth factor receptors and the platelet-derived growth factor receptor-beta. Although sorafenib is effective in regressing HCC, it shows serious side-effects including increasingly pruritic and painful skin changes (cutaneous eruption).

An example of unnecessary surgery is the removal of all the armpit lymph nodes after a biopsy when a sentinel node shows early stage breast cancer; removal of only the sentinel node may be needed. Limiting the surgery to the sentinel node avoids painful surgery of the armpit lymph nodes, which can have complications such as swelling and infection (such limited surgery is already being practiced at the Memorial Sloan-Kettering Cancer Research Center). Radiation-induced second cerebral tumors constitute a significant risk for persons undergoing radiotherapy for the management of cerebral neoplasms. High-grade gliomas are the most common radiation-induced tumors in children (Pettorini et al. 2008). The actual incidence of this complication is not known, although it is thought to be generally low.

Medical Radiation

Chromosome aberrations induced by ionizing radiation are well-known. Medical radiation-induced tumors are well-documented. For example, several types of tumors (sarcomas, meningiomas) can develop in the CNS after irradiation of the head and neck region (Parent 1990). Tumorigenic mechanisms underlying the radiation therapy of the CNS are discussed by Amirjamshidi and Abbassioun (2000) (See below).

Radiation therapy is commonly used to treat, for example, patients with primary and secondary brain tumors. Unfortunately, ionizing radiation has limited tissue specificity, and tends to damage both neoplastic and normal brain tissues. Radiation-induced brain injury, in fact, is a potential, insidious later cerebral side-effect of radiotherapy. Most commonly it consists of damage in small arteries and capillaries, resulting in secondary processes of ischemia.

After radiation therapy, imaging techniques (CT, MRI, SPECT) can be used to assess treatment response and detect radiation-induced lesions and recurrent tumors. Optical spectroscopy has also been used for detecting radiation damage (Lin et al. 2005). The F_{500} nm spectral peak allows accurate selection of tissues for biopsy in evaluating patients with new, contrast enhancing lesions in the setting of previous irradiation. This peak is highly correlated with a histological pattern of radiation injury. Deep lesions require a stereotactic biopsy to be conclusive. Also, much of the radiation effect is mediated by acute and chronic inflammatory cellular reactions. Biopsy samples supplement pathological differentiation of radiation effect from tumor progression. It should be noted that most of the biopsies show radionecrosis as well as scattered tumor cells.

Women treated with therapeutic chest radiation may develop cancer. This possibility becomes exceedingly serious considering that 50,000–55,000 women in the United States have been treated with moderate to high-dose chest radiation (~20 Gy). This possibility is much more serious for pediatric or young adult cancer patients, because these women are at a significantly increased risk of breast cancer and breast cancer mortality following cure of their primary malignancy (Mertens et al. 2008). A recent study also indicates that such young women develop breast cancer at a young age, which does not appear to plateau (Henderson et al. 2010). In this high-risk population, ironically there is a benefit associated with early detection. In other words, young women with early stage breast cancer following chest radiation have a high likelihood for favorable outcome, although life-long surveillance is needed.

Presently, although approximately 80% of the children with cancer are cured, the curative therapy could damage a child's developing organ system; for example, cognitive deficits following cranial radiotherapy are well known. Childhood survivors of malignant diseases are also at an increased risk of primary thyroid cancer (Sigurdson et al. 2005). The risk of this cancer increases with radiation doses up to 20–29 Gy. In fact, exposure to radiation therapy is the most important risk factor for the development of a new CNS tumor in survivors of childhood cancer, including leukemia and brain tumors.

The higher risk of subsequent glioma in children subjected to medical radiation at a very young age reflects greater susceptibility of the developing brain to radiation. The details of the dose-response relationships, the expression of excess risk over time, and the modifying effects of other host and treatment factors have not been well defined (Neglia et al. 2006).

A recent study indicates that childhood brain tumor survivors are at an increased risk of late endocrine effects, particularly the patients treated with cranial radiation and diagnosed at a younger age (Shalitin et al. 2011). Among children with cancer, the application of radiotherapy, therefore, should not be taken lightly, and it should be administered only when absolutely necessary to successfully treat the primary tumor. When radiotherapy is administered, use of the minimum effective dose tends to minimize the risk of second CNS neoplasms (late effect). Prolonged follow-up of childhood cancer survivors (particularly those treated with radiation) is necessary because of the long period between treatment and the development of malignancy. This practice should be a part of the effective therapy of the primary disease.

It is well established that radiation doses are related to risk for subsequent malignant neoplasms in children with Hodgkin's disease. It has been reported that increasing radiation dose was associated with increasing standardized incidence ratio ($p=0.0085$) in survivors of childhood Hodgkin's disease (Constine et al. 2008). Approximately, 75% of subsequent malignancies occurred within the radiation field. Although subsequent malignancies occur, for example, in breast cancer survivors in the absence of radiotherapy, the risk increases with radiation dose.

The pertinent question is: Is it always necessary to practice tumor surgery, radiotherapy, chemotherapy or hormonal therapy or a combination of these therapies? Although the conventional belief is that cancer represents an "arrow that advances unidirectionally", it is becoming clear that for cancer to progress, it requires cooperative microenvironment (niche), including immune system and hormone levels. However, it is emphasized that advanced (malignant) cancers do not show regression, and require therapy. In the light of the inadequacy of standard treatments of malignancy, clinical applications of the stem cell technology need to be expedited.

Prostate Cancer

There were an estimated 217,730 new cases of prostate cancer in the United States in 2010 with 32,050 deaths, making it the second leading cause of cancer deaths in men. Currently, there are more than 2,000,000 men in the United States who have had radical or partial prostate surgery performed. Considering this huge number of prostate surgeries and the absence of a cumulative outcome data, it seems appropriate to carefully examine the benefits of radical surgery, especially in younger men.

Clinical prostate cancer is very rare in men of ages younger than 40 years. In this age group the frequency of prostate malignancy is 1 in 10,000 individuals. Unfortunately, the incidence of malignancy increases over the ensuing decades, that is, the chance of prostate malignancy may reach to 1 in

7 in men between the ages of 60 and 79 years. Reactive or aging-related alterations in the tumor microenvironment provide sufficient influence, promoting tumor cell invasion and metastasis. It has been shown that nontumorigenic prostate epithelial cells can become tumorigenic when cocultured with fibroblasts obtained from regions near tumors (Olumi et al. 1999).

Prostate cancer treatment is one of the worst examples of overtreatment. Serum prostate specific antigen (PSA) testing for the early detection of prostate cancer is in wide use. However, the benefit of this testing has become controversial. The normal cut-off for serum levels of PSA is 4 ng/ml, so a man presenting with a PSA above this level is likely to require a rectal biopsy, but only 25% of men with serum levels of PSA between 4 ng and 10 ng/ml have cancer (Masters 2007). The PSA threshold currently being used for biopsy ranges between 2.5 and 3.4 ng/ml. Up to 50% of men presenting with prostate cancer have PSA levels within the normal range. It is apparent that screening of prostate cancer using PSA has a low specificity, resulting in many unnecessary biopsies, particularly for gray zone values (4 ng–10 ng/ml). According to one point of view, the risks of prostate cancer overdetection are substantial. In this context, overdetection means treating a cancer that otherwise would not progress to clinically significant disease during the lifetime of the individual. Overdetection results in overtreatment. The advantages and limitations of PSA test in diagnosing prostate cancer were reviewed by Hayat (2005, 2008).

Androgen deprivation therapy (ADT) is an important treatment for patients with advanced stage prostate cancer. This therapy is carried out by blocking androgen receptor or medical or surgical castration. Although ADT is initially very effective, treated tumors inevitably progress to androgen-independent prostate cancer (AIPC), which is incurable. One possible mechanism responsible for the development of AIPC is modulation of the tissue microenvironment by neuroendocrine-like cancer cells, which emerge after ADT (Nelson et al. 2007).

Recently, Pernicova et al. (2011) have further clarified the role of androgen deprivation in promoting the clonal expansion of androgen-independent prostate cancer. They reported a novel linkage between the inhibition of the androgen receptor activity, down-regulation of S-phase kinase-associated protein 2, and the formation of secretory, senescent cells in prostate tumor cells. It is known that several components of the SASP secretome, such as IL-6, IL-8, KGH, and epidermal growth factor, are capable of transactivating androgen receptor under androgen-depleted conditions (Seaton et al. 2008). It needs to be pointed out that androgen deprivation therapy, used in high-risk patients with prostate cancer, may cause reduced libido, erectile dysfunction, fatigue, and muscle loss; osteoporosis is also a late complication. Therefore, periodic bone density scanning needs to be considered.

Recently, the FDA cleared the use of NADiA (nucleic acid detection immunoassay) ProsVue prognostic cancer test. This proprietary nucleic acid detection immunoassay technology identifies extremely low concentrations of proteins that have not been routinely used as a diagnostic or prognostic aid. It is an in vitro diagnostic assay for determining the rate of change of serum total PSA over a period of time. The assay can quantitate PSA at levels <1 ng/ml.

This technique can be used as a prognostic marker, in conjunction with clinical evaluation, to help identify patients at reduced risk for recurrence of prostate cancer for years following prostatectomy. It targets the early detection of proteins associated with cancer and infectious diseases. This technique combines immunoassay and real-time PCR methodologies with the potential to detect proteins with femtogram/ml sensitivity (10–15 g/ml). Additional clinical information is needed regarding its usefulness in predicting the recurrence.

A significant decrease in the risk of prostate cancer-specific mortality is observed in men with few or no comorbidities. Indeed, active surveillance in lieu of immediate treatment (surgery or radiation, or both) is gaining acceptance. Most men with prostate cancer, even those with high-risk disease, ultimately die as a result of other causes (Lu-Yao et al. 2009). Debate on this controversy is welcome, but narrow opinions and facile guidelines will not lead to facts and new information; men worldwide deserve it (Carroll et al. 2011). Automatically linking positive diagnosis with treatment, unfortunately, is a common clinical practice. Unfortunately, even men who are excellent candidates for active surveillance in the United States often undergo some treatment. Deferment of treatment is advised in men with low-risk disease, especially of a younger age.

Active surveillance is proposed for patients with low-risk prostate cancer in order to reduce the undesirable effects of overdiagnosis. Prostate specific antigen serum level lower than 10 ng/L and Gleason score lower than seven are the main criteria to select patients for active surveillance. The correct use of these two criteria is essential to differentiate between aggressive and non-aggressive prostate cancer. Autopsy studies indicate that approximately one out of three men older than 50 years show histological evidence of prostate cancer (Klotz 2008). Thus, a large proportion of prostate cancers are latent, never destined to progress, or affect the life of the patient. It is estimated that the percentage of low-risk prostate cancer is between 50% and 60% of newly diagnosed cases. A large number of patients die having prostate cancer, but not because of this cancer (Filella et al. 2011).

First whole genome sequences of prostate tumors were recently published online in *Nature* journal (vol. 470: 214–220, 2011). This study revealed that rather than single spelling errors, the tumor has long "paragraphs" of DNA that seem to have broken off and moved to another part of the genome (rearrangement of genes), where they are most active. These portions of DNA contain genes that help drive cancer progression. The mutated genes involved include PTEN, CADM2, MAG12, SPOP, and SPTA1. This information may lead to the development of more efficient, less invasive ways to diagnose and treat this cancer. Such information, in addition, should lead to personalized therapeutics according to sequencing results of different gene mutations or chromosomal rearrangements. The urgent need of such studies becomes apparent considering the huge number of new cases of prostate problems reported every year.

In contrast to prostate cancer, cardiovascular disorders take the heavier toll of life. In other words, the risk of death for men in the United States between the ages of 55 and 74 years due to cardiovascular disease surpasses that of prostate cancer. Cardiovascular disease is the most common of the chronic

non-communicable diseases that impact global mortality. Approximately, 30% of all deaths worldwide and 10% of all healthy life lost to disease are accounted for by cardiovascular disease alone.

In conclusion, initial treatment with standard surgery, irradiation, chemotherapy, or hormonal therapy, or combination of these protocols can result in both local and systemic sequelae. Therefore, surveillance for late recurrence and secondary primary malignancies is recommended for most cancer patients. Patients with breast, lung, prostate, colorectal, and head and neck cancers constitute the largest groups requiring long-term monitoring and follow-up care.

Eric Hayat

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Preface

Cellular dormancy refers to the cell entering a state of quiescence where growth is arrested in the G0-G1 phase of cell cycle. In this phase the cells are inactive and asymptomatic. The micrometastasis model defines tumor cell dormancy as a state of balanced apoptosis and proliferation of micrometastasis resulting in no net increase in the tumor mass. Cancer dormancy is referred to (in clinical terms) in connection with recurrence of cancer systemically or locally a long time after removal of the primary tumor in a patient who has been clinically disease free. Occurrence of cancer dormancy is a characteristic of all migrating tumor cells. Once tumor cells disseminate and start migrating to a new site to metastasize, the interaction of the tumor cells with the microenvironment determines whether the cells will proliferate and form metastases or undergo growth arrest and enter cancer dormancy. The disseminated cells will opt for dormancy if the new environment is not permissive such as absence of available growth factors and angiogenesis, and cellular stress. However, such dormant cells can exist in a quiescent state for many years, but start proliferating and form metastases that are incurable. For example, in breast cancer, 20% of clinically disease-free patients relapse 7–25 years after mastectomy.

It is suggested that metastasis-initiating cells are cancer stem cells or such cells revert to this functional state upon infiltrating a target organ. It seems plausible that primary tumors shed tumor cells at an early stage into the blood circulation, and a subset of these disseminated cells may persist in a state of dormancy. Molecular characterization of disseminated tumor cells in bone marrow and circulating tumor cells in blood opens a new avenue for understanding cancer dormancy and might contribute to the identification of metastatic stem cells.

Cellular quiescence is opposite to cell proliferation and is considered to be in a non-dividing state, but is reversible. It is a reversible growth/proliferation arrest process induced by diverse anti-mitogen signals, each of which regulates a group of genes; these genes play a key role in the cessation of cell growth and division. Different genes involved in this process can be identified.

In contrast to quiescence, senescence is essentially an irreversible cell growth arrest, which occurs when cells that can divide encounter oncogenic stress. Cellular senescence is a crucial anticancer mechanism. Premature senescence functions as a tumor suppressor mechanism in response to oncogenic stimuli. It is characterized by irreversible cell cycle arrest mediated

by tumor suppressors such as p53, Rb, and the promyelocytic leukemic (PML) protein. However, senescence is both tumor promoter and tumor suppressor. With the exception of embryonic stem cells and some tumor cells, most division-competent cells can undergo senescence when stimulated. DNA double strand breaks are one of the potent senescence inducers. Other stresses implicated in the induction of senescence include oxidative damage, telomerase dysfunction, and aberrant oncogene-dependent proliferative signaling. Senescence cells increase with age, which may give rise to cancer.

Although some evidence indicates that autophagy induction accelerates the development of senescence, the opposite has also been reported, i.e., autophagy suppression does not alter senescence induction. In other words, presence of senescence does not depend on the prior induction of autophagy. Some other studies report that autophagy and senescence occur in parallel, but are not interdependent. It is apparent that the exact relationship between autophagy and senescence is very complex, and contradictory results have been reported. Furthermore, because apoptosis is induced when autophagy is inhibited, the correct interpretation of the relationship between autophagy and senescence becomes difficult.

This is volume 3 of the multivolume series discussing Tumor Dormancy, Quiescence, and Cellular Senescence. The role of tumor dormancy and senescence in a number of diseases, including breast cancer, ovarian cancer, and leukemia, is discussed. Also is discussed the role of senescence and autophagy in the age-related cardiovascular diseases. The enchantment of autophagy seems to retard cardiac senescence. The role of cancer dormancy in breast cancer is discussed, indicating that tumor cells are able to persist in the dormant state until they become active and cause distant metastases. It is pointed out that cancer stem cells exist within breast tumors, which possess the ability of self-renewal and differentiation in a deregulated manner, resulting in tumor progression. The wingless related protein (Wnt) pathway plays a significant role in the “awakening” of dormant tumors.

Ovarian cancer is the most deadly of gynecological malignancies, and the long-term survival rate is quite poor, especially in patients having Type II high grade ovarian cancer with defective TP53. It is pointed out that Type II tumors are phenotypically heterogeneous, and a subpopulation of tumor cells is relatively resistant to chemotherapy. The resistant tumor cell population persists after chemotherapy in a state of dormancy, with recurrent tumors arising upon transformation of such dormant cells back to malignant growth. How lineage, histological subtypes, and grade influence the differential response of ovarian cancer resistance to platinum drugs is explained. Also is discussed the role of angiogenesis, growth arrest and autophagy in human ovarian cancer xenograft models for tumor dormancy.

By bringing together a large number of experts (oncologists, neurosurgeons, physicians, research scientists, and pathologists) in various aspects of this medical field, it is my hope that substantial progress will be made against a terrible human disease and injury. It is difficult for a single author to discuss effectively the complexity of cancer diagnosis, therapy and prognosis. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of cancer metastases and

therapy. I hope these goals will be fulfilled in this and other volumes of the series. This volume was written by 26 contributors representing 7 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights their writings, which should build and further the endeavors of the readers in these important areas of disease and injury. I respect and appreciate the hard work and exceptional insight into the role of dormancy, quiescence, and cellular senescence in various diseases and stem cell functions provided by these contributors. The contents of this volume are divided into five subheadings: General Applications, Role in Breast Cancer, Role in Ovarian Cancer, Role in Leukemia and Role in Cardiovascular Disease.

It is my hope that subsequent volumes of the series will join this volume in assisting in the more complete understanding of the major human diseases and their treatments. There exists a tremendous, urgent demand by the public and the scientific community to address to cancer diagnosis, treatment, cure and hopefully prevention. In the light of existing cancer calamity, government funding must give priority to eradicating deadly malignancies over military superiority.

I am thankful to Dr. Dawood Farahi and Phil Connelly for recognizing the importance of medical research and publishing through an institution of higher education. I am thankful to my students for their contribution to the preparation of this volume.

Union, NJ, USA M.A. Hayat
May 2014

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Part I

General Applications

1 Active, Dormant, or on the Path to Elimination: What Does a Senescent Cell Do?

Ittai Ben-Porath

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Abstract

Views of the physiological significance of cellular senescence, a coordinated program activated by cells exposed to stress, have been evolving dramatically in recent years. Senescence involves a cell cycle arrest accompanied by morphologic and metabolic changes, and by enhanced cytokine secretion. There is much evidence to indicate that senescence is central in suppressing tumor development, acting to block the proliferation of cells expressing an active oncogene or suffering from damaged DNA. The detection of senescence in additional settings, including inflammation and wound healing, as well as in aging tissues, suggests that this cellular program is involved in a variety of physiologic processes, mostly associated with pathology. Importantly, however, the fundamental nature of this program remains poorly understood. Does senescence represent a state of dormancy or dysfunction, or do senescent cells play an active, designated role within normal, aging and tumorigenic tissues? Are senescent cells retained within tissues, or are they rapidly removed? Is the function of senescence to counter tissue pathology, or is it an aberrant state primarily contributing to disease? Recent studies of senescence in the *in vivo* setting have provided some important insights into these questions and have highlighted areas requiring further study.

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Introduction: Cellular Senescence, What Is It Good For?

Views of cellular senescence and its biological roles have evolved dramatically since the first description of this phenomenon in 1961 by Leonard Hayflick. Senescence is a complex phenotype acquired by cells exposed to various types of stress, notably DNA damage and oncogene activation (Kuilman et al. 2010; Rodier and Campisi 2011). It involves an irreversible arrest of mitotic division, accompanied by complex changes in cell morphology, metabolism, chromatin structure and protein secretion. The predominant view of senescence, emerging in recent years, is that of a coordinated cellular program through which cells respond to a stress or damage, and which, once activated, can affect the function and structure of the tissue in which it occurs (Kuilman et al. 2010; Rodier and Campisi 2011). This current view is supported in large part by studies in which senescence was detected and studied in the living tissue. However, research of senescence *in vivo* is relatively recent, and therefore in most cases in which it is detected, the physiologic functions of this program are poorly understood.

In fact, answers to many fundamental questions regarding senescence are lacking. Prominent among these is the question of the very nature and function of this complex phenotype. Are senescent cells in a state of dysfunction – "sick" cells that are either rapidly eliminated or remain to interfere with their healthy environment – or is senescence a component of normal physiological processes, senescent cells executing designated functions to which their unique characteristics are suited? Additional, more specific questions remain: how do senescent cells interact with their environment, what is their eventual fate, what is the exact nature and function of their various phenotypes, and, ultimately, how do they affect disease.

Senescence was originally viewed as a barrier for cell division, a means to prevent immortality and thereby tumorigenesis. Subsequent work revealed that the gradual shortening of telomeres is often the trigger for senescence of propagated normal human cells, supporting the idea that cell division history is monitored through an inherent internal molecular clock. The accumulation of findings demonstrating rapid entry of cells into senescence in response to various types of acute stress, such as DNA damage, disruption of chromatin structure and suboptimal growth conditions led to the subsequent view of senescence as a general stress response program (Ben-Porath and Weinberg 2004). The discovery that constitutively activated oncogenes such as *RAS* can induce senescence shed a new light on the role of senescence in tumor suppression: it was now seen as a direct cellular response to oncogenic signaling, acting to block tumor cell proliferation. Tumor suppression may thus represent a primary function of senescence, and a central means by which the p53 and Rb proteins act to suppress tumorigenesis. The molecular mechanism by which oncogenes activate senescence has not been fully elucidated; interestingly, there is evidence to indicate that in fact this occurs through the induction of DNA damage (Halazonetis et al. 2008).

These evolving views of senescence have all focused on the irreversible arrest of proliferation that this program entails as its primary function. If senescence is viewed only as a means to block the expansion of an aberrant or damaged cell, then this goal can indeed be achieved through cell cycle arrest. However, the question arises of whether a cytostatic response is indeed as effective as cell elimination. Furthermore, such a view does not take into account the many additional phenotypic traits acquired by senescent cells. In culture, a flattened enlarged morphology is a hallmark of senescence, and such cells undergo dramatic changes in contact with neighboring cells and with the ECM. Chromatin structure in senescence is modified, and has been suggested to adopt a closed heterochromatic pattern and a distinct nuclear organization (Narita 2007). Aspects of protein turnover and nucleotide metabolism are also altered in senescent cells, and aspects of autophagy appear to be integrated in to this program (Narita and Young 2009; Aird

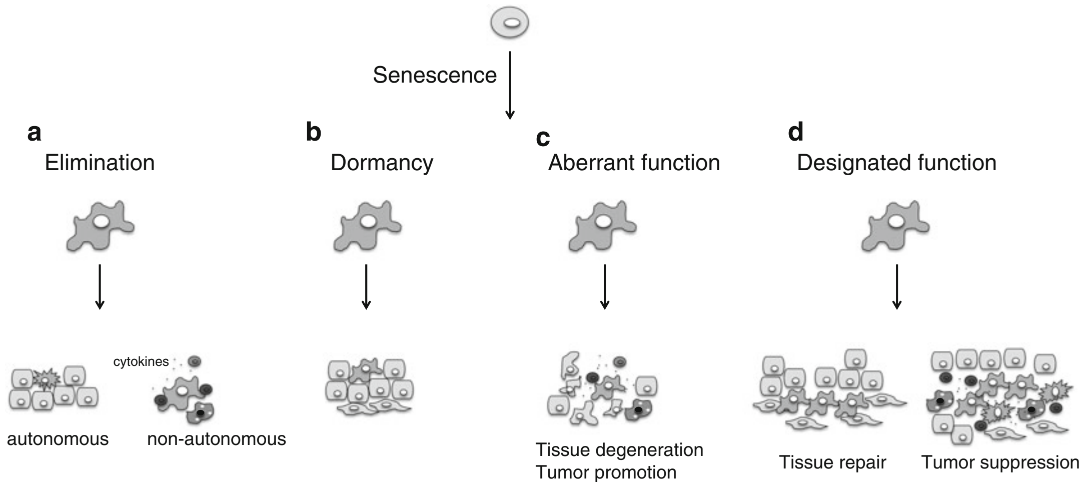

Fig. 1.1 Potential functional views of senescence. (**a**) A path to elimination. Cell autonomous elimination (*left*), through cell death subsequent to senescence, or non-cell-autonomous (*right*), induced, potentially, by recruited immune cells. (**b**) A state of dormancy (non-function) within tissues, with potential passive effects through accumulation. (**c**) A state of aberrant function. The aberrant properties of retained senescent cells can promote tissue degeneration and tumorigenesis. (**d**) A state executing designated functions within physiologic processes, such as wound healing and resolution of fibrosis (*left*), and tumor suppression, acting through the cell autonomous arrest of tumor cells as well as, potentially, by the generation of a tumor suppressive environment (*right*)

et al. 2013; Deschenes-Simard et al. 2013). Most suggestive of the potential effects of senescent cells on their environment is the senescence-associated secretory phenotype (SASP). Senescent cells, especially those carrying an active oncogene and suffering from DNA damage, secrete a wide range of cytokines known to influence many types of immune and non-immune cells, including IL1α, IL6, IL8, CXCL1, IGFBP7, VEGF and many others, as well as matrix modifying enzymes, such as matrix metalloproteases (Coppe et al. 2009). The local and systemic influences of the SASP are therefore potentially broad. Taking this information into account, it is possible to chart at least four descriptions of the basic nature of senescence (Fig. 1.1):

(a) **A path to elimination:** damaged cells that conduct no function and are on the course to rapid elimination.
(b) **A dormant state:** damaged cells that conduct no specific function, yet can accumulate in tissues and may influence their structure/function by virtue of their mere presence.
(c) **A state of aberrant function:** cells that are retained in the tissue for short or long term, which due to their aberrant phenotype exert negative effects on tissue structure and function.
(d) **A state of designated function:** cells retained in the tissue for short or long term, which conduct specific designated functions, within physiologic processes.

These four descriptions, which are further discussed below, are not mutually exclusively, and senescence may function differently in distinct settings.

How Long Are Senescent Cells Retained in Tissues?

Cultured senescent cells can remain viable for long periods, although, depending on cell type, gradual death may be observed in such cultures. Senescent cells could therefore remain viable also in tissues. Indeed, many studies have demonstrated that the numbers of senescent cells in

tissues increase with aging, in human and various animal models (Rodier and Campisi 2011), indicating that such cells, once formed, can persist within living organs. For detection of senescence the senescence-associated β–galactosidase activity (SA-βGal) assay is often used, as well as the expression of several senescence-associated molecular markers. The two *Ink4a* (*Cdkn2a*) locus products, p16^{Ink4a} and p19^{Arf}, which are central molecular activators of this program, and are not normally expressed in most tissues, are seen as markers of senescence (Caldwell et al. 2011). DNA damage markers are often also detected in the same tissues, suggesting that at least some aging-associated senescent cells represent DNA damaged cells. The accumulation of senescent cells in tissues is recapitulated in mouse models of premature aging, such as mice suffering from DNA damage induced, for example, by *Brca1* mutation (Cao et al. 2003; Baker et al. 2004). Senescent cells have also been detected in various pathological settings, for example in cirrhotic or virally infected livers (Paradis et al. 2001), once again indicating that such cells can be retained.

The duration for which senescent cells remain in tissues is, however, often difficult to directly assess. Whole body irradiation induces DNA damage foci in various tissues and increased expression of p16^{Ink4a} (Le et al. 2010); in this setting, in which the timing of senescence induction is known, it was found that p16^{Ink4a} levels were still elevated 3 months after irradiation, suggesting that at least some senescent cells were retained for this period.

We recently addressed this question using transgenic mice in which p14^{ARF} (the human ortholog of mouse p19^{Arf}) was inducibly activated in the skin of adult mice (Tokarsky-Amiel et al. 2013). In this system the senescence program is activated directly at the molecular level, through p53, without induction of DNA damage or oncogenesis. Senescent cells appear in the epidermis after 1 week of transgene induction. By subsequently silencing the p14^{ARF} transgene, we could quantify the numbers of remaining senescent cells in the skin at different timepoints. One month after transgene silencing, most senescent cells – 61% – were still present in the skin, supporting the notion that such cells can remain within tissues for periods of weeks or even months.

At the same time, this experiment also demonstrates that these cells gradually disappear from the tissue. Which forces act to eliminate senescent cells? Little is known about this process. Elimination could occur in a "passive" process, through tissue dynamics: proliferating cells in the epidermis, for example, could "push" non-dividing senescent cells towards their shedding. Elimination could also occur actively, either through a cell-autonomous mechanism, such that senescent cells proceed to die after a given period, or through a non-cell-autonomous mechanism: the environment or surrounding cells actively eliminating cells in a directed and specific manner.

The same possibilities apply also to senescent cells generated within tumor lesions. Early tumor lesions of various tumor types were shown to contain senescent cells, but such cells are often not detected upon progression (Collado and Serrano 2009). For example, in a Ras-induced pancreatic cancer model, senescent cells are detected in very early lesions – pancreatic intraepithelial lesions (PanIN1) – but are absent in the more progressed PanIN2/3 lesions (Guerra et al. 2011). It is unclear whether these cells are taken over "passively" due to the selective advantage of proliferating on-senescent cells, or whether they are actively eliminated.

Much interest has been directed towards the potential role of the immune system in actively eliminating senescent cells. It is attractive to hypothesize that senescent cells recruit immune cells through cytokine secretion, and this leads to their own destruction by the recruited cells. The first demonstration of such a mechanism was provided using a model in which p53 was re-activated in liver cancer xenografts (Xue et al. 2007). This caused widespread senescence of tumor cells that was accompanied by immune infiltration, and the tumors underwent a dramatic decrease in size within 2–3 weeks. The elimination of the tumor

cells in this setting was occurred through the activity of innate immune cells, since inhibition of macrophages, neutrophils or NK cells all attenuated tumor regression. Similarly, induction of tumorigenic lesions in the mouse liver by expression of a mutant *N-Ras* gene leads to senescence of oncogene expressing hepatocytes, and lymphocytic infiltration was observed within lesions (Kang et al. 2011). The senescent cells in such lesions were mostly absent 2–3 weeks after oncogene activation, as assessed by scoring of $p16^{Ink4a}$ expressing cells, and the rate of their disappearance was attenuated in immune deficient mice. It was apparently monocytes/macrophages that eliminate senescent tumor cells in this system; interestingly, however, CD4+ Th1 T-helper cells were essential in this response, and T cells reactive towards mutant *N-Ras* protein were detected in the mice, indicating a role for the adaptive immune system in the reaction against the tumor cells. In both of these models, preferential targeting by the immune system of the senescent tumor cells versus non-senescent cells within the same lesions, was difficult to directly assess.

A different setting in which senescent cells appear to be eliminated by immune cells is that of liver fibrosis (Krizhanovsky et al. 2008). Following chemically induced liver damage, hepatic stellate cells expand and differentiate into myofibroblasts, generating fibrosis, and these cells subsequently undergo senescence. Here it was shown that NK cells act to clear the senescent cells from the tissue, and elimination occurs within approximately 3 weeks. That NK cells specifically target senescent cells was directly demonstrated in co-culture experiments, and it was shown that killing occurs through the granule exocytosis pathway (Krizhanovsky et al. 2008; Sagiv et al. 2013).

Together, this early information indicates that in some settings senescent cells may be designated to be removed by the immune system, and that this occurs within approximately 7–20 days of their formation. It should be noted though that even in these systems it is possible that not all senescent cells were removed, and the efficiency of removal rates remain to be fully assessed.

The context of senescence is likely important in determining retention times. For example the activity of oncogenes could act as a stimulatory force for immune cells to remove senescent cells, and Ras activity and persistent DNA damage have been shown to amplify the SASP (Coppe et al. 2008; Rodier et al. 2009). Therefore, senescence within tumor lesions could possibly lead to quicker elimination. Tissue type is likely also important, and an immune protected environment could conceivably allow longer retention times of senescent cells.

Consistent with the idea that immune cells remove senescent cells, it appears that active inflammation can result in complete absence of senescence in situations where it otherwise occurs. Induction of pancreatitis in mice by cerulein treatment, which promotes tumorigenesis on the background of *K-Ras* mutation, prevented the appearance of senescent cells within PanIN lesions (Guerra et al. 2011); anti-inflammatory treatment of the cerulein-treated mice allowed the appearance of such cells. Similarly, treatment of skins with the inflammation-inducing phorbol ester TPA prevented the appearance of senescent cells upon $p14^{ARF}$ induction (Tokarsky-Amiel et al. 2013). In both cases the absence of senescent cells could be attributed to the inflammatory process preventing the ability of the epithelial cells to acquire the senescent phenotype to begin with; alternatively, inflammatory cells could act swiftly to remove senescent cells immediately upon their formation.

Inflammation: Eliminating or Inducing Senescence?

The relationship between senescent cells and the immune system cannot, however, be interpreted only in this antagonistic light. Several studies have revealed instances in which immune cells appear to promote cellular senescence. Cerulein-induced pancreatitis was shown to induce senescence of pancreatic ductal cells, in a $p16^{Ink4a}$ (but not p53) dependent manner,

and quite rapidly: within 3 days of treatment (Lee and Bar-Sagi 2010). Here, the Ras oncogene was shown to suppress the senescence machinery. This offers a striking reversal of roles of oncogene activity and inflammation in regards to senescence.

In the tumorigenic setting, there have been several demonstrations of recruitment of immune cells to the tumor site, in which these cells appear to induce the senescence of tumor cells. In the Eμ-Myc lymphoma model, the Myc oncogene has been shown to induce both apoptosis and senescence in tumor cells, both responses delaying tumor growth; however, senescence is induced indirectly: macrophages recruited due to the lesion secrete TGFβ, leading to senescence within the tumor (Reimann et al. 2010). Senescence can thus be triggered in tumor cells also in *trans*, through the activity of immune cells.

The growth of tumors in the RIP-Tag2 insulinoma model, in which the SV40 Large T-antigen (Tag) oncogene is expressed in pancreatic islet β-cells, is limited by the activity of CD4+ Th1 T-helper cells reactive against the Tag antigen (Braumuller et al. 2013). It appears that these cells inhibit cancer growth by inducing senescence of tumor cells, through direct action of TNF and IFNγ.

In another example, Myc-induced lymphomas exhibit cellular senescence upon Myc silencing: in this case it is not oncogene-induced senescence, but, rather, senescence upon withdrawal due to oncogene addiction (Rakhra et al. 2010). Interestingly, CD4+ T-helper cells were implicated in this setting in inducing senescence itself: in mice lacking these cells senescence was muted.

These and other findings illustrate that innate and adaptive immune components may in some settings act to induce senescence, while in others act to remove such cells. The case of CD4+ T-helper cells appears to be particularly interesting in executing both of these contrasting functions. The anti-senescent or pro-senescent action of immune cells is context dependent, and the factors determining this action would be of great interest.

Can Cellular Senescence Promote Tumorigenesis?

Whether brought about by oncogene activity or by oncogene withdrawal, whether induced in a cell-autonomous manner or in *trans* through the activity of immune cells, in the studies discussed above senescence activation went hand in hand with tumor suppression, and its blockage led to accelerated tumor growth or recurrence, or to decelerated tumor regression. However, compelling data exists to indicate that senescent cells could also act as tumor promoting agents. Senescent fibroblasts co-injected with carcinoma cells into mice promote their tumorigenic capacity and invasive traits (Coppe et al. 2009). Many of the cytokines secreted as part of the SASP are known to be able to promote cancer, in some cases through direct stimulation of the cancer cells and in others through generating a tumor supportive stroma. These experiments are taken to suggest that senescent cell accumulation during aging, particularly in tissue stroma, could act to encourage tumorigenic transformation of neighboring cells (Coppe et al. 2009). Another interesting potential interpretation of these findings, further discussed below, is that fibroblast senescence represents a form of fibroblast activation; thus senescent cells could share traits with tumor supportive cancer-associated fibroblasts, which also display NFκB-controlled cytokine secretion (Erez et al. 2010; Kojima et al. 2010).

Consistent with this, a recent study indicates that senescence, or a senescent-like state, may be a general feature of tumor stroma. Mice in which the luciferase reporter gene was placed under control of the p16^{Ink4a} promoter display luminescence in organs in which p16^{Ink4a} is expressed (Burd et al. 2013), presumably correlating with sites of senescence. In these mice high levels of luminescence were observed in developing tumor lesions, but, interestingly, not only in the neoplastic cells but also in the stroma. Thus, senescence of immune, fibroblastic or endothelial cells in tumor stroma, may be a widespread feature.

Whether senescence in the stroma acts to support or inhibit the tumor or to repress its

growth remains to be studied. An interesting demonstration of the latter case has been recently provided: in the liver, senescence of hepatic stellate cells appears to suppress transformation of hepatocytes, acting in *trans* by influencing macrophages (Lujambio et al. 2013). The senescent cells promoted an M1 anti-tumorigenic phenotype in the macrophages, thus generating a tumor suppressive, rather than tumor supportive, environment for transformed hepatocytes.

This leads to the question of whether senescent carcinoma cells (as opposed to senescent stromal cells) can also influence neighboring non-senescent carcinoma cells, either stimulating or suppressing their tumorigenicity. A recent study has provided such an example of senescence acting in a tumor promoting role: in the MMTV-Wnt breast cancer model, doxorubicin treatment inhibits tumor growth and causes senescence of the tumor cells; surprisingly, prevention of senescence by elimination of p53 leads to better tumor response to therapy, i.e. enhanced tumor regression (Jackson et al. 2012). The p53-expressing tumor cells respond to the drug-induced DNA damage by senescence accompanied by cytokine secretion, while p53-deficient cells die. In this case, the presence of the senescent cells and the cytokines they secrete appear to assist the remaining viable tumor cells.

Senescent Cell Function: Designated or Aberrant?

Presented above were four main potential descriptions of senescence and its functions. Senescent cells could affect the tissue in which they are formed even if immediately eliminated, by virtue of their absence or by the damaged caused by their elimination. However, as discussed above, it is obvious that senescent cells can be retained in tissues for days, weeks or longer periods. This leads to the question that is probably most difficult to dissect, both conceptually and experimentally: do senescent cells execute designated functions, or do their effects merely represent a state of dysfunction, a pathology at the cellular level that can lead to a pathology at the tissue or organismal level.

What can we deduce to be normal, designated functions of senescence? In the cancer setting, as discussed above, it seems to be beyond doubt that the cell cycle arrest occurring at senescence acts to block cancer development. However, it is also clear that a cytostatic effect may be less effective than actual elimination of tumor cells by apoptosis, raising the question of why senescence is employed for this task rather than apoptosis. The self-promoted elimination of senescent cells through recruitment of immune cells helps resolve this question, allowing senescence to be viewed basically as a program for cell elimination, yet not very rapid or fully efficient. If so, why is it beneficial for senescent tumor cells to remain in the lesion, even for short periods? The possibility that senescence could be propagated from cell to cell through cytokine secretion or other means (Nelson et al. 2012) could point to this program as having a field effect of tumor suppression, blocking not only the cells in which it was originally activated but also, for additional safety, surrounding cells.

The case for a designated contribution of cellular senescence to the processes of wound healing and fibrosis appears, on the basis of recent reports, most compelling. It is the myofibroblast, or activated fibroblast, being the cell of function. In chemically induced liver fibrosis, senescence of the hepatic stellate cells and the subsequent removal of these cells is important in resolving fibrosis, and when senescence of these cells is prevented, excessive fibrosis occurs (Krizhanovsky et al. 2008). Senescence appears to carry out a similar role during wound healing in the skin: SA-βGal-positive cells are detected in the granulation tissue at a time window of 7–9 days after wounding (Jun and Lau 2010). These senescent cells express anti-fibrotic genes, namely matrix metalloproteases, and act to limit fibrosis during the healing process. Senescence is absent in mice carrying a mutation in the *Ccn1* gene, which is involved in matrix modification, and such mice display increased matrix deposition and fibrosis. It is thus possible that senescence is integrated in certain settings into the process of fibroblast

activation and its resolution. In fact, expression profiling of senescent fibroblasts has suggested that these cells represent an activated fibroblast state (Shelton et al. 1999).

The "dark" aberrant side of senescence seems to be most appropriately discussed in the context of aging. As noted above, a host of studies demonstrated increases in senescent cells during aging. This is supported by the p16^{Ink4a} promoter-driven luciferase reporter mice, which show dramatically increased luminescence in multiple tissues as the mice age (Burd et al. 2013). Senescence could contribute to aging by non-function: should stem cells in different tissues undergo senescence, due to telomere shortening or other types of damage or stress, their lack of function could induce tissue degeneration. Alternatively, cytokine secretion by senescent cells could negatively influence tissue function, and chronic low level inflammation induced by such cytokines could represent one means of ongoing damage to the tissue (Rodier and Campisi 2011).

A recent study has provided an elegant demonstration of the negative effect exerted by senescent cells on their environment. Mice harboring a hypomorphic mutation in the mitotic checkpoint protein BubR1 suffer from premature aging phenotypes, including kyphosis, cachexia, cataracts, dermal thinning, as well as a shortened lifespan (Baker et al. 2004). Cellular senescence is detected in tissues of these mice (Baker et al. 2004, 2011). To test the contribution of cellular senescence to the observed aging-like phenotypes, the BubR1 mutant mice were crossed to mice engineered such that cells that express p16^{Ink4a} can be eliminated. This was done by expression, under control of the p16^{Ink4a} promoter, of a transgene encoding a Fkbp protein fused to Caspase 8 (termed *INK-ATTAC*). Administration of a dimerizing drug leads to caspase activation and selective killing of p16^{Ink4a}-expressing cells, which were preferentially senescent. Strikingly, continued drug treatment of mice from 3 weeks to 8 months of age led to the reduction in senescence marker expression in affected tissues, including SASP components such as IL6 and MMPs, and to a significant delay in the onset of many of the aging-like phenotypes, including sarcopenia, cataracts and fat loss (Baker et al. 2011).

These remarkable results provide the important insight that the presence within tissues of the senescent cells is itself of detriment. The effects of senescent cell elimination during normal aging in this and other models is likely to provide additional important insights into this question.

In light of these results the question of why senescent cells are retained rather than eliminated naturally becomes more prominent. In the BubR1 model, no detrimental effects of senescent cell removal were observed. Based on this, retention of senescent cells, and their degeneration-promoting effects, can be seen as an aberrant, pathological event.

Does a Reversible, Partially Senescent State Exist?

Am important question related to the above discussed topics is whether the senescence program is always brought about in its full form, and in an irreversible manner, or can cells acquire a partial, senescent phenotype. The existence of such a partial state may have important functional implications. It is in fact likely that not all cells expressing p16^{Ink4a} or p19^{Arf} are fully senescent.

Induction of p14^{ARF} in the epidermis for short periods revealed that cells respond rapidly to this senescence-inducing signal by activating the endogenous senescence machinery (i.e. p19^{ARF} and p16^{Ink4a}); however, re-silencing of p14^{ARF} prior to the completion of entry into senescence allowed cells to avert it (Tokarsky-Amiel et al. 2013). During the period in which the senescence program is activated but not yet completed, the process is thus still reversible. This could guarantee that only a persistent signal or damage will lead to this irreversible fate. Additionally, partially senescent cells, which may function differently from fullysenescent cells could exist in tissues. Such partially senescent cells in which an oncogene is active may find a way to evade senescence and continue to proliferate and give rise to a tumor. In *N-Ras*-driven liver carcinomas developing in immune deficient mice, despite the

reduced immune-mediated elimination of cancer cells, the numbers of p16^{Ink4a} expressing cells decreased as lesions progressed, a finding that was taken to suggest reversal of such cells to a proliferating state (Kang et al. 2011). The potential for the existence of such partial and/or reversible senescent states is highly interesting and requires further investigation.

Summary

Senescence has been studied for decades almost exclusively in the culture dish. The advances in studying this phenomenon *in vivo* have provided new and important viewpoints of the nature of this fundamental cellular program. Four potential ways to view the functionality of this program were discussed above: a route towards elimination, a non-functional state, a state of aberrant function, and a state of designated function. As made obvious, experimental data supporting all of these options, in different settings, exists. Novel active functions for senescent cell in various settings are continuously emerging, and no doubt that our understanding of this program will continue to evolve in the near future.

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2 Hypoxia and Tumor Dormancy: Can the Two Tango?

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Abstract

Majority of cancer patients die of metastatic disease that can develop decades after primary tumor removal. During this period the disseminated tumor cells (DTCs) may stop proliferating and survive in a dormant state, a phenomenon also referred to as minimal residual disease (MRD). MRD is a well-known clinical phenomenon and studying the mechanisms behind this stage of tumor progression specifically the contribution of the microenvironments are areas of active investigation. Hypoxic microenvironments, which result from a decrease in oxygen levels, are common during tumor progression, but its role in the induction and maintenance of the dormant state remains unclear. This chapter focuses on some of the experimental as well as theoretical evidence supporting how tumor hypoxia both in the primary tumor as well as at target organ sites can influence disseminated tumor cells (DTCs) to enter dormancy. Furthermore, the interplay between hypoxic and the unfolded protein response (UPR) signaling in promoting the survival of dormant tumor cells, which is critical for both long term survival as well as therapy resistance is also reviewed. Lastly, the chapter emphasizes on the parallels between the concept of tumor dormancy and cancer stem cells (CSCs) and the overlapping roles of hypoxia mediated signals in the maintenance of quiescence of CSCs as well as and

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dormant tumor cells. This information is essential to design urgently needed therapeutic strategies aimed at either maintaining these DTCs in a dormant state or eradicating them before they progress to overt metastasis.

Introduction

Metastatic disease rather than the primary tumor itself is one of the major causes of morbidity and mortality among cancer patients. Traditionally metastasis was thought to happen during later stages of cancer progression. However, accumulating evidence suggests that tumor cells disseminate from the primary lesion at early stages of tumor development even before the acquisition of full malignant properties (Aguirre-Ghiso 2007). Although disseminated tumor cells (DTCs) are highly prevalent among cancer patients (up to ~70% in prostate cancer patients), only a fraction of patients with DTCs will carry overt metastasis at the time of or shortly after primary tumor resection (Aguirre-Ghiso 2007). Others may remain free of clinical evidence of the disease for years or even decades after primary tumor removal. The appearance of tumor relapse after such a prolonged disease free period implies that these DTCs remain in the body in a dormant state.

Classically, tumor dormancy is described as the time it takes for the cancer to relapse after clinical remission. It refers to a protracted stage in which DTCs can remain undetected and asymptomatic for prolonged periods of time by entering either (1) growth arrested state (Cellular dormancy) or (2) exist as micrometastases (small masses of tumor cells), which results from a balance between proliferation and cell death due to the lack of angiogenesis (angiogenic dormancy). The definitions and mechanisms regarding cellular and angiogenic dormancy are discussed in detail in other excellent reviews ((Aguirre-Ghiso 2007) and references therein). Although we have made progress in our understanding of the underlying mechanisms responsible for tumor dormancy, there still remains some unanswered questions. The first question is in regards to whether the cells are already dormant at the time of dissemination. Another question is whether cues from the primary tumor microenvironment pre-program DTCs and micrometastases to enter dormancy. Alternatively, the stress of dissemination process, or the stress of seeding in a non-permissive target organ microenvironment itself can be growth suppressive favoring the induction of dormancy either at the solitary cell level or after some initial proliferation (Sosa et al. 2013). A third question is whether dormant tumor cells are equivalent/similar or distinct from cancer stem cells. This review will focus on role of the hypoxic niche and the activation of stress signals such as the unfolded protein response in determining the dormant phenotype. The role of hypoxia in dormant tumor cell survival following therapy will also be addressed. Discussions regarding the striking parallels between dormant tumor cells and cancer stem cells and their relevance to clinical tumor dormancy will be highlighted as well.

Influence of Tumor Hypoxia on Angiogenic Dormancy

In most normal mammalian tissues the oxygen tension ranges from 2 to 9% O_2. Tumor tissue hypoxia arises due to an imbalance in the supply and demand of oxygen. Both chronic or diffusion-limited hypoxia (≤ 0 0.05% O_2), which arises due to hyper-proliferation of cancer cells that outstrips its vascular supply and acute or perfusion limited hypoxia (0.05–2.3% O_2), which is due to structural and functional abnormalities in the tumor microvessels co-exist in tumors. Unlike normal healthy cells, tumor cells can adapt to such oxygen fluctuations by decreasing energy consumption and increase anaerobic metabolism. Hypoxia presents a “Janus face” in tumor biology. On one hand it promotes adaptive processes that leads to tumor aggressiveness, metastatic potential and enhanced resistance to conventional cancer therapies that adversely affect overall patient survival (Hockel and Vaupel 2001). But on the other hand, it is also associated with restrained proliferation and apoptosis. It has been shown to alter gene

expression that impairs neoplastic growth through molecular mechanisms that result in cellular quiescence, differentiation, apoptosis and necrosis (Hockel and Vaupel 2001).

Hypoxia via activation of hypoxia-inducible factor-1α (HIF-1α) stimulates the transcription of angiogenic mediators such as vascular endothelial growth factor (VEGF), growth factors like transforming growth factor-β, platelet derived growth factor, enzymes such as urokinase-type plasminogen activator, which are all involved in the invasion and expression of several factors shown to enhance resistance to therapy (Hockel and Vaupel 2001). In contrast, hypoxia can also induces apoptotic cell death by a number of HIF-1α dependent and independent pathways. These include the HIF-1α mediated increase in expression of pro-apoptotic proteins NIX and BNIP3 (Sowter et al. 2001), which mediate cell death through activation of both apoptotic and necrotic pathways. HIF-1α also promotes apoptosis via increased p53 levels involving Apaf-1 and caspase-9 as downstream effectors (Hockel and Vaupel 2001). These findings indicate that although most of the hypoxia- induced pathways promote tumor growth, apoptosis is also induced by hypoxia. Therefore the balance between these pathways is critical for hypoxia to effect tumor outcome- proliferation, death or dormancy.

Microscopic tumors (<2 mm diameter) often found during early stages of primary tumor development or as micrometastatic lesions, can remain dormant by their inability to expand beyond a microscopic size. Such microscopic tumors have been shown to be avascular and enter dormancy known as tumor mass or angiogenic dormancy due to the inability of the nascent tumor mass to recruit blood vessels. Characterized by a balance in proliferation and apoptosis, these microscopic tumors maintain a small but constant metabolic demand (Aguirre-Ghiso 2007). In comparing a small cohort of microscopic melanoma metastases and macroscopic melanoma metastases obtained from sentinel lymph nodes of patients, Barnhill et al. demonstrated that while the micrometastases demonstrated balanced rates of proliferation and apoptosis i.e. angiogenic dormancy, the macrometastases showed significantly higher rates of proliferation than apoptosis consistent with progressive growth (Barnhill et al. 1998).

But these pre-angiogenic micrometastases can emerge from dormancy if they acquire the ability to become vascularized either by down-regulating angiogenesis inhibitors or by upregulating proangiogenic factors (Aguirre-Ghiso 2007). While there is overwhelming evidence that hypoxia is one of the primary physiological regulators of this angiogenic switch (Bergers and Benjamin 2003), it remains unclear whether tumor hypoxia has a role in the angiogenic dormancy process. For instance, it is unknown whether these microscopic tumors albeit being avascular bear regions of hypoxia. Studies have shown that microscopic tumors that are <1 mm diameter were largely hypoxic (hypoxic fraction >90%) with little or no blood perfusion while those of a greater size (~1–4 mm diameter) were relatively well vascularized and well perfused and showed little to no hypoxia. Such microscopic tumors bearing a single or multiple hypoxic foci remained either avascular (Li and O'Donoghue 2008) or showed a vascular network characterized by low blood flow velocity, high vessel tortuosity (Simonsen et al. 2010). Li et al. observed that while the non-hypoxic rim of these microscopic tumors exhibited proliferation, the hypoxic core of these tumors were not, suggesting that dormant tumors may be hypoxic and that hypoxia may be causally related to low proliferation rates and thus tumor dormancy (Li and O'Donoghue 2008). Also is it possible that the hypoxic/anoxic core of these microtumors undergo apoptosis that balances the proliferation of the vascularized rim of these tumors to promote a balance in proliferation and apoptosis and thus angiogenic dormancy. For instance microtumors that overexpress BNIP3 instead of VEGF might undergo slower growth because the cells would undergo high levels of apoptosis and a small amount of proliferation (Sowter et al. 2001). Further studies are needed to analyze the expression pattern of hypoxic response genes in these microtumors to determine if the balance in the levels of apoptotic vs. proliferative signals would favor dormancy or metastatic outgrowth.

Hypoxia Regulation of Cellular Dormancy

Cellular dormancy occurs when DTCs enter a state of quiescence through a G_0/G_1 arrest. Experimentally, these dormant DTCs are characterized not only by the absence of proliferation but also by their ability to survive the interlude between entry to and exit from the dormant state. Studies using various tumor model systems highlight the important role of microenvironment/niche in governing cellular dormancy. These studies have unraveled niche signals such as loss of integrin and/or extra cellular matrix (ECM) function, reduced urokinase receptor expression as well as activation of stress signals as mediators of cellular dormancy. These signals not only promote cell-cycle arrest but also activate stress adaptive responses that promote survival of dormant tumor cells (Aguirre-Ghiso 2007).

It is interesting to note that one of the early responses to oxygen deficits in tumor tissue is to reduce the rate of oxygen consumption. It does so by decreasing proliferation of hypoxic cells, hence tumor cells in hypoxic areas are usually dormant, dividing far more slowly but remaining viable for prolonged periods (Gatenby et al. 1985). In fact the dividing fraction in tumors is low (5–20%) suggesting that large fractions are either dying or quiescent. Studies have shown that cells exposed to hypoxia have disproportionately long G1 phase or arrest in G0/G1 phase of cell cycle. Using imunocytochemical techniques, investigators have demonstrated that hypoxic tumor cells are in a non or slow proliferating state as a vast majority of these cells are negative for proliferation markers (e.g. PCNA, BrdU). The absence of proliferation in hypoxic cells has been attributed in part to the upregulation of cell cycle inhibitors such as p21 (WAF1) and KIP1 (p27) (Carmeliet et al. 1998). Similar studies from our laboratory have also demonstrated the lack of proliferation in hypoxic regions of tumors (Kumar et al. unpublished results).

Recently, using dormancy expression profiles from a variety of cancer types Kim et al. identified a dormancy gene signature. They found that this signature was significantly overrepresented in estrogen receptor positive breast cancer patients (HDS-high dormancy score) vs. estrogen receptor negative breast cancer patients that had a low dormancy score. Moreover they found that the estrogen receptor positive patients whose tumor had high dormancy score experienced longer metastasis-free periods. In contrast, when this score was underrepresented patients recurred with metastasis more frequently (Kim et al. 2012). This suggests that DTCs from Estrogen receptor positive tumors with a high dormancy score were more likely to enter a dormancy state. Several of the genes in the dormancy signature are regulators of quiescence and angiogenesis. Furthermore, several of the genes in the dormancy signature are regulated during hypoxia. Taken together, these data highlight the possibility that if the DTCs originated from within the hypoxic niche of the primary tumor then they may already be pre-programmed to be growth-arrested and enter a dormant state.

Another possibility is that the microenvironment in the target organs where the DTCs arrive can induce a dormant state. There is plenty of evidence for the role of hypoxic signaling in establishing the premetastatic niche, at distant sites to allow for engraftment of DTCs. However, whether hypoxia signaling also prepares the “metastatic soil” in distant organs such that the disseminated tumor cells that arrive can remain present in a prolonged state of dormancy is unclear. For instance, hypoxia sensitive metastasis suppressor genes such as MMK4, GPR56, KISS1 and CD82 (KAI1) suppress growth and expansion of DTCs at the target organ without affecting primary tumor growth suggesting a possible mechanisms for hypoxia in creating a dormancy favorable niche in the target organ (Aguirre-Ghiso 2007). Similarly, in several different types of cancers the bone marrow (BM) is a common homing organ for DTC. In most instances DTCs are detected in the BM at a much higher rate (15–70%) than the frequency at which metastasis (10–30%) occurs in the BM. Despite the hostile environmental conditions such as hypoxia, hypoglycemia etc. these DTCs can persist in the BM over many years with the potential

to recirculate into other organs. Pantel and his colleagues showed that a vast majority of these DTCs were in a quiescent state. Recently, they showed that these DTCs in the BM activate markers of the unfolded protein response (UPR) or the endoplasmic reticulum (ER) stress response as cellular adaptation strategy to survive the hypoxic microenvironment in the BM from where they originate (Bartkowiak et al. 2010). These findings suggest that the hypoxic milieu in the BM may not only prime the DTCs to enter a quiescent state but could also activate adaptive mechanisms such as the UPR to survive.

UPR Signaling Regulates the Growth Arrest and Survival Programs During Cellular Dormancy

Previous studies from the Aguirre-Ghiso laboratory discovered that HEp3 head and neck squamous carcinoma cells (HNSCC) that display a high ERK1/2 to p38α/β signaling ratio showed enhanced proliferation *in vivo*. They further demonstrated that a reversion of this ratio resulting a low ERK1/2 to p38α/β signaling ratio results in a spontaneous reprogramming of these cells into dormancy (D-HEp3 cells) *in vivo*. D-HEp3 cells inoculated *in vivo* enter a G_0-G_1 arrest characterized by the induction of p21, p27, p18 and p15 (Adam et al. 2009). Additionally activation of at least three transcription factors p53, BHLHB3/Sharp-1 and NR2F1 is required for the dormancy of these cells *in vivo* (Adam et al. 2009).

While exploring the mechanisms that drive the reprogramming of HEp3 cells into dormancy, the same group showed that these D-HEp3 cells develop an UPR that is required for the dormancy of these cells *in vivo*. ER stress has previously been shown to induce G_0/G_1 arrest and cell survival, the two components of dormancy. While all the three sensors of UPR—PERK, ATF6α and IRE1α are activated in these dormant cells; only PERK activation contributes towards the growth-arrest of D-HEp3 cells. This occurs by attenuating translation of G1-S transition regulators cyclin-D1, -D3 and CDK4 (Ranganathan et al. 2008). Moreover inducible activation of PERK signaling in tumorigenic HEp3 cells using a dimerizable Fv2E-PERK was sufficient to fully abrogate tumorigenicity and induce a "dormancy-like" state that results in a transient growth arrest and delayed tumor growth *in vivo* (Ranganathan et al. 2008).

In addition to activating these three arms of UPR, these dormant cells also induce the expression of chaperone BiP/Grp78, whose upregulation is linked to survival against several stresses that affect the ER homeostasis including chemotherapeutic drugs (Ranganathan et al. 2006). While the upregulation of BiP expression and PERK activation was not advantageous to basal survival of these dormant cells *in vivo*, they protected these cells from therapy induce cell death both *in vitro* as well as *in vivo* (Ranganathan et al. 2006) (and Ranganathan et al. unpublished results). Mechanism analysis revealed that this occurred via a BiP dependent inhibition of Bax activation (Ranganathan et al. 2006). On the other hand activation of both IRE-1α and ATF6α were required for the basal adaptation and survival *in vivo*. This occurred in part via the ATF6α mediated Rheb induction and a strong inhibition of mTOR signaling (Schewe and Aguirre-Ghiso 2008).

Another pathway that has emerged as an important regulator of tumor dormancy and is activated by microenvironmental stressors such as the UPR is autophagy, a process of "self-eating" of intracellular materials, to temporarily sustain energy production during starvation or stress conditions. Recent studies show that autophagy may be the survival mechanism of the dormant cells. Using models of ovarian cancer, investigators have shown that autophagy is critical for the survival of dormant tumor cells (Lu et al. 2008). Studies have also demonstrated that impaired integrin signaling, which can promote tumor dormancy also induces autophagy that protects cells from anoikis (detachment induced apoptosis) (Fung et al. 2008). Since such impairment in integrin signaling is possible in DTCs, not engaging efficiently in a foreign ECM, it has been speculated that this could stimulate autophagy and promote survival and maintenance of a dormant state.

In summary the studies presented above highlight an overlooked aspect of dormancy, the ability of these cells either from early primary lesions or from advanced tumors to survive in the long interlude between dissemination and metastatic relapse. Because chemotherapy targets mostly dividing cells, it is commonly assumed that the lack of proliferation of dormant cells could lead to chemotherapy treatment failure (Aguirre-Ghiso 2007). However, the above results suggest that dormant cells have survival mechanisms in place that are uncoupled from proliferation programs that selectively protect them from a hostile microenvironment or from stress imposed by cancer therapies.

One of the well-known inducers of ER stress is tumor hypoxia. Elegant studies from Koumenis' laboratory have demonstrated that PERK-eIF2α pathway is activated in response to hypoxic stress independent of HIFs (Koumenis et al. 2002). They showed that increased PERK signaling enhanced the tolerance to chronic hypoxic stress ($<0.02\%$ O_2) and promoted tumor growth *in vivo* that was dependent on eIF2α and ATF4 expression (Bi et al. 2005). In addition, studies from Wouters laboratory have shown that during tumor hypoxia PERK activation is also critical for the induction of the essential autophagy genes MAP1LC3B and ATG5. They further demonstrated that this induction was critical for the enhanced resistance of tumor cells to treatment such as radiation (Rouschop et al. 2010). In addition to PERK, BiP is also linked to resistance to various stresses that affect the ER homeostasis, serves as a survival factor and is upregulated in the hypoxic regions of several primary tumors (Kaufman 2002). A role for hypoxic signaling in promoting UPR mediated survival of dormant tumor cells was recently demonstrated in BM DTCs. Using cell lines derived from breast cancer BM DTCs as well as fresh BM samples from breast cancer patients it was demonstrated that similar to the dormant D-Hep3 cells, these DTCs upregulate UPR genes such as grp78, grp94, PDI but not their cytoplasmic homologues. The levels of these UPR markers were further induced upon exposure to hypoxia suggesting that these changes were an adaptive response to the hypoxic microenvironment of the BM (Bartkowiak et al. 2010). Moreover, these investigators also found that these UPR- positive DTCs also shared a $CD44^{high}/cd24^{low}$ cancer stem cell (CSC) phenotype, which was also upregulated upon exposure to hypoxia. These findings raise three important questions: (1) Do metastasis founding DTCs that survive long-term in the BM arise from quiescent CSCs, (2) how does hypoxia regulate CSC behavior and (3) can non-stem cells be converted towards a cancer stem cell phenotype? These questions are fundamentally important to our understanding of tumor dormancy. In fact there is striking parallels between dormant tumor cells and CSCs, which can also undergo periods of dormancy and preferentially resist therapy and survive in hostile microenvironments.

Influence of Hypoxia on Cancer Stem Cell Maintenance and Plasticity

Just as pluripotent stem cells are critical for the maintenance of regenerative tissues in the body, increasing evidence has accumulated to suggest that cancer stem cells (CSCs) also known as tumor initiating cells (TICs) also exist within a heterogeneous tumors and are important for initiation and maintenance of tumor progression. The term CSC refers to the functional trait of the cancer cell rather than its cellular origin and biological properties within normal tissues. The existence of these CSCs was first demonstrated in human acute myeloid leukemia (AML) by pioneering work from John Dick and his colleagues. Since then using specific surface markers such CSC sub-populations have been identified in a number of solid tumor types including those of the breast, bladder, central nervous system, colon, head and neck, ovaries, pancreas and the skin (Lobo et al. 2007).

Classically cellular differentiation of normal stem cells has been perceived to follow a unidirectional, hierarchical and irreversible progression. It was therefore hypothesized that tumor growth follows a hierarchical cancer "stem" cell (CSC) model. This suggests that only a specific

subpopulation of cancer cells have "unlimited" tumor initiating/CSC properties and hence the ability to sustain cancer growth while the rest of the tumor cells have limited or no growth potential (Lobo et al. 2007). Since tumor cells have greater plasticity it is possible that they can change their phenotypes depending on microenvironmental cues. Reports have suggested a more dynamic regulation of CSC phenotype and function allowing for a bidirectional interconversion between the CSCs and non-CSCs in response to signals from the microenvironment (Chaffer et al. 2011). This implies that non–stem cell subpopulations could dedifferentiate into stem cells—leading to reversibility.

How then are these different behaviors of CSCs regulated/controlled? Similar to normal stem cells CSC behavior is critically dependent on contextual and configurational cues from the surrounding niche environment. Several reports have shown that the presence of low oxygen tension (hypoxia) in stem cell niches offers a selective advantage to maintain a more stem-like and undifferentiated phenotype. While the reasons why a hypoxic niche favors the maintenance of stem cells is largely unknown it is postulated that a low oxygen environment reduces the extent of ROS induced DNA damage during radiation or chemotherapy (Mohyeldin et al. 2010).

Recent reports have demonstrated that HIFs through signaling pathways such as Notch and Oct4 are crucial regulators of the stem cell phenotypes (Mohyeldin et al. 2010). Consistent with these findings hematopoietic stem cells have been shown to reside in hypoxic niches and rely on HIFs to maintain an undifferentiated phenotype. Similarly, presence of hypoxic areas within a growing tumor suggests possible niches for CSCs. Recently, excellent studies from different laboratories have shown that the fraction of cells positive for CSC markers or the side population in established cancer cell lines or cultures from human tumors increases following exposure to low oxygen conditions (Li and Rich 2010). While hypoxia clearly promoted the undifferentiated state in CSC population, the molecular mechanisms underlying these observations remained obscure until recently.

Studies have shown that prolonged exposure of several cancer cell lines as well as glioma non-stem cells to hypoxia resulted in the induction of genes essential for stem cell function such as Oct4, Nanog and c-myc and augmented the tumorigenic potential of non-stem cells (Li and Rich 2010). Recently, it was identified that both HIF-1α and HIF-2α are critical for CSC maintenance. However, HIF-2α is selectively induced only in CSC and not normal stem cell populations and is critical not only for the maintenance of CSCs in an undifferentiated state but also for their plasticity and correlated with poor patient outcome (Li and Rich 2010). These findings establish a clear link between hypoxia, HIFs and molecules that are crucial for the regulation of CSC behaviors.

Additional evidence for the role of hypoxia in the dynamic behavior of CSCs was provided by findings from Roesch et al. who identified that histone demethylases JARID1B is essential for the maintenance of a small subpopulation of slow cycling melanoma cells and is essential to both initiate and sustain melanoma growth. They further demonstrated that JARID1B$^-$ cells could however switch to JARID1B$^+$ cells and support tumor growth. Also these studies further emphasized the significance of low oxygen environment for the dynamic regulation of CSC phenotype as JARID1B expression in JARID1B$^-$ melanoma cells was rapidly enhanced at low oxygen and steadily reverted to normal expression intensity (Roesch et al. 2010). These landmark studies suggest that hypoxia could potentially serve as the trigger that allows the reprogramming of non-CSC to CSC.

Recently, using experimental HEp3 model of HNSCC, Bragado and colleagues also demonstrated the existence of such marker enriched CSC subpopulation of G0-G1 arrested slow cycling, ALDHA1^{high}/α6 integrin^{high} expressing tumor cells (Bragado et al. 2012). They found that this sorted ALDHA1^{high}/α6^{high} subpopulation had immediate and enhanced tumorigenic potential *in vivo*. In agreement with the above findings they also demonstrated that with time even the non-CSC subpopulation regained tumorigenic capacity *in vivo*, which was linked to the

restoration of ALDHA1^{high}/α6^{high} cells. In addition, they also found that the tumorigenic potential was functionally dependent on α_6 expression.

They further demonstrated that α_6^{high} CSCs were associated with low levels of the repressive histone modification H3K27me3 and H3K9me3. In contrast, the α6^{low} cells were associated with high levels of these repressive marks. This suggests that the CSCs maybe held in a transcriptionally ready state, while the non-CSCs are primarily in a transcriptionally repressed state demonstrating a strong correlation between epigenetic mechanisms and the dynamic states that dictate tumorigenic capacity.

Although in these studies the ALDHA1^{low}/α6^{low} were able to form tumors they had a longer latency period *in vivo* (15–20 days) before they regenerated ALDHA1^{high}/α6^{high} expressing tumors. While these studies did not examine the role of hypoxia in this dynamic regulation, it is possible that during this latency period *in vivo*, these cells could have experienced periods of hypoxia that may have resulted in the induction of markers such as α_6 essential for its "stem-like" potential. To this end we examined whether tumor hypoxia can serve as a micro-environmental "trigger" that, allows for the reprogramming of α_6^{low} cells to α_6^{high} cells and recover tumorigenic capacity. Our studies found that *in vitro* exposure of α_6^{low} non-CSCs from these HEp3 tumors to low oxygen environment (0.1–1% O_2) resulted in a significant increase in the α_6 mRNA and protein levels, which was comparable to that seen in the α_6^{high} cells and is sustained as long as the α_6^{low} cells are maintained in hypoxia. In correlation with these findings, we found that a vast majority of α_6^{high} cells resided within or near the vicinity of hypoxic regions (Kumar et al. unpublished observations). These results imply that tumor hypoxia may serve as a haven for CSCs. This hypoxia induced shift in α_6^{low} subpopulation to a α_6^{high} expressing cells could represent a true phenotypic switch to an enhanced tumorigenic program rather than a mere stress adaptation mechanism.

Alternatively it is plausible that a reversion in the chromatin states of the non-CSCs to a more permissive state for transcription may require the erasing and/or writing of new histone 3 post-translational modifications, which could explain why longer periods are required for non-CSCs to fully restore their tumorigenic potential. Such epigenetic mechanisms can also be regulated by a hypoxic niche as recent studies have found that a majority of histone demethylases including JMJD2B/KDM4B and JMJD2C, which are H3K9me3 demethylases are upregulated in response to hypoxia, which can have profound implications in cancer biology (Yang et al. 2009).

All of the data discussed above show that as previously postulated cancer stem cells may not strictly adhere to the traditional hierarchical model, where the stem cell competence is only restricted to a discrete subset of tumor cells as characterized by the expression of certain markers. But rather they suggest that the different subpopulations within a tumor are in a highly dynamic state with respect to CSC phenotype and functions and can undergo bidirectional interconversion between CSC and non-CSC states while still maintaining a hierarchy with the CSC at the top of the hierarchy and the non-CSC are the "differentiated" progeny. While the transition between the two state is still under investigation the data discussed above point to a crucial role of tumor hypoxia in regulating this phenotypic heterogeneity within tumors.

Hypoxic Signaling in the Regulation of CSC Quiescence and Tumor Dormancy

Alongside self-renewal and mulitpotency, stem cell potential is frequently associated with dormancy/quiescence, which can also be applied to CSCs. Since CSCs by definition are known for their ability to initiate robust tumor growth, and are competent to differentiate into various non-self renewing tumor bulk populations, it is quite paradoxical to associate them with dormancy. However, there are striking parallels between CSC quiescence and tumor dormancy. For instance, dormant tumor cells and CSC alike have the ability to survive non-permissive microenvironments that are incapable of nurturing tumor growth and survive cancer therapy (Aguirre-Ghiso 2007;

Schillert et al. 2013). Recent evidence indicates that CSC population itself comprises of heterogeneous subpopulations including a dormant, slow-cycling fraction. Using fluorescent markers to specifically label quiescent cells (label retaining cells, LRCs), elegant studies from different groups provide strong evidence for the existence of such slow cycling, dormant cells as a subpopulation of CSCs in tumors of the breast, colon, pancreas, ovary, head and neck and skin that contributed towards CSC mediated tumor progression ((Schillert et al. 2013) and references there in). Additionally this slow cycling/quiescent population were more resistant to chemotherapy and retained the capacity to proliferate after withdrawal of chemotherapy (Schillert et al. 2013). This is analogous to the reversible quiescence observed in dormant tumor populations following therapy. Unlike dormant tumor cells that can remain quiescent for prolonged periods, it is unclear if CSCs undergo long phases of quiescence. While in the hematopoietic stem cells (HSCs) such long-term quiescent stem cells are present, such populations have not been described yet in solid tumor CSCs (Mohyeldin et al. 2010). Pantel and colleagues have demonstrated that DTCs from BM of cancer patients are quiescent and can persist in the BM for several years with the potential to give rise to late recurrences. This coupled with the findings that these DTCs express putative CSC markers: $CD44^{high}/CD24^{low}$, suggest that DTCs may arise from CSCs at least in breast cancer patients that similar to HSCs can remain quiescent for prolonged periods (Bartkowiak et al. 2010). Taken together these findings suggest that these slow-cycling CSC compartment may contribute towards the latent disease and delayed recurrences caused by the dormant tumor cells.

It is clear from the preceding discussion that a hypoxic microenvironment clearly promotes the induction and maintenance of tumor dormancy and that it is also critical for maintaining the CSCs self-renewal capacity and plasticity. But whether hypoxia also contributes towards the quiescence of CSC subpopulations and if these same mechanisms also operate to maintain the slow cycling or quiescent state of dormant tumor cells needs to be investigated. Several investigators have demonstrated that slow-cycling HSCs are more likely to localize in the low oxygen areas of the BM away from the blood vessels whereas the fast cycling hematopoietic progenitors with limited self renewal capacity reside in areas much closer to the vasculature (Mohyeldin et al. 2010). In contrast CSCs have been proposed to reside in the perivascular niche, in an intimate relationship with the tumor vasculature (Mohyeldin et al. 2010). But in recent studies by Li et al. CSCs have also been proposed to exist in a secondary niche within cancers that is far away from the vasculature and as a consequence more hypoxic (Li et al. 2009). CSCs found in these hypoxic niches were maintained in an undifferentiated, quiescent state in several solid tumors including neuroblastoma, breast and cervical cancers (Axelson et al. 2005). These studies suggest that hypoxic/angiostatic niche environments could be conducive for the maintenance of CSC dormancy. For instance hypoxic induction of p21, which leads to cell cycle arrest in dormant tumor cells is also activated in quiescent CSCs (Carmeliet et al. 1998). In mouse models of cancer Felsher and his colleagues demonstrated that MYC inactivation induced tumor regression that required the enhanced expression of antiangiogenic protein Tsp-1. In addition, MYC inactivation also uncovered stem-like properties that induced differentiation but some cells remained in a dormant state and turned cancerous upon reactivation of the oncogene (Bellovin et al. 2013). Furthermore, independent studies have shown that hypoxic stabilization of HIF-1 can cause cell-cycle arrest via inhibition of myc expression and can also induce Tsp-1 expression (Keith et al. 2012). These findings demonstrate that there is a direct link between tumor dormancy and CSC quiescence and suggest a possible role for the angiostatic/hypoxic niche in regulating this process. Similarly, NDRG1 (N-myc downstream regulated gene-1) a metastasis suppressor gene, and a target of hypoxia, has also been implicated in the quiescence of CSCs in prostate cancer as well as tumor dormancy (Kobayashi et al. 2011). An additional link between hypoxic niche environments and CSC

and dormant tumor subsets, is their use of mTOR signaling to promote survival and growth inhibition. Activation of mTOR signaling is essential for the survival of dormant tumor cells as well as for CSC quiescence (Gaur et al. 2011; Schewe and Aguirre-Ghiso 2008). Taken together these findings highlight the critical importance of a hypoxic microenvironment in regulating the quiescence of CSC and dormant tumor populations.

Conclusion

Our knowledge on the mechanisms of tumor dormancy, which makes cancer cells refractory to conventional therapies, is limited. Even less understood is the role of the microenvironment in regulating the dormant phenotype. This chapter gives an overview of the role of tumor hypoxia in regulating the dormancy program during the initial stages of tumorigenesis as well as in micrometastatic disease and in DTCs. The genomic state of the tumor cells, microenvironmental epigenetic factors and the degree of hypoxia are all factors that determine whether the net phenotypic result of hypoxia induced changes in gene expression would result in tumor dormancy. One can hypothesize that in the early stages of primary tumor progression when the tumor cells have not acquired genetic alterations that lead to immortalization such as loss of tumor suppressors such as TP53, RB1 (retinoblastoma 1) etc., hypoxia induced growth arrest programs may favor tumor dormancy. Since dissemination occurs early in tumor progression microenvironment restrictions such as hypoxia or stress signals resulting from hypoxia could influence the genetic progression of DTCs and slow down the metastatic progression by keeping the cells in a growth arrested state. Alternatively, even in genetically progressed DTCs that carry oncogene activation, signals from the hypoxic niche of the primary tumor could induce dormancy gene signature and activate survival mechanisms in DTCs that can overcome the pro-growth oncogenic signals and allow them to survive in a dormant state. Traditionally, tumor hypoxia has been considered a therapeutic hindrance as it renders solid tumors more resistant to therapy. The findings that dormant tumor cells as well as DTCs can tap into hypoxia induced UPR survival signals to survive and persist for prolonged periods holds potential for developing therapies that target specific components of the UPR machinery to eradicate dormant DTCs.

There are interesting parallels between tumor dormancy and the role of CSCs in tumor propagation such as the ability to engage several survival-promoting mechanisms to resist therapy. Furthermore the commonalities in the molecular pathways that are integral to CSC biology and maintenance of tumor dormancy, point to a partial overlap between these two populations and that the hypoxic niche could serve as a direct link between these two populations. The studies discussed in this chapter highlight the important role of hypoxia and HIFs in the maintenance, plasticity and quiescence of CSCs. Therefore, disruption of the hypoxic niche while being detrimental to the maintenance of CSC properties, could serve as a critical therapeutic target to kill tumor cells. These studies also underscore the fact that the standard culture conditions using ambient air may fail to maintain the heterogeneity present within the tumors and may not represent *in vivo* situation. Hence understanding the importance of hypoxia and the role of factors such as HIF2α in the maintenance of CSCs and facilitating ways to destroy the niche are important from both a basic science standpoint as well as for drug development. For instance, improving oxygenation through "vessel normalization" via repair of tumor vessel abnormalities might be able to disrupt CSC phenotype by normalizing the CSC microenvironment. Furthermore disrupting endothelial cell homeostasis in the CSC niche could also reduce tumorigenesis by depleting the CSC pool as these niche endothelial cells release trophic 'angiocrine factors' that can activate dormant CSCs (De Bock et al. 2011).

The existence of dormant/slow-cycling cell sub-population in CSCs suggest that these cells may survive treatment but still retain the ability to proliferate in response to a yet to be identified stimuli and cause disease recurrences. Accordingly it has been postulated that low dose metronomic chemotherapy can in fact induce tumor dormancy in cancer cells. In agreement with this

Martin-Padura et al. demonstrated that following low dose antiangiogenic metronomic therapy of hepatocellular carcinoma, the residual population identified in the liver parenchyma were CSCs that remained dormant after treatment and is responsible for the tumor regrowth (Martin-Padura et al. 2012). Since such anti-angiogenic strategies could induce tumor hypoxia, one can speculate that the emergence of a dormant CSC subpopulation could be a result of hypoxia-induced shift in the tumor cell phenotype. The existence of such plastic states could pose both obstacles and opportunities. For instance, activating dormant tumor cells prior to treatment could sensitize them to conventional therapy and favor the eradication of minimal residual disease. An alternative strategy would be to keep these cells long-term in a dormant state, which could prevent relapses and potentially convert cancer into an asymptomatic-chronic condition.

Therefore, the successful eradication of cancer requires a better understanding of the mechanisms that regulate the onset of dormancy and dormant cell drug-resistance. Given the significant overlap between CSCs and dormant tumor fractions and the crucial role of hypoxic niche in governing the CSC fate and dormancy maintenance, unraveling the molecular complexities that dictate dormant state will lead to a better understanding of tumor progression in cancer patients with important implications for improved diagnosis and treatment.

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Progeroid Syndromes: Role of Accelerated Fibroblast Senescence and p38 Activation

3

Terence Davis

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Abstract

Human ageing studies are problematic due to their complex nature so genetic progeroid syndromes that manifest a subset of ageing phenotypes are used as proxies to dissect out specific ageing processes. Many such processes are believed to involve cellular senescence, as senescent cells gradually build-up during life. Two forms of cellular senescence exist; replicative due to telomere dysfunction and stress-induced via activation of p38 MAP kinase. As progeroid syndromes show premature ageing, they are useful for cell ageing studies that may provide support for the linkage between cellular senescence and ageing. For several progeroid syndromes, notably Werner, ATR-Seckel, Hutchinson-Gilford, Ataxia-Telangiectasia, Nijmegen Breakage and Dyskeratosis congenita, there is clear evidence that fibroblasts undergo rapid or premature ageing. For other syndromes such as Cockayne, Rothmund-Thomson and Bloom there is no such clear evidence. In addition, no clear relationship between the severity of ageing features and premature fibroblast senescence is seen. However, as some of these syndromes result in early death from non-age related causes, it may be that insufficient lifespan is available for significant premature ageing to occur. For example in Nijmegen Breakage syndrome, fibroblasts age rapidly but the progeroid features are slight, possibly due to early death from

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cancer. In addition, it may be that premature cell senescence occurs in cell types other than fibroblasts. The accelerated cell ageing that does occur results from accelerated telomere dysfunction in some syndromes, activation of p38 in others, or a mix of both mechanisms. In this chapter, I provide a summation of the evidence for accelerated cellular senescence in progeroid syndromes and attempt to relate this to the severity and tissue specificity of the progeroid phenotypes. Finally, I discuss a model that may underlie the accelerated ageing in a subset of these syndromes that may be relevant to normal ageing processes.

Introduction

Human ageing is a process of the gradual build-up of deleterious changes that eventually impinge sufficiently upon normal tissue function to cause failure of that tissue leading to death. An example of this process is the gradual weakening of cardiac function prior to heart failure. As the impairment of normal biological function can result from both the processes of ageing and disease, it would be unsurprising if the basic ageing process itself eventually lead to the onset of those diseases specifically associated with ageing (Burton 2009). Such diseases include, amongst others, type II diabetes, cardiovascular disease (atherosclerosis), osteoporosis, dementia, sarcopenia (muscle wasting) and cancer. If this premise is correct, then an understanding of the basic mechanisms underlying the ageing process should lead to advances in the pathophysiology of much age-related illnesses. This, in turn, should lead to novel therapeutic interventions that are aimed at the basic ageing process itself leading to the amelioration, or even prevention, of age-related disease leading to an improved quality of later life.

To date, basic ageing processes have been considered as a scientific speciality in their own right (biogerontology) that is quite distinct from the process of disease progression in the elderly that has often been the province of the various medical specialities such as geriatrics. The reasons for this are complex, but may be related to the idea that disease can be treated, but ageing cannot (Bagley et al. 2011). However, it has been increasingly recognised that a significant overlap exits between the fields of biogerontology and disease processes.

Much remains to be discovered regarding the pathophysiology of human ageing (Puzianowska-Kuznicka and Kuznicki 2005), a complex process involving genetic and environmental factors affecting several physiological pathways. Ageing is associated with loss of function of many cells, tissues and organs of the body. Since every tissue and organ is made up of many billions or even trillions of cells, it is necessary to understand how cells age and what contribution old cells make to the tissue in which they lie in order to discover the impact of cell ageing on frailty and disability. As mitotic human tissues consist of cells that have the ability to divide when stimulated, one mechanism commonly postulated as underlying human ageing is cellular senescence, or the observation that many normal human somatic cells are capable of only a finite number of divisions (Burton 2009). How often these cells proliferate is dependent upon how frequently cells become damaged and lost, the so-called 'wear and tear' hypothesis of ageing. As lost cells need to be replaced the remaining stock needs to enter division, eventually leading to a senescent phenotype. Although until recently controversial, it is becoming increasingly accepted that cell ageing (also called *replicative senescence*) is a major player in organismal ageing.

Practical difficulties underlie human ageing studies, most importantly the biological complexity and polygenic nature of the ageing pathologies. An alternative to the study of whole body ageing in normal humans is the study of progeroid syndromes (PSs) whose phenotypes show specific characteristics of ageing (Puzianowska-Kuznicka and Kuznicki 2005). These hereditary disorders are often monogenic and affect only a subset of normal ageing phenotypes, i.e. they are segmental, with different tissues showing accelerated ageing in different PSs. However, the process and pathology of the premature ageing that occurs appears remarkably similar to that seen in

normally aged individuals (Hofer et al. 2005). Thus if these syndromes are reflective of normal ageing processes their study allows distinct ageing pathways to be dissected out from the whole body ageing background leading to the identification of causative genes underlying ageing processes.

Cellular Senescence

Mechanisms of Cellular Senescence

Human mitotically competent cells (e.g. fibroblasts, endothelial cells, lymphocytes) have a limited division capability after which they enter a non-dividing state termed senescence, or mortality stage 1 (M1). Cellular senescence is not death, but a stress response resulting in permanent withdrawal from the cell cycle, with the cells capable of long-term survival. In this regard, senescence resembles terminal differentiation as the cells have distinct morphological and functional changes that impair cellular homeostasis (Kipling et al. 2004). For example, senescent fibroblasts adopt an enlarged and granular morphology with the presence of large arrays of F-actin stress fibres and show high expression of senescence-associated β-galactosidase activity (SAβ-gal). In addition they have an increased expression of inflammatory cytokines such as IL-1α and inflammatory molecules such as ICAM-1, which has been termed the senescence associated secretory phenotype or SASP (Freund et al. 2011).

Cellular senescence is categorised into two distinct types, replicative (or intrinsic) and stress-induced, although this is mostly a definition dependent upon the *mode* of senescence as the resulting cells are remarkably similar in phenotype. Replicative senescence in fibroblasts is largely the consequence of progressive telomere shortening that results from the mechanism of DNA replication being unable to synthesise the 5′ ends of linear chromosomes – the so-called "end-replication problem." Telomeres are capped by a protein complex (the shelterin complex) whose function is to protect the DNA terminus that would otherwise be recognised as a double strand DNA break (DSB) and activate the DNA repair pathway. As fibroblasts divide their telomeres shorten until a critical length is reached, whereupon they lose the shelterin capping function exposing the DSB. This results in a DNA damage response (DDR) that activates the p53/p21^{WAF1} cell cycle arrest pathway inducing senescence (d'Adda di Fagagna et al. 2003).

In addition to replicative senescence, human fibroblasts can undergo stress-induced premature senescence (SIPS) via activation of the MAP kinase p38 (and possibly other mechanisms) that responds to endogenous and exogenous cellular stress (Freund et al. 2011). The p38 MAP kinase is involved in growth arrest in response to the expression of oncogenes such as *ras*, exogenous stress such as arsenite treatment or oxidative stress, ongoing DDR, difficulties in completion of DNA replication (so-called replication stress), and also in telomere-dependent senescence: indeed, p38 defines a common senescence signalling pathway linking both replicative senescence and SIPS (Iwasa et al. 2003). The p38 MAP kinase pathway is important for cell growth arrest and senescence due to its ability to activate both the p53/p21^{WAF1} and the pRb/p16^{INK4A} cell cycle arrest pathways (Fig. 3.1). Activation of p38 leads to the expression of inflammatory molecules that is typical of senescent cells via activation of the kinase MK2 and phosphorylation of the mRNA binding protein TTP, and also the altered morphology of senescent cells via phosphorylation of the small heat shock protein HSP27 and the subsequent formation of F-actin stress fibres. Thus p38 plays a role in both inducing cell senescence and the SASP.

Role of Cellular Senescence in Human Ageing

Many human tissues require extensive and continuous cell turnover throughout life to maintain homeostasis (e.g., small intestine, immune system, skin), and cell division is central to normal function or repair. Tissue function not only requires cells and cell division, but also the

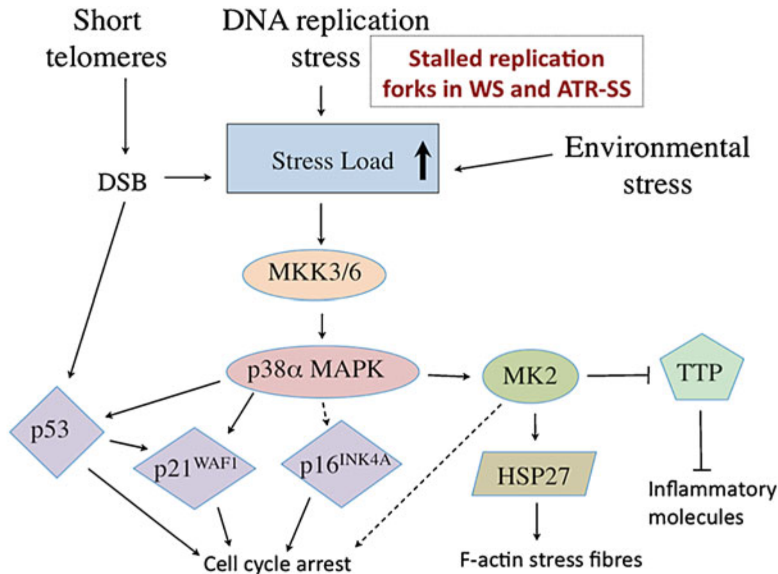

Fig. 3.1 The p38 mitogen-activated protein kinase pathway. This pathway is activated by endogenous and exogenous stress signals including oxidative stress, DNA damage (including short or dysfunctional telomeres), expression of cancer-inducing proteins (oncoproteins), various environmental stresses and possibly psychosocial stress. The actual mechanism whereby the stress is detected as a stress load is currently unknown, but stress results in the activation of MKK3 or MKK6 that activate p38. This leads to inhibition of the cell cycle via activation of the inhibitor proteins $p21^{WAF1}$ and/or $p16^{INK4A}$, or by activation of p53, leading eventually to stress-induced cellular senescence (SIPS). Additionally, p38 activates MK2 that controls the inflammatory response that typifies senescent cells. In both WS and ATR-SS fibroblasts the stress signal is thought to be due to a decreased ability to restart stalled DNA replication forks

extracellular strata (or matrix) that maintains tissue integrity. When division competent cells reach senescence this will impinge upon tissue integrity in two basic ways: failure to replace cell loss, and the deleterious biochemical features displayed by senescent cells (Kipling et al. 2004). For example, senescent cells secrete inflammatory cytokines such as IL-1α and tumour necrosis factor (TNFα), and express cell surface molecules such as ICAM-1 that are involved in the recruitment of leukocytes during inflammation (Davis and Kipling 2006). In addition, senescent cells up-regulate the expression of matrix degradative enzymes suggesting that, instead of maintaining matrix integrity, they actively degrade it. Moreover, senescent fibroblasts are known to aid the malignant progression of pre-malignant keratinocytes and breast epithelial cells; thus, although cellular senescence has been suggested as a possible tumour suppressive mechanism, the presence of senescent stromal fibroblasts may be particularly adept at creating a tissue environment that can promote the development of age-related epithelial cancers. By these mechanisms, cellular senescence may contribute to age-related degenerations in division competent tissues and/or the genesis of certain age-related pathologies (Kipling et al. 2004).

Evidence for replicative senescence *in vivo* has been difficult to acquire leading to criticism of this postulate. However, as most human tissues lack detectable telomerase activity the cells in these tissues show progressive telomere shortening with age *in vivo*. Even in tissues with detectable telomerase activity, such as immune cells, this activity is often insufficient to maintain telomere length. Shortened telomeres are correlated with the progression of age-related diseases including immunosenescence, cardiovascular disease, sarcopenia, osteoporosis, osteoarthritis and skin ageing. In addition, there is an association between telomere length and mortality in

people aged 60 years or older [reviewed in (Davis et al. 2009)]. These data provide circumstantial support for a continual build-up of replicatively senescent cells *in vivo* through telomere shortening.

As well as replicative senescence, SIPS may occur in human tissues that are exposed to chronic stress. For example, cells of the vascular system are chronically exposed to a variety of oxidative burdens that would induce apoptosis or premature senescence (i.e. telomere-independent senescence). In addition, endothelium cells in areas of vascular transitions (e.g., blood vessel bifurcations) are under intense haemodynamic stress resulting in low-level (but chronic) injury again leading to premature senescence and/or cell loss through apoptosis. Several lines of evidence indicate that a build up of senescent cells does indeed occur in atherosclerosis-prone areas of the vasculature that may be due to SIPS (Erusalimsky and Kurz 2005). This SIPS then leads to increased proliferation of surrounding cells leading ultimately to their replicative senescence. Similarly, human skin is under mechanical stress and suffers chronic injury due to abrasion that leads to cell loss and/or SIPS. The resulting wound healing process to replace these lost cells may lead to significant cell turnover and ultimately cellular senescence and skin ageing (Wall et al. 2008).

Cellular senescence may have relevance in mental health, incidence of which increases with age, with depression being likened to a state of accelerated ageing (Wolkowitz et al. 2010). Depressed individuals have high plasma levels of inflammatory markers such as TNFα, suggesting elevated inflammation and stress signalling that could lead to enhanced cellular turnover and SIPS. This SIPS may then underlie the increased rates of type II diabetes, osteoporosis, and cardiovascular disease often seen in depressed individuals. However, although psychosocial stress has been proposed to prematurely age cells this has not been formally demonstrated (Wolkowitz et al. 2010).

Overall, data exist suggesting that several ageing phenotypes may be related to the build-up of cells throughout life that have undergone replicative and/or stress-induced senescence. That senescent cells may contribute to the ageing process in mammals has been shown in mice by eliminating such cells, or preventing their build-up: this slowed or even reversed the acquisition of age-related pathologies in several tissues, including fatty tissue, skeletal muscle, spleen, intestine, the nervous system and the eye (Jaskelioff et al. 2011). Thus, although it may not yet be formally proven, there is strong evidence supporting a link between the build-up of senescent cells and biological ageing processes.

Progeroid Syndromes

Phenotypic Characteristics of Progeroid Syndromes

One of the more intensively studied progeroid syndromes (PSs) is Werner syndrome (WS) due to null mutations in the *WRN* gene encoding the DNA helicase RECQ3 (WRN). Werner syndrome is often first suspected during the teenage years as affected individuals lack the pubertal growth spurt resulting in shortness in height. Major characteristics are bilateral juvenile cataracts, skin atrophy and sclerosis, hair-greying, thymic atrophy and soft tissue calcification, together with age-related diseases such as type II diabetes, atherosclerosis and osteoporosis, all diseases that are inflammatory in nature (Davis et al. 2009). Premature ageing is segmental as no nervous system pathology is noted and there is no obvious immune system dysfunction, i.e. premature ageing affects some tissues but not others. An elevated incidence of cancer is observed, however, not all cancer types are affected, and there is an emphasis on rare non-epithelial cancers such as mesenchymal and soft-tissue sarcomas. Whether this is due to an active mechanism to promote these cancer types, or a suppression of epithelial cancers, is unclear. WS individuals die with a median age of 54 mainly due to cardiovascular disease or cancer.

A second widely studied PS is the extremely rare Hutchinson-Gilford progeria (HGPS) due to splice defects in the *LMNA* gene encoding the nuclear protein lamin A/C. Hutchinson-Gilford

individuals appear normal at birth, but within a year display the effects of premature ageing (Davis et al. 2009). Initial symptoms include severely reduced growth rate and HGPS individuals are short and below average weight. As the condition progresses individuals develop alopecia, skin of wrinkled and aged appearance reminiscent of scleroderma, and inflammatory conditions such as arteriosclerosis, osteoporosis, and atherosclerosis. These individuals show very rapid ageing and appear many decades older than they actually are, having a similar respiratory, cardiovascular and arthritic condition to a senior citizen. Average age at death is 13, with 90% of individuals dying of heart failure and cerebrovascular accident (strokes).

Cockayne syndrome (CS) is of two varieties, CSA and CSB, due to mutations in the proteins ERCC8 and ERCC6 that are involved in DNA repair pathways (Kraemer et al. 2007). However, CS individuals of both types are phenotypically very similar and have characteristic aged facial features, thin hair, cachexia, thin dry skin, retinal degeneration, hearing loss, neurodegeneration (cerebellar ataxia), and cataracts. Their clinical course typifies premature ageing and usually results in early death, with CSA individuals dying in the second or third decade and CSB individuals usually in the first decade of life.

Rothmund-Thomson (RTS) individuals show moderate ageing characteristics, although lifespan is not shortened in the absence of cancer (Davis et al. 2013). Features include poikiloderma, alopecia, grey hair, and short stature, with juvenile cataracts reported in some individuals, and an elevated cancer incidence in others. The syndrome is highly pleiotropic with not all individuals showing all symptoms, probably due to RTS being of two types (I and II) resulting from mutations in (at least) two separate genes. The observed pleiotropy may be due to insufficient historic phenotypic characterisation of the two types as the aetiology is known only recently and only for Type II (due to mutations in the gene encoding the helicase RECQL4), although juvenile cataracts appear to be specific for Type I and osteosarcoma to Type II, whereas poikiloderma is common to both RTS types.

Individuals with ataxia-telangiectasia (AT) show moderate features reminiscent of premature ageing, such as grey hair, wrinkled skin, skin atrophy and sclerosis (scleroderma), and show a reduced lifespan with death usually occurring in the third and fourth decades (Davis et al. 2009). However, AT individuals do not show such inflammatory features as atherosclerosis. Death is usually from recurrent respiratory infection in adolescence or early childhood. It is caused by null mutations of the gene encoding the checkpoint kinase ataxia-telangiectasia, mutated (ATM).

The progeroid features described for Nijmegen Breakage syndrome (NBS), such as sparse grey hair and distinctive 'bird-like' facies, do increase with age, but are relatively mild and there are few inflammatory features (Davis et al. 2009). However, the data on NBS are potentially confounded by many individuals dying at a young age as a result of cancer, so premature ageing has little time to manifest itself. In addition, NBS is complicated by its resemblance to AT, and to other human syndromes, leading to possible misdiagnosis. Those NBS individuals with a known aetiology have hypomorphic mutations in the gene encoding nibrin (*NBN*; sometimes referred to as *NBS1*).

Seckel syndrome (SS) results from mutations in a least six genes that impinge upon centrosome function and affect the activation of the ataxia-telangiectasia and Rad3-related (ATR) checkpoint kinase, with classical SS having hypomorphic mutations in ATR itself (referred to here as ATR-SS, with the term SS referring to non-ATR SS). Seckel syndrome individuals show moderate ageing with few inflammatory features, although accelerated ageing is clearly present in the ATR-SS mouse model (Tivey et al. 2013b). Features include, bird-like facies, short stature, sparse hair, café-au-lait spots, impaired cardiovascular function and type II diabetes, all symptoms that occur in normal ageing.

Dyskeratosis congenita (DC) individuals show many ageing characteristics, such as alopecia, grey hair, wrinkled skin, abnormal skin pigmentation, poikiloderma, osteoporosis and cancer (Davis et al. 2009). The aetiologies of DC

are mutations in members of the telomerase protein complex, with classical X-linked DC mutated for the protein dyskerin. Other mutations occur in the proteins NOP10 and TERT, and the RNA subunit TERC.

Bloom syndrome (BS) individuals have a moderately reduced lifespan (Hofer et al. 2005); however, although classified as a PS, there is little evidence of a premature ageing defect apart from a high incidence of type II diabetes in young BS individuals, and an elevated cancer incidence. Bloom syndrome is due to null mutations in the gene encoding the DNA helicase RECQ2 (BLM).

Are Progeroid Syndromes Associated with Accelerated Fibroblast Senescence?

Associated with premature ageing, accelerated cellular replicative senescence is found in greater than 90% of WS fibroblast strains (Davis et al. 2009). Normal human dermal fibroblasts (NDFs) frequently have replicative capacities greater than 50 population doublings (PDs); in contrast WS fibroblasts usually do fewer than 25 PDs. This accelerated senescence of WS cells *in vitro* has been postulated to correspond to a similar process *in vivo*, and thus contribute to the accelerated ageing of division-competent tissues (Davis et al. 2009). Likewise, many strains of HGPS fibroblasts show reduced replicative capacity, although increased apoptosis is also prevalent, and HGPS individuals undergo rapid ageing and have very short lifespan (Hofer et al. 2005). However, many HGPS fibroblast strains have what appears to be a normal replicative capacity despite the extensive apoptosis that can be present (Davis et al. 2009). These differences may be due to the extensive heterogeneous division capability of cells taken from different individuals at different times of life, and/or due to heterogeneous genetic backgrounds.

Two strains of SS fibroblasts have been examined; the strain with a hypomorphic mutation in *ATR* has a significantly reduced replicative capacity compared to NDFs (Tivey et al. 2013b); the other strain that does not have an *ATR* mutation appears to have a normal replicative capacity. These differences reflect that SS is a very heterogeneous syndrome resulting from mutations in six different genes.

Nijmegen breakage syndrome fibroblast strains either have no replicative defect (cell life spans greater than 40 PDs), or have a much-reduced replicative lifespan (Ranganathan et al. 2001; Tivey et al. 2013a). The reasons for this large difference are not known, but NBS strongly resembles other PSs (or genomic instability syndromes) in clinical features, notably AT and ataxia-telangiectasia-like disorder (ATLD). It is thus possible that some NBS cases may be misdiagnosed. As the fibroblast strain with a reduced replicative capacity is known to have an *NBN* mutation (Ranganathan et al. 2001), it may be that the fibroblast strains with normal replicative capacities are from an, as yet, unknown syndrome that shares clinical features with NBS. I use the terms NBS and NBSL (NBS-like) respectively, here, for these subtypes solely for ease of clarity.

Cockayne Syndrome has two variants (CSA and CSB), and it has been shown that CSB fibroblasts do not have a replicative capacity defect with the strains used managing greater than 50 PDs (Tivey et al. 2013a). The situation with CSA fibroblasts is unclear, however, but the CSA strains used to date cluster at the low end of the normal range that is suggestive of a reduced division capacity. Those RTS fibroblasts that are mutated for RECQL4 (Type II) do not show an obvious mean replicative defect, although RTS strains cluster at the low end of the normal range for fibroblast lifespan (Davis et al. 2013) that may correlate with the observation that RTS individuals have a normal lifespan (Hofer et al. 2005). The replicative capacity for Type I RTS fibroblasts is unknown. With ataxia-telangiectasia, the replicative capacity of fibroblasts is significantly reduced compared to NDFs almost to the same degree as seen in WS fibroblasts, and most AT fibroblast strains appear to have replicative capacities less than 25 PDs (Davis and Kipling 2009). The only DC fibroblasts that I have studied are from the X-linked variant that has mutations in *DKC1* (dyskerin) and these fibroblast

strains have a very reduced replicative capacity (Davis et al. 2009; Tivey et al. 2013a). Reduced replicative capacity is also seen in fibroblasts from DC cases due to mutations in the other causative genes (Davis et al. 2009). Finally, in the case of BS, there is no apparent replicative defect in any of the fibroblast strains examined to date (Tivey et al. 2013a).

Role of p38 in Accelerated Senescence in Progeroid Fibroblasts

Fibroblasts senesce primarily as a result of telomere erosion that activates p53 and $p21^{WAF1}$. However, telomere erosion rates in WS fibroblasts have been shown to be similar to that seen in NDFs, although some telomere dysfunction does occur, in particular a low level of sudden telomere truncation (Davis et al. 2005, 2009), suggesting an alternative pathway is involved in the premature WS cell senescence. In addition to a short replicative capacity, WS fibroblasts have very slow growth rates and an enlarged morphology with extensive arrays of F-actin stress fibres that strongly resembles senescent normal cells (Davis et al. 2005). They also have high levels of activated p38, phosphorylated HSP27, and $p21^{WAF1}$. The stress-associated MAP kinase p38 is involved in cellular senescence processes resulting from both the erosion of telomeres and to endogenous and exogenous stress, and its activation can lead directly to cell cycle arrest by activating either the $p53/p21^{WAF1}$ or the $p16^{INK4A}$ pathways (Iwasa et al. 2003).

Overall, WS fibroblasts resemble cells that have undergone p38 driven stress-induced premature senescence (SIPS). Treatment with the p38 inhibitor SB203580 had a remarkable effect on these cells, more than doubling their replicative capacity to greater than 40 PDs, increasing their growth rate and changing their morphology to that seen in young NDFs. In contrast, p38 inhibition resulted in only a small extension of the replicative lifespan of NDFs (Tivey et al. 2013a). These data suggest that the shortened WS replicative lifespan results from a robust telomere-independent p38-driven SIPS.

A large increase in replicative lifespan for ATR-SS fibroblasts resulted from inhibition of p38 by three different inhibitors, with the increase directly proportional to the extent of p38 inhibition achieved (Tivey et al. 2013b). Untreated ATR-SS fibroblasts show an aged morphology with F-actin stress fibres, activated MK2 (a p38 target and the major HSP27 kinase) and phosphorylated HSP27, and elevated $p16^{INK4A}$, all features that were corrected by p38 inhibition. These data are consistent with SIPS in ATR-SS cells.

Smaller increases in replicative capacity were found for NBS fibroblasts, although whether p38 is activated in NBS cells is undetermined and the cells may not have an aged morphology (Ranganathan et al. 2001). Increased telomere dysfunction (particularly telomere fusions) is prevalent in NBS cells, but the telomere erosion rates appear not to be significantly elevated (Hou et al. 2012; Ranganathan et al. 2001). These data suggest that the shortened replicative capacity may result from the combined effects of telomere dysfunction and SIPS; however, NBS is not well understood at this time. For the NBSL strains with unknown aetiology, however, the effect of p38 inhibition was similar to that seen in NDFs (Tivey et al. 2013a).

With AT, whilst fibroblast replicative capacity is reduced (Naka et al. 2004; Tchirkov and Lansdorp 2003), there is clear heterogeneity in the response to p38 inhibition, with some AT fibroblast strains showing lifespan extension and increased growth rates greater than seen in NDFs (although much smaller than seen with WS cells), whereas other strains showed a much reduced response (Davis and Kipling 2009). In addition, although some AT fibroblast strains had an altered morphology, they showed no increased level of F-actin stress fibres, and no activated p38. This lack of p38 activation in AT cells agrees with (Naka et al. 2004), but contrasts with the study of (Barascu et al. 2012) who showed activated p38 in AT lymphoblasts and fibroblasts, although the fibroblasts used did not appear to have F-actin stress fibres. The AT fibroblasts in this study did have some features characteristic of senescent cells, such as high SAβ-gal levels, that were corrected by p38 inhi-

bition, or siRNA knockdown (Barascu et al. 2012). These differences suggest that loss of ATM produces a stress signal that activates p38 leading to a low level of SIPS that results in some of the premature senescence seen in AT cells. However, this stress response is much reduced when compared to that seen in WS, ATR-SS and NBS cells, and the major cause of the shortened replicative capacity of AT fibroblasts appears to be the dysfunctional telomeres and accelerated telomere shortening seen in AT cells (Tchirkov and Lansdorp 2003).

Activated p38 and phosphorylated HSP27 are also seen in Type II RTS, CSA and some CSB fibroblasts (Davis et al. 2013; Tivey et al. 2013a). However, these are at a much-reduced level compared to that seen in WS and p38 inhibition produces only a small fibroblast lifespan extension similar to that seen in NDFs. In addition, few enlarged cells with F-actin stress fibres are observed. This suggests only a low level of stress is occurring in these cells and any reduced replicative lifespan for the CSA cells results from a process other than SIPS. The protein mutated in Type II RTS (RECQL4) is believed to play a role in telomere maintenance so the activated p38 may result from a low level of telomere dysfunction (Davis et al. 2013). Alternatively these cells show elevated oxidative stress that would also activate p38. For CSA cells, a defective DNA repair process may lead to cell cycle arrest via p53 activation.

For fibroblasts from HGPS, BS, and SS no p38 activation is seen, the cells have a normal morphology, and p38 inhibition does not extend replicative lifespan beyond that seen in NDFs (Tivey et al. 2013a), suggesting that SIPS is not present in these cells. This is supported by the observation that none of these cells strains show F-actin stress fibres. Thus the premature senescence seen in the HGPS cells used in the study is not due to p38 activation or SIPS. Finally, with DC fibroblasts p38 inhibition had only minimal effects on either replicative lifespan or cellular morphology, but as the cells used were almost at replicative senescence when p38 inhibition began and the activation of p38 was not assessed, no real conclusions could be drawn. However, the premature senescence of DC cells is thought to be due primarily to accelerated telomere erosion (Davis et al. 2009).

Discussion

General Discussion

Human ageing is a gradual process that reduces normal tissue function eventually resulting in tissue failure leading to death; an example being the gradual weakening of cardiac function prior to heart failure. As both ageing and disease impair biological function, it would be unsurprising if basic ageing processes lead eventually to the onset of those diseases specifically associated with ageing. Indeed, biogerontologists have long been aware that an understanding of the basic ageing mechanisms should lead to advances in the pathophysiology of much age-related illnesses, and possibly novel therapeutic interventions. This view is increasingly being accepted amongst medical professionals in addition to biogerontologists (Bagley et al. 2011). Due the polygenic nature and biological complexity of the ageing pathologies, biogerontologists often make use of the group of disorders known as progeroid syndromes (PSs) whose phenotypes show specific characteristics of ageing (Puzianowska-Kuznicka and Kuznicki 2005). As these disorders are often monogenic and affect only a subset of normal ageing phenotypes, these syndromes are useful as their study may allow distinct ageing pathways to be dissected out from the whole body ageing background leading to the identification of causative genes underlying ageing processes.

One possible mechanism that may underlie human ageing is that of cellular senescence as division competent tissues require continuous proliferative capacity throughout life to maintain function due to continuous cell loss. In addition, senescent cells display deleterious biochemical features such as the expression of degradative enzymes and inflammatory molecules (Davis and Kipling 2006; Kipling et al. 2004). Thus instead of their normal function to support tissue function, senescent cells may actively do the opposite

and contribute to the age-related decline in tissue structure and the genesis of age-related pathologies (Kipling et al. 2004). Although evidence for replicative cellular senescence *in vivo* has been difficult to acquire, it has become increasingly evident that a linkage between cellular senescence and the age-related decline of tissue function does indeed exist. Human cells senesce as a result of two basic processes, replicative senescence due to telomere erosion, and SIPS resulting from various stressors throughout life. However, in either case the cells have a strikingly similar phenotype and behaviour. As cellular senescence is proposed as a major causation of human ageing, it is useful to look possible roles for cellular senescence in the various PSs where specific ageing processes may be dissected out from the background of whole human ageing. In addition, as many of the age-related pathologies are inflammatory in nature and senescent cells express high levels of the stress-related kinase p38, a role for p38 in the premature ageing seen in PSs is predicated.

Although fibroblasts from several of the PSs show premature fibroblast senescence, there appears to be no clear relationship between the replicative cellular capacity of the cell strains used and the presence of premature ageing features of PSs. It may be that the presence of accelerated cellular senescence correlates with the severity of the ageing phenotype, e.g. in WS, HGPS and DC the ageing features are marked and the lifespan of individuals is reduced, and the replicative capacity of fibroblasts is much reduced compared to NDFs. In comparison, the ageing characteristics of individuals with RTS and BS are few and the replicative lifespan of fibroblasts is not significantly reduced. However, there are PSs with relatively mild ageing features such as NBS, AT and ATR-SS, where the replicative capacity of fibroblasts is much reduced. However, there is a complicating issue in that individuals with these syndromes have very short lives that do not result from premature ageing, thus it may be that they do not live long enough to manifest significant accelerated ageing phenotypes. This contrasts with other short-lived PSs such as HGPS, where the short lifespan of individuals results directly from the premature ageing. Finally, in CS significant accelerated ageing is not related to significant accelerated fibroblast senescence in CSB, but may be related to premature cellular ageing in CSA. It should be noted that there appear to be no genes that specifically cause ageing; the processes that affect ageing involve gene products that have diverse additional functions in the body, so mutations in such genes will have broad-ranging phenotypic consequences.

Alternatively, tissue specificity may be important, since dermal fibroblasts from WS, HGPS, DKC, AT and possibly CSA show premature senescence and a notable feature of these PSs is skin ageing, in particular the inflammatory scleroderma seen in WS, HGPS and AT. However, the other PSs with premature fibroblast ageing (NBS and ATS-SS) do not show accelerated skin ageing, although they do manifest the skin conditions of telangiectasias and café-au-lait spots. Moreover, BS also manifests telangiectasias and café-au-lait spots and no accelerated fibroblast ageing. However, a second aged phenotype, that of premature grey hair, is found in all the PSs that have premature fibroblast senescence, but in none of the others.

It should be noted that the only cell type that has been extensively studied are fibroblasts and the premature cellular phenotypes may affect other cell types such as endothelial cells or lymphocytes, although lymphocytes from WS have a normal replicative capacity (Davis et al. 2009). That other cell types may play a role in accelerated ageing phenotypes comes from the observation that large arteries in HGPS individuals are severely depleted in endothelial and vascular smooth muscle cells, with many others thought to be senescent (Stehbens et al. 2001). Senescent endothelial cells have been found extensively in, and are thought to be causative of, atherosclerotic plaques that frequently occur in HGPS individuals. It is possible that features of ageing seen in other PSs may result from the premature ageing of other cell types. Thus, the observed lack of accelerated ageing of fibroblasts in some PSs does not suggest that the observed ageing features are not due to replicative cellular senescence, or the presence of senescent cells of other cell types.

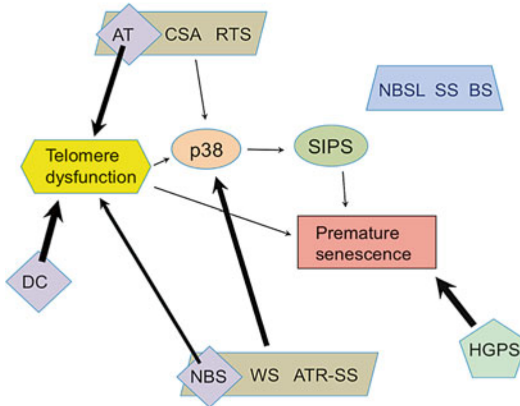

Fig. 3.2 Integrated model for induction of premature senescence in fibroblasts from PSs. Progeroid syndromes induce premature senescence via multiple processes; some via telomere dysfunction (indicated by *purple diamonds*), others directly activate p38 and SIPS (indicated by *brown parallelograms*) and some do both (AT, and possibly NBS) or neither (HGPS). Other PSs do not show premature senescence (*blue trapezium*). The intensity of the inducing signal and thus the degree of premature senescence induced by this mechanism is indicated by the *thickness of the arrow* from the relevant PS

As for a role for p38 in cellular senescence and ageing in PSs, it is clear that no obvious relationship exists between the replicative capacity of fibroblasts and the activation of p38 and SIPS; although it may be a matter of the degree of p38 activation (Fig. 3.2). This is illustrated for WS and ATR-SS cells that have high levels of activated p38 resulting in an altered cellular morphology with F-actin stress fibres: in these cells the loss of replicative capacity appears to be mostly (if not entirely) due to p38 and SIPS. With NBS the situation is more complicated with the cells having increased telomere dysfunction (Ranganathan et al. 2001) and, possibly, SIPS, although which effect predominates is unknown (Fig. 3.2).

For CSA, CSB and RTS, however, the levels of activated p38 seen suggest that only a low level of SIPS is occurring. This SIPS may have little or no effect upon replicative capacity, although any small effects would be hard to detect due to the large variation in replicative lifespan seen in human NDFs (Davis et al. 2013; Tivey et al. 2013a), and is insufficient to have major effects on cell morphology. Moderate p38 activation is also seen in AT and the cells have a much-reduced replicative capacity. In this case a low level of SIPS synergises with a strong telomere dysfunction signal (Tchirkov and Lansdorp 2003) to produce the shortened lifespan (Fig. 3.2). The much-reduced replicative lifespan seen with HGPS cells is not due to activated p38 and SIPS, and it may be independent of dysfunctional telomeres (Davis et al. 2009). In DC cells premature senescence results from accelerated telomere erosion. This leaves NBSL, SS and BS that do not appear to have premature cell senescence, do not activate the p38 pathway and have not been reported to show accelerated telomere dysfunction.

A possible complication in the scenario shown in Fig. 3.2 is that telomere dysfunction can also activate p38 (Iwasa et al. 2003), although it predominantly signals through p53 (d'Adda di Fagagna et al. 2003). Thus some of the p38 signal seen in various PSs (e.g. RTS and WS) may originate from a low level of telomere dysfunction.

As well as a poor relationship between p38 activity and fibroblast premature senescence, there appears to be no clear relationship between p38 activity and the presence of inflammatory disorders in the various PSs. For example the disorder scleroderma has been linked with p38 activity (Ihn et al. 2005), and this condition is seen in the PSs WS, AT and HGPS that show variable p38 activity. Likewise PSs that show high p38 activity such as ATR-SS do not show scleroderma. One possible caveat here is that not all cases of scleroderma show high p38 activity (Ihn et al. 2005), suggesting that others factors are at play that may interact with p38. A similar situation exist with the severity of cardiovascular disease that is noted in HGPS that shows no p38 activation in fibroblasts, compared to WS that does, although as has been stated previously this may be cell type dependent.

Overall it is clear from this work that, whilst there is no overall correlation between premature fibroblast senescence and p38 activity, and the ageing characteristics seen in various PSs, there may be a clear role for these activities in a distinct subset of these disorders. This subset of PSs has defects in a certain type of DNA repair

processes, namely that involving the replication of the so-called common fragile sites: these syndromes are WS, ATR-SS (and possibly NBS) that will be discussed in detail below. For some syndromes (AT, RTS, CSA and some CSB strains), it is possible that the low levels of p38 activity and fibroblast senescence may play a role in the milder ageing characteristics seen in these syndromes. Finally for the other PSs a larger role for these activities may occur in other cell types that have not been studied, although that is speculation. Alternatively, it is possible that SS and NBSL may not actually show premature ageing, as these have not been sufficiently characterised.

Proposed Model for Accelerated Cell Ageing in WS and ATR-SS

Common DNA fragile sites (CFSs) are observed as non-staining gaps or breaks in metaphase chromosomes of cells cultured under conditions of replicative stress. These sites are difficult to replicate and frequently cause replication fork stalling during normal replication (Tivey et al. 2013b). An important function of the kinase ATR is the co-ordination of checkpoint control responses to replication fork stalling (Ozeri-Galai et al. 2008) (see Fig. 3.3). It appears that ATR responds to stalled replication forks in at least two ways: (1) by activating the BLM DNA helicase and MUS81 endonuclease that create transient DNA DSBs (Shimura et al. 2008); (2) by recruiting the WRN DNA helicase to process the DNA DSBs and prevent fork collapse (Ammazzalorso et al. 2010; Pirzio et al. 2008). By keeping these pathways in balance, ATR leads to replication fork stability and coordinates an error-free repair and replication fork restart in a manner that does not involve DNA recombination (Franchitto et al. 2008), although the exact mechanism is not known. Cellular proliferation can then continue normally with the cells eventually reaching senescence that, incidentally, contributes to tissue and whole body ageing (Fig. 3.3, left panel). It is important to note that the function of this pathway is one of tumour suppression as CFS expression is common to many tumour types, and not ageing *per se* (Arlt et al. 2006; Tivey et al. 2013b).

During this repair process p38 is activated to a low level through an, as yet unknown, pathway leading to a low degree of SIPS. That this SIPS does occur is supported by the observation that normal fibroblasts do show an extension (albeit small) of replicative capacity when treated with p38 inhibitors (Tivey et al. 2013a). It may be that p38 activation results from the formation of the transient DSBs, as both transient DSBs and activated p38 are absent in BS that lacks the BLM protein (Shimura et al. 2008). These DSBs activate ATM; however ATM is not the upstream kinase for p38 in this scheme as ATM inhibition does not prevent p38 activation in ATR-SS cells (my unpublished observations). Thus the actual mechanism whereby p38 is activated is not fully understood.

In WS, lack of the WRN helicase leads to an inability to process the transient DSBs and stabilise the replication fork, and the creation of large numbers of stable DSBs due to ATR activation of BLM and MUS81 (Fig. 3.3, middle panel). Subsequently the fork collapses leading to an error-prone fork repair and restart via ATR activation of CHK1 and RAD51-induced recombination that leads to genomic instability and CFS expression. The increased DSB level results in a much elevated p38 activation and a significant level of SIPS. Likewise, with ATR-SS, the lack of ATR results in the failure to recruit WRN and subsequently large DSB creation (Ammazzalorso et al. 2010) and replication fork collapse (Fig. 3.3, right panel). However, with the lack of ATR, the checkpoint kinase ATM is used in the error-prone fork restart by activating CHK1 (the usual ATM target being CHK2) and CFS expression (Ozeri-Galai et al. 2008). As with WS, the level of p38 activity is elevated leading to extensive SIPS. This idea is supported by the observation that ATR deficiency does not synergise with WRN deficiency in the elevated frequency of CFS expression, which is suggestive of a common pathway (Pirzio et al. 2008). Further support is provided by the extensive similarity between fibroblasts from both ATR-SS and WS, in that they grow slowly, have slow cycling time, increased chromosomal instability, and show increased replication fork

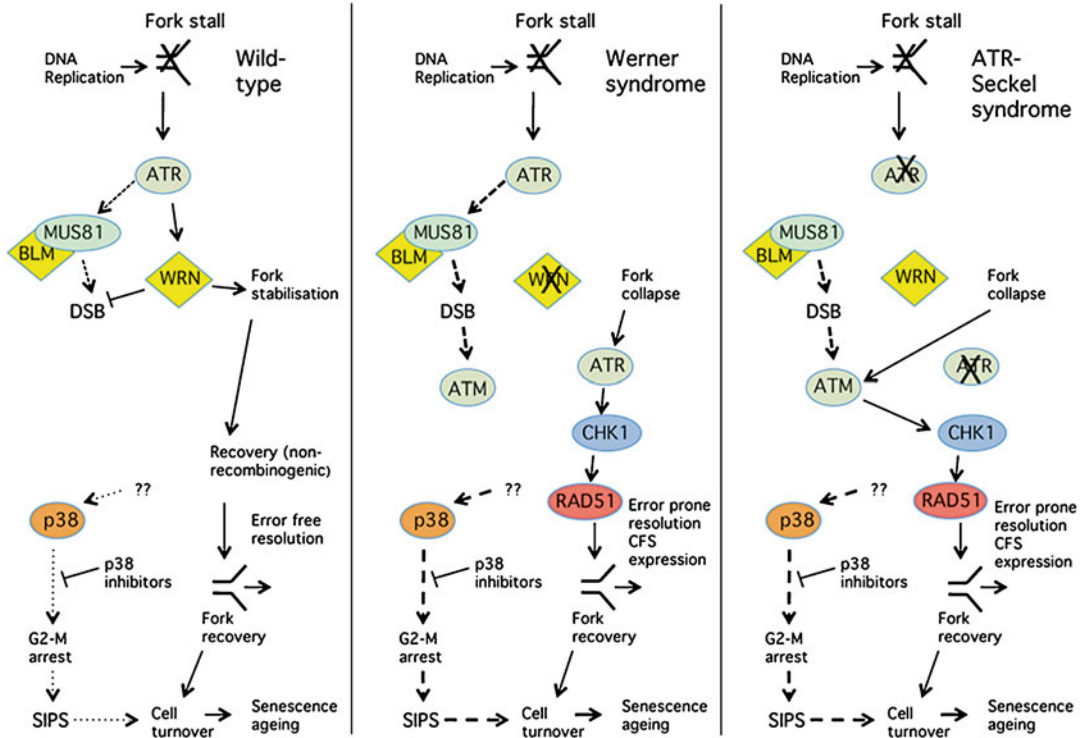

Fig. 3.3 Model for the premature cell senescence and ageing in WS and ATR-SS. Under normal conditions (*left panel*) the DNA replication fork has difficulties processing CFS regions of DNA. This results in a temporary fork stall and the activation of ATR. ATR recruits the WRN helicase resulting in fork stabilisation and an ATR dependent error-free fork recovery, and the restart of DNA synthesis. This allows cellular turnover that leads eventually to senescence and, incidentally, tissue ageing. During this process, p38 is activated leading to a low level of SIPS increasing cellular turnover, although the mechanism by which it is activated is unknown. Loss of either WRN or ATR (*middle* and *right panels*) negatively impinges upon this process resulting in fork collapse, an error-prone fork recovery and CFS expression, and an increased activation of p38. Thus, although fork restart is enabled, the elevated p38 activation results in a significant level of SIPS, increased cell turnover and accelerated ageing. Note that many details of this pathway are still poorly understood

stalling especially at CFSs (Davis et al. 2005; Mokrani-Benhelli et al. 2012; Pirzio et al. 2008).

Overall there is a strong overlap in the cellular phenotype of WS and ATR-SS cells as related to SIPS. This SIPS may lead to aspects of the whole body phenotypes of both ATR-SS and WS such as growth retardation and premature aging due in part to a reduction in cellular division capacity and an accelerated rate of build up of senescent cells. The chronic activation of p38 may also contribute to accelerated aging and the disease predisposition spectrum of these patients – so-called “inflamm-aging” (Franceschi et al. 2000).

Although ultimately ATR-SS and WS are “private” mechanisms of aging (insofar as they are driven by mutations not found in normal individuals), both pathways rapidly converge on a core signalling pathway (p38 MAP kinase) that is subject to substantial regulation by cell intrinsic and extrinsic factors. This in turn raises the possibility that normal human ageing might be affected, even if temporarily, by differential activation of the p38 pathway as a result of other activating circumstances, for example increased oxidative stress. Indeed, the observations of low levels of p38 activation and elevated oxidative stress in other human progerias strengthen the potential relevance of the p38 pathway to human ageing, even though the p38 activity doesn’t result in obvious premature cellular senescence

in these syndromes (Davis et al. 2005; Tivey et al. 2013a). It is possible that the gene defects in other PSs can impinge on this pathway at a low level but in a chronic fashion leading to a degree of premature ageing. One such PS is NBS that has *NBN* mutations, as nibrin forms part of the MRN complex that first recognises the DNA replication fork stall and subsequently recruits ATR (Lee and Dunphy 2013). It is not known if fibroblasts from NBS do show p38 activation, but they may undergo a level of SIPS, suggesting a failure in the early part of the pathway shown in Fig. 3.3. It may be that increased telomere dysfunction plays a greater role in NBS premature cellular senescence than in WS or ATR-SS (see Fig. 3.2). It is interesting to note the putative role for BLM in this pathway to create the DSBs that might be responsible for increased p38 activity, as neither SIPS nor p38 activation is seen in BS and premature ageing is minimal.

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[1] Note: due to the reference limitations imposed in this chapter (a maximum of 35 permitted), an exhaustive review of the literature is not possible. Therefore I have predominantly cited papers that review the information quoted (many of which are my own works) rather than the primary source. This does not denigrate the primary sources in any way whatsoever (these are listed in the cited references), nor does it imply any form of priority or enhanced importance of the cited references, and sincere apologies are herein expressed for all the authors of the primary literature that I have been unable to cite due to these limitations. If readers wish further information on any of the topics discussed I recommend that they read the original sources that can be found in the cited references.

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Induction of Cancer Cell Senescence: Role of Caffeic Acid 3,4-Dihydroxy-Phenethyl Ester

4

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Abstract

Compared with traditional cytotoxic cancer therapy, therapy-induced cancer cell senescence represents a novel promising strategy because it has similar efficacy, but fewer side effects. In this chapter, we briefly review the basic concept of cellular senescence and therapy-induced senescence and discuss the signalling pathway of cellular senescence, especially, Ras and Twist signalling. Moreover, we focus on Caffeic Acid 3,4-Dihydroxy-Phenethyl Ester (CADPE), a natural compound from the medicinal plants *Sarcandra Glabra* (Sarcandra) and *Teucrium pilosum* (Decne), that suppresses human cancer cell growth and colony formation by the induction of cellular senescence, not by apoptosis or autophagy. Furthermore, we discussed the underlying mechanisms and show that CADPE suppressed the Twist1-related senescence signaling pathways. Together, our results demonstrate that CADPE induces human cancer cell senescence by suppressing Twist expression and that CADPE could be a novel effective agent in the treatment of tumors.

Introduction

Cell senescence, firstly described by Hayflick and Moorhead, was discovered in normal human fibroblast cells after prolonged in vitro culture and replicative constraint (Hayflick and

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Moorhead 1961). This phenomenon was induced or accelerated by at least three reasons: DNA damage, oxidative stress and activation of oncogenic signaling (Collado et al. 2007). In-vitro cultured senescent cells were characterized as having large cell size, flattened morphology, prominent nuclei and increased cytoplasmic granularity. Most notably, the cells can be visualized with an extensively used assay, senescence-associated β-galactosidase (SA-β-gal) staining, which stains the perinuclear compartment blue (Dimri et al. 1995; Dong et al. 2011). In contrast to cell apoptosis, senescence led to permanently arrested cell growth through maintaining the cell cycle at the G1 stage (Dimri et al. 1995).

The molecular machinery that initiates cell-cycle is depended on the activation of a cyclin-dependent kinases (CDKs) protein family (Bringold and Serrano 2000). When quiescent cells are induced to proliferate, CDK4 and CDK6 are firstly activated by association with cyclin D protein. Activation of CDK4-6/cyclin D kinase complex leads to subsequent activation of the CDK2/cyclin E and CDK2/cyclin A kinases, which in turn mediate DNA replication and cell cycle progression (Bringold and Serrano 2000). On the other side, there is a group of small proteins known as Cyclin-dependent kinase inhibitors (CKIs), which reversely regulates cell cycle progression. CKIs separate into two families, the INK4 family and the Cip/Kip family. Four members, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ are belong to INK4 family, while three members, $p21^{WAF1/CIP1}$, $p27^{Kip1}$, and $p57^{Kip2}$ are composed of Cip/Kip family. The INK4 family proteins specifically interact and block the CDK4 and CDK6 kinases, which suppresses cell cycle initiation. The Cip/Kip family proteins associated to all the CDK4-6/cyclin D kinase complexes and CDK2/cyclin E and A kinases complexes, but the effect are totally different. Although Cip/Kip proteins inhibit CDK2/cyclin E and A kinases to cause cell-cycle arrest, they act as positive regulators of CDK4-6/cyclin D kinase through stabilization of the association between the kinase and the cyclin subunits. Most of the CKI proteins are of special relevance to senescence through regulation of cell-cycle progression (Bringold and Serrano 2000; Canepa et al. 2007).

Oncogene-induced senescence has been found in nonmalignant human tissues as one mechanism of tumor inhibition (Ewald et al. 2010). The antagonistic relationship between senescence and oncogenic transformation is central for tumor initiation (Ewald et al. 2010). Several signaling pathways regulate cellular senescence (Dimri 2005). Oncogenic Ras represents the gold standard of oncogenes. Activated Ras is more oncogenic than any other mutated proto-oncogenes in most tumors (Bringold and Serrano 2000). In addition to the pathogenic role of oncogenic Ras in cancer initiation and progression, induction of senescence is another important function. Specifically, oncogenic Ras initially triggers an "aberrant" proliferation in cells. After a few doublings, the cells go into a permanent cell-cycle arrest reminiscent of senescence, rather than sustaining proliferation (Bringold and Serrano 2000; Serrano et al. 1997). Ras-induced senescence suppresses the molecular machinery of cell-cycle progression by increasing the expression of specific CKIs, including $p21^{WAF1/CIP1}$, $p16^{INK4a}$, and $p19^{ARF}$, which leads to Rb hypophosphorylation and cell-cycle arrest (Itahana et al. 2004).

Twist proteins are transcription factors with a basic helix-loop-helix domain, which have well documented functions in embryogenesis, cancer migration and epithelial-mesenchymal transition (Yang et al. 2004). Recently, it has been shown that induction of Twist1 is sufficient to override oncogene-induced senescence in both mouse and human cells by blocking the p53- $p21^{WAF1/CIP1}$ tumor suppressor pathway through inhibition of Ras (Ansieau et al. 2008). Furthermore, Twist1 negatively regulates $p16^{INK4a}$ expression which accompanied the induction of senescence (Lee and Bar-Sagi 2010). Therefore, Twist1 efficiently induces cellular senescence in cancer cells and could be a promising target to induce cellular senescence.

Therapy-Induced Cancer Senescence

The traditional cancer therapy strategy is cytotoxic treatment, which relies on the complete destruction of tumor cells (Ewald et al. 2010). Based on this viewpoint, treatment with toxic compounds and high dose radiation are general options taken by oncologists to kill tumor cells (Collado and Serrano 2010; Ewald et al. 2010). These approaches usually lead to severe side effects and drug or radiation resistance. Therapy-induced senescence, which operates by permanently disabling cell proliferation, provides an alternative therapy method to treat cancer. It was found that some tumor cells can be forced into cellular senescence by agents used in the management of human cancers in the 1990s (Ewald et al. 2010). This response of cancer cells to undergo senescence has been noted with both radiation and chemotherapeutic drugs (Ewald et al. 2010). When equitoxic levels of different agents were applied to fibrosarcoma cells in vitro, the DNA-interactive agents, doxorubicin and cisplatin, showed the strongest induction of the senescent phenotype (Ewald et al. 2010), suggesting that therapy-induced cancer senescence may provide an important determinant of treatment outcome and a target for augmentation in cancer therapy. Clinical results have yielded promising results (Desai and Stadler 2006; Martin and Schilder 2006; Winquist et al. 2006), which have shown targeting senescence to be as effective as cytotoxic therapy but with fewer side effects (Dimri 2005; Ewald et al. 2010). Additionally, it has been documented that senescent cells often have a gene expression signature that includes the upregulation of inflammatory cytokines and other immune modulators (Minamino et al. 2003; Xue et al. 2007). Accordingly, macrophages, neutrophils and natural killer cells were increased in tumors for tumor clearance, which prominently led to tumor regression (Xue et al. 2007). Therefore, therapy-induced senescence is a new promising angle in cancer therapy.

Caffeic Acid 3,4-Dihydroxy-Phenethyl Ester (CADPE) and Existing Pharmacological Research

Caffeic acid 3,4-dihydroxyphenethyl ester (CADPE), also named teucrol, was originally isolated from the medical plant *Teucrium pilosum* in 2000 (El-Mousallamy et al. 2000). It is considered a synthetic derivative of Caffeic acid as well. CADPE has attracted a great deal of interest in multiple biological activities. In human renal carcinoma cells, CADPE suppresses tumor angiogenesis by blocking STAT3-mediated VEGF expression. This results also confirmed in the in vivo mice model that CADPE suppressed tumor growth and suppressed STAT3 phosphorylation, HIF-1α expression, vascularization and STAT3-inducible VEGF gene expression in tumors (Jung et al. 2007). In human gastric carcinoma, it has been reported that CADPE inhibits PMA-stimulated cell invasion and matrix metalloproteinase-9 expression by suppression of FAK/MEK/ERK-mediated AP-1 activation (Han et al. 2010). In human hepatocellular carcinoma, CADPE also suppresses cancer cell proliferation by blocking the IL-6 induced STAT3/cyclin D1 signaling pathway (Won et al. 2010). In addition, CADPE effectively prevented STAT3 recruitment to the cyclin D1 promoter then suppressed the expression of cyclin D1 (Won et al. 2010). Furthermore, our previous research also showed that CADPE suppressed RANKL-induced osteoclast differentiation and prevents ovariectomy-induced bone loss through inhibition of the MAPK/AP1 and Ca^{2+}-NFATc1 signaling pathways (Wu et al. 2012). Recently, pharmacokinetic and metabolic properties of CADPE on antitumour activity were elucidated (Guo et al. 2013). The results showed that after tail intravenous administration in mice, CADPE was rapidly detected in blood and the organs including liver, kidney, heart, spleen, and brain in 1 min (Guo et al. 2013). CADPE could be quickly hydrolyzed both in mice and in vitro in mouse plasma, but was much stable in vitro in human plasma

(Guo et al. 2013). The major metabolites of CADPE were caffeic acid, hydroxytyrosol, and a CADPE glucuronide in mice (Guo et al. 2013). However, the anti-tumor molecular mechanism of CADPE remains largely unknown.

Effect of CADPE on Human Cancer Cell Senescence

CADPE Inhibits Human Cancer Cell Growth, but Slightly Affects Normal Human Cell Growth

Our previous research employed three separate approaches to analyze cancer cell growth (Dong et al. 2011), the sulforhodamine B (SRB) assay, cell number counting assay and colony formation assay. All of the results demonstrated that CADPE is an effective small molecule inhibitor of human cancer cell growth in a dose-dependent manner.

CADPE Induces Cellular Senescence in Human Cancer Cells, but Not Apoptosis and Autophagy

CADPE-treated cells were characterized by an enlarged cellular size and nucleus, and flattened and vacuolated cellular morphology, which are strongly correlated with cell senescence. Our previous findings (Dong et al. 2011) demonstrated that CADPE increased SA-β-gal expression and activity in a dose-dependent manner in all seven human cancer cell lines used (Fig. 4.1). CADPE also concentration-dependently enhanced the *DEC1* expression level, another cellular senescence marker, suggesting CADPE induces cellular senescence in human cancer cells.

Cellular senescence is characterized by flattened and vacuolated cell morphology, increased senescence-associated SA-β-gal activity, induction of the cellular senescence marker DEC1, and finally, G1 phase cell-cycle arrest (Qian et al. 2008). Our previous data (Dong et al. 2011) showed that CADPE induces cell-cycle arrest in G1 phase (Fig. 4.2). We also investigated whether CADPE also induces apoptosis in human cancer cells. Our previous results indicated that CADPE has little effect on human cancer cell apoptosis (Fig. 4.3) (Dong et al. 2011). To determine whether CADPE induces cell autophagy, our previous data also indicated that CADPE has little effect in inducing cancer cell autophagy (Fig. 4.4) (Dong et al. 2011).

CADPE Regulates the Twist1-Modulated Senescence Signalling Pathway

The basic helix-loop-helix transcription factor Twist1 has been well documented in regulating cancer cell senescence (Yang et al. 2004). We focused on whether CADPE regulates Twist1 expression (Dong et al. 2011). By Western blot analysis, Real Time-PCR, and luciferase reporter gene assay, our previous data demonstrated that CADPE inhibited the expression of Twist1 in a dose-dependent manner (Dong et al. 2011). The IC50 for the inhibitory effect of CADPE on Twist1 expression and promoter activity was ~10 μM, which is consistent with the IC50 of CADPE on human cancer cell growth. We also showed that CADPE strikingly enhanced expression of $p21^{WAF1/CIP1}$ and $p16^{INK4a}$ in a dose-dependent manner, while mildly increasing Ras and p53 expression levels. As a result, CADPE inhibited the phosphorylation of Rb in a dose-dependent fashion.

We used a “rescue” strategy to investigate whether overexpression of Twist1 prevents CADPE-induced cellular senescence (Dong et al. 2011). The results showed that CADPE-induced cellular senescence was sharply decreased in cells overexpressing Twist1 in two human cancer cell lines (AGS and H1299), suggesting that CADPE regulates cellular senescence through the Twist1 signaling pathway.

Toxicity of CADPE

CADPE, a natural compound, is abundant in some medicinal plants such as *Sarcandra Glabra* (Sarcandra). In traditional medicine, Sarcandra has been used for treating inflammation and certain malignant tumors in Southeast Asia, the Pacific, Madagascar, Central & South America,

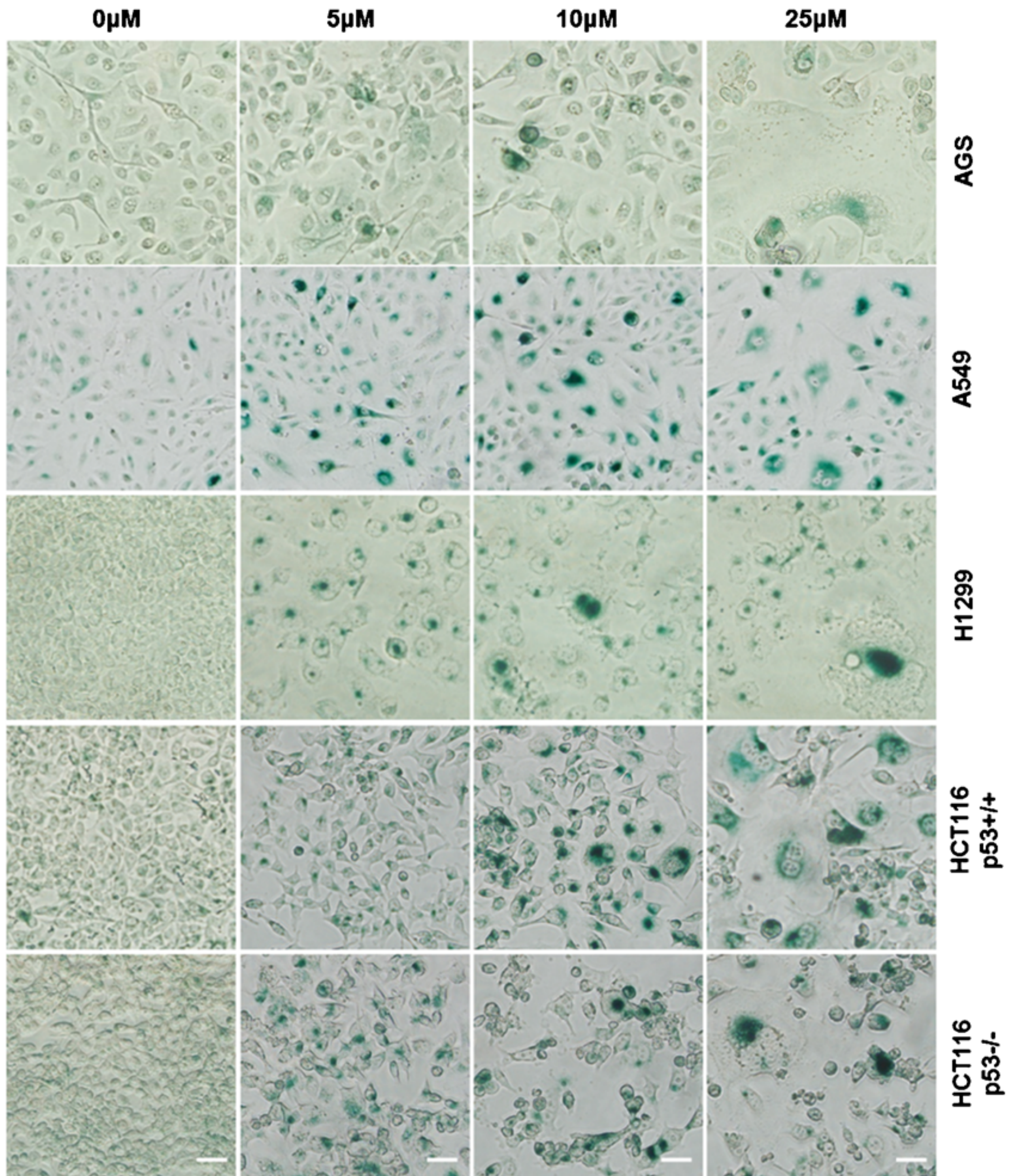

Fig. 4.1 CADPE induces cellular senescence in human cancer cells. SA-β-gal staining of AGS, A549, H1299, HGC27, HCT116 (p53WT) and HCT116 ($p53^{-/-}$) cancer cells after treatment with the different concentrations of CADPE for 72 h. Cells (2×10^4 per well) were fixed and stained with fresh SA-β-gal using a standard protocol. The *scale bar* represents 50 μm

and the West Indies for thousand years (He et al. 2009). It is reasonable to speculate that CADPE is at most minimally toxic. In our previous data, the growth of primary cultured normal human umbilical vascular endothelial cells (HUVECs) were only slightly affected by CADPE treatment, with an IC50 of >50 μM, which is far more than the IC50 in human cancer cells (IC50 of ~10 μM).

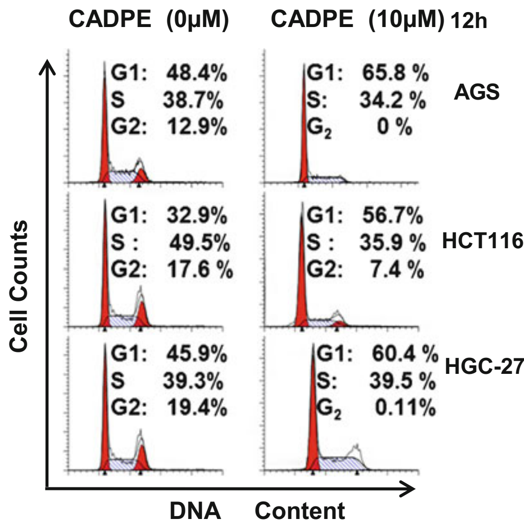

Fig. 4.2 CADPE causes cell-cycle arrest at G1 phase in human cancer cells. CADPE induces G1 phase cell-cycle arrest in AGS, HCT116, and HGC27 cells. Cancer cells were treated with or without CADPE (10 μM) for 12 h, fixed and stained with PI (25 μg/ml), then subjected to flow cytometry analysis (Cell count versus PI/DNA content)

In hepatoma and sarcoma tumor-bearing mouse models, CADPE (2.5 mg/kg) significantly decreased tumor growth without affecting mouse body weight, suggesting minimal toxicity of CADPE in vivo (Guo et al. 2013). Similar results were obtained from the ovariectomized mice that CADPE (10 mg/kg/day) exerted little effect on the body weight of the mice over 3 months (Wu et al. 2012). Taken together, all of the data indicated that CADPE is only minimally toxic in vivo.

Conclusion

Between 1980 and 2010, approximately 70% of all drugs approved by the Food and Drug Administration to treat cancer were derived from traditional medicine or natural sources (Dong et al. 2011). However, traditional medicines have not been widely accepted mainly because of their poorly understood pharmacological mechanisms (Yang et al. 2010). CADPE, a natural compound,

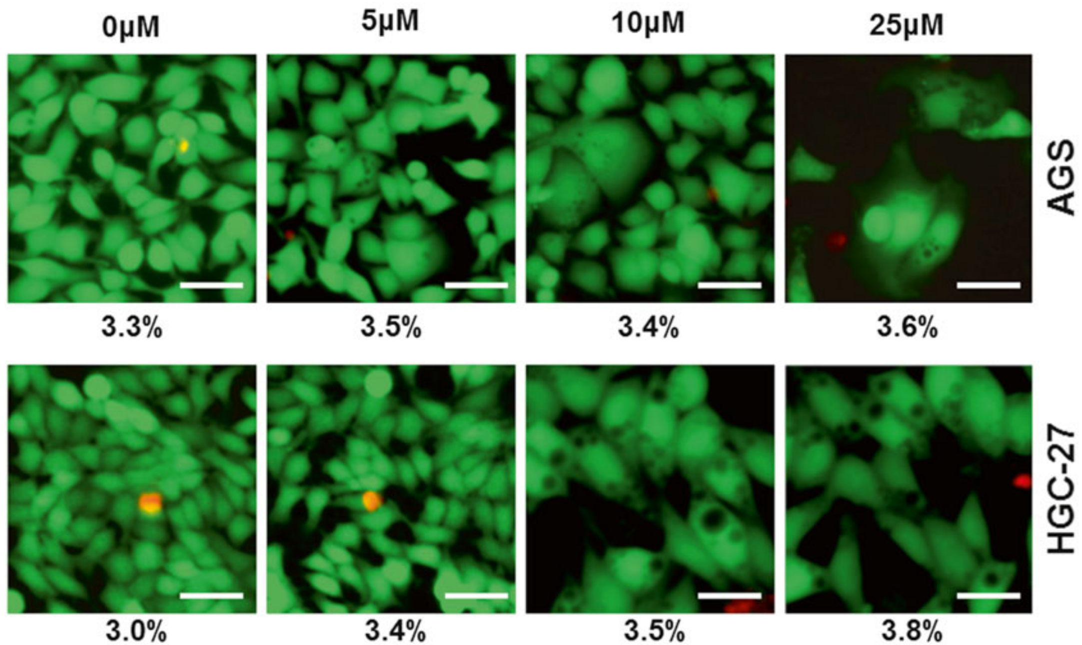

Fig. 4.3 CADPE has little effect on apoptosis in different human cancer cells using Live/Dead assays. Cells (5×10^3 per well) were treated with the indicated concentrations of CADPE for 72 h. The live (*green*) or dead (*red*) cells were stained with Live and dead assay kit, and then analyzed under a fluorescence microscope. The *percentage* indicates the ratio of dead/live cells. The *scale bar* represents 50 μm

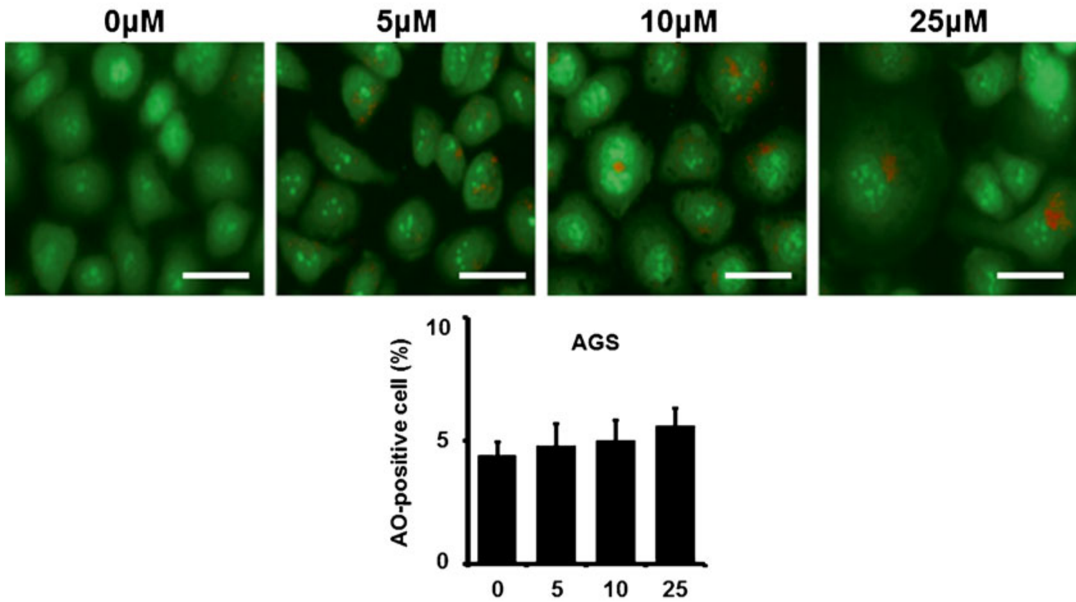

Fig. 4.4 CADPE has little effect on cell autophagy. Cells (8×10^3 per well) were treated with different concentrations of CADPE for 72 h, then fixed and stained with acridine orange. Photographed pictures (*top*) and statistical graphs (*bottom*) were shown. The *scale bar* represents 50 μm. *Columns*, mean; *bars*, SD

which is isolated from the medicinal plants *Sarcandra Glabra* (Sarcandra) and *Teucrium pilosum* (Decne) has been reported to suppress hepatocellular carcinoma growth (Won et al. 2010), tumor angiogenesis (Jung et al. 2007), and gastric cancer metastasis (Han et al. 2010). However, its molecular mechanism remains poorly understand. Our results demonstrated that CADPE induces human cancer cell senescence, but not apoptosis or autophagy by modulating the Twist1 signaling pathway.

Our data demonstrate that CADPE strongly induces human cancer cell senescence by causing cellular senescence-related morphological changes, increasing senescence-associated SA-β-gal expression and activity, elevating cellular senescence marker DEC1 expression, suppressing the Twist1-mediated senescent signaling pathway, and blocking the cell cycle at G1 phase. However, whether CADPE induces human cancer cell senescence in vivo requires further investigation. At present, the direct target(s) of CADPE remain completely unknown. Moreover, it has been reported that senescent tumor cells are more efficiently cleared by immune cells (Lee and Bar-Sagi 2010; Ventura et al. 2007). Therefore, whether CADPE affects immune system clearance of senescent tumor cells also needs further research. In summary, the identification of additional CADPE properties in future studies will provide a promising agent for the treatment of human tumors.

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Part II

Role in Breast Cancer

Various Factors Contributing to Tumor Dormancy: Therapeutic Implications in Breast Cancer

5

Natalia Krawczyk, Malgorzata Banys, Hans Neubauer, and Tanja Fehm

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Abstract

Cancer dormancy describes a stage in tumor progression where tumor cells survive in a quiescent state. Breast cancer is especially known for prolonged asymptomatic periods (up to 15–20 years) followed by a recurrence. Two main mechanisms of tumor cell dormancy are under discussion: tumor cells cease dividing completely or persist proliferating at a slow rate counterbalanced by apoptosis. In the last decades, major efforts have been made to understand the process of interaction between circulating tumor cells (CTCs) in the bloodstream and their extravasation into distant sites, where CTCs may survive in a dormant state or acquire the ability to build metastases. Despite remarkable progress in this field, factors that determinate the fate of a single tumor cell remain to be clarified.

An important hypothesis explaining the phenomenon of tumor cell dormancy is the stem cell theory. This theory indicates stem cell-like characteristics of at least a fraction of dormant CTCs that allows them to persist in the secondary sites and resist standard chemotherapies. Since dormant tumor cells are considered to cause relapse and metastases after long asymptomatic period, an understanding of their biology may be crucial for development of new therapeutic strategies to eradicate this distinct cell population and prevent recurrence. In this chapter we discuss biological

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mechanisms and clinical implications of tumor cell dormancy in breast cancer patients.

Introduction

Earliest hypotheses of hematogenous spread of solid malignancies and its role in the metastatic process originate from the nineteenth century (Ashworth 1869; Paget 1889). In the "seed and soil" theory from 1889, Paget indicated the interactions between tumor cells and microenvironment of secondary homing sites (Paget 1889). Tumor cell dissemination is a common phenomenon in breast cancer (BC) patients. In 30–40% of BC patients disseminated tumor cells (DTCs) can be detected in bone marrow (BM) at the time of primary diagnosis (Braun et al. 2005). According to a large pooled analysis of more than 4,700 patients with primary breast cancer, DTC presence in BM is associated with poor clinical outcome (Braun et al. 2005). Furthermore, several studies showed that DTCs can be found in BM after completion of adjuvant systemic treatment; these persistent DTCs were also shown to be of negative prognostic value (Janni et al. 2005). Beyond detection in bone marrow, isolated tumor cells can be observed in peripheral blood as circulating tumor cells (CTCs). Depending on stage of disease and sensitivity of method used the CTC positivity rates range from 10 to 80% of BC patients. Recent studies revealed a prognostic impact of CTC in both metastatic and primary setting; however, data on CTC prevalence and their clinical significance are to date incoherent (Cristofanilli et al. 2004; Rack et al. 2010).

Daily spread of isolated tumor cells into blood circulation of cancer patients has been reported by several researchers (Liotta et al. 1974). A significant percentage of these cells are already apoptotic or dead, while others are assumed to be eliminated by shear forces of the bloodstream or inadequate interaction between tumor cells and microenvironment. However, in up to 30% of patients CTCs are able to persist in the blood circulation after extirpation of primary tumor (Krag et al. 1999; Banys et al. 2012). Some of these CTCs possess the ability to extravasate and migrate through endothelial cell layer (Luzzi et al. 1998; Cameron et al. 2000). Merely a small fraction of those is able to survive at secondary sites and cause tumor growth ("metastatic inefficiency") (Mehes et al. 2001). These observations are consistent with clinical findings; 50% of patients do not suffer from a relapse despite tumor cell dissemination into bloodstream or bone marrow (Braun et al. 2005). Factors that determinate if isolated tumor cells form macrometastases at distant sites or are seeded as DTCs throughout the body remain yet unclear. In spite of prognostic relevance of tumor cell dissemination, detection of tumor cells in blood or bone marrow does not inevitably predict distant metastasis or local recurrence. This suggests that a significant portion of tumor cells in secondary homing sites are in a state of dormancy.

Cancer Dormancy

Cancer dormancy is a clinical phenomenon defined as a quiescent state of tumor disease; tumor cells are present in peripheral blood or secondary homing sites in the absence of clinical progression. Breast cancer is characterized by lengthy asymptomatic time intervals followed by disease recurrence (Demicheli et al. 1996). In these latency periods a subclinical evidence of tumor can be observed for decades without clinical manifestation of disease (Karrison et al. 1999). This observation led to hypothesis that tumor cells are able to persist in the so-called dormant state until they become active and cause distant metastases (Fehm et al. 2008b). However, after a certain period of time, the mortality in patients with dormant tumor cells is comparable to that of the general population ("the limit of dormancy") (Karrison et al. 1999). Although the phenomenon of tumor cell dormancy plays an important clinical role in breast cancer, many biological aspects of this state remain to be explained.

In the study of Meng et al. persistent CTCs could be detected in 36% of breast cancer patients without any clinical evidence of tumor for 8–22 years after the surgical treatment (Meng et al. 2004a). As the risk of relapse in this patients'

group is very low (about 1% per year) (Demicheli et al. 1996; Karrison et al. 1999), the tumorigenic potential of these cells seems to be limited. Moreover, the CTC counts remain stable over time suggesting a certain balance between cell proliferation and cell death (Meng et al. 2004a). Similar findings were described by several researchers for BCL1 murine lymphoma (Vitetta et al. 1997) and prostate cancer (Morgan et al. 2009).

Many hypotheses have been proposed to account for the cellular homeostasis observed in cancer dormancy. Holmgren et al. implied that suppression of angiogenesis could be responsible for this phenomenon (Holmgren et al. 1995). Sufficient immune response inhibiting growth of tumor cells, normal cell cycle regulation of CTCs or preponderance of tumor growth inhibitors in microenvironment that arrest CTCs in G0-1 have been suggested by other researchers (Pawelec et al. 2000).

Due to the state of dormancy CTCs may elude systemic therapies, that eradicate proliferating cells, and persist in secondary homing sites for a long time (Becker et al. 2007). However, Meng et al. observed in their comprehensive analysis that a half-life time of CTC in the blood after removal of primary tumor is very short (estimate 1–2.4 h) (Meng et al. 2004a). This is consistent with previous observations that epithelial cells enter the process of apoptosis as soon as they lost contact with tumor stroma and bordering cells (Frisch and Francis 1994). Based on the fact that half-life time of CTC is no more than hours and many of detected cells are apoptotic, there must be a source of replicating tumor cells at secondary sites which constantly replenish the CTCs and keep them at the stable level for a long period of time (Meng et al. 2004a). A strict regulation of proliferation and apoptosis allows control of the level of persistent tumor cells.

Tumor Cell Dormancy: Potential Mechanisms

Factors determining the cell's decision to enter a dormant state and controlling its duration still need to be clarified (Fehm etal. 2008b). Based on current studies, the phenomenon of tumor cell dormancy can be explained by two experimental models. It is assumed that dormant cancer cells may persist either by ceasing cell division completely (mitotic arrest) or by staying in a slow proliferation state compensated by apoptosis (Aguirre-Ghiso 2007; Hussein and Komarova 2011). Since these two models are not mutually exclusive, both dormancy forms can be observed in tumor cell population of a particular patient.

Single-Cell Dormancy

According to the single-cell dormancy model, isolated tumor cells or cell clusters separate from the primary tumor and arrive at the secondary homing sites to achieve a prolonged arrested state; dormant cells suspend their division completely until conditions favor their re-entry into the next cell cycle. These latency periods can last years or even decades. Hypotheses that address the metabolic status of minimal residual disease (MRD) during dormancy, remain to be validated. The mechanisms responsible for the regulation of the cell cycle and determination of its proliferative status are highly complex.

The phenomenon of dormancy as a result of division arrest of a single tumor cell has been supported by detection of dormant tumor cells in tissues where primary tumors are developing or tissues with normal vasculature harboring disseminated cells (Townson and Chambers 2006). According to numerous studies dormant CTCs stain negative for markers of proliferation like Ki67 or PCNA (Naumov et al. 2006). This fact supports the hypothesis that dormant CTC are arrested in G0/1 cell cycle phase. Moreover, dormant cancer cells have been found to cause a blockade of receptors for tumor necrosis factor-related inducing ligands (TRAIL) leading to arrest of apoptosis pathway. Two theories describing this process have been proposed: TRAIL-receptor can be blocked by osteoprotegerin, a glycoprotein belonging to the tumor necrosis factor receptor family (Holen et al. 2005) or proto-oncogene tyrosine protein kinase c-SRc can confer resistance to TRAIL and support cancer cells survival in BM microenvironment (Zhang et al. 2009).

Micrometastatic Dormancy

In contrast to single tumor cell dormancy caused by the arrest of cell division, micrometastatic dormancy seems to be a result of a balance between cell proliferation and programmed cell death that allows certain control of the tumor size. A strict coordination of these two processes is modulated by angiogenic and antiangogenic factors produced by tumor and stromal cells, as well as hormonal, immunologic or other microenvironment stimuli (Indraccolo et al. 2006). Based on an animal model, Naumov et al. reported that tumor cells that failed to activate the angiogenic switch may gain the ability to enter the state of dormancy (Naumov et al. 2006). According to data from genomic studies, minimal residual disease is genetically heterogeneous, comprising "active" and "dormant" cell populations, in which an advantageous mutation is acquired shortly before the appearance of a highly aggressive metastatic clone (Klein et al. 2002).

Which of these two models best describes the phenomenon of tumor dormancy in breast cancer patients remains yet unclear. According to Hussein et al. aggressive breast cancers might fit into the micrometastatic model of dormancy, while indolent tumors are linked to the single cell dormancy model (Hussein et al. 2011). These findings could be supported by an analysis demonstrating that more aggressive breast cancer cell lines tend to proliferate rapidly, while the estrogen receptor positive cells remain in the state of mitotic arrest (Barkan et al. 2008).

Tumor Cell Dormancy: Clinical Relevance

Clinical cancer dormancy is a state with no clinical evidence of disease despite the persistence of tumor cells in peripheral blood or secondary sites. This phenomenon has been described in several solid tumors, such as melanoma, renal carcinoma, non Hodgkin lymphoma and breast cancer (Meng et al. 2004a). About 20% of breast cancer patients suffer from a relapse 7–25 years after surgery. Interestingly, between 10 and 20 years after surgery the rate of recurrence stays stable at about 1% per year (Demicheli et al. 1996; Karrison et al. 1999). These late relapses are thought to arise due to interruption of the state of dormancy attributed to disseminated tumor cells. After a certain period of time a mortality rate in breast cancer patients with detected tumor cells does not differ from that of the healthy population ("limit of dormancy") (Karrison et al. 1999).

Despite major advances in therapy of breast cancer leading to improvement in survival, a population of tumor cells in BC patients is able to survive standard cytostatic treatment and persist in blood circulation or secondary sites after completion of chemotherapy (Janni et al. 2005). DTCs that are resistant to standard antiproliferative agents are considered to be in a prolonged state of dormancy. Moreover, a relevant fraction of these cells demonstrates stem cell-like characteristics, such as CD44 positive/CD24 negative phenotype or a presence of ALDH1 (Balic et al. 2006). Some of these features, like immunophenotype, growth characteristics or low rate of proliferation may contribute to their resistance to standard systemic therapies (Becker et al. 2007), These long persisting, dormant tumor cells are currently considered to be a surrogate marker for MRD. An understanding of the biology of dormant tumor cells and their ability to enter or maintain this state as well as to induce their programed death may be crucial for development of new treatment strategies. Based on current studies in the field of tumor cell dormancy, several therapeutic strategies are potentially possible: altering the microenvironment, targeting angiogenesis, disrupting signal transduction, and activating the immune system.

Microenvironment of Dormant Tumor Cells

The question at which point of dissemination tumor cell enters a dormant state still remains to be solved. A changing environment of the bloodstream after the cell has been separated from the tumor stroma may initiate tumor cell dormancy

and thereby enable its survival in hostile conditions. This would mean that DTCs reach the bone marrow in a dormant state. On the other hand, dormancy can be induced in tumor cells after arrival at the site of its seeding, as the non-permissive environment of the secondary site may favor its activation.

The processes of cell proliferation, angiogenesis and invasion in secondary homing sites can be influence by specific interactions between cancer cells and their microenvironment; hence, it is reasonable to develop therapies targeting not only tumor cell but also surrounding environment. A well-established treatment which targets microenvironment is bisphosphonate therapy. Beyond the inhibition of osteoclast-mediated bone resorption, several studies have shown their anticancer activity and confirmed their efficacy in prophylaxis of bone metastases as well as their positive impact on survival in some subgroups of breast cancer patients (Holen and Coleman 2010). An in vitro analysis by Clezardin et al. has shown that bisphosphonates are able to affect the microenvironment by modified secretion of cytokines and to inhibit cell proliferation, adhesion and invasion as well as to induce apoptosis (Clezardin et al. 2005). Moreover, a small trial by Solomayer et al. reported that bisphosphonate may eradicate dormant tumor cells from BM of BC patients (Solomayer et al. 2012).

Angiogenesis

According to the micrometastatic theory of tumor cell dormancy, certain balance between angiogenic and anti-angiogenic factors allows to control the size of the micrometastasis. After this steady-state level in the pathway of tumor development the initiation of angiogenesis or "angiogenic switch" may appear and cause the exponential growth of the tumor (Bergers and Benjamin 2003). Treatment with angiogenesis inhibitors may, therefore, represent an important therapeutic strategy to prevent tumor progression. Promising results have been shown in the clinical studies on monoclonal antibody against VEGF in combination with chemotherapy in metastatic breast cancer patients (Robert et al. 2011). The role of anti-angiogenic agents in treatment of BC patients remain to be evaluated in ongoing clinical trials.

Signal Transduction

The use of cell-modulating drugs, such as antibodies or small molecules, in breast cancer therapy has gained increasing importance in the recent years. The human epidermal growth factor receptor 2 (HER2) is the most common target of these specific approaches. Interestingly, there are phenotypic discrepancies between cells of primary tumor and those isolated in peripheral blood and secondary sites, as we and others demonstrated before (Krawczyk et al. 2009). Furthermore, HER2 negative patients may acquire HER2 gene amplification in MRD in the course of their disease, despite the HER2 negative primary tumor at the time of primary diagnosis (Meng et al. 2004b). However, the anti-HER2 therapy is intended only for patients with HER2 positive primary tumors. Patients with HER2-negative primary lesion and HER2 positive MRD can possibly benefit from such a treatment (Rack et al. 2012).

Phenotypic discrepancies between primary tumor and MRD have been also reported with regard to hormone receptor status. Inversely, MRD is generally hormone receptor negative, in spite of the hormone receptor positive primary tumor (Fehm et al. 2008a; Banys et al. 2012). This discrepancy may explain the failure of the endocrine therapy in a subgroup of breast cancer patients with positive estrogen receptor status. Since the phenotype of MRD, as a crucial target of adjuvant treatment approaches, may differ from this of a primary tumor, characterization of residual tumor cells is becoming increasingly important. Finally, the aim of successful adjuvant therapy must be the complete eradication of residual tumor cells, since tumor cell persistence in secondary sites has been shown to be of negative prognostic value (Janni et al. 2005). According to the recent interventional study by Rack et al. an extended post-adjuvant HER2

targeted therapy is able to eradicate persistent HER2 positive DTCs in breast cancer patients. These results support the findings demonstrated in previous trials (Bozionellou et al. 2004).

The number of others agents targeting signal transduction of the tumor cell, such as lapatinib (targeting the HER1/2-dependent tyrosine kinase), RAD001 (targeting mammalian target of rapamycin), sunitinib (targeting c-kit, platelet-derived growth factor, and VEGF), enzastaurin (targeting protein kinase C) and lonafarnib (targeting farnesyltransferase) has increased in the recent past. Those molecules allow an individual, MRD targeted systemic treatment. However, the prognostic significance of MRD eradication is still to be evaluated in prospective randomized trials.

In conclusion, cancer dormancy is a crucial stage of tumor progression. Its mechanisms are yet to be clarified; dormant cells can persist either in mitotic arrest (i.e. by completely leaving the cell cycle), or by continuing to proliferate at a slow rate counterbalanced by apoptosis. Better understanding of the phenomenon of tumor dormancy might help to develop new targeted treatment strategies to control this step of the disease in the future.

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6 Progression of Hormone-Dependent Mammary Tumors After Dormancy: Role of Wnt Pathway

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Abstract

The cancer stem cell theory suggests the existence of cells within breast cancers that possess the ability to self-renew and differentiate, albeit in a deregulated manner, which sustains tumor progression. Therefore, latent breast tumors and/or their metastasis may eventually resume growth thorough signals impacting on cancer stem cells and their niche. Since it has been determined that the Wingless Related Protein (Wnt) signaling is a likely niche factor and regulator of Mammary Stem Cells dynamics, it is conceivable that this pathway play a significant role in the "awakening" of dormant tumors. We have previously shown that in virgin females, MMTV-induced pregnancy-dependent (ER+PR+) tumor transplants were able to remain dormant for up to 300 days, but were able to resume growth after hormone stimulation. In a subsequent transplant generation, all these tumors became ER−PR− and grew in virgin females, indicating that cancer dormancy facilitated progression to hormone-independence. Our data also showed that mutations altering expression of genes involved in the Wnt pathway were prone to be selected during progression. To gain more insight into the mechanisms underlying these observations, we compared the gene expression profile of tumors that either underwent or not dormancy before progressing to hormone-independency. Confirming our

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previously reported data, we found that the most significant up-regulated gene in hormone-independent tumors that progressed after dormancy was *Wnt1*. In addition, in this group we have determined a systematic down-modulation of previously described mediators of normal pubertal mammary gland development. Using a hierarchical clustering analysis to classify breast cancer patients, we have also identified a specific group of breast carcinomas with significant modulation of genes also deregulated in the MMTV-induced tumors that resumed growth after dormancy. Interestingly, that group of human samples was mainly composed by patients with basal-like breast carcinomas, which also showed down-regulation of genes associated to pubertal mammary development. Therefore, we believe that the cluster of co-regulated genes in basal human breast cancer and mouse mammary tumors resuming growth after dormancy might be mechanistically associated to the activation of Wnt pathway, which might induce proliferation from mammary progenitor basal cells.

Introduction

Breast Cancer

Cancer diseases result from the accumulation of mutations, chromosomal instabilities and epigenetic changes that together facilitate an increased rate of cellular evolution and damage, which progressively impairs the detailed and complex system of regulation of cell growth and cell death. Changes in gene activities are further influenced by the microenvironment within and in the vicinity of tumor cells as well as by exogenous factors. When one combines all of these aspects with inborn genetic variations among individuals, there is every kind of reason to expect tumors to display prodigiously diverse phenotypes. Breast tumors, like most solid cancers, are heterogeneous and consist of several pathological subtypes with different histological appearances of the malignant cells and different clinical presentations and outcomes, and the patients show a diverse range of responses to a given treatment. Furthermore, breast tumor tissue shows heterogeneity with respect to its microenvironment, including the types and numbers of infiltrating lymphocytes, adipocytes, stromal and endothelial cells. The cellular composition of tumors is a central determinant of both the biological and clinical features of an individual's disease (Sorlie 2007).

The management of breast cancer has been dramatically changed with the advent of widespread screening programs and the systematic use of adjuvant hormonal therapy and chemotherapy. Recent data have shown that these changes are having a major impact in outcome, and despite increasing incidence breast cancer mortality is decreasing in most of the Western world. The overview of randomized adjuvant therapy trials has confirmed that systemic therapies (hormone therapy and chemotherapy) are producing cures and has also shown that tamoxifen is of benefit in only patients with estrogen positive (ER+) disease, effectively representing a form of targeted therapy. On the other hand trastuzumab therapy, either concurrent or sequential with adjuvant chemotherapy, is helping patients with HER2-positive tumors. These examples give credence to the old idea that breast cancers are a heterogeneous group of diseases. This has been further confirmed by molecular profiling of breast cancers using array technology showing the biologic and clinical heterogeneity of breast cancer is explained by differences in the genetic composition of the primary tumors (Brenton et al. 2005).

A straightforward interpretation of the recurrent appearance of several different patterns of gene expression among tumors of similar anatomical origin is to regard each as representing a different biological entity. One possible basis for the consistent differences in these patterns between tumor subtypes might be that they originate from different cell types. In fact, it has been found breast tumor subtypes with patterns of gene expression similar to those of luminal epithelial cells (the cells that line the duct and give rise to the majority of breast cancers) and patterns of at least one other subtype (termed basal) that

resembles the pattern found in basal epithelial cells of the normal mammary gland characterized by expression of cytokeratins 5, 6 and 17 (Roarty and Rosen 2010). Advances in microarray technology and pathology have led to improved techniques of sub-classifying breast tumors. Global gene expression profiling enables a subdivision into five individual subclasses (known as the Sorlie–Perou subtypes) found to convey a distinct prognostic and biological message in breast cancer above and beyond established clinical markers (Eriksson et al. 2012). The five groups are the Luminal A, Luminal B, Basal-like, ErbB2, and the Normal breast-like subtypes. Luminal A tumors are mostly ER+, have a low proliferation rate, and are of low grade, whereas Luminal B tumors are also mostly ER+, but may express low levels of hormone receptors, and are usually of high grade and have a higher proliferation rate than Luminal A. The Basal-like subtype, on the other hand, is often characterized by triple-negative tumors (ER-, PR-, and HER2-negative) and a certain cytokeratin pattern, and the ERBB2þ subtype shows amplification and high expression of the ErbB2 gene (also known as HER2 or HER2-neu). Lastly, there is the Normal breast-like subtype, which shows expression of many genes expressed by adipose tissue and other non-epithelial cell types, strong expression of basal epithelial genes, and low expression of luminal epithelial genes. It is, however, unclear whether the latter subtype is a distinct group or represents poorly sampled tissue (Sorlie 2007). Since breast cancers are a heterogeneous group of diseases, the signaling pathways involved in their progression may be different and unique for each type. In this chapter we will show that activation of the wnt pathway would be particularly relevant for ER+ PR+ mammary tumors to resume growth and progress after long dormancy periods.

Breast Cancer Dormancy and Hormone-Dependency

Clinical cancer dormancy is defined as an unusually long time between removal of the primary tumor and subsequent relapse in a patient who has been clinically disease-free. The condition is frequently observed in certain carcinomas (e.g. breast cancer), B-cell lymphoma, and melanoma, with relapse occurring 5–25 years later. Clinical data suggest that a majority of breast cancer survivors have cancer cells for decades but can remain clinically cancer-free for their lifetime. “A long time” has not been precisely defined, but is meant to exceed the time when recurrence is at a lower rate. In breast cancer, 20% of clinically disease-free patients relapse 7–25 years after mastectomy and, from 10 to 20 years, the rate of relapse is relatively steady at about 1.5%/year (Uhr and Pantel 2011).

A dormancy score based on gene signatures developed by combining dormancy expression profiles from a variety of cancer types was recently generated. Although neither recurrence information nor ER status was used to select these genes or to refine the scores, it was found that luminal, ER+ breast cancers were more likely to have a high dormancy score. Then, it was applied to both breast cancer cell line expression data as well as four published clinical studies of primary breast cancers and it was determined that ER+ breast cell lines and primary tumors had significantly higher dormancy signature scores ($P<0.0000001$) than ER- cell lines and tumors. Interestingly, positive dormancy genes were more synchronously up-regulated in patient tumors than in cell lines grown *in vitro*, demonstrating the importance of the tumor microenvironment on dormancy properties (Kim et al. 2012). In addition, these authors noticed that the rate of recurrence was significantly reduced for patients whose tumors were ER+ and had a high dormancy score. These results were consistent with the observed clinical outcome that ER+ tumors tend to recur later than ER- tumors (EBCTCG 2005). It has been reported that women taking estrogen and progesterone for menopausal symptoms (hormone replace treatment: HRT) showed an increased risk of breast cancer development, and it has been argued such an effect might be due to the growth of dormant ER+PR+ tumors. Therefore, these women would already had breast cancer at the start of HRT but were unaware of it. In fact, breast autopsy studies of women

who had died of non-breast cancer-related causes and who had exhibited no evidence of breast disease during life found that, on average, 8.9% had undiagnosed ductal carcinoma *in situ* and 1.3% had undiagnosed invasive breast cancer. Remarkably, it was estimated that 82% of these micro-tumors would have been mammographically undetectable and these studies were restricted to women over 40, when they became candidates for HRT. Therefore, these undiagnosed microdiseases, if they fall into the ER+, PR+ luminal subtype, are susceptible to cancer stem cell reactivation and expansion by exposure to hormones. The hypothesis proposed by Horwitz and Sartorius (2008) would also explain why the increased breast cancer risk observed with E+P treatment is restricted to ER+PR+ disease. These ideas also have direct impact on the use of HRT in breast cancer survivors, because any residual disease would be subject to the same hormonal effects. In fact, when HRT safety was analyzed in 447 breast cancer survivors, after 5 years, there were 39 recurrences among 221 women on HRT, compared with 17 recurrences among 221 no-hormone controls (Horwitz and Sartorius 2008).

Stem Cells and Breast Cancer Dormancy

Traditionally, the mechanisms proposed to account for tumor dormancy have hinged on interactions between cancer cells and host cells within the tumor microenvironment. For example, dormancy has been suggested to arise from a requirement to either switch off host immune surveillance or switch on angiogenesis at sites of latent disease. However, there is little or no evidence that immune suppression of the host, as occurs in organ transplant recipients, triggers reactivation of minimal residual disease and breast cancer relapse Likewise, in vivo evidence for an angiogenic switch regulating reactivation of dormant breast cancer is lacking. Alternatively, the tumor dormancy phenomenon might be explained by an emerging concept that places cancer stem cells at the root of solid tumors. Particularly, in breast cancer it is conceivable that mechanisms governing survival and quiescence in normal mammary stem cells may likewise govern survival and quiescence within dormant tumors. This supports the notion that dormant mammary cancers may harbor transformed mammary progenitor cells (Gestl et al. 2007).

It has been postulated that the tumor cells in G0/G1 may be the precursors of the population of tumor cells underlying clinical cancer dormancy. Initially, this appeared to be a reasonable assumption, particularly because dormant tumor cells are the ones that are resistant to conventional therapy and persist. However, studies on circulating tumor cells (CTCs) in breast cancer survivors 7–22 years after mastectomy and clinically disease-free challenge this notion. The short half-life of these CTCs (1–2 h) indicates that there must be a replicating population of tumor cells at secondary sites that replenishes the CTCs and keeps them at the same low level for many years (Meng et al. 2004). Similarly, our experimental data from MMTV-induced pregnancy dependant mammary tumors show that in the absence of hormone stimulus ER+ PR+ tumors remain dormant, but this status does not result from cells remaining in G0, but cells dividing slowly and dying at similar rates (Gattelli et al. 2004).

Mammary stem cells (MaSCs) are the key drivers of self-renewal and differentiation throughout development, particularly in active growth phases, but these cells are also essential for the maintenance of tissue homeostasis. The niche, hypothesized as the local tissue microenvironment, is essential to maintain and regulate stem cells within the mammary gland. The existence of MaSCs was demonstrated by reconstitution of the mammary gland after transplantation of a mammary epithelial fragment into the cleared fat pad (Kordon and Smith 1998). More recent studies have identified functional MaSCs by surface marker expression followed by mammary reconstitution assays, leading to a preliminary understanding of the hierarchical organization of epithelial subtypes that comprise the mammary ductal tree. This has facilitated the investigation of the molecular signaling pathways regulating MaSC self-renewal

and lineage commitment. It has been determined that the Wingless Related Protein (Wnt) signaling is a likely niche factor and regulator of MaSC dynamics, yet how these signals integrate with systemic hormones and local growth factors remains unclear. Similar to normal tissues, the cancer stem cell theory suggests the existence of cells within breast cancers that possess "stem-like" qualities in their ability to self-renew and differentiate, albeit in a deregulated manner, ultimately sustaining tumor progression and driving tumor heterogeneity. The fact that tumorigenesis, in many ways, may follow the hierarchical nature of an adult tissue, suggests that similar or related pathways are involved in both MaSC and cancer stem cell (CSC) dynamics. Specifically, Wnt signaling pathways play important roles in multiple aspects of both MaSC and CSC biology (Roarty and Rosen 2010).

Somatic stem cells maintain tissue homeostasis in the face of wear and injury. To perform this function over the lifetime of the organism, somatic stem cells possess both a strong intrinsic cell survival program and a capability to enter into, and emerge from, extended quiescence. Robust survival and reversible quiescence are not only biological hallmarks of putative mammary stem cells; they are also clinical hallmarks of minimal residual disease (MRD) as encountered in breast cancer patients. Therefore, mechanisms governing survival and quiescence in normal mammary stem cells may likewise govern survival and quiescence within dormant MRD. Viewed this way, the failure of adjuvant therapy to cure the majority of breast cancer patients at risk for relapse might be blamed on a population of malignant progenitor cells resident within MRD that persists despite treatment and ultimately reconstitutes the malignancy (Gestl et al. 2007). The hierarchical model of mammary gland development proposes the existence of stem and progenitor cells, which are under tight control of both cell intrinsic and extrinsic cues, and give rise to the mature mammary epithelium of either the luminal or basal/myoepithelial lineage by a series of lineage-restricted events. Currently, significant challenges exist in understanding the complex interactions between MaSCs, their more committed progeny, and differentiated epithelial cells, all of which are required to maintain MaSC activity and the functional integrity of the mammary gland. To date, only a few of the paracrine mediators of hormonal action have been identified, including Amphiregulin, receptor Activator for Nuclear Factor κB Ligand (RANKL) and Wnt4. Other Wnt proteins likely represent important mediators of hormone action, although their specific functional roles have yet to be defined (Roarty and Rosen 2010).

The Wnt Signaling Pathway

Activation of Wnt signaling is initiated by the binding of Wnt ligands to Frizzled (FZD) 7 transmembrane receptors in combination with Lrp5/6 or Ror1/2 co-receptors. The binding of Wnt ligands to these receptor complexes is regulated by a number of proteins that either bind to the receptor component (such as DKKs, SOST, or Wise/SOSTDC1) or to the Wnt ligand itself, such SFRPs. Multiple downstream signaling pathways are activated following Wnt ligand binding. The best characterized is the *canonical Wnt pathway*. In the absence of Wnt ligand, the cytoplasmic β-catenin protein is degraded through the action of a multi-protein complex, centered on the axin protein, which phosphorylates β-catenin amino-terminal residues, marking it for ubiquitin-dependent degradation. Instead, following Wnt binding to the FZD-Lrp5/6 receptor complex, β-catenin turnover is inhibited leading to its accumulation and subsequent translocation into the nucleus where it forms a complex with members of the T-cell factor/lymphoid enhancing factor (Tcf/Lef) transcription factor family, inducing Wnt target gene expression, such cyclin D1, axin2 and c-myc (Saito-Diaz et al. 2013).

In the *non-canonical Wnt pathways*, the Wnt signal is transduced in a FZD-dependent but Lrp5/6-independent manner, and activates two downstream signaling branches. Wnt induces cytoskeletal changes via activation of the small GTPases RHOA and RAC1 regulating cell polarity (*WNT/PCP pathway*). Wnt also modulates cell adhesion, motility and gene transcription by NFAT

via activation of the heterotrimeric G-proteins, calcium calmodulin dependent kinase II, and protein kinase C (*WNT/Ca++ pathway*).

The Wnt Signalling Pathway in the Mammary Gland

Wnt signals regulate a variety of key processes, to bring about tissue-level effects including cell proliferation, differentiation, polarity and migration. Given the importance of the pathway in so many tissues and its links to the regulation of cell proliferation, it is not surprising that the mutations which induce canonical Wnt signalling drive carcinogenesis, whilst inactivation of the Wnt pathway leads to loss-of-tissue architecture. This highly complex pathway is tightly regulated by positive and negative feedback signals and through tissue-specific interactions with other signal transduction pathways.

The prototype member of the Wnt family, Wnt-1, was originally identified as a site of integration by the mouse mammary tumor virus (MMTV). Although MMTV expression of Wnt-1 drives the formation of mouse mammary tumors it is not expressed at detectable levels in the normal mammary gland. However, other members of the Wnt family are naturally expressed in the breast (Wnt-2, Wnt-4, Wnt-5a, Wnt-5b, Wnt-6 and Wnt-7), which are differentially regulated during the phases of mouse mammary gland development including puberty, pregnancy, lactation and involution (Jarde and Dale 2012).

Following embryonic establishment of a mammary placode, later embryonic stages lead to the invasion of mammary epithelium into the underlying fatty stroma. These minimal epithelial ducts then remain quiescent until the initiation of postnatal ductal development that follows the rise of estrogen levels immediately prior to and during puberty. Postnatal development of the mammary gland is classified into two different steps: ductal development followed by lobular development, which primarily occurs during pregnancy. During sexual maturation, the end of each duct forms a terminal end bud (TEB), a highly proliferative epithelial structure enriched in mammary stem cells. The end bud elongates and ramifies to form the mammary ductal architecture. Definitive evidence for a role for Wnt signaling in mammary ductal elongation came from loss-of-function studies of the Wnt co-receptors Lrp5 and Lrp6 (Lindvall et al. 2006, 2009). In Lrp5−/- and Lrp6+/, the number of TEBs per gland and the branching complexity were reduced, whilst over-expression of Lrp6 from the MMTV promoter induced increased ductal branching in the young virgin animal's mammary glands (Zhang et al. 2009). These results suggest that the absence of Wnt-responsive mammary cells delays the normal development of the gland and that active Wnt signals and Wnt co-receptor drive the formation of a mature ductal tree. During pregnancy, rapid growth and differentiation of lobulo-alveolar structures fills the stroma with a dense network of epithelial ducts and alveoli (Richert et al. 2000). In the mouse, the Wnt reporter Tcf/Lef1-βgal was prominently expressed during mid-pregnancy within the epithelial cells of the developing alveoli (Boras-Granic et al. 2006) suggesting a role for the Wnt pathway during lobulo-alveolar development.

To demonstrate the requirement for Wnt signaling during mammary gland lobular-alveolar development, studies were conducted using suppressor components of the pathway. The lactating mammary gland from mice over-expressing axin from the MMTV promoter showed severe hypoplasia of the lobulo-alveolar structures, associated with reduced β-catenin and cyclin D1 expression and increased apoptosis (Hsu et al. 2001). Similarly, the over-expression of a dominant negative β-catenin-engrailed fusion protein that lacks the C-terminal β-catenin signaling domain revealed a severe inhibition of lobulo-alveolar development in pregnant mice, associated with reduced proliferation and increased apoptosis (Tepera et al. 2003). These results suggest that the Wnt pathway play a major role during pregnancy and that Wnt signals may provide survival and proliferative signals.

The Wnt pathway may also be involved in the remodeling of the mammary gland during involution. Some Wnt genes were re-expressed during involution, including Wnt-2, Wnt-5a,

Wnt-5b and Wnt-7b. The activation of Wnt signaling in the absence of survival factors may drive involution-induced apoptosis, as previously observed with myc expression in the absence of other growth factors (Jarde and Dale 2012). Taken together, these studies have demonstrated the involvement of the Wnt canonical signaling pathway in the mammary gland development. On the other hand, although crucial for embryo development (Seifert and Mlodzik 2007), the involvement of non-canonical Wnt signaling pathways in normal mammary development is not well understood.

Mammary Stem Cells, Target of the Wnt Signals?

Several lines of evidence suggest that Wnt signaling is involved in the maintenance of the stem/progenitor pool in the mammary gland. It has been demonstrated that the stem/progenitor fraction was increased in the hyperplastic mammary glands of MMTV-Wnt-1 and MMTV-stabilized β-catenin transgenic mice as well as in primary cultures of mammary epithelial cells treated with Wnt-3a (Li et al. 2003; Liu et al. 2004; Shackleton et al. 2006). It has also been demonstrated that MMTV-Wnt-1 and MMTV-stabilized b-catenin induced Wnt signaling in distinct progenitor cell populations (Teissedre et al. 2009).

Combinations of cell surface markers, namely Lin^{-}, $CD24^{+}$, $CD29^{high}$ or Lin^{-}, $CD24^{+}$, $CD49f^{high}$, have been described that allow the isolation of cell populations that are highly enriched for mouse mammary stem cells. The majority of stem/progenitor cells in the virgin mouse mammary epithelium were localized in the basal compartment. Interestingly, histological analysis of Wnt-reporter transgenic lines indicated that Wnt-responsive cells were also located in the basal compartment. Isolation of Axin2-lacZ positive cells stained with a live-cell fluorogenic β-galactosidase substrate allowed the characterization and isolation of Wnt-responsive cells in combination with stem cell surface markers. Zeng and Nusse (2010) demonstrated that Wnt-responsive cells generated mammary outgrowths more efficiently than Axin2-lacZ- negative cells. They also noted that Axin2-lacZ+, Lin-, CD24+, CD29high cells expressed high levels of Lrp5 and Lrp6, in contrast to Axin2-lacZ + equivalent cells. Accordingly, Lrp5−/− mammary epithelial cells exhibited little to no stem cell activity in limiting dilution transplants, whilst Lrp5-high mammary cells were enriched for stem cell activity. In addition, by using s-SHIP promoter-GFP transgenic model that selectively identifies mammary stem cells it has been shown that Wnt-1 increased the number of SMA+; s-SHIP-GFP+, providing more support for the idea that Wnt signaling stimulates the size of this population (Bai and Rohrschneider 2010). Interestingly, although Wnt ligands are expressed by luminal and basal cells, only the basal/myoepithelial cells express Frizzled receptor genes (Kendrick et al. 2008), indicating a directionality of Wnt signaling. This has been supported by the strong induction of Wnt-4 mRNA by progesterone in the luminal population, suggesting that Wnt signals would be paracrine effectors of progesterone-induced mammary stem cell expansion both during pregnancy and at the diestrum phase of the estrus cycle (Jarde and Dale 2012).

Recently, it has been demonstrated an up-regulation of differentiation genes and a marked decrease in the Wnt/Notch signaling ratio in basal stem/progenitor cells of parous mice. This was associated with down-regulation of potentially carcinogenic pathways and a reduction in the proliferation potential of this cell subpopulation both in vitro and in vivo. These observations may identify why early pregnancy has a strong protective effect against breast cancer in humans and rodents, and suggest that inhibitors of the Wnt pathway may be used to mimic the parity-induced protective effect against breast cancer (Meier-Abt et al. 2013).

Wnt signaling would be relevant not only for normal mammary stem cells, but also for survival and expansion of breast cancer stem cells and may contribute to tumor invasion at distant organs. It has been recently reported that periostin (POSTN), a component of the extracellular matrix, is expressed by fibroblasts in the normal tissue and in the stroma of the primary tumors.

Infiltrating tumor cells need to induce stromal POSTN expression in the secondary target organ (*e.g.* the lung) to initiate colonization. The expression of this protein is required to allow cancer stem cell maintenance, and blocking its function prevents development of metastasis. Interestingly, it has been postulated that POSTN exerts this effect recruiting Wnt ligands and thereby increasing Wnt signalling in cancer stem cells (Malanchi et al. 2011).

Association Between Hormone-Dependency and Wnt Activation in Dormant Breast Cancer

To understand the biology of dormant breast cancer *in vivo*, diverse mouse models have been employed (Aguirre-Ghiso 2007; Brackstone et al. 2007) being the Wnt signaling pathway pointed out as relevant in at least some of them (Gestl et al. 2007), particularly in hormone-dependent mammary tumors (Gattelli et al. 2004). We have previously shown that in virgin females, MMTV induced pregnancy-dependent (PD) tumor transplants were able to remain dormant for up to 300 days. During that period, these tumors synthesized DNA, expressed high levels of estrogen and progesterone receptors (ER+ PR+) and were able to resume growth after hormone stimulation. Surprisingly, in a subsequent transplant generation, all these tumors were fully able to grow in virgin females, they expressed very low levels of ER and PR (ER− PR−) and had a monoclonal origin; then, they behaved as hormone-independent (pregnancy-independent: PI) tumors. Therefore, our data indicated that although hormone stimulation was required to rescue PD tumors from dormancy, PI clones prevailed once tumor growth was re-established after long dormancy periods.

In the MMTV as in other hormone-dependent breast cancer animal models, it has not been possible to foresee when and how tumor growth would be released from hormone control, making it very difficult to pinpoint the pathways involved. Interestingly, leaving virgin females carrying latent pregnancy-dependent tumor cells without hormone treatment for more than 4 months allowed us to predict hormone independent behavior in the following passage.

We determined that PI tumor passages arising after a dormant phase usually displayed a lower level of glandular differentiation together with epithelial cell trans-differentiation (squamous metaplasias were frequently detected in those samples). Interestingly, it has been shown that squamous differentiation is the most characteristic histological pattern of mammary tumors of genetically engineered mice with activated Wnt/b-catenin signaling components. Our morphological data showing a higher tendency to form solid cords together with trans-differentiated foci and a very low incidence of pure acinar structures in tumors arising from long dormancy periods suggested to us that mutations altering expression of genes involved in the Wnt pathway were prone to be selected in the progression of PD dormant tumors towards hormone-independence (Gattelli et al. 2004).

Genes Affected by Dormancy Period in Mouse Mammary Tumors: Rationale

In order to gain more insight into the effects triggered by dormancy in hormone-dependent mammary tumors, we compared the gene expression profile of PD and PI MMTV-induced tumors that either underwent (D) or not (ND) long periods of latency. To this purpose, RNA samples were obtained from ER+ PR+ tumors growing in pregnant females, which had been either impregnated immediately (PD/ND) or more than 4 months after tumor transplantation (PD/D) and from ER− PR− transplants that came from either PD/ND (called PI/ND) or PD/D (called PI/D) tumors. To clarify our strategy, Fig. 6.1a, b shows an example of each pattern of tumor growth.

Microarray Data Processing, Statistical and Data Mining Analyses

Total RNA from mammary tumors was obtained using the RNeasy Mini Kit (Qiagen) and hybridized to Affymetrix GeneChip Mouse Gene 1.0 ST

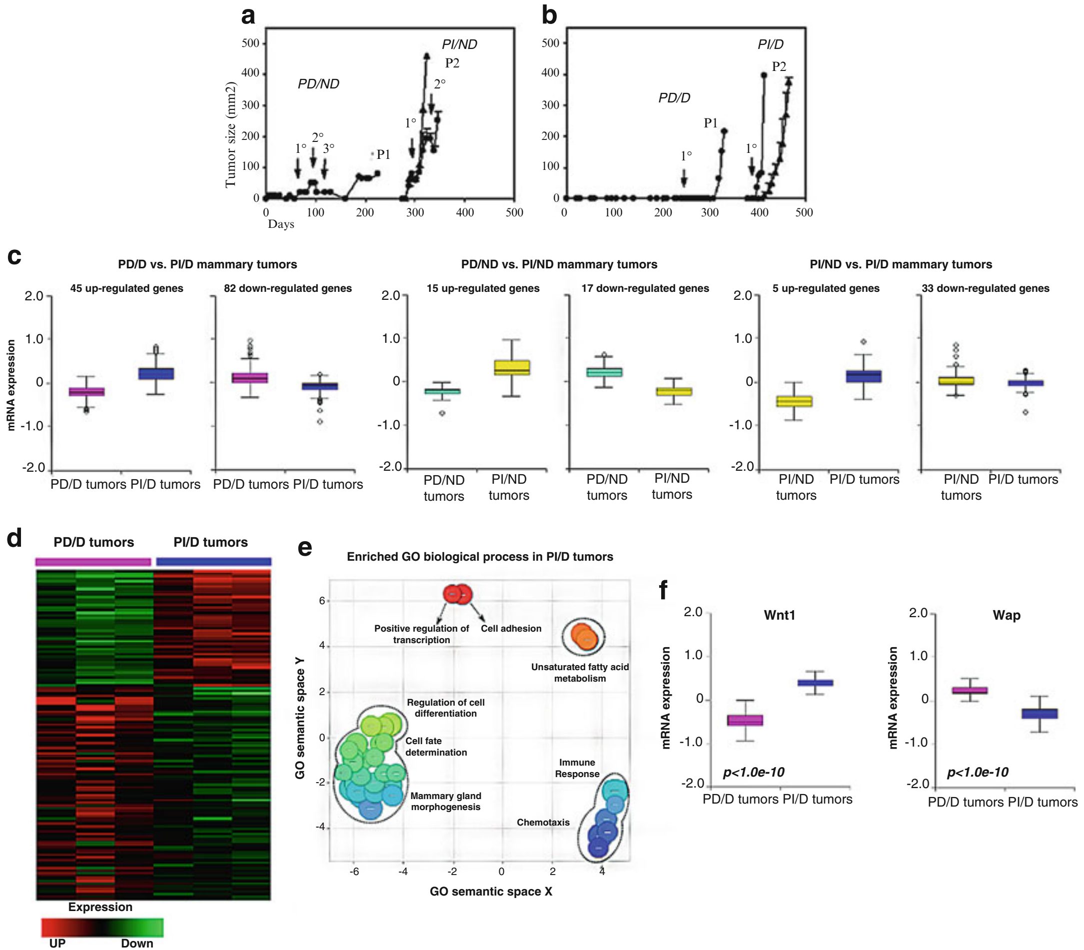


Fig. 6.1 Impact of hormone-dependency and dormancy on the expression profile of MMTV-induced mouse mammary tumors. (**a**) Growth profile of a PD/ND (pregnancy-dependant without dormancy) tumor and the following PI/ND (pregnancy-independent without dormancy) passage. (**b**) Growth profile of a PD/D (pregnancy-dependant with dormancy) tumor and the following PI/D (pregnancy-independent with dormancy) passage. *Arrows* with 1^0, 2^0, 3^0 indicate deliveries of pregnant female mice. *Circles*: parous females. *Triangle*: virgin females. (**c**) Comparison of gene expression profiles obtained from the MMTV-induced tumors shown in Table 6.1: PD/D, PI/D, PD/ND and PI/ND. (**d**) Heatmap of 127 differentially expressed genes of PD/D vs. PI/D mouse mammary tumors (q-value $<$ 0.05). *Color scale* at *bottom* of picture is used to represent expression level: low expression is represented by *green*, and high expression is represented by *red*. (**e**) Scatterplot graph showing the representative clusters, after redundancy reduction of the statistical significant GO terms ($p < 0.025$) enriched in the PI/D gene expression signature, in a two dimensional space related to GO terms' semantic similarities. *Bubble color* indicates the p-value of GO terms (expressed as Log10 p-value) and *bubble size* indicates the frequency of the GO term in the underlying GOA database (bubbles of more general terms are larger). (**f**) Box plots showing increased expression of *Wnt1* and decreased expression of *Wap* mRNA expression levels in PI/D tumors when compared to PD/D tumors ($p < 0.00001$)

Array (Affymetrix) according to the standard Affymetrix protocols. For the analysis, two to three biological replicates of each tumor pattern: PD/ND, PD/D, PI/ND and PI/D were used (Table 6.1). A total of ten tumor samples were analyzed. Importantly, these tumors belonged to ten different independent tumor lines. Before to perform the microarray, the quality of the RNA from tumors was analyzed using Eukaryote Total RNA Nano assay (2,100 Expert). Briefly, we carried out QC and normalization procedures in R/Bioconductor using the simpleaffy package

Table 6.1 MMTV-induced tumors used in the gene expression profiling analysis

	Tumor	Status
1.	LA13	PD/ND
2.	LA0	PD/ND
3.	LACAYB	PD/D
4.	LA4	PD/D
5.	LA	PD/D
6.	2216	PI/ND
7.	LA45	PI/ND
8.	2312	PI/D
9.	2178	PI/D
10.	2314	PI/D

(Wilson and Miller 2005). We employed the Robust Multichip Average algorithm for background adjustment, quantile normalization and probe set values summarization (Irizarry et al. 2003). To compare the mammary gland tumor expression profiles, we utilized the Rank Products test (Breitling et al. 2004). Statistical analysis and heat-map visualization of differentially expressed transcripts ($p<0.01$; $q<0.05$) were done with the MultiExperiment Viewer software (MeV 4.8) (Saeed et al. 2003).

For automated functional annotation and gene enrichment analysis, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al. 2008). The DAVID resource calculates over-representation of specific biological themes/pathways with respect to the total number of genes assayed and annotated. REViGO resource was employed to summarize and visualize the enriched GO terms in an scatterplot graph based on the p-values obtained by DAVID. This allows the identification of biological themes/pathways within a specific list of differentially expressed genes.

To identify the molecular pathways that were mainly affected in hormone-independent tumors that evolved after long dormancy periods (PI/D), we looked for protein/gene interaction networks using the STRING resource ('Search Tool for the Retrieval of Interacting Genes/Proteins') (von Mering et al. 2005). The database STRING aims to collect, predict and unify most type of protein-protein associations, including direct and indirect associations. STRING runs a set of prediction algorithms, and transfers known interactions from model organisms to other species based on predicted orthology of the respective proteins. In order to identify each gene in the database, we used both mouse gene name and Entrez gene ID in the 'protein-mode' application. The analysis input options were 'co-occurrence', 'co-expression', 'experiments', 'databases', and 'text mining' data.

To perform a comparative analysis of PI/D associated genes in human breast cancer, we analyzed 1,114 primary carcinomas obtained from four independent studies available in public databases. The frozen RMA preprocessing algorithm was applied to the Affymetrix HGU133 Plus2 platform based studies (GSE26639 $n=226$, GSE21653 $n=266$ and GSE20685 $n=327$) to generate a compiled dataset of 819 breast carcinomas. In addition, we analyzed the van de Vijver dataset that included 295 early-stage breast cancer samples. This gene expression profile was derived by researchers from the Netherlands Cancer Institute and Rosetta Inpharmatics—Merck using Agilent Hu25K oligonucleotide (60mer) microarray (Agilent Technologies, Palo Alto, CA-USA).

Results and Discussion

Transcriptomic Changes Affected by Dormancy in Mouse Mammary Tumors

Our first goal was to identify gene expression changes that occurred during transition from hormone-dependent tumors that remained dormant in the absence of pregnancy and resumed growth upon impregnation (PD/D) to those that arose after transplantation of PD/D tumors showing hormone-independent behavior (PI/D). The statistical analysis of the gene expression profiling data identified 127 genes differentially expressed between these tumor types ($q<0.05$; 2 fold changes). Among the 127 transcripts, 45 were up-modulated and 82 were down-modulated

in the PI/D tumors (Fig. 6.1c, d). Interestingly, when we examined the transcripts that were differentially expressed in hormone dependent tumors that did not go through long latency periods (PD/ND) and the hormone-independent passages that evolved from other PD/ND tumors (PI/ND), our statistical analysis revealed that only 32 genes were differentially expressed (Fig. 6.1c). Among these transcripts, 15 were up-modulated and 17 were down-modulated in the PI/ND tumors.

We used the DAVID resource for automated annotation and enrichment analysis of the differentially expressed genes based on GO database. In addition, we employed REVIGO resource for summarization and visualization of the significant GO term semantic similarities ($p<0.05$). Among the statistically significant over-represented categories under "Biological Process," we found the mammary gland morphogenesis/development, cell fate determination, differentiation and cell adhesion related genes (Fig. 6.1e). In addition, categories of genes found in the immune and inflammatory response were also highly enriched related genes in the PI/D mammary tumor expression profiles. Table 6.2 shows the top statistically significant deregulated transcripts in dormancy during the progression from hormone dependency to the independency. Interestingly, the most significant up-regulated gene was *Wnt1* (*Wingless-related MMTV integration site 1*) (Fig. 6.1f). We have also identified up-modulation of *Stra6* (*Stimulated by retinoic acid gene 6*) and Enpp2 (*Ectonucleotide pyrophosphatase/phosphodiesterase 2*). *Stra6* and *Enpp2* genes encode cell surface antigens that are known to be synergistically induced by Wnt-1 and RAR signaling in the mouse mammary epithelial cell line C57MG/Wnt-1 (Tice et al. 2002). *Stra6* mRNA up-modulation was also observed in hyperplastic mammary gland and mammary tumors from transgenic mice expressing Wnt-1 compared with normal mammary tissue (Szeto et al. 2001). Enpp2 (also known as *Autotaxin*) has been implicated in human cancer progression as factors contributing to the motility, angiogenic properties, and metastatic spread of cancer cells (Nam et al. 2000). *Enpp2* mRNA is over-expressed in human breast carcinomas compared to adjacent normal breast tissue, with the lowest frequency of expression in luminal A breast cancer subtype (Popnikolov et al. 2012). It is important to point out that none Wnt-induced gene was identified as deregulated tumors that did not go through dormancy periods were compared (*i.e.* in PD/ND vs. PI/ND analysis), suggesting that Wnt pathway is particularly affected during progression of hormone dependent tumors that resume growth after dormancy.

Interestingly, in PI/D tumors a systematic down-modulation of previously described mediators of normal pubertal mammary gland development (McBryan et al. 2008) were identified. For example, *Areg* (*Amphiregulin*), *Cited1* (*Cbp/p300-interacting transactivator 1*), *Cxcl15* (*Chemokine C-X-C motif ligand 15*), *Foxa1* (*Forkhead ox A1*), *Lalba* (*Lactalbumin alpha*), *Mmp3* (*Matrix metallopeptidase 3*), *Pdk4* (*Pyruvate dehydrogenase kinase isoenzyme 4*), *Igfals* (*Insulin-like growth factor binding protein acid labile subunit*), *Sftpd* (*Surfactan associated protein D*), *Lpl* (*Lipoprotein lipase*), *Retnla* (*Resistin like alpha*), *Bche* (*Butyrylcholinesterase*), *Efemp1* (*Epidermal growth factor-containig fibulin-like extracellular matrix protein 1*), *Ubd* (*Ubiquitin D*), *Car13* (*Cabonic anhydrase 13*). Therefore, these genes would be involved in hormone-controlled (mainly estrogen and progesterone) mammary epithelial proliferation. In the transition to hormone-independent behavior their participation in tumor development, together with expression of ER and PR, would be lost.

Noteworthy, several proteins involved in the Wnt signalling pathway (e.g. *Wnt2*, *Wnt5a*, *Fzd1*, etc.) have been identified as modulators of pubertal mammary gland development (Kouros-Mehr and Werb 2006). However, differently from pregnancy-associated mammary development during which Wnt family members are thought to act as paracrine mediators of progesterone signaling (Brisken et al. 2000), it has not been proposed the mechanism by which ovarian hormones and wnt-related factors are related during mammary development of pubertal mice.

The development of the mouse mammary epithelium in virgin females is regulated by

Table 6.2 Top up-regulated and down-regulated transcripts in mammary cells from PI/D tumors

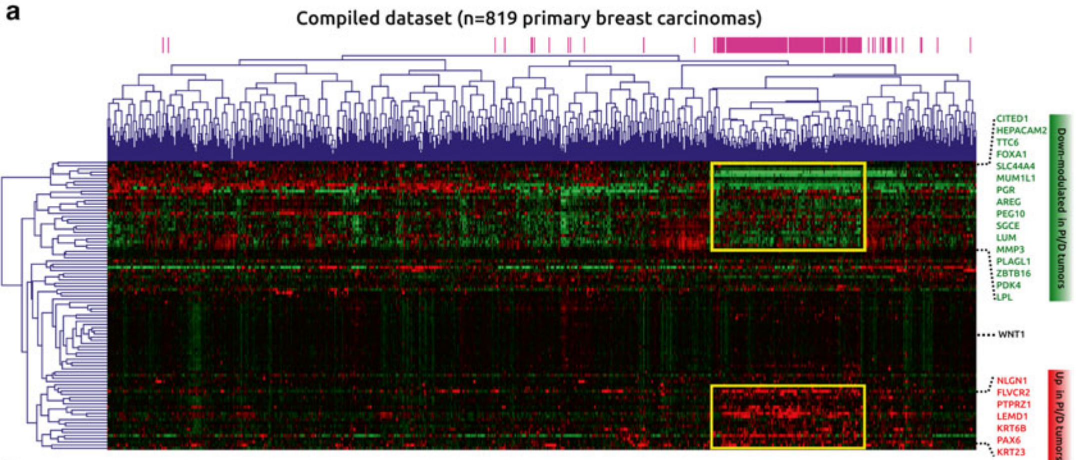
Gene name	Entrez ID	Fold change	p-value
Up-modulated transcripts			
Wnt1 (Wingless-realted MMTV integration site 1)	22408	+2.48	<1.0e-8
Hbbt1 (Hemoglobin subunit beta-1-like)	100503605	+2.48	<1.0e-8
Serpinb11 (Serine peptidase inhibitor, clade B 11)	66957	+2.39	<1.0e-8
Lrrn1 (Leucine rich repeat protein 1 neuronal)	16979	+2.38	<1.0e-8
Serpinb10 (Serine peptidase inhibitor, clade B 10)	241197	+2.34	<1.0e-8
Krt6b (Keratin 6B)	16688	+2.24	6.31e-5
Dsg3 (Desmoglein 3)	13512	+2.23	7.53e-6
Hbb-b1 (Hemoglobin beta adult major chain)	15129	+2.22	1.88e-6
Lhfpl3 (Lipoma HMGIC fusion partner-like 3)	269629	+2.21	1.41e-6
Gabrg1 (Gamma-aminobutyric acid receptor 1)	14405	+2.20	2.59e-5
Dact1 (Dapper homolog1)	59036	+2.19	9.42e-7
Gabrg3 (Gamma-aminobutyric acid receptor 3)	14407	+2.19	3.29e-5
Nlgn1 (Neuroligin 1)	192167	+2.19	7.06e-6
Snord61 (Small nucleolar RNA C/D box 61)	353374	+2.18	1.32e-5
Cst6 (Cystatin E/M)	73720	+2.18	6.97e-5
Lemd1 (LEM domian containing 1)	213409	+2.18	3.58e-5
Asxl3 (Additional sex combs like 3)	211961	+2.17	8.01e-6
Cyp2b19 (Cytochrome P450 family 2b)	13090	+2.17	1.18e-5
Ptprz1 (Protein tyrosine phosphatase receptor Z1)	19283	+2.17	1.13e-5
Pde3a (Phosphodiesterase 3A)	54611	+2.16	7.06e-6
Stk32b (Serine/threonine kinase 32B)	64293	+2.16	6.97e-5
Down-modulated transcripts			
Cxcl15 (Chemokine C-X-C ligand 15	20309	–2.42	<1.0e-8
Tph1 (Tryptophan hydroxylase 1)	21990	–2.36	<1.0e-8
Igf2 (Insulin-like growth factor 2)	16002	–2.35	<1.0e-8
Olah (Oleoyl-ACP hydrolase)	99035	–2.34	<1.0e-8
Saa2 (Serum amyloid A2)	20209	–2.33	<1.0e-8
Wap (Whey acidic protein)	22373	–2.32	<1.0e-8
Wfdc12 (WAP four-disulfide core domain 12)	192200	–2.32	9.44e-7
Peg10 (Paternally expressed 10)	170676	–2.30	<1.0e-8
Lao1 (L-amino acid oxidase 1)	100470	–2.29	<1.0e-8
Csn1s2b (Casein alpha s2-like B)	12992	–2.28	<1.0e-8
Ttc6 (Tetratricopeptide repeat domain 6)	100503562	–2.28	3.15e-5
Hepacam2 (HEPACAM family member 2)	101202	–2.27	4.7e-6
Foxa1 (Forkhead box A1)	15375	–2.26	9.42e-7
Glycam1 (Glycosylation dependent cell adhesion 1)	14663	–2.26	<1.0e-8
Tcfap2b (Transcription factor AP-2 beta)	21419	–2.26	1.41e-6
Hpgd (Hydroxyprostaglandin dehydrogenase 15)	15446	–2.25	<1.0e-8
Defb1 (Defensin beta 1)	13214	–2.25	1.41e-5
Retnla (Resistin like alpha)	57262	–2.23	6.36e-5
Ubd (Ubiquitin D)	24108	–2.20	1.77e-4
Cxcl13 (Chemokine C-X-C motif ligand 13)	55985	–2.19	5.93e-5
Itih2 (Inter-alpha trypsin inhibitor 2)	16425	–2.19	5.32e-5
Areg (Amphiregulin)	11839	–2.18	4.15e-5

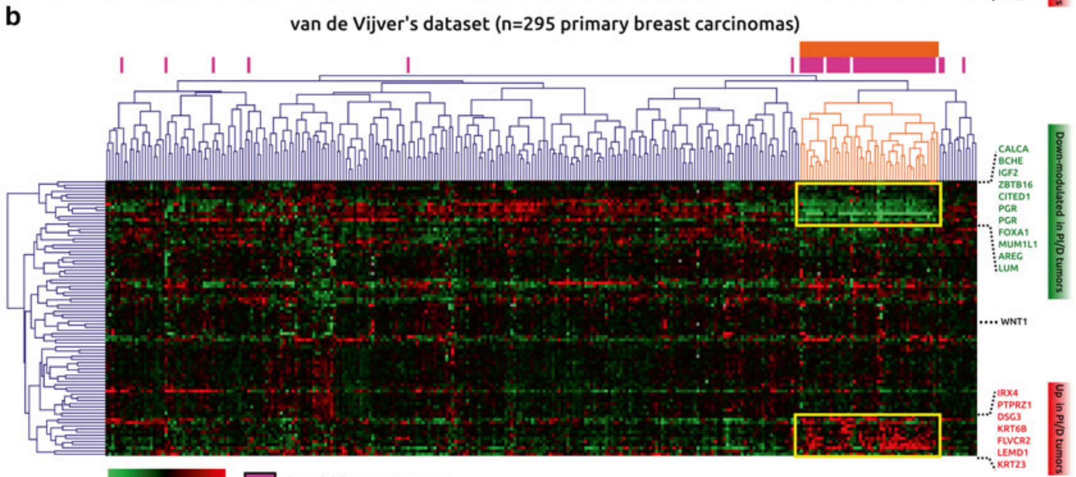
estrogen, progesterone and prolactin, together with a myriad of co-regulators and downstream paracrine effectors. Cited1 is one of these factors since it is a co-regulator of estrogen (McBryan et al. 2008) signaling during puberty and it is also a mediator of pubertal ductal growth. Importantly, it has been demonstrated that Cited1 is primarily regulated in vitro and in vivo by the Progesterone receptor (Sriraman et al. 2009), a gene whose expression resulted down-modulated in the PI/D mammary tumors.

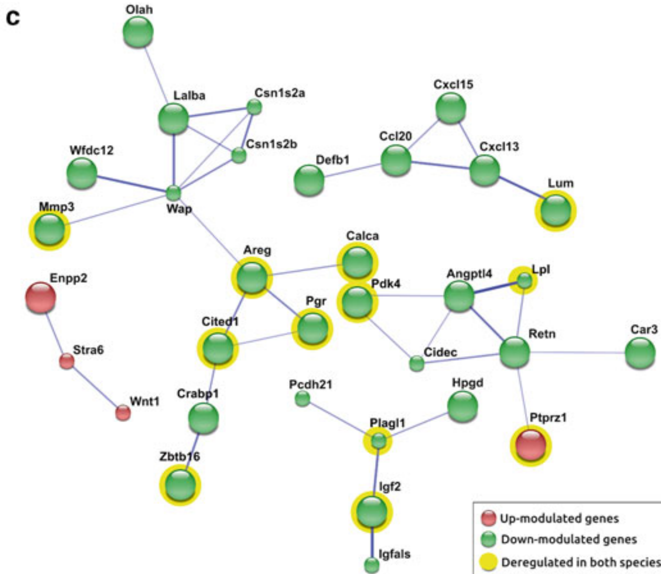
One of the most early gene expression changes induced by estrogen during pubertal mammary gland development is the induction of *Areg*. Anphiregulin is a member of the epidermal growth factor receptor family of ligands that binds exclusively to ErbB1. The AREG secretion activates ERK and AKT intracellular pathways to regulate cell proliferation (Kariagina et al. 2011). In normal mammary glands, estrogen and progesterone induce the expression of *Areg* involving the transcriptional co-activator Cited1. *Areg* and *Cited1* KO mice phenocopy ER KO mice as ablation of these genes result in severely impaired ductal development during puberty, suggesting that they are essentials for ductal elongation as well as estrogen-induced proliferation and terminal end-bud formation. Furthermore, *AREG* and *CITED1* genes are co-expressed with ER-alpha receptor in a subgroup of primary breast carcinomas from women exhibiting good prognosis (McBryan et al. 2008). This observation is consistent with the well-established correlation between well-differentiated carcinomas (low tumor grade) and good outcome.

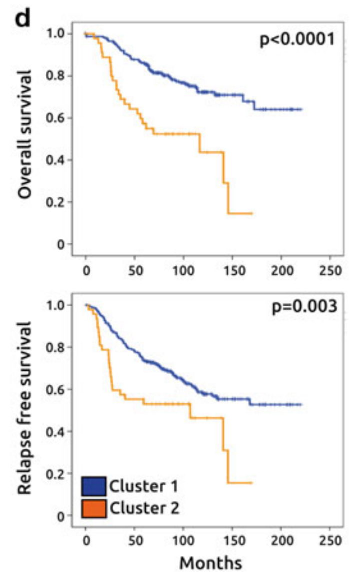
Another common PI/D down-regulated gene encodes the whey acidic protein, WAP (Fig. 6.1f). This mouse milk protein, was widely described as a typical marker of mammary gland differentiation that plays a negative regulatory role in the cell-cycle progression of mammary epithelial cells through an autocrine/paracrine mechanism (Ikeda et al. 2004). Interestingly, expression of WAP and β-casein was reduced in *Areg* KO mice (McBryan et al. 2008) and overexpression of the WAP transgene in the mammary gland depressed proliferation and advanced functional differentiation of mammary epithelial cells. In addition, it has been shown that WAP exerted an inhibitory function in the degradation of laminin, which depressed proliferation, tumorigenesis, and invasion of human breast cancer cells (Nukumi et al. 2007). These data suggest that WAP down-regulation in the PI/D tumors would be associated not only to the loss of terminal differentiation, but also to an increase in the proliferative capacity and invasiveness of these tumors.

To further explore the relevance and the prognostic value of PI/D gene expression signature, we analyzed a total of 1,114 human primary breast carcinomas derived from 4 independent publicly available gene expression-profiling studies. We used hierarchical clustering (HCL) analysis to classify the patients into groups according to the human orthologous genes of the mouse PI/D gene expression signature and then determined the overall and relapse-free survival rates for these groups. This procedure allowed us to identify a specific group of breast carcinomas with significant up- or down-modulation of genes deregulated in PI/D mammary tumors (Fig. 6.2a, b). Interestingly, this group of samples was mainly composed by patients with basal-like breast carcinomas ($p<0.0001$). The heat maps shown in Fig. 6.2 display 28 transcripts commonly deregulated (in the same direction) in human breast carcinomas and PI/D mouse mammary tumors. Among these transcripts, 19 were down-modulated and 9 were up-modulated in both species. Within the first list, we found some genes of which little is known as well as genes previously described to be associated with the pubertal mouse mammary development (*AREG*, *CITED1*, *FOXA1*, *MMP3*, *PDK4*, *LPL* and *BCHE*), as described above. In addition, Fig. 6.2c shows a protein-protein interaction network associating the common core of modulated genes across human and PI/D mouse mammary tumors. Kaplan–Meier analysis revealed that breast carcinomas that expressed the PI/D signature (cluster 2) were particularly associated with shorter overall ($p<0.0001$) and relapse-free ($p=0.003$) survival comparing with the rest of patients (cluster 1) (Fig. 6.2d). Taking together, these data suggest that tumors, in which pathways relevant to normal mammary physiology and development are maintained, may have a


a
Compiled dataset (n=819 primary breast carcinomas)
CITED1
HEPACAM2
TTC6
FOXA1
SLC44A4
MUM1L1
PGR
AREG
PEG10
SGCE
LUM
MMP3
PLAGL1
ZBTB16
PDK4
LPL
WNT1
NLGN1
FLVCR2
PTPRZ1
LEMD1
KRT6B
PAX6
KRT23
Down-modulated in PI/D tumors
Up in PI/D tumors


b
van de Vijver's dataset (n=295 primary breast carcinomas)
CALCA
BCHE
IGF2
ZBTB16
CITED1
PGR
PGR
FOXA1
MUM1L1
AREG
LUM
WNT1
IRX4
PTPRZ1
DSG3
KRT6B
FLVCR2
LEMD1
KRT23
Down-modulated in PI/D tumors
Up in PI/D tumors
Down
Up
Basal-like carcinomas


c
Olah
Lalba
Csn1s2a
Csn1s2b
Wfdc12
Wap
Mmp3
Cxcl15
Ccl20
Cxcl13
Defb1
Lum
Areg
Calca
Enpp2
Pdk4
Angptl4
Lpl
Pgr
Cited1
Stra6
Cidec
Retn
Car3
Wnt1
Crabp1
Pcdh21
Hpgd
Plagl1
Ptprz1
Zbtb16
Igf2
Igfals
Up-modulated genes
Down-modulated genes
Deregulated in both species


d
p<0.0001
Overall survival
1.0
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
p=0.003
Relapse free survival
Cluster 1
Cluster 2
Months

significantly better patient outcome than those tumors in which these pathways have been lost such as occurs in basal-like breast carcinomas and in PI/D mammary tumors.

In summary, the results showed herein confirm our previous observations about the relevance of the wnt pathway activation for the progression of ER+PR+ mammary cancer cells towards a more aggressive hormone-independent behavior upon resuming growth after long dormancy periods (PI/D tumors). In addition, here we demonstrate that these mouse mammary tumors display a transcription profile that resembles the pattern of expression found in basal human breast cancer with bad prognosis. In both cases, genes associated with puberal, hormone-dependent mammary growth are down-regulated. Although the list of genes commonly regulated do not include wnt pathway activators, based on the previously reported data cited herein, we believe that the cluster of co-regulated genes in basal human breast cancer and PI/D mouse mammary tumors might be mechanistically associated to the activation of wnt pathway, which would play a significant role in re-activating tumor growth from progenitor basal cells after long dormancy periods.

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Fig. 6.2 PI/D gene expression signatures in human breast cancer. (**a**, **b**) Heatmaps of the PI/D gene expression signature in 1,114 human primary breast carcinomas obtained from four independent studies available in a public database. (**c**) Graph of interactions among the core of genes modulated in PI/D mammary tumors. Pathways are discriminated by different colors based on up-modulated (*red node*) or down-modulated (*green node*) transcripts. (**d**) Kaplan–Meier curves of overall and relapse-free survival among the 295 early-stage breast cancer patients obtained from van de Vijver et al. study (2002) based on groups of carcinomas with (cluster 2) or without (cluster 1) expression of the PI/D gene signature

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Part III

Role in Ovarian Cancer

7 Chemoresistance, Dormancy and Recurrence in Platinum Drug Therapy of Ovarian Cancers

Tamara A. Kalir and D. Stave Kohtz

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Abstract

Ovarian cancer is the most deadly of gynecological malignancies. Lineage analyses have suggested broadly classifying ovarian cancers into two types: Type I, which includes low grade cancers with intact TP53, and Type II, which comprises high grade cancers with defective TP53. If detected in early stages, surgical resection of ovarian tumors results in a high rate of long-term survival; however, most ovarian cancers are detected at advanced stages. The standard first line treatment for advanced stage ovarian cancer is maximal surgical cytoreduction followed by platinum-based combination chemotherapy. Although the overall prognosis for less aggressive Type I ovarian cancers is better, their response to chemotherapy is generally weaker than that of the Type II ovarian cancers. Despite an initially favorable response of optimally debulked Type II ovarian cancers to platinum-based chemotherapy, the rate of recurrence is high, making the long-term survival rate quite poor. The dynamics of the response of high grade ovarian cancers platinum suggest that the tumors are phenotypically heterogeneous, and that a subpopulation of tumor cells is relatively resistant to chemotherapy. The resistant tumor cell population persists after chemotherapy in a state of dormancy, with recurrent tumors arising upon transformation of the dormant cells back to malignant growth. This chapter will consider

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how lineage, histological subtype, and grade influence the differential responses of ovarian cancers to platinum-based chemotherapy. In addition, mechanisms contributing ovarian cancer resistance to platinum drugs and to tumor cell entry into and exit from dormancy will be discussed.

Introduction

Developmental Origins and Anatomy of the Ovaries

An understanding of the pathogenesis of ovarian cancer requires an appreciation of the embryologic development and anatomy of the ovaries. The ovary initially takes form as an 'indifferent gonad,' which first becomes apparent as a thickening of the intermediate mesoderm along the dorsal body wall, termed the urogenital ridge. The urogenital ridge eventually becomes two distinct structures, a gonadal ridge and a mesonephros. Some cells of mesonephric origin remain joined to the gonadal ridge, and this connecting remnant is the mesovarium. The gonadal ridge mass differentiates into a central part, the ovarian medulla, and a covering of a surface layer of flat to cuboidal, single layered cells called the germinal epithelium. Local steroid hormone action and other parenchymal microenvironmental cues are postulated to be involved in gonadal ridge differentiation. While the ovarian surface epithelial cells appears morphologically identical to other mesothelial cells lining the celomic cavity and shares common surface microenvironment by virtue of facing the abdominopelvic cavity, the ovarian surface epithelium is distinctive in being the only derivative of celomic mesothelium that does not express the marker CA125. This surface glycoprotein of unknown function is, in the adult an epithelial differentiation marker and tumor marker for ovarian and Mullerian duct-derived tumors. It is expressed by epithelial cells of the fallopian tube, endometrium and endocervix, and also by mesothelial cells lining the visceral and parietal peritoneum, pericardium and pleura. Its lack of expression in ovarian surface epithelium suggests that this epithelium may be subject to specific local microenvironmental influences that render it less committed to mature mesothelial phenotypic differentiation when compared to the rest of the pelvic peritoneal lining cells. However, CA125 expression re-emerges in a number of ovarian carcinomas, suggesting plasticity of the ovarian surface epithelium, at least under pathologic conditions.

Between the ovarian medulla and the surface epithelium, a number of ova are found. The immature ova originate from cells from the dorsal endoderm of the yolk sac. These cells migrate from near the allantois, along the hindgut to the gonadal ridge, where they undergo mitosis to become diploid stem cells called oogonia. In colonizing the primordial gonads, the oogonia migrate into the germinal epithelium, and are carried into the underlying stroma by bud-like ingrowths. The oogonia become surrounded by a layer of connective tissue cells, forming rudimentary follicles. The origin of the granulosa cells is still controversial. It is possible that mesonephric cells closely associated with the oogonia proliferate throughout development to form the granulosa cells, or that the granulosa cells develop from cells from the surface epithelium of the ovary and break apart into cell clusters that undergo follicular development. A tunica albuginea develops between the surface germinal epithelium and the ovarian medulla. The ovaries are formed in the abdominal cavity, and then descend into the pelvic cavity. This involves the gubernaculum, a fibrous tissue band that runs from the abdominal wall to the fundus of the uterus and limits descent of the ovary to the appropriate level. The portion of the gubernaculum that lies between the ovary and the uterus becomes the ovarian ligament.

Concurrently with the development of the ovaries, the celomic epithelium in the vicinity of the gonads invaginates to give rise to the left and right paramesonephric, or Mullerian ducts. The Mullerian ducts differentiate to eventually form the fallopian tubes, uterus and upper vagina. Thus, the perigonadal celomic epithelium represents an embryonic field with the ability to differentiate along multiple different pathways, which

include the mucosal epithelium of the fallopian tubes, endometrium, and endocervix. The close development of ovarian germinal epithelium and Mullerian epithelium are noteworthy for our discussion of ovarian and peritoneal surface carcinogenesis, specifically in relation to the 'field effect' hypothesis. This will be explicated further below.

Anatomically, the ovaries are paired, almond-shaped organs which lie within the pelvic cavity on either side of the uterus, at the level of the bifurcation of the common iliac artery. Each ovary is attached to the uterus by an ovarian ligament, and to the pelvic side wall by a suspensory ligament which additionally houses the ovarian blood and lymphatic supply. The ovary lies in an ovarian fossa, or shallow depression in the posterior part of the broad ligament of the uterus. As previously mentioned, the ovary is unique in the abdomino-pelvis in not being covered by the peritoneum that invests all other organs. The ovarian surface lining, a modified peritoneum called the germinal epithelium, is in continuity with the visceral peritoneum covering adjacent organs. The absence of a peritoneal covering is to allow an egg cell after to gain access after ovulation to the infundibulum of the closely apposed fallopian tube. It is this association that is implicated among the various pathways of ovarian carcinogenesis.

Risk Factors for Ovarian Cancer

Cancer is not a new disease. As early as 3000–1500 B.C. in ancient Egypt, there were documented descriptions of cancers of the breast, among other tumors, and the disease was attributed to acts of the gods. In the fifth century B.C., Hippocrates broke from tradition by postulating that cancers are due to natural causes, specifically resulting from imbalances in bodily 'humors'. A further development occurred in the Middle Ages, when identification of families and villages with high incidence of cancer brought forth the concept that cancer may be due to either inherited or environmental causes. A new frontier was entered when Rudolf Virchow examined tumors under the microscope, and made the observation that "every cell is born from another cell." He established cancer as a cellular disease. Twentieth- and twenty-first century advances in cancer biology are largely the result of developments in the field of molecular genetics. Indeed, cancer today is considered a genetic and epigenetic disorder, with the identification of its association with disease-specific gene mutations and heritable defects in chromatin organization and modification.

While ovarian cancer is rare, it is the most common cause of death from gynecologic malignancy in women in the United States, and is the fifth leading cause of cancer deaths among American women. Despite its lethality, the etiology of ovarian cancer is poorly understood. Family history is the most important and best defined risk factor to date, although it accounts for only 5–10% of cases. Women with two or more affected relatives or a relative diagnosed under 50 years of age, have the highest risk. There are three types of hereditary ovarian cancer syndromes with autosomal dominance:

(i) Hereditary breast/ovarian cancer syndrome, which shows the highest risks (up to 50%) for women with family histories of breast or ovarian cancer and mutations in the BRCA1 and BRCA 2 genes. The BRCA1 gene is present in two copies, one located on paternal chromosome 17, and the other on maternal chromosome 17. The BRCA2 gene is similarly present in two copies, one paternal and the other maternal, located on each chromosome 13. An individual who inherits one mutated copy of either of these genes from a parent, may never develop cancer but has an increased risk of up to ~50%, if the other chromosomal copy of the gene acquires mutation and, additional mutational events occur.

(ii) Lynch Syndrome II or hereditary nonpolyposis colon cancer/ovarian cancer, refers to those ovarian cancers which occur in families with a high incidence of cancers of the colon and endometrium. It is associated with mutations in DNA mismatch repair genes: hMSH1, hMSH2, hPMS1, and hPMS2.

(iii) Hereditary site specific ovarian cancer refers to cases for which there is a family history

of ovarian cancer but no specific gene has been identified. Some of these cases may not be a direct result of a gene mutation, but rather of mutation resulting from group exposure to a specific environmental carcinogen by virtue of a common habitat or other shared practices.

There are other, less strongly associated risk factors for ovarian cancer. Endometriosis has a rare, roughly 1% incidence of malignization which, when it occurs results in the development usually of either endometrioid or clear cell carcinomas. Co-existing with endometriosis, infertility is also considered a risk factor for ovarian cancer. Early menarche and late menopause are weakly associated risks. Factors considered to decrease a woman's risk for ovarian cancer include: pregnancy (inverse relationship, with more pregnancies conferring greater protection), breastfeeding, and oral contraceptive use (inversely related, with longer use conferring greater protection). These reproductive, menstrual and hormonal factors have been associated with lifetime number of ovulations, which are felt to be directly related to ovarian cancer risk. It has been postulated that the disruption of the ovarian surface epithelium is the inciting event for development of cancer, particularly in the case of high-grade serous carcinomas. More specifically, the damage sustained by the surface tissue during ovulation, and the resulting repair process and inflammation that necessarily ensue, provide the opportunity for genetic aberrations including mutations that may eventually result in cancer. Conversely, decreased ovarian function resulting from surgical factors such as hysterectomy or fallopian tubal ligation, which aore thought to lead to partial devascularization and fewer ovulations, are protective and have been found to be associated with decreased ovarian cancer risk. Other risk factors that have been proposed in the past, but have not been proven to be of significant risk, include: talc exposure to diaper area in female infants, dietary fat, smoking, obesity, use of menopausal hormones, alcohol, caffeine, and environmental and occupational risk factors.

Histopathogenesis of Ovarian Cancer

Current thinking is that ovarian epithelial cancer arises in one of two ways: (1) by de novo transformation without an identifiable precursor lesion, believed to occur in the development of high-grade serous carcinomas; or, (2) by a stepwise progression from hyperplasia to adenoma, to borderline tumor to cancer, believed to occur in the development of low-grade serous carcinomas, mucinous carcinomas, and endometrioid and clear cell carcinomas. Most endometrioid and clear cell carcinomas are believed to arise through a stepwise series of transformations, and many of these tumors are found with co-existing endometriosis which, though largely a benign condition, is thought to be a precursor. Only a small fraction of endometriosis cases, less than 1%, undergo malignant transformation. In many endometriosis-associated cancers, the diagnostic pathologist is able to find associated hyperplasia, adenofibroma, or borderline malignant areas which are suggestive of an antecedent process. In contrast high-grade serous carcinomas, previously thought to occur via this transformation spectrum, are currently believed to arise de novo from the surface epithelium of the ovary or, in some cases from the fallopian tube mucosal epithelium. Molecular biological evidence supports this dualistic model of ovarian carcinogenesis: the genesis of high grade serous carcinomas appears to be driven by early mutations that promote genomic instability, in particular TP53 mutations (Shih Ie and Kurman 2004), whereas low-grade serous carcinomas exhibit mutations in K-ras and BRAF genes, endometrioid tumors harbor pTEN gene mutations, and mucinous tumors show mutations in the K-ras gene, all in the context of nominal TP53.

Recently, it has been proposed that many high-grade serous carcinomas of the ovary or peritoneal surface may actually arise from the mucosa of the fimbriated end of the fallopian tube. Lee et al. (2007) found eight cases of ovarian cancer with co-existing in situ carcinoma of the fallopian tube mucosa, and identified the same TP53 gene mutations in both sites. Further,

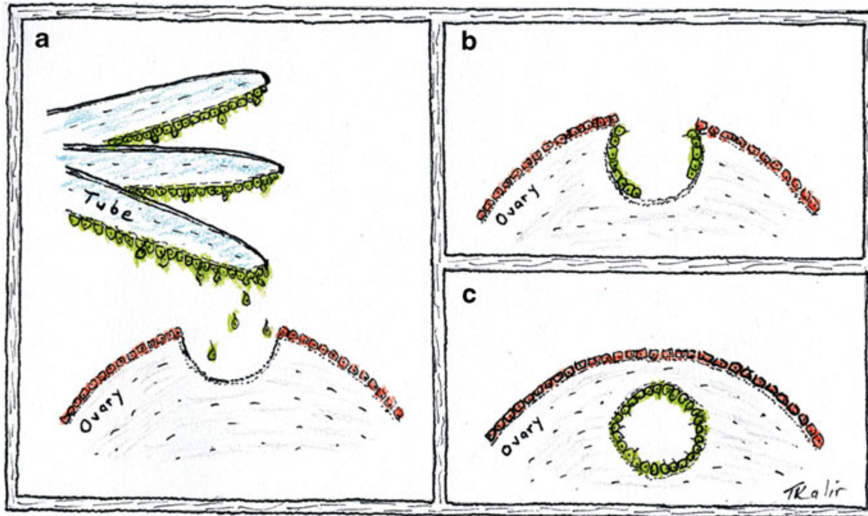

Fig. 7.1 *Panel A* shows exfoliation of some fallopian tube mucosal epithelial cells onto the surface of the ovary in an area disrupted by a recent ovulation. *Panel B* shows the proliferation of the fallopian tube epithelial cells along the disrupted ovarian focus. *Panel C* shows that, under the influence of proliferating ovarian surface epithelial cells causing reepithelialization of the ovarian surface and possible epithelial-to-mesenchymal cell conversion inducing formation of a matrix, the fallopian tube cells become walled off, forming a cystic 'inclusion'

Kindelberger et al. (2007) showed that in cases of primary peritoneal surface carcinoma, more than half of the fallopian tubes displayed mucosal involvement, often with in situ carcinoma. In a series study of risk-reducing salpingooophorectomy specimens from women with BRCA gene mutations, Medeiros et al. (2006) found clusters of TP53 defective cells and intraepithelial carcinomas in the mucosa of the fallopian tubes of roughly 30% of the women, while these changes were not present on the ovarian surface. Crum et al. (2007) studied prophylactic salpingooophorectomies of high-risk patients, and found that for incidental cancers, more than half occurred in the distal fallopian tube, supporting the idea that the distal fallopian tube is the site of origin of the majority of ovarian/fallopian tube cancers in high-risk women. Figure 7.1 illustrates the process, and the mechanism involved is as follows: After ovulation and prior to repair of the disrupted ovarian surface focus, the fimbriated end of the fallopian tube which is closely apposed to the ovarian surface, may exfoliate cells from its mucosal epithelium that implant in the unrepaired, disrupted site on the ovarian surface. As a result of ovarian surface germinal epithelial cell proliferation and synthesis of connective tissue type components of the extracellular matrix, the implanted fallopian tube epithelial cells also proliferate and become walled off from the native ovarian parenchyma, forming a cystic epithelial inclusion. Notwithstanding epigenetic and genetic changes that could impede this process from occurring, surface germinal epithelial cells could also contribute to formation of epithelial inclusions of ovarian surface epithelial origin, and these may subsequently undergo metaplastic change to resemble tubal epithelial cells. Hence, it remains unclear whether intraovarian epithelial inclusions which morphologically resemble fallopian tube cells, are truly of tubal origin.

It is noteworthy that high-grade serous carcinomas comprise the vast majority of ovarian cancers (roughly 80%). These tumors are quite unlike the simple, low-cuboidal normal ovarian surface epithelial cells, and in fact more closely resemble Mullerian-duct epithelia. Specifically, high-grade serous carcinomas of the ovary, fallopian

tube, endometrium and endocervix appear histologically and phenotypically indistinguishable. This supports the hypothesis of the tubal origin of at least a subset ovarian cancers. Because most patients with high-grade serous carcinomas present with advanced stage disease and bulky tumors involving multiple organs, the decision as to whether a tumor is fallopian tubal or ovarian in origin can, for a small subset of cases, be a daunting task for the diagnostic pathologist. Because the gross and microscopic appearances, and the patterns of spread are similar for these two entities, the determination of the organ of origin may at times be arbitrary. Historically, in the absence of other helpful findings, the organ with the greatest amount of tumor, usually the ovary, was considered the primary. However, Crum et al. (2007) have recently challenged this convention by proposing that fallopian tube fimbrial cancers may exfoliate cells which disseminate to the peritoneum, and that secondary sites of deposition may be larger than the primary.

The sequential histological changes that precede development of fallopian tube carcinoma have not been well elucidated. Fallopian tube mucosal epithelial hyperplasia, demonstrated by a proliferation of the pseudostratified columnar cells in the absence of marked cytologic atypia and mitotic activity, has been \traditionally considered to a variant of normal and not precancerous. Its incidence is fairly low. Fallopian tube preinvasive carcinoma (dysplasia or carcinoma in situ) was a rare diagnosis until the advent of risk-reducing salpingooophorectomy for women with BRCA gene mutations (Medeiros et al. 2006). The diagnosis of in situ carcinoma of the fallopian tube requires the presence of marked cytologic atypia, multilayered epithelium containing cells with large, pleomorphic nuclei and prominent nucleoli, and interspersed mitotic activity.

Multifocal carcinomas that involve the fallopian tube show three patterns: (1) synchronously detected multifocal carcinomas within the fallopian tube or tubes; (2) multifocal carcinoma involving various genital organs including the fallopian tube or tubes; and (3) direct spread of cancer, frequently intraepithelial carcinoma along the mucosa of the cervix and endometrium, to the fallopian tube. Roughly 20% of patients with fallopian tube cancer have bilateral involvement. It is not clear whether these additional foci of carcinoma represent synchronous tumors or metastasis. Woodruff et al. (1985) have proposed that multifocal disease may reflect neoplastic transformation of the common embryologic field, which includes the coelomic epithelium that covers the ovary, fallopian tube, and other pelvic peritoneum. The idea of a so-called field effect was first proposed by Slaughter et al. (1953) in his ‘field cancerization’ hypothesis, put forth to explain the development of multiple primary tumors and locally recurrent cancer. Initially a stem cell acquires genetic alterations and forms a patch, or clonal unit of altered daughter cells. Such patches can be recognized by TP53 mutations. The patch is converted into an expanding field, after additional genetic alterations are acquired and the cell displays a growth advantage over its neighbors; its proliferative field eventually displacing neighboring normal mucosal epithelial cells. Also in favor of this hypothesis is the observation that noncontiguous endometrioid carcinomas are not infrequently found simultaneously in the endometrium, ovary and fallopian tube mucosa. The frequent finding in these cases of associated endometriosis has implicated it as the site of multifocal neoplastic ‘field’ transformation. However, the debate remains unsettled as to whether these are multiple primary tumors or a single primary with metastases.

Ovarian cancers have a characteristic tendency to spread via the mechanism of exfoliation into the peritoneal cavity. Exfoliated cells are propelled by the normal circulation of the peritoneal fluid upwards along the right paracolic gutter to the undersurface of the right hemidiaphragm. Here they may implant and grow as surface nodules. The omentum is also a favored site of involvement, although all intraperitoneal surfaces are at risk. Exfoliation and implantation are one of two primary modes of spread of ovarian cancer. The other mode is lymphatic. Ovarian cancer cells metastasize via the retroperitoneal lymphatic spaces that drain the ovary. These follow the ovarian blood supply in the infundibulopelvic ligament and terminate in aortic and vena caval

lymph nodes, up to the level of the renal vessels. Lymphatic channels also pass laterally, through the broad ligament of the uterus and parametrial channels alongside the uterus, to terminate in the pelvic sidewall lymphatics, including the external iliac, obturator, and hypogastric chains. Spreading may also occur along the round ligament of the uterus, with resultant involvement of the inguinal lymphatics. Lymph node metastases are correlated with stage of the disease, and retroperitoneal lymph node involvement is found in a number of cases of advanced ovarian cancer.

Platinum Chemotherapy of Ovarian Cancer

Mechanisms of Platinum Cytotoxicity

Platinum drugs (carboplatin and cisplatin) have been employed with surgical debulking as adjuvant chemotherapy in combination with taxanes (docetaxel or paclitaxel) or cyclophosphamide for advanced ovarian cancers, and combinations with taxanes have been shown to be superior as a first line treatment (Piccart et al. 2000). Platinum chemotherapeutic reagents such as cisplatin form mostly intrastrand and some interstrand DNA adducts by crosslinking N7 positions of purines. Three intrastrand crosslink (1,2-d(GpG), 1,2-d(ApG) and 1,3d(GpNpG)) comprise 90% of the DNA lesions generated by cisplatin, while interstrand crosslinks comprise around 5% (Eastman 1986). The intrastand crosslinks are repaired by nucleotide excision repair (NER), whereas interstrand crosslinks (ICLs) are repaired by ICL repair, which is less well understood. Mechanisms of ICL repair have been proposed in which mammalian cells use novel excision repair reactions (requiring the XPF and ERCC1 proteins) to uncouple the crosslink (McHugh et al. 2001). The abundance of unrepaired interstrand crosslinks may be more closely associated with induction of apoptosis in cancer cells by cisplatin than the abundance of intrastrand crosslinks. Defects in ERCC1 (Damia et al. 1996) or low expression of ECRR1-XPF endonuclease (Arora et al. 2010), the latter of which is observed in metastatic testicular germ cell tumors, renders cancer cells exquisitely sensitive to cisplatin (Usanova et al. 2010).

The presence of platinum drug DNA adducts blocks both the progression of DNA replication forks and transcription, resulting in the activation of ATR; ineffective repair of crosslinks can lead to double strand breaks, resulting in activation of ATM signaling pathways. DNA replication forks stalled by intranuclear or internuclear crosslinks recruit ATR through ATRIP, resulting in assembly with TopBP1 and the 9-1-1 (Rad9, Rad1, Hus1) complex (Yan and Michael 2009). Depending on the cellular context, activated ATR kinase will phosphorylate several proteins, including effectors of cell cycle arrest (e g., H2AX, p53, Chk1), DNA repair (BRCA1/2), and apoptosis (p53, FANCJ). An early event in the response to DNA damage is the phosphorylation of H2AX by ATR, giving rise to intranuclear foci of phosphorylated histone H2AX. Accumulation of phosphorylated H2AX foci occurs within 4 hours after exposure to cisplatin, and depending on the dose and duration of cisplatin exposure, apoptosis is observed 18 or more hours later (Manju et al. 2006). Poly (ADP-ribose) polymerase (PARP-1) cleavage has been shown in some cancer cells to occur between 16 and 24 h after treatment with cisplatin (Manju et al. 2006). This lag time is an important characteristic of cisplatin-induced cell death. The events that precipitate apoptosis in ovarian carcinoma cells after exposure to cisplatin are not completely understood, but cytotoxic sensitivity to cisplatin is maintained, and possibly enhanced, in the face of defects in TP53 that are characteristic of the high grade forms of ovarian cancer. Implicated in the cytotoxic response of ovarian carcinoma cells to cisplatin is activation of ERK, and long-term sustained activation of SAPK/JNK and p38 kinases, followed by downstream induction of AP-1 and FAS-L expression (Mansouri et al. 2003). Enhanced accumulation of Fas-L and its binding to Fas (receptor) leads to activation of caspase 8, downstream activation of other caspases, and activation of mitochondrial death pathways (Muzio et al. 1998). Cells with defects in nucleotide excision repair cross complementing genes (ERCC1-4) display cytotoxic hypersensitivity to

cisplatin, suggesting that accumulation of DNA lesions also plays a role in the induction of cell death (Damia et al. 1996). Some studies have indicated that cisplatin lesions in DNA directly block transcription, leading to apoptosis (Ljungman and Lane 2004).

Molecular Lineages in Ovarian Cancer: Impact on Tumor Behavior and Outcome of Platinum Chemotherapy

As with most solid tumors, the majority of ovarian cancers and their metastasis have clonal origins (Khalique et al. 2009). A clonal origin for the tumor does not necessarily indicate that all the tumor cells are genetically identical, as intratumor genetic heterogeneity arises during tumor development (Khalique et al. 2007). In addition, epigenetic mechanisms can contribute phenotypic variability within the tumor cell population, including overt changes in cellular morphology of subpopulations of tumor cells, such as epithelial to mesenchymal transitions (Wu et al. 2012). Ovarian cancers differentiate into histologically distinct subtypes that resemble normal tissues: serous, endometrioid, and mucinous ovarian cancers resemble cells that line the glandular fallopian tube, endometrium, and endocervix, respectively. Clear cell ovarian cancers resemble cells found in nests in the vagina, and this histological relationship is suggested by the development of vaginal clear cell adenocarcinoma in daughters whose mother took diethylstilbestrol during pregnancy (Laronda et al. 2012). A slew of rarer histological subtypes, such as mixed and transitional, also appear as ovarian cancers. Within histological subtypes, macro-architecture also may impact tumor clinicopathological behavior (Veras et al. 2009).

The resemblance between histological subtypes of ovarian cancers and normal tissues is more than a coincidence. Patterns of gene expression in different histological subtypes of ovarian cancer correlate with those in the normal tissues they resemble. Colinear expression of *Hoxa9* (follapian tube primordia), *Hoxa10* (developing uterus) and *Hoxa11* (low uterine and cervical primarodia) genes is observed in Mullerian duct development in the mouse embryo (Taylor et al. 1997), but these genes are not normally expressed in adult epithelial cells lining the ovaries or inclusion cysts (Naora 2005). Expression of *HOXA9*, *HOXA10* or *HOXA11* are observed in serous, endometrioid, and mucinous ovarian cancers respectively (Cheng et al. 2005), indicating that histological phenotypes are driven by aberrant activation of normal developmental pathways. Over 90% of ovarian cancers are thought to arise from the epithelium that lines the surface of the ovary, ovarian inclusion cysts, or the fimbriae of the fallopian tubes, and are referred to collectively as epithelial ovarian cancers. This epithelium has been described as coelomic, mesothelial or transitional, but gene expression profiling studies have suggested that it is multipotent, expressing genes that maintain stem cell characteristics (Bowen et al. 2009). As such, these cells would have the potential to give rise to neoplasias with different histological subtypes, and although neoplastic ovarian cells lose expression of some stem cell genes, why they develop into and sustain specific histological subtypes is not understood. As will be discussed below, a fraction of the tumor cells may maintain a stem cell phenotype (cancer stem cell), and in contrast to the larger mass of histologically differentiated cells, this population may be responsible for tumor growth and recurrence after therapy.

Although ovarian cancer is often viewed clinically as one disease, histological (or morphological) subtypes of ovarian cancer can exhibit different behaviors, prognoses and responses to therapeutic intervention. The standard treatment for epithelial ovarian cancers is surgical debulking and chemotherapy with platinum analogues plus paclitaxel (Piccart et al. 2000). In contrast to advanced ovarian carcinomas displaying serous and endometrioid histology, advanced stage ovarian carcinomas displaying clear cell and/or mucinous histologies have been associated with shorter progression-free intervals and worse overall survivals after platinum/taxane-based regimens (Bamias et al. 2010; Goff et al. 1996). It is not known whether histological parameters

per se affect tumor behavior, including the responses to platinum/taxane therapy, but as manifestations of intrinsically different molecular diseases they could be considered as semaphores in determining appropriate therapeutic courses or in highlighting the need for development of more effective regimens. For example, the resistance of the ovarian clear cell subtype to conventional platinum-based chemotherapy has encouraged further investigation into the unique aspects of its molecular pathogenesis (Tan et al. 2013). When using histological subtype or grade to determine the therapeutic course or progress of therapy, it is important to consider that chemotherapy itself can significantly alter the histological of ovarian tumors (McCluggage et al. 2002).

A recent classification of ovarian cancers into two types considers grade and histology, but emphasizes molecular lineage as the prime determinant of tumor behavior (Shih Ie and Kurman 2004). Type I ovarian cancers are of lower grade, often confined to the ovary, and are thought to arise from a precursor lesion (e.g., adenofibromas or borderline tumors) by stepwise progression through a relatively well-defined series of mutations (Lim and Oliva 2013). Type I tumors in comprise low-grade serous, low-grade endometrioid, clear cell, and mucinous carcinomas, and Brenner tumors. Type II tumors are of higher grade, and are thought to arise from an initial event causing genomic instability, followed by clonal expansion and additional mutational hits (Landen et al. 2008). The factors initiating genomic instability may be associated with changes in the status of TP53, which is predominantly abnormal in Type II ovarian tumors, whereas in Type I tumors it is normal (Ayhan et al. 2009; Roh et al. 2010). In addition, genomic instability may be promoted in Type II tumors by defects in BRCA1 or BRCA2. In a study of high grade serous ovarian cancers with TP53 abnormalities, defects in BRCA caused by either germline mutations, somatic mutations, or methylation was observed in 50% of cases (McAlpine et al. 2012). Further, defects in BRCA were not observed in a group of non-high-grade serous cases. Although TP53 abnormalities could be considered a signature for high grade serous ovarian tumors, only a subset of these tumors display BRCA abnormalities, and the functional relationship between BRCA and TP53 abnormalities in ovarian cancers is not established. It is reasonable to suggest that loss of BRCA DNA repair pathways through genetic or epigenetic mechanisms may promote the generation of TP53 defects in the precursor cell population of high grade ovarian carcinomas. As genomic destabilization appears to be an essential early event for development of Type II ovarian carcinomas, defects in TP53 may be considered driver mutations in these malignancies.

Overall, Type I ovarian tumors are more indolent than Type II, with those presenting clear cell histology being the most aggressive. A two tier grading system of serous ovarian cancers (Malpica et al. 2004) revealed that survival of patients with low grade tumors (Type I) was significantly higher than those with high grade tumors (Type II). In addition, patient with Type I tumors have a better prognosis following surgery (Braicu et al. 2011). On the other hand, clinical and in vitro evidence has revealed that primary and recurrent Type I ovarian cancers are less responsive to platinum/taxane-based chemotherapy than Type II (Santillan et al. 2007). As indicated above, advanced clear cell or mucinous ovarian cancers respond poorly to cisplatin/taxane treatment (Bamias et al. 2010; Goff et al. 1996). Because of the more indolent nature of many Type I ovarian cancers, however, some studies have shown median overall survival time of patients treated with platinum/taxane is higher for Type I than Type II ovarian cancers (Bamias et al. 2012), an observation significantly affected by the higher baseline survival of patient with Type I cancers. The prevalence of TP53 defects in Type II ovarian cancers, and their absence from Type I cancers, suggests that in ovarian cancer cells TP53 function may be enigmatic, and protect cancer cells against the cytotoxic effects of platinum chemotherapy. Depending on the cellular setting, TP53 promotes apoptosis, senescence or cell cycle arrest. Functional TP53 cooperates with chemotherapy when the therapeutic agent activates its functions in apoptosis or senescence. Chemotherapeutic regimens that

activate the cell cycle arrest function of TP53, however, may be more effective in TP53-defective tumors, as cell cycle arrest by TP53 can confer resistance (Bunz et al. 1999; Johnson and Fan 2002). While TP53 activation may contribute to the cytotoxic response of normal cells such as kidney epithelium to platinum (Wei et al. 2007), the cytotoxic response of Type II ovarian cancers to platinum reagents must involve TP53-independent pathways. In fact, the wild-type TP53 characteristic of Type I ovarian cancers may induce cell cycle arrest and provide a protective effect. Consistent with this notion, some studies have shown TP53 defects to be positively associated with survival in the short term (Hall et al. 2004).

Low grade Type I ovarian cancers are associated with a prevalence of defined genetic changes in certain signaling pathways, including mutations in *KRAS*, *BRAF* and *ERBB2* and activation of MAPK signaling in low grade ovarian serous carcinoma (Anglesio et al. 2008; Sundov et al. 2013); mutations of *PIK3CA*, inactivation of *PTEN*, and activation of phosphatidylinositol 3-kinase/AKT pathways in ovarian clear cell carcinoma (Tan et al. 2013); and *CTNNB1* (β-catenin), *PTEN* mutations, and activation of Wnt/β-catenin signaling pathways in low grade ovarian endometrioid cancers (Geyer et al. 2009). As Type I ovarian cancers respond poorly to platinum chemotherapy, arise from identifiable progenitors, alter specific signaling pathways during progression, and display genetic stability, efforts to profile genetic or expression markers that would predict outcomes of individual Type I ovarian cancers after platinum chemotherapy have been limited. In contrast, Type II ovarian cancers arise from cryptic precursors, activate a wider range of signaling pathways and display genetic instability. Type II ovarian cancers initially respond well to platinum/taxane adjuvant therapies, but eventually recur after a range of disease-free intervals. Identification of gene expression or immunochemical markers with predictive value in determining disease free interval or survival in independent or multiplex analyses would provide a means of tailoring treatment regimens or of identifying patients that would require novel therapies. Studies of platinum drug resistance often do not distinguish between Type I and Type II ovarian cancers, and therefore end up characterizing molecular differences between the cancer types rather than specific differences that may contribute to variations in the responses of individual tumors.

As TP53 mutations are present in most type II ovarian carcinomas, and the few that have nominal TP53 activity may have copy number gain in MDM2 or MDM4, TP53 status is not a useful predictor of drug resistance or clinical outcome in these cancers (Ahmed et al. 2010). On the other hand, defective BRCA 1 or 2 is observed in only 10% of all ovarian cancer cases, and defects in BRCA 2 are associated with better survival and therapeutic response than those bearing defective BRCA 1 or wild-type BRCA genes (Liu et al. 2012; Yang et al. 2011). Further prognostic breakdown of high grade cancers using gene expression profiling approaches, however, has proved challenging. The expression pattern of a panel of 11 genes has been shown to have predictive value in determining survival of patients with high grade serous treated with carboplatin and paclitaxel (Gillet et al. 2012). A connection between clinical endpoints and genetic alterations in high grade serous type ovarian carcinomas has noted eight regions of amplification or deletion on five chromosomes that clustered into subgroups, suggesting that high grade serous cancer may be segregated into clinically distinct subgroups (Engler et al. 2012).

Platinum Resistance and Ovarian Cancer Recurrence

Mechanisms of Platinum Drug Resistance

Diverse molecular mechanisms have been proposed to contribute to the acquisition of resistance to organoplatinum compounds and other therapeutic agents, including modulation of drug uptake and efflux, enhanced mechanisms of detoxification, inhibition of apoptosis, and recovery or enhancement of DNA repair mechanisms.

Genes or gene products observed to affect cisplatin sensitivity of cancer cells include: metallothionein, thought to be an intracellular metal sink; CCND1, a G_1 cyclin (Noel et al. 2010); ERCC1, an enzyme involved in DNA excision repair (Damia et al. 1996); glutathione S-transferase (GST), thought to modulate signal transduction kinase cascades in response to stress (Townsend et al. 2005); and interleukin 6, a cytokine (Wang et al. 2010). Modulators of apoptosis, including TP53, X-linked inhibitor of apoptosis protein, and Akt have been found in cultured ovarian cancer cells to be interdependent determinants in the response to cisplatin (Abedini et al. 2009), and alterations in the TP53 gene have been associated with variable responses to cisplatin of cultured ovarian cancer cell lines (Perego et al. 1996). Since the majority of type II ovarian cancers contain defective TP53, the relevance of cell culture studies of TP53 in differential responses of high grade ovarian carcinoma cells to cisplatin remains unclear. Finally, in addition to cell autonomous mechanisms, the tumor microenvironment is thought to play an important role in determining chemoresistance of ovarian cancers (Chien et al. 2013).

Defects in FANC-BRCA pathways are associated with genomic instability and enhanced sensitivity to platinum drugs in a subset of ovarian carcinomas. During oncogenic progression, methylation of FANCF or BRCA1 occurs in a subset of ovarian carcinomas (perhaps as high as 20%), resulting in genomic instability, and thereby promoting other neoplastic changes (Taniguchi et al. 2003). Subsequent reversion of these genes to demethylated forms restores drug-resistance to this subset of cancers. Familial forms of ovarian carcinoma represent 10–15% of cases, are associated with inherited defects in BRCA1/2 that compromise DNA repair functions, and are hypersensitive to platinum reagents. While the prognosis of cisplatin treatment is better for patients developing BRCA1/2 defective ovarian carcinomas, these cases relapse into drug-resistant forms, frequently driven by secondary (reversion) mutations in BRCA1/2 (Sakai et al. 2008; Swisher et al. 2008). Somatic mutation of BRCA1/2 in sporadic ovarian carcinomas is rare, and while changes in BRCA1/2 expression also should be considered, other distinct mechanisms undoubtedly contribute to drug resistance.

Epigenetic Contributions to Platinum Drug Resistance

The epigenetic machinery of the cell includes DNA methylation, histone modifications, non-coding RNAs, and chromatin remodeling and organization, the latter being affected by the first three as well as by the architecture of the nucleus. Epigenetic regulatory mechanisms may work in concert with somatic mutations to drive tumor progression and promote tumor cell survival. An association has been noted between CpG island methylation of the MLH1 mismatch repair gene and relapsed invasive ovarian cancers, and this can be reversed in xenographic models by demethylating agents (Zeller et al. 2012). There is clinical evidence that the hypomethylating agent azacitidine can partially reverse platinum resistance in patients with ovarian cancer (Fu et al. 2011). Low dose decitabine administered before platinum to resistant ovarian cancers has been shown to alter methylation of MLH1, RASSF1A, HOXA10 and HOXA11 and positively correlate with progression free interval (Matei et al. 2012). Intriguingly, recent studies have suggested that HDAC4 is enriched post-treatment in cisplatin resistant cells of high grade serous ovarian cancers, and HDAC4 promotes cisplatin resistance through deacetylation of STAT1 (Stronach et al. 2011). Contingent on gene and tissue type, metazoan genes localized adjacent to the nuclear envelope generally tend to be suppressed, whereas genes localized centrally in the nucleoplasm tend to be transcriptionally active (Malik et al. 2010). Chromatin organization appears to be influenced by its proximity to the NPC, as channels of euchromatin interrupt the lamina and extend from the NPC into the nucleus. Alterations in nuclear pore structure, possibly by affecting the architecture of underlying chromatin, may play an important role in determining sensitivity or resistance of ovarian

cancer cells to platinum-based chemotherapy (Kinoshita et al. 2012).

Regrowth of tumors from remnant tumor cells present an important challenge to the success of cancer chemotherapy. Adding to the complexity of this challenge, the expression of drug resistance by remnant tumor cells does not appear to depend on genetic modifications of tumor cells, but rather may be conferred by ephemeral epigenetic changes that can disappear upon regrowth of the tumor. Thus, growth of remnant cells does not necessarily result in the generation of chemoresistant tumors. A study of retreatment of ovarian cancer patients with platinum drugs after remission and relapse with the same regimen of chemotherapy revealed surprisingly high probabilities of success (Seltzer et al. 1985). A review of retreatment of relapsed tumors of different types revealed that transient resistance to chemotherapy is common (Cara and Tannock 2001), and statistically discounted somatic mutation as the prevailing mechanism, suggesting instead epigenetic modalities. Recent studies of tumor cells in culture have shown that populations of drug-tolerant cells persist after treatment with different chemotherapeutic agents, and that these cells are mostly quiescent and express surface markers in common with cancer stem cells (Sharma et al. 2010). When these cells remain in culture in the presence of drug, a fraction give rise to colonies of cells with relatively stable tolerance, and that cell population remains tolerant in the presence of drug and for many generations after drug removal. The frequency with which tolerant and stably tolerant cells appeared in these experiments also suggest mechanisms that do not involve genetic mutations, and further studies indicated that chemical agents that inhibit histone demethylation also block acquisition of drug tolerance, suggesting an epigenetic mechanism (Sharma et al. 2010).

Profiling of microRNA (miR) expression in ovarian cancer patients treated with platinum-taxane therapy identified a signature of miRs 484, 682, and 214 that predict sensitivity, and that miR484 operates by modulating tumor vasculature through modulation of VEGFB and VEGFR2 pathways (Vecchione et al. 2013). Although no difference was observed among serous tumors, miR-141 (which targets KEAP1, a gene involved in oxidative stress) levels were higher in non-serous ovarian tumors that did not respond well to therapy (van Jaarsveld et al. 2012). miR130a is upregulated in cisplatin-resistant cultured cell lines, and may be indirectly associated with MDR1 and P-glycoprotein conferred resistance (Yang et al. 2012) Two microRNAs, miR-152 and miR-185, increase the sensitivity of cultured ovarian carcinoma cell lines to cisplatin in vitro and in vivo, and have been found to target DNA methyltransferase 1 directly (Xiang et al. 2014).

Cancer Stem Cells, Cellular Dormancy and Recurrence of Ovarian Cancer

The cancer stem cell (CSC) hypothesis arose from the concept that tumor cells of a single genotypic origin can display different cancer phenotypes. Historically, transplantation of tumor cells between animals or between cultures plates and animals was observed to require a larger number of cells than would be predicted from the clonal nature of the tumor, and further studies revealed that subpopulations of cells could be selected or sorted from the total tumor cell population that were more efficient at initiating new tumors. These cells comprised a small fraction of the total tumor cells, and similar to embryonic stem cells, have the capacity for self-perpetuation as well as for giving rise to other types of cancer cells. Cell surface marker, transcriptomic, and signaling systems analyses revealed similarities as well as differences between CSCs from different tumor types, as well as similarities between CSCs and embryonic stem cells or induced pluripotent stem cells (Schoenhals et al. 2009). To account for the self-renewal of CSCs and the accumulation of the tumor mass of non-CSCs, CSCs are thought to undergo asymmetric mitoses, yielding both CSCs and the "differentiating" cells that comprise the majority of the tumor load (Fig. 7.2). The latter may proliferate more rapidly than CSCs, but eventually become terminal. Positive correlations between the prevalence of cells

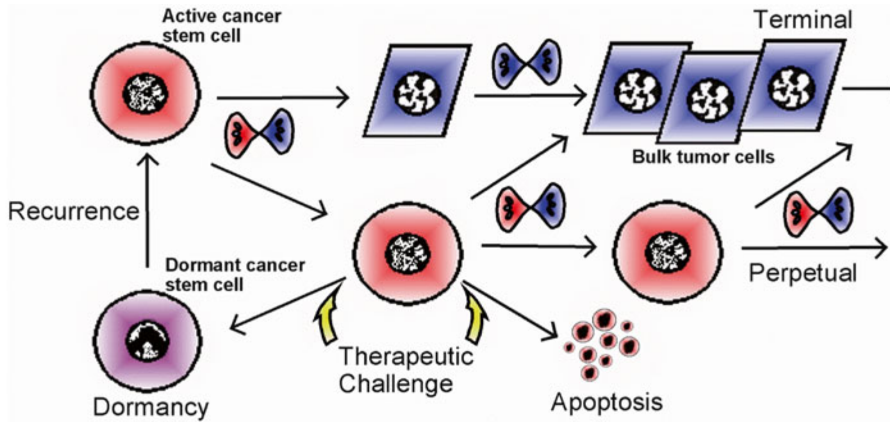

Fig. 7.2 Defining the relationships between cellular dormancy, tumor recurrence, and cancer stem cells. *Red*: actively growing cancer stems cells; *blue*: bulk tumor cells; *purple*: dormant cancer stem cells. Asymmetric mitoses are indicated as *red/blue*

bearing CSC markers in ovarian cancers and recurrence have been reported (Steffensen et al. 2012). Cancer stem cells for epithelial ovarian cancers may be the source of progenitor cells for metastases. Metastasis of CSCs is promoted by epithelia to mesenchymal transition, a process evidenced by their ability to form spheroids, and subsequent mesenchymal to epithelial transitions, and both processes appear to be mediated by changes in the intracellular stability of TWIST1 (Yin et al. 2013).

Cancer stem cells have been identified in epithelial ovarian malignancies by several methods. In FACS analyses, a side population of ovarian tumor cells that excludes Hoechst dyes has been shown to display enhanced tumor-initiating properties in xenografts (Szotek et al. 2006). Cell populations with tumor-initiating properties have been isolated from ovarian cancers using positive selection with surface markers such as CD44. Marker analyses have revealed that tumor initiating cells phenotypically resemble CSCs from other tissues, expressing some of all of the following: CD44, CD24, Epcam, CD133, ALDH1, MyD88, and CD117 (Kryczek et al. 2012; Meirelles et al. 2012; Steffensen et al. 2012), as well as of pluripotency markers such as β-catenin, Oct-4, and SSEA-4 (Alvero et al. 2009b). Loss of expression of other markers, such as Ecadherin (Meirelles et al. 2012), also are associated with the CSC phenotype in ovarian tumors. While genetic defects in β-catenin have been linked with low grade endometrioid cancers, they are rare in serous and other ovarian carcinomas (Geyer et al. 2009). Nonetheless, activation of Wnt/β-catenin pathways in epithelial ovarian cancers are associated with the CSC phenotype (Arend et al. 2013), and a correlation between activation of signaling pathways involved in epithelial to mesenschymal transitions and those activated in CSCs is apparent (Talbot et al. 2012). Recent studies have found that blocking the Notch signaling pathway, which is involved in epithelial to mesenchymal transitions, improves the response of ovarian cancer to platinum therapy (McAuliffe et al. 2012).

The CSCs phenotype is relatively chemoresistant in compared to the bulk tumor cell population, and epigenetic mechanisms appear to function in both chemoresistance as well as in perpetuation of the stem cell phenotype (Alvero et al. 2009a; Crea et al. 2009; Steg et al. 2012). Primary/recurrent pairs of high grade ovarian adenocarcinomas were analyzed for expression of cancer stem cell markers (ALDH1A1, CD44, CD133). Immediately after primary therapy (combination: cisplatin or carboplatin and taxane (either paclitaxel or docetaxel), the percentage of remnant tumor cells expressing one or more of these markers was enriched, with

CD133 displaying the greatest degree of enrichment. Some stem cell transduction pathways also were observed to be enriched the recurrent tumors (Steg et al. 2012). It has been suggested that the enhanced expression of selective transporter ABCG2 and MDR1, as well as expression of toll-like receptor signaling may function in chemoresistance of ovarian CSCs (Fong and Kakar 2010).

Exit of chemoresistant tumor cells from the cell cycle is sometimes referred to as cellular dormancy in order to distinguish it from other events that can result in blocked or retarded tumor growth, such as insufficient vasculature or attack by the immune system (Aguirre-Ghiso 2007). Tumor cellular dormancy can follow therapeutic intervention, but is also observed in primary tumors, as their growth can lag or stall before they become clinically significant (Harach et al. 1985). Protracted tumor dormancy often occurs after treatment; and tumor cells that resist therapy can persist in an occult or asymptomatic state for years before causing a recurrence of disease (Udagawa 2008). How cancer cells produce a chemoresistant dormant population, such as through asymmetric tumor growth or through induction by exposure to therapeutic agents, is not understood. Equally elusive are the mechanisms that induce dormant (quiescent) cancer cells to awaken and proliferate, resulting in recurrence.

It is important to define tumor cellular dormancy in the context of the CSC hypothesis (Fig. 7.2). The activity of CSCs would likely be impacted by factors that restrict tumor growth, such as vascular insufficiency or immunological intolerance, but in these cases dormancy results from the equilibrium reached between tumor growth and attrition due to lack of nutrients or negative selection by the immune system. In contrast, tumor cellular dormancy after therapeutic intervention is associated with protracted cell cycle arrest, and is a state associated with tumor cells that have survived therapeutic challenge. By definition, dormant tumor cells are dormant CSCs. For tumor cells in this state to be properly referred to as dormant and to be distinguished from terminal senescent cells, they must have the capacity to reenter the cell cycle and clonally support regrowth and recurrence of the tumor. Thus, merely spotting quiescent tumor cells in tissues after chemotherapy and remission does not necessarily identify them as dormant, as the capacity of these cells for regrowth would remain uncertain.

The CSC hypothesis predicts that bulk and other types of tumor cells arise from asymmetric division of CSCs into CSC and bulk tumor cells, followed by rapid but limited growth of the resultant bulk tumor cells (Fig. 7.2). Some evidence has suggested that prevalence of cells bearing CSC markers in an epithelial ovarian cancer correlates with positively with recurrence (Steffensen et al. 2012). Little is known, however, of the relative frequency of symmetric and asymmetric divisions of CSCs, whether these frequencies vary at different periods of tumor growth, or whether the growth parameters of CSCs are similar to the those of dormant tumor cells in the early stages of tumor recurrence. Increases in the serum CA125 antigen have been used to detect early recurrence of ovarian cancers, but no survival benefit was found for early versus delayed treatment of recurrent tumors (Rustin et al. 2010). Since the tumors in this study were recurrent rather than newly diagnosed, it is unlikely that the lack of a survival benefit is due to overdiagnosis and/or overtreatment (Klotz 2012). Rather, this surprising outcome suggests an absence of knowledge of the kinetics of tumor growth, in particular the growth of tumors from dormant cells. For example, does malignant tumor growth from single dormant cells produce consistent fractions of bulk tumor cells and CSCs, or does growth proceed through phases in which growth of one population outpaces the growth of the other? Enhanced accumulation of the CSC fraction during early tumor growth could explain the lack of a survival benefit for early treatment of recurring tumors.

The lack of controlled and defined model tumor cell systems has precluded directly studying the cellular kinetics and dynamics of tumor growth from dormant cells. After drug treatment of a tumor in a rodent model, investigators may need to wait 300 days or longer to observe recurrence, and as such are limited by the practical

duration of the experiment as well as the natural lifespan of the animal. As indicated above, the high probability of recurrent tumors responding to retreatment with the original platinum-based chemotherapeutics suggests that transitions between drug-resistant, dormant, and malignant states are driven by epigenetic events, and this make sense as dormant cells are unlikely to undergo rounds of somatic mutation and selection as would be required these transition were driven by genetic events. Studies of some ovarian cancer cell lines have suggested that alterations to nuclear pore architecture, and underlying changes in chromatin architecture, directly affect sensitivity to platinum drugs, and can be employed to drive transitions between cellular dormancy and malignancy in an experimental system (Kinoshita et al. 2012). These results are consistent with epigenetic modalities regulating platinum drug resistance and cellular dormancy, and suggest that variation in expression of nucleoporins can produce phenotypic diversity within the tumor cell population. Although dormant tumor cells must function as CSCs in order to fuel recurrence of a tumor, it is not necessarily clear that they arise or are selected from the pool of CSCs. Little is known of the potential overlap in phenotypic markers between dormant tumor cells and CSCs, nor whether an abundance of cells expressing CSC markers in a particular tumor would predict a greater likelihood of generating dormant tumor cells in response to therapy. It has been reported that ovarian cancers with a greater percentage of cells expressing certain CSC markers (e.g., CD133; (Zhang et al. 2012)) have a poorer prognosis; however, it not known whether this is because of their intrinsic chemoresistance or because, in response to therapy, they yield a greater fraction of dormant cells with tumor-initiating properties.

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8 The Role of Angiogenesis, Growth Arrest and Autophagy in Human Ovarian Cancer Xenograft Models for Tumor Dormancy

Margie N. Sutton, Zhen Lu, and Robert C. Bast Jr.

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Abstract

The concept of tumor dormancy has been developed to explain a prolonged interval between primary therapy and the recurrence of metastatic disease. The process of dormancy may be driven or supported by several different mechanisms including angiogenesis and the tumor microenvironment, cell cycle arrest, immune surveillance, and autophagy. One xenograft model for dormancy in ovarian cancer emphasizes the importance of an effective and stable tumor microvasculature. In another xenograft model of dormancy in ovarian cancer, autophagy appears to be important for sustaining dormant cancer cells under nutrient poor conditions. Autophagy plays an ambiguous role in cancer pathophysiology. As a cellular defense mechanism, loss of autophagy has been implicated in tumor progression in several models, but autophagy can also provide a survival mechanism for dormant cancer cells. ARHI (DIRAS3) is a maternally imprinted tumor suppressor gene that is downregulated in 60% of human ovarian cancers associated with decreased progression free survival. Re-expression of ARHI blocks proliferation, inhibits migration, prevents angiogenesis and induces autophagy and tumor dormancy. Treatment of mice bearing ARHI-induced dormant xenografts with chloroquine, a functional inhibitor of autophagy, delays the

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outgrowth of tumor transplants, consistent with a role for autophagy in sustaining nutrient deprived cancer cells. This mechanism may extend beyond ovarian cancer, as downregulation of ARHI has been found in breast, lung, prostate, pancreatic, and thyroid cancers. This review considers the role of multiple mechanisms for dormancy in epithelial ovarian cancers that may provide new targets for eliminating dormant cancer cells.

Introduction

The concept of tumor dormancy has been developed to explain the prolonged interval – sometimes many years – between primary therapy and the recurrence of metastatic disease. This phenomenon was noted as early as 1934 by Willis and has been observed in several different malignancies including carcinomas of the breast, ovary and kidney, as well as lymphomas and melanomas, where recurrence can be observed 5–20 years following initial removal of the primary tumor (Willis 1934). Possible mechanisms that could contribute to tumor dormancy include: (1) an exit from the cell cycle or cell cycle arrest; (2) a balance between cell proliferation and cell death; (3) a prolonged block in effective angiogenesis, (4) a requirement for additional mutations or epigenetic events to permit the progression of metastatic clones, and (5) immune suppression of cancer cell growth. Dormancy can occur following primary surgery or radiation therapy, but is now observed most frequently after systemic chemotherapy or hormonal therapy. By definition, dormant cancer cells have survived primary therapy and generally contain a subpopulation of drug resistant tumor initiating cells.

For epithelial cancers that arise from several different sites, attempts to eliminate dormant cancer cells with additional maintenance chemotherapy have not been effective. This is particularly true for ovarian cancer where maintenance therapy with paclitaxel (Markman et al. 2009) or bevacizumab (Perren et al. 2011) can prolong progression free survival, but not overall survival or the rate of cure.

Clinical Biology of Ovarian Cancer

Ovarian cancer is neither a common nor a rare disease. The lifetime risk for a woman in the United States is 1 in 70 compared to 1 in 8 for breast cancer. Among women, approximately 3% of cancers arise from the ovary and these malignancies account for 6% of cancer deaths (Jemal et al. 2002). Ovarian cancers have been thought to develop from epithelial cells that cover the ovary or that line cysts immediately below the ovarian surface. Recent studies suggest that a fraction of high grade serous "ovarian" cancers actually arise from the fimbriae of the fallopian tube (Crum et al. 2007). Like breast or lung cancer, ovarian cancer can metastasize through blood or lymph. Most frequently, however, ovarian cancer cells metastasize from the ovary or fallopian tube across the abdominal cavity, producing multiple tumor nodules on the parietal and visceral peritoneum. When ovarian cancers are detected prior to metastasis, more than 90% can be cured with currently available therapy. When disease has spread throughout the abdominal cavity or above the diaphragm, long term survival falls to less than 30%. Despite promising research, there is at present no proven strategy for early detection and less than a quarter are diagnosed while still limited to the ovaries (Stage I) (Lu et al. 2013; Menon et al. 2009).

Primary treatment of newly diagnosed ovarian cancer involves "cytoreductive surgery" followed by combination chemotherapy. Gynecologic surgeons with special training remove not only the ovaries, fallopian tubes, uterus and omentum, but as much metastatic cancer as possible, even when complete resection is not feasible. Small metastatic deposits less than 1 cm in diameter are often left after surgery and chemotherapy is required for their elimination. Ovarian cancer is chemo-responsive, but much less chemo-curable. Treatment with six cycles of carboplatin and paclitaxel will produce a response in 70% of patients, but less than 30% remain free from recurrent disease. In the majority, disease recurs within 1–3 years, but in some cases recurrence has been documented 5–10 years following primary therapy. Median survival now extends to 4–5 years

with optimal and aggressive care, but the fraction of women cured remains less than 30% and has not changed over the last two decades.

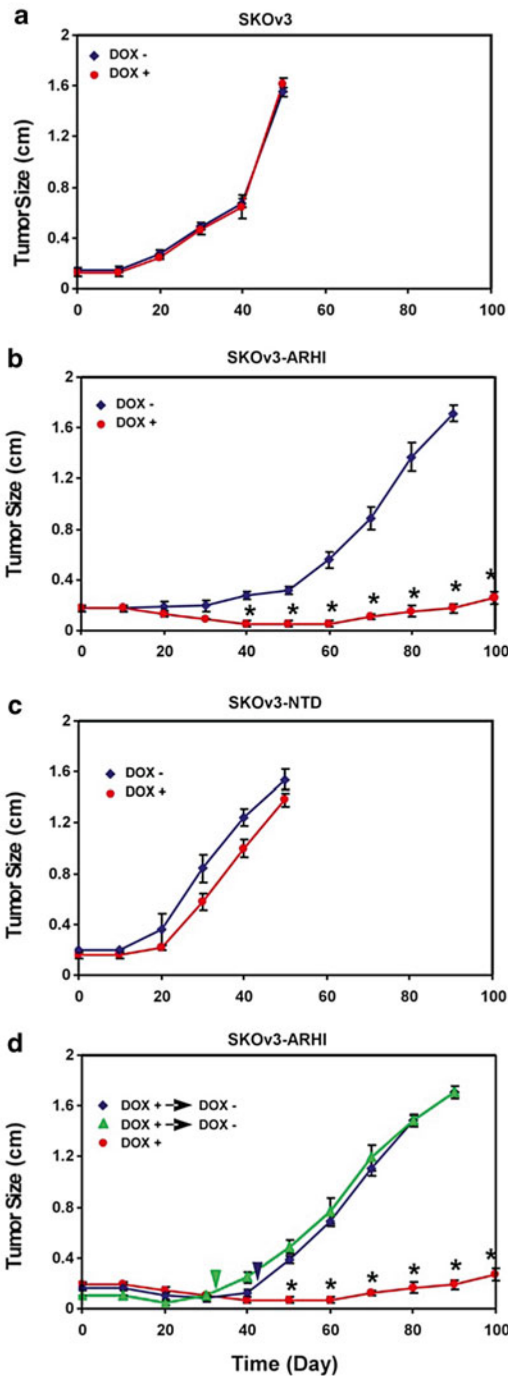
Recurrence of metastatic disease occurs most often within the peritoneal cavity. In the past, "second look" operations have been performed after primary surgery and chemotherapy to detect residual disease. Ovarian cancer cells have been found in small, poorly vascularized fibrotic nodules on the peritoneal surface. In this setting, dormant ovarian cancer cells are likely to be hypoxic and nutrient deprived. Survival of dormant cancer cells in this setting is likely to depend upon multiple mechanisms, including autophagy. Compartmentalization and self-digestion of long-lived cellular proteins and organelles yield amino acids and fatty acids that can provide needed energy under nutrient poor conditions.

Models for Human Ovarian Cancer Dormancy

Studies with two human ovarian cancer xenograft models in immunosuppressed mice suggest that the induction or maintenance of dormancy relates to a block in the development of stable vasculature. Gilead and colleagues grew spheroids from human MLS ovarian cancer cells in culture. Ovarian cancer spheroids were injected subcutaneously into genetically immunosuppressed female CD-1 nu/nu mice. Tumor growth and angiogenesis were monitored sequentially with magnetic resonance imaging (MRI). Maturity of vessels was judged by their response to oxygen and carbon dioxide which depends upon the growth of pericytes and stromal cells around the endothelial cells that line nascent tumor vessels. In three of ten xenografts, tumors grew promptly and progressively. In seven of ten xenografts, small subcutaneous tumor nodules remained stable from 13 to 54 days before growing progressively, providing a model for tumor dormancy (Gilead et al. 2004; Gilead and Neeman 1999). Vessels appeared at the periphery of nodules within 2 days after injection of spheroids. Both mature and immature vessels formed, but underwent up to six cycles of growth and regression. Emergence from dormancy and progressive tumor growth correlated with penetration of new vessels and stroma into the cancer spheroids. These observations are compatible with a model where VEGF is produced by hypoxic ovarian cancer cells, attracting endothelial cells and pericytes. Development of tumor vessels provides sufficient oxygen to suppress VEGF expression. In the absence of tumor-derived VEGF, tumor vessels regress, deceasing oxygen delivery and inducing VEGF once again.

Our own group had developed the first inducible model for tumor dormancy in ovarian cancer. *ARHI* (*DIRAS3*) is a maternally imprinted tumor suppressor gene that encodes a 26 kD GTPase with 50–60% homology to Ras and RAP. ARHI is downregulated in ovarian cancers as well as many other tumor types including breast, lung, prostate, pancreatic and thyroid carcinomas (Dalai et al. 2007; Huang et al. 2010; Lin et al. 2011; Lu et al. 2001; Wang et al. 2003; Wu et al. 2013; Yu et al. 1999). ARHI expression was down-regulated in 63% of 407 invasive cancers and could not be detected in 47%, associated with decreased progression free survival but not overall survival (Rosen et al. 2004).

Sublines of SKOv3 and HEY ovarian cancer cells – designated SKOv3-ARHI and HEY-ARHI – have been developed that re-express ARHI at physiologic levels in the presence of doxycycline. Re-expression of ARHI blocks proliferation, upregulates p21, arrests the cell cycle in G1, and inhibits motility (Badgwell et al. 2012). Doxycycline-driven, re-expression of ARHI at physiologic levels eliminates clonogenic growth of SKOv3-ARHI through autophagic type II cell death (Lu et al. 2008). Subcutaneous xenografts of the SKOv3-ARHI ovarian cancer cell line were established in Balb/c nu/nu mice. Oral administration of doxycycline in drinking water to tumor bearing mice increased ARHI in xenografts, arrested tumor growth, blocked angiogenesis and induced autophagy, but did not kill ovarian cancer cells. When doxycycline was withdrawn after 6–8 weeks, ARHI levels declined, tumor vasculature was established and SKOv3-ARHI xenografts grew rapidly and progressively (Fig. 8.1).



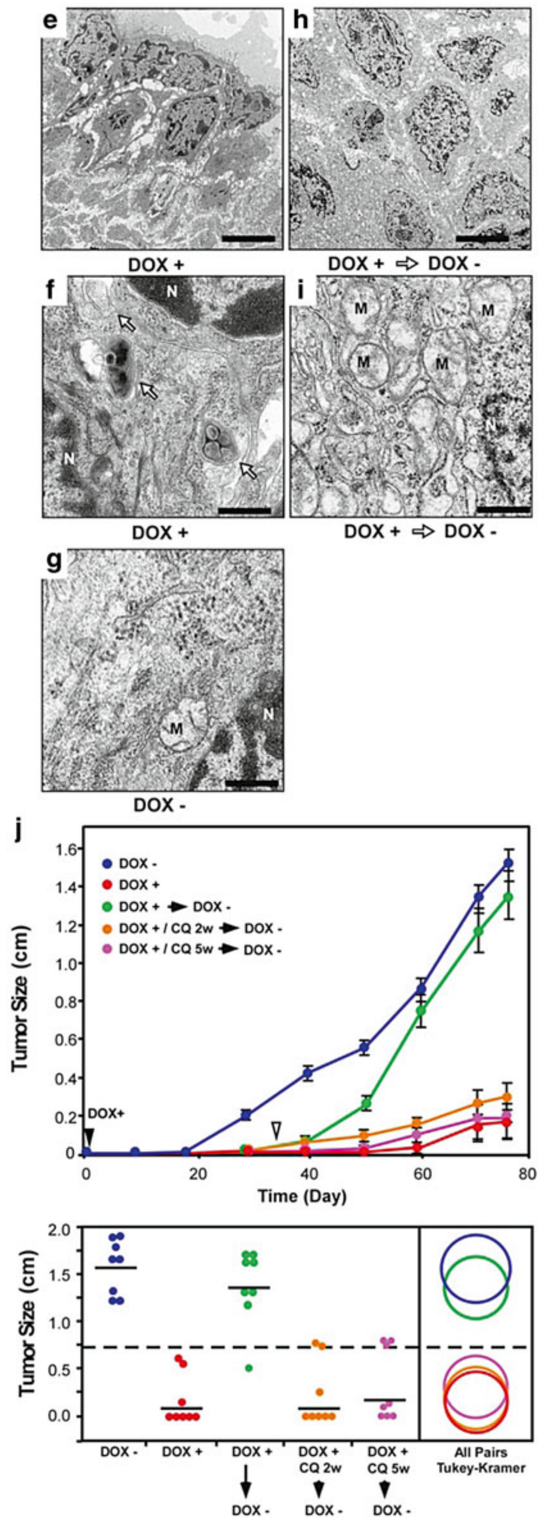


Fig. 8.1 ARHI-induced autophagy results in tumor dormancy in xenografts. (**a**–**d**) BalB/c nu/nu mice were injected with SKOv3 (**a**), SKOv3-ARHI (**b**) or SKOv3-NTD (**c**) cells and provided with or without DOX in their drinking water. (**d**) DOX withdrawn after 32 (*green triangles*) or 42 (*blue* diamonds) days of treatment shows re-growth of tumor. (**e**–**i**) TEM Images of tumor xenografts indicating autophagosomes. (**j**) Inhibition of autophagy blocks tumor dormancy (Lu et al. 2008)

Once angiogenesis occurred, re-institution of doxycycline treatment failed to inhibit xenograft growth (Lu et al. 2008; In preparation: Established angiogenesis will inhibit ARHI (DIRAS3) induced tumor dormancy), consistent with the importance of a block in angiogenesis to maintain tumor dormancy.

Angiogenesis and the Tumor Microenvironment in Ovarian Cancer Dormancy

The process of angiogenesis has been well studied and has been found to be a vital component of tumor progression. Without the recruitment and formation of functional blood vessels to support tumor growth by providing oxygen and nutrients, tumors remain in a dormant state. If successful angiogenesis is inhibited, the tumors remain avascular and microscopic in size (Gimbrone et al. 1972; Hart 1999; Holmgren et al. 1995; O'Reilly et al. 1996). Small tumor size is not, however, necessarily due to lack of proliferation. Dormant tumor cells typically exhibit increased apoptosis to counteract the elevated proliferation, producing angiogenic dormancy (Holmgren et al. 1995; Naumov et al. 2006). Thus, the tumor microenvironment is a key component in maintaining this dormant state. The balance between proangiogenic factors (e.g., PDGF, VEGF and FGF) and antiangiogenic factors (e.g., angiostatin, endostatin, and vasculostatin) regulate the switch which controls angiogenic dormancy. Re-expression of ARHI downregulates, but does not eliminate VEGF expression.

The tumor microenvironment can also contribute to regulating autophagic cell death. In the SKOv3-ARHI dormancy model, VEGF, IL-8 and IGF have all been detected in tumor xenografts. VEGF and IL-8 are expressed by the human ovarian cancer cells and released into the peritumoral space, whereas IGF is produced by the murine stroma. Addition of these peptide growth factors and cytokines to SKOv3-ARHI cells cultured in the presence of doxycycline, recues ARHI-induced ovarian cancer cells from autophagic death (Lu et al. 2008). Thus, VEGF, IL-8 and IGF may act as survival factors, regulating and limiting autophagic damage to cancer cells. Survival of dormant cells may require a balance where sufficient autophagy is maintained to meet minimal energy requirements, but where autophagy stops short of inducing cell death (Fig. 8.2).

Cell Cycle Arrest in Ovarian Cancer Dormancy

Cancer dormancy has been attributed to a balance between cell proliferation and cell death that limits expansion of the tumor cell population. Alternatively, dormancy could result from growth arrest where cancer cells enter and remain in G0-G1 and do not proliferate (Aguirre-Ghiso 2007). Non-proliferating cancer cells can exhibit a low metabolic rate. Computer simulations have been used to explore whether balanced proliferation or growth arrest was more likely to underlie dormancy (Wells et al. 2013). Growth arrest and quiescence were found to be more likely to occur in dormant cancer cells. If growth arrest occurs more frequently, cytotoxic chemotherapy that targets cycling cells is unlikely to eliminate dormant cancer cells and different strategies will be required to achieve cure. In the two ovarian cancer xenograft models for tumor dormancy, cell cycle arrest is likely to play a role. Although cell cycle events were not studied after injection of MEL spheroids into nu/nu mice (Gilead et al. 2004), other reports with large pancreatic cancer cell spheroids (500–600 uM) indicate that cells proliferate only in outer cell layers and not at the center of spheroids (Laurent et al. 2013). Induction of ARHI in the SKOv3-ARHI model produced growth arrest in G1 (Lu et al. AACR 2013 Abstract #1680, ARHI induces autophagy and enhances chemosensitivity to cisplatin in ovarian cancer cell lines and xenografts).

Immunity in Ovarian Cancer Dormancy

Nu/nu mice are deficient in T and B cell function. Consequently, studies in xenograft models cannot assess the influence of antigen specific immunity on dormancy. Primary autochthonous ovarian cancers have been induced in immuno-competent

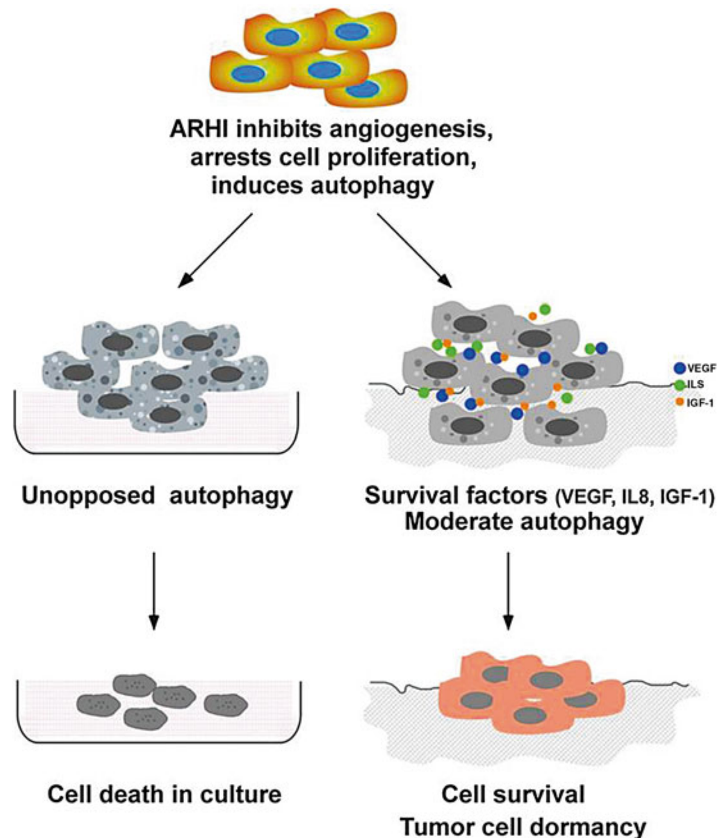


Fig. 8.2 Model of the survival factors in the tumor microenvironment that prevent autophagy-induced death of dormant cancer cells by ARHI (DIRAS3)

genetically engineered mice (Mullany and Richards 2012) and these models may permit evaluation of the immune response in regulating dormancy. Although studies of ARHI as a modulator of immune surveillance have not yet been performed, recent reports suggest that autophagy can affect both innate and adaptive immunity, as well as, inflammation though several different mechanisms. Autophagy has been shown to increase resistance to $CD8^+$ T lymphocyte-mediated lysis of human breast cancer cells (Akalay et al. 2013). Autophagy is important for cell-autonomous elimination of intracellular microbes by modulating activation of inflammatory cytokines, Toll-like and Nod-like receptors, antigen presentation, and mature T cell development and homeostasis (Deretic 2012). When tumor bearing mice were treated with chemotherapy, autophagy-competent, but not autophagy-deficient, cancers attracted dendritic cells and T lymphocytes into the tumor bed to establish tumor specific immunity. Autophagy was found to be essential for the release of ATP from dying cancer cells to attract dendritic cells and T lymphocytes and to establish immunity that could enhance the effects of chemotherapy (Michaud et al. 2011).

Autophagy in Ovarian Cancer Dormancy

Autophagy is a physiological process that digests warn organelles and long lived proteins, releasing amino acids and fatty acids which are catabolized yielding ATP. During nutrient deprivation, autophagy is induced, providing energy and promoting cell survival. Excessive autophagy can, however, result in cell death. The process of autophagy has been conserved in eukaryotes from yeast to

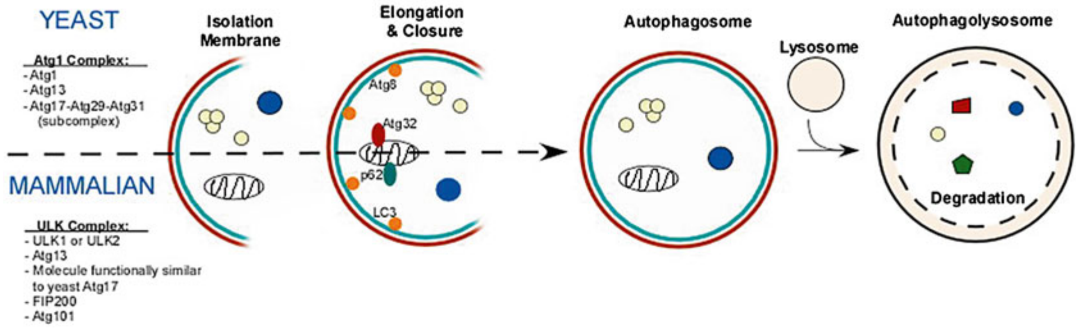


Fig. 8.3 The process of autophagy has been conserved in eukaryotes. Yeast, plants and mammalian cells all undergo a similar process of self-digestion of damaged or worn-out organelles

mammals (Fig. 8.3) (Reggiori and Klionsky 2002). Autophagy is induced by downregulation of mammalian target of rapamycin (mTOR) which results in decreased phosphorylation of ATG13. Inhibition of mTOR is regulated in mammalian cells by the tuberous sclerosis complex (TSC1/2) which is upregulated by inhibition of Class I PI3K or by activation of AMP kinase. Once autophagy is induced, an autophagy inhibition complex (AIC) is assembled to nucleate autophagic vesicles. The AIC contains Beclin1, UVRAG and Vps34 Class III PI3K. Prior to induction, Beclin1 exists as a homo-dimer bound to Bcl2. An important step in regulating autophagy is the dissociation of Beclin1 from Bcl2 and the monomerization of Beclin1. Prompted by the AIC, double membrane structures elongate, enclosing warn mitochondria, endoplasmic reticulum and long-lived proteins. Autophagic vesicles are decorated with Atg5 and Atg12. Atg4, a cysteine protease, is activated and upregulated, cleaving microtubule associated protein (MAP) LC3-1. Cleaved LC3-1 is then lipidated, forming LC3-2 (LC3-II) that attaches to the developing autophagosome, displacing Atg5. Mature autophagosomes then dock and fuse with lysosomes forming autophagolysosomes, a step requiring lamp 1, lamp 2 and Rab7. Autophagolysosomes are acidified, activating proteases and lipases which digest proteins and lipids. Chloroquine passes readily into these structures and raises the pH, functionally inhibiting autophagy.

ARHI (DIRAS3) enhances autophagy at several critical steps. Through inhibition of the PI3K/Akt signaling pathway, ARHI upregulates TSC1/2 and inhibits mTOR inducing autophagy. ARHI acts on several additional targets in the autophagic process, as knockdown of ARHI blocks mTOR induced autophagy. Recently, our laboratory has found that ARHI acts as a critical switch in the displacement of Bcl-2 from Beclin1 and in dissociating the Beclin1 homodimer (Lu et al. 2014b). ARHI further contributes to forming the AIC by increasing the association of Vps34 and ATG14 with Beclin1. Moreover, ARHI induces the Atg4 cysteine protease that cleaves LC3-1 (LC3-I), while co-localizing directly with LC3-II in the membrane of the developing autophagosome during elongation. ARHI-mediated downregulation of PI3K/Akt and Ras/ERK signaling decreases phosphorylation of FOXO3a and sequesters the transcription factor in the nucleus, upregulating Atg4 and MAP-LC3-I required for autophagosome maturation and upregulating Rab7 required for fusion of the autophagosome with the lysosome (Lu et al. 2014a). Thus, re-expression of ARHI in ovarian cancer can contribute to the induction of autophagy though several different mechanisms and clinically is expressed in small deposits of ovarian cancer that persist following primary surgery and chemotherapy, associated with punctate LC3. This phenotype is consistent with autophagy in resistant, dormant ovarian cancer cells and is the main mechanism by which ARHI induces tumor dormancy (Figs. 8.4 and 8.5).

The clinical relevance of these observations with cell lines and xenografts is supported by observations with primary and "second look"

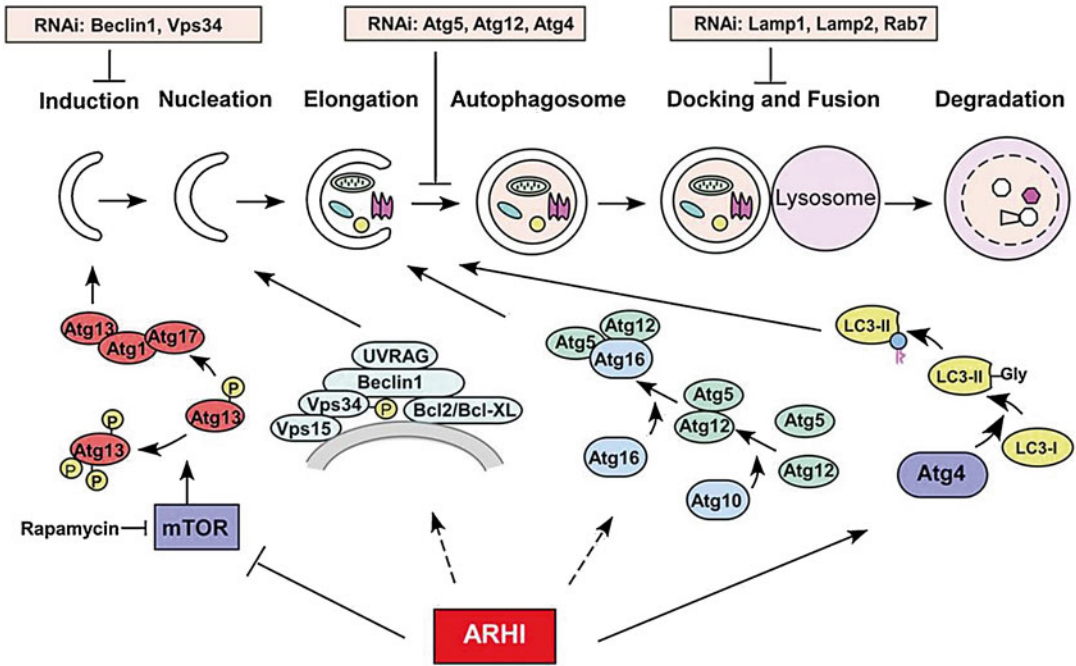


Fig. 8.4 ARHI (DIRAS3) plays a critical role in many steps of autophagosome formation. ARHI inhibits mTOR through suppression of the PI3K pathway initiating the induction of autophagosome formation. ARHI affects the autophagosome initiation complex (*AIC*) by disrupting the Beclin-1 homodimer and displacing Bcl-2. ARHI directly affects the elongation stage of autophagosome formation by up-regulating ATG4

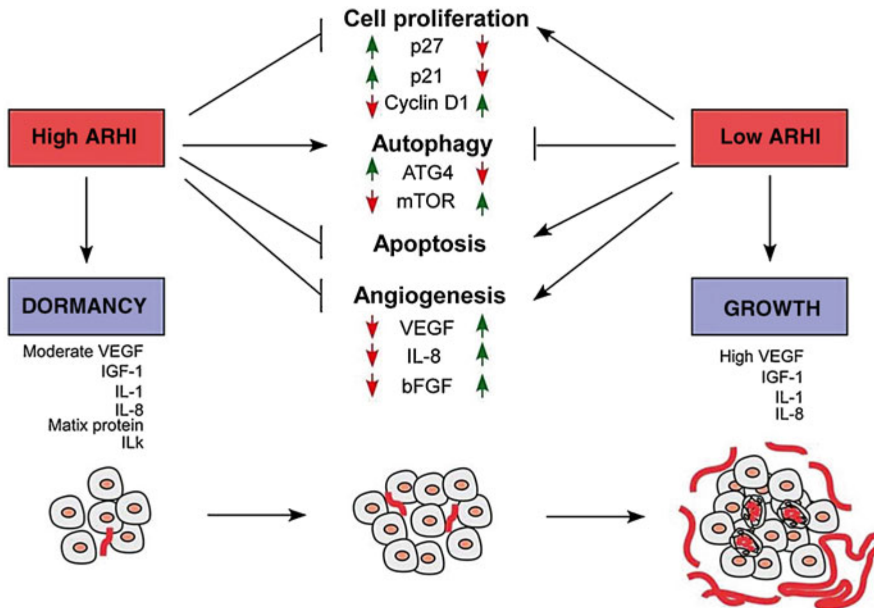


Fig. 8.5 ARHI (DIRAS3) regulates tumor dormancy through several mechanisms

surgical specimens (Lu et al. 2014b). All patients were in a complete clinical remission at the completion of adjuvant chemotherapy defined by normal CA-125 level, normal CT scans of the abdomen and pelvis, and a normal physical exam. When immunohistochemical analysis was performed, ARHI staining (>2–3) was observed in 41% of primary cancers and 97% of second-look cases. LC3 staining (>2–3) was observed in 35% of primary cancers and 85% of second-look cases. Punctate ARHI staining was observed in 23% of primary cancers and 84% of second-look cases, whereas punctate LC3 staining was observed in 21% of primary cancers and 81% of second-look cases. Positive staining of both total and punctate ARHI and LC3 was significantly higher in second-look than in primary cases ($P<0.0001$).

Despite substantial homology to Ras, ARHI inhibits Ras transformation (Sutton et al. In preparation; The role of DIRAS family tumor suppressors in Ras transformation), blocks growth and motility of transformed cells while inducing autophagy and tumor dormancy. At a structural level, ARHI is distinguished from Ras by a 34 amino acid N-terminal extension that is required for most functions, including autophagy (Lu et al. 2008; Luo et al. 2003). Re-expression of ARHI at physiologic levels leads to programmed cell death of ovarian cancer cells in culture with, at most, low levels of apoptosis. This cell death was preceded by the development of autophagy as judged by increased catabolism of long-lived vesicles that are decorated with LC3-II, and visualization of autophagosome formation by electron microscopy. Re-expression of an N-terminal deleted construct with a Dox-inducible promoter did not induce autophagy or suppress tumor growth. This structure-function correlation also observed in vivo. Induction of the full length ARHI at physiological levels in ovarian cancer xenografts suppressed remained the growth of dormant ovarian cancer cells for weeks and xenografts grew promptly when ARHI was downreguated. R-expression of the N-terminal deleted ARHI mutant protein had no effect on xenograft growth compared when compared to controls that were not fed doxycycline (Lu et al. 2008).

Autophagy plays an ambiguous role in cancer pathophysiology. As a cellular defense mechanism, dysregulated autophagy has been implicated in tumor progression for many disease sites. Enhanced development of breast cancers occur in genetically engineered hemizygous Beclin +/− mice with impaired autophagy, suggesting that the process of autophagy can suppress carcinogenesis (Qu et al. 2003; Yue et al. 2003). In pancreatic cancer, unresolved inflammation and disrupted regulation of autophagy are common features in the pathogenesis of pancreatitis and subsequently pancreatic cancer (Gukovsky et al. 2013). Altered expression of other autophagy proteins have been observed in human cancer specimens. In prostate carcinomas, Atg5 expression was markedly up-regulated in prostate intraepithelial neoplasms and prostate cancer cells determined by immunohistochemical analysis, whereas, but no somatic mutations of ATG5 were found (Kim et al. 2011). Cytoplasmic LC3 punctate staining has been observed in a variety of human cancers, including lung adenocarcinomas, breast adenocarcinomas, hepatocellular carcinoma, testicular seminoma, and melanoma. These authors observed increased LC3 punctate staining in the majority of human cancers compared with normal tissues from corresponding organs. Cancers of the same histological type showed substantial heterogeneity in the number and intensity of LC3 puncta per cell (Ladoire et al. 2012).

Conclusion

Thus, autophagy can suppress the induction of cancer and can cause death of transformed cells, but can also serve as a survival mechanism contributing to the survival of dormant cancer cells in nutrient poor conditions. Context matters. Manipulation of autophagy in either direction, positive or negatively, could plausibly be utilized as a therapeutic approach to eradicate cancer (Ryter and Choi 2010). Clinically, these strategies have been employed by using chloroquine and its derivative, hydroxychloroquine, (inhibitors of lysosomal acidification) to enhance chemotherapeutic

efficacy in many clinical trials across several disease sites including prostate, pancreatic, breast and non-small-cell lung cancers (Choi et al. 2013). For ovarian cancer, the modulation of autophagy for a therapeutic effect has recently been observed where VEGF therapies have been effective in delaying tumor re-growth, possibly by affecting dormant cells. The role of ARHI (DIRAS3) in promoting autophagy and tumor dormancy may very well extend beyond ovarian cancer as downregulation of this imprinted tumor suppressor gene has been found in breast, lung, prostate, pancreatic and thyroid cancers (Huang et al. 2010; Lin et al. 2011; Lu et al. 2001; Wang et al. 2003; Yu et al. 1999). Overall, a greater understanding of the mechanisms surrounding autophagy and tumor dormancy as related to cancer pathogenesis will provide insight to new targets for therapeutic and diagnostic approaches.

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Part IV

Role in Leukemia

Regulation of the Promyelocytic Leukemia Protein and Its Role in Premature Senescence

9

Alan W. Lau, Adriana E. Tron, and Wenyi Wei

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Abstract

Premature senescence functions as a tumor suppressor mechanism in response to oncogenic stimuli. It is characterized by irreversible cell cycle arrest mediated by tumor suppressors such as p53, Rb and the Promyelocytic Leukemia (PML) protein. PML mainly localizes in sub-nuclear structures known as PML nuclear bodies. These nuclear bodies accumulate in senescent cells largely due to increased PML gene transcription driven by p53 and/or the interferon pathways. PML exerts its pro-senescence activity by modulating both the p53 and Rb pathways, the major regulators of the cellular senescence program. Mechanistically, PML binds and promotes p53 modifications to generate a positive feedback loop, thereby triggering the senescence program. In addition, PML associates with Rb and may function in Rb/E2F-mediated gene silencing in senescent cells. Moreover, PML bodies recruit DNA damage sensing and repair proteins, thereby linking PML to the activation of the DNA damage response pathway, a pathway frequently activated in senescence. Therefore, elucidation of key factors controlling PML protein abundance will help to better understand how cells become transformed by avoiding senescence and allowing continued cellular proliferation in the presence of oncogenic signals. These findings will also be crucial in aiding scientists and

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physicians in the development of novel anticancer therapies by restoring PML to trigger senescence.

Introduction

Cellular senescence is the phenomenon by which cells undergo an irreversible cell cycle arrest, losing the ability to further divide and proliferate. Several stresses have been implicated in the induction of senescence, including oxidative damage, telomerase dysfunction, aberrant oncogene-dependent proliferative signaling, and DNA damage (Collado and Serrano 2010). Together, these mechanisms converge on the two major tumor suppressor genes *p53* and *Rb*, whose mutations or inactivation are most common in human cancers. Biologically, the onset of the senescence program limits excessive or aberrant cellular proliferation, such that the state of senescence protects against the development of cancer (Vijg and Campisi 2008), and could have further impact on organismal aging and contribute to the development of age-related pathologies (Baker et al. 2011). One particularly relevant source of stress in tumor cells is derived from the aberrant proliferative signals of oncogenes, which may trigger premature senescence through a process termed as oncogene-induced senescence (OIS). Recent studies from multiple groups clearly showed that OIS functions as a potential tumor suppressor mechanism (Di Micco et al. 2006).

Notably, in response to various oncogenic stimuli, such as Ras signaling, there is increased expression of the promyelocytic leukemia protein (PML) (Ferbeyre et al. 2000; Pearson et al. 2000; de Stanchina et al. 2004), which subsequently results in the onset of a premature senescent state (Ferbeyre et al. 2000). PML is a protein initially identified as a fusion product with retinoic acid receptor alpha (RARα) in acute promyelocytic leukemia (APL) (de The et al. 1991; Goddard et al. 1991; Pandolfi et al. 1991). Consistent with a tumor suppressor role for PML, disruption of PML in vivo sensitizes mice to tumorigenesis induced by physical or chemical carcinogens, and accelerates tumor onset in several mouse cancer models (Scaglioni et al. 2006). PML is expressed ubiquitously and is usually accumulated at PML nuclear bodies. Importantly, PML nuclear bodies are found to be significantly elevated in response to several cellular stresses as well as when cells enter the senescent state. Mechanistically, PML plays a key role in senescence mainly acting through the Rb and p53 pathways, two proteins known to physically interact with PML and accumulate in PML nuclear bodies. It was observed that PML levels in senescent cells are largely dependent on DNA damage signals and accumulate near unrepaired DNA damage regions, indicating that senescence is coupled to the incapacity of cells to deal with unrepaired lesions in the genome (Dellaire et al. 2003). In further support of a possible link between senescence and pre-malignancies, PML bodies were frequently found in benign prostate tumors but not in prostate cancers (Vernier et al. 2011). Moreover, PML nuclear bodies are absent in cancer cells (Shen et al. 2006). The intrinsic connection between PML and the onset of senescence suggests a general and central role for PML in tumor suppression.

Transcriptional Modulation of the PML Tumor Suppressor

Recent studies indicate that PML is accumulated in senescent cells in part due to enhanced *PML* mRNA translation (Ferbeyre et al. 2000; Scaglioni et al. 2012). *PML* mRNA expression is induced by interferon (IFN) via the Jak/Stat signaling pathway and functions through IFN-stimulated response elements and IFNγ-activated sites present in the PML gene promoter (de Stanchina et al. 2004). Interferon also regulates many other components of PML nuclear bodies to mediate their anti-viral activities (Regad and Chelbi-Alix 2001). The induced expression of IFN-target genes during the establishment of senescence suggest that the IFN pathway might also control the senescent program. In keeping with this notion, treatment of primary human cells with β-IFN leads to the elevated expression of p53, PML, and subsequent onset of premature

senescence (Moiseeva et al. 2006). In addition, constitutive STAT5 signaling, a critical signaling component downstream of Jak can also enhance PML expression and induce senescence (Mallette et al. 2007). These findings are in agreement with the idea that cytokine-mediated PML expression is associated with the onset of the senescence program.

Notably, the first intron of the *PML* gene also contains p53 responsive elements that physically associate with p53 and activate PML transcription during Ras-induced senescence or the DNA damage response (de Stanchina et al. 2004). These findings suggest that PML also functions downstream of the p53 tumor suppressor to mediate senescence, a process that may also involve the activation of the Rb/E2F pathway to halt normal cell cycle progression (Vernier et al. 2011). However, the Scaglioni group recently showed that oncogenic K-RAS could induce PML upregulation even in p53-null cells, suggesting the presence of a p53-independent mechanism of PML induction. Further studies revealed that under these experimental conditions, the PML 5′ untranslated mRNA region (5′ UTR) mediates selective uploading of PML mRNA onto polyribosomes and its selective translation, leading to p53 independent PML upregulation (Scaglioni et al. 2012). In addition to these upstream regulatory routes, PML gene expression can also be activated by β-catenin in a LEF/TCF-independent manner (Shtutman et al. 2002). Hence, it has been proposed that β-catenin may operate on the basal transcription machinery on the PML promoter. However, more studies are required to further dissect the potential crosstalks or synergies among various transcription factors in governing PML transcription in different stresses or tissue contexts.

Post-Translational Regulation of PML

Besides transcriptional induction of the *PML* gene during the onset of senescence, PML is also regulated via several posttranslational modifications (Fig. 9.1). For example, PML is phosphorylated by ERK2 on several residues and these modifications have been implicated in promoting the degradation of PML upon treatment with arsenic trioxide. Furthermore, ATR and CHK2 also phosphorylate PML in a DNA damage-dependent pathway (Bernardi et al. 2004). In addition, Big MAP kinase 1 (BMK1) was also found to drive suppression of PML function through its phosphorylation of PML on S403 and T409 (Yang et al. 2010). Moreover, phosphorylation by CK2 induces PML ubiquitination and subsequent degradation by the 26S proteasome (Scaglioni et al. 2006), while the physiological E3 ligase responsible for CK2-induced PML ubiquitination still remains uncharacterized.

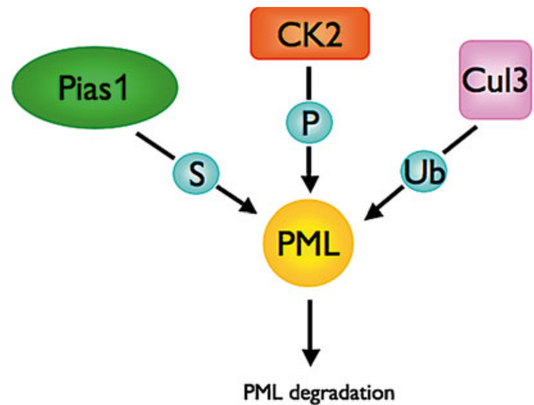

Fig. 9.1 Mechanisms controlling PML protein turnover. Several post-translational modifications trigger PML ubiquitination and proteasomal degradation. *S* SUMOylation, *P* Phosphorylation, *Ub* Ubiquitination. *Arrow* indicates potential cross-talk

Besides phosphorylation-dependent modifications, PML is also modified by the covalent binding of ubiquitin like modifiers, such as SUMO1 and SUMO2/3. A recent study showed that PML is SUMOylated by the E3 SUMO ligase Pias1 (Rabellino et al. 2012). Importantly, SUMOylation of PML has been previously reported to regulate the formation of PML nuclear bodies (Shen et al. 2006). On the other hand, the SUMOylation of PML is also controlled by SUMO proteases (Gong et al. 2000), whose role in senescence remains unknown. Interestingly, overexpression of the SUMO protease SENP1 in the prostate leads to the development of prostatic intraepithelial neoplasia (PIN) at an early age

(Cheng et al. 2006). Further studies showed that androgen suppresses PML protein levels in prostate cancer cells and induces the expression of SENP1, which in turn, enhances prostate epithelial cell proliferation (Bawa-Khalfe et al. 2010). Notably, acetylation of PML has been linked to an increase in PML SUMOylation, suggesting that acetylation primes PML for SUMOylation (Hayakawa et al. 2008). However, additional studies are required to further reveal the intrinsic interaction between SUMOylation and acetylation in modulating PML stability.

Consistent with its tumor suppressive role, down-regulation of PML is frequently observed in diverse types of human tumors and is correlated with tumor progression. Because no major noticeable changes in *PML* transcripts were observed in human cancers, it has been proposed that aberrant degradation of the PML protein may be the major mechanism accounting for the reduction of PML abundance in tumors. Moreover, arsenic trioxide, an effective APL treatment, induces PML proteasomal degradation through a process that requires PML SUMOylation by Pias1 (Rabellino et al. 2012). In a broad spectrum of cancers, PML was found to undergo ubiquitin-mediated degradation primed by CK2 phosphorylation, but the responsible E3 ligase for this process was unidentified. However, this group did determine that Pias1-dependent SUMOylation of PML promoted CK2 interaction, triggering PML ubiquitination and turnover by the proteasome (Rabellino et al. 2012). Interestingly, the SUMO-targeting ubiquitin E3 ligase RNF4 has been found to ubiquitinate poly-SUMO chains on PML leading to degradation of the protein (Lallemand-Breitenbach et al. 2008; Tatham et al. 2008). However, it is still unknown whether Pias1, CK2 and RNF4 are part of an integrated cellular network that leads to aberrant degradation of PML in cancer cells. Furthermore, RNF111/Arkadia has also been identified to be a SUMO-targeting E3 ligase that is involved in PML degradation in response to arsenic treatment (Erker et al. 2013). Unfortunately, similar to RNF4, it is still unknown whether Arkadia functions with Pias1 and CK2 to promote PML destruction in cancer cells. Nevertheless, inhibition of this proteolytic process enhances the tumor suppressor properties of PML (Scaglioni et al. 2006), supporting the rationale for developing anti-CK2 therapies for cancer.

In further identifying upstream regulatory pathways that may control PML stability, the E3 ubiquitin ligase E6AP was also found to interact with PML and can promote PML degradation in a proteasome-dependent manner (Louria-Hayon et al. 2009). Remarkably, E6AP-deficient cells show an increase of DNA damage-dependent apoptosis, due to an accumulation of PML in the nucleus causing an increase in PML-dependent responses. Additionally, partial loss of E6AP attenuates MYC-induced B-cell lymphomagenesis. In this model, tumor suppression is achieved by the induction of cellular senescence but not apoptosis. Accordingly, partial loss of E6AP leads to PML restoration and subsequent induction of PML-dependent senescence (Wolyniec et al. 2012).

In a recent paper, Yuan and colleagues reported that hypoxia induces PML proteasomal degradation by the $Cul3^{KLHL20}$ ubiquitin ligase complex (Yuan et al. 2011). Sequential modifications of PML by CDK1/2 and Pin1 facilitate the recruitment of PML to $Cul3^{KLHL20}$ for ubiquitination. Under hypoxic conditions, the drastic up-regulation of KLHL20 leads to a robust PML ubiquitination through this pathway, thereby reducing PML abundance. Biologically, PML down-regulation correlates with HIF-1α, Pin1, and KLHL20 up-regulation in human prostate cancer, and hyperactivation of this pathway correlates with high-grade tumors (Yuan et al. 2011). These studies also clearly showed that Pin1 binds to PML and induces PML down-regulation. This association largely depends on phosphorylation of PML by the MAP-kinase ERK2 (Lim et al. 2011), therefore revealing a CK2-independent pathway controlling PML ubiquitination. Given that the MAP-kinase cascade is activated by several extracellular signals, including oncogenic stress, future studies are needed to elucidate its role in PML-induced senescence. Additionally, it remains to be addressed whether the CDK1/2-Pin1 and the ERK2/Pin1 signaling pathways that mediate the timely ubiquitination of PML are

complementary, mutually exclusive, or tumor type specific.

While the proteasome has been well documented to control intracellular levels of PML, autophagy can also promote the degradation of PML-RARα, the oncogenic fusion version of PML implicated in the pathogenesis of acute promyelocytic leukemia. Induced by treatment with all-trans retinoic acid (ATRA) or arsenic trioxide (As_2O_3), the ubiquitin-binding adaptor protein p62/SQSTM1 directs ubiquitinated PML-RARα to autophagosomes for subsequent degradation (Wang et al. 2011). Unfortunately, additional studies are still required to elucidate whether non-fused PML, which functions largely as a tumor suppressor, is similarly targeted for autophagy, but these findings do suggest that other cellular degradation pathways may also regulate PML stability.

PML Induces the p53 Tumor Suppressor Pathway

The role of PML in activating the p53 pathway was first discovered in the context of Ras-induced senescence (Ferbeyre et al. 2000; Pearson et al. 2000). These studies suggested that PML stimulated p53 transcriptional activity by recruiting p53 into PML bodies, inducing p53 modifications. Specifically, PML promotes the acetylation of p53 at lysine 382 in a process catalyzed by the acetyl transferase CBP, which localizes to PML bodies (Pearson et al. 2000). In addition, PML induces the phosphorylation of p53 at serine 15, a modification that is usually catalyzed by the DNA damage response kinases ATM and ATR (Ferbeyre et al. 2000). Interestingly, proteins that inhibit senescence can reverse these modifications. For example, SIRT1, a NAD-dependent deacetylase, inhibits PML-induced senescence by deacetylating p53 at lysine 382 in PML bodies (Langley et al. 2002). The adenoviral oncoprotein E1A blocks cellular senescence by impairing the ability of PML to form PML bodies and preventing p53 phosphorylation in cells expressing oncogenic *ras* (Ferbeyre et al. 2000).

Notably, p53 activation requires the whole PML body macromolecular complex. The site of interaction between p53 and PML was mapped to the DNA binding domain of p53 (Fogal et al. 2000). Therefore, p53 may not bind simultaneously to PML and DNA. Taking all the data together, a plausible model is that p53 recycles between promoters and PML bodies undergoing cycles of reversible post-translational modifications (Fig. 9.2). Among the PML isoforms, PML IV binds p53 more efficiently than PML I or PML-RARα, a fusion oncoprotein found in acute promyelocytic leukemia. Consistent with this finding, only PML IV relocalizes p53 into PML bodies when co-transfected with p53 (Fogal et al. 2000). However, expression of PML IV is not sufficient to induce senescence in *PML*$^{-/-}$ MEFs, suggesting that other PML isoforms might be required for this process. It is possible that a particular combination of PML isoforms, possibly with a predominant role of PML IV drives p53 recycling and/or modification. Further studies are required to fully understand the contribution of each PML isoform in the onset of premature senescence.

Furthermore, PML may activate p53 by binding MDM2, an E3 ligase that targets p53 for ubiquitination and proteasomal degradation (Wei et al. 2003). MDM2 can shuttle PML from the nucleus to the cytoplasm in transfected cells, showing preferential binding to the non-SUMOylated form of PML. Trimeric complexes between PML, p53 and MDM2 were found in unstressed cells. Interestingly, upon DNA damage, these complexes segregated into PML-p53 and PML-MDM2 complexes (Kurki et al. 2003). Given that PML was unable to activate a p53 fusion protein lacking its PML binding domain, PML's ability to promote p53 activity seems to rely primarily on the formation of a p53-PML complex. An alternative mechanism proposed is that PML recruits MDM2 into the nucleolus, thereby promoting the stabilization and activation of p53 (Bernardi et al. 2004). This activity of PML depends on PML phosphorylation by ATR and interaction of PML with the nucleolar protein L11. ARF, a positive regulator of p53 and senescence that can also sequester MDM2 into the nucleolus, does not seem to be required for PML and MDM2 translocation (Weber et al. 1999). On the contrary, PML

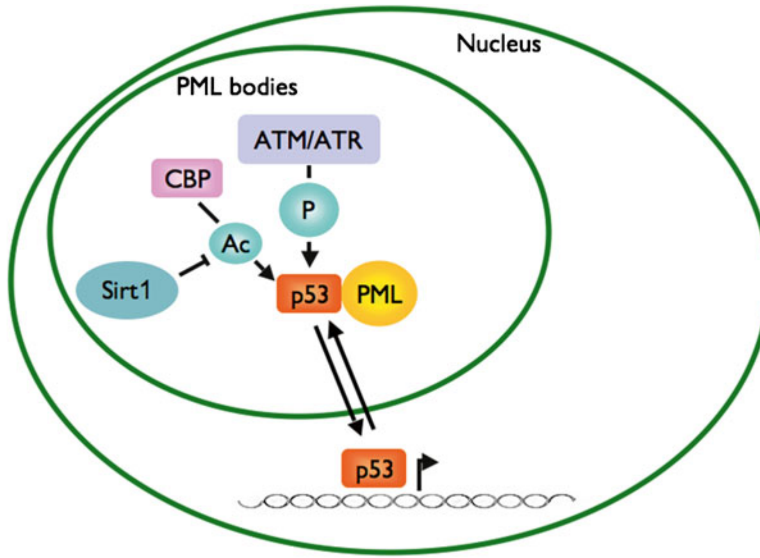

Fig. 9.2 Schematic representation of p53 recycling between PML bodies and DNA promoters. P53 is acetylated in the PML bodies by CBP in a process reversed by the NAD-dependent deacetylase Sirt1. In addition, PML promotes the phosphorylation of p53 by the DNA damage responsive kinases ATM and ATR

activates transcription of the ARF promoter by forming a complex with β-catenin, which potentially explains its role in activating p53. Finally, previous work also suggested that PML controls the nucleo-cytoplasmic transport of several growth-promoting mRNAs, including MDM2 (Culjkovic et al. 2006).

In summary, these studies clearly demonstrate that PML promotes p53 transcriptional activity. Given that p53 induces the expression of PML, these two tumor suppressors are interlinked in a positive feedback mechanism that potentiates the functions of p53 and/or PML (Fig. 9.2). Additionally, PML was found to suppress growth and tumorigenesis in the absence of p53, indicating that other pathways are also involved in the functions of PML, especially in the onset of cellular senescence.

PML Promotes Rb-Dependent Senescence

While PML was initially found to regulate senescence via p53, further studies demonstrated that PML was also capable of inducing cell cycle arrest and senescence in cells where the p53 pathway was inactivated by the papillomavirus oncoprotein E6 or the dominant negative p53 allele p53H175R. These studies suggested that PML might engage an Rb-dependent senescence program that does not require p53. Further work demonstrated that PML associates with Rb and that the PML-Rb complex formation requires the pocket region of Rb, and the B boxes and the C-terminal region of PML (Alcalay et al. 1998). Rb was also found to be essential for the formation of senescence associated heterochromatin foci (SAHF). Although SAHF does not colocalize with PML bodies, the Adam's group identified PML bodies as key components of SAHF formation, as the heterochromatin protein HP1 and the histone chaperone HIRA transiently localize to PML bodies before moving to SAHF (Zhang et al. 2005). These events were observed upon replicative senescence, enforced expression of $p16^{INK4A}$, or Ras-induced premature senescence, before cell cycle arrest or detection of senescence markers. Recently, the Ferbeyre group reported that Rb/E2F complexes were relocalized into PML bodies along with heterochromatin proteins when senescence was induced by

forced PML expression by retroviral vector, or by induction of endogenous PML by β-interferon, oncogenic *ras*, or short telomeres (Vernier et al. 2011). Relocalization of Rb/E2F complexes into PML bodies resulted in the inhibition of E2F transcriptional activity and a corresponding reduction in E2F regulated genes. Consequently, blocking the expression of E2F target genes leads to cell cycle arrest and accumulation of DNA damage signals that are essential for activation of p53 and the senescence process. Additionally, PML bodies may also be the site of "nucleation" or initiation of chromatin condensation on E2F target genes that later become the detectable sites of SAHF (Vernier et al. 2011).

Furthermore, indirect connections were also identified between the Rb pathway and PML through the regulation of the Myc and Cyclin D1 oncoproteins. In this regard, it is well known that Myc and Cyclin D1 promote inactivation of Rb via phosphorylation catalyzed by the CDK4/6-Cyclin D complex. PML enhances the inhibitory function of Mad, an antagonist of Myc (Khan et al. 2001). In addition, PML controls the protein stability of Myc, possibly by binding and recruiting it into PML bodies along with the 26S proteasome (Buschbeck et al. 2007). On the other hand, PML prevents the expression of Cyclin D1 in part by blocking the transport of *Cyclin D1* mRNA to the cytoplasm (Lai and Borden 2000).

It is noteworthy that PML cannot induce senescence in the absence of functional Rb and p53 pathways. However, PML can still slow cellular growth and inhibit tumor formation in these conditions. It is plausible that PML activity must be carried out by PML in association with other cellular factors apart from the p53 and Rb pathways, as well as translocation of PML to the nucleolus, or defects in PML body formation, among other mechanisms.

PML Contributes to the DNA Damage Response

Several proteins involved in DNA damage response have been found to localize to PML bodies. These proteins range from DNA damage sensing proteins such as p53, ATM, ATR, topoisomerases and helicases, to DNA repair proteins involved in both double-strand breaks and homologous recombination. It has been reported that treatment with DNA damaging agents increases the number and size of PML bodies and causes relocalization and/or modification of PML bodies-associated proteins. Studies by Carbone and colleagues proposed that PML bodies function as sensors of DNA damage lesions contributing to the recognition of the sites of damage and/or their processing (Carbone et al. 2002). It has been postulated that the increase in PML bodies is part of the cellular response to DNA damage.

Given that the formation of DNA damage foci and the DNA damage response pathway are important features of the onset of senescence (Di Micco et al. 2006), it is possible that the association of PML bodies with DNA damage sensing or DNA repair proteins contributes to the activation of the DNA damage response pathway detected in senescence and to the proper onset of permanent cell cycle arrest to avoid the transference of damaged DNA to daughter cells.

Targeted Therapies to Restore PML Tumor Suppressor Function

PML is slowly emerging as a key tumor suppressor whose expression is down regulated in multiple types of human carcinomas (Gurrieri et al. 2004; Lee et al. 2007; Vincenzi et al. 2010). As such, identifying pharmacological means to restore its expression becomes increasingly more vital. In this review, we detail the proteasomal degradation pathway as a major route by which PML expression can be regulated, thereby making it an attractive therapeutic target when attempting to manipulate cellular PML levels. Currently, multiple small molecule inhibitors have been designed that may prove to be useful for future therapeutic regimens. As E6AP has been identified to be an E3 ligase that promotes the ubiquitination and degradation of PML (Louria-Hayon et al. 2009), inactivation of this ligase's function may enhance expression of PML in pathological conditions where PML

levels may be compromised. Interestingly, a product-like macrocyclic *N*-methyl peptide inhibitor has been designed that can inhibit E6AP-catalyzed polyubiquitination of target proteins. While this peptide has not been tested on PML ubiquitination mediated by E6AP, it has shown robust inhibitory activity against E6AP-catalyzed polyubiquitination of two characterized target proteins, Prx1 and p53 (Yamagishi et al. 2011). Although obstructing the E3 ligase activity of E6AP may be the most direct means to prevent PML degradation, inhibition of upstream signaling pathways may also prove to have merit as pharmacological interventions. As PML must first be phosphorylated by upstream kinases before it can be recognized by E3 ligases, inhibiting these kinases may also elevate PML expression and function. Scaglioni et al. demonstrated in a lung cancer xenotransplant model that emodin, a pharmacological inhibitor of CK2 kinase, could re-establish PML function in vivo (Scaglioni et al. 2006). Furthermore, a recent study elegantly described the in vivo anti-tumor effects of using a newly developed BMK1 inhibitor (XMD8-92) to restore PML activity (Yang et al. 2010). CK2, BMK1, and E6AP are three proteins known to play integral roles in PML degradation. Based on the studies described in this review, other attractive targets may also include additional E3 ligases such as KLHL20 or the SUMO E3-ligase Pias1.

While there has been some headway made in treating cancers deficient in the PML tumor suppressor, this field is still in its infancy. Additional studies identifying new and novel upstream regulators of PML function would significantly improve our understanding of this protein while also identifying new nodes of intervention when attempting to treat tumors deficient in PML.

Conclusion

The promyelocytic leukemia tumor suppressor PML plays a critical role in the onset of premature senescence, preventing the proliferation of cells with malignant potential. Mechanistically, the contribution of PML to senescence involves the p53 and Rb tumor suppressor pathways. PML physically interacts with the DNA-binding domain of p53 to promote p53 modifications. Additionally, p53 activation is driven by PML binding to MDM2, an E3 ligase that catalyzes the ubiquitination and subsequent degradation of p53, and by PML-mediated sequestration of MDM2 into the nucleolus. PML also interacts with Rb and recruits the Rb/E2F complex into PML bodies. These interactions may play a role in Rb/E2F-mediated gene silencing and heterochromatin formation during senescence. Likewise, PML bodies associate with DNA damage response and repair proteins, which might be components of the DNA damage response frequently found aberrantly activated in senescent cells. Importantly, replicative senescence has been proposed as a result of accumulated chronic DNA damaging signals and tumorigenesis may occur when the DNA damage response is inactivated. Therefore, bypassing senescence or reversion of this process is considered a critical step towards cellular transformation. Consistent with the notion of PML being a tumor suppressor, *PML* inactivation in mice leads to cancer susceptibility, and PML deficiency occurs in a broad spectrum of human cancers through mechanisms that involve its aberrant ubiquitination and turnover. Therefore, understanding the molecular mechanisms of PML degradation during tumorigenesis should provide the rationale for developing novel therapeutic strategies to trigger PML restoration as an approach to induce premature senescence to suppress tumor formation.

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Part V

Role in Cardiovascular Disease

10 Cardiac Senescence and Autophagy

Ken Shinmura

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Abstract

The biology of aging has not been fully clarified, but increasing evidence demonstrates that mitochondria play a critical role in bioenergetics, oxidant production, and regulation of cell death, all of which contribute to the aging process. The decline in cardiac performance that is observed in aging may, in part, be due to the decline in cardiac mitochondrial function that is associated with accumulation of oxidative damage. The process of degradation of dysfunctional or unnecessary cellular components with lysosomal machinery, known as autophagy, is vital for longevity and maintenance of normal cardiomyocyte function. However, aging is associated with reduced efficiency of autophagy. Therefore, interventions aimed at improving mitochondrial turnover by enhancing autophagy may be important therapies to retard cardiac senescence and manage age-related cardiovascular diseases. Lifelong caloric restriction (CR) can attenuate the decline in cardiac function with aging and retard cardiac senescence in rodents. The effect of CR appears to be related to a reduction in cellular damage induced by reactive oxygen species and an enhancement in autophagic flux. Promising CR mimetics with autophagy-inducing properties are those that intersect with the critical signaling pathways including the insulin/insulin-like growth factor-1 signaling, sirtuin pathway, AMP-activated protein kinase pathway, and mammalian target of rapamycin pathway.

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Introduction

The process of tissue deterioration with age leads to a decrease in overall health of the aging population. While the biology of aging is not well understood, cardiovascular disease (CVD) is known to be a common problem in the elderly (Lakatta 2003; Lakatta and Levy 2003) For example, left ventricular hypertrophy (LVH), atrial fibrillation (AF), and congestive heart failure (CHF) are essential cardiovascular problems, the risk for which increase dramatically with aging (Lakatta and Levy 2003).

The prevalence of LVH is associated with elevated blood pressure and increased body mass index, which are common issues in many developed countries. LVH evaluated by either echocardiography or electrocardiography has been shown to be associated with increased risk for coronary heart disease, sudden death, stroke, and overall cardiovascular events (Lakatta and Levy 2003).

In the Framingham study, AF without identified cause ("lone" AF) was present 16.8% of men and 6% of women with AF at a mean age of 70 years (Lakatta and Levy 2003). During long-term follow-up, individuals with lone AF showed stroke more than four times as frequently as did control subjects (most of them being patients with cerebral embolism).

The morbidity of CHF increases with aging, as does mortality (Lakatta and Levy 2003). Approximately half of older patients with CHF show normal LV systolic but abnormal LV diastolic function. The prognosis of CHF associated with LV diastolic dysfunction is similar to that associated with LV systolic dysfunction (Chatterjee and Massie 2007). However, therapeutic strategies aimed at improving LV diastolic dysfunction have not been established yet. Myocardial infarction (MI) is the most common cause of CHF associated with systolic dysfunction, and not surprisingly, the incidence of MI also increases with aging (Shih et al. 2011). Clinical evidence demonstrates an increase in the rate of cardiac rupture after MI with aging, as well as an increase in both in-hospital and post-discharge mortality rates (Shih et al. 2011).

The poor outcome of cardiovascular disease in the elderly may be explained, at least in part, by cardiac senescence at the cellular and organ levels (Lakatta 2003; Lakatta and Levy 2003). Therefore, better understanding of cardiac senescence is needed to develop novel therapeutics for regulating cardiac senescence. This is particularly important since the progress in medical care in developed countries has improved the quality of life and lengthened the average lifespan, and these countries are now confronted with "an aged society".

Age-Associated Changes in Cardiac Structure and Function

The heart exhibits a continuum of structural and functional alterations during the aging process. These age-associated alterations are likely to be related to the sharp increase in the incidence of LVH, AF, and CHF observed in the elderly population (Lakatta and Levy 2003).

Structural Changes

In cross-sectional studies including subjects without hypertension or clinically apparent CVD, the left ventricular (LV) wall thickness was found to increase with aging in both sexes (Lakatta and Levy 2003). Elderly patients without apparent CVD did not show an overall increase in LV mass at autopsy; however, as compared with younger patients, elderly patients showed cardiomyocyte enlargement associated with a robust decrease in the estimated cardiomyocyte number (Lakatta and Levy 2003). Increased collagen is observed in the aged myocardium, associated with an increase in the formation of collagen cross-linking. In addition, the accumulation of metabolite wastes is observed in the aged myocardium. Molecules such as lipofuscin, a brown granular pigment that consists of cross-linked lipids and proteins produced during lysosomal digestion, and amyloid, aggregates of insoluble fibrous protein, are evident in the aged myocardium. Figure 10.1 presents age-associated changes in the cardiac structure.

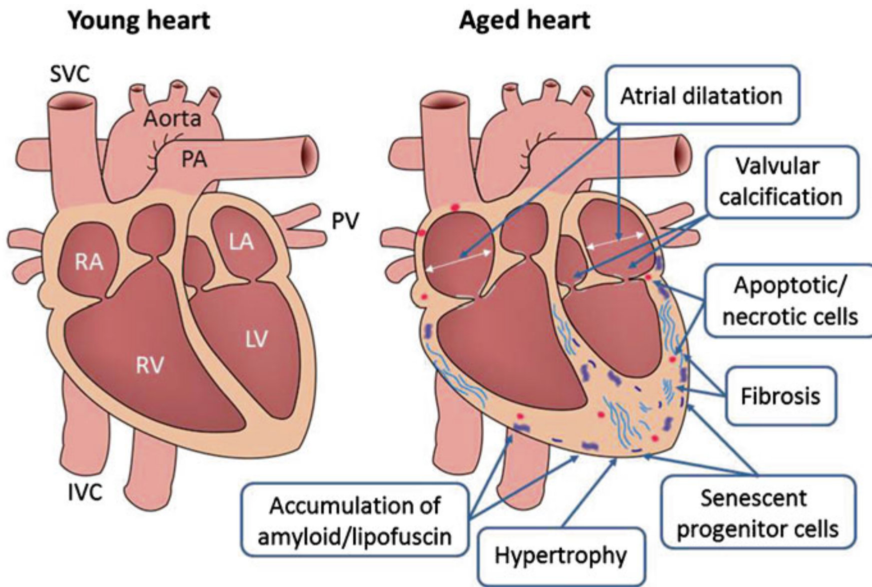

Fig. 10.1 Age-associated changes in the cardiac structure. *IVC* inferior vena cava, *LA* left atrium, *LV* left ventricle, *PA* pulmonary artery, *PV* pulmonary vein, *RA* right atrium, *RV* right ventricle, *SVC* superior vena cava

Functional Changes

Parameters of LV systolic performance at rest are preserved during aging without apparent CVD (Lakatta and Levy 2003). However, the maximum ejection fraction (EF) during exhaustive upright exercise decreases with aging in healthy subjects. This age-associated decrease could be explained by the decrease in coronary flow reserve due to coronary arteriosclerosis and the impaired response in contractile function and heart rate (HR) to β–adrenergic stimulation during exercise in the elderly.

In contrast, the aged heart exhibits impaired LV diastolic function (Chatterjee and Massie 2007; Lakatta and Levy 2003; Meyer et al. 2006). The age-associated impairment in LV diastolic function is complicated. Senescent cardiomyocytes are characterized by prolonged relaxation, diminished contraction velocity, a decrease in β-adrenergic response, and increased myocardial stiffness (Lakatta and Levy 2003). This impairment in LV diastolic function contributes to the increased incidence of CHF and AF in the elderly (Lakatta and Levy 2003). Evidence from studies using senescent rats demonstrates slowed relaxation and altered Ca^{2+} handling in the isolated cardiomyocyte (Lakatta 2003; Shinmura et al. 2011). In particular, impaired sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity, which is mainly responsible for controlling $[Ca^{2+}]_i$ by uptake of Ca^{2+} into the sarcoplasmic reticulum (SR) during relaxation, has been shown to contribute to the abnormalities in cardiac relaxation (Schmidt et al. 2000). The decrease in SR Ca^{2+} uptake during relaxation, resulting in prolonged contraction, is associated with decreased SERCA2 content and activity in experimental models of senescence. More recently, SERCA2a protein levels have been reported to be significantly decreased in the senescent human myocardium (Cain et al. 1998).

In addition, the accumulation of myocardial collagen and extracellular matrix increases with aging, contributing to myocardial stiffness, and cardiac diastolic dysfunction (Lakatta and Levy 2003). Furthermore, increase in the size of cardiomyocytes contributes to LV diastolic dysfunction associated with aging. Cardiomyocyte hypertrophy is associated with changes in the cytoskeletal proteins that could alter the microtubule architecture and heighten organization of sarcomeres within individual myocytes. Increased

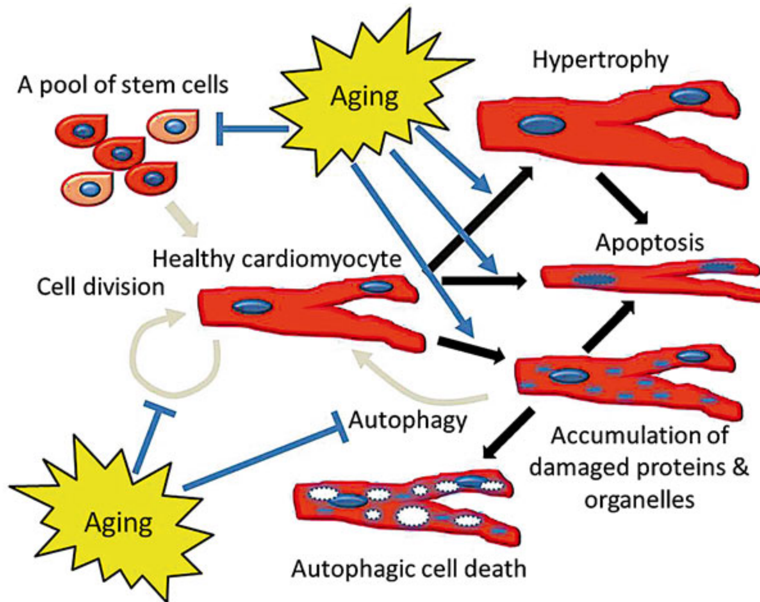

Fig. 10.2 Age-associated changes in cardiomyocytes

collagen volume fraction, larger cardiomyocyte diameter, and higher resting cardiomyocyte tension have all been correlated with LV diastolic stiffness. With aging, the myosin heavy chain isoform was found to shift from α to β in the rodent heart (Lakatta 2003). Lieber et al. (2008) demonstrated that the levels of α– and β–tubulin were significantly increased and those of desmin were decreased in aged rats, and this finding might explain the observed cardiac dysfunction with aging. Posttranslational modification of myofilament proteins, such as titin may also play an important role in LV diastolic dysfunction associated with aging (Shinmura et al. 2011).

The HR in healthy subjects does not change in elderly patients in the supine position at rest (Lakatta and Levy 2003). However, the maximal HR during exercise decreases with aging. The decrease in HR response to exercise is responsible, at least in part, for the reduction in the maximum acute cardiac output reserve in the elderly. Fibrosis in the cardiac conducting system increases with aging, while the number of specific cells consisting of the cardiac conducting system decreases with aging (Lakatta and Levy 2003).

Age-Associated Changes in Cardiomyocytes and Non-cardiomyocytes

Cardiac senescence can be characterized by both quantitative alterations, whereby a decrease in cardiomyocyte number is observed with aging, and qualitative alterations, whereby cardiomyocyte properties and the extracellular matrix change with aging. Figure 10.2 presents age-associated changes in cardiomyocyte properties.

Decrease in Cardiomyocyte Number in the Aged Heart

The number of cardiomyocytes in both the rodent and human heart is known to decline with aging (Centurione et al. 2002; Kajstura et al. 2010). The rate of cell death by apoptosis and necrosis in the adult heart is very low, but this rate increases proportionally with age (Olivetti et al. 1997). Old paradigm of human cardiomyocyte growth is that proliferation occurs only during the developmental stages shortly after birth.

The growth of the heart later in life is mainly mediated by an increase in cell size, known as cardiomyocyte hypertrophy. Cardiomyocyte hypertrophy and fibrosis in the aged heart play a role in compensatory mechanisms for cell loss. The discovery of adult cardiac stem cells in 2003 by Anversa's group (Beltrami et al. 2003) caused a paradigm shift in the interpretation of cardiac senescence. Now it appears that the renewal of cardiomyocytes occurs actively even in the aged heart. Two processes have thus been identified for the renewal of cardiomyocyte pool: by division of existing adult cardiomyocytes, and by the differentiation of progenitor/stem cells into new cardiomyocytes (Shih et al. 2011). However, both processes are likely to be impacted by the aging process.

Then, the important issue is how and why the number of cardiomyocytes declines with aging despite the high capacity of cardiomyocyte renewal. Two possibilities exist for the decline in the number of cardiomyocytes: either (1) a robust increase in cardiomyocyte death occurs by both apoptosis and necrosis associated with aging that cannot be covered by the renewal of cardiomyocytes, or (2) senescence of stem cells occurs, preventing the formation of new cardiomyocytes. Increasing evidence demonstrates that aging predisposes cardiomyocytes to apoptosis (Centurione et al. 2002; Kajstura et al. 2010). Cardiomyocytes are exposed to oxidative stress from a high level of metabolic and mechanical stress from constant beating, and yet the low turnover rate of cardiomyocytes, compared to other mitotic cells, requires the majority of them to survive for a long time (Shinmura 2013). The accumulation of cellular oxidative footprints including nuclear and mitochondrial DNA damage increases with aging. Together, these molecular alterations lead to the activation of pro-apoptotic genes, ultimately increasing cardiomyocyte death.

Kajstura et al. (2010) demonstrated that the human heart is a highly dynamic organ regulated by a pool of resident cardiac stem cells that modulate cardiac homeostasis and condition organ aging because cardiomyogenesis involves a large and progressively increasing number of parenchymal cells with aging. However, the ability of regenerate cardiomyocytes cannot prevent the manifestations of myocardial aging. Myocardial aging has been characterized by activation of resident cardiac stem cells with decreased telomere length, rather than by activation of cardiac stem cells with long telomeres, generating an old progeny that contributes to the manifestations of aging myopathy (Kajstura et al. 2010). Therefore, newly developed cardiomyocyte originating from cardiac stem cells with shortened telomeres are more susceptible to cardiac death, resulting in a failure to maintain cardiomyocyte homeostasis in the aged heart.

Sahin et al. (2011) have provided experimental evidence linking telomere-shortening and mitochondrial dysfunction. Targeted deletion of telomerase reverse transcriptase (Tert$^{-/-}$) in mice caused severe telomere dysfunction when backcrosses for ≥ 4 generations (G4) were performed, and resulted in pathological phenotypes not only in highly proliferative tissue but also in postmitotic organs, such as the heart. These G4 Tert$^{-/-}$ mice developed dilated cardiomyopathy, which is associated with mitochondrial dysfunction. These abnormalities are linked to p53-mediated repression of proliferator-activated receptor-γ coactivator (PGC)-1α and PGC-1β and their downstream genes, nuclear respiratory factor-1 and mitochondrial transcription factor A. Therefore, senescence-induced telomerase dysfunction might represent a primary instigator of mitochondrial dysfunction associated with aging, thereby reducing the cardiomyocyte population as the heart ages.

Impaired Autophagy in the Aged Heart

The autophagy and ubiquitin/proteasome system are two major proteolytic systems in the cell that ensure normal cellular function. The autophagy pathway is responsible for the degradation of intracellular components. Depending on the pathway along which cellular components are delivered to lysosomes, three types of autophagy can be distinguished: macroautophagy, microautophagy,

and chaperone-mediated autophagy (Dutta et al. 2012). Among them, macroautophagy is the most prominent and best-studied form of autophagy in the heart (Yamaguchi and Otsu 2012).

Autophagy is essential for cardiomyocytes, since the relative longevity of cardiomyocytes and their higher energy requirements expose cells to a high level of oxidative damage accumulation (Dutta et al. 2012; Shih et al. 2011; Yamaguchi and Otsu 2012). Proliferative cells remove biological wastes from oxidative damage by cell division. However, since most cardiomyocytes do not divide, halving cellular waste through mitosis is not possible. Therefore, effective autophagy is vital to maintain normal levels of metabolism and cellular function. Autophagic failure is reported to cause cardiomyopathy in experimental and clinical models. For example, targeted disruption of the autophagy gene *atg5* restricted in the mouse heart caused an accumulation of damaged proteins, ultimately causing cardiac hypertrophy, subsequent dilated cardiomyopathy, and heart failure at the age of 10 months (Taneike et al. 2010). The hearts of these mice exhibited severe mitochondrial dysfunction. Since mitochondrial biogenesis and destruction are regulated by mitochondrial fusion/fission and selective-autophagy of a mitochondrion, named mitophagy, these results strongly suggest that basal Atg5-dependent autophagy is required to maintain mitochondrial metabolism in the heart (Taneike et al. 2010).

The requirement for autophagy in cardiomyocytes increases with aging. An age-related increase in oxidative damage has been well documented. Reactive oxygen species (ROS) mainly produced by mitochondria damages surrounding mitochondrial as well as nuclear DNA, membranous lipids, and constitutive proteins. Oxidized DNA may undergo mutation, lipid peroxidation that may attenuate integrity of cellular and intracellular membrane, and oxidized proteins that may lose their enzymatic activity. These events all negatively affect mitochondrial and cellular function and contribute to the decline in cardiac function with aging (Shinmura 2013). To prevent cytotoxicity, the oxidative damage should be eliminated by autophagy. Since accumulation of oxidative damage in mitochondria further results in mitochondrial dysfunction, characterized by structural disorganization, enlargement, disability for efficient energy production, and susceptibility to ROS leakage under stress, autophagy is particularly important. Moreover, senescence-related cellular damage, including mutation accumulation and protein aggregation, impairs mitochondrial fission, which also serves as a waste-clearing mechanism by halving the amount of cytosolic waste (Shih et al. 2011).

Despite the increased need for autophagy in the aged heart, aging has also been associated with reduced efficiency of autophagy (Shinmura et al. 2011; Taneike et al. 2010; Wohlgemuth et al. 2007). Increasing age decreases the formation of autophagosomes and the activity of proteolysis, including autophagy, in some tissues. The activity of autophagy assessed by the protein levels of light chain3-II in the hearts of aged rodents was lower than that in the hearts of young rodents (Shinmura et al. 2011; Taneike et al. 2010; Wohlgemuth et al. 2007). In addition, intralysosomal accumulation of lipofuscin is responsible, at least in part, for the gradual inhibition of autophagy in aging (Dutta et al. 2012; Shih et al. 2011). Lipofuscinogenesis results from peroxide-induced Fenton reactions elicited by intralysosomal materials that produce highly reactive hydroxyl radicals. Subsequently, ROS-derived modification of proteins and lipids causes cross-linking within lysosomes/autolysosomes, leading to the generation of lipofuscin. The accumulation of intracellular garbage eventually overburdens the autophagosomal-lysosomal degradative capacity by acting as a sink for lysosomal hydrolases. The failure to digest the accumulating lipofuscin from the growing number of lysosomes leads to a decreased capacity of macroautophagy to cope with the cellular demand. This series of events trigger a vicious cycle in which autophagic failure and the accumulation of damaged mitochondria perpetuate each other, leading to the further oxidative stress and lipofuscinogenesis (Dutta et al. 2012; Shih et al. 2011).

In addition to removing cellular waste, macroautophagy contributes to the elimination of dysfunctional mitochondria, which can lead to

the induction of apoptosis (Dutta et al. 2012; Shih et al. 2011). As mentioned above, cardiomyocyte removal via apoptosis increases with aging (Centurione et al. 2002; Kajstura et al. 2010); however, it is still unclear if autophagic failure is directly attributable to the increased apoptosis in the aged heart. Regardless of this issue, mitochondrial dysfunction is likely to play a pivotal role in cardiac senescence (Shinmura 2013). Mitochondrial dysfunction is also implicated in the pathogenesis of a host of CVD that are highly prevalent in old age, including coronary artery disease, CHF, LVH, and diabetic cardiomyopathy (Dutta et al. 2012). Altered regulation of macroautophagy has also been shown to contribute to the pathogenesis of these conditions, providing further support for a role of the mitochondrial-lysosomal axis in cardiac senescence.

Assorted Alterations in the Aged Heart

Cardiomyocyte hypertrophy is pronounced in the aged heart and contributes to the development of a dysfunctional heart (Shih et al. 2011). Increased protein synthesis in response to hypertrophic signaling under inefficient autophagy and cytosolic saturation of oxidative damage causes accumulation of damaged proteins and organelles. In addition, energy demands are increased in hypertrophied cardiomyocytes, thus requiring more energy production from a pool of dysfunctional mitochondria. Subsequently, ROS production from dysfunctional mitochondria further increases and the release of cytochrome *c* leads to cardiomyocyte apoptosis.

Cardiac senescence is not only confined to the aging of cardiomyocytes, but also to that of non-cardiomyocytes. Although cardiomyocytes mainly frame the heart, non-cardiomyocytes constitute more than half of the cell population in the heart (Shih et al. 2011). Cardiac fibroblasts are responsible for providing the cardiac scaffold through the production, maintenance, and remodeling of extracellular matrix (ECM). Fibroblasts obtained from aged hearts have impaired proliferative capacity and a blunted response to profibrotic stimuli in vitro (Lindsey et al. 2005). Aged hearts subjected to MI exhibited reduced collagen deposition in the scar, delayed stabilization of scarring, and reduced but prolonged inflammation, suggesting that cardiac fibroblasts become dysfunctional with aging (Bujak et al. 2008). This may contribute to increased risk of cardiac rupture following MI in the elderly. In addition to cellular changes with aging, ECM also changes with aging (Shih et al. 2011). All of these cellular and molecular changes impact cardiac function during the aging process and likely contribute to the higher incidence of CVD in the aging population.

Effects of CR on Cardiac Senescence and Autophagy

Numerous experimental interventions designed to regulate the aging process have been attempted. To date, an established intervention that has been consistently shown to reduce the rate of aging and to increase both mean and maximal lifespan in various species is lifelong caloric restriction (CR) (Shinmura 2013; Shinmura et al. 2011; Speakman and Mitchell 2011). The beneficial effects of lifelong CR may be exerted, at least in part, through a reduction of oxidative damage in organs and tissues.

In contrast, although overexpression of antioxidant enzymes and antioxidant supplement diets have shown some degree of success in attenuating age-associated physiological dysfunction and extending mean lifespan, they have not extended maximal lifespan (Shinmura 2013). Clinical investigations demonstrated that antioxidant treatment has either no effect or detrimental effects on beneficial health outcomes in cancer, diabetes, CVD, and overall mortality (Shinmura 2013). These results suggest that modest ROS production promotes longevity by inducing the innate adaptive response against oxidative stress; this mechanism may be essential for the development of overall stress resistance and lifespan extension.

In addition to the remarkable lifespan extension, CR profoundly affects age-related physiological and pathophysiological alterations (Shinmura

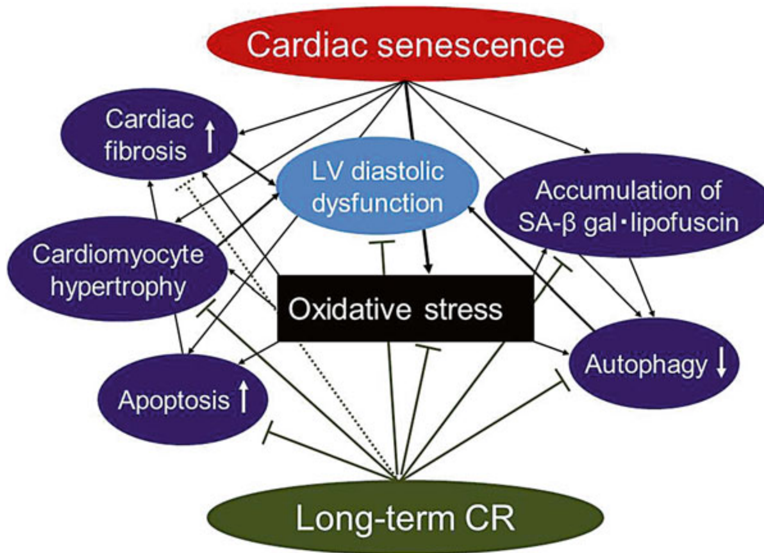

Fig. 10.3 Effects of caloric restriction (CR) on cardiac senescence. *SA-β* gal senescence-associated β galactosidase

2013; Shinmura et al. 2011; Speakman and Mitchell 2011). The effect of CR is clearly distinct from that of malnutrition and starvation. In general, there is no evidence of malnutrition or cachexia in animals treated with standard CR regimens if caloric intake is restricted to 50–70% of ad libitum (AL) quantities. CR decreases oncogenesis and apoptosis, and some experimental studies suggest that CR could attenuate the incidence of dementia and neurodegenerative diseases in humans (Speakman and Mitchell 2011). CR is also anticipated to reduce cardiovascular morbidity and mortality by conferring cardiovascular protection. However, the impact of long-term CR on cardiovascular senescence has not been fully evaluated. Therefore, we investigated the impact of long-term CR on cardiac senescence in male Fischer344 rats, starting from the age of 8 months until 30 months, focusing on the effects of CR on cardiac diastolic dysfunction associated with aging (Shinmura et al. 2011).

The major findings of our study were as follows: (*1*) long-term CR improved LV diastolic function without affecting LV systolic function; (*2*) long-term CR attenuated cardiomyocyte hypertrophy, apoptosis, and the cardiac expression of markers of senescence, such as β-galactosidase, lipofuscin, and p16^{INK4a}; (*3*) long-term CR failed to reduce cardiac fibrosis and to prevent decreases in phosphorylated troponin I and phosphorylated phospolamban levels; (*4*) long-term CR attenuated the decrease in the levels of sarcoplasmic reticulum calcium ATPase (SERCA2) protein and its activity, and ameliorated age-associated deterioration of intracellular Ca^{2+} handling; (*5*) long-term CR suppressed the mammalian target of rapamycin (mTOR) pathway; and (*6*) long-term CR enhanced autophagic flux in the heart (Fig. 10.3) (Shinmura et al. 2011). These results clearly demonstrate that CR can impact cellular function that is related to CVD in the aging population.

Decreased size of cardiomyocytes observed with CR may contribute to the amelioration of LV diastolic dysfunction in CR rats (Shinmura et al. 2011). Cardiomyocyte hypertrophy is associated with changes in the cytoskeletal proteins that could alter Ca^{2+} sensitivity. However, our results indicate that cardiomyocyte responsiveness to Ca^{2+} estimated from the relationship between Ca^{2+} transient and myocyte shortening is similar between isolated myocytes obtained from AL and CR rat hearts (Shinmura et al. 2011).

Therefore, we speculated that inhibition of the SERCA2 expression decline is a major factor in preserving LV diastolic function by CR. However, it is also possible that CR mediates age-associated alterations in cytoskeletal proteins.

The accumulation of myocardial collagen and extracellular matrix increases with aging, contributing to increased cardiac fibrosis, myocardial stiffness, and cardiac diastolic dysfunction. Dhahbi et al. (2006) demonstrated that long-term CR reduced myocardial collagen and extracellular matrix content and attenuated cardiac fibrosis associated with aging. Thus, CR-induced changes in cardiac connective tissue may contribute, in part, to the amelioration of diastolic function, especially late diastolic function, as observed by Taffet et al. (1997). However, we did not observe a significant decrease in cardiac fibrosis in CR rat hearts (Shinmura et al. 2011). We speculate that the discrepancy between the study of Taffet et al. and our study could be due, at least in part, to differences between species and the formula of CR diet since functional differences such as metabolic rate and heart rate differ between species, and our study did not include mineral restriction. These differences must be considered with regard to fibrosis, extracellular matrix composition, and rennin-angiotensin-aldosterone system (RAS) interactions. Recent investigations revealed an essential role of RAS in the development of cardiac fibrosis with aging (Shinmura et al. 2011). Thus, our results may suggest that CR is not sufficient for the suppression of RAS activation with aging when minerals are not restricted during CR.

The mechanisms by which long-term CR retards cellular senescence and attenuates the physiological decline of organ function have not been fully elucidated. In general, CR decreases the age-associated accumulation of oxidative damage to lipids, proteins, and DNA (Shinmura 2013; Speakman and Mitchell 2011). We found that the expression of protein carbonyls was lower in CR hearts than in AL hearts (Shinmura et al. 2011). In addition, the number of cardiomyocyte apoptosis was reduced in the aged heart treated with CR. Thus, it is possible that long-term CR retards cellular senescence and attenuates the degree of cell death by attenuating oxidative damage in the aged heart. However, direct evidence that attenuation of oxidative damage is the primary means by which CR prevents cardiac senescence is still lacking.

Another important mechanism by which long-term CR retards cardiac senescence is enhancement of autophagy. Enhancement of autophagy in the aged heart is considered to confer protection through degradation and removal of damaged organelles and protein aggregates accumulated during the aging process. Increasing evidence suggests that the modulation of the autophagic response represents a primary mechanism underlying the extension of lifespan by CR (Hars et al. 2007; Jia and Levine 2007; Shinmura et al. 2011; Speakman and Mitchell 2011; Wohlgemuth et al. 2007). Indeed, the inhibition of autophagy abolishes the anti-aging effect of CR in lower organisms (Jia and Levine 2007). Our results indicate that autophagic flux is enhanced in CR hearts (Shinmura et al. 2011) and this finding is consistent with that of previous studies in which CR enhanced the autophagic activity in the aged heart (Wohlgemuth et al. 2007). Inuzuka et al. (2009) demonstrated that suppression of phosphoinositide 3-kinase preserved cardiac function and attenuated the expression of senescence makers associated with enhanced autophagy. Because autophagy is not inhibited but is only somewhat impaired in the aged heart, the accumulation of impaired SR and mitochondria is sublethal and may result in mild functional decline. Impaired autophagy in the aged heart may contribute, in part, to the accumulation of lipofuscin, which further inhibits autophagy. We found that long-term CR attenuated the accumulation of lipofuscin (Shinmura et al. 2011), suggesting that long-term CR disrupts this cycle in the aged heart. In addition, we demonstrated that enhanced autophagy was associated with suppression of the mTOR pathway in the heart of mice treated with CR (Shinmura et al. 2011). CR can induce macroautophagy via different pathways: the insulin/insulin-like growth factor signaling, sirtuin pathway, AMP-activated protein kinase (AMPK) pathway, and mTOR pathway (Dutta et al. 2012; Speakman and Mitchell 2011; Yamaguchi and Otsu

2012). These pathways are closely interconnected, and all of them play important roles in mediating different aspect of the autophagic response.

Similarly, clinical studies have demonstrated that CR, when accompanied by significant body weight loss, exerts cardiac-specific effects that ameliorate LV diastolic function in healthy subjects (Meyer et al. 2006), as well as in patients with type 2 diabetes mellitus (Hammer et al. 2008). Whether enhanced autophagy during CR is actually responsible for the improvement in LV diastolic dysfunction remains to be confirmed. However, clinical application of CR and the development of CR mimetics that can replicate the effects of CR have considerable potential as novel therapeutic approaches for the treatment of patients with diastolic dysfunction, because no successful therapeutic strategies aimed at improving LV diastolic dysfunction have been established yet.

Pharmacological Interventions to Prevent Cardiac Senescence Through Autophagy

Promising CR mimetics with autophagy-inducing properties are those that intersect with the critical signaling pathways identified above. They include biguanides such as metformin, which targets the AMPK and insulin signaling pathways; resveratrol, which affects sirtuin activity; and rapamycin, which interacts with the mTOR signaling (Dutta et al. 2012; Yamaguchi and Otsu 2012). In addition, the polyamine molecule spermidine has been reported to extend the lifespan in rodents (Yamaguchi and Otsu 2012). Moreover, autophagy has been shown to mediate the lifespan extension through either the effects of rapamycin or spermidine in *Caenorhabditis* (*C*) *elegans* and *Drosophila melanogaster* (Yamaguchi and Otsu 2012).

mTOR: Rapamycin

Treatment with rapamycin, a specific inhibitor of TOR kinase complex 1 (TORC1), induces an increase in autophagic activity, as well as, starvation. Rapamycin-induced autophagy is independent of the effects of Sirt1, suggesting that rapamycin and Sirt1 enhance the autophagic activity via distinct mechanisms (Yamaguchi and Otsu 2012). TORC1 phosphorylates Atg13 and phosphorylated Atg13 exhibits a lower affinity for Atg1. Thus, inhibition of TORC1 by rapamycin induces dephosphorylation of Atg13 and binding of Atg13 to Atg1, which leads to the induction of autophagy. AMPK acts as an upstream kinase of mTOR to promote autophagy. Inhibition of mTOR by rapamycin reportedly prolongs the lifespan in nematodes, flies, and mice (Speakman and Mitchell 2011; Yamaguchi and Otsu 2012). Rapamycin also reduced the levels of phosphorylated S6 kinase, a target substrate in the mTOR signaling pathway, resulting in further enhancement of autophagy. In *C. elegans* and yeast, rapamycin can extend the lifespan only if autophagy is not inhibited (Yamaguchi and Otsu 2012). Rapamycin has been shown to reverse existing cardiac hypertrophy induced by pressure overload (McMullen et al. 2004), but it remains unclear whether enhanced autophagy is actually attributed to the lifespan extension by rapamycin in rodents.

Sirt 1: Resveratrol

Sirt1 and its orthologs (Sir2 in yeast and *Drosophila*, and Sir2.1 in *C. elegans*) are members of the conserved sirtuin family of nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase. Sirt1 is reported to regulate cellular senescence and lifespan extension in yeast, nematodes, and flies (Yamaguchi and Otsu 2012). Overexpression of Sirt1 induces autophagy in human cells and in *C. elegans*. Long-term CR increases the intracellular levels of NAD^+ and subsequently activates Sirt1 (Speakman and Mitchell 2011). It is likely that extension of lifespan by CR is related to increasing Sirt1 expression. Sirt1 deacetylates not only histone H3 and H4, but also various transcriptional factors including p53, nuclear factor-κB, forkhead box O1, O3, and O4, and PGC-1α. Sirt1 can induce autophagy through deacetylation of the *Atg* gene

products, Atg5, 7, and 8. Sirt1 exists both in the nucleus and the cytoplasm. Because the cytoplasm-restricted mutant of Sirt1 can stimulate autophagy (Yamaguchi and Otsu 2012), Sirt1 may induce autophagy independent of its nuclear effects through deacetylation of cytoplasmic target proteins.

Resveratrol activates Sirt1 directly and indirectly (Petrovski et al. 2011). Resveratrol prolongs the lifespan in yeast, nematodes, and flies. In contrast, resveratrol extends the lifespan in mice only when they are fed with a high-fat diet. The knockdown or knockout of Sirt1 prevents the induction of autophagy by resveratrol and by nutrient deprivation. These data suggest that CR stimulates autophagy via the activation of Sirt1. Both CR and resveratrol inhibit gene expression profiles associated with cardiac aging in mice (Barger et al. 2008). Studies in rodents have shown that resveratrol inhibits cardiomyocyte apoptosis, prevents LVH, improves endothelial function, and enhances autophagy at a low dose (Petrovski et al. 2011). In contrast, higher dose of resveratrol inhibits macroautophagy in H9c2 heart myoblasts (Petrovski et al. 2011). In addition, higher levels of Sirt1 overexpression (12.5-fold) increase the number of cardiomyocytes undergoing apoptosis and the degree of cardiomyocyte hypertrophy, resulting in the development of cardiomyopathy associated with mitochondrial dysfunction in mice (Alcendor et al. 2007).

In addition to Sirt1-activating property, resveratrol has various properties (Petrovski et al. 2011). Thus, discovery of other sirtuin-activating compounds those are more specific for Sirt1 with higher affinity would be highly beneficial in the development of CR-mimicking therapies.

AMPK: Metformin

AMPK is clearly an important element for regulating autophagy. AMPK exerts a negative regulatory effect on mTOR and triggers Sirt1-dependent deacetylation of the transcriptional factors and *Atg* gene products, resulting in enhanced autophagy (Yamaguchi and Otsu 2012). Metformin is widely prescribed as an anti-diabetes drug and activates AMPK. It is reported that metformin treatment enhances cardiac autophagy and protects against cardiomyocyte apoptosis in diabetic mice (He et al. 2013). Metformin treatment prolongs the lifespan and prevents cancer in mice, but its effects vary, depending on mice strain and gender (Anisimov et al. 2010). The effect of metformin on cardiac autophagy in aged animals is yet to be elucidated.

Spermidine

Intracellular concentration of polyamines declines with aging, yet administration of an exogenous polyamine spermidine prolongs the lifespan in yeast, nematodes, and flies in an autophagy-dependent manner (Yamaguchi and Otsu 2012). A diet enriched with polyamines also prolongs the lifespan in mice, but the involvement of autophagy in this effect is yet to be elucidated. Spermidine treatment induces deacetylation of histone H3 through the inhibition of histone acetyltransferases (HAT). The altered acetylation status of the chromatin leads to significant upregulation of the *Atg* genes including those encoding Atg7, 11, and 15, triggering autophagy in yeast, nematodes, flies, and human cells (Yamaguchi and Otsu 2012). Deletion of Atg7 in yeast and flies abolished the lifespan extension by spermidine. Recent studies have shown that spermidine reverses vascular aging in mice by increasing nitric oxide bioavailability, reducing oxidative stress modifying structural factors and enhancing autophagy (Larocca et al. 2013).

Conclusions

Cardiac senescence is characterized by a decrease in cardiomyocyte number and qualitative alteration in cardiomyocyte properties and extracellular matrix with aging. The number of cardiomyocytes is determined by the balance between cardiomyocyte death and the renewal of cardiomyocytes, both of which are greatly influenced by aging. Quantitative alterations with aging include cardiomyocyte hypertrophy and autophagy. Because most

cardiomyocytes do not divide and their higher energy requirements expose cells to a high level of oxidative damage accumulation, autophagy is essential for cardiomyocytes to maintain their function and survival for a long time. Despite an increased need for autophagy to eliminate dysfunctional cellular components, aging is associated with reduced efficiency of autophagy. Failure to eliminate dysfunctional mitochondria by autophagy could lead to further accumulation of oxidative damages and induction of apoptosis by cytochrome *c* released from impaired mitochondria. Although the mechanisms involved in impaired autophagy in the aged heart are not well understood, intralysosomal accumulation of lipofuscin is likely involved. Lipofuscin is associated with ROS-derived modification of proteins and lipids, and is responsible, at least in part, for the gradual inhibition of autophagy with aging.

Since mitochondria-driven oxidative damage plays an essential role in cardiac senescence and the development of CVD, the administration of antioxidants might mitigate the burden of cardiomyocyte injury with aging; however, the efficiency of antioxidant supplementation is still a matter of debate. Alternatively, exploitation of the cellular machinery to repair oxidatively damaged molecules and organelles has potential for controlling cardiac senescence and associated pathologies. Here, we discussed interventions aimed at improving mitochondrial turnover through autophagy through CR and CR mimetics, which would be especially relevant to retardation of cardiac senescence and management of age-related CVD. However, most autophagy mediators have multiple functions, suggesting that their manipulation might produce unrelated and sometimes undesirable effects. Therefore, deeper understanding of various effects induced by autophagy-related factors is warranted before their clinical use.

Disclosures *None declared.*

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