Tumor Dormancy, Quiescence, and Senescence
Volume 1

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Tumor Dormancy, Quiescence, and Senescence

Aging, Cancer, and Noncancer Pathologies

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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 (Guinet 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 intradurallipoma (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 (Ursinoet 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 (Martens 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 rise 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 the ages younger than 40 years. In this age group the frequency of prostate malignancy is one in 10,000 individuals. Unfortunately, the incidence of malignancy increases over the ensuing decades, that is, the chance of prostate malignancy may reach to one in seven 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 in 25% of men with serum levels of PSA between 4 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 (4–10 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). Automatic linking of 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 7 are the main criteria to select patients for active surveillance. The correct use of these two criteria is essential to differentiate between aggressive and nonaggressive 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

Little is known regarding the factors that regulate entry of residual cancer into a dormant state or the subsequent reinitiation of growth. The prognostic factors present in the primary tumor are imprecise in predicting which patients will be cured by local treatment and which patients will have metastatic recurrence.

Although much progress has been made in identifying many of the genetic factors that contribute to cancer development, much remains to be learned about genetic and epigenetic factors that influence both tumor dormancy and the growth of metastasis. A majority of us have in situ tumors that may remain dormant or may progress into a lethal form of cancer; the former are prevented from recruiting their own blood supply.

This is volume 1 of the multivolume series discussing Tumor Dormancy, Quiescence, and Cellular Senescence. The role of tumor dormancy in a number of diseases, including breast cancer, melanoma, prostate cancer, liver cancer, and lung cancer is discussed. It is also pointed out that quiescent state regulates hematopoietic stem cells and muscle stem cells. The mediation of reversible quiescent state in a subset of ovarian, pancreatic, and colon cancers by the kinase is detailed. Molecular mechanisms underlying stress-induced cellular senescence and accumulation of reactive oxygen species and induction of premature senescence are presented. The importance of the role of microRNASE in oxidative stress-induced apoptosis and senescence and the effect of microRNA as a modulator of cell proliferation in lung cancer are detailed. Suppression of cellular senescence in glioblastoma brain tumor is also explained.

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 diseases and injuries. It is difficult for a single author to discuss effectively the complexity of diagnosis, therapy, including tissue regeneration. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of cancer cure and tissue regeneration. I hope these goals will be fulfilled in this and other volumes of the series. This volume was written by 60 contributors representing 11 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 the volume are divided into three subheadings: Dormancy, Quiescence, and Cellular Senescence for the convenience of the readers.

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.

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M.A. Hayat

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Part I

Tumor Dormancy

Dormancy, Quiescence, and Cellular Senescence

1

M.A. Hayat

Contents

Keywords

Dormancy • Quiescence • Cellular senescence • Hibernation

Dormancy (Hibernation)

Dormant tumors are defined as microscopic (diameter of ~1 mm) human cancers, either primary, recurrent or metastatic, and can remain in an asymptomatic, non-detectable, and occult form for a long period of time. In other words, this phenomenon of long-term persistence of cancer cells that do not grow is called tumor dormancy (hibernation). Patients can carry cancer cells for a very long time, in some cases indefinitely without relapsing. Based on tumor type and stage, the dormancy period may range from years to even decades between the initial therapy and the occurrence of relapsed tumors or recurrent metastatic disease. As seen in autopsies of adults who have died from non-cancer causes, tumors can be dormant as long as a lifetime without ever becoming clinically evident. Dormant tumors represent the earliest stages in tumor development and are highly prevalent in humans. This phenomenon occurs early in tumor development, indicating that tumor growth is not continuous, and may pass through a long period of subclinical equilibrium. Dormant tumors have been identified in many organs, including thyroid, breast, and prostate, and are being described as cancer without disease.

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Therefore, clinical relevancy of tumor dormancy is important.

Tumor dormancy is attributed to the long latency periods frequently observed in cancer patients between the primary diagnosis and treatment and the potential clinical evidence of local recurrence or distinct metastasis. It may happen after treatment in the form of minimal residual disease. Late relapse after treatment is well-documented in breast cancer patients, and dormancy is a common phenomenon in these patients. These dormant micrometastases escape the treatment and can lead to late relapse.

It is possible to induce tumor dormancy in immunoincompetent hosts by prior immunization against tumor cells. Equilibrium between immune response and tumor cells leads to a long-term tumor dormancy. This equilibrium is also observed early in tumor development, and adaptive immunity may help contain tumor outgrowth. However, after variable times, tumor dormancy ends with the disease progress. The presence of immunoescape mechanisms in tumor cells from relapsing patients also suggests that the immune equilibrium which maintained dormancy has broken down. Identification of such mechanisms would offer new leads to favor the immune balance, and thus clear minimal residual disease from patients.

Although the clinical implication of tumor dormancy in prevention and treatment of tumors has intrigued the medical community for years, there is a paucity of molecular markers and mechanistic understanding. A critical limitation confronting the field of tumor dormancy is the lack of suitable experimental models as well as consistent and abundant sources of dormant tumor cells. It is still unclear what keeps these tumors in a microscopic size, preventing their expansion, and what triggers their proliferation. Genetic and epigenetic factors responsible for these two phases will be explained in the proposed four volumes of Tumor Dormancy and Cellular Quiescence.

Tumor dormancy is the result of angiogenesis suppression and some other environmentally imposed limitations to growth. Dormant tumors can become malignant and aggressive after undergoing an angiogenic switch, receiving appropriate molecular signals, and overcoming genetic and environmental constraints to tumor progression. Understanding how to prevent conversion of such lesions from harmless dormant nodules into malignant tumors may lead to the development of improved cancer treatments. Preventing the development of metastases is perhaps achievable more readily than curing patients with overt metastases. It is estimated that two out of three humans never develop cancer.

As stated above, angiogenesis plays a major role in the growth promotion of dormant micrometastasis because blood vessels deliver oxygen and nutrients into the tumor microenvironment. However, it is accepted that human tumors can arise in the absence of angiogenic activity and exist in a microscopic dormant stage for months to years without neovascularization. The disease stage of cancer, therefore, seems to be a late event in tumor development.

Cellular Quiescence

Hematopoietic stem cells are responsible for blood cell production throughout the lifespan of an organism. Millions of blood cells are used every second in humans, and hematopoietic cells must replenish these cells. The properties of these cells include relative quiescence, self-renewal capacity, and the ability to differentiate into multiple lineages. In fact, adult hematopoietic stem cells exist in a relatively quiescent state in the bone marrow microenvironment to fulfill long-term self-renewal and multilineage differential functions, an event that is tightly regulated by extrinsic and intrinsic cues. In general, hematopoietic stem cells either stay in quiescence or proliferate toward differentiation for the production of mature blood cells or toward self-renewal for giving rise to themselves. In other words, the state of quiescence in hematopoietic stem cells is reversible and differs from quiescence associated with senescence, differentiation, or growth factor deprivation.

In order to both maintain a supply of mature blood cells and not to exhaust themselves throughout the lifetime of an individual, under steady state, most hematopoietic stem cells remain quiescent, and only a small number enters the cell cycle. However, in response to hematopoietic stress, such as blood loss, these cells exit quiescence and rapidly expand and differentiate to repopulate the peripheral hematopoietic compartments. When quiescence is disrupted, hematopoietic stem cells display defective maintenance in G_0 phase of cell cycle, leading to premature exhaustion of the stem cell pool under conditions of hematopoietic stress, impaired self-renewal, and loss of competitive repopulating capacity. This eventually causes hematological failure. Quiescence of hematopoietic stem cells is critical not only for protecting the stem cell compartment and sustaining stem cell pool during late periods, but also for protecting stem cells by minimizing their accumulation of replication-associated mutations.

As stated above, understanding the regulation of hematopoietic stem cells quiescence is of great importance not only for undertaking the physiological functions of these cells, but also pathological origins of many related disorders. Under standing quiescence regulation in hematopoietic cells will also enable directed manipulation of the function of these cells, which will improve the efficiency of bone marrow transplantation and treatment of various hematopoietic disorders. The current advances in quiescence regulators and related pathways for hematopoietic stem cells will be discussed.

Relative quiescence is a defining characteristic of hematopoietic stem cells, while their progeny has dramatic proliferative ability and inexorable progress toward terminal differentiation. The balance between quiescence and proliferation is tightly controlled by both hematopoietic stem cell intrinsic mechanisms and the interaction of these cells with their specific microenvironments (known as stem cell niches). This control is carried out through cell-cell, cell-extracellular matrix, and receptor-ligand interactions, and involves both positive and negative regulators. This information increases the efficiency of both bone marrow transplantation and treatment for hemato-deficiency and hematopoietic cancers.

Despite the enormous proliferative capacity of hematopoietic stem cells, most of these cells reside in a non-cycling quiescent state at any given time point. This ensures lifelong-hematopoiesis and protection of hematopoietic stem cell pool from myelotoxic insult and premature exhaustion under conditions of hematopoietic stress. The naturally quiescent state of hematopoietic stem cells is controlled by negative regulators of cell proliferation. For example, endogenous levels of the cyclin-dependent kinase inhibitor p21 is crucial in order to maintain quiescence of hematopoietic stem cells and to protect stem cell pool from exhaustion during stressed conditions. In the absence of p21, increased cell cycling leads to stem cell exhaustion and hematopoietic failure. Under conditions of stress, restricted cell cycling is crucial to prevent premature stem cell depletion and hematopoietic death.

Is Tumor Dormancy Clinically Relevant?

2

Dieter Hölzel, Renate Eckel, Rebecca Emeny, and Jutta Engel

Contents

Abstract

Late progressions can be observed with all solid cancers. With the term dormancy a potential cause is offered for these observations. In this article we present a point of view from a cancer registry and analyze clinical data about metastasis (MET)-free survival, post-MET survival, and overall survival to quantify late progressions. If dormancy is a characteristic of the MET process then all types of MET, including local recurrences, regional MET in the lymph nodes, or distant MET in organs, must be considered. First, it can be deduced from clinical data that the initiation of secondary foci is a temporally sequential process, which can begin years, or days, before a R0-resection. Second, the growth time of these different MET can be estimated from the survival time and generally takes years. Third, remarkable growth differences of these secondary foci must be considered which already can be correlated, in part, with molecular subgroups. Within these subgroups, growth is quite homogeneous. These three factors of MET growth largely explain the variability of observed relapse-free survival times. In contrast, the term dormancy is vague. It is an appealing metaphor with strong analogies such as circulating tumor cells of hematological neoplasms or dormant tumor cells in transplanted organs. But late MET can be the result of a number of very different causes. Where a disseminated tumor cell

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lodges, in niches or in specific organs, how long a tumor cell circulates before settling and establishing a focus, or whether the tumor cell has differential growth or even cell quiescence phases, determined by a dynamic equilibrium of divisions and apoptosis, could all contribute to the differential occurrence of MET. MET detection may also be delayed by adjuvant treatment, and all causal variants can be functionally equivalent, delay MET diagnosis, and appear as a slow growing tumor. But time of initiation and the growth of tiny foci are inaccessible and impossible to measure in humans. Therefore, the term tumor dormancy conceals our ignorance of the multi-step MET process. Because it is such a cloudy and elusive term it cannot be clinically relevant. It is a hypothetical construct that fails to offer new research perspectives, additional prognostic factors or an opportunity for novel therapy.

Keywords
Tumor dormancy • Model • Breast cancer • Colorectal cancer • Metastasization • Long-term survial

Introduction

Tumor progression after an above-average, long, metastasis-free survival can be observed in all solid tumors. In many publications frequencies, characteristics, and survival of intact or fragmented tumor cells (TC) in blood, bone marrow, lymph nodes (LN) and organs are described. These facts are correlated with progressions and should show the relevance of dormant TC for late metastases (MET) (Meng et al. 2004; Naumov et al. 2002; Uhr and Pantel 2011; Weinberg 2008). The observation of a bimodal distribution of the MET-free survival time in breast cancer (BC) in one cohort study was already interpreted in 1990 as a result of a rest period of disseminated TC before growing up to a detectable MET (Demicheli et al. 2010). Since then, longer MET-free survival times for BC, e.g. beyond 5 years, are connected today with the term "TC dormancy" (Aguirre-Ghiso 2007).

In the following pages we describe, with the results of experimental and clinical studies, and with data from a cancer registry, the growth of secondary foci with MET-free, post-MET and overall survival. The Munich Cancer Registry (MCR) collects data about local, regional and distant relapses during the course of disease as important outcome criteria (Munich Cancer Registry). We present population-based data from patients who were registered from 1988 to 2009, did not have earlier or synchronous second malignancies and were followed-up during this period. It is important to note that the data about the courses of disease are not complete and therefore the percentage of primary MET of all cancer-related death is slightly overestimated. Additionally, MET is diagnosed during the course of disease if symptoms require clarification or a palliative chance exists. Therefore, any MET pattern is a selected perspective. Nevertheless, population-based data can add generally valuable aspects to the alternative view of heavily selected study cohorts.

Correlations of MET relapses with prognostic factors of the primary tumor (PT) reveal growth differences and an order of late progressions. Well known survival curves with adjuvant treatments describe further aspects of the MET process. Nonetheless, such a registry-based viewpoint does not contribute new results. Only additional facts can be considered and supplemental questions arise with our attempt to align known clinical outcomes with the hypothesis of dormant TC. But we have not been able to achieve this: therefore the current clinical relevance of the term tumor dormancy has to be questioned.

Basic Characteristics of the MET Process

Generally, MET is a secondary focus established by a disseminated TC of the PT. MET foci can arise locally, near the PT, regionally in the LN, or in distant organs. They are the result of a complex multistep process (Talmadge and Fidler 2010; Valastyan and Weinberg 2011). Three characteristics of the MET-process will be distinguished for the sake of reasoning (Hölzel et al. 2010); the temporally sequential initiation of MET, the

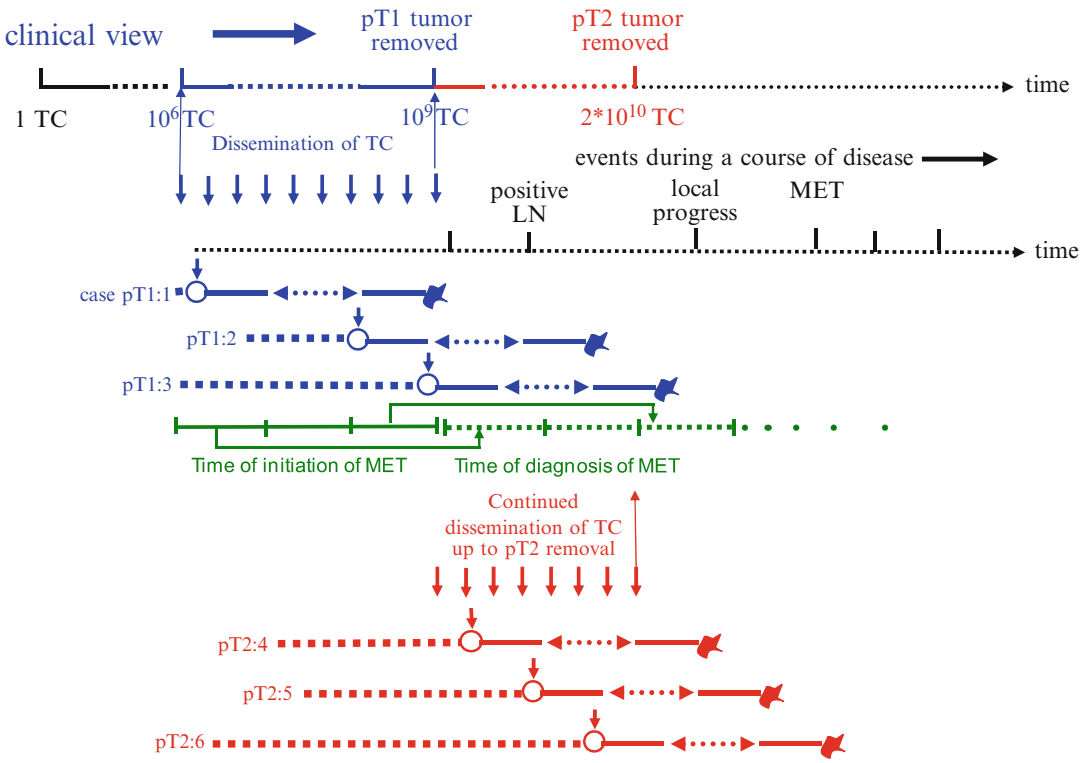

Fig. 2.1 Risk, initiation and detection of secondary foci in solid tumors. Local, regional and distant MET are initiated sequentially. Already tiny tumors – for breast cancer about 1 mm diameter – can initiate MET. The risk ends with the R0-resection of the primary tumor. The extremes are shown with case pT1:1, a primary advanced tumor and case pT1:3 with an initiation just before removal and a resulting long MET-free survival. If the tumor is removed only with a pT2 size (*red part of the figure*) the proportion of primary M1 rises and some cases (case pT1:2) may be diagnosed earlier due to symptoms of MET. Case pT1:3 shows a short MET free interval despite normal MET growth. Also with pT2 a long MET free interval can be observed (case pT2:6). The notation e.g. pT2:x for cases indicate an initiation of MET before pT2 and after pT1 ▪▪▪, ○, ◂···▸, 🐀

mean growth time of the different foci, and the subgroup-specific, homogeneous growth of all MET.

Temporally Sequential Initiation

To begin with, the initiation of TC dissemination that may become a detectable MET occurs in a temporally sequential process. MET foci could have been initiated by even small, 1 mm PT either years or days before a R0 resection. A very early MET initiation can be diagnosed as a primary M1, whereas the initiation that occurs shortly before R0-resecction may only be detectable years after the primary diagnosis. In colorectal cancer with a pT1 diagnosis, 1.4% MET and 8.1% pLN are observed. If the PT is first detected as a pT3 (Fig. 2.1 a delayed detection is shown in red) then in the course of further growth from pT1 to pT3, an additional 39.5% LN will have been infiltrated and primary M1 will be diagnosed in 17.0% of these pT3 patients. Most of these M1-MET foci were very likely initiated before pT1 was achieved. Together with the additional foci detectable at pT1, a total of 47.6% pLN and 18.4% M1 diagnoses are observed at pT3 (Table 2.1). Ten years after a pT1 or pT3 diagnosis, approximately 10.3 or 46.5% tumor associated deaths are observed, respectively. That means that after a M0 diagnosis, distant MET occurs in 8.9/26.2% for either pT1/pT3 tumors. In BC with an interval

Table 2.1 Terciles of the survival time from diagnosis of the primary tumor of metastasized and deceased patients with breast or colorectal cancer and the distributions of prognostic factors within the terciles

		Survival time of metastasized and deceased patients				
Subgroup	Characteristics (%)	1. Tercile	2. Tercile	3. Tercile	all	All patients (%)
Breast pT1	Time interval (ys or n)	<3.8	3.8–7.0	≥7.0 [12.3]	n = 1.255	n = 19.612
	M1	24.8	10.4	2.6	12.4	1.4
	0/≥4 pLK	39.3/34.9	44.1/30.6	57.8/17.2	47.3/27.3	76.1/5.7
	Grade 3–4	48.1	39.1	26.4	37.8	19.6
	HR negative	29.9	16.4	9.6	19.1	9.0
	age < 50 years	31.6	34.2	37.8	34.6	20.0
Breast pT2	Time interval (ys or n)	<2.9	2.9–5.8	≥5.8 [10.8]	n = 2.159	n = 11.088
	M1	33.0	15.1	5.8	17.7	4.3
	0/≥4 pLK	25.8/51.7	30.9/41.4	38.1/33.1	31.8/41.7	49.2/21.3
	Grade 3–4	69.4	56.7	42.2	56.1	39.4
	HR negative	34.7	19.4	8.4	21.1	13.6
	age < 50 years	22.6	27.6	34.7	28.4	18.0
Colon rectum pT2	Time interval (ys or n)	<2.2	2.2–4.7	≥4.7 [7.7]	n = 393	n = 4.265
	M1	76.3	29.2	11.4	38.9	4.7
	0/≥4 pLK	40.0/25.6	67.6/9.5	77.2/2.0	62.5/11.8	80.4/3.7
	Grade 3–4	32.3	13.7	19.5	21.8	14.6
	age (mean, ys)	66.9	64.1	62.2	64.3	69.7
Colon rectum pT3	Time interval (ys or n)	<1.2	1.2–2.8	≥2.8 [5.6]	n = 3808	n = 13.591
	M1	91.7	64.9	29.5	61.9	18.4
	0/≥4 pLK	18.6/54.8	27.6/43.5	54.1/21.4	33.7/39.7	52.4/19.4
	Grade 3–4	41.8	30.3	24.1	32.1	25.7
	age (mean, ys)	69.3	65.1	63.6	65.9	70.2

Data of all patients in the corresponding strata are presented in the last column. The value in brackets after the lower limit of the third tercile is the 90% percentile of the MET free survival time for all patients. Minor changes between the weighted terciles and the sum arise from missing data

of approximately 5–50 mm tumor diameter, there is a linear association between the tumor size and the occurrence of pLN ($y(\%) = 12 + 1.2 * d$, d = tumor diameter in millimeter). With every millimeter, regional and distant MET increases by approximately 1.2% (Engel et al. 2012).

Growth Time of Foci

Secondly, the time required for MET growth can be estimated from very different observational points of view. For BC MET, the time distribution of MET free survival is shown in Fig. 2.2a, b, whereby for 80% of pT1 patients, between approximately 1 and 8 years of growth time may occur before MET is detected. The double of the median MET free survial time is about 6 years and an estimator of the mean growth time. The variability is due to the temporally sequential initiation of MET approximately 6 years earlier, which would have occurred at the 10% limit approximately 5–6 years earlier, and at the 90% limit only days before PT diagnosis. However, there are longer times observed, apparently because the growth of the foci continuously slows. Figure 2.2c shows the survival post diagnosis and results from the MET-free survival time plus the survival following MET. The median survival following MET is about 2 years for receptor positive tumors (Fig. 2.2d). It is remarkable in Fig. 2.2b and c that with increasing survival, both survival times up to MET and after MET increase continuously and are not relevantly correlated. Such positively skewed distributions describe natural growth variations and

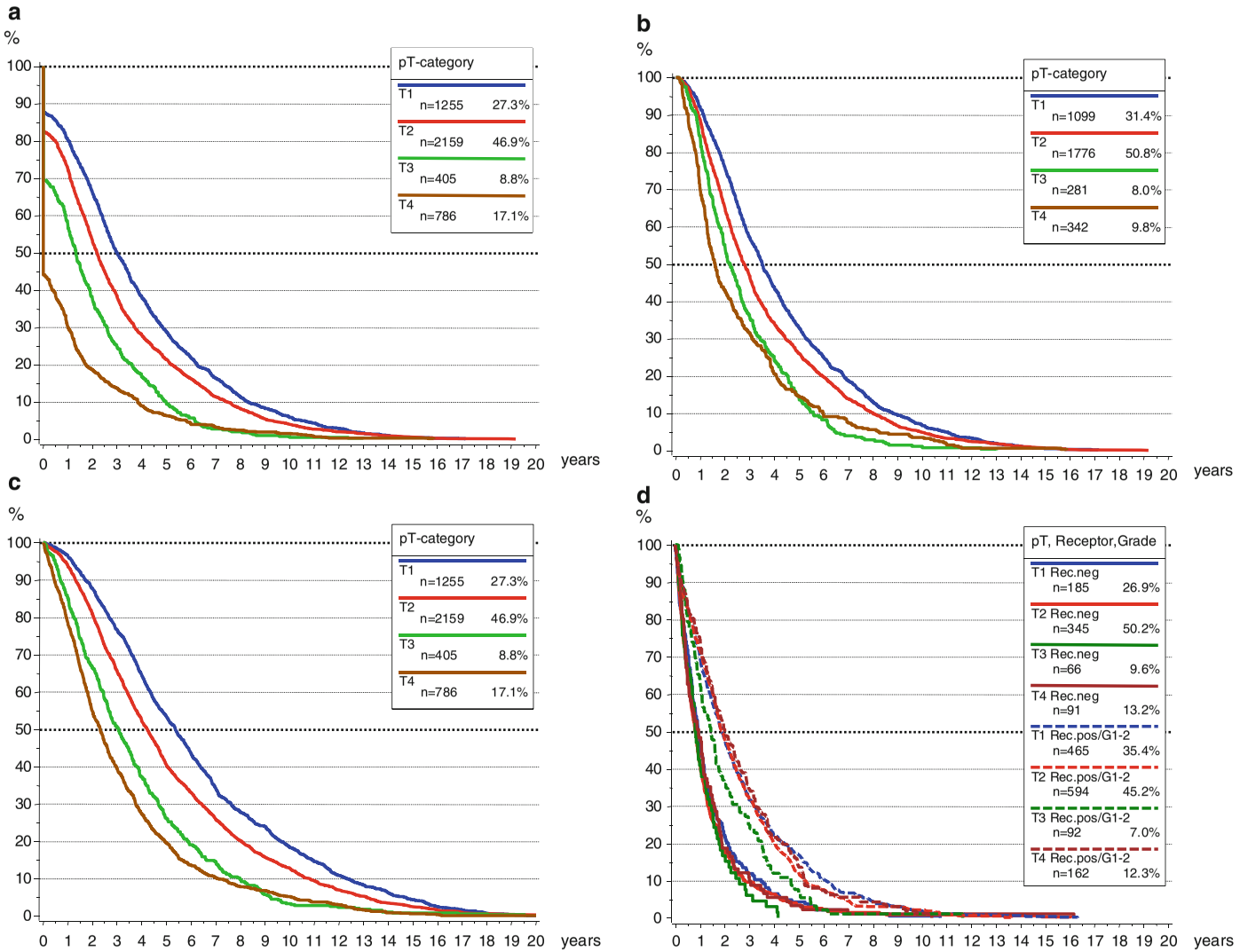

Fig. 2.2 Breast cancer: Distribution functions of the MET-free survival time with (**a**) and without (**b**) primary advanced diseases and of the survival time of all metastasized and deceased patients (**c**) and relapse related survival time after MET for receptor negative and positive tumors (**d**). All distributions are additionally stratified according to the pT-category. For convenient comparisons with survival curves all time distributions are presented as 1-empirical distribution function. In this article MET-free survival means the time from diagnosis to MET and events like death from any cause or second malignancies are not considered

therefore do not need an additional explanation by dormancy.

From the local recurrence (LR)-free survival time after breast conserving therapy with and without irradiation one can estimate about 5 years for the growth time of true LR from initiation to detection (Early Breast Cancer Trialists' Collaborative Group 2005; Hölzel et al. 2011). Indirectly, the growth of LN foci can be estimated from the distribution of ITC and micro MET in SLN which results in the same approximate 5 years as LR and MET (Engel et al. 2012). For colorectal cancer the estimation of growth time is approximately 4 years for the MET-free time, also in the subgroup of pT1-2 metastasized and deceased patients. The survival following MET for colorectal cancer in a population-based setting is about 13 months (data of the MCR not shown).

Homogeneous Growth Within Molecularly Distinct Subgroups

Thirdly, it is important to note that the growth of tumor-specific MET is very homogeneous. For example, the 10 year survival rates for colon cancer with pT1/pT3 diagnoses are at 89.7/53.5%, whereas for diagnoses with 0/3/6 pLN rates are at 75.0/39.4/19.7%. The corresponding numbers for BC are 89.0/48.7 for pT1/pT3 and 90.5/68.0/47.5% for each respective pLN status. For the survival following MET these classic prognostic factors have little additional influence (Fig. 2.2d). Therefore, MET is largely an autonomous process, independent of whether a rare MET from a pT1 tumor or a frequent MET from a pT3 tumor, already with multiple pLN, is observed. The similar growth time of all MET will become more apparent when, for example with BC, the molecular biologically defined subgroups of ER positive and negative tumors are compared. Figure 2.2d demonstrates how subgroup growth times may vary by a factor of almost 2, yet within each subgroup, a very homogeneous growth is observed.

Figure 2.1 shows 90% of the MET free survival times depicted in terciles (dotted green line). Homogeneous growth implies that this time interval can be transposed in the time before diagnosis of PT. That means normally late initiated MET will not appear as primary M1 and early initiated MET not after a long MET-free survival time. The arrows which connect the initiation and detection time of one MET illustrate the homogeneous growth. Therefore, for the most part, the variability of the MET-free survival time results from the sequential initiation with a comparable growth afterwards and not from an approximate 10- to 20-fold growth variation.

Homogeneous MET growth means, that for example in Fig. 2.2b, for all PT diagnosed at pT2, only a small portion of the 10% of MET diagnosed in the first year and of the 10% diagnosed after 8 years may have noticeably shorter or longer volume doubling times (VDT). Even M1 diagnoses or short MET-free intervals as shown in Fig. 2.1, case pT1:3, could have a delayed growth, particularly if the MET was initiated long before pT1 was reached, and then would be detected as a primary M1 at the time of pT2 discovery. Acceleration and retardation of growth cannot reliably be assessed from a short or long length of time after diagnosis of the PT.

An important consequence of these three aspects of MET growth is the sequential acquisition of the MET potency of a PT. More and more, this is being demonstrated with whole genome sequencing and the reconstruction of the evolution of the PT. In addition, the acquisition of MET-potency over time is a prerequisite for the prediction of outcomes with gene expression profiles from the PT (van't Veer et al. 2002). The time of dissemination of the first TC, of the first TC with MET-potency and of its first initiation of a MET are all unknown. The time difference of the last two events, and if not zero, the residence of the disseminated TC with MET potential, are the important questions.

Volume Doubling

Large foci and their changes over time can be well measured with modern imaging. Primary or secondary tumor foci with approximate 1 cm

diameters contain about 10^9 TC. Thirty VD are required to achieve such a size. Nonetheless, the heterogeneity of TC within the PT or a focus and their individual development, in particular during treatment, are not considered, not least because they may be comparable for PT and secondary foci. Therefore, time estimates and their reciprocal transformation from MET to PT, according to Fig. 2.1, seem to be plausible. With BC the VDT for all secondary foci are equivalent within 5–6 years. From these estimates the VDT of secondary foci of about 60 days and of the PT of about 140 days (obtained from screening Peer et al. 1993; Weedon-Fekjaer et al. 2008) result in a ratio of the PT to MET VDT of over two. This ratio may even be independent of varying VDT of the PT, that is, faster growing PT initiate even faster growing MET as seen in Figs. 2.2d and 2.4a. From the sequential initiation of MET and the long growth time follows, among others, that at least the first local, regional or distant MET that may be detectable at the PT diagnosis may be initiated independent of each other.

Relapse Related and Overall Survival

The presented MET process can be gleaned from survival data. The conclusions apply to all solid tumors. Nonetheless, BC is particularly suitable because of the large number of patients and the typically available mm size of the PT as well as the tumor spread in a homogeneous tissue, in contrast to e.g. colon cancer. Figure 2.2a demonstrates the MET-free time with attention towards primary advanced diseases. With increasing pT, the portion of patients with primary MET increases, even when the portion of M1-diagnoses in the MCR are slightly over estimated.

The distributions of MET-free time after a primary M0-diagnosis are comparable for different pT. This is easier to recognize in Fig. 2.2b, where primary MET have been excluded. No fundamentally different distributions result from overall survival after diagnosis of PT (Fig. 2.2c). Only the later occurring MET show an increasing MET-free survival and also concurrently increasing post-MET survival. The median overall survival is simply 2 years longer than the mean of the length of survival until MET. For the approximate 12/88% ER negative/postive diagnoses it can be seen that the mean survival after MET is 1/2 years, and nearly independent of classic prognostic factors such as pT (Fig. 2.2d). The PT reveals a comparable ratio for receptor neg/pos tumors e.g. at 90% with 1.5/4.3 years or at 70% with 5.0/13.6 years (Fig. 2.4a). A bimodal time distribution, that in extreme cases would produce a parallel to the time axis if there were non-overlapping distributions, is not observed in our registry data. It is possibly an artifact from heterogeneous selected groups according to the pT-category or receptor status (Demicheli et al. 2010).

Characteristics of Early and Late MET

Table 2.1 shows the survival time after a BC or colorectal cancer diagnosis of metastasized and deceased patients, grouped according to terciles of the survival times. The tercile limits are, of course, dependent on the follow-up time. For all surviving patients, the 50/90% percentiles of follow-up time are 6.1/15.1 years for BC and 5.2/14.4 for colorectal cancer.

The reduction of the upper tercile limit with increasing PT is notable. This is a lead time effect, because the biology of MET is not changing but rather the risk of initiating a MET. If the MET growth is homogenous, then the growth time of the PT accounts for the time difference of tercile limit, e.g. for BC from pT1 to pT2 of more than 1 year correspondent 3 VD (14–28 mm). The comparison of distributions of selected prognostic factors with all patients in one cohort shows the importance of each factor. The effect of differential growth can be seen in the portion of the primary advanced cancer (M1). Even in the third tercile there are patients who live longer with MET without there being any evidence of dormant TC or stagnating MET.

An unfavorable histologic grade for both cancers and receptor negative tumors for BC are also correlated with growth rate, therefore the proportion is decreasing in the third tercile (Table 2.1). The pLN are remarkable in that the high proportion

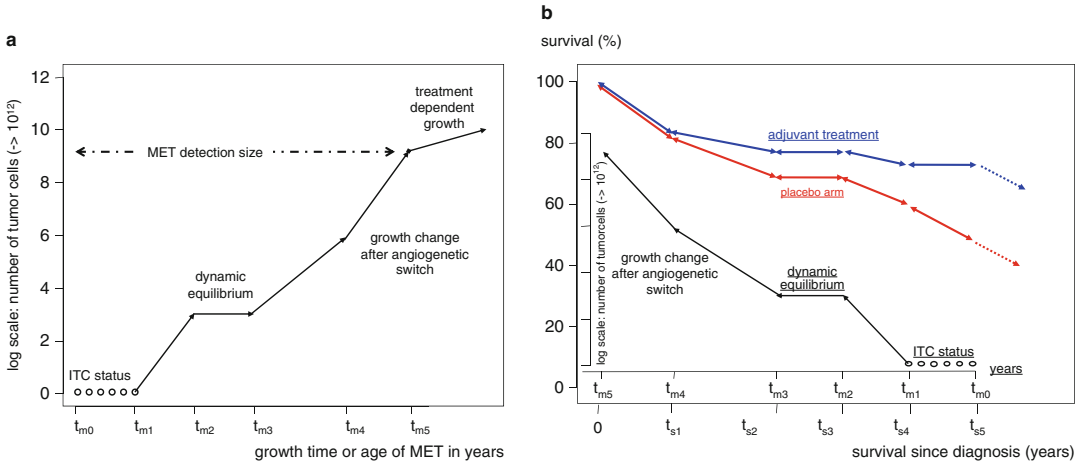

Fig. 2.3 (**a**) A growth trajectory for MET beginning with assumed dormant TC, a dynamic equilibrium and four phases with different VDT. The growth time tmx is also the age of the MET. (**b**) A fictive interaction of a placebo controlled adjuvant treatment with the different growth phases of a MET. An alternative interpretation of Fig. 2.3a is the distribution of prevalence of MET development stages. For the interaction, the trajectory from Fig. 2.3a is flipped horizontally and then shows the remaining time up to the detection of the MET in the placebo arm (*blue line*) and the interaction with the treatment (*red line*). If the treatment is longer than [t_{m1}–t_{m0}], each TC division would happen under treatment

of LN negative patients, particularly in colorectal cancer, demonstrate the MET risk without LN positivity. The increase in pLN with larger tumors is continuous and demonstrates that the number of pLN is an excellent chronometer for the duration of TC dissemination from the PT. The more pLN the more primary M1 can be observed and therefore the survival time is shorter. The differences in survival time in Fig. 2.2 are explained by a lead time effect and differences in biological VDT. The last aspect is also known as length time effect and results in a higher detection probability of prognostically favorable tumors with early detection. The trichotomy of the time intervals for MET initiation and detection supports the view of the three aspects of MET growth, namely the sequential initiation and long-lasting and homogeneous growth of secondary foci.

The superposition of the differential growth of biological or prognostic subgroups is transparent in Fig. 2.2d. The second aspect, the natural variability of VDT, is demonstrated particularly well in BC. From mammography screening (Weedon-Fekjaer et al. 2008) the 25/50/75% percentiles of VDT have been estimated at 65/143/308 days for 60–70 year old patients. This is a factor of 5 that with 30 VD makes a difference of 20 years for the growth of PT. These differing VDT of a PT are passed on to the disseminated TC and determine the growth of the MET. The example of receptor status shows this even when the reason for the growth acceleration of more than a factor of two has not yet been explained. That means that long MET free intervals in BC can be explained by natural differences in growth of the foci. There are no discontinuities in the survival curves and in distributions in Table 2.1, nor in the third tercile which could be most influenced by tumor dormancy phases.

Growth Trajectory

A growth trajectory is the path that a growing focus follows as a function of time. A fictive one is outlined in Fig. 2.3a. Due to the logarithmic y-axis for the number of TC of a MET, differential exponential growth phases are represented by straight lines with size dependent differential slopes (Fig. 2.3a: [t_{m1},t_{m2}] or [t_{m4},t_{m5}]). A possible turning point of growth velocity could happen at the angiogenetic switch (Naumov et al. 2006).

In this trajectory, growing MET reach the detection size with the age of t_{m5}. TC clusters in which cell division and apoptosis would compensate each other over a longer time constitute a dynamic equilibrium and result in a growth trajectory parallel to the x axis as any pause in growth $[t_{m2},t_{m3}]$. Isolated TC that reside in an organ in a dormant state for longer periods of time before the first division are depicted with a series of circles $[t_{m0},t_{m1}]$. This last status is functionally equivalent to disseminated TC with longer circulating times, or that reside in niches, or have a delayed extravasation and thereafter a rapid initiation of a MET in an organ.

Effects of Adjuvant Treatment

To understand possible interactions of different MET status and treatment, the growth trajectory is flipped horizontally in Fig. 2.3b. Thereby survival events are synchronized with the growth trajectory. All of these stages of MET development are represented in a patient cohort. The remaining growth time up to the time of MET detection is then tm5-tmx. How an adjuvant therapy works in dependence of the size and growth characteristics of a MET focus can be seen in the shape of the MET-free survival curve. Because of the homogeneity of MET growth, the respective time delay of a therapeutic effect is reflected in the growth phases. If the MET are too advanced, they are apparently irreversible, as many clinical studies support. Smaller foci, perhaps under 10^6 TC, that are not yet supplied by blood vessels (Naumov et al. 2006), maybe partly reversible, which results in opening survival curves ($[t_{m4},t_{m3}]$).

If, over a longer phase, foci are maintained in a dynamic equilibrium (Fehm et al. 2008; Udagawa 2008) $[t_{m3},t_{m2}]$, and were potentially all reversible with therapy, then the survival in the treatment group should have a phase that is parallel to the x axis. The interaction of an adjuvant therapy with dormant, solitary TC (Townson and Chambers 2006) could result in differing effects ($[t_{m1},t_{m0}]$). If adjuvant therapies take longer than the dormant phase, then all TC would begin with cell division and effective therapies would be recognized by a risk reduction. If the dormancy phase was longer than the therapy, then a change in the MET risk would be apparent (blue dotted line). Only therapies that continuously block the signal transduction of mitotic pathways would have survival curves that open like scissors if dormant TC initiated MET. Current adjuvant therapies show a less complex structure. In the beginning they run parallel, for a defined time the curves open in a scissor-like fashion, and thereafter they run parallel. If all TC have reached the location of focal initiation before the beginning of adjuvant therapy, then we see the outcome of treatment and there is no evidence of late, post R0 resection initiating TC.

Distant MET and Dormancy

If dormancy were a characteristic of the MET process the effect should occur in local, regional, and distant foci. Figure 2.2 shows that for distant MET, later occurring MET is less frequent with increasing PT size. Since on the other hand, MET are comparable for either large or small tumors, then the characteristics of being a late MET must have little to do with the size of the PT. An observation "the less favorable prognostic the PT, the shorter the dormancy" would require new characteristics of the initiated TC. Later MET that occur under 10 years are predominantly the result of a lead time effect since survival after MET is mostly independent of PT. Figure 2.1 illustrates that such misinterpretations can occur from the wrong association with the date of PT diagnosis. With the reference to the initiation of MET, a long-standing growth or dormancy could very likely be associated with a very short MET-free interval or even with primary M1 (Fig. 2.1 case pT2:4b). Figure 2.4 more likely suggests that the differential survival with ER+ and ER-tumors in BC can be explained by different VDT and not by differing lengths of dormancy phases dependent on receptor status. Late MET that occur after 10 years are characteristic for BC MET and need no explanation by tumor dormancy. A small portion of MET in BC may have particularly fast or

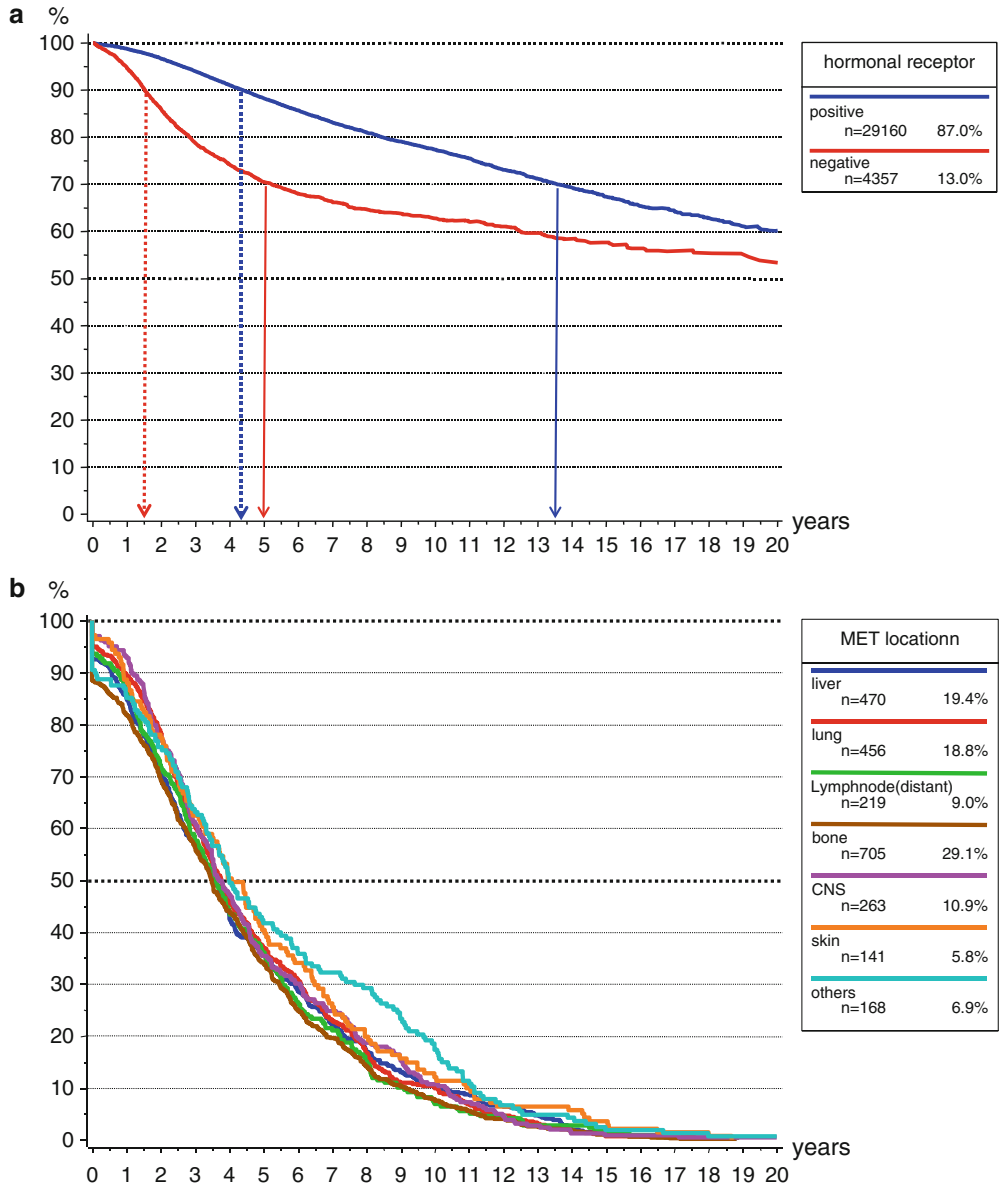

Fig. 2.4 (**a**) Tumor specific survival for breast cancer patients stratified according the receptor status without any indication of a discontinuity due to a dormant phase. (**b**) MET free survival time for different distant MET for breast cancer with a pT2 primary tumor. Primary advanced cases are also included, as indicated by the initial step

particularly slow growth. In this regard, different organs show different VDT with the same PT.

There are no facts that dormancy is involved if MET-free survival occurs after 5 years (Aguirre-Ghiso 2007; Brackstone et al. 2007). Additionally, it is important to assess the quality of data. For BC the risk of a second malignancy in 20 years is 40% (Kaplan Meier estimate from MCR data), among those 20% contra- or ipsilateral second breast cancer occurs, the latter after breast conserving treatment (Hill-Kayser et al. 2006). Such frequent second malignancies require critical assessment of the cause in respect to the cancer related death for every late MET.

Regional MET and Dormancy

How does it look with the infiltration of LN? Even in this case, pLN can occur long after the diagnosis of PT. If the delay were explained by dormant TC, then a high prevalence for ITC must be evident already with the meticulous dissection of the SLN. This is probably not the case. ITC, micro and macro foci in LN appear to be so distributed that the infiltration of the LN net through continuous dissemination and a growth without a dormancy phase seems plausible. However, ITC means that the whole range from ITC up to TC clusters of less than 0.2 mm can be detected in a SLN. A reseeding from TC in the LN would put into question the prognostic relevance of SL and the observable infiltration of the LN net from the effluent stream of the PT. There are no robust data for dormancy in LN.

Local MET and Dormancy

Even with LR there are no clinical data that would suggest dormancy. If one observes breast conserving therapy, the first point to make is the subdivision of residual tumors, true recurrences (TR, that have no contact with the PT), and de novo carcinomas. The growth rate of TR can be estimated from the time distribution of a metaanalysis of studies with and without radiation after breast conserving therapy, and results in approximately 5 years for 30 VD (Early Breast Cancer Trialists' Collaborative Group 2011). Even here, a sequential initiation and a homogeneous growth of over 5 years before R0-resection can be assumed. From this also follows the analogy to MET in distant organs and pLN, which can be detected simultaneously with the PT. These are the multifocal findings which are detected near the PT at diagnosis and are initiated from migrating TC. Late LR are most likely de novo cancer that occur in other quadrants and have other histologies. Late TR that occur near to the PT also exist. In comparison to the MET, it is possible that after 6–8 years only a few percentage are concordant with the variability of the VDT (Weedon-Fekjaer et al. 2008). Genomic tests of the PT and the local focus will hopefully soon clarify the type of synchronous or recurrent foci. For dormancy of local MET, no robust data are available either.

Implausible Implications for Dormant Tumor Cells

TC arrive very soon after hematogenous and lymphogenous dissemination in all organs (Fisher and Fisher 1966). The first steps of the MET process are very efficient. The inefficiency does not occur until TC growth after extravasation (Cameron et al. 2000; Luzzi et al. 1998). The challenging question is whether dormancy is a common characteristic of this step.

TC even from very small PT have MET potency. The linear relationship between size and MET confirms that the dissemination increases with the duration of the MET-risk. But the PT does not disseminate increasingly genetically potent cells. If gene signatures can predict the organ tropism, then these properties must be a very early property of the PT and not the product of an evolutionary development or even of maturation after dissemination. Since millions of TC can be disseminated by a PT, which passively reach all organs through lymph and blood vessels, survive there and show typical tumor-specific MET patterns, then a cascade like initiation from focus to focus can be excluded. This is shown in Fig. 2.4b with the distributions of the organ specific MET-free survival time for BC. The small differences between the distributions must be compared with the growth time from initiation of a MET up to the detectable size lasting for years. Therefore, MET foci arise independently from one another.

Also the assumption that the PT disseminates a TC, predetermined in its evolutionary development, and equipped with a specific gene signature for different organs, is not compatible with Fig. 2.4b. A predestined TC location cannot be confirmed by any MET-pattern in other solid tumors. Moreover, this would reduce the importance of the environment, which, as we know from embryology and wound repair, constrains

pluripotent cells to selective functions by changing gene-expression for organ or tissue formation.

A simple thought experiment reveals fundamental problems with dormancy. If dormancy were a characteristic of the MET process, then TC would exist in organs which were disseminated from the PT in different sizes. Because a different MET probability is associated with each tumor size, an intelligent mechanism of the TC and/or the environment must exist which knows among others the size at dissemination and the present duration of dormancy. This is because the PT size is correlated with MET frequency which has to be coordinated within the subset of all disseminated TC and even within a patient cohort. Ultimately, the disseminated TC would be equipped by the PT with a signature for a definite MET in the sense of a final destination and would reopen the discussion about entelechy for a teleological process from the Middle Ages. Furthermore, the remote control of TC by the PT up to the R0-resection with a signal protein also seems implausible but does not contradict colonisation by the PT. Such a thought experiment shows that MET is also, up to the initiation of a secondary focus, an autonomous mechanical process (Michaelson et al. 2005) with a probability of success which increases with the number of disseminated TC and therefore with time.

An evolutionary development of the PT with delayed dissemination of more aggressive or organ specific TC cannot be deduced from MET patterns. Also a change of the pattern during the course of disease cannot be observed. The determination of the prognosis, or even the location of the MET, from the PT would otherwise not be possible (Bos et al. 2009; van't Veer et al. 2002). That is to say, it is questionable whether successful TC did not have any dormancy phase a posteriori. Because TC lack a memory mechanism of their own history the number of long-lived, viable TC in all organs can only have a minor role. There are no convincing data about systematic initiation of MET after R0 resection, as there are no convincing data about a dormancy phase before initiation of a focus. However, this is not inconsistent with the existence of dormant TC.

Seed and Soil Observations

The lack of evidence of the importance of dormancy for MET does not conflict with observations from Paget (1889); that the distribution of secondary growth is not determined by the blood flow and random sampling. A tumor-specific organ tropism exists. A TC of liver adenocarcinoma remains unsuccessful initiating MET in the lung although it transits through the pulmonary capillaries, but it is very successful in all liver segments. In contrast to the liver, a primary lung adenocarcinoma shows a strong tropism for its homeland organ, which is evidenced by the frequent multifocal PT of the lung. The circulation path of the TC is identical in both cases. Because there is no correlation between organ tropism and blood flow, and TC are ubiquitous in all organs (Suzuki et al. 2006) tumor-specific driver genes must be diversified. The metastatic propensity of small cell lung cancer TC to the CNS or to adrenal gland points to the importance of the seed and the selection of the soil. The little overlap between genes identified as a signature for lung and bones fit with these observations (Landemaine et al. 2008). This correlation also explains the striking success of local or site-specific MET in the environment of the PT.

An adjuvant radiation of the CNS in patients with small cell lung cancer reduces CNS-MET and therefore TC must already be at that location at the time of diagnosis of the PT. The hypothesis that radiation destroys dormant TC instead of already growing foci, because their repair mechanisms are not activated, seems implausible. The analogy to the LAG Phase of bacterial growth, during which bacteria adapt themselves to growth conditions of a new environment, is fitting. Especially, CNS MET should have longer LAG phases or slow and differential growth of tiny foci. Perhaps a successful MET is correlated with the density of isolated TC in an organ if separately disseminated TC find each other and can then more easily activate nearby vessels together. Video microscopy suggests such an equilibrium of newly arriving TC and disappearing older ones (Kienast et al. 2010).

Last but not least, genome sequencing of a PT and different secondary foci confirms the temporal sequence of focus initiation (Campbell et al. 2010; Yachida et al. 2010). Additionally, the parallel evolution in primary sites must be considered as the result of very heterogeneous PT. It is noteworthy that the additional mutations shown by the phylogenetic reconstruction of the evolution of PT are not driver mutations for the respective organ. Also from this point of view, organ tropism should be established very early without noticeable delay between organs (Fig. 2.4b).

Conclusion

Millions of continuously disseminated TC and their ubiquitous detectability in animal models with different cell lines suggest the imprecise term of tumor dormancy for solid tumors. Historically, the metaphor may have been inspired by the knowledge of hematological cancers, hematopoietic stem cells, their treatment, and the development of recurrences. Also the transfer of TC with organ donations and the mobilization for MET initiation suggest the existence of TC in niches.

In reality, the term dormancy conceals our ignorance of the MET process of solid tumors, beginning with circulation up to the growth of isolated TC or clusters of local, regional and distant MET (Aguirre-Ghiso 2007). The detection of TC and their possible delayed division or apoptosis cannot explain or quantify our observations. It is only a surrogate for dissemination of a PT. For this reason, the adverse prognostic value of bone marrow TC detected at diagnosis of PT in early BC is not to be questioned. Nonetheless, the detection of dormant TC does not implicate them as MET precursors nor as causes of late recurrences. The dormancy concept opens no new line of vision, shows no promising research approach and is therefore not helpful (Klein 2011). It is even counterproductive that the term communicates timeliness and scientificness, so that in spite of our limited knowledge, a search for so-called dormant TC and their characterization is offered to patients as being useful.

Also the expectations of improving prognosis with dormant TC as a treatment target are not likely to be of clinical importance. The evidence of the heterogeneity of PT, the resulting lack of monoclonal TC, and the plasticity of each focus makes the therapeutic failure with currently available agents very likely, or chronifications of METs, unlikely. In addition, adjuvant therapies are likely to affect foci already growing in organs. Their improved interception with foci below the detectable size would be of clinical importance. In summary, it follows that the detection, properties, representativeness or interference of dormant TC in cell systems and animal models and the transferability to humans are certainly of great interest. But the significance of dormant TC for the scientific development and application of future therapies appears at present to be still very low. Thus far, tumor dormancy is a theoretical construct with marginal relevance for patients and with no contribution to the differentiation of thought and action and is therefore clinically meaningless.

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Microenvironmental Influence on Breast Cancer Dormancy and Metastasis

3

George R. Nahas, Jacqueline M. Park, and Pranela Rameshwar

Contents

Abstract

Breast cancer remains a serious public health issue; this despite early diagnosis and aggressive treatment. This chapter discusses the different aspects that are contributors in the evasion of cancer. The review discusses the emerging role of cancer stem cells in dormancy. Included in the discussion are studies on mesenchymal stem cells as protection for the cancer cells from immune clearance. We reviewed the role of hormones and the intracellular pathways in metastasis. Overall, this review provides a 'snapshot' of breast cancer subset in dormancy and immune evasion.

Keywords

Breast cancer • Stem cell • Bone marrow • Dormancy • Metastasis

Abbreviations

ABC	ATP-binding cassette
BC	breast cancer
BCC	breast cancer cell
CCL5	chemokine C-C ligand 5
CSC	cancer stem cells
CSF	colony stimulating factor
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
CTC	circulating tumor cells

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EMT	epithelial to mesenchymal transition
FOXP3	forkhead lineage-specific transcription factor
GJIC	gap junctional intercellular communication
miRNA	microRNA
MSC	mesenchymal stem cell
NK	natural killer
NKT	natural killer T cells
NOD	non-obese diabetic
PTHrP	parathyroid hormone-related protein
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
SDF-1	stromal cell-derived factor 1
SCID	severe combined immunodeficiency
TGF-β	transforming growth factor beta
T_{regs}	regulatory T cells
VEGF	vascular endothelial growth factor

Introduction

Breast cancer (BC) remains a major public health challenge in the United States and nearly all deaths from BC are due to metastatic disease (Castano et al. 2011). As a result of these figures, the predisposition for BC to metastasize to bone marrow has become an intense topic of research in order to elucidate mechanisms of metastasis and to develop new treatment modalities.

Cancer Metastasis

Approximately a century ago, Paget (1889) introduced his 'seed and soil' hypothesis, describing a necessary compatibility between the tumor cell and the microenvironment for successful metastasis. For example, prostate cancer's tendency to metastasize to the bone is a well known phenomenon, which consequently piques interest as to why these cancer cells hold a predisposition for bone metastasis. In the case of breast cancer cells (BCCs), there appears to be a metastatic predilection to bone and bone marrow, suggesting that BCCs play the role of the 'seed' and implant into the bone marrow, otherwise thought of as the 'soil.' Further complicating the situations, BCCs frequently remain dormant in the bone marrow and may not activate until years later. This phenomenon introduces the interesting notion that tumor cells may successfully metastasize to the bone marrow, and then proceed to merely survive without any active malignancy for years. What then allows the cells to survive in an inactive state after successful metastasis and what eventually provokes the dormant cell to later activate and to behave like a tumor cell? This chapter will attempt to characterize the BCC and its interaction with the environment, thus illustrating metastasis and dormancy.

Breast Stem Cells

Cancer is caused by genetic mutations that render cells with properties similar to that of stem cells—most notably, self-renewal. Originally, it was believed that every cell in a tumor was capable of obtaining this stem cell-like quality through mutations. Current theories, however, embrace the concept that cancer stem cells (CSCs), deriving from normal stem cells or progenitor cells that regain stem cell-like properties, compose a small proportion of tumors and may serve as the nidus for tumor initiation. The clinical importance of identifying CSCs is underlined by the failure of current treatment modalities to fully eradicate solid tumors. Normal stem cells, as opposed to differentiated cells, seem to have resistance to chemotherapeutics as a result of a variety of mechanisms including but not limited to ATP-binding cassette (ABC) transporters such as the multidrug resistance gene (Zhou et al. 2001). If these characteristics hold for CSCs, it may explain why a small number of cells with chemo-resistance may potentially metastasize. It may also explain why tumors may initially regress in response to chemotherapy, but re-grow at a later time due to expansion of escaped chemo-resistant CSCs. Therapy, therefore, may need to be directed specifically towards CSCs as opposed to the bulk of the tumor.

Most tissues in complex organisms, such as the human body, contain resident stem cells that

differentiate into daughter cells and maintain themselves into adulthood. In addition to stem cells throughout other tissues, studies have identified the existence of normal mammary stem cells. In an attempt to characterize the phenotype of the breast stem cell, it was demonstrated that a single cell with markers $CD29^{high}/CD24^{+}$ or $CD49F^{high}/CD24^{+}$ formed a complete and functional mammary gland when transplanted into a cleared mouse mammary fat pad (Shackleton et al. 2006). Evidence of the existence of stem cells in normal adult tissue constructed a foundation for the CSC theory.

The possibility of CSCs as an initiating point for cancer was demonstrated in the relationship between normal hematopoietic cells and leukemic stem cells. The acute myeloid leukemia (AML) initiating cell was identified, purified, and then transplanted into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Lapidot et al. 1994). 0.1–1% of the newly formed tumor was composed of leukemia initiating $CD34^{+}CD38^{-}$ cells rather than $CD34^{-}$ and CD $34^{+}CD38^{+}$ cells, which composed the bulk of the tumor. Although the bulk of the tumor contained no leukemia initiating cells, it did contain clonogenic leukemic progenitors capable of growth. This conclusively revealed that a hierarchy of cells exists and a rare subset of cells—less mature than colony forming cells—comprises leukemia initiating cells (Lapidot et al. 1994).

The premise underlying identification of the leukemia initiating cell was then extrapolated to the characterization of breast CSCs. After identifying breast CSCs as $CD44^{+}CD24^{-/low}$, as little as 100 cells of this phenotype were able to form tumors in mice, unlike the thousands of other phenotypes which failed to form tumors (Al-Hajj et al. 2003). The $CD44^{+}CD24^{-/low}$ cell, thus, indicates a more immature cell lineage than those with the CD24 marker. Cells with the CD24 marker, on the other hand, still preserve self-renewing properties as observed in the previous study involving the formation of a fully functional mammary gland with $CD29^{high}/CD24^{+}$ or $CD49F^{high}/CD24^{+}$ cells. This is similar to the relationship between $CD34^{+}CD38^{-}$ cells and $CD34^{-}$ and CD $34^{+}CD38^{+}$ of AML initiating cells and normal hematopoietic progenitor cells. With the existence of CSCs, research could then focus on its role in tumorigenesis and metastasis.

Reasons/Hypothesis for Metastasis

Why tumor cells metastasize is a topic of research due to the high mortality rates associated with metastasis. Metastasis is defined as the spread of tumor cells from the primary tumor site to distant sites. First proposed by Stephen Paget in 1889, successful metastasis is a result of compatibility between tumor cells and environment. Paget's 'seed and soil' hypothesis states that metastasis depends on both the characteristics of selected cancer cells (the seeds) and their compatibility with certain microenvironments (the soil) (Paget 1889). Circulating tumor cells (CTCs) do not necessarily 'seed' themselves into the first site they encounter. In order to extrapolate this idea to the human model, a group decided to observe ovarian metastasis in patients with peritoneovenous shunts, a palliative intervention for those with ascites. The study revealed that viable cancer cells were introduced into the venous system and shunts did not increase the risk of metastasis outside the peritoneal cavity nor did they observe seeding into the lungs, the first capillary bed the cancer cells encountered (Tarin et al. 1984). The results demonstrated that metastasis is not random and is compatibility with the 'seed' and the 'soil' hypothesis for successful metastasis.

Proving tumor heterogeneity became imperative as Paget's theory depended on a subpopulation of tumor cells that acquired metastatic properties over time, allowing only those cells to metastasize. This step was crucial because there needed to be an explanation for only particular cells metastasizing. As time passed and more research techniques developed, it was shown that tumors are indeed heterogeneous and contain cells that have a variety of different biological profiles, including various and advanced metastatic potentials. Further supporting this premise, a study involving induced melanoma cells in mice was performed, and it was observed that

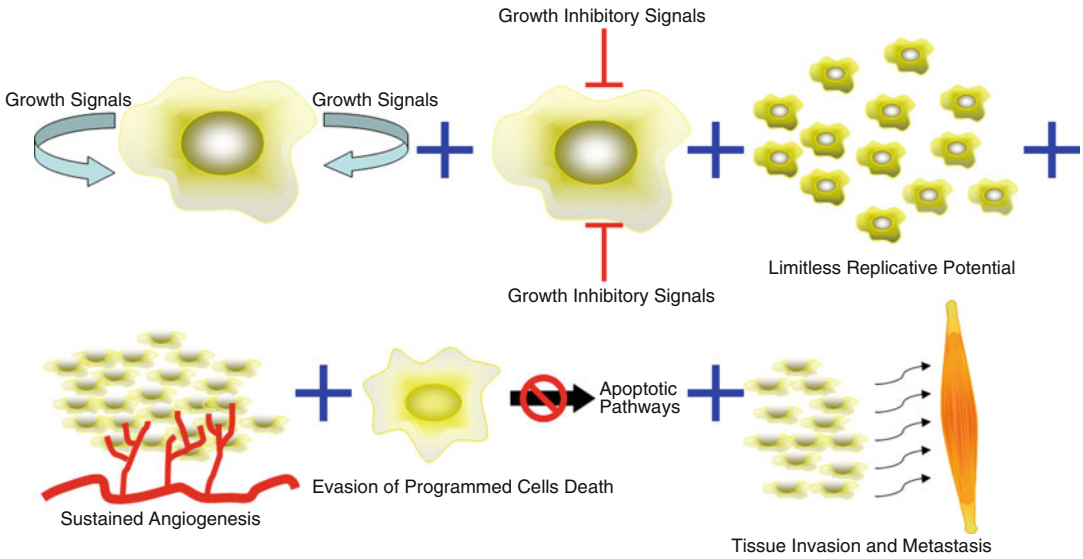

Fig. 3.1 Proposed characteristics for successful metastasis. Cartoon shows requirement for successful metastasis. These include self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion (Fidler 1973; Hanahan and Weinberg 2000; Talmadge 2007)

the tumors demonstrated size and pigmentation variability, implicating tumor heterogeneity (Fidler et al. 1981). With the establishment of tumor heterogeneity, the stage was set for evolutionary theories of metastasis.

Currently there are two prominent models describing metastasis. The first model, known as clonal evolution, describes single cells accumulating a broad spectrum of changes over time, thus, creating cells with variable phenotypic and behavioral characteristics, which includes the ability to metastasize. This attests to the fact that given the right circumstances any cell has the potential to transform into a cancer cell with metastatic capabilities. On the other hand, the stem cell cancer theory describes that only a specific subset of multi-potent cells that composes the CSC compartment is initiated, and thus drives the ability to metastasize to different tissues. Contrary to the clonal model, this stem cell model defends the idea that only a particular subset of cells within a tumor can have the potential for metastasis. Again, the importance in distinguishing between the two is clinically significant and will direct future treatment modalities.

Both models, nevertheless, describe a scenario in which only cells that have acquired those abilities to metastasize do so and proceed to form secondary tumors at distant sites. Metastatic cells require a very particular and specific phenotype in order to metastasize successfully. A recent model proposed six characteristics imperative to malignant tumor growth: self sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000). The necessity to have all of these characteristics may explain why tumors are generally rare in humans. The final step in the complex series of events that metastasis requires is tumor growth at the distant site (Fig. 3.1). A tumor, therefore, contains a broad spectrum of cells, including those that do not have metastatic potential, those that have metastatic potential, and a variety of cells that make up those in between.

Metastatic Factors

The six characteristics aforementioned necessary for successful metastasis—self sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000)—can be divided into two categories, differentiated those that are intrinsic and those that are extrinsic to the tumor cell. While intrinsic factors include those that are built into the cell machinery, such as factors that control cell cycle regulation, extrinsic factors involve those that influence the cell from the microenvironment. While there are many different qualities that compose intrinsic and extrinsic factors, gene regulation and external influence on cellular adhesion are most notable.

TAC1

Gene expression is a particular example of intrinsic factors that may regulate and dictate metastasis. BCCs show preference for metastasis to bone marrow. Recent studies have provided insights into the role of the *TAC1* gene in the development of BC and other cancers (Corcoran et al. 2008). While *TAC1* produces a variety of peptides through alternative splicing and posttranslational modification, its most prominent peptide is substance P (Rameshwar et al. 2003). Substance P has been shown to mediate cancer proliferation, impart radiation resistance, protect from apoptosis, induce growth-and angiogenic-promoting factors, and to facilitate metastasis to the bone marrow (Corcoran et al. 2008). Recently, *TAC1* regulation has become an intense focus of research due to it's linked to oncogenesis. For example, studies have demonstrated that stromal-cell derived factor-1α (SDF-1α) regulates *TAC1* expression (Corcoran et al. 2008). Current research is exploring *TAC1* at the level of translation by investigating post-translational regulating factors.

Extrinsic Factors

Detachment of tumor cells is vital for metastasis. It is prudent, therefore, to explore factors that contribute to an altered phenotype that allows for detachment. Epithelial to Mesenchymal Transition (EMT) represents an alteration of cellular adhesion. Providing architectural support for normal epithelia, epithelial cells are normally strongly attached to the basement membrane through a variety of different proteins, including but not limited to E-cadherins, desmosomes, claudins, and occludins. Through EMT, cells lose polarity and attain a mesenchymal-like or fibroblastoid phenotype at the primary site of the tumor (Chaffer et al. 2007). In particular, loss of cellular polarity is shaped by the loss of E-cadherin, occludin, and β4-integrin along with gain of vimentin, fibronectin, N-cadherin, and α-smooth muscle actin (Dohadwala et al. 2010). Downregulation of E-cadherin has been associated with a poorer prognosis in cancer (Dohadwala et al. 2010). Further understanding of EMT may lead to understanding of this distinct metastatic pathway.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are important to define due to their interaction with and contribution to the immune system, and thus, their possible role in tumor survival under particular conditions. Abundant in the adult bone marrow and adipose tissue, MSCs are ubiquitous, multipotent cells and can be found in fetal organs and amniotic fluid (Ida 2006). Over the last decade, MSCs have gained significant interest due to their plasticity to develop into a number of cell types, their immunosuppressive properties, and their ability to be given across an allogenic barrier (Garcia-Olmo et al. 2005). Considering the 'plastic' functions of MSCs, it should not be a surprise that dysfunctional or misplaced MSCs may create a nidus for tumors.

MSCs have a particular role in BC metastasis as they encounter BCCs at an early stage when

the BCCs metastasize to bone marrow (Corcoran et al. 2008). Currently, MSCs appear to serve a bimodal role in BC progression as they can inhibit BCC proliferation but can also promote its progression. Two proposed mechanisms by which MSCs can inhibit BCC progression are: direct cell cellular contact and cell-in-cell internalization as demonstrated by apoptosis of tumorigenic BCCs when binding to MSCs was observed (Chao et al. 2011). The role of MSCs in tumor inhibition, however, is only half of the story, which is why MSC involvement in tumor progression should be further explored.

MSCs have tumor promoting qualities at the primary cancer site and at distant sites as well. As previously discussed, MSCs help create a tumor microenvironment through derivation of tumor associated fibroblasts (Mishra et al. 2008). BCCs and MSCs operate through reciprocal interactions and paracrine mechanisms as MSC derived fibroblasts harbor BCC growth through stromal cell-derived factor 1 (SDF-1) (Mishra et al. 2008). Another cancer promoting pathway contends that exosomes, membrane vesicles that contain RNA and protein, are able to convert adipose tissue-derived MSCs to tumor-associated myofibroblasts, inducing increased expression of tumor promoting factors SDF-1, vascular endothelial growth factor (VEGF), chemokine C-C ligand 5 (CCL5), and tumor growth factor beta (TGF-β) (Cho et al. 2012). Exosomes have also been shown to contain microRNA (miRNA) from stroma, thus promoting BC quiescence (Lim et al. 2011). Although most of the discussion so far has pertained to MSCs at the distant site of metastasis, there is also evidence that MSCs may travel to the original tumor site as a result of tumor inflammation invoking cytokines that attract MSCs to the tumor stroma (Gehmert et al. 2011).

MSCs have immunosuppressive qualities that suppress inflammation and the overall immune response to tumors. Tumors have been shown to attract MSCs to the tumor microenvironment via secreted chemokines. Once MSCs incorporate themselves into the tumor stroma, they may serve to suppress local NK and cytotoxic T lymphocytes (CTLs), which normally function in killing tumor cells (Patel et al. 2010). Furthermore, MSCs have been shown to induce the proliferation of a particular type of CD4+ derived T-cell, the T regulatory cell (T_{regs}), which suppresses the immune response (Fig. 3.2). Among other influences, T_{regs} work to suppress the differentiation of natural killer T-cells (NKT), cells that are instrumental in antitumor activity (Azuma et al. 2003). Therefore, elucidating the functions and characteristics of T_{regs} warrants further research.

Regulatory T-Cells (T_{regs})

Dysregulation of the immune system, in particular T-cell subsets, has been shown to promote tumor progression. In order to understand this mechanism, it is important to first explore the dichotomy of specific immune suppressive and immune promoting cells. Th17 cells, a subset of CD4+ T-helper cells, promote immune response toward inflammation, while another type of T_{reg} suppresses the response. The immune suppressive cells identified by cells markers: forkhead lineage-specific transcription factor (FOXP3), CD25, and cytotoxic T lymphocyte antigen 4 (CTLA-4) T_{regs} also serve an immunosuppressive role while controlling the immune system from reacting against self antigens (Sakaguchi 2004). The infiltration of T_{regs} in tumors could protect cancer cells due to the immunosuppressive role of these T-cell subsets. Among the various cells suppressed by T_{regs} is the suppression of NKT cells, known to exhibit anti-tumor qualities.

There is evidence, however, that T_{regs} may promote metastasis through manipulating the receptor activator of nuclear factor-kB (RANK) and receptor activator of nuclear factor-kB ligand (RANKL), otherwise known as the RANKL-RANK signaling system, in addition to its immunosuppressive effects. Similar to the previously known RANKL-RANK control of mammary lobuloalveolar cells during pregnancy through inhibitor of nuclear factor-κB (IκB) kinase-α (IKK-α)—a protein kinase that is needed for the self-renewal of mammary cancer progenitors—the RANK pathway was also found to be significant in pulmonary metastasis in Erbb2 over expressing mammary carcinoma cells (Tan et al. 2011). Moreover, metastasis was

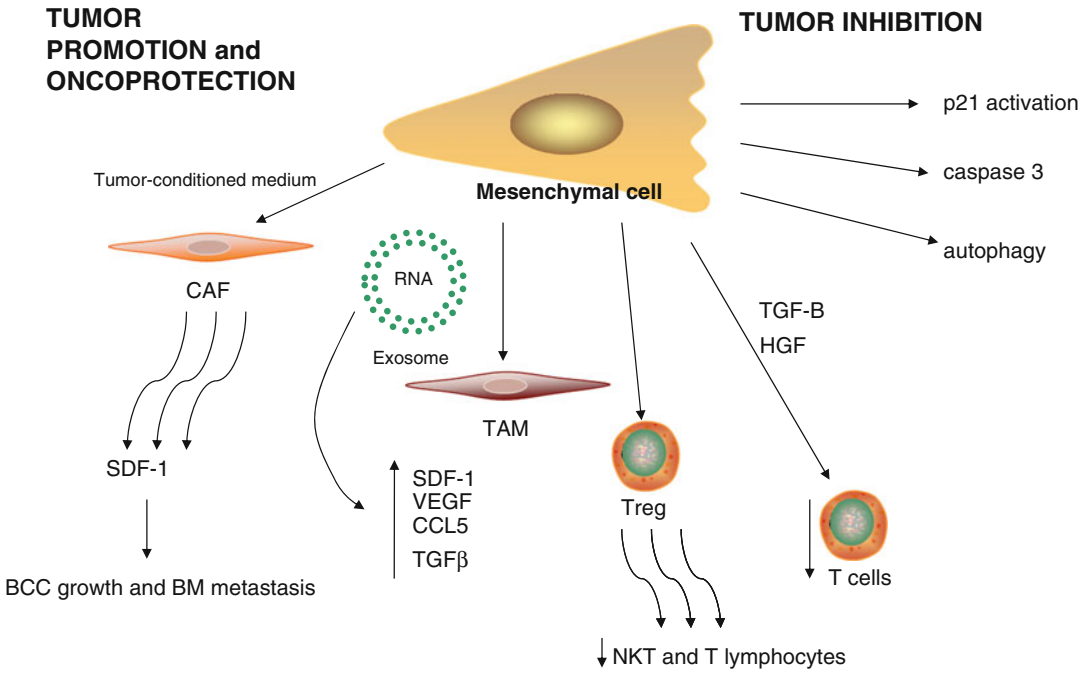

Fig. 3.2 The bimodal roles of MSCs in tumorgenesis. Studies have demonstrated a role in arresting cancer cell proliferation through activation of the p21, caspase3, and promoting autophagy. MSCs also display tumor promoting qualities. When exposed to tumor-conditioned medium, MSCs assume a CAF state which has been shown to promote BCC growth and metastasis. Adipose tissue-derived MSCs are converted to TAM through exosomes containing RNA and proteins. Exosomes have also been shown to induce tumor promoting factors SDF-1, VEGF, CCL5 and TGF-β. MSCs also demonstrate oncoprotection as they promote immunosuppression. T_{regs} have been shown to suppress NKTs and CTLs. In addition, the production of TGF-β and HGF induces MSCs to suppress the proliferation of T-cells. *MSC* mesenchymal stem cell, *CAF* carcinoma-associated fibroblast, *BCC* breast cancer cell, *TAM* tumor-associated myofibroblasts, *SDF-1* stromal cell derived factor 1, *VEGF* vascular endothelial growth factor, *CCL5* chemokine C-C ligand 5, *TGF-β* tumor growth factor beta, *NKT* natural killer T-cells, *CTL* cytotoxic T lymphocyte, *HGF* hepatocyte growth factor, T_{regs} regulatory T-cells

contingent on CD4+CD25+ regulatory T-cells, which produce RANKL, suggesting that possible therapeutic interventions in the future may include targeting the RANKL-RANK pathway (Tan et al. 2011).

Bone Metastasis

Perhaps to fully appreciate bone metastasis, an understanding of normal bone homeostasis is due. Bone homeostasis is accomplished through a delicate balance between bone resorping osteoclasts and bone forming osteoblasts. Osteoblasts are derived from mesenchymal stem cells, whereas osteoclasts are members of the monocyte/macrophage family. Induction of osteoclasts from monocytes relies on receptor activation of RANKL and macrophage colony stimulating factor (M-CSF), both expressed by osteoblasts. Parathyroid hormone-related protein (PTHrP) is also known to be a stimulator of RANKL.

Bone metastasis may be either osteolytic or osteoblastic. Osteolytic bone metastasis is initiated by the release of osteoclastic agents in the bone microenvironment. For example, expression of IL-8 from BCCs was shown to induce the expression of RANKL mRNA and protein in osteoblastic cells, stimulating the formation of osteoclasts (Bendre et al. 2003). It is important to note that osteolysis is caused by osteoclast stimulation, not by direct effects of tumor cells on

bone. Moreover, metastasis of BC to bone tends to be osteolytic. On the other hand, research has revealed that regardless of whether metastasis is predominantly osteolytic or osteoblastic, each has components of the other as a primarily osteolytic period may be followed by an osteoblastic period (Pelger et al. 1998). This may provide clues to the worth of the paradoxical treatment of bone resorption inhibiting agents in an osteoblastic lesion. The leading candidate for osteolytic lesions in BC metastasis is osteoclast activating factor PTHrP released by tumor cells. Its expression has been shown to be higher when tumor cells are present at the metastatic bone site compared to when tumor cells are present at soft-tissue sites, or the breast (Powell et al. 1991). At any rate, densely mineralized bone represents a highly unsuitable environment for tumor cells, suggesting that bone resorption is imperative to provide tumor space and expansion, further implicating the harmony that exists between osteolytic and osteoblastic metastasis and cytokines and other signaling factors that influence these processes.

Cytokines play a significant role in bone metastasis. As aforementioned, TGF-β is known to have a suppressive effect on the immune system, creating an environment conducive to the progression of cancer metastasis in bone. TGF-β is a potent inducer of EMT, allowing for cellular depolarization, detachment and possible metastasis. TGF-β can be released from bone, as a result of bone resorbing factors IL-8 and PTHrP secreted by BCCs, invoking the cycle with breast tumor cells previously addressed. To this end, TGF-β plays a major and integrative role in the possible development of cancer.

Bone is ideal in support of cancer metastasis. High blood flow in red marrow and the production of angiogenic factors, such as VEGF and, bone reacting cytokines such as PTHrP, are elevated as compared to soft tissue; creating a conducive environment for metastasis (Van Der Pluijm et al. 2001). These interactions serve as a precursor to the up-regulation of angiogenic and bone-resorbing factors, such as PTHrP, mediated by BCCs in bone as opposed to in other tissue (Van Der Pluijm et al. 2001). Bone also provides a nourishing environment for tumor cells with the presence of TGF-β, insulin-like growth factors I and II, fibroblast growth factors, platelet-derived growth factors, bone morphogenetic proteins, and calcium. In particular, research has demonstrated a role of TGF-β based on the observation that fibroblasts secrete small amounts under the influence of tumor cells as tumor cells co-cultures with fibroblasts increased migration and scattering of tumor cells in vitro and accelerated tumor growth and expanded metastatic patterns in vitro (Stuelten et al. 2010). Mechanisms of metastasis, particularly to the bone, in addition to the description of a microenvironment conducive to overall tumor survival are important for overall tumorigenicity. Whether or not treatment and research is tailored to thwart any number of the pathways or mechanisms aforementioned, BC dormancy continues to be problematic.

Dormancy

Dormancy does not necessarily only apply to single cells with a lack of apoptosis or cells cycle arrest, but may also apply to the balance of apoptosis and proliferation, revealing a constant tumor size (Holmgren et al. 1995). Regardless, research has demonstrated that dormant BCCs are found to be in the G1 phase of the cell cycle and the ability to stay senescent in a particular stage of the cell cycle renders anti-cycle therapeutics ineffective against dormant cells, since the efficacy of these treatments require cancer cells to be proliferating. In order to fully eradicate tumor cells, and not merely place cancer into remission, after which cancer can be reactivated and grow in the future, understanding of the characteristics mechanisms, and possible locations of dormant cells warrants further investigation.

After identifying that bone marrow is a particular site for metastasis, pinpointing the exact site in the bone marrow became priority. Experimental evidence suggests that dormant cells exist near the bone marrow endosteum, where they form gap junctional intercellular communication (GJIC) with hematopoietic-supporting cells and bone marrow stoma

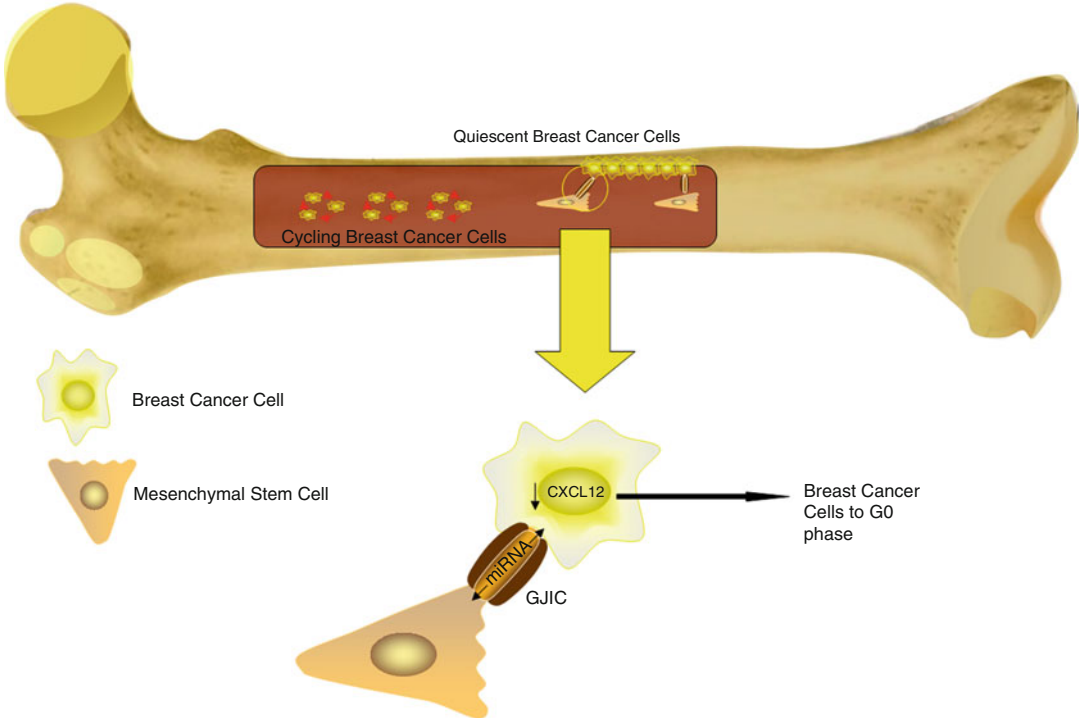

Fig. 3.3 Potential contributing factors in BC dormancy. Dormant, non-cycling cells have been shown to exist proximal to the bone marrow endosteum, where they form GJIC with BM cells. In contrast cycling cells have been shown to exist within the cellular compartment of the BM. GJICs form between MSCs and BCCs and allow crossing of miRNA that have were found to reduce CXCL12 levels. CXCL12 is normally constitutively expressed and is a known regulator of hematopoiesis. It has been demonstrated that decreased levels of CXCL12 correlate with decreased BCC proliferation. *BC* Breast Cancer, *GJIC* gap junctional intercellular communication, *BM* bone marrow, *MSC* mesenchymal stem cell, *BCC* breast cancer cell, *miRNA* microRNA, *CXCL12* chemokine C-X-C motif ligand

(Kiel and Morrison 2008). Supporting evidence for this location was demonstrated as non-cycling BCCs were found close to the endosteum of a xenogenic mouse model, while proliferating cells were found within the cellular compartment to the bone marrow (Lim et al. 2011). In addition to cellular communication through gap junctions, cytokines are a particular topic of interest with regard to BC. C-X-C motif chemokine 12 (CXCL12), normally constitutively expressed and a known regulator in hematopoiesis, is downregulated when contact is made between the same stroma and BCCs; it was discovered that decreased levels correlated with decreased proliferation of BC (Moharita et al. 2006). In search of possible mechanisms for decreased levels, it was found that particular miRNAs that cross GJICs between BC and stromal cells specifically reduce CXCL12 levels and transition BCCs to the G0 phase (Lim et al. 2011). The importance of CXCL12 is outlined in its interaction with C-X-C chemokine receptor type 4 (CXCR4), found on MSCs, the first cells to interact with metastatic BCCs in the bone marrow (Corcoran et al. 2008). This contact, inducing a variety of different states including but not limited to immunosuppression, may form the beginning of a microenvironment conducive to BCC survival in the bone marrow. These mechanisms may outline specific targets for future treatments and therapeutic interventions (Fig. 3.3).

Discussion

The development and evolution of the CSC theory has introduced a novel way of approaching cancer research particular with regards to metastatic pathways and quiescent mechanisms. Clinically, treatment regimens currently take a classical route and are primarily concerned with killing cells that are rapidly dividing, yet treatment modalities must evolve and embrace the concept that CSCs drive tumorigenesis. Further understanding and research of CSCs and their role metastasis may force practicing physicians and scientists to direct their attention towards therapies for CSCs rather than to reduce the bulk of the tumor through radiation and/or the use of drugs that target the proliferating cancer cells. As the science progress on examining CSCs in tumor biology, this would lead to an understanding of the mechanisms used by cancer cells to remain dormant and to evade detection and treatment.

BCC dormancy in bone marrow is achieved a complex mechanism such as factors intrinsic to the cell. These include ABC drug transporters and GJICs, and those that are extrinsic to the cells including MSC-mediated oncoprotection such as evasion of the immune response. An understanding the underlying mechanisms in dormancy will have a tremendous impact on cancer. Perhaps the most interesting parameter in dormancy and cancer propagation is the role of MSC in oncoprotection. This is particularly intriguing due to recent attention of MSC for a variety of different therapies transcending many different fields of medicine. Regardless, cancer metastasis and dormancy continue to be a major research and clinical dilemma.

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Determination of Breast Cancer Dormancy: Analysis of Circulating Free DNA Using SNP 6.0 Arrays

4

Jacqui Shaw, Kevin Blighe, Karen Page, David Guttery, and R. Charles Coombes

Contents

Abstract

New biomarkers are needed in breast cancer to monitor minimal residual disease. Specific point mutations, promoter methylation and loss of heterozygosity have been demonstrated previously in paired tumor and circulating cell-free DNA (cfDNA) isolated from plasma. Moreover, acquired alterations unique to cfDNA have also been found, suggesting disease progression. This prompted us to characterize the "circulating" breast cancer genome, thus testing the hypothesis that cfDNA acts as a surrogate liquid biopsy of breast cancer. This was achieved using Affymetrix SNP 6.0 technology and bioinformatics to map single nucleotide polymorphism (SNP) and copy number variation (CNV), comparing cfDNA with matched normal leucocyte and primary tumor DNA in breast cancer patients and paired normal leucocytes and cfDNA in healthy female controls. Our results show that

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concordance of SNP genotype calls in paired leucocytes and cfDNA can distinguish between primary breast cancer patients and healthy controls ($p<0.0001$), and between pre-surgical breast cancer patients and patients on follow-up after surgery and treatment ($p=0.0016$). In 50 patients on follow-up, a significant difference ($p=0.0006$) was seen between cfDNA samples taken an average of 3 years apart, suggesting disease progression. Considering CNVs, amplification was observed in matched tumor and cfDNA at multiple loci on different chromosome arms but was quiet or absent in normal DNA. Many of these tumor-specific CNVs contributed significantly to disease through binary logistic regression analysis. Furthermore these CNVs remained detectable in cfDNA up to 12 years after diagnosis and treatment despite no other evidence of disease. Taken together these cfDNA results suggest breast cancer dormancy in the majority of the patients on follow-up.

Keywords
Breast cancer • Cell-free DNA (cfDNA) • SNP 6.0 arrays • Copy number variation (CNV)

Introduction

Recent decades have been marked by significant advancements in nucleic acid analysis. The development of sensitive molecular techniques to detect tiny quantities of nucleic acids has been welcomed in many areas of research. Increasingly, attention is being drawn to nucleic acids circulating in blood and their potential diagnostic and prognostic value.

The presence of circulating nucleic acids, now widely termed circulating cell free DNA (cfDNA), in blood plasma was first described by Mandel and Métais (1947). The potential diagnostic implications of Mandel and Métais' discovery was realized in the 1970s, when Leon et al. (1977) reported that cancer patients showed increased levels of cfDNA in serum compared to healthy controls, with greater amounts of cfDNA found in the serum of patients with metastases, compared to localized disease. They showed levels of cfDNA decreased by 90% after radiotherapy in lymphomas, ovarian, lung, endometrial and cervical cancers, while high or increasing concentrations of cfDNA were associated with a lack of response to treatment (Leon et al. 1977). However, owing to limitations in technology available at the time, the precise cellular origin of this cfDNA in the circulation of cancer patients could not be determined. Subsequent studies showed that increased levels cfDNA were not only found in patients with cancer, but also in patients with inflammatory conditions (Shapiro et al. 1983).

Origin and Nature of cfDNA

In healthy individuals, it can be assumed that cfDNA originates from leucocytes or other nucleated cells; however, its origins in malignancy are less clear. One possible mechanism is via cell necrosis, as higher amounts of cfDNA have been found in the plasma of patients with large or advanced/metastatic tumors (Nawroz et al. 1996). A second alternative is by apoptosis, as evidenced by the typical pattern of apoptotic DNA fragmentation known as an 'apoptotic ladder', which results from inter-nucleosomal cleavage of genomic DNA (Jahr et al. 2001). However, as many proliferating cancer cells lose their ability to induce apoptosis; other mechanisms such as spontaneous and active release of DNA by proliferating cancer cells may be more important (Anker et al. 1975). This may also explain the presence of very low concentrations of cfDNA in some cancer patients, where the cancer may have been quiescent at the time of sample collection.

Jahr et al. (2001) suggested that in cancer higher levels of cfDNA are derived from cells that disintegrate by apoptosis and/or necrosis in expanding tumor tissue. They noted that several complex processes determine the fate of DNA released from degenerating tumor cells and that the differing efficiencies of these processes may explain the varied distribution in the amounts and composition of circulating

cfDNA present in the blood. The variation in the lifetime of cfDNA in blood plasma, reported as ranging from 15 min to several hours (Schwarzenbach et al. 2011), and differences in DNase activity in the plasma may also help to explain this.

Another possible mechanism leading to generation of cfDNA is lysis of circulating tumor cells (CTCs) in the blood (Alix-Panabières et al. 2012). However, the number of CTCs typically observed is not sufficient to explain total cfDNA levels. Chen et al. (1999) calculated that there would need to be 1,000–10,000 cancer cells per milliliter of blood to directly account for the typical amounts of cfDNA detected. Turnover of disseminated tumor cells (DTCs) in micrometastatic deposits could also lead to release of cfDNA. Recently, our group demonstrated that the presence of viable DTCs in the bone marrow was inversely related to that of circulating cfDNA in blood plasma of patients in the post-adjuvant setting (Payne et al. 2012), indicating that there may be an inverse relationship between these during the dormancy phase of breast cancer. Although the precise origins of cfDNA are not known, we and others are continuing the investigation of the relationship(s) between cfDNA and both CTCs and DTCs (Fig. 4.1).

The stability of cfDNA in blood plasma and its clearance from the circulation has not been extensively studied. However, a study by Lo et al. (1999), showing the rapid clearance of fetal DNA from the maternal circulation, gives us some insight into how this may work in cancer. Lo et al. (1999) concluded that that the rapid kinetics of circulating cfDNA would potentially make plasma-based molecular diagnostics a useful monitoring tool in cancers. Silva et al. (2002) first investigated the persistence of tumor DNA in the plasma of breast cancer patients following mastectomy and showed that patients post mastectomy with detectable tumor DNA in plasma had characteristics of poor prognosis, such as vascular invasion. They suggested that the persistence of cfDNA after mastectomy identified a group of patients with more aggressive disease, and potentially reflected micrometastatic dissemination.

Molecular Analysis of cfDNA

Molecular analysis of cfDNA is one attractive approach to interrogate the 'circulating cancer genome'. The main advantages of cfDNA are its ease of availability and its stability. Both cfDNA concentration and size/integrity have been suggested previously for diagnosis of cancer (Wang et al. 2003; Page et al. 2006). Using a TaqMan qPCR assay targeted to the *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene, we found that both the mean plasma DNA concentration and integrity were significantly higher in late stage (metastatic) breast cancer – but not early stage breast cancer – when compared to healthy female controls. However, elevated levels are sometimes seen in benign disease. Specific patterns in cfDNA (e.g. mutations, loss of heterozygosity (LOH), and hypermethylation) have the potential to provide tumor specific markers and have been more widely investigated (Jung et al. 2010). In our first study, using just two markers we showed that cfDNA was more sensitive than either CTCs or DTCs for detecting evidence of minimal residual disease in primary breast cancer patients (Shaw et al. 2000). We therefore sought to characterize cfDNA in more detail in the follow-up of primary breast cancer and first demonstrated amplified *HER2* in cfDNA up to 10 years after treatment in asymptomatic patients, suggesting persistent micrometastases and a dormant state (Page et al. 2011). Moreover, *HER2* amplified cfDNA was detected in two HER2 negative patients following primary breast cancer treatment, suggesting emergence of a *HER2* amplified sub-clone and indicating disease progression. Recently, we also described molecular portraits of cfDNA using whole genome approaches (Shaw et al. 2012).

Genomic Profiling of cfDNA with Single Nucleotide Polymorphism (SNP) Arrays

A single nucleotide polymorphism (SNP) is defined as a polymorphic single base change in a DNA sequence that has a frequency of at least 1% in the population (Haimovich 2011). It is

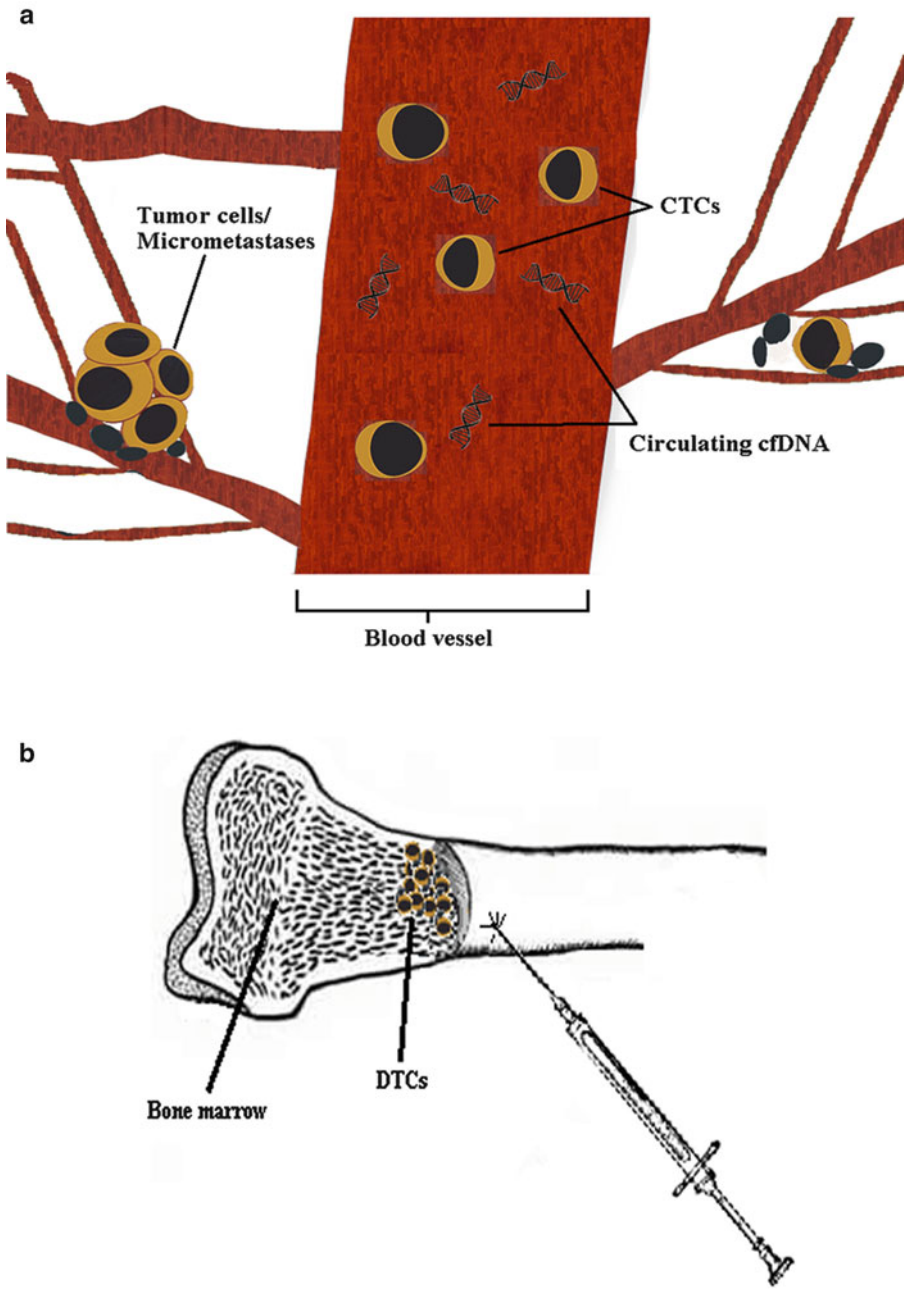

Fig. 4.1 Determining the relationship(s) between cfDNA and CTCs in the blood circulation (**a**) and DTCs in the bone marrow (**b**)

estimated that there are more than 10 million SNPs in the human genome that have a minor allele frequency of 5% (LaFramboise 2009). These SNPs are the most common type of genetic variation and contribute to the approximately 1% of the human genome that differs between individuals. Whole genome studies such as the International HapMap Project (International HapMap Project, http://hapmap.ncbi.nlm.nih.gov/) aim to map SNPs in different healthy

populations. Although variance in the human genome was originally thought to be mostly due to SNPs, copy number variation (CNV) is also recognized as a major source of normal human genome variability (Sebat et al. 2004) and contributes significantly to phenotypic variation (Redon et al. 2006). These CNVs include duplications, losses, and inversions, and are generally regarded as being ≥1,000 bases in length but can vary in size ranging from a few kilobases (Kb) to megabases (Mb). The commonality and abundance of CNV can be inferred when, for example, any two healthy individuals' samples are compared to reveal gains and losses between each (Van de Wiel et al. 2011). Inherited CNVs have been suggested as possible susceptibility factors for neurological disorders, obesity (Sebat et al. 2004), and autoimmune disorders. Copy number variation in somatic cells is an important and emerging area of cancer research (Van de Wiel et al. 2011), as CNVs are potential causal variants in cancer owing to the fact that a larger section of DNA can be affected.

Jin et al. (2011) aimed to identify CNV and related SNPs that could both be used to distinguish between types of aggressiveness in prostate cancer using the process of tagging SNPs for CNVs. They identified a 32.3Kb deletion that was 'tagged' with SNP rs2209313 on 20p13. One other notable study, which compared CNV in *BRCA1*-associated ovarian cancer to that in sporadic ovarian cancer, suggested that the two were distinct from each other based on their respective CNV profiles (Yoshihara et al. 2011). However, other studies relating CNVs to cancer – particularly in breast cancer – are lacking.

DNA Array Technology

DNA SNP arrays have the potential to characterize genomes by picking-up SNP, CNV, and other genomic variation through taking a whole-genome approach, whilst also having the potential to compare differences in these variants across different samples. They improve upon targeted/candidate gene studies by analyzing many genes and other genomic regions in tandem (Quackenbush 2002).

Affymetrix was the first company to produce oligonucleotide-based arrays with just 1,494 SNP markers. Further releases by Affymetrix incorporated 10,000, 100,000, 250,000, and later 500,000 SNP markers (LaFramboise 2009). The sixth generation of Affymetrix SNP arrays is the *Genome-Wide Human SNP Array 6.0* (Affymetrix SNP 6.0). The array chip is capable of genotyping 906,600 SNPs and contains an additional 945,826 probes for detecting CNVs. Of the copy number (CN) probes, 115,000 target previously-known CNVs. Overall, markers are distributed evenly across the genome, with a median marker spacing of 1–5 Kb. The main advantage of the Affymetrix SNP 6.0 over the current releases by Illumina Inc. and Agilent Technologies Inc. is that a much smaller starting template of DNA is required (50 ng/μl compared to 400 and 500 ng/μl, respectively). However, Illumina's current release offers higher density, being capable of scanning for around 4.3 million markers with a median marker spacing of just 0.36Kb.

As well as genotyping SNPs, Affymetrix SNP 6.0 arrays are also capable of detecting LOH, which can reveal the presence of a recessive mutation in a tumor suppressor gene. However, this can be confounded by the phenomenon of copy neutral-LOH (CN-LOH), which can occur where a chromosomal region is subject to LOH but is then duplicated, leaving a "neutral" CN state of two. However, this could still prove to be a tumorigenic event that would remain undetected through other genomic array technologies. The Affymetrix SNP 6.0, however, is equipped to unearth CN-LOH as it scans the genome with both CN and SNP probes. Additionally, the SNP probe signal intensities can – along with those of the CN probes – be used to estimate CN – i.e. CNVs can be revealed by SNPs and thus be indirectly inferred from genotyping (Korn et al. 2008; Jin et al. 2011). This owes to the fact that signal intensity is merely dependent on the amount of target sequence present (LaFramboise 2009). For example, Jin et al. (2011) noted that although studies had identified CNVs at three loci associated with Crohn's disease, rheumatoid arthritis, and diabetes, these CNVs had already been identified in SNP-based studies. Additionally,

SNP analysis can be used to determine which allele has been either amplified or deleted (Korn et al. 2008) – for example, knowing that a section of DNA within a gene has been amplified does not provide information on the particular allele in question. Combining SNP and CN data can help determine this allele-specific CN (Korn et al. 2008).

A general problem associated with arrays (especially those involving a high-density of probes) is that a large starting quantity of DNA is often required for analysis. For example, even though the SNP 6.0 requires much less starting template of DNA than its competitors, a total of 250 ng DNA (at 50 or 100 ng/µl recommended) is still required. The process of whole genome amplification (WGA) can be used to generate high quantities of DNA where limiting amounts of template are available, such as those obtained from either laser capture microdissection (Rook et al. 2004) or from cfDNA. However, in order to be successful, the WGA process must both accurately amplify the target DNA and also provide sufficient quantities. Using the *Affymetrix Early Access Mendel Nsp 250K GeneChip* array platform, Jasmine et al. (2008) compared the SNP call-rates between genomic DNA and whole genome amplified DNA of 14 healthy controls and reported excellent concordance (>99%) between the two sets of samples on a paired basis. We found similar high concordance using the earlier 10K array (unpublished observations).

Sequential Analysis

Due to the inherent problems in obtaining sequential samples in the adjuvant and post adjuvant setting, little is known about the nature of dynamic changes in the cancer genome over time. However, sequential analyses may prove to be most important for the following-up of cancer patients. In breast cancer, gene expression analysis revealed that multiple genetic changes from the primary tumor can occur in micrometastases in the bone marrow (Gangnus et al. 2004). We hypothesize that in patients on follow-up who are otherwise disease free, evidence of tumor DNA detected in cfDNA would suggest the presence of dormant micrometastases, possibly deriving from – or related to – DTCs in the bone marrow. Hence, it would be hugely advantageous to be able to detect these specific changes indicative of both minimal residual disease and, potentially, disease progression in cfDNA.

Results of our recent study (Shaw et al. 2012) suggest that cfDNA is a useful liquid biopsy that can be analyzed to determine a suite of useful diagnostic and prognostic biomarkers. Furthermore, results of our study show that cfDNA 'evolves' over time and can give indications of an impending metastasis. We utilized the Affymetrix SNP 6.0 microarray to investigate: normal leucocyte DNA, a reference to help discriminate benign and pathogenic SNPs and CNVs (Conrad et al. 2009); microdissected primary tumor DNA, to help define the 'mutation state' of the primary tumor (Schwarzenbach et al. 2011); and, finally, sequential cfDNA of 50 breast cancer patients on follow-up in the post adjuvant setting to provide a tool for analysis of circulating tumor-specific and de novo markers in cfDNA.

Results

Sample Information

We profiled 251 genomes using Affymetrix SNP 6.0 arrays to determine SNP profiles and CNVs in the following patients and samples: (1) healthy female volunteers (healthy control, n = 8), for whom we compared matched leucocyte and plasma cfDNA; (2) breast cancer patients who had surgery and were on follow-up either with or without endocrine therapy in the post adjuvant setting (on follow-up, n = 50), matched leucocyte, tumor (for 40/50 patients), first plasma cfDNA (mean 6 years post-surgery), and second plasma cfDNA (mean 9 years post-surgery); (3) primary breast cancer patients who were awaiting surgery (pre-surgical, n = 15), matched leucocyte, tumor, and pre-surgery plasma cfDNA (mean 14.7 weeks prior to -surgery).

High SNP call-rates were achieved for the majority of samples, highlighting the efficiency

of the array technology. Of note, the normal leucocyte DNA samples across all three groups had the highest overall mean SNP call-rate (96.89%, range 70.14–99.32%), compared to other samples. In the on follow-up group, first plasma cfDNA, second plasma cfDNA, and tumor DNA had means of 91.37% (range 85.04–98.51%), 88.62% (range 85.21–98.33%), and 88.52% (range 86.65–89.62%), respectively. The means for pre-surgical breast cancer patients' plasma cfDNAs (86.80%; range 85.85–88.36%) and those of the healthy controls (83.06%, range 69.62–92.33%) were comparable. Finally, the pre-surgical patients' tumor DNA samples exhibited a mean of 93.61% (range 87.06–98.70%). These high values for SNP call rate, similar to those seen for normal leucocyte DNA, reflect the fact that the majority of these tumor samples were from the fresh frozen (FF) tumor tissue rather than from formalin-fixed, paraffin-embedded (FFPE) tissues, hence yielding better quality DNA.

As all samples in the study were processed and hybridized in two batches only, it was possible to determine intra-assay reproducibility. To this end, 13 samples were repeated for the entire process – from DNA extraction to array hybridization – across the two batches. They showed excellent correlation between contrast QC ($p < 0.0001$), QC call-rate ($p = 0.0001$), median of the absolute values of all pairwise differences ($p = 0.0005$), and in the total number of derived CNVs ($p < 0.05$), using paired t-tests. The results also exhibited good correlation (average ρ 0.727, Spearman's rank) when comparing unfiltered probe intensity values between replicate samples. The frequency of each specific CNV detected between the two batches also showed good agreement.

Comparison of SNP Calls

Various comparisons were carried out between patients and samples, within and between groups, based on the concordance (agreement) of SNP genotype calls between paired DNA samples from different individuals. As such, the concordance of SNP genotype calls between paired cfDNA and matched leucocyte DNA samples was compared between the breast cancer patients and healthy controls. The highest mean concordance of 89.35% (range 81.10–94.08%) existed in the healthy controls, suggesting that the two genomes were closely related, as would be expected if the cfDNA in healthy individuals was derived from apoptosis of normal cells. In addition, this level of concordance was not significantly correlated to sample quality (using the SNP call-rate as indicator of quality) (Spearman's rank). By group, the SNP concordance was lowest for the 15 pre-surgical primary breast cancer patients (mean 44.88%, range 36.00–68.27%), but also remained low for the 50 patients on follow-up in both the first (mean 69.10%, range 33.17–99.44%) and second (mean 54.22%, range 33.31–97.96%) cfDNA samples. A point to note is that 28 and 12% of the first and second plasma cfDNA samples from the on follow-up group, respectively, exhibited a concordance at or above the mean value obtained for the healthy control group (89.35%). This potentially indicates a 'normal' genomic plasma cfDNA profile for these patients and cfDNA samples. Potentially then, a SNP profile of cfDNA may allude to either the presence or absence of minimal residual disease. The mean SNP concordance by group was also compared, which revealed a significant difference between the healthy controls and the pre-surgical patients ($p = 0.0001$, Mann–Whitney unpaired *t*-test).

In the on follow-up group, the mean SNP concordance of the first cfDNA samples was not significantly different to that of the healthy controls ($p = 0.0612$), but those of the second cfDNA were ($p = 0.0004$), which suggests genomic changes with time in cfDNA. The level of SNP concordance was also compared between each patient's cfDNA sample and each of the healthy controls. This revealed a significant difference between the first and second cfDNA samples of the patients on follow-up ($p = 0.0006$, paired Wilcoxon signed-rank *t*-test), and between the cfDNAs of the patients on follow-up and pre-surgical patients ($p = 0.0016$, Kruskal-Wallis ANOVA). The SNP genotype calls were also compared between each cfDNA sample and

matched tumor, where available (available for 15 pre-surgical and 40 patients on follow-up), which revealed low concordance (overall mean 46.89%, range 31.04–66.20%), highlighting genomic differences between cfDNA and matched primary tumor DNA. However, comparing these concordances again revealed a significant difference between the pre-surgical and on follow-up groups ($p < 0.05$, Kruskal-Wallis ANOVA).

Copy Number Variations

Using 1Kb as the minimum size, the number of CNVs called for each sample/group was identified, and the percentage of overlap with the Toronto database of genomic variants (DGV) was determined. A total of 9,301 CNVs were identified in the leucocyte DNA samples and 9,341 in the plasma cfDNA of the 8 healthy controls. Of these CNVs, 57.07 and 55.95% respectively were found to overlap either partially or completely with the Toronto DGV, and are thus known/confirmed genomic variants. The remainder – 42.93 and 44.05%, respectively – did not overlap and were thus identified as being novel. When looking at the leucocyte DNA samples in the pre-surgical patients and those on follow-up, 40.38 and 40.52% respectively were identified as novel. However, in the plasma cfDNA samples, 50.05% of the pre-surgical plasma cfDNA CNVs were identified as novel, compared to 45 and 46.48% in the on follow-up first plasma and healthy control plasma cfDNA. Finally, in the tumor DNA samples, 50.05% of CNVs called in the FFPE tumor DNA of patients on follow-up were novel, and 40.12% were novel in the pre-surgical group.

The number of CNVs called in each sample was compared between paired plasma and matched leucocyte DNA samples. In the patients on follow-up, there were eight patients who relapsed and these showed a significant difference between their first cfDNA sample and matched leucocytes ($p < 0.01$, paired Wilcoxon signed-rank *t*-test). A statistically-significant difference ($p < 0.05$) was also found for relapsed second cfDNA compared to the matched normal leucocyte DNA. These second cfDNA samples were from the final blood sample taken prior to the time of relapse. Hence, we believe that genetic 'signatures' of relapse might be detected by looking at these samples. When results for the other 42 patients on follow-up who did not relapsed were stratified by size and node-status (i.e. T_1N_0 ($T \leq 20$ mm) and $T > 20$ mm/node-positive), there were no significant differences for either the first or second cfDNA sample. The difference in the total number of CNVs called between the first and second cfDNA samples was also analyzed: overall, 30 patients showed a decrease in CNVs from their first to second cfDNA, and 20 showed an increase; while, of the relapsed patients, five showed a decrease and three an increase. Hence, there were no obvious trends with time or with disease progression in terms of the number of CNVs.

In relation to amplifications and deletions, a 'sweep' of all samples in the study revealed 750 different cytobands that exhibited amplification using a high but arbitrary threshold of $CN > 6.0$ by Gaussian-smoothed signal. Many of these amplifications, however, were unique to a single sample, indicating genomic heterogeneity. The presence or absence of amplification at each of the 750 cytobands by sample type and patient group revealed, through clustering, two main groups: one group contained all leucocyte DNA samples and both the first cfDNA samples of patients on follow-up and the cfDNA of pre-surgical patients; whereas the other group contained all tumor DNA samples and the second cfDNA samples of patients on follow-up. However, of interest, the second plasma cfDNA samples from the relapsed patients also clustered at the left with the normal leucocytes and first plasma samples.

Next, the results were filtered to remove uniquely-affected cytobands (i.e. cytobands showing amplification in a single DNA sample), which reduced the total to 634. These 634 were filtered further to show only those with amplification in >10% of samples only (per sample group), giving 23 cytobands. Eighteen of these 23 cytobands showed amplification in plasma and tumor DNA of breast cancer patients but little or no amplification in the cfDNA of healthy controls. The remaining 5 cytobands, whilst showing

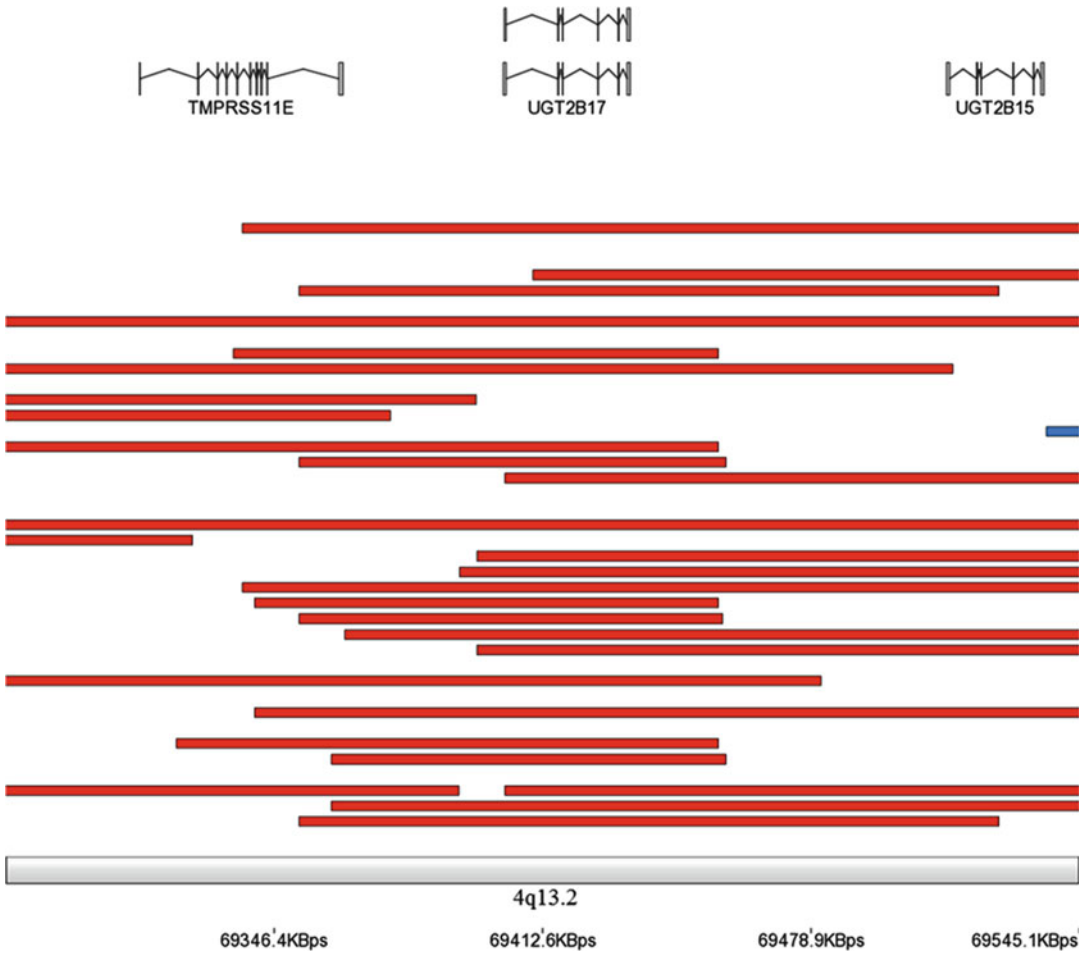

Fig. 4.2 High level amplification in tumor DNA of breast cancer patients on follow-up, showing commonly-amplified cytoband 4q13.2 in tumor, based on a six-state HMM. Hidden Markov CN segment states per sample are represented by horizontal bars. Amplification, *red*; deletion, *blue*

amplification in plasma and tumor of breast cancer patients, also showed some amplification in the leucocyte and/or plasma cfDNA of healthy controls. Eighteen of the 23 regions also have known overlapping genes and the remaining five have none. Further analysis, performed by raising the threshold to CN > 8.0 revealed 352 regions, while lowering the threshold to CN > 4.0 revealed amplification in at least one sample at virtually every known cytoband. These CNV levels support results of Redon et al. (2006) that CNV is common in the human genome.

Interestingly, at this lower threshold (CN > 4.0), 8 cytobands exhibited high-frequency (>90%) amplification in the tumor DNA samples of patients on follow-up. These 8 cytobands also showed amplification in breast cancer cfDNA, but at a lower frequency (>25%). Figure 4.2 shows an example of amplification detected at one of these cytobands (4q13.2) in patients on follow-up, in this case generated using a hidden Markov model (HMM) to verify the initial findings. There was clear evidence of local amplification in tumor DNA.

Logistic Regression to Reveal Significant Copy Number Variant Regions

Binary logistic regression analysis was used to correlate the prevalence of the 23 common CNVs to the molecular phenotype of the primary tumor. Based on cancer status (i.e. cancer or healthy), five CNVs significantly-contributed to disease in the first cfDNA samples in the patients on follow-up (5q13.2, 9p11.2, 10q11.22, 14q11.2, and 15q25.2), while nine CNVs significantly-contributed in the second cfDNA samples (5q13.2, 9p11.2, 9q12, 10q11.22, 14q11.1, 14q11.2, 15q25.2, 16p11.2, and 19p13.3). Whereas, in the cfDNA samples of the pre-surgical patients, 12 CNVs significantly-contributed to disease. Of these, 6 were unique to the pre-surgical cfDNA samples (1p36.33, 1p36.21, 2p11.2, 7q11.21, 10q11.23, and 14q32.33).

In the on follow-up group, we also compared results for patients who relapsed with those who did not. Two CNVs (1p36.33 and 9q12) were correlated significantly with relapsed status based on the first cfDNA samples, and 11 (1p36.33, 1q21.1, 9p11.2, 9q12, 10q11.22, 10q11.23, 14q11.1, 14q11.2, 15q11.2, 16p11.2, 19p13.3) based on the second. Whereas, in the tumors, six CNVs contributed significantly to relapse, four of which (1p36.33, 1q21.1, 9p11.2, and 9q12) were significant at a value of $p < 0.0001$. Of interest, these 6 cytobands also distinguished between $T > 20$ m/node-positive tumors compared to those that were T1N0 ($T \leq 20$ mm). Significant differences also arose based on endocrine therapy received (tamoxifen, anastrozole (Arimidex®), Goserelin (Zoladex®), or tamoxifen/anastrozole combined) compared to no therapy received, predominantly in the second cfDNA comparisons. Finally, based on receptor status in the on follow-up group, no CNVs contributed significantly to either ERBB2- or PR-positive status in either tumor or cfDNA. However, 5p11 contributed significantly to ER-positive status in the second plasma cfDNA samples, and to the triple-negative status of the primary tumor.

Principal Component Analysis and Hierarchical Clustering to Interrogate Both SNP and CN Data

Comparison of both SNP and CN data was investigated through principal component analysis (PCA), which is a method used for separating a set of related data into groups (or principal components). In the leucocyte DNA samples, PCA analysis of SNP/CN data revealed no stratification between cancer patients and healthy controls; however, analysis revealed a clear division between the cfDNA samples of the pre-surgical patient group compared to those of the healthy controls. This was also evident using SNP and then CN markers separately. The 3 resulting plots (SNP+CN, SNP, and CN data separately) were very similar and subsequent examination of the component loadings for each sample from each plot revealed a Spearman's rank ρ correlation of 0.972, suggesting similarities between the SNP and CN profiles. Potentially, then, these SNP and CN intervals are linked. In the patients on follow-up, PCA revealed clear separation between tumor and leucocyte DNA samples. However, plasma cfDNA samples were widely distributed: some clustered close to the tumors; others close to the leucocytes; while others were scattered in-between. Overall, there were more second cfDNA samples clustered close to the tumor samples; cfDNA samples that clustered with the leucocyte cluster had high SNP concordances when compared to their matched leucocyte.

Hierarchical clustering of data for the 50 patients on follow-up revealed a similar separation of samples as PCA, with the tumor and leucocyte DNA samples clearly separated and cfDNA samples mostly between these (Fig. 4.3). Two main clusters were identified: tumor with mostly second cfDNA samples; and leucocyte with mostly first cfDNA samples. When observing just the first and second cfDNA samples, there was a clear division in the dendrogram structure in each case, but no differences between the T_1N_0 ($T \leq 20$ mm) and $T > 20$ mm/node-positive patients were apparent. When observing all tumor DNA samples, the dendrogram lacked any high-level branching structure, indicating that these tumor samples comprised a homogenous group.

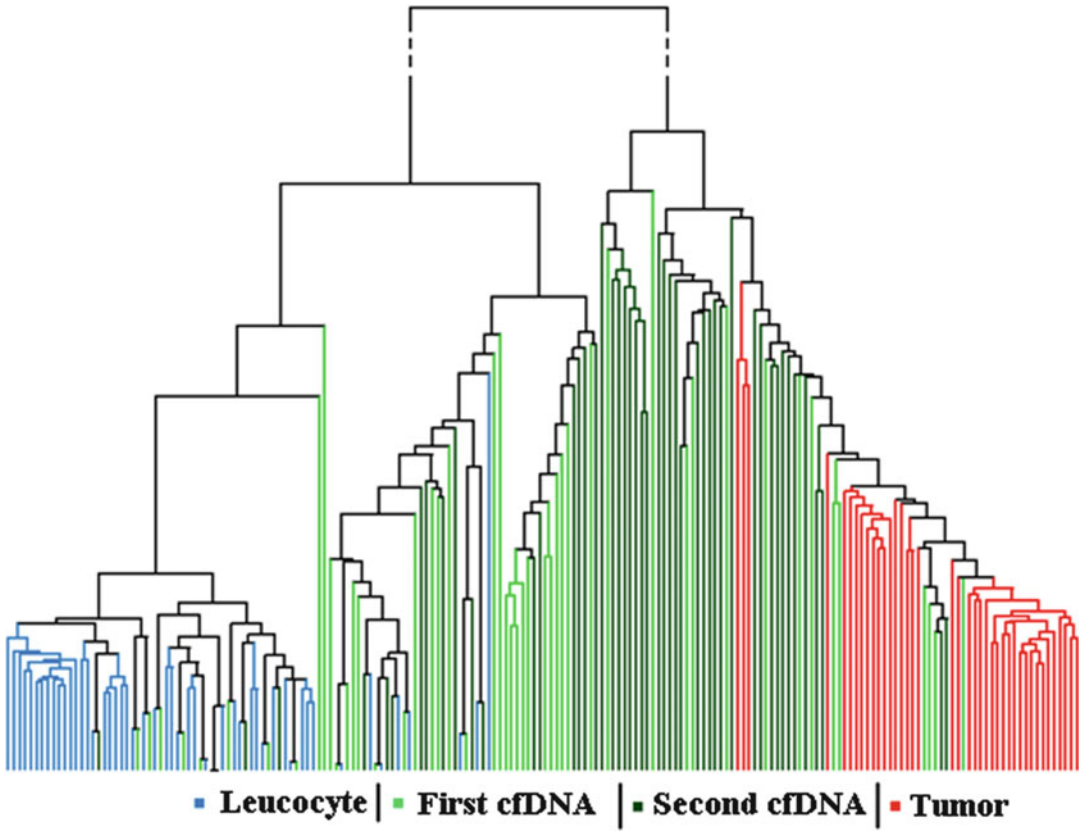

Fig. 4.3 Hierarchical clustering of on follow-up group revealed a division between tumor and leucocyte DNA samples

Loss of Heterozygosity Analysis

Examining the on follow-up group's samples for LOH revealed many that were de novo in the cfDNA samples taken after surgery: between plasma cfDNA and matched tumor, the extent of overlapping LOH ranged from just 10–35%. When solely focusing on exons in cfDNA, there were 34 LOH events that occurred in 2 or more of the 50 patients on follow-up (first and second cfDNA samples combined into one group), whilst 36 occurred in two or more of the 15 pre-surgical patients' cfDNA samples. There was generally more LOH detected in $T > 20$ mm/node positive patients than T_1N_0 ($T \leq 20$ mm) patients, and an overall increase in LOH detected between the first and second cfDNA samples. Through analyzing both CN and LOH data concurrently, it was revealed that 1.47% of CNVs exhibited CN-LOH.

Discussion

Comparing Formalin-Fixed and Fresh Frozen Tissue

In our SNP 6.0 study, we found a difference in quality of data generated between FFPE and FF tumor tissue. Only 389 CNVs, on average, were called per sample in the FFPE on follow-up tumors. However, in the pre-surgical FF tumors, the average was 2,515 per sample. This was also reflected by differences in the QC

data. Hence, higher molecular weight DNA fragments obtained from FF tissue appear to result in greater efficiency, with regard to CNV calling, whereas SNP calls were generally less variable, with high call rates in all samples. All CNV data generated from FFPE tissues was therefore regarded as preliminary, with careful comparison to FF and cfDNA results. It is thus important to determine the optimum methods for using FFPE tissue that can give the greatest confidence in results. For example, WGA might not accurately amplify FFPE DNA (Jasmine et al. 2008). However, another study also involving the Affymetrix SNP 6.0 microarray has shown good concordance between FF and matched FFPE tissues (Tuefferd et al. 2008). Overall, FF is a useful reference against which FFPE tissue can be compared in future studies.

Concordance of SNP Genotype Calls Segregated cfDNA of Healthy Controls from Breast Cancer Patients

Our results demonstrate for the first time that bioinformatic analysis of SNP 6.0 array profiles of cfDNA can significantly distinguish between women with breast cancer and healthy female controls ($p < 0.0001$). This could potentially pave the way for a blood test to diagnose breast cancer if these results were validated in other, larger cohorts. In healthy controls, the high % SNP concordance between paired leucocytes and cfDNA confirmed that these genomes were essentially the same, as would be expected if the cfDNA in "healthy" plasma DNA was derived from normal cells. Also, using healthy control plasma cfDNA data as baseline, results showed that the first and second cfDNA samples of the patients on follow-up differed significantly from each other ($p = 0.0006$), highlighting that the plasma cfDNA profiles of breast cancer patients on follow-up change with time. These data support other research that showed that the tumor genome can evolve with metastatic disease (Gangnus et al. 2004). It therefore follows that cfDNA analysis can be used to monitor the evolving circulating cancer genome.

Copy Number Variation

Copy number variation is common in the human genome (Redon et al. 2006). In cancer, instability at a particular CNV could confer an advantage and promote disease progression. Thus, by mining CNV data it is possible to identify CNVs that are associated with disease and not found in healthy, non-cancer genomes.

By overlapping CNVs discovered in our study with known variants listed in the Toronto DGV, 45 and 46.48% of CNVs identified in the first and second plasma cfDNA samples, respectively, of the patients on follow-up were novel. For the tumor samples of these patients, the figure was slightly greater than half (50.05%). However, when interpreting these results, it is important to note that each DNA sample was considered as a single entity due to the method of genomic segmentation making calls on a per sample basis and not correlating segments intra-patient. Hence, CNVs that overlapped with each other across samples in the same group would have been counted separately. However, the extent of overlap can be elucidated better with more defined breakpoints between segments (Conrad et al. 2009), which would also help the development of targeted approaches such as qPCR.

The amount of novel variation found in our study was surprising. Much of the data in the Toronto DGV is derived from projects that have used single technologies with no reproducible, parallel studies to confirm findings. Additionally, there is a misrepresentation of small (<1Kb) CNVs in such online databases (Conrad et al. 2009), so much of the data listed in the Toronto DGV requires validation. This is exacerbated by the fact that genomic positions of genes can differ with progressive releases of the human genome and makes for a database that is in need of continual updating. As array technology improves in resolution and high throughput sequencing becomes more common, the data will continue to be refined.

Amplifications and Deletions

Amplifications comprised the majority of CNVs for the majority of samples analyzed in our study. The exact mechanisms that lead to gene amplification are uncertain, and the extent to which gene amplification plays a causal role in cancer is equally uncertain. Nevertheless, mapping amplifications in cancer can help to identify putative genes for targeted therapy. Throughout our study, amplifications were more prevalent than deletions, and the implication is that certain regions of the genome are predisposed to be affected by CNV (due to their sequence) indirectly through events such as non-allelic homologous recombination, variable number of tandem repeats formation, and retrotranspositions. In such a mechanism, increased genomic instability will result in an increased number of amplifications. Could a variant SNP be the driver of such events? – mapping SNPs to CNVs could provide insight into what role SNPs have in driving CNV.

One important finding from these data, which demonstrate amplified and deleted areas of the genome, is that plasma cfDNA characterization may provide important information for clinicians in choosing subsequent therapies through the identification of which gene products to target. The dosage level of a candidate oncogene could be increased due to high level amplification and have consequences on the resulting phenotype (Uchida 2006); conversely, a large deletion could silence gene transcription.

Commonly-Amplified Intervals Persist in cfDNA: Evidence for Dormancy

Through the analysis of the commonly-amplified CNVs, it was shown that tumor-specific CNVs were able to persist in the cfDNA extracted from blood plasma up to 12 years after surgery to remove the primary tumor. The fact that these alterations were similar in >90% of the tumor DNA samples indicates their importance in breast cancer. These intervals could therefore provide novel diagnostic information to be used in the future, especially as some of these CNVs were shown to contribute significantly to cancer status by binary logistic regression analysis in both the first and second cfDNA samples. Moreover, some of these CNVs were found to be significant in discriminating between disease and healthy, relapsed and non-relapsed, and size and nodal status. In our study, it was shown that this could be achieved conclusively through focusing on certain altered regions in the genome.

Profiles of Relapsed Patients

When taking the number of CNVs into account (amplifications and deletions combined), there were few differences between groups. The number of CNVs called in a sample could be a reflection of sample DNA quality, with an average of 1,163 and 1,168 being called in healthy control leucocyte and plasma cfDNA respectively. However, when comparing the number of CNVs called, a significant difference was identified on a paired sample basis between the leucocytes and first and second cfDNA samples ($p = 0.0016$ and <0.05 respectively) of the eight patients who relapsed.

Importantly, binary logistic regression analysis of the 23 commonly-amplified CNVs also revealed significant differences: six of the CNVs contributed significantly to relapsed status based on tumor comparisons (four were at a significance of $p < 0.0001$). The same 6 cytobands also contributed significantly to the $T > 20$ mm/node-positive status compared to T_1N_0 ($T \leq 20$ mm). Also, the CNVs at 9q12 and 1p36.33 contributed significantly to relapsed status in the first cfDNA samples, while both of these and nine others contributed significantly in the second cfDNA samples, again suggesting evolving genomic instability. This suggests that these 11 intervals/CNVs may be involved in the development of metastasis. Moreover, they show that metastatic events can potentially be picked-up by analyzing serial plasma cfDNA samples in patients on follow-up and stratifying differences based on disease progression.

Breast Cancer Dormancy

Relapse in breast cancer can occur up to 20 years after primary treatment (Karrison et al. 1999), a length of time that suggests a period of apparent dormancy where there may be growth restriction of unseen micrometastases. Although dormancy is common in breast cancer, the associated mechanisms behind it are unknown. Reasons why tumors remain in a dormant state have been hypothesized as being due to an inability to invade blood and/or lymph vessels, apoptosis predominating over growth, an inability to induce angiogenesis and/or vascularization, cell-cycle regulatory proteins functioning normally, and a balance between growth-inducing and -inhibiting factors in the tumor microenvironment, favoring inhibition (Karrison et al. 1999; Meng et al. 2004).

Of the 50 patients on follow-up, 42 remained 'disease-free' by standard clinical measures some 10–13 years after surgery and treatment. However, results of our study shows that tumor associated CNVs persisted in the cfDNA many years after surgery and adjuvant treatment for the majority of these 42 patients who were disease free, suggesting tumor dormancy and persistence of minimal residual disease. However, what does all of this say about long term prognosis? The fact that tumor-specific alterations remain detectable in the blood of patients on follow-up years after surgery and treatment infers that their blood is constantly being replenished by replicating tumor cells and/or fragments (Meng et al. 2004).

It has been shown previously that CTCs can persist for many years after end of treatment or mastectomy (Meng et al. 2004). Therefore, death of these CTCs may steadily release cfDNA. However, in direct relation to this – and of critical importance – are the findings by Meng et al. (2004), that the half-life of CTCs in blood is 1–2.4 h, whilst Schwarzenbach et al. (2011) report that cfDNAs half-life in the same medium of healthy individuals is between 15 min and several hours. Therefore, in the post adjuvant setting, detection of tumor specific alterations in cfDNA, as identified in our study, more likely reflects release of cfDNA from micrometastatic cells rather from occasional CTCs in the circulation.

Genomic instability and dormancy can be seen as having a certain level of interplay: genomic instability drives oncogenesis and metastasis (Uchida 2006), whilst a dormant micrometastatic tumor clone concurrently persists in the body without any apparent destructive effects on the individual. However, tumor cells are under selective pressure to acquire increasing mutations (through genomic instability) that permits them to grow uncontrollably and metastasize. Thus, is a dormant micrometastatic clone merely "silently" accumulating mutations until such a time when the balance of driver mutations permit it to "awaken"?

In conclusion, we have shown that tumor-specific alterations are able to persist in the cfDNA of breast cancer patients up to 12 years after surgery and treatment, despite no other clinical evidence of disease, inferring breast cancer dormancy in the majority of patients. Further, it has been shown that by focusing on SNP profiles in cfDNA, one can distinguish between breast cancer patients and healthy individuals, and between patients who have breast cancer but are at different stages of treatment. Our results show that SNP array technology can detect subtle alterations in cfDNA with good inter-assay reproducibility ($p < 0.0001$, 0.0001, and 0.0005 for CQC, QC call-rate, and MAPD respectively; and an average Spearman's rank correlation of 0.727). Finally, eight amplified CNVs were identified in the 50 patients on follow-up, each with a prevalence of >90% in tumor DNA, suggesting their importance in breast cancer.

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5 Clonogenicity of Cultured Prostate Cancer Cells Is Controlled by Dormancy: Significance and Comparison with Cell Culture Models of Breast Cancer Cell Dormancy

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Contents

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Abstract

The metastatic disease determines the cancer-specific death of most patients suggesting that decreasing the rate of metastasis should translate into increased cancer-specific survival. Several experiments in mice suggest that a major limiting step of the metastatic process is the ability of single cancer cells to proliferate at distant sites. Most dispersed cancer cells disseminated in distant organs seem to remain dormant for long period before eventually dying or resuming cell proliferation to give rise to micrometastases. The study of these dispersed dormant cells is made difficult by their rarity and the difficulty to isolate them into a viable cell population. Our recently published work shows that a dormant state can be easily induced in prostate cancer cells ex vivo in cell culture. Indeed, if and only if cells are cultured at low clonal density, slightly hypertonic conditions will induce a dormant state leading to an almost 1,000-fold reduction in clonogenicity. Our data suggest that a full dormant state is a stable anergic state actively generated in dispersed cells in response to specific growth conditions, and which may require special growth stimuli to be reversed. Here we compare this model with two examples of breast cancer cell dormancy induced in vitro in order to highlight their convergences and discuss the link between dormancy, epithelial-mesenchymal transition and stemness. We suggest that despite some

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similarities with stem cell dormancy, dormancy of epithelial cancer cells may be related to a reversible differentiation-like process. We also discuss the applications of the culture model of prostate cancer cell dormancy with emphasis on the development of new tools to fight the metastatic disease at the clonogenic step.

Keywords
Prostate • Cancer • Clonogenicity • Cell culture • Osmotic pressure • Osmolality • Dormancy • p53 • smad7

Introduction

Clonogenicity is the ability of single cells to expand by division into new cell population. It is a property shared by both stem cells and cancer cells. In the latter case, clonogenicity is a key property for the metastatic process. Indeed, clinical and experimental data suggest that metastases result from the clonal cell expansion of single cancer cells which have been disseminated at distant sites from the primary tumor through hematogenous or lymphatic routes. Experiments with the D2-HAN breast cancer cell derivatives model (Morris et al. 1994; Rak et al. 1992) have convincingly demonstrated that a limited step of the metastatic process can be the clonal growth of single cancer cells disseminated in the parenchyma of colonized organs (Cameron et al. 2000; Morris et al. 1994; Naumov et al. 2002). Indeed, after injection of cancer cells suspension into the venous circulation, most cells derived from lines with low metastatic potential enter into a quiescent state for prolonged periods after their extravasation into the parenchyma of target organs. This is at variance with highly metastatic cells which start cell proliferation to form micrometastases at high frequency after their extravasations. These experiments suggest that entry into a long term but nonetheless reversible quiescence (i.e. a dormant state) could be the main factor limiting the clonogenicity of metastasized dispersed cancer cells. This view is strongly supported by several clinical observations (Vessella et al. 2007). Indeed, a metastatic relapse can occur several years (and up to decades) after the resection of the primary tumors as commonly observed for hematological malignancies and melanoma, prostate or breast cancers. In the case of prostate and breast cancers, bone-marrow disseminated tumor cells can be detected in a large fraction of patients several years after the resection of the primary tumor and are indicative for the probability of future metastatic recurrence (Braun et al. 2000; Janni et al. 2005; Wiedswang et al. 2004). However, the study of dormant cancer cells is a difficult task due to their paucity in vivo, which also makes their isolation as viable cell populations very difficult. The development of cell culture models of dormancy is therefore highly needed to facilitate the investigation of the mechanisms underlying the dormant state.

The *in vitro* cloning efficiency, the ability of cells seeded at low density to give rise to clonal populations, can be used as a surrogate for the capacity of dispersed tumor cells to expand into metastases. We have been investigating the factors which can modulate the cloning efficiency of prostate cancer cells. Unexpectedly, we found that slight changes in the osmotic pressure of the culture medium can dramatically affect cloning efficiency of a subline derived from LNCaP cells (Havard et al. 2011), a cellular model representative of the majority of human prostate cancers. Upon further investigation we found that small variations of the osmotic pressure was sufficient to induce a dormant state in the overwhelming majority of the cells provided that they are cultured at a sufficiently low cell density (Havard et al. 2011). In this chapter, we discuss the significance of the main results obtained through this model and compare the characteristics of this dormant state with those of two cell culture models of breast cancer cells dormancy to highlight potentially interesting issues. We conclude this chapter by suggesting applications of the cell culture model of prostate cancer cell dormancy.

Description of the Cell Culture Model of Prostate Cancer Cell Dormancy

Clonogenicity of Prostate Cancer Cells Is Modulated by Osmotic Pressure of the Culture Medium

Our initial observation was carried out with an LNCaP cell subline, LNCaP* which has been derived by antibiotic selection after transfection of a plasmid expressing the murine ecotropic retrovirus receptor. At variance with the initial LNCaP cells which were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS) and antibiotics (RPMI-FCS), LNCaP* cells were routinely cultured in Dulbecco-Modified Eagle's Medium supplemented with 10% FCS and antibiotics (DMEM-FCS). Both LNCaP* and the initial LNCaP cells are strongly dependent on their endogenous androgen receptor activity for their growth as shown by the strong inhibition obtained upon treatment with an androgen receptor antagonist such as bicalutamide (unpublished). Nevertheless LNCaP* cells cultured in DMEM-FCS have cloning efficiencies 100–1,000-fold lower than initial LNCaP cells cultured in RPMI-FCS. We showed that there was little difference between the intrinsic clonogenicity of LNCaP and LNCaP* cells, but that the choice of the growth medium fully accounts for the difference of cloning efficiency. Indeed, the cloning efficiency of the initial LNCaP cells is strongly reduced in DMEM-FCS whereas LNCaP* cells recover a high cloning efficiency (around 0.1) when cultured in RPMI-FCS. Further investigation allowed us to demonstrate that the slight difference of osmotic pressure between RPMI (310 mOsm/L) and DMEM (360 mOsm/L) is the key parameter determining cloning efficiency. Reducing osmotic pressure of DMEM-FCS with water restores a high cloning efficiency for LNCaP or LNCaP* cells in DMEM and conversely, increasing osmotic pressure of RPMI-FCS with sodium chloride or sorbitol decreases the cloning efficiency of LNCaP or LNCaP* cells in RPMI-FCS. Cloning efficiency was high in isotonic medium (305 mOsm/L), maximal in slightly hypotonic medium (twofold increase in 270 mOsm/L) and strongly reduced above 350 mOsm/L (more than a 100-fold decrease), showing that slight variations of osmotic pressure can drastically affect clonogenic properties. Such variations of osmotic pressure are much smaller than those usually used to induce an osmotic stress (with shift of 300 mOsm/L or greater) but only slightly exceed the known physiological range. For comparison, the threshold for thirst is around 10 (to 20) mOsm/L above normal plasma level. A dependence of clonogenicity on osmotic pressure could be observed in another prostate cancer cell line, Du145, albeit with smaller effects (see below for further discussion concerning this point). The dependence of clonogenicity upon osmotic pressure is cell type specific since the clonogenicity of NIH-3T3 fibroblasts is regulated in the opposite way: cloning efficiency is maximal in DMEM-FCS and decreased upon decreasing osmotic pressure of the growth medium.

High Osmotic Pressure Induces Reversible Cell Quiescence (Dormancy) Only at Low Cell Density

The decrease of the clonogenic potential of the LNCaP or LNCaP* cells in slightly hypertonic medium like DMEM-FCS was unexpected taking into account that LNCaP* cells had been maintained in DMEM-FCS since their isolation. In fact, when LNCaP* cells are plated at high cell density (about 2.4×10^4 cells/cm^2) they can be regularly passaged every 3–4 days and grow equally well in DMEM-FCS or DMEM-FCS medium supplemented with 25% water (270 mOsm/L). This suggests that a slight hypertonicity specifically affects the clonogenic potential without affecting the proliferation of LNCaP* cells. This prompted us to examine in more details the behavior of cells plated at low cell density in slightly hypertonic growth medium. Both microscopic examination and cell counting show that the number of living cells remains close to the

number of seeded cells during the first week after plating. Beyond 7 days, cell death eventually occurs. Analysis of cell cycle by flow cytometry show that after 7 days of culture in slightly hypertonic medium, there is a nearly complete disappearance of cells in S phase, most cells being blocked in the G1 phase and less than 15% in the G2 phase. By contrast, cells plated at low cell density in hypotonic medium show a cell cycle distribution more similar to that of cells plated at high cell density and in exponential growth. This clearly shows that cells plated at low cell density under slightly hypertonic conditions are growth-arrested.

An important issue is the reversibility of this quiescent state. We first observed that the length of exposure to hypertonic conditions determines the ability of cells plated at low cell density to resume proliferation when changed to hypotonic conditions. Thus, after 6 days of exposure, the cloning efficiency was reduced tenfold. We took advantage of our previous observation that citric acid and glutathione can strongly enhance the cloning efficiency of LNCaP* cells in DMEM-FCS and tested whether these compounds could help to reverse the quiescent state induced by prolonged exposure to hypertonic conditions at low cell density. Indeed, we found that citric acid plus glutathione could strongly cooperate with hypotonicity to reverse the quiescent state induced by a 6-days exposure to hypertonic conditions, demonstrating that this quiescent state is reversible and thus can be qualified as a dormant state.

Prostate Cancer Cell Dormancy as a Stable Cellular Anergy

As reported above, dormancy of prostate cancer cells can be efficiently induced in culture by plating cells at low cell density in the growth medium (DMEM-FCS) which is routinely used to passage them at high cell density. Since changing the osmotic pressure is enough to prevent this entry into dormancy, it is not due to a lack of growth factors but to a loss of response to them. This loss of response is achieved within the first 5 days of culture in DMEM-FCS at low cell density. We have further extended this observation by showing that prostate cancer cells infected with retroviral vectors expressing various growth factors (including basic or acidic Fibroblast Growth Factor, Interleukin-6, -8, -11, Leukemia Inhibitory Factor, Parathyroid Hormone-Related Protein, Platelet Derived Growth Factor, Stem Cell Factor, Tumor Necrosis Factor α and β, as well as Osteopontin, Plasminogen Activator-Inhibitor-1 and Vitronectin) cannot escape dormancy induced by slightly hypertonic conditions at low cell density. Similarly, acute treatment of the dormant cells with recombinant growth factors proteins such as the Epithelial Growth factor, basic Fibroblast Growth factor Interleukin-1α, -6, -11, Stem cell factor, Stromal-Derived Factor 1 or the Stem Cell factor cannot reverse the dormant state under hypertonic conditions (unpublished observations). This unresponsiveness, or anergy, of dormant cells to common growth factors is remarkable, as it seems unlikely that the slight change in osmotic pressure would inhibit the major signaling pathways involved in cell growth. Indeed, hyperosmolarity by itself is not sufficient to induce dormancy and is only active on cells at low density. More likely, at low cell density, small changes of osmolality could trigger a predetermined program of cell quiescence or dormancy which renders cells unresponsive to usual growth factors. This view is strongly supported by the observation that the induction and/or the maintenance of the dormant state depends on the activity of growth inhibitory intracellular signaling. Indeed, we have shown that disruption of two inhibitory signaling pathways, the p53 and the smad signaling pathways (by ectopic expression of dominant negative p53 and smad7, an inhibitory smad) significantly increases the clonal cell growth under hypertonic conditions while they have no effect under optimal hypotonic conditions.

Another salient feature of the dormant state of cultured prostate cancer cells is its stability. As reported in the previous section, once induced after a few days of culture at low cell density under hypertonic conditions, the dormant state cannot be efficiently reversed by placing the cells under hypotonic conditions. This suggests that the mechanisms stabilizing the dormant state are self-sustained. Interestingly, we observed that

overexpression of smad7 allows a very efficient rescue from dormancy when cells are changed to hypotonic conditions. Thus the smad signaling, which is triggered by growth factors of the TGFbeta family, appears to be critically involved in the maintenance of the anergic state. This suggests that under low cell density conditions, hypertonicity triggers a stable autocrine loop involving members of the TGFbeta family, which helps to maintain the dormant state even when cells are changed to permissive (hypotonic) conditions. In agreement, we observed that the treatment of cells with Activin A, a member of the TGFbeta family, significantly decreases clonal cell growth under hypotonic conditions. However, as Activin A is less efficient than hypertonicity to inhibit clonogenicity and as this inhibitory action does not seem to be self-sustained, it is likely that the efficiency of hypertonicity to induce dormancy involves the coordinated action of others cell signaling pathways (Havard et al. 2011). However, a puzzling issue comes from the observation that about 15% of the dormant LNCaP* cells are blocked in the G2 phase of the cell cycle -and about 85% in the G0/G1 phase of cell cycle (Havard et al. 2011). The apparent anergy and stability of the G2 growth-arrested state may result from an irreversible cell cycle arrest in the G2 phase. In this case, G2-growth arrested cells would not be true dormant cells because of this irreversibility. Alternatively, this may imply that the anergy and stability of the growth-arrested state in dormant prostate cancer cells is not dependent on the entry into a G0/G1 phase of the cell cycle (even if most dormant cells are found in the G0/G1 phase of the cell cycle). Solving this issue may help to understand of the mechanisms underlying cell dormancy.

Synthesis and Issues of the Model of Prostate Cancer Cell Dormancy

We have inferred that prostate cancer cell dormancy in cell culture reflects the induction of a predefined program which leads to a cellular anergy. This model implies that other stimuli could induce this dormant state. For instance, prostate cancer cells which have metastasized to a distant site could activate the same dormancy program to adapt to unfavorable growth conditions at the metastasis site due to extracellular matrix components, poor availability of proper growth factors or slight hypertonic conditions. It is worth noting that plating at low density *de facto* prevents homotypic cell interactions, a situation which might be similar to that of disseminated cells in ectopic tissues. As a predetermined program, dormancy could share common characteristics in various cell types. In our study, we evidence a role of the smad and p53 pathways. Very interestingly, we note that these two pathways have been previously implicated in the dormancy of stem cells, in particular from bone marrow and prostate (Liu et al. 2009; Salm et al. 2005; Yamazaki and Nakauchi 2009). We further observe that this stable anergic state can be disrupted by exposure to some metabolites such as citric acid and thiols. It may not be surprising *at posteriori* that factors able to stimulate clonal cell growth under hypertonic (dormancy-inducing) conditions may not be classical growth factors since after a few days under these conditions cells seem to become unresponsive (anergic) to growth stimulation by classical growth factors. The mechanisms by which these metabolites can disrupt the dormant state are still an issue.

If dormancy is an anergic state relying on the self-activation of growth inhibitory pathways, one can anticipate that it could overlap with the activity of tumor suppressor genes. As indicated above, in LNCaP cells, disruption of the p53 pathway significantly reduces the entry into dormancy. Moreover, we observed that Du145 cells which harbor a deletion of the RB and a mutation of the TP53 suppressor genes have a cloning efficiency about 100-fold higher than LNCaP cells (harboring wild type TP53 and RB genes) in hypertonic growth medium, but only a fivefold higher cloning efficiency in an optimal hypotonic growth medium (Havard et al. 2011). This suggests that difference in cloning efficiency between Du145 and LNCaP* cells in DMEM-FCS are mostly accounted by different efficiency of dormancy induction and that tumor suppressor genes may be involved in the process.

Comparison with Cell Culture Models of Breast Cancer Cell Dormancy

Similarities with Different Cell Culture Models of Breast Cancer Cell Dormancy

A first model of breast cancer cell dormancy in vitro relies on a 'clonogenic' assay in plastic culture plates (Barrios and Wieder 2009; Korah et al. 2004). In this model, treatment with basic Fibroblast Growth Factor (bFGF) was shown to be sufficient to induce the quiescence of more differentiated and poorly metastatic breast cancer cells such as MCF-7 or T-47D, but not of undifferentiated and highly metastatic MDA-MB-231 cells. As long term survival of the quiescent cells is enhanced by fibronectin, bFGF-induced quiescence in the presence of fibronectin was considered as an *in vitro* paradigm for breast cancer cell dormancy in bone marrow in view of the high expression of these factors at this site. Additionally, as this cell quiescence is obtained in normal growth medium with 10% fetal calf serum, it suggests that bFGF induces some insensitivity (anergy) to usual growth factors (a phenomenon which had been in fact previously noted in Fenig et al. 1997). The cell quiescence was suggested to be determined by an intrinsic genetic program which is independent of the inducing conditions (Barrios and Wieder 2009), as stated above for prostate cancer cells. Interestingly, it was proposed that TGFbeta is a mediator of this dormant state (Barrios and Wieder 2009) on the basis of previous experiments in MCF-7 (Fenig et al. 2001). However, this state of quiescence is not efficiently self-sustained as most dormant cells reinitiate proliferation upon removal of bFGF, at variance with what observed in our prostate cancer cell model of dormancy. This could result in part from the cell density used in this assay which might be above the threshold for a complete suppression of intercellular cooperation, allowing the dormant phenotype to be destabilized in a way similar to what we observe with prostate LNCaP cells (see above). An issue is to determine whether stability of the dormant state induced by bFGF is enhanced upon seeding cells at truly clonal cell density.

A complementary model is based on a 3-dimensional (3D) cell culture of breast cancer cells on a matrix basement membrane extract derived from Engelbreth-Holm-Swarm mouse sarcoma cells (Cultrex or Matrigel tradename) (Barkan et al. 2008). The authors observed a strong correlation between the ability of cell lines to proliferate on this substrate and the ability to escape dormancy when cells are injected into animals. As in the first model above, only highly metastatic cells like MDA-MB-231 or D2.A1 can actively proliferate on this substrate after a short latency period. MCF-7 or D2.OR cells, which are prone to enter into a dormant state in vivo, are strongly growth-inhibited. Moreover, it was shown that extracellular matrix components, including fibronectin and collagen I, could stimulate cell growth in correlation with actin cytoskeleton changes (Barkan et al. 2008, 2010). Along this line, promotion of a fibrotic environment was shown to promote cell escape from dormancy in vivo. However, stimulation of D2.OR cells, which overexpress the EGF receptor, with EGF could significantly enhance their growth in 3D culture (Wendt et al. 2011). Thus, it appears that at least part of the D2.OR cells cultured in 3D cell culture have not acquired a complete anergic state. Moreover, cells may not even be completely growth-arrested as some cell growth of D2.OR could be observed after 8 days in 3D culture (Wendt et al. 2011). In fact, in the initial setting of the 3D culture model of dormancy, cells were maintained in reduced (2% versus 10%) FCS concentration (Barkan et al. 2008), presumably to decrease basal growth rate of D2.OR cells. Nevertheless, the remarkable correlation between cell dormancy in vivo and cell growth rate in this 3D cell culture system is unlikely to be a coincidence but should reflect common basic mechanisms regulating both dormancy in vivo and cell growth under 3D conditions. Notably, it has been observed that the ability of D2.OR cells to proliferate in 3D cultures was strongly dependent upon the initial cell density (Shibue and Weinberg 2009). The absence of proliferation was only observed when D2.OR cells were seeded at a cell density low enough to preclude homotypic cellular interactions. Correlatively, the incomplete

growth arrest of D2.OR observed in 3D culture (Wendt et al. 2011) was obtained upon seeding at higher cell density. This dependence of dormancy induction/stability on cell density highlights a strong similarity with the culture model of prostate cancer cell dormancy. It suggests that dormancy is deeply linked to clonal cell growth conditions and that intercellular cooperation efficiently prevents or destabilizes the dormant state.

Inverse Correlation Between Dormancy and Epithelial-Mesenchymal Transition (EMT)

Interestingly, in both models of breast cancer cell dormancy, transition from the dormant to the proliferative state correlates with morphologic and actin cytoskeleton changes suggestive of an epithelial-mesenchymal transition (EMT) (Barkan et al. 2008, 2010; Barrios and Wieder 2009). Conversely, the induction of dormancy by bFGF is associated with morphologic and actin cytoskeleton modifications towards a more epithelial morphology (Barrios and Wieder 2009). Moreover, poorly metastatic cells such as MCF-7 and D2.OR, which are prone to enter into a dormant state, express epithelial markers such as E-cadherin while highly metastatic cells like MBA-MD-231 or D2.A1, which tend to escape entry into dormancy, constitutively express mesenchymal markers such as vimentin. Thus, in breast cancer cells, dormancy is associated with an epithelial phenotype and the escape from it with a more mesenchymal one. This issue was further investigated in a recent work based in part on the 3D cell culture model (Wendt et al. 2011). Expression of E-cadherin showed a strong association with growth inhibition in the 3D cell culture and dormancy upon injection into the animal. In addition, it was shown that ectopic expression of E-cadherin or Twist – a master gene regulating EMT – respectively induces and reverses the dormant state in 3D culture (Wendt et al. 2011).

These observations are at variance with our results on prostate cancer cells which indicate a role of the TGFbeta family in the induction of dormancy (Havard et al. 2011), since TGFbeta is a potent inducer of EMT in breast epithelial cells (see for instance Lindley and Briegel 2010 and references in Wendt et al. 2011) as well as in some prostate epithelial cells. Moreover, we found that acute treatment of prostate cancer LNCaP* cells with recombinant TGFbeta or stable transduction with a constitutively active HIF-alpha subunit (HIF transcription factor has also been described as an inducer of EMT in LNCaP (Luo et al. 2006)) does not promote escape from hypertonicity-induced dormancy (unpublished data). This divergence between breast and prostate cancer cells is further supported by their response to the PP1 and PP2 inhibitors which target both the src-family kinases and the TGFbeta receptor (Ungefroren et al. 2011). We found that PP2 at 5 μM does not impair at all clonal cell growth of LNCaP* prostate cancer cells under hypotonic conditions and may possibly promote their clonal cell growth under hypertonic conditions (unpublished observation). By contrast, PP1 at 10 μM inhibits the collagen-I-induced transition to a proliferative state in 3D culture of breast cancer cells (Barkan et al. 2010).

Several explanations may account for these differences between prostate and breast cancer cells. One possibility is that the mechanisms controlling dormancy are different in these two cell types, with EMT promoting dormancy escape in breast cancer cells whereas not affecting it or even displaying opposite effects in prostate cancer cells. However, there are more subtle possibilities relying on the complexity of the EMT phenomenon and on the variability of the response to EMT inducers in different cell lines. Indeed, in cancer cells, mesenchymal and epithelial states are not mutually exclusive, cells with an hybrid phenotype being frequently observed (Armstrong et al. 2011; Chao et al. 2011), and the description in terms of mesenchymal or epithelial phenotype may not be adequate. This is supported by the observation that TGFbeta has no growth-promoting effect on the breast D2.OR cell line in 3D culture, despite the induction of cytoskeleton changes indicative of EMT. In fact the lack of effect of TGFbeta was correlated with a lack of down-regulation of E-cadherin by this cytokine, suggesting that E-cadherin expression is more

relevant than EMT to predict dormancy of breast cancer cells (Wendt et al. 2011). Similarly, the possibility that induction of dormancy in MCF-7 by bFGF involves TGFbeta but no EMT (see above) may provide another example of the variability of cell response to EMT inducers. Thus, it would be interesting to analyze in prostate cancer cells the precise role of EMT/MET mediators such as Activin A or E-cadherin, FAK, Twist and integrin β1, which have been previously shown to be involved in the regulation of breast cancer cell dormancy (Barkan et al. 2008, 2010; Barrios and Wieder 2009; Shibue and Weinberg 2009; Wendt et al. 2011). This may help to identify molecular mechanisms directly regulating dormancy and to decipher whether these mechanisms could really differ in different cell types.

Is Dormancy of Cancer Epithelial Cells Linked to a Reversible Differentiation-Like Process?

Interestingly, in breast cancer cells, data obtained from the models reported above suggest that dormancy is a feature of the most differentiated and less metastatic breast cancer cells, but not of the highly metastatic cells harboring mesenchymal features which correlate with an undifferentiated and breast cancer stem cell phenotype (Mani et al. 2008; Morel et al. 2008). Thus, dormancy does not appear to be restricted to normal stem cells but rather seems to be also a property of partially differentiated cancer cells. In fact, taking into account the ability of cytokines of the TGFbeta family to induce prostate cancer cell dormancy and to promote prostate epithelial cell differentiation towards a luminal cell phenotype (Danielpour 1999; Salm et al. 2005), we propose that cell dormancy could rely in part on the process of cell differentiation. This would account for the opposite effects of TGFbeta on cell dormancy in some breast and prostate cancer cells due to its opposite action on cell differentiation in the corresponding cell lines. Furthermore, the possibility to induce cancer cell dormancy in 3D-Matrigel/Cultrex culture may be linked to the synergic ability of extracellular matrix components to induce and/or to maintain a differentiated phenotype as observed for normal hepatocytes (Bissell et al. 1987). This may also relate to the observation that Cultrex 3D-culture promotes formation of branched organoid structure with the D2.OR breast cancer cells which are prone to enter into a dormant state but not with the proliferating D2.A1 cells (Wendt et al. 2011).

Conversely, a high clonogenic potential would be linked to a less differentiated state and reversal of dormancy to a process of dedifferentiation. This would account for the correlation observed between EMT and dormancy escape in 3D culture of breast cancer cells since there is strong evidence linking EMT to acquisition of stem cell properties (Mani et al. 2008; Morel et al. 2008), suggesting that EMT may be linked to a dedifferentiation process at least in some epithelial cell types. This would fit a model assuming that after their extravasation, metastatic cells would acquire a more epithelial phenotype (Chao et al. 2011; Oltean et al. 2006; Wells et al. 2008) and then become quiescent, eventually resuming cell division after an EMT and acquisition of a stem cell phenotype. However, data from the cell culture models of dormancy suggest that loss of some features associated with a differentiated epithelial cell phenotype may be more relevant than acquisition of stem cell properties to account for the escape of dormancy and clonal cell proliferation. For instance, in the breast cancer cell models, it is expression of E-cadherin rather than EMT itself which is more predictive of the dormancy of breast cancer cells (Wendt et al. 2011). Additionally, unpublished data obtained from the clonogenic properties of LNCaP* cancer cells suggest there are no cancer cells related to normal epithelial prostate stem cells in this model. Indeed, upon seeding at low cell density under hypotonic conditions, cloning efficiency is around 0.1, suggesting that at least 10 % of the LNCaP* cells harbor clonogenic properties. However, in the presence of an androgen receptor antagonist added to the growth medium (such as bicalutamide), cloning efficiency is decreased more than tenfold due to inhibition of cell proliferation and nearly all grown clones display a reduced size

(unpublished observations). This shows that most if not all LNCaP* cells harboring clonogenic properties are dependent on a functional androgen receptor for their (clonal) cell growth, a feature characteristic of at least partially differentiated epithelial prostate cells but not of prostate epithelial basal stem cells or intermediate cells (Lee et al. 2012; van Leenders and Schalken 2003). In fact, the biological relevance of prostate cancer stem cell is still an issue since in most natural human prostate cancers, cancer cells are highly dependent on the expression and activity of their androgen receptor for their growth and/or survival and they continuously express the Prostate Specific Antigen (PSA) marker, characteristic of the differentiated luminal phenotype (van Leenders and Schalken 2003) even after progression to a castration-resistant phenotype (Balk 2002). Altogether, this strongly supports the view that LNCaP* cells and most natural human prostate cancer cells originate from partially differentiated or dedifferentiated luminal cells rather than directly from normal prostate epithelial basal stem cells (van Leenders and Schalken 2003) and that escape from dormancy may not necessarily involve formation of cancer cells with stem cell properties.

Applications: Fighting Metastasis by Targeting Clonogenicity

The metastatic disease is at the origin of most cancer-related death in humans but therapies devised to specifically target the clonogenic step of the metastatic process are lacking. This is all the more unfortunate that the initial clonogenic growth step of dispersed cancer cells may constitute the Achilles' heel of metastasis. At least three strategies could be devised for the development of new therapeutic approaches. One could focus on the characterization of drugs specifically cytotoxic against solitary cancer cells -see for instance (Gil-Bernabe et al. 2012; Najmi et al. 2005). Another solution, taking into account that dormancy of cancer cells has been reported to confer an increased resistance to chemotherapy (Braun et al. 2000; Najmi et al. 2005; Naumov et al. 2003), could be to reverse the dormant state of isolated cancer cells and then to apply some chemotherapy effective against cycling cells to eradicate them as previously proposed for hematopoietic malignancies (Essers and Trumpp 2010). Maintenance and/or induction of cancer cell dormancy could be another alternative to the killing of cancer cells for improving cancer-specific patients' survival. We will briefly describe salient features of the cell culture model of prostate cancer cell dormancy which may help for the development of these new strategies.

Set Up of Screenings for Identification of Drugs Targeting Dispersed Cancer Cells

Prostate cancer cells are notoriously resistant to chemotherapy and radiotherapy, and up to now most chemotherapies confer only a very modest increase in patient survival. Preliminary observations indeed confirmed that prostate cancer LNCaP* cells are highly resistant to drug-induced cell killing when grown under high cell density conditions (unpublished). However, when cells are seeded at low clonogenic conditions, several drugs used in human were found to be efficient in inhibiting cloning efficiency under permissive conditions (hypotonic growth medium; unpublished). Microscopic examination of the treated cells shows that most of the drugs do not induce cell death but rather promote cell quiescence. An interesting issue is whether some of these drugs induce a stable dormant state of dispersed cancer cells under hypotonic conditions similar to the hypertonicity-induced dormant state. Additionally, we observed that combinations of some of these active drugs could be effective in the killing of dispersed prostate cells cultured under hypotonic conditions (unpublished). In parallel, we are investigating the response of dormant prostate cancer cells (obtained after a few days culture of dispersed prostate cancer cells under hypertonic conditions) to these drugs either singly or in combination.

These preliminary data show that refined drug screenings targeting one of the most critical steps

of the metastatic process could provide information not accessible with more classical drug screening relying on high cell density cultures. The cell culture model of prostate cancer cell dormancy is well suited for drug screening for compounds targeting clonogenicity and/or dormancy due to its simplicity and remarkable high sensitivity. One obvious drawback however is that by nature it cannot be extensively miniaturized since the very low seeding density is at the core of its specificity. However, the simplicity of the culture conditions compare favorably with other techniques used to study cell dormancy.

Identification of Natural Factors Modulating Dormancy

During the course of our investigations, we have shown that the conditioned medium harvested from cultures of several prostate cancer cell lines contained low molecular weight compounds (molecular weight inferior to 1,000 Da) which promote LNCaP* cell growth under dormancy-inducing conditions (Havard et al. 2011). Screening of low molecular weight chemicals has led us to identify citric acid and several thiols as active compounds. However, mass spectroscopic analyses suggest that the concentration of citric acid in conditioned media is too low to account for their biological activity. The identity of the low molecular weight compounds secreted at high cell density is still a pending issue which could give important clues on the mechanisms involved in reversion of the dormant phenotype. In addition, we have shown that the conditioned medium harvested from U2-OS osteosarcoma cell culture contained high molecular weights compounds that also promote clonal cell growth of LNCaP* cells under hypertonic (dormancy-inducing) conditions. These results may be relevant to the strong prevalence of bone metastatic lesions in prostate cancer as local production by osteoblasts of factors preventing entry of prostate cancer cells into a dormant state may drastically enhance the frequency of metastatic outgrowth in bones. The factors produced by the osteoblastic U2-OS cells could constitute a new target for the development of antimetastatic therapies in the case of prostate cancer.

In conclusion, we have presented here a cell culture model for the dormancy of prostate cancer cells and discussed some of its features. Interestingly, this model shares important similarities with those of breast cancer cell dormancy but at the same time differs from them in some specific aspects such as the role of TGFbeta. The availability of cell culture models for dormancy not only opens the way to studying the mechanism of dormancy but also allows to perform screenings specifically designed to identify drugs active on dormant cells, an essential step to fight tumor recurrences. The simplicity, sensitivity and robustness of our model make it particularly amenable to these studies.

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Dormancy and Metastasis of Melanoma Cells to Lymph Nodes, Lung and Liver

6

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Abstract

Hematogenous and lymph node metastasis of melanoma cells is a major cause of death in the United States and Canada. Melanoma cells have a propensity for spreading to lymph nodes via the lymphatics. However, little is known about regional growth patterns of draining lymph node metastases arising from dermal melanomas. In a mouse model, by 10–14 days following intradermal injection, melanoma cells were replicating as discrete, evenly spaced lymph node metastases. When the injection site was excised at 4 days post intradermal injection, neither primary dermal tumors nor lymph node metastases were observed, indicating that metastasizing cells did not come directly from the initial injection and that the primary dermal tumor was required for lymph node metastases. While 23.1% of melanoma cells were proliferating in the lymph node, only 0.9% of these cells were undergoing apoptosis. We never observed metastasizing cancer cells replicating in blood or lymphatic vessels. When melanoma cells undergo hematogenous metastasis after portal vein injection, they are initially arrested in the liver by size constraints. However, they extravasate as an active process involving pseudopodial projections; during this process the vasculature remains intact. The metastasizing cells can then migrate to preferred sites for replication. In the lung, after intravenous injection, melanoma cells become arrested by size constraint at sites directly proportional to the available lung

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volume. By 10 days post injection, cancer cell replication preferentially occurs at the lung surface with 80% coverage. The vast majority of single, extravasated melanoma cells in the lung and liver are dormant. However, metastatic efficiency and dormancy of melanoma cells can vary widely with the melanoma cell line injected and/or the organ involved. The percentage of inoculated cells that remain as single, dormant cells at 2 weeks post inoculation is tenfold higher for B16F1 cells in liver compared with B16F10 cells in the lung. In contrast, metastatic efficiency of B16 F10 cells in lung is >500-fold higher than for B16F1 cells in liver.

Keywords

Melanoma cells • Lymph node metastasis • Hematogenous metastasis • Dormancy • Metastatic efficiency • In vivo videomicroscopy • Allogenic host metastasis

Introduction

While *in situ* cancers can generally be dealt with by conventional treatments such as surgery, chemotherapy, and/or radiation, cancers pose a much more difficult problem once they metastasize. The metastasis of cancer cells involves detachment from the primary tumor, entrance into the lymphatics or blood stream (hematogenous metastasis), spread to a secondary site, exit from the lymphatics into lymph nodes or from blood vessels (extravasation) into surrounding tissue in target organs, and growth at the secondary site. While the general outline of these processes has long been known, the elucidation of details of how this process occurs is far from complete. In particular, little is known of the pattern of distribution and fate of metastasizing melanoma cells in lymph nodes. These topics are discussed in this chapter.

A second area of investigation is the characteristic inefficiency of the metastatic process. It has long been known that if an animal is injected intravenously with tumor cells, not all of those cells go on to form tumors, even in a syngeneic host. It was assumed that most of the cells were destroyed by intravascular hemodynamic forces and that extravasation of the cancer cells was a rate limiting step. In fact, it has been shown that most of the cells survive passage through the circulation and that extravasation is not a rate limiting step; however, many of these cancer cells enter a state of dormancy after extravasation (see reviews MacDonald et al. 2002; Morris et al. 1997). This dormant state of cancer cells has become a major source of investigation because it can potentially explain why cancers can recur in patients who were thought to be cured. Dormancy provides yet another challenge in cancer treatments, in that conventional methods are generally ineffective against these cells (Naumov et al. 2003). Because the cancer cells remain as single cells, they can not be effectively detected or removed by surgery. Also because they are not actively replicating, they are generally unaffected by chemotherapy or radiation therapy. Melanoma cells potentially add another dimension to dormancy, in that they can survive in an allogeneic host through localized immunosuppression (Duff et al. 2003; Lee et al. 2005; Polak et al. 2007). Thus factors affecting metastatic efficiency and dormancy are also discussed in this chapter.

Lymphatic Metastasis of Melanoma Cells

Introduction – Lymph Node Metastasis

Malignant melanoma is the seventh most common cancer in the United States and Canada (Diepgen and Mahler 2002). While malignant melanomas can spread to lymph nodes by both blood and lymphatic vessels, they have a propensity for early spread by the lymphatics, which act as a conduit for invasion of lymph nodes by the melanoma cells (Dadras et al. 2003). In fact, increased lymphangiogenesis in the immediate vicinity of a primary melanoma tumor is an indicator for an increased risk of lymph node metastasis (Dadras et al. 2003).

It has been shown in a syngeneic mouse model that 80–90% of the draining lymph nodes develop melanoma metastases by 10–14 days after intradermal (ID) injection of melanoma tumor cells (Trites et al. 2000). However, in this study by Trites and coworkers, the pattern of melanoma growth within the lymph node was not described in detail nor was the percentage of metastatic melanoma cells that were actively proliferating or undergoing apoptosis reported (Trites et al. 2000); these topics are explored in this chapter.

The structure of the lymph node has been well described by Willard-Mack (2006). Lymph nodes characteristically contain lobules surrounded by a capsule. Lymph, which can carry antigenic molecules, particles and cells from nearby tissues, enters via afferent lymphatic vessels on one side of the node, flows through sinuses located between the lobules and beneath the surface of the capsule, and leaves by an efferent lymphatic vessel, which leads to downstream lymph nodes and eventually to the venous system. At the hilus where the efferent vessel leaves the node, arterioles and venules enter and branch within the lobules. The lobules are conical in shape with wider cortices on the afferent side of the node and narrow medullae converging at the hilus. Within the entire lymph node is a framework of ligand coated fibroblastic reticular cells forming a complex mesh of fibers that compartmentalize the lymph node and interact with the lymph borne cells. In the superficial cortex adjacent to the capsule on the afferent side is a layer of evenly distributed spherical follicles. B lymphocytes home to the follicles where they interact with antigen presenting cells and, when stimulated, proliferate to form distinctive germinal centers. In the deeper paracortex is a single deep cortical unit where T lymphocytes home and proliferate. Although melanoma cells from a primary tumor are transported in the afferent lymphatic vessels in the same manner as lymphocytes, macrophages and dendritic cells from inflamed tissues, it has not been shown if they home to or preferentially proliferate within specific regions of the lymph node.

PCNA is an auxiliary protein of DNA polymerase delta, a vital enzyme for DNA replication (Foley et al. 1991); PCNA staining allows for detection of cells that are in G1, S, G2 and M phases of the cell cycle (Connolly and Bogdanffy 1993). Commercially available antibodies can be used to detect intracellular PCNA. In addition, apoptosis in cells can be detected using a TUNEL assay, which detects DNA breaks. A newly developed procedure used in our study allows the simultaneous detection of proliferation by PCNA staining and apoptosis by TUNEL on the same section of tissue.

In this study we have characterized the pattern of growth of melanoma cells that spontaneously metastasized from dermal tumors to draining inguinal lymph nodes as well as their spread from inguinal to axillary lymph nodes in both syngeneic and allogeneic hosts. We have also simultaneously quantified the proliferation and apoptotic death of melanoma cells that had spontaneously metastasized from a dermal tumor.

Techniques Used in This Study

Spontaneous Metastasis

B16F10 cells (American Type Culture Collection, Manassas, VA) have been previously modified to stably express β-galactosidase, (B16F10-LacZ) to provide positive histological identification (Kirstein et al. 2009). Using the same procedure, these cells were also stably transformed with tdTomato cDNA (B16F10-LacZ-tdTomato) for in vivo fluorescence identification. B16F10-LacZ cells were transfected with a pcDNA3.1 mammalian expression vector containing a cDNA for tdTomato for easy localization of tumor cells via fluorescence imaging. These cells have been tested in vivo and had a similar ability to form primary tumors and metastases as the parental cell lines. C57BL/6 female mice, aged 6–8 weeks, underwent intradermal (ID) injection approximately half way between the abdominal midline and the inguinal lymph node with 10^5 B16F10-LacZ or B16F10-LacZ-tdTomato mouse melanoma

cells in a volume of 100 μL α-MEM (minimal Eagle's medium); the cancer cells formed ID tumors that metastasized into the ipsilateral inguinal and axillary lymph nodes via the draining lymphatics as was previously described (Trites et al. 2000). For controls, some mice were either injected with media only or were injected with lysed cells, or had the site of injection of B16F10 cells excised at day 4 after injection, before a primary tumor had formed. Mice were cared for in accordance with the Canadian Council on Animal Care, under a protocol approved by the University of Western Ontario Council on Animal Care.

In Vivo Videomicroscopy

This procedure has been previously published (Trites et al. 2000). Briefly, the mice were positioned in a prone position on the stage of an epifluorescence inverted microscope (Olympus IX70). The lymph nodes were exposed, positioned on a cover slip window and imaged using oblique transillumination from a fiber-optic light source to view unstained tissues. In addition, episcopic fluorescence illumination was used to visualize cellular (tdTomato) fluorescence. Images were recorded in real time using a Basler A602F CMOS camera for digital video capture. Representative single images from the video files were used for some of the analysis. Representative images were also taken from whole mounts of freshly excised or stained tissues using similar procedures, as well as from conventional histological sections. Twenty C56BL/6 mice were injected with syngeneic B16F10-LacZ or B16F10-LacZ-tdTomato melanoma cells and were used to determine the timing and pattern of lymph node metastases; six mice were used at 4–7 days post injection (p.i.), four mice were employed at 8–10 days p.i. and 10 mice were used at 11–28 days p.i. Six additional C57BL/6 mice had their site of ID injection with B16F10-LacZ cells removed at 4 days p.i. to determine the role of the primary tumor in lymph node metastases. A total of seven allogeneic BALB/c mice were injected ID with B16F10-LacZ melanoma cells to examine the ID tumors and lymph node metastases in this host. Seven C57BL/6 mice also were injected with B16F10-LacZ cells to investigate the proliferation and apoptosis of melanoma cells that had metastasized to lymph nodes.

Tissue Preparation and X-Gal Staining

C57BL/6 mice were euthanized at selected times (6–28 days) post-injection. B16F10-LacZ or B16F10-LacZ-tdTomato primary tumors and draining ipsilateral inguinal and axillary lymph nodes containing metastases were removed as previously described (Trites et al. 2000). Excised tissues were fixed in 10% formaldehyde and embedded in paraffin wax. Serial sections (4 μm) were cut from each tissue and mounted on glass slides. For some experiments the tissues were fixed in a gluteraldehyde solution (0.2% gluteraldehyde, 5.0 mM EGTA pH 7.3, 2 mM $MgCl_2$ in 100 mM sodium phosphate buffer pH 7.3), and an X-gal staining protocol was employed following published procedures (Hedley et al. 2008).

Dual PCNA and TUNEL Immunofluorescent Staining Protocol

Tissue sections were de-paraffinized using xylene and a graded series of ethanol and distilled water by standard histological procedures. Antigen retrieval was performed by heating slides in 10 mM sodium citrate, pH 6 at 95°C for 5 min and then allowing them to cool for 20 min to room temperature. The slides were then subjected to a membrane permeabilization step, in which they were immersed in fresh phosphate-buffered saline (PBS) with 0.01% Tween-20 detergent (twice for 2 min each) and then equilibrated in PBS (twice for 3 min each). Tissues were then incubated in 20 μg/ml proteinase-K (Millipore, Billerica, MA) for 15 min. The sections were then blocked with an equilibration buffer from the TUNEL staining kit (Millipore) before being incubated with a 1:4 solution of terminal-deoxynucleotide-transferase enzyme from the TUNEL staining kit for 1 h at room temperature. Tissues were then blocked with a 2% solution of goat serum albumin (GSA), followed by an overnight incubation of 1:100 rabbit-anti-goat PCNA (Abcam, Cambridge, MA) in 2% GSA at 4°C. Tissues for isotype control were incubated with 1:100 non-specific rabbit primary antibody. The secondary antibody was prepared by centrifuging

a solution of 1:4 anti-digoxigenin (Millipore) and 1:100 anti-goat Alexa 594 (Invitrogen, Carlsbad, CA) in 2% GSA at 14,000 × g for 14 min. Tissues were then blocked with 2% GSA and incubated with the secondary antibody solution for 1 h at room temperature. Sections were then counterstained with 4′, 6-diamidino-2phenylindole (DAPI) to visualize nuclei and mounted in Vectashield (Vector Laboratories, Burlingame, CA) fluorescent mounting medium. For each immunostained section a corresponding serial section was stained with haematoxylin and eosin (H&E). Mouse esophageal tissue was used as a positive control for proliferation, and involuting rat mammary fat pad was used for a TUNEL positive control for staining.

Quantitative Analysis

From tissues collected at the various stages of the metastatic process, digital images of the lymph nodes were manually segmented to identify regions of tumor tissue. The number of tumor cell clusters and the total area fraction of tumor tissue were calculated using ImageJ computer software (NIH, Bethesda, MD). Raw data or averages and standard deviations are reported.

Description of Lymph Node Metastasis

Pattern of Spontaneous Metastasis to Lymph Nodes

To determine the pattern of growth of melanoma cells that had spontaneously metastasized from dermal tumors, we injected syngeneic C57BL/6 mice intradermally with B16F10-LacZ or B16F10-LacZ-tdTomato melanoma cells. Dermal tumors began forming by 6–7 days at the site of ID injection of B16F10-LacZ melanoma cells (Fig. 6.1a). At that time individual melanotic cells were observed in the ipsilateral inguinal node (Fig. 6.1b). By 8–10 days post-ID injection, the ID tumors had grown to approximately 5–15 mm, and melanotic cells were present in the ipsilateral inguinal lymph nodes in small clusters (Fig. 6.1c, d). Using in vivo videomicroscopy, optical sectioning of whole tissues revealed tumor cells at depths between 30 and 115 μm from the surface of the node, corresponding to the superficial cortex layer. Melanin containing cells were not observed in the lymphatic vessels leading to the lymph nodes but were seen within the draining lymph nodes where the vessels entered (Fig. 6.1e). By 10–28 days p.i., the B16F10-LacZ melanoma cells were apparent as discrete, evenly spaced clusters of blue-green cells staining positive for β-galactosidase in ipsilateral inguinal nodes (Fig. 6.2a, b). The ipsilateral inguinal micrometastases were also visualized as fluorescent images of B16F10-LacZ-tdTomato melanoma cells (Fig. 6.2c). At day 10, 11% of the surface of the lymph node was covered by melanoma cells. By days 16 and 23 p.i., this percentage had increased to 22–17%, respectively, in nodes from separate mice. Similar patterns of B16F10 metastases were also seen in ipsilateral axillary lymph nodes (Figs. 6.2d, e, f). By days 14–28 p.i. the ipsilateral axillary tumor coverage ranged from 9 to 28% in nodes from three separate mice. Thus the melanomas spread to cover a large portion of the surface of both the ipsilateral inguinal and axillary lymph nodes. As a control, four α-MEM media ID injected mice showed no similar patterns of staining (Fig. 6.2g). To ensure that the staining was not due to phagocytic cells picking up melanin debris, two mice were injected ID with 10^5 lysed B16F10-LacZ melanoma cells; 14 days p.i., no tumor had formed at the site of injection and no pattern of staining was observed in the draining lymph nodes (Fig. 6.2h).

Role of Primary Tumor in Metastasis

To determine the role of the primary dermal tumor in the metastasis to the draining lymph nodes and to determine if melanoma cells directly moved into draining lymph nodes after ID injection rather than being released by the primary tumor, we removed the site of injection 4 days after injection of the melanoma cells. For these mice, no primary tumor was observed after injection (up to 19 days). No melanoma cells were observed in the draining lymph nodes at 19 days post injection in mice with the site of injection removed; metastasis to

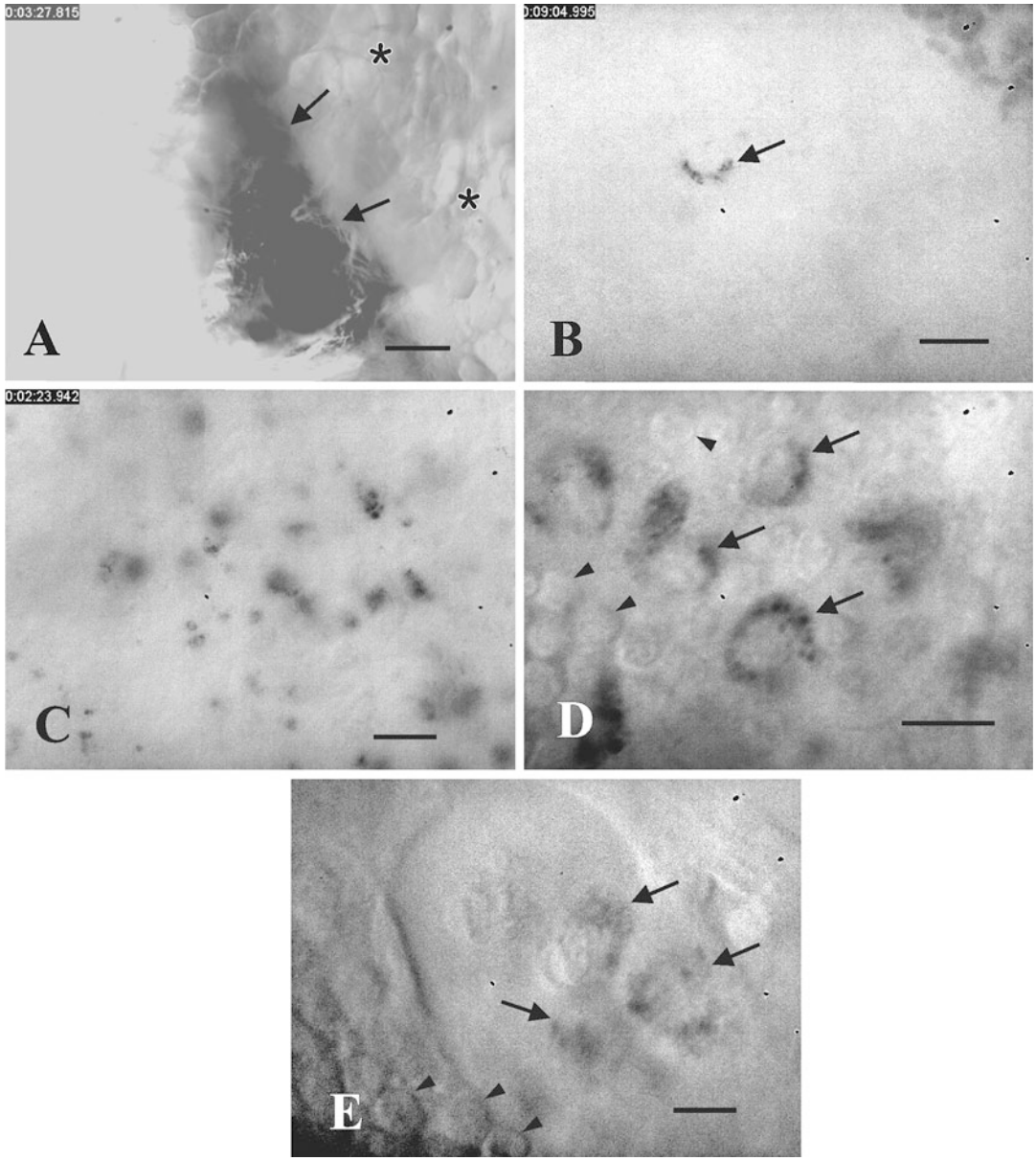

Fig. 6.1 Primary melanoma tumor and metastases in C57BL/6 mice imaged by in vivo videomicroscopy. *Panel A* Primary dermal tumor 6 days after ID injection with 10^5 B16F10-LacZ cells per mouse. * = normal dermal tissue, *arrows* = dark melanotic tumor. Bar = 1 mm. *Panel B* Melanotic cell (*arrow*) present in ipsilateral inguinal lymph node 7 days after ID injection. *Panel C* Melanotic projections (*black spots*) showing at surface of ipsilateral inguinal lymph node 8 days after ID injection. *Panel D* Melanotic cells (*arrows*) present within ipsilateral inguinal lymph node 10 days after ID injection. Optical section also shows lymphocytes (*arrow heads*) in subcapsular region. *Panel E* Melanotic cells (*arrows*) 10 days after ID injection adhering in ipsilateral inguinal lymph node where lymphatic vessel enters. Lymphocytes are also indicated (*arrow head*). The *bars* for Panels *B*–*E* = 20 μm

draining lymph nodes was observed in mice whose site of injection was not resected, consistent with our previous observations (data not shown). Histological examination of draining lymph nodes from mice whose site of injection was not resected also revealed clusters of B16F10-LacZ cells as evidenced by blue X-gal staining, consistent with what was observed in the whole

mounts; however, no such staining was observed in sections of lymph nodes from resected mice or uninjected mice (data not shown).

Patterns of Spontaneous Metastasis to Lymph Nodes in an Allogeneic Host

It is known that melanoma cells can suppress the immune response of its host (Duff et al. 2003; Lee et al. 2005; Polak et al. 2007). We thus investigated whether B16F10-LacZ melanoma cells injected ID into allogeneic BALB/c mice would form a primary dermal tumor, metastasize to draining lymph nodes, and replicate in a pattern similar to that observed in a syngeneic C57BL/6 mouse. By 14 days 7/7 ID injected allogeneic mice formed primary dermal tumors. In addition, ipsilateral inguinal nodes showed many black foci of melanotic cells (data not shown). For example, when one node was stained with X-gal at 14 days post ID injection, many large clusters of the blue-green staining cells were observed that covered 9% of the ipsilateral inguinal lymph node, which is similar to the 11% coverage observed at 10 days with syngeneic C57BL/6 mice (data not shown, Fig. 6.2a). In addition, the melanoma cells had also metastasized to the ipsilateral axillary nodes where they covered 11% of the surface area. They again formed discrete foci of metastases as observed with syngeneic C57BL/6 mice.

Proliferation and Apoptosis of Metastatic Melanoma Cells

Seven C57BL/6 mice received ID injections of B16F10-LacZ melanoma cells. Seventeen days later all of the injected mice had developed primary dermal tumors at the site of injection as previously observed (Fig. 6.1). The ipsilateral inguinal lymph nodes of all the mice contained melanoma cells, consistent with our previous finding (Fig. 6.2). Two of these nodes contained small tumors of approximately 1–2 mm in diameter. Both tumors spread out along the subcapsular sinus, growing inward into the stroma of the lymph node. Tissues were stained for TUNEL, PCNA, and H & E for the primary tumor (Fig. 6.3a–c) and for lymph node metastases (Fig. 6.3d–f). A total of 1,655 melanoma cell nuclei from these nodes were assessed; a total of 383 (23.1%) nuclei stained positive for PCNA whereas only 15 (0.9%) nuclei were TUNEL positive (Fig. 6.4). The positive nuclei were not evenly distributed over the lymph node (Fig. 6.3). The primary dermal tumors had an average of 31.6% PCNA positive nuclei and only 2.3% TUNEL positive nuclei (Fig. 6.4). Thus for both the primary tumors and the lymph node metastases at 17 days post ID injection of melanoma cells, the number of proliferating cells greatly exceeded the number of cells undergoing apoptosis by an average of over tenfold (Fig. 6.4).

Discussion – Lymph Node Metastasis

We have characterized the timing and pattern of replication of melanoma cells that have metastasized from a dermal tumor into draining lymph nodes of syngeneic C57BL/6 mice. Primary dermal tumors were present by 6–7 days post ID injection. At this time individual melanin containing cells were observed in ipsilateral inguinal lymph nodes. With the growth of the dermal tumors, small clusters of melanin containing cells became apparent in the ipsilateral inguinal node, suggesting that the individual cells had begun to replicate. By 14 days many melanoma cells had replicated into large clusters separated by areas with no apparent cancer cells. It is known that the nodal stroma, which is mainly comprised of fibroblastic reticular cells, forms a three dimensional framework and compartments that can serve as a conduit system for lymphocytes and soluble molecules such as chemokines (Crivellato et al. 2004). It is also known that lymph node stroma can promote the growth of cancer cells through the release of growth factors (Lebedis et al. 2002). Thus the pattern of melanoma cell replication in the lymph nodes could result from the migration of melanoma cells into the stromal compartments where they could be induced to replicate. The melanoma cells enter the node via afferent lymphatics in the superficial cortex layer in the vicinity of the follicles. They appear to arrest and replicate in discrete compartments at this site. Not only do the melanoma cells in the ipsilateral inguinal node begin to replicate, but some melanoma cells make their way to the efferent lymphatic and

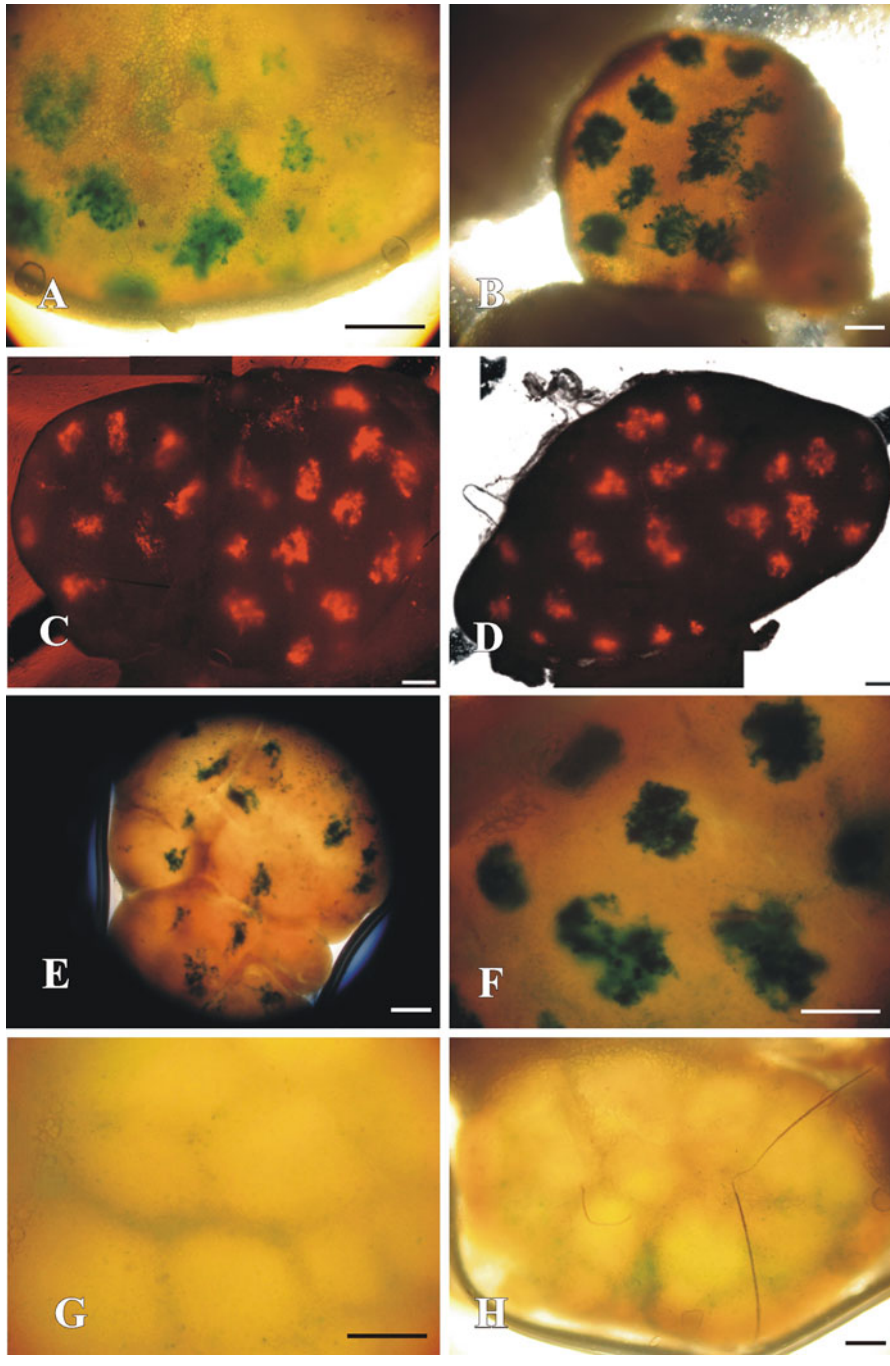

Fig. 6.2 Metastasis of B16F10-LacZ and B16F10-LacZ-tdTomato cells into lymph nodes visualized by LacZ staining and fluorescence in whole mounts. B16F10-LacZ cells in draining ipsilateral inguinal lymph node at 10 days (*Panel A*) and 16 days (*Panel B*) after ID injection of 10^5 cells into C57BL/6 mice. B16F10-LacZ-tdTomato cells in ipsilateral inguinal (*Panel C*) or ipsilateral axillary (*Panel D*) lymph nodes at 23 days post ID injection. B16F10-LacZ cells in draining ipsilateral axillary lymph nodes at 28 days (*Panel E*) and 14 days (*Panel F*) after ID injection of 10^5 cells. *Panel G* Lac-Z stained normal inguinal lymph node from an α-MEM media ID injected C57BL/6 mouse. *Panel H* Lac-Z stained ipsilateral inguinal lymph node 14 days after injection ID with 10^5 lysed B16F10-LacZ cells. Scale bars = 100 μm

spread to the draining ipsilateral axillary node where the pattern of replication is similar. While the melanoma cells can become arrested and replicate in the nodes, they were never seen to attach or replicate in the lymphatic vessels leading to the nodes. B16F10 melanoma cells are known to express integrin $\alpha_v\beta_3$ on their surface, and lymph nodes contain the $\alpha_v\beta_3$ ligand vitronectin (Kim and Lim 2007; Nip et al. 1992). Melanoma cells adhere in vitro to frozen sections of lymph nodes via integrin $\alpha_v\beta_3$ (Nip et al. 1992). Furthermore, a melanoma cell line that was isolated from lymph nodes has a greater expression of integrin $\alpha_v\beta_3$ than the parental cell line maintained in culture (Nip et al. 1992). In addition, B16F10 cells express integrin $\alpha_5\beta_1$ on their surface (Ratheesh et al. 2007), and lymph nodes contain fibronectin, which is a ligand for integrin $\alpha_5\beta_1$ (Reilly et al. 1985). Thus the melanoma cells may bind to the lymph nodes via specific integrin-ligand bonds.

In previous studies, to rule out the possibility that melanin containing cells were phagocytes that had engulfed melanin, we used histological and immunohistochemical (S-100) stains, which revealed that the melanin containing cells were in fact melanoma cells (Trites et al. 2000). In this study we have provided additional evidence. When 10^5 melanin containing B16F10 melanoma cells were lysed before ID injection, no melanin containing cells were subsequently observed in the lymph nodes. Also the B16F10 cells were marked with both LacZ and tdTomato fluorescent markers to specifically identify these cells. Expression of these markers will only be maintained in the viable cancer cell, thus assuring the specificity of our results.

We next observed that the presence of the primary tumor was required for the metastasis of melanoma cells to the lymph nodes. Thus when the site of injection was excised 4 days after injection, no melanoma cells were observed in the lymph nodes. An intact primary tumor could be continuously supplying cancer cells to the draining lymph nodes. Another possibility is that the primary tumor secretes factors that aid in the establishment of metastases. Immunohistochemical analysis has indicated that matrix metalloproteinase-2 and transforming growth factor-β have been detected at high levels in human melanomas and in matched plasma samples (Malaponte et al. 2010). These plasma levels were at significantly higher concentrations than in normal controls. Furthermore, the levels were elevated in patients with metastatic melanoma. In addition, it has been shown that vascular endothelial growth factor-C expression in primary dermal melanoma is correlated with the localization of melanoma metastases to the lymph nodes (Schietroma et al. 2003). Thus, in addition to supplying cancer cells for metastasis, the expression of the primary tumor can potentially play a role in directing and supporting the growth of cancer cells.

One of the reasons melanomas are such deadly tumors is their ability to suppress the immune system. We have shown that melanomas can not only replicate in an allogeneic host but also metastasize to ipsilateral inguinal and ipsilateral axillary nodes forming a pattern of growth similar to what was observed with the syngeneic host. Two molecules that are known to be immunosuppressive are indoleamine 2,3-dioxygenase (IDO) and cyclooxygenase-2 (COX-2). IDO is a catabolic enzyme that degrades tryptophan to kynurenine (Prendergast 2008). Resulting tryptophan starvation of T cells prevents them from dividing, and thus they are not activated by the presence of tumor antigens (Munn et al. 2005). In addition, T cell suppression is induced by kynurenine and other downstream catabolites generated by the IDO pathway (Fallarino et al. 2002). Increased expression of IDO in melanoma metastases is associated with a high density of T_{reg} cells and shorter patient survival (Brody et al. 2009). Expression of IDO can also be increased in tumor-draining lymph nodes (Munn et al. 2004). In addition, COX-2 is a rate limiting enzyme in the synthesis of prostaglandins (PGs) and mainly accounts for increased PG synthesis during inflammation (Kurumbail et al. 1996; Smith and Dewitt 1996). Melanoma conditioned media treatment of macrophages in vitro increases their synthesis of COX-2 (Botella-Estrada et al. 2005). Inhibition of COX-2 reverses melanoma induced suppression of macrophage function; also, COX-2

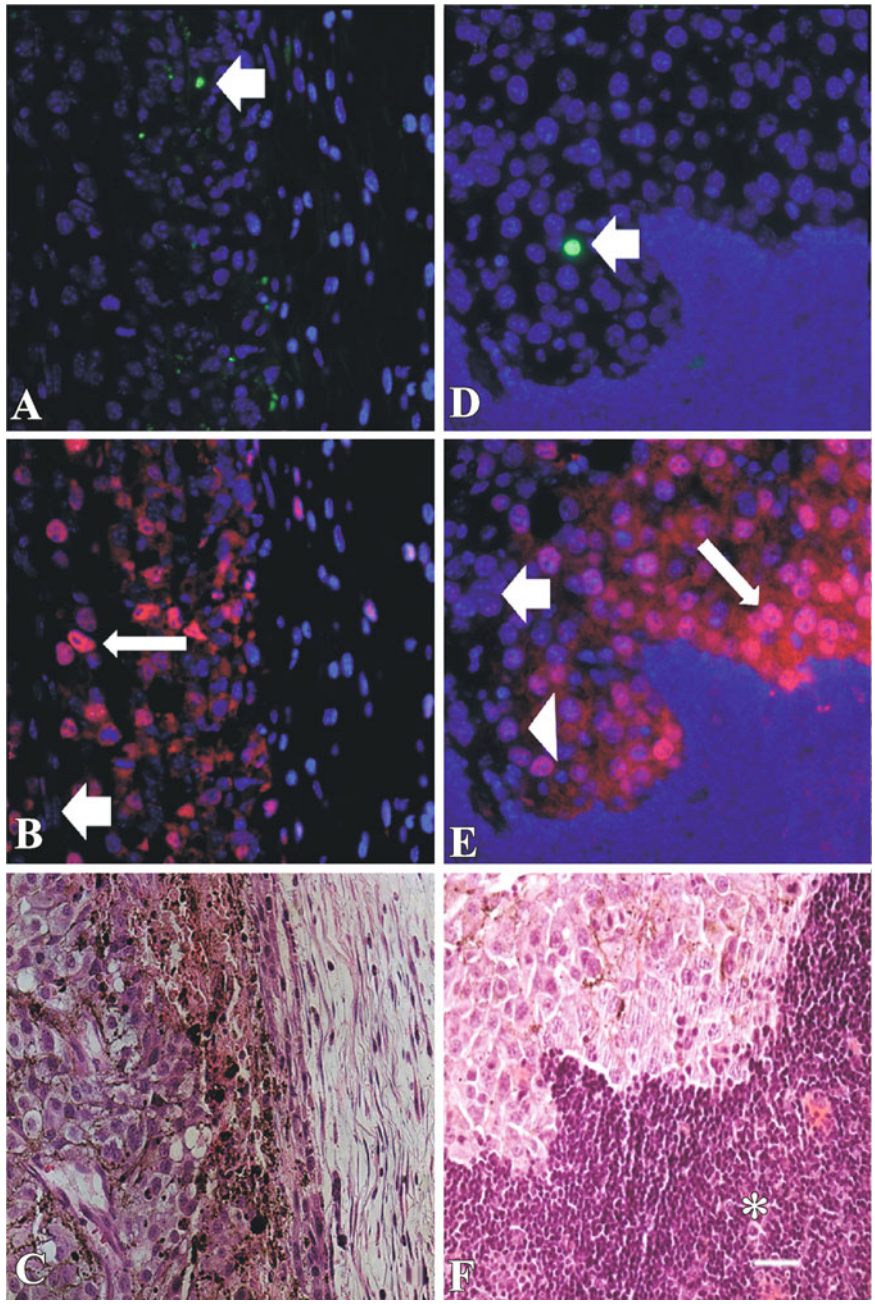

Fig. 6.3 Proliferation and apoptosis of melanoma cells in a primary tumor (*Panels A–C*) and in a metastasis to an inguinal lymph node (*Panels D–F*). *Green* (FITC) TUNEL stain (apoptosis) is shown in *Panels A* and *D*. *Red* (RITC) PCNA stain (proliferation) is shown in *Panels B* and *E*. *Blue* (DAPI) nuclear stain is shown in *Panels A*, *B*, *D* and *E*. *Panels C* and *F* are stained by H&E. Normal lymphatic tissue is indicated in the *lower right* of *Panel F* by an *. The *large arrows* in *Panels A* and *D*, identify nuclei that stained positive for TUNEL. In *Panels B* and *E thin arrows* are examples of nuclei that stain for PCNA (counted as positive), and the *arrowhead* shows a cell that weakly stains for PCNA (also counted as positive). *Large arrows* show nuclei staining for DAPI only. Scale bars = 24 μm

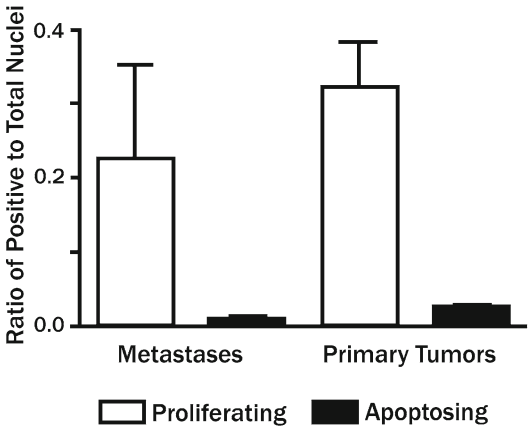

Fig. 6.4 Ratios of B16F10-LacZ cells undergoing proliferation (PCNA stain positive) or apoptosis (TUNEL stain). Primary dermal tumors and metastases to draining ipsilateral inguinal lymph nodes were removed at 17 days post ID injection of B16F10-LacZ cells into C57BL/6 mice and stained for proliferation (PCNA) and apoptosis (TUNEL). The ratio of positive to total nuclei was plotted for both lymph node metastases and primary skin tumors. For the lymph node metastases, 1,655 nuclei from two mice were examined; for the primary skin tumors 9,142 nuclei from seven mice were examined. Bars = SD

inhibition enhances the effect of interferon-γ on the reduction of melanoma growth and survival of melanoma bearing mice (Botella-Estrada et al. 2005; Duff et al. 2003). In addition, significantly higher COX-2 mRNA synthesis was found in melanoma sentinel lymph nodes compared with nonsentinel nodes (Botella-Estrada et al. 2005). Thus IDO and/or COX-2 may be involved in allowing melanoma cells to survive and replicate in an allogeneic host.

We have observed that the B16F10 melanoma cells replicate in the lymph nodes to form large clusters of cells following their spontaneous release from a primary tumor. However, it was not known if the cancer cells simply replicated or if their increase in numbers was the net result of both replication and apoptosis. Using a newly developed technique in which replication and apoptosis could be quantified on the same histological section, we determined that the average rate of replication in lymph nodes was approximately 15-fold higher than for apoptosis. Furthermore, the rate of apoptosis in lymph nodes of approximately 0.9% is similar to that normally observed when growing cells in tissue culture. Thus the B16F10 cells replicate in lymph nodes with little or no increase in apoptosis above what is normally observed in replicating cells.

We have thus determined that melanoma cells form discrete, compartmentalized clusters when they metastasize to lymph nodes. Once present in the inguinal node, melanoma cells have the ability to further metastasize to the draining axillary node. The primary tumor plays an essential role in establishing metastases in lymph nodes, and once the metastasis is established in the lymph node, approximately 20% of the cells are actively replicating at any given time with only minimal levels of apoptosis. It is hoped that these observations increase our ability to prevent nodal metastasis by increasing our knowledge of this process. As an example, our results indicate that melanoma cells in lymph nodes undergo little or no apoptosis. PRIMA-1 met (APR-246) could potentially reverse this finding since it can reactivate p53 in melanoma cells and thus make them susceptible to apoptosis (Wiman 2010). We have observed that in vitro treatment of melanoma cells with PRIMA-1 met while the cells are undergoing a prolonged time in culture without feeding has resulted in 93% cell death as opposed to 4.8% cell death for untreated cells (unpublished results authors). Thus increasing the susceptibility of melanoma cells to apoptosis could provide an attractive therapeutic target.

Hematogenous Metastasis of Melanoma Cells

Cancer cells that intravasate into the blood stream will most often first encounter the liver or the lungs. This section of the chapter will thus also focus on experimental hematogenous metastasis to these two organs.

Liver Metastasis

Initial Arrest

The hepatic portal vein drains blood from the gastrointestinal tract and spleen and together with

hepatic arteries carries the blood to sinusoids, which form the capillary bed of the liver. Sinusoids are small blood vessels with a fenestrated epithelium that forms multiple, highly branched vessels that are bounded by hepatocytes. After flowing through the sinusoid bed, the blood exits the liver via a hepatic vein. The cancer cells are generally 15–18 μm in diameter and are trapped in the sinusoids by size constraint (see review, Morris et al. 1997). Initially the cells completely block flow in the sinusoid involved; however, because of the highly branched nature of the sinusoids, blood continues to flow through the liver and the pressures do not build up behind the arrested cell. The maximal length to width ratio of 5.6 for B16F10 mouse melanoma cells in liver is much less than the 28 observed for these cells in muscle where perfusion pressure is much higher. Perhaps that is one reason one does not often observe cancer cells metastasizing to muscle. Thus over 97% of cancer cells maintained membrane integrity for at least the first 2 h of their arrest in the liver. These arrested cells generally remain clustered close to the terminal portal vein in acinar zone 1.

Extravasation, Migration, and Growth

It was initially thought that cancer cells extravasated by replicating intravascularly and then digesting away large sections of blood vessels allowing ovoidal cancer cells to passively move into the surrounding tissues. However, in vivo videomicroscopy allowed direct observation of cells undergoing extravasation, revealing a very different alternative. The cancer cells that were initially arrested by size constraints moved away from one side of the vessel and began to spread out along the vessel wall, allowing blood flow to resume in the vessel (see review, Morris et al. 1997). The cancer cells then extend long projections into the hepatocyte layer and eventually move entirely out of the sinusoids and into a space between the sinusoids. Importantly, no disruption of the microcirculation or loss of blood was observed during this process. Thus the extravasation of cancer cells appears to resemble diapedesis of lymphocytes. This process takes approximately 2–4 days in mice. At least some of the cancer cells have been observed to extend long pseudopodial-like projection 20–30 μm long up to the subcapsular layer of the liver. The body of the cells then follows and squeezes between subcapsular hepatocytes. We have never observed cancer cells replicating intravascularly. The subcapsular region of the liver is the favored site of replication of the extravasated cancer cells. When the location of melanoma metastatic tumors was examined in mice, only one internal tumor was observed versus 76 at the surface (Luzzi et al. 1998). In the metastatic tumors, ~90% of the cells were replicating while 6% were undergoing apoptosis (Luzzi et al. 1998). In the case of B16F1 melanoma cells, the α6β1 integrin, which binds to laminin, is involved in the extravasation process (Hangan et al. 1997). When B16F1 melanoma cells were preincubated with antibodies against α6β1 integrin before they were injected into the hepatic vein of syngeneic C57BL/6 mice, the extravasation of these cells from the hepatic circulation was reduced by approximately 40% at 4–7 h post injection (p.i.) compared with controls (Hangan et al. 1997).

Lung Metastasis

The pulmonary circulation supplies the entire output of the right ventricle of the heart to the lungs. The bronchial circulation makes a lesser contribution. The pulmonary arteries branch and follow the bronchi until arterioles form capillary networks in alveolar walls. From these capillary networks pulmonary veins form and in mice they follow the bronchi. The pulmonary arterial pressure is only about 1/6 of the systemic arterial pressure. Thus, like the sinusoids in the liver, the pulmonary circulation has a relatively low-pressure.

As in the liver, B16F10 melanoma cells initially become arrested by size constraints after intravenous injections (Cameron et al. 2000). The initial distribution of the cancer cells is random and is directly proportional to the available lung volume. Thus most cancer cells are located in the vicinity of arterioles, which are also distributed uniformly throughout the lung volume. By day 4 after intravenous injection, no cancer cells could be

observed within arterioles or venous vessels. Thus, as with the liver, no intravascular replication of cancer cells was observed. At day ten p.i., the cancer cells preferentially replicated at the surface of the lung, which had over 80% coverage. Thus, as with the liver, the tumor cell growth tends to occur at preferred sites to which at least some of the cancer cells migrate after extravasation. It is not known if the cancer cells actively migrate to sites that are conducive to their growth or if this is a random process. In the lung metastases approximately 90% of the cells were replicating and only 6% were undergoing apoptosis, in agreement with what was observed in the liver (Cameron et al. 2000; Luzzi et al. 1998).

Metastatic Efficiency and Dormancy in Liver and Lung Metastases

Liver

At day 3 after injection of B16F1melanoma cells via the portal vein, over 82% of the injected cells had successfully extravasated (Luzzi et al. 1998; MacDonald et al. 2002), and by day 13 p.i. over 36% of injected cells still remained as single cells. Of these single cells, only 2% were replicating as detected by Ki-67 staining and only 3% were undergoing apoptosis as shown by TUNEL staining. Thus approximately 95% of these single melanoma cells were dormant. In contrast, only approximately 3% of melanoma cells in hepatic tumors were dormant. By day 13 p.i., ~41% of extravasated melanoma cells remained as single cells. By day 3 p.i., only ~2.3% of extravasated B16F1melanoma cells formed micrometastases as defined by a cluster of 4–16 cells. By day 13 only 3.4% of these micrometastases remained as micrometastases and approximately 1% of them went on to form tumors. The total loss of cells by day 13 p.i. was 63.8%. Thus a decrease in metastatic efficiency appears to have two causes as follows: only approximately 2.3% of extravasated cancer cells begin to divide by day 3 p.i. and only approximately 1% of them went on to form tumors by day 13. Thus in the liver the inability of extravasated cells to start dividing is a major source of a decrease in metastatic efficiency.

Lung

In the lung approximately 3.5% of injected B16F10 melanoma cells remain as single cells 12–14 days p.i.; this is in contrast to 36.1% of single B16F1melanoma cells remaining after 13 days in the liver (Cameron et al. 2000; Luzzi et al. 1998). Therefore, only approximately 1/10 of the injected cells remain as dormant single cells in lung compared with liver. However, by 12–14 days p.i., over 500-fold more extravasated B16F10 melanoma cells develop into tumors in the lung compared with extravasated B16F1 cells in the liver. Therefore, the lung appears to be a much more fertile environment for the development of B16F10 metastatic tumors than does the liver for metastatic B16F1 melanoma tumors.

Discussion and Conclusions

In vivo videomicroscopy indicates that metastasizing melanoma cells do not replicate in blood vessels or lymphatics. The cells are initially arrested in lungs and liver by size constraints. However, while the initial arrest of the cancer cells is passive, the extravasation process is active. The cells attach onto the side of the vessel and send out long pseudopodial projections into the surrounding cellular layer and even up to the surface of the organ. The cell body then moves out from the vessel into the surrounding tissue without apparent damage to the vessels. Once the cells have extravasated, they can further migrate to areas of the organ that are conducive to their replication. Thus initial replication of the metastasizing melanoma cells does not occur randomly within an organ but at preferred distinct sites.

The distribution of the initial replication of metastasizing cancer cells is not even. For mouse lung and liver, the replication initially occurs preferentially at the surface of the organ. For mouse lymph nodes, the melanoma cells replicate at discrete, evenly spaced sites within the node. The relative importance of cancer cell replication and apoptosis in the growth of metastasizing tumor cells in target organs has also been determined. In both lung and liver melanoma tumors, approximately 90% of the cells were replicating while

only 6% were undergoing apoptosis (Cameron et al. 2000; Luzzi et al. 1998). For the lymph node metastases, 23.1% of the cancer cells were replicating and 0.9% were undergoing apoptosis. Thus 15–20 times more cancer cells were actively replicating than were undergoing apoptosis.

Melanoma cancer cells are not only able to form dermal tumors in an allogeneic host, but they are able to metastasize to draining lymph nodes. This observation adds a new dimension when considering dormancy. Not only can one ask why a cancer cell remains dormant and does not replicate in its target organ but also how does this cancer cell survive, particularly in an allogeneic host. The survival of the cancer cell in lymph nodes is all the more remarkable, since lymph nodes are supposed to be a bastion of host cell defense.

The vast majority of single melanoma cells remaining in lung and liver are neither replicating nor undergoing apoptosis. Thus for single melanoma cells, only 2% in liver and 3% in lung are actively replicating 2 weeks p.i. In liver 3% and in lung 0% of single melanoma cells at 2 weeks p.i. are undergoing apoptosis. Thus the vast majority of single melanoma cells at 2 weeks p.i. are dormant. Dormancy and metastatic efficiency varies with the organ involved and/or the melanoma cell line employed (B16F1 versus B16F10). Thus the number of dormant cells remaining after 2 weeks p.i. is approximately tenfold higher in the liver using B16F1 cells compared with the lung using B16F10 cells. Metastatic efficiency for B16F10 cells in the lung is >500-fold higher than for B16F1 cells the liver. In contrast, for just metastasis to the lung, the metastatic efficiency for B16F10 cells is only 13-fold higher than for B16F1 cells (Kobayashi et al. 1998). These two results suggest that both the nature of the metastasizing melanoma cells and the organ involved can play a role in determining metastatic efficiency.

Key Concepts

1. Metastatic cells are not observed to replicate in blood or lymphatic vessels.
2. While initial arrest of cancer cells is by size constrains, extravasation is an active process involving pseudopodial projections with no visible damage to the blood vessels.
3. Melanoma cells undergo post-extravasation migration; at least some of these cells go to preferred sites for initial replication.
4. The initial replication of metastasizing melanoma cells in its target organ is not uniform but occurs at preferred sites.
5. For metastasizing melanoma tumors in their target organs, relatively few of the cells are undergoing apoptosis compared with those actively replicating.
6. In allogeneic hosts, melanoma cells can not only grow to form dermal tumors, but they can also metastasize and replicate in draining lymph nodes.
7. The vast majority of single, extravasated melanoma cells in lung and liver are dormant for at least 2 weeks p.i.
8. Metastatic efficiency and dormancy of melanoma cells can vary drastically depending on the melanoma cell line and/or the organ involved.

Future Work

Despite all that has been found out about metastasis and dormancy, a great deal remains to be discovered. The signaling pathways and attractive forces involved in migration of the cancer cells during extravasation are largely unknown. Melanoma cells replicate at preferred sites within an organ; however, whether cancer cells actively or passively migrate to these sites for their growth is also unknown. The factors at certain sites that stimulate cells growth also require further study. Why some melanoma cells begin to replicate while others remain dormant requires further work. It is of interest that during our studies on mouse liver, a single, non-replicating cancer cell (as identified by its maintenance of an internal dye) was observed in the body of a metastatic tumor. Why did this cell fail to divide when all the cells surrounding it were actively replicating? Further work is also required to determine if differences in the number of cells that remain as single, dormant cells in liver and lungs were due to organ differences and/or differences between

B16F1 and B16F10 melanoma cells. It is also important to determine the relative importance of differences in these cell lines versus different target organs in determining metastatic efficiency. Thus a major question is, what governs dormancy? The mechanism involved in the ability of melanoma cells to survive in an allogeneic host and in particular in lymph nodes requires attention. Metastasis of cancer cells is the primary cause of cancer death; dormancy poses great challenges since it confounds the traditional methods of combating cancer. Thus research in these areas is of paramount importance if we are to make headway in dealing with cancer.

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7 Late Recurrence Is a Sign of Melanoma Dormancy: Need of Life-Long Follow-Up of Melanoma Patients

Uwe Wollina

Contents

Abstract

Melanoma is the malignant tumor of melanocytes. The most common type is cutaneous melanoma on what we will focus. The demonstration of early spread of melanoma cells in animal models and the detection of circulating melanoma cells in patients with thin tumors argue against the dogma of metastasis as a late tumor event. On the other hand, late (≥10 years) recurrence has been observed in up to 3.5% of patients. The paradoxon of early spread and late metastasis argues for melanoma cell dormancy. Because late recurrence is not related to increased tumor thickness but may occur in tumors thinner than 0.5 mm, a life-long follow-up for all melanoma patients seems mandatory. Improved patient education in skin self-examination and easy availability of outpatient dermatology service are the most important tools.

Keywords

Melanoma • Dormancy • Late metastasis • Follow-up

Introduction

Melanoma is the malignant tumor of melanocytes. It occurs in skin, neighbouring mucous membranes and in extracutaneous sites such as the eye. In this chapter we will focus on cutaneous melanoma, the most common type of this malignancy. Melanoma incidence in Caucasians has

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been raised since the last century reaching 10–12 cases per 100,000 inhabitants and year for Central Europe, 10–25 for the United States, and 50–60 for Australia (Garbe and Leiter 2009). The major factors contributing to melanoma risk include natural and artificial (i.e., tanning beds, etc.) exposure to ultraviolet radiation (UVR) and fair skin complexion. Melanoma course and prognosis is dependent upon tumor stage at primary diagnosis. Major negative prognostic factors are tumor thickness, older age and metastasis. Males tend to present with thicker primary cutaneous melanomas than females, which contributes to gender differences in prognosis (Balch et al. 2004; Lasithiotakis et al. 2008).

The Paradox of Early Tumor Cell Spread but Late Recurrence

Because melanoma prognosis is dependent upon tumor thickness, tumor cell spread was thought to occur as a late event. Comparing patients with primary cutaneous melanoma for early (≤3 years, n = 320) and late metastases (>8 years, n = 70), tumor thickness, ulceration and history of other non-melanoma skin cancer were identified as risk factors for early metasases (Brauer et al. 2010). In contrast, single melanoma cells have been detected in lymph nodes of patients with thin tumors (<1 mm tumor thickness). Circulating melanoma cells were reported in a small proportion of cutaneous melanomas independent from tumor stage, suggesting early dissemination (De Giorgi et al. 2010; Ireland et al. 2011).

Animal models such as transgenic RET.AAD mice argue in favour of a very early spread of melanoma cells before histopathologic signs of vascular invasion are evident, i.e., within 3 weeks after oncogenic transformation. On the other hand, metastases occur much later. i.e., up to 1.5 years, suggesting tumor cell dormancy. The metastatic cell shows the identical genetic signature of early melanoma development (Eyles et al. 2010). The same authors conducted a CD8-T-cell depletion leading to earlier metastases. They found that CD8-T-cell controls tumor cell growth by cytotoxic and cytostatic effects (Eyles et al. 2010). The results argue for T-cell induced tumor dormancy without induction of cell killing or apoptosis. Identified messengers for such pathways are interferon-γ (IFNγ) and tumor necrosis factor (TNF) (Shankaran et al. 2001). The proof of this concept is also derived from clinical trials. In humans, type I IFN's are capable of inhibiting melanoma progression and help prolonging relapse-free survival (Kirkwood et al. 1996; Hansson et al. 2011).

On the other hand, we can find patients with a late melanoma recurrence. Late recurrence in melanoma has been defined as first signs of metastasis ≥10 years after complete excision of primary melanoma. The frequency of late recurrences has been reported from 0.5 to 3.5% of cases (Shaw et al. 1985; Crowley and Seigler 1990; Tsao et al. 1997; Peters et al. 1997; Schmid-Wendtner et al. 2000; Leman and Mac Kie 2003; Hohnheiser et al. 2011) (Table 7.1).

We investigated late recurrent cutaneous melanoma in 1881 patients with a follow-up of 10 years or more. The frequency in our study was 1.1%. There was no clear gender difference. The mean age of these patients was 44 years. Most primary tumors were classified as superficial spreading melanoma. Tumor thickness was between 0.33 and 9.5 mm (median 2.0 mm). The largest period observed for late recurrence was 25.1 years. The major type of

Table 7.1 Frequency of late recurrence of cutaneous melanoma in studies with >1,000 melanoma patients

Number of patients	Percentage of late recurrence (%)	References
1,283	2.7	Shaw et al. (1985)[a]
2,766	0.7	Tsao et al. (1997)[b]
1,015	3.5	Peters et al. (1997)[c]
6,298	0.5	Schmid-Wendtner et al. (2000)
3,822	0.7	Leman and Mac Kie (2003)
1,881	1.1	Hansel et al. (2010)
2,487	0.7	Hohnheiser et al. (2011)

[a]Stage I melanoma only
[b]Recurrence after ≥ 15 years
[c]Stage I and II (IUCC)

metastasis was loco-regional (n=12) (Hansel et al. 2010). Obviously, small tumor thickness does not prevent late recurrence.

Early Spread and Late Recurrence – Features of Melanoma Cell Dormancy: Consequences for Follow-Up of Patients

Tumor cell dormancy has been defined as disease-free period between primary cure and subsequent metastasis (Hadfield 1954). Only a proportion of patients with dormant melanoma cells will develop metastatic disease. Detailed analysis of current understanding of tumor dormancy in melanoma is found in another chapter in this volume.

An interesting view on melanoma cell dormancy is the observation of early spread of melanoma cells from small primary tumors. This seems to be a rare event in cutaneous melanoma compared to uveal melanoma. There is another clinically important difference between cutaneous and uveal melanoma: tumor mass dormancy is very unusual in the former with the exception of brain metastasis leading to the assumption that tumor cell dormancy is the predominant type for cutaneous melanoma (Ossowski and Aguirre-Ghiso 2010).

Late recurrence of melanoma occurs after tumor dormancy escape. A critical issue for tumor dormancy escape is the interaction of dormant or quiescent cells with the extracellular matrix. For this microenvironmetal interaction, β1-integrins and laminin are components expressed by tumor cells after escaping from dormancy (premetastatic niche). Matrix stiffening by deposition of collagen I and expression of matrix metalloproteinases (MMPs) by tumor cells are markers for a micrometastatic stage (Barkan and Chambers 2011).

Epoxyeicosatrienoic acids (EETs) are cytochrome P450 metabolites of arachidonic acid that are produced by the vascular endothelium in responses to various stimuli. They are regulators of angiogenesis. Investigations in Tie2-CYP2J2, Tie2-CYP2C8, and Tie2-sEH transgenic mice with B16F10 melanoma demonstrated that epoxyeicosanoids can stimulate melanoma dormancy escape by induction of vascular endothelial growth factor (VEGF). Multiorgan metastases are the consequence of this pathway. Interruption of metabolization of epoxyeicosanoids by soluble epoxide hydrolases (sEH) by sHE-inhibitors also promotes tumor growth and spread (Panigrahy et al. 2012).

Current guidelines for regular follow-up of melanoma patients suggest a 5–10 year period (Garbe et al. 2008; Marsden et al. 2010; Dummer et al. 2010). There is no international consensus on frequency, tools, and time of follow-up. Because melanoma patients may develop metastases and secondary malignancies, a follow-up seems plausible but cost factors have raised concerns (Wolff and Wollina 2000). One argument for a regular follow-up is the earlier detection of second melanomas with improved prognosis due to significantly thinner tumors (Uliasz and Lebwohl 2007).

In a recent analysis of 33,384 melanoma patients with stage I to III disease the hazard rates for recurrence have been evaluated. The hazard rates were constantly low (~1:125 per year) for stage IA. The hazard rates increased in stage IB to 1:40 for the first three years of follow-up. In other stages the hazard rates were constantly higher. The findings suggest, that intensified follow-up in stage IB or higher is justified but not for stage IA (Leiter et al. 2012). Such a view is shared by other groups (Turner et al. 2011). In view of the tumor dormancy independency from the melanoma stage, the education of melanoma patients for life-long skin self examination becomes more important. More efforts are needed to increase compliance with skin self-examination (Hull et al. 2011).

Discussion

In the light of recent data and tumor biology of melanoma, dormant melanoma cells seem to develop in early stages of the disease. The mechanisms are becoming better understood but prevention of melanoma dormancy yet is not a reality. Late metastases are the consequence of tumor dormancy escape. Both pathways, initiation

of dormancy and escape from dormancy, contribute to late recurrence of melanoma. Late melanoma recurrence has been observed in 0.5–3.5% of patients, even in those with thin primary tumors (<1 mm tumor thickness). Hopefully, in the future we might have tools to identify patients at risk and possibly to prevent tumor dormancy. Small molecules and antibodies interfering with β1-integrins such as ATN-161, volociximab, and JSM6427 may be a first step to control dormant tumor cells (Barkan and Chambers 2011). Some concern has been expressed regarding drugs that potentially interfere with escape from tumor dormancy such as sHE-inhibitor but further studies are needed (Xu et al. 2011). Today, however, the only consequence is the life-long follow-up of melanoma patients. Due to cost explosion in health systems, a hospital-based follow-up is unrealistic. Skin self-examination of educated patients and easy availability of dermatologic outpatient service are the cornerstones of melanoma patient care (Shih et al. 2009).

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Part II

Quiescence

Hematopoietic Stem Cell Quiescence and Long Term Maintenance: Role of SCL/TAL1

8

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Contents

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Abstract

The life-long production of blood cells depends on the maintenance of the hematopoietic stem cell (HSC) pool. The design of quantitative transplantation assays has been instrumental in providing insight into HSC functions, i.e. sustained self-renewal and multipotentiality: competitive transplantation, limiting dilution analysis to estimate stem cell frequency and stem cell activity as well as serial transplantation assays. These assays indicate intrinsic stem cell heterogeneity and reveal the quiescence of adult HSCs with long term reconstituting capacity, contrasting with fetal HSCs that actively proliferate. Importantly, adult HSCs are maintained in quiescence by their interaction with specialized niches in the bone marrow, via cell surface receptors such as KIT, MPL and CXCR4 expressed on HSCs and membrane-bound ligands present on stromal cells. In response to hematopoietic stress, quiescent HSCs transiently switch into

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active division to produce progenitors and mature blood cells. This regulation of HSC quiescence is critical for the preservation of long-term stem cell functions as shown by genetic studies, which also demonstrated that the process is controlled by a significant number of transcription factors. Among these, the basic helix-loop-helix transcription factor SCL/TAL1 which is essential for the onset of hematopoiesis, has been shown to regulate HSC quiescence and self-renewal, together with its protein partners, E47, GATA2 and LDB1. The SCL complex controls the transcription of genes such as *KIT*, establishing a functionally important axis in stem cell regulation.

Keywords
Hematopoietic stem cells • Quiescence • SCL/TAL1 • Stem cell niche

Introduction

The hematopoietic system is composed of various organs and tissues as the bone marrow, the spleen, the thymus and the lymph nodes. In the adult, the bone marrow is the main site of production and maturation of blood cells, while the other organs have more specialized functions. Mature blood cells have finite life spans and are produced throughout life by hematopoietic stem cells (HSCs) via successive rounds of expansion, lineage commitment and differentiation. Mature blood cells such as B lymphocytes, macrophages and red blood cells are produced from highly proliferative progenitors in the bone marrow whereas the production of T lymphocytes transits through the thymus (Fig. 8.1). Thus, one of the functional characteristics of HSCs is this multilineage potential. The life-long production of blood cells depends on the maintenance of HSCs, through their self-renewal capacities. Upon division at least one of the daughter cells will inherit the functional characteristics of its mother HSC, namely multipotency and self-renewal potential. On the other hand, the other cell is engaged into the differentiation process following asymmetric division. Commitment into the differentiation process is associated with restriction in lineage potential and self-renewal capacities (Fig. 8.1). Therefore, HSCs are characterised by two essential properties, multipotency and self-renewal capacity, that can be assessed in transplantation assays, as discussed below.

Stem cell transplantation represents the first successful cell therapy. Today, several diseases are cured with stem cell transplantations, including certain type of leukemias and lymphomas. This therapeutic approach consists in destroying the sick bone marrow with high dose chemotherapy or radiotherapy and then transplanting healthy stem cells to rescue the hematopoietic system of the recipient.

Hematopoietic Stem Cell Assays

Basic Concepts Underlying the Transplantation Assay

The design of quantitative transplantation assays has been instrumental in refining stem cell concepts in the hematopoietic system and defining their cellular as well as their molecular attributes using the mouse model. Prior to transplantation, the entire hematopoietic system of the host mouse is destroyed by a lethal dose of irradiation, which turns out to be a key aspect that allows for successful engraftment. As a consequence, the bone marrow microenvironment where stem cells reside, the stem cell niche, is free to receive the donor stem cells. Generally, donor stem cells are injected in the circulation of the host and migrate into the bone marrow where they can occupy the free niche. The reconstitution potential of HSCs, as assessed by the contribution of mature blood cells in the peripheral blood, is thus dependent on the capacity of stem cells to home in the bone marrow, attach to the microenvironment (discussed in section "Extrinsic Regulators of Quiescence: The Niche") and to differentiate in all lineages. In order to discriminate the contribution to hematopoiesis of donor cells and host cells, scientists usually take advantage of congenic mouse strains that carry differential CD45 isoforms, namely CD45.1 and CD45.2. The presence of cells expressing CD45.1 or CD45.2 is determined using flow cytometry.

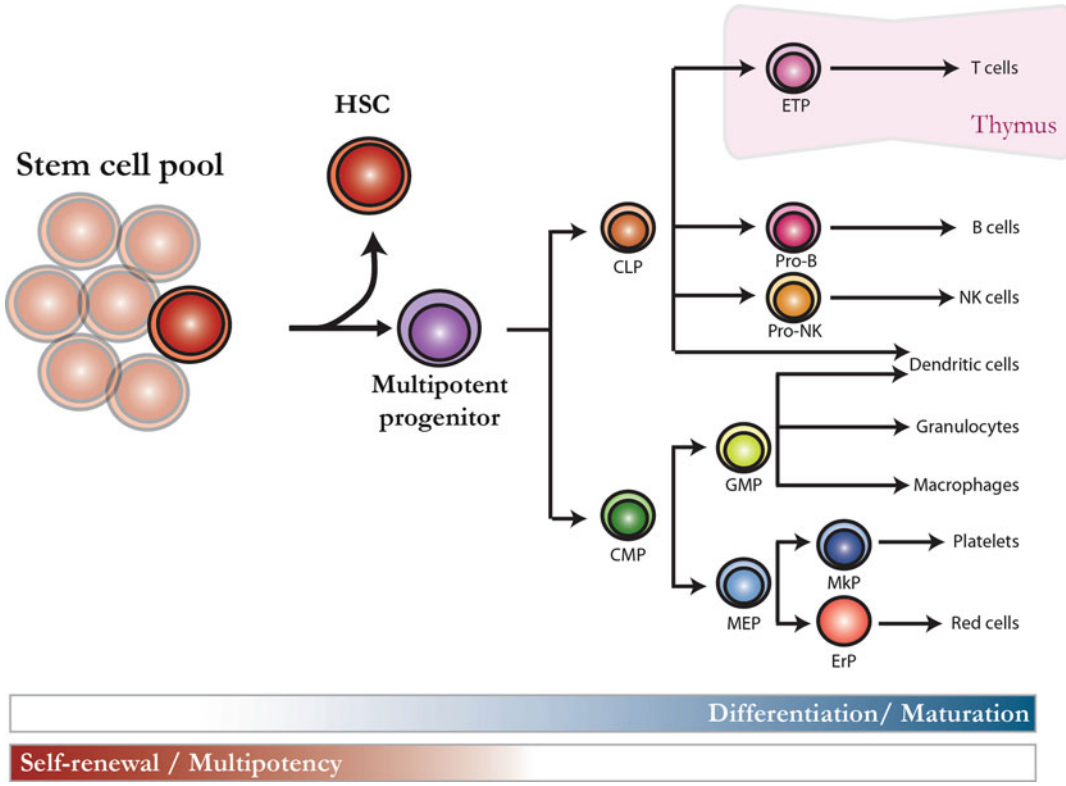

Fig. 8.1 The hematopoietic system. Hematopoietic stem cells (HSC) are at the apex of the hematopoietic system. Through asymmetric self-renewal division, HSCs give rise to one daughter HSC and one daughter multipotent progenitor. Self-renewal capacity and multipotent differentiation potential are inversely correlated with the differentiation and maturation processes. *CLP* common lymphoid progenitor, *CMP* common myeloid progenitor, *ETP* early T-cell progenitor, *NK* natural killer cells, *GMP* granulocyte-macrophage progenitor, *MEP* megakaryocyte-erythrocyte progenitor, *MkP* megakaryocyte progenitor, *ErP* erythrocyte progenitor

Competitive Transplantation

Competitive transplantation is used to compare the functional potential of two sources of HSCs. For example, one can evaluate the competitive potential of test HSCs against wild-type competitor HSCs (Harrison 1980; Szilvassy et al. 1990). Briefly, the relative competitive advantage (RCA) of test HSCs is evaluated based on the initial proportion of test cells versus the final proportion recovered in transplanted mice, as evaluated by the expression of CD45.1 or CD45.2 antigens on the surface of hematopoietic cells (Lacombe et al. 2010). Thus, if the initial proportion of cells is the same as the one recovered, one can conclude that test HSCs have an equivalent reconstitution potential as competitor HSCs, reflected by a RCA value of 1. This transplantation strategy can also reveal impaired or improved competitive advantage of test HSCs over competitor HSCs with RCA value of >1 and <1, respectively. Competitive transplantation is limited by its incapacity to distinguish between the number of HSCs present in the test populations or the quality of individual HSC to produce progeny (Purton and Scadden 2007).

Limiting Dilution Assay

The limiting dilution assay is the gold standard to evaluate the frequency of HSCs in a given population, by transplanting several groups of mice with different dilutions of test HSCs. After a given

period of time, the contribution of test cells to the hematopoietic system of host mice is evaluated based on CD45 antigens. A mouse is considered reconstituted when test cells contribute to at least 1% of all lineages (B cells, T cells and myeloid cells) in the peripheral blood. Based on Poisson statistics and the percentage of positively reconstituted mice for each cell dilution, one can calculate the competitive repopulating unit (CRU) in a given population (Szilvassy et al. 1990). The L-Calc software (Stem Cell Technologies Inc.) is frequently used to calculate CRU frequencies.

One consideration with this strategy is the fact that the graft at limiting dilutions does not provide sufficient mature blood cells and progenitors capable to rescue recipient mice from the short-term effects of irradiation. Thus, support cells, generally total bone marrow, are co-transplanted with each cell dilution to even out the immediate survival of mice after irradiation. Usually, support cells carry the same CD45 allele as recipient mice, allowing for unambiguous distinction of test cells.

Mean Activity of Stem Cells

While the above assays allow for a quantitation of stem cell frequency, another assay was designed to evaluate the quality of individual HSC to produce progeny. The mean activity of stem cell (MAS) is estimated by transplanting a limiting number of functional stem cells, near the CRU frequency, to larger cohorts of host mice (Ema and Nakauchi 2000). Thus, MAS reflects the functional quality of an individual stem cell and its capacity for clonal expansion.

Serial Transplantations

Another assay that has been used to evaluate the self-renewal capacity of HSCs consists in serial transplantations. Reconstitution potential decreases with serial transplantation and fall behind the level of detection after four to five rounds of transplantations (Cheng et al. 2000; Harrison 1973), suggesting that HSC self-renewal potential is extensive but not unlimited (Siminovitch et al. 1964).

In stem cell assays based on transplantation, it is important to distinguish cell autonomous from cell non-autonomous functions, as exemplified by the *W* and *Sl* mutations discussed in the section "Extrinsic Regulators of Quiescence: The Niche", as well as homing efficiency (Benveniste et al. 2003), which in itself is not intrinsic to self-renewal (Purton and Scadden 2007).

Phenotypic Characterization of Hematopoietic Stem Cells

The combination of flow cytometry and transplantations is a very powerful method to identify cell populations enriched in stem cell activity. In the mouse, HSCs lack the expression of markers expressed at the surface of mature cells (lineage negative) and display a high level of KIT and SCA-1 markers ($Kit^+Sca\text{-}1^+Lin^-$or KSL) (Ikuta and Weissman 1992). Within the KSL population, HSCs are enriched in the population expressing low levels of THY1.1 marker (Spangrude et al. 1988) or CD49b marker (Benveniste et al. 2010). Furthermore, several expression markers allow for a distinction between functionally different immature populations (discussed in section "Long-Term, Intermediate-Term and Short-Term Reconstituting Hematopoietic Stem Cells") such as long-term repopulating HSCs ($CD150^+$, $CD34^-$, $CD48^-$, $FLT3^-$), short-term repopulating HSCs ($CD150^+$, $CD34^+$, $CD48^+$, $FLT3^-$) (Trumpp et al. 2010) and lymphoid-myeloid primed progenitors ($CD34^+$, $FLT3^+$) (Adolfsson et al. 2001). In addition, HSCs exhibit the ability to efflux Rhodamine-123 (Benveniste et al. 2010) and Hoecsht 33342 (Venezia et al. 2004).

Even if we can isolate bone marrow cell populations highly enriched in HSCs, in the best circumstances, only half of the population are functionally competent for long-term reconstitution. Thus, the reconstitution activity of a cell population is heterogeneous and it is essential to combine phenotypic analysis with functional assays.

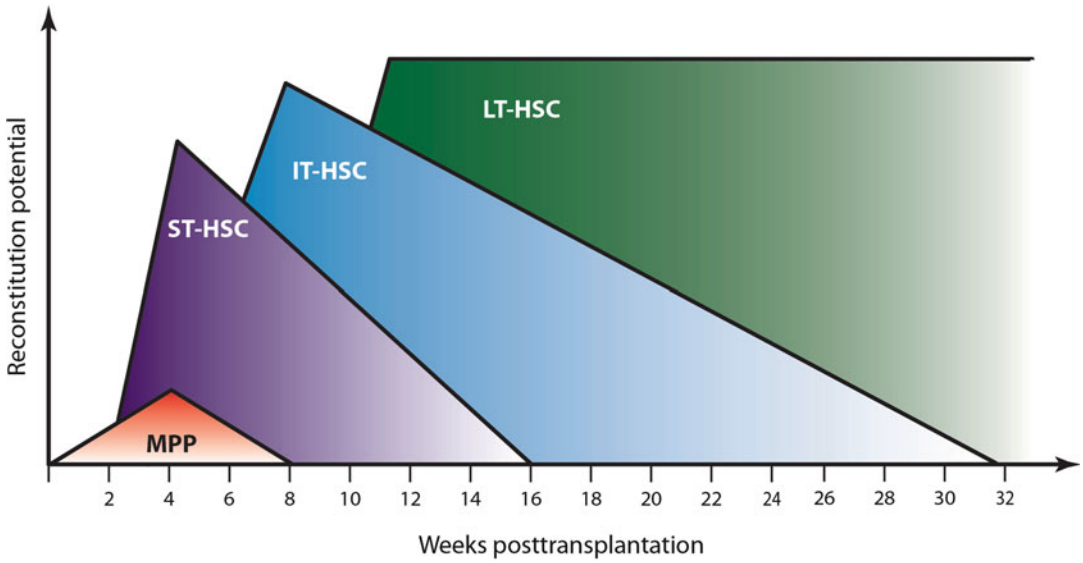

Fig. 8.2 Schematic of reconstitution kinetics. Following transplantation, donor hematopoietic cells reconstitute the recipient mouse with different efficacies and kinetics depending on the nature of the cells. Multipotent progenitors (MPP) contribute rapidly and modestly to hematopoiesis of the host. Short-term reconstituting HSCs (ST-HSC) contribute with high efficacy for up to 16 weeks to the host hematopoietic system after an initial lagging time. Intermediate-term HSCs (IT-HSC) contribute to hematopoiesis up to 32 weeks after transplantation. Finally, long-term reconstituting HSCs (LT-HSC) contribute with high efficacy to the host hematopoietic system. The quiescence state characterizing LT-HSCs may partially explain the lag time before which contribution to the host reconstitution is detected

Heterogeneity in Hematopoietic Stem Cells

Long-Term, Intermediate-Term and Short-Term Reconstituting Hematopoietic Stem Cells

Transplantation assays have unravelled several classes of multipotent cells based on the how long they can sustain hematopoiesis in host mice. Long-term repopulating stem cells (LT-HSCs) are the most primitive HSCs. They can sustain hematopoiesis for the entire lifespan of the host (Harrison 1973; Morrison and Weissman 1994) and after transplantation, they can sustain hematopoiesis in the recipient for more than 32 weeks (Benveniste et al. 2010), reflecting an extensive self-renewal potential. The existence of HSCs with intermediate potential was demonstrated by Benveniste et al. (2010), defined by their capacities to sustain hematopoiesis for 6–8 months before exhaustion. Short-term repopulating stem cells (ST-HSCs) are less primitive and have a repopulation potential not exceeding 8–16 weeks (Morrison and Weissman 1994; Benveniste et al. 2010), reflecting a limited self-renewal potential.

Beside the time of contribution to hematopoiesis, HSC classes also have distinct reconstitution kinetics. The more primitive HSCs exhibit delayed onset of repopulation, compared to less primitive HSCs or progenitors that contribute rapidly to hematopoiesis, as shown by retroviral mediated clonal marking strategy (Jordan and Lemischka 1990). It was later confirmed by fluorescence imaging that mice transplanted with LT-HSCs show a high level of reconstitution that reaches a plateau around 8 weeks and remain high whereas mice transplanted with ST-HSCs also have high levels of reconstitution but this activity peaks at about 6 weeks and decreases thereafter. Finally mice transplanted with multipotent progenitor (MPP) show low level of overall reconstitution that peaks at 4 weeks (Fig. 8.2) (Cao et al. 2004).

Quiescence Marks the Difference Between Fetal and Adult Hematopoietic Stem Cells

In the mouse embryo, HSCs capable of long-term reconstitution of recipient mice first appear in the aorta-gonad-mesonephros (AGM) region. These cells transit by the fetal liver before reaching the bone marrow, the place where HSCs will reside during adult life. One important difference between fetal and adult HSCs resides in their cell cycle status. It is well documented that fetal HSCs are actively cycling with less than 5% found in quiescence (G0 state), whereas more than 70% of adult HSCs are in G0 phase of the cell cycle (Lacombe et al. 2010; Passegué et al. 2005; Wilson et al. 2008). Even though the time required to complete a cell cycle is about the same between fetal and adult HSCs (Bowie et al. 2007), the frequency of cell cycle entry is absolutely different. It was shown that all fetal HSCs divide within 48 h (Nygren et al. 2006), whereas adult HSC divide every 36 or 145 days in vivo (Wilson et al. 2008). This quiescence of adult HSCs is associated with maintenance of the stem cell pool throughout adult life whereas the pool of HSCs expands dramatically during fetal development. Therefore under homeostasis, only a small proportion of adult HSCs need to proliferate to ensure the daily demand in blood cells. The developmental switch of fetal to adult HSCs occurs between 3 weeks and 4 weeks postnatally, driven by SOX17 (Bowie et al. 2007; Kim et al. 2007), clearly indicating a cell-intrinsic difference in gene expression programs underlying key HSC properties. Therefore, gene function in HSCs can be very different according to their developmental stages. For example, *Bmi1* deficiency affects both fetal and adult HSC self-renewal (Park et al. 2003), whereas decreased *Scl* gene dosage affects adult HSCs only (Lacombe et al. 2010).

Quiescence is important for stem cells. This state is thought to protect adult HSCs from proliferative stresses such as DNA damage, elevated levels of ROS, mutations and telomere shortening. At the functional level there is a link between quiescence and stem cell function. In the adult bone marrow, stem cell functions are enriched in the G0 fraction compared to the G1/S/G2/M fraction (Bowie et al. 2007). Furthermore, genetic studies have established a strong correlation between quiescence disruption and defect in self-renewal capacity in adult HSCs, accompanied most of the time with exhaustion of the stem cell pool (Orford and Scadden 2008). There was a single discordance dissociating quiescence from stem cell maintenance, but the latter was examined in fetal HSCs (Ku et al. 2012). Thus, the tight regulation of HSC quiescence and proliferation is indispensable for long-term maintenance of stem cell function in the adult. Quiescence is a reversible state controlled both extrinsically by the stem cell niche and intrinsically by molecular regulators.

Cell Cycle Analysis by DNA and RNA Staining

DNA content is quantified by staining with a stoichiometric dye such as Hoechst 33342 or DAPI (4′, 6-diamidino-2-phenylindole). However, quiescent cells (G0) and cells in G1 phase of the cell cycle carry *2n* DNA content and cannot be differentiated by this labelling technique. Because quiescent cells exhibit low metabolic activity, G0 and G1 phase of the cell cycle can be differentiated according to the RNA content using the Pyronin Y (PY) fluorescent dye. Thus, within the *2n* population, quiescent cells fall in the PY negative population whereas cells in G1 phase are PY positive. Alternatively, cells in G0 and G1 can also be distinguished on the basis of Ki-67 immunostaining detected by flow cytometry. DNA/RNA content analysis is use to evaluate the proportion of cells in each phase of the cell cycle at a given time (Lacombe et al. 2010; Lacorazza et al. 2006; Tipping et al. 2009).

Label Retaining Assays

DNA/RNA labelling assays reflect a “snap-shot” image of cell cycle distribution. Another assay based on label retention is commonly used to investigate the cell cycle kinetics of primitive

hematopoietic cells. The principle behind these assays is to stain cells with a label that will be diluted at each cell division. Therefore, cells that have divided extensively do not exhibit a detectable level of label whereas label-retaining cells represent cells that have not or rarely divide.

Bromodeoxyuridine (BrdU) is a thymidine analog that is incorporated in newly synthesized DNA and can be detected by a specific antibody. With a short exposure of mice to BrdU (drinking water for 2 days), a correlation between quiescence (G0) and BrdU staining has been shown (Lacorazza et al. 2006). In the context of label-retaining assays, mice are exposed to BrdU for a long period of time (10–13 days in drinking water (Wilson et al. 2008; Zhang et al. 2003)) in order to stain HSCs which divide infrequently. The period of staining is followed by a BrdU-free chase period. It has been shown that BrdU retaining cells are detected in the bone-marrow for up to 306 days of chasing period (Wilson et al. 2008).

A transgenic mouse model that expresses the fusion protein histone H2B-GFP under the control of a tetracycline-responsive regulatory element has been used for label-retaining assays. In this assay, the label (H2B-GFP transgene) is turned-on in a given cells population genetically engineered to express tetracycline-controlled transactivator (tTA). For the chase period, the transcriptional activity of tTA is inhibited by continuous exposition of mice to Doxycycline and five divisions are sufficient for the GFP signal to fall below the detection limit (Wilson et al. 2008).

Interestingly, the kinetics of extinction of BrdU and H2B-GFP labelling are similar, suggesting that both techniques are equivalent. Moreover, long-term HSCs reside in the label retaining population (Wilson et al. 2008). Nonetheless, BrdU seems to impose an initial proliferative stress to quiescent HSCs (Wilson et al. 2008), allowing for HSC labelling within 10–13 days, despite their low division frequencies during steady state.

5-Fluorouracil Treatment

5-fluorouracil (5-FU) is an anti-metabolite that incorporates into DNA and RNA and kills cells by several mechanisms, including inhibition of *de novo* synthesis of deoxythymidine monophosphate (dTMP). Because of their low metabolic activity, quiescent HSCs are relatively less affected by 5-FU treatment compared to proliferating cells such as progenitors. Therefore, 5-FU induced lethality in HSC population has been used to evaluate the proportion of proliferating versus quiescent HSCs in mice (Arai et al. 2004). Moreover, as a consequence of massive decrease of the bone marrow cellularity following 5-FU injection, quiescent HSCs are forced into proliferation and this treatment has been used to compare quiescent and proliferative gene signature in purified HSCs (Venezia et al. 2004). Therefore, a second dose of 5-FU administration kills proliferating HSCs but also causes lethality in mice, starting around 10 days after the first injection (Cheng et al. 2000). Even if a correlation with HSC cell cycle has been observed, this early lethality of mice limits the possibility of assessing long-term HSC activity.

Extrinsic Regulators of Quiescence: The Niche

Adult HSCs reside within stem cell niches in the bone marrow and more precisely in the trabecular region of long bones (Zhang et al. 2003), although the exact localisation of HSCs is unclear. Two distinct regions have been proposed as stem cell niches: the endosteal niche located near the bone surface and the peri-vascular niche located more centrally in the bone (Trumpp et al. 2010). Quiescent HSCs, as defined by BrdU retaining cells, are mostly found in the endosteal niche (Zhang et al. 2003). The stem cell niche is extremely important for HSCs and it is proposed that extrinsic regulators responsible for the interaction between HSCs and their niche are important to maintain HSCs in quiescence (Trumpp et al. 2010). Several genes have been functionally implicated in cell-cell interaction between HSCs and their niches.

Attachment to the Niche Maintains Hematopoietic Stem Cells in Quiescence

In mid 60s, it was demonstrated that extrinsic regulators are required for stem cell functions.

The steel locus (*Sl*) encodes the KIT ligand (KitL, also referred to as SCF or SF). Using a mouse model with bi-allelic mutations in the *Sl* locus (Sl/Sl^d), McCulloch et al. (1965) demonstrated that the *Sl* gene is required in the recipient mouse to support the growth of donor cells. On the other hand, bone marrow cells from Sl/Sl^d mice were able to engraft and contribute to hematopoiesis as well as wild-type cells, showing a non-cell-autonomous effect. Furthermore, membrane-bound KitL is more efficient in supporting hematopoietic progenitors in vitro compared to the soluble form of KitL (Toksoz et al. 1992), indicating the importance of cell-cell interaction in the niche. In agreement with an important role of KitL in the HSCs behaviour, it has later shown by Driessen et al. (2003) that the transmembrane form of KitL is required for stem cell localisation in the endosteal niche.

Interestingly, the hematopoietic deficiency in *W/W* mutant mice mirrors that of *Sl/Sl* mice, except that the defect is cell-autonomous (McColloch et al. 1964) and was later attributed to hypomorphic *Kit* mutations. In adult hematopoiesis, the interaction between KIT and its ligand is important to maintain HSCs in quiescence. It was reported by Thorén et al. (2008) that in W^{41}/W^{41} mice with a mild hypomorphic *Kit* mutation, LT-HSCs show more BrdU staining compared to wild-type after 2 weeks of BrdU administration in drinking water, suggesting increased proliferation. The authors also observed that W^{41}/W^{41} mice show a decreased proportion of HSCs and progenitors (KSL $Flt3^-$ and KSL $Flt3^+$ respectively) in G0 phase of the cell cycle. This decreased quiescence was accompanied by increased proportion of cells in G1 and S/G2/M phases of the cell cycle in HSCs population specifically. At the functional level, *Kit* is required for long-term maintenance of HSC reconstitution potential in competitive and non-competitive transplantation setup (Thorén et al. 2008).

HSC quiescence is also controlled by the interaction between MPL, a tyrosine kinase receptor, and its ligand, thrombopoietin (THPO), expressed by osteoblastic cells (Yoshihara et al. 2007). Interestingly, Yoshihara et al. (2007) have shown that inhibition of the MPL receptor with a neutralizing antibody decreased the proportion of HSCs in quiescence and at the opposite, exogenous administration of THPO to the mouse transiently increased the proportion of quiescent HSCs.

The interaction between CXCR4 and its ligand CXCL12 is important for the attachment of HSCs to the niche. Indeed, deletion of *Cxcr4* in hematopoietic cells caused an important decrease in the numbers and the frequency of functional HSCs, as assessed by LDA (Sugiyama et al. 2006). The authors also observed an increased proportion of proliferating cells suggesting that the interaction with a specialized niche helps maintain HSCs in quiescence. Interestingly, CXCR4 and CXCL12 are critical for engraftment of HSCs and up-regulation of *Cxcr4* has been reported to enhance the engraftment potential of transplanted HSCs (Peled et al. 1999).

TIE2 is a tyrosine kinase receptor interacting with angiopoietin-1 (ANG-1). It was demonstrated by Arai et al. (2004) that TIE2 is expressed at the surface of HSCs and allows for the interaction between HSCs and osteoblastic cells. Interestingly, stimulation of the TIE2/ANG-1 axis increases the proportion of KSL in G0 in vivo, suggesting a positive regulation of quiescence. Furthermore, it is proposed that TIE2/ANG-1 signalling suppresses cell cycle progression in leukemic cells by maintaining these cells in G0/G1 phase (Ichihara et al. 2011).

Circulating and Mobilized Hematopoietic Stem Cells

In homeostasis condition, most of HSCs are in the bone marrow, but a small proportion of cells circulate in the peripheral blood according to a circadian rhythm and the number of circulating HSCs is two to three fold higher at the peaks compare to the nadir of circadian oscillation (Méndez-Ferrer et al. 2008). The authors found an inverse correlation with the level of CXCL12 found in the stem cell niche and the retention of HSCs. These observations suggest that HSC mobilisation for the purpose of stem cell harvesting and transplantation may be more efficient at

night time (Lucas et al. 2008). In accordance with this uniform exit from quiescence, Forsberg et al. (2010) reported that mobilised HSCs adopt a proliferating signature with an upregulation of genes implicated in cell cycle regulation, translation, RNA processing factor and metabolic processes and a downregulation of negative regulators of the cell cycle, transcription factors and signalling proteins.

Interestingly, the EGR1 transcription factor has also been implicated in the retention of HSCs in their niches. EGR1 is the founding member of a family of zinc-finger transcription factors. Min et al. (2008) showed that *Egr1*$^{-/-}$ HSCs exhibit increased proliferation and spontaneous mobilization in the peripheral blood and in the spleen of mice. The tight correlation between HSC proliferation and their release from the niche raises the question whether proliferation is a cause or consequence of HSC mobilization. Interestingly, induction of HSC proliferation does not automatically release HSCs from their niches, since no mobilized HSCs are observed in *Cdkn1a*$^{-/-}$ animals (discussed in section "*Cdkn1a* and *Id1*"). Thus, EGR1 is a transcription factor that can both regulate HSC quiescence and localization.

Intrinsic Regulators of Quiescence

Several intrinsic regulators of HSC quiescence have been described (Table 8.1), including cell cycle regulators, components of signal-transduction pathways and mostly transcription factors. Within the latters, we find hematopoietic specific (SCL, GATA2), ubiquitous (E47, MYC and STAT5), developmental related (HOXB4) and stress response related (FOXOs, JUNB and NURR1) transcription factors. Interestingly, several of these are deregulated in cancer. Within the transcription factors implicated in regulation of HSC quiescence, one is of particular interest, SCL, because of its essential contribution to the generation of HSCs during embryonic development (Robb et al. 1995; Shivdasani et al. 1995). SCL turns out to be a key regulator of the hematopoietic system at multiple levels.

Importance of *Scl* for Hematopoietic Stem Cells

SCL is a key regulator of hematopoiesis (reviewed by Lécuyer et al. 2002) and, depending on the lineage and stage of differentiation, SCL controls cell survival (Chagraoui et al. 2011; Ema et al. 2003; Krosl et al. 1998; Martin et al. 2004; Souroullas et al. 2009), differentiation (Chagraoui et al. 2011; Curtis et al. 2004; Goardon et al. 2006; Mikkola et al. 2003) or proliferation (Chagraoui et al. 2011; Dey et al. 2010).

Herein, we will review the role of SCL in HSCs. *Scl* is highly expressed in purified LT-HSCs (Lacombe et al. 2010) and within this population, the transcript is more abundant in quiescent cells (G0 phase) compared to non-quiescent cells (G1/S/G2M phase) (Lacombe et al. 2010), in agreement with the finding that *Scl* is part of an HSC quiescence gene signature (Venezia et al. 2004). Accordingly, diminished expression levels of *Scl* (as in *Scl*$^{+/-}$mice, by *Scl* RNA interference (Lacombe et al. 2010), or in Mx1-Cre *Scl*$^{+}$*/flox* mice (S.R.S. and T.H., unpublished results)) decreased the proportions of HSCs found in G0 and increased the proportion of HSCs in G1 phase of the cell cycle, suggesting that SCL levels control the G0-G1 transition. Interestingly, this cell cycle perturbation was specifically observed in adult LT-HSC-enriched populations (Lacombe et al. 2010) and was not found in progenitors or in fetal LT-HSCs. This decreased quiescence status was also supported by increased BrdU incorporation in *Scl*$^{+/-}$ HSCs compared to controls (Lacombe et al. 2010). Finally, decreased quiescence is associated with impaired long-term stem cell activity. This activity is cell-autonomous since *Scl* gene dosage does not affect the capacity of recipient mice to support the self-renewal of donor HSCs (Lacombe et al. 2010). Together, these observations support a role for *Scl* gene dosage in specifically controlling the quiescence and long-term maintenance of adult HSCs.

The importance of *Scl* for stem cell function has been a matter of debate. SCL dosage was independently reported to be important in murine LT-HSCs (Lacombe et al. 2010) or

Table 8.1 Regulators of hematopoietic stem cell quiescence. Intrinsic and extrinsic regulators of hematopoietic stem cell (HSC) quiescence are classified according to their functions

Function	Gene	Protein	Effect	Pathway/Comments	References
Cell cycle regulators	*Ccnc*	Cyclin C	–		Miyata et al. (2010)
	Cdc42	CDC42	+	Rho subfamily, CXCR4 pathway	Yang et al. (2007)
	Cdkn1a	p21	+		Cheng et al. (2000) and van Os et al. (2007)
	Cdkn1c	p57	+		Matsumoto et al. (2011) and Zou et al. (2011)
	Cdkn2c	p18	+		Yuan et al. (2004)
	Rb1	RB	+		Viatour et al. (2008)
Chromatin remodeling	*Bmi1*	BMI1	–	PcG family, upstream of Hox genes	Lessard and Sauvageau (2003) and Park et al. (2003)
	Chd4	MI-2B	+		Yoshida et al. (2008)
DNA repair	*Fancg*	FANCG	+	Nuclear FA core complex	Barroca et al. (2012)
E3 ubiquitin ligase	*Cbl*	CBL	+	Binds phopshorylated SRC family kinases	Rathinam et al. (2008)
	Fbxw7	FBW7	+	Negative regulator of mTORC1, NOTCH1, cMYC	Thompson et al. (2008)
Niche interaction	*Cd81*	CD81	+	Tetraspanin, complex with integrins, upstream of AKT	Lin et al. (2011)
	Cxcr4	CXCR4	+	CXCL12/SDF receptor	Sugiyama et al. (2006)
	Grp78	GRP78	+	Binds Crypto. Metabolic regulation	Miharada et al. (2011)
	Kit	KIT	+	KitL receptor, independent from CXCR4	Kiel and Morrison (2006)
	Mpl	MPL	+	TPO receptor	Qian et al. (2007) and Yoshihara et al. (2007)
	Tek	TIE2	+	ANG-1 receptor	Arai et al. (2004)
RNA binding	*Rbm15*	OTT1	+		Xiao et al. (2012)
Signal transduction	*Irgm1*	IRGM1	+	Interferon-inducible GTPase	Feng et al. (2008)
	Bid	BID	+	Downstream of ATM, regulates ROS levels	Maryanovich et al. (2012)
	Sh2b3	LNK	–	JAK2-STAT pathway, downstream of MPL	Bersenev et al. (2008)
	Tsc1	TSC1	+	mTOR pathway, interacting with PI3K/AKT, Negative regulator of mTORC1	Chen et al. (2008)
	Akt	AKT	–	PI3K/AKT pathway	Juntilla et al. (2010)
	Stk11/Lkb1	LKB1	+	AMP kinase, glycolytic flux in response to stress	Gan et al. (2010)
	Pten	pTEN	+	PI3K/AKT pathway	Yilmaz et al. (2006) and Zhang et al. (2006)

Transcription Factor	*Cited2*	CITED	+	Negative regulator of HIF1, regulates *Egr1, Cdkn1c, Hes1*	Du and Yang (2012)
	Egr1	EGR1	+	Independent from CXCR4, retention in the niche	Min et al. (2008)
	Elf4	MEF	–		Lacorazza et al. (2006)
	Foxo1	FOXOs	+	AKT pathway/Regulates ROS levels	Tothova et al. (2007)
	Foxo3	FOXOs	+	AKT pathway/Regulates ROS levels	Miyamoto et al. (2007) and Tothova et al. (2007)
	Foxo4	FOXOs	+	AKT pathway/Regulates ROS levels	Tothova et al. (2007)
	Gata2	GATA2	+	May be part of the SCL complex	Tipping et al. (2009)
	Gfi1	GFI-1	+		Hock et al. (2004) and Zeng et al. (2004)
	Hif1	HIF1	+	Regulates ROS levels	Takubo et al. (2010)
	Irf2	IRF2	+		Sato et al. (2009)
	JunB	JUNB	+		Santaguida et al. (2009)
	Mecom	PRDM3	+	Also known as Mds1-Evi1	Zhang et al. (2011)
	Myc	c-MYC	–		Wilson et al. (2004)
	Nr4a2	NURR1	+		Sirin et al. (2010)
	Pbx1	PBX1	+		Ficara et al. (2008)
	Stat5a	STAT5	+	JAK2-STAT pathway	Wang et al. (2009)
	Tal1	TAL1/SCL	+	May be part of the SCL complex	Lacombe et al. (2010)
	Tcf3	E12-E47	+	May be part of the SCL complex	Semerad et al. (2009) and Yang et al. (2008)
	Trp53	p53	+		Liu et al. (2009)
Transcription regulator	*Id1*	ID1	+		Jankovic et al. (2007) and Perry et al. (2007)
	Pml	PML	+	Negative regulator of mTORC1	Ito et al. (2008)
Niche	*Ctnnb1*	β-catenin	+	WNT pathway	Nemeth et al. (2009)
	Dkk1	DKK1	–	WNT pathway	Fleming et al. (2008)
	Spp1	Osteopontin	+		Nilsson et al. (2005) and Stier et al. (2005)
	Wnt5a	WNT5	+		Nemeth et al. (2007)

Positive (+) or negative (–) effect on HSC quiescence is indicated

human HSCs (Brunet de la Grange et al. 2006; Reynaud et al. 2005). In contrast, the conditional deletion of the *Scl* gene in adult hematopoietic cells did not affect stem cell activity (Mikkola et al. 2003; Curtis et al. 2004). This apparent discordance could be due to differences between intermediate or long-term repopulating cells (Benveniste et al. 2010) or, alternatively, to the functional redundancy between *Scl* and *Lyl1*, the latter encoding another hematopoietic bHLH transcription factor which is more than 80% identical to SCL (Souroullas et al. 2009). Nonetheless, redundancy could have been evoked by the conditional deletion of *Scl*. In support of this, Wilson et al. (2010) showed that SCL and LYL1 do not occupy the same regions of the genome in a genome-wide analysis of SCL target genes in multipotent cells. Hence, SCL peaks are about equally distributed between promoter, intragenic and intergenic regions whereas LYL1 peaks are almost exclusively found in intragenic and intergenic regions. Nevertheless, LYL1 could potentially substitute for SCL on these promoters when the *Scl* gene is deleted.

Members of the SCL Complex Regulate Hematopoietic Stem Cell Quiescence

It is well documented that SCL is part of multifactorial transcriptional complexes, in which the core components are SCL, E2A, GATA1/2, LMO2 and LDB1 (Wadman et al. 1997). Apart from LMO2, the regulation of HSC quiescence and/or functions has been reported for all other partners.

E Proteins

E proteins are a subclass of bHLH protein family that includes in mammals E12, E47 (both encoded by *Tcf3* gene), HEB and E2-2 that can form homodimers and heterodimers with other E proteins or with tissue specific bHLH such as SCL (Hsu et al. 1994). To our knowledge the importance for HSCs function and quiescence has only been explored for E47 using the $E47^{-/-}$ mouse model. Interestingly, $E47^{+/-}$ HSCs did not show any defect in HSC functions, possibly due to a high level of redundancy between E proteins (Zhuang et al. 1998).

It was demonstrated by several studies that *E47* deletion causes a cell-autonomous loss of the quiescent HSCs (Semerad et al. 2009; Yang et al. 2008, 2011) associated with increased proliferation as demonstrated by high BrdU incorporation (Semerad et al. 2009; Yang et al. 2008). In transplantation, this results in a loss of competitive potential and eventually an impaired long-term contribution to the KSL pool (Semerad et al. 2009; Yang et al. 2011), suggesting a progressive exhaustion of HSCs in the absence of *E47*. In serial transplantation, $E47^{-/-}$ cells show minimal contribution to the hematopoietic system of recipient mice with the consequence that mice transplanted with $E47^{-/-}$ *HSCs* exhibit increased lethality compared to control conditions (Semerad et al. 2009; Yang et al. 2008).

GATA2

GATA proteins are a family of zinc-finger transcription factors known to be important for specification and differentiation of numerous tissues. GATA1 is redundant with GATA2 both in transcriptional assays (Lécuyer et al. 2002) and in the development of primitive hematopoiesis in vivo (Fujiwara et al. 2004). No role for *Gata1* has been reported in HSCs. In contrast, several studies point to the importance of *Gata2* in adult HSCs, correlating with higher expression levels for *Gata2* in quiescent HSCs (Tipping et al. 2009; Venezia et al. 2004). Surprisingly, both loss of function and gain of function resulted in increased proportions of HSCs found in quiescence (Rodrigues et al. 2005; Tipping et al. 2009), suggesting that *Gata2* expression level has to be tightly regulated to preserve a physiological level of quiescence. Indeed, enforced expression of *Gata2* was associated with impaired chimerism in recipient mice compared to control cells, possibly due to improper proportions of cells in quiescence and impaired proliferative capacity (Tipping et al. 2009). At the

opposite, *Gata2* loss of function also impaired the competitive potential of HSCs as well as their self-renewal capacity (Rodrigues et al. 2005). Altogether, studies with *Scl* and *Gata2* indicate that HSC quiescence is a highly regulated process controlled by gene dosage or gene expression levels.

LDB1

LDB1 is a nuclear protein that does not have the ability to bind DNA directly. Rather, LDB1 is recruited to the SCL complex via binding to LMO2. Similar to *Scl*, *Ldb1* deletion in fetal HSCs did not affect their proliferation status (Li et al. 2011). However, conditional *Ldb1* deletion in the adult caused an exhaustion of HSCs in competitive transplantation experiments (Li et al. 2011). The activity of *Ldb1* in maintaining the pool of HSCs is cell autonomous since *Ldb1* gene deletion in recipient mice did not alter bone marrow chimerism (Li et al. 2011).

SCL Target Genes Regulate Hematopoietic Stem Cell Quiescence and Maintain Stem Cell Functions

SCL is a tissue specific member of the helix-loop-helix (HLH) super-family of transcription factors. SCL heterodimerizes with E proteins (Hsu et al. 1994), through its first alpha-helix (Lécuyer et al. 2002) and this is required for DNA binding to the consensus E box, AACAGATGGT (Hsu et al. 1994), through its basic domain. SCL can also regulates the transcription of its target genes by association with transcription factors that bind GC-rich sequences, as shown for *Kit* regulation (Lécuyer et al. 2002) or GATA sites (Wadman et al. 1997). Thus, SCL has many potential target genes, some of which are confirmed by various techniques that include transcriptional assays or chromatin immunoprecipitation (ChIP) in primary cells. Among the genes encoding receptors that control HSC quiescence, the *Kit* gene has been extensively studied using these different techniques (Lécuyer et al. 2002). This occupancy of the *Kit* locus by SCL was also observed in genome-wide studies of SCL target genes in multipotent cells by ChIP-sequencing (ChIP-Seq), which also revealed that the *Cxcr4* and *Mpl* loci are bound by SCL (Wilson et al. 2010). Together, these studies suggest that SCL can favour quiescence in HSCs by regulating the expression of receptors responsible for the attachment of HSCs to their microenvironment.

Kit

It was reported by Lécuyer et al. (2002) that KIT is coexpressed with SCL in lineage negative bone marrow cells, and that SCL occupies the *KIT* promoter in the human $CD34^{+}TF1$ hematopoietic cell line. Furthermore, enforced *SCL* expression is sufficient to induce ectopic *Kit* expression in murine B cells (Lécuyer et al. 2002). At the opposite, *SCL* downregulation (expression of dominant negative *Scl* or *Scl* antisense) decreases *KIT* mRNA and protein levels in TF1 cells (Krosl et al. 1998) and impairs the survival of TF1, showing that *SCL* is important for TF1 cell survival in vitro (Krosl et al. 1998). At the opposite, enforced *Scl* expression in BaF3, an IL-3-dependent cells line that does not express either *Kit* or *Scl*, is sufficient to induce *Kit* expression and survival responsiveness to KitL stimulation (Krosl et al. 1998). Furthermore, all members of the SCL complex co-occupy the Kit locus in TF-1 cells and in multipotent cells: SCL, E2A (Lécuyer et al. 2002), LMO2 and GATA2 (Wilson et al. 2010). In summary, these studies indicate that *Scl* is upstream of *Kit* and controls *Kit* transcription in primitive hematopoietic cells.

In addition to SCL, LMO2 or LMO2 and GATA2 were also found on the *Mpl* and *Cxcr4* loci, respectively (Wilson et al. 2010) and SCL was also found on the *Cd81* locus (Wilson et al. 2010). Based on transcriptional studies with the *Kit* promoter, these ChIP-Seq data support the view that the SCL complex regulates the transcription of these genes, which remains to be confirmed in transcriptional assays. Therefore, SCL potentially regulates the expression of four

of the six genes reported to control HSC retention in the niche (Table 8.1).

Cdkn1a and *Id1*

In addition to these niche interacting receptors, Lacombe et al. (2010) showed by immunoprecipitation of the chromatin that SCL binds in vivo to the *Cdkn1a* promoter in lineage depleted bone marrow cells. CDKN1A (also known as p21) is part of the family of cyclin-dependent kinase inhibitors and regulates the G1 check-point. The binding of SCL on DNA occurs in a region where two E boxes matching the SCL consensus sequence are found (Lacombe et al. 2010). Cheng et al. (2000) reported that *Cdkn1a*$^{-/-}$ bone marrow cells enriched for progenitors and HSCs (Lin^{-} cells) were less quiescent compared to wild-type cells and that HSC activity is exhausted after five serial transplantations. Nonetheless, with improved purification protocols for HSCs and the use of H2B-GFP label retention, increased cycling was not observed under steady state conditions in highly purified HSC populations (Foudi et al. 2009). Rather, *Cdkn1a* deficiency impaired hematopoietic reconstitution under proliferative stress (van Os et al. 2007), as described for *Scl* (Lacombe et al. 2010). These observations, therefore, suggest that *Cdkn1a* is a functional SCL target gene in stress response.

ID1 is a member of the HLH super-family known to associate with E proteins with high affinity. Because ID proteins lack the DNA binding domain, they act as dominant negative regulators of E protein transcription activity. Lacombe et al. (2010) showed by immunoprecipitation of the chromatin that SCL binds in vivo to the promoter region of the *Id1* gene in bone marrow cells enriched for progenitors and HSCs, which was also identified in genome-wide studies (Wilson et al. 2010). Taking advantage of *Id1*$^{-/-}$ mouse model, Jankovic et al. (2007) showed that the initial rate of expansion in culture of KSL from *Id1*$^{-/-}$ mice was accelerated compared to wild-type KSL, and in vivo, *Id1*$^{-/-}$ KSLs accumulated more BrdU compared to wild-type cells (Jankovic et al. 2007) in line with an increased proportion of cycling stem cells. At the functional level, *Id1* deficiency impaired HSCs reconstitution potential. More precisely, in secondary transplantation experiment, *Id1*$^{-/-}$ HSCs showed an impaired reconstitution potential and reduced contribution to the peripheral blood (Jankovic et al. 2007) and bone marrow compared to control cells (Jankovic et al. 2007; Perry et al. 2007), suggesting that HSCs are prematurely exhausted in absence of *Id1*. Thus, *Id1* deficiency mirrors *Scl* deficiency in HSCs. Unlike *Scl* however, *Id1* also controls HSC by a cell non-autonomous mechanism (Suh et al. 2009).

Conclusion

In summary, molecular and cellular studies indicate that HSC quiescence is controlled by a transcription factor network that involves SCL, E47, GATA2, LDB1 and potentially LMO2, controlling the expression of a set of genes that have been implicated in HSC quiescence and long-term maintenance. This network could possibly be extended to other transcriptional regulators. For example, ERG and the closely related FLI1 transcription factor were often found in the vicinity of SCL-LMO2-GATA2 in several loci that include *Kit*, *Cxcr4*, *Mpl*, *Cdkn1a* and *Id1* (Wilson et al. 2010). Since ERG function is required for HSC maintenance and self-renewal under proliferative stress similarly to SCL (Ng et al. 2011; Taoudi et al. 2011), we anticipate that ERG may also control HSC quiescence.

Functional studies so far have associated the control of adult HSC quiescence with long-term maintenance. Furthermore, genetic disruption of the network controlling HSC interaction with the niche indicates an essential role of the microenvironment for HSC quiescence. These genetic studies have unravelled the importance of adhesion molecules and cell surface receptors in controlling niche retention and HSC functions, as well as the major contribution of transcription regulators in this process. Several signal transduction pathways have been implicated downstream of MPL or other cell surface molecules (Pietras et al. 2011). How the intricate wiring of signal transduction pathways interacts with transcriptional

regulators to control HSC quiescence remains an area of investigation.

Current chemotherapeutic approaches target cell division and have been efficient at reducing tumor burden but are likely to spare quiescent cancer stem cells. An understanding of HSC quiescence will shed light into the dormancy of cancer stem cells and leukemia initiating cells, thereby opening new therapeutic opportunities.

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Regulation of Muscle Stem Cell Quiescent and Undifferentiated State: Roles of Hesr1 and Hesr3 Genes

9

Masahiko Yamaguchi and So-ichiro Fukada

Contents

Abstract

Skeletal muscle stem cells, which are called "muscle satellite cells", are tissue-specific monopotent stem cells in skeletal muscle that play central roles in postnatal muscle growth and regeneration. Similar to other stem cells in the body, muscle satellite cells are maintained in an undifferentiated quiescent state in adult skeletal muscle. Loss or dysfunction of satellite cells is observed in neuromuscular disorders and during the aging processes, which result in the loss of myofibers and muscle force. Hence, investigation of the molecular maintenance mechanism of muscle satellite cells is one of the important areas of interests in skeletal muscle biology. In this chapter, we introduce muscle satellite cells and discuss the maintenance mechanisms of muscle satellite cell based on our Hesr1 and Hesr3 results.

Keywords

Skeletal muscle • Stem Cell • Quiescence • Hesr family genes

Introduction

Skeletal muscle is one of the tissues that have a great regenerative potential. Muscle stem cells, which are called "muscle satellite cells", play essential roles in this process. Similar to other stem cells in the body, satellite cells are maintained in an undifferentiated and quiescent state. When

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skeletal muscle is damaged, satellite cells are activated and start to proliferate. In stem cell research, studies of satellite cells have yielded some advantages. First, the location of satellite cells is clearly defined. In uninjured muscle, satellite cells are detected beneath the basal lamina and upon myofibers (Mauro 1961). Second, methods for direct isolation of satellite cells have been established (Fukada et al. 2004). Third, it is known that the skeletal muscle differentiation system is under the control of a powerful cell-fate determining gene, MyoD (Davis et al. 1987). MyoD alone can convert fibroblasts into myogenic cells. Satellite cells do not normally express MyoD protein, but they start to express it after they are activated. Therefore, we can easily distinguish undifferentiated and differentiated satellite cells by MyoD protein expression. Finally, in vivo functional analyses of genes of interest are possible because we can specifically delete them in satellite cells using Pax7-CreERT2 mice (Lepper et al. 2009).

The study of satellite cells began when Alexander Mauro (1961) identified satellite cells in frog muscle by electronic microscopy. Since then, accumulated studies have revealed some of the molecular markers of satellite cells (M-cadherin, c-met, syndecan3, 4, integrin α7, CD34, calcitonin receptor, CXCR4, etc.). However, some fundamental questions about satellite cells remain unanswered. One of them is the molecular mechanism that maintains the satellite cell pool through a lifetime. Loss or dysfunction of satellite cells is considered to be responsible for the loss of muscle mass and force in muscular dystrophies and during aging processes. Therefore, we must determine how satellite cells are maintained in an undifferentiated and quiescent state throughout the lifetime, and establish a therapeutic methodology to treat these diseases. To reveal the answer, we identified 'quiescence genes', which are highly expressed in quiescent satellite cells but downregulated after activation/proliferation. In this chapter, we introduce muscle satellite cells including their characteristics as stem cells, their contribution to diseases, their cell determination genes and inhibitory factors, and discuss the molecular mechanism by which two 'quiescence genes', Hesr1 and Hesr3 maintain satellite cells in an undifferentiated and quiescent state.

Muscle Satellite Cells as Stem Cells

During embryonic development, satellite cells first appear in the limbs of the mouse embryo between embryonic days 16–18 (E16-18) and actively proliferate and fuse with myofibers to accumulate myonuclei of myofibers. During postnatal development as well, satellite cells divide to provide new myonuclei to growing muscle fiber (Moss and Leblond 1971). It is reported that this contribution of satellite cells to the increase in myonuclei continued until postnatal day 21 (P21) (White et al. 2010). Paired box 7 (Pax7) is specifically expressed in muscle satellite cells, and Pax7-null mice exhibit an almost complete lack of satellite cells in their muscles (Seale et al. 2000). In these mice, skeletal muscle growth is dramatically diminished. Therefore, satellite cells are essential for skeletal muscle growth. White et al. also indicated that the number of satellite cells is constant after P21. Intriguingly, the requirement for Pax7 in satellite cells is restricted until P21. Thus, P21 seems to be a turning point for muscle satellite cells and myofibers.

During postnatal development, perhaps after P21, satellite cells become quiescent, and satellite cells are maintained in an undifferentiated and quiescent state in adult skeletal muscle. When activated by muscle damage, they proliferate, differentiate, and fuse with each other to make myotubes. Mature myofibers are eventually rebuilt by maturation of myotubes. In the past 15 years, many studies have demonstrated that stem cells distinct from satellite cells contribute to making myofibers (Ferrari et al. 1998). However, two recent independent studies have indicated that skeletal muscle regeneration absolutely requires Pax7-expressing cells. Using Pax7-CreERT2 and DTA (diphtheria toxin A) mice, Fan's or Galy's team depleted Pax7-expressing cells and then injured skeletal muscles. In the skeletal muscles, a complete lack of regenerative potential was observed (Lepper et al. 2011; Sambasivan et al. 2011). Because Pax7 is specifically expressed in muscle satellite cells, these studies indicated the absolute necessity of satellite cells in skeletal muscle regeneration. In addition, it is reported that a single satellite

cell contributes to both myofiber generation and the satellite cell pool (Sacco et al. 2008). Therefore, the functions and characteristics of satellite cells satisfy the criteria of stem cells, and there is no doubt that satellite cells are physiological stem cells for the purpose of skeletal muscle homeostasis.

Previous studies have reported that muscle satellite cells can differentiate into the mesenchymal lineage: adipocytes, osteogenic cells, and myofibroblasts. Therefore, satellite cells were considered to be multipotent stem cells in skeletal muscle. However, using highly purified satellite cells, our group demonstrated that satellite cells could not differentiate into adipogenic cells (Uezumi et al. 2010). Instead, skeletal muscle resident PDGFRα+ mesenchymal progenitors contribute to ectopic fat formation in skeletal muscle. Clonal analyses also indicated that PDGFRα+ mesenchymal progenitors could differentiate into adipocytes, osteogenic cells, and myofibroblasts (Uezumi et al. 2010, 2011, unpublished data). Therefore, satellite cells are considered to be the monopotent stem cells that are fated specifically produce myogenic-lineage cells in the physiological condition.

Muscular Disorders and Muscle Satellite Cells

The representative disease of skeletal muscle is muscular dystrophy. Among the muscular dystrophies, Duchenne muscular dystrophy (DMD), which is caused by mutations of the dystrophin gene, is the most common inherited muscular disorder. A lack of dystrophin causes instability of the muscle membrane and leads to muscle degeneration. In DMD patients, satellite cells and their progeny, myoblasts, are thought to be exhausted by repeated cycles of degeneration and regeneration. It has been considered that the exhaustion of satellite cell numbers and/or performance leads to the loss of regenerative potential and results in the loss of myofibers and muscle force.

Satellite cells are also expected to provide a source of cells for cell therapy in DMD. In a mouse model, the transplantation of satellite cell-derived myoblasts produced many dystrophin-positive myofibers (Partridge et al. 1989), and this therapeutic approach had been expected to treat DMD patients. However, transplantation of cultured myoblasts into humans DMD patients has not been successful. Our group compared the in vivo muscle reconstitution potential of satellite cells and cultured satellite cell-derived myoblasts, and demonstrated their loss of in vivo myofiber-producing potential following in vitro culture (Ikemoto et al. 2007). These results clearly indicated that the cultured myoblasts did not possess the in vivo regenerative potential of satellite cells. Therefore, at present we cannot use the bona fide potential of satellite cells for cell therapy after expanding them in vitro.

Our bodies change with age, and a reduction in muscle mass is observed. This decrease of muscle mass is referred to as ‘sarcopenia’. Age-related loss of muscle mass is inevitable, and likely contributes to the decline of muscle strength with aging. Sarcopenia differs from acute disuse atrophy (bed rest, space flight, immobility, etc.) in several points. Among them, a decreased in the number of myofibers is one characteristic of sarcopenia but not of acute atrophies. As described above, muscular dystrophy patients also exhibit a reduction of myofiber numbers. A myofiber is a terminally differentiated cell and cannot produce new myofibers; instead, the satellite cell is the physiological cell source for newly generated myofibers. Therefore, the loss or dysfunction of satellite cells is considered to be one cause for the progression of these diseases. Several studies have indicated a decreased number of satellite cells, especially in sarcopenic muscle. Hence, there is a possibility that a loss of satellite cells leads to the development of sarcopenia. Why are the numbers or functions of satellite cells decreased in these diseases? We do not currently know the answer. To overcome this problem, we have to learn more about the physiological molecular regulation of satellite cells. In the next section, we will focus on the genes that are essential for skeletal muscle development and discuss the relationship between them and satellite cells.

Myogenic Regulatory Factors and Muscle Satellite Cells

MyoD is the myogenic determination gene that converts fibroblasts into myogenic cells (Davis et al. 1987). Together, MyoD, Myf5, myogenin, and Mrf4 comprise the myogenic regulatory factors (MRFs), a group of skeletal muscle-specific basic helix-loop-helix transcriptional factors, that play essential roles in myogenic differentiation. Myogenin and Mrf4 are critical for the late stages of myogenic differentiation (Sabourin and Rudnicki 2000), but satellite cells do not express either myogenin and Mrf4 in their dormant state. MyoD and Myf5 are thought to direct the myogenic program during embryonic development because, in these double-mutant mice, myogenic progenitors retain their capacity for multipotent differentiation and can change a cell's fate. It is reported that Mrf4 is also works as a myogenic determination gene during embryonic development (Kassar-Duchossoy et al. 2004).

On the other hand, when activated, satellite cells initially express MyoD. MyoD-null mice do not show any developmental or morphological abnormality. However, when their muscles are injured, MyoD-null mice exhibit a severe regenerative defect. In addition, MyoD-null myoblasts show a striking reduction of proliferating myogenic cells during regeneration in vivo (Megeney et al. 1996). In contrast, the phenotype of Myf5-null muscle is much milder than that of MyoD-null muscle when muscles are injured (Gayraud-Morel et al. 2007). Thus, the role of Myf5 in regeneration processes seems to be weak compared with MyoD. In addition, recent study demonstrated that most satellite cells express Myf5 protein in their quiescent state (Gayraud-Morel et al. 2012). Intriguingly, Myf5 heterozygous muscle satellite cells are more primed for myogenic commitment than wild-type satellite cells. Therefore, both Myf5 and MyoD are myogenic determination genes during embryonic development, but the suppression of MyoD in quiescent satellite cells seems to be essential for maintaining the satellite cell pool in an undifferentiated state. Myf5, on the other hand, may play a role in maintaining the myogenic cell fate in adult satellite cells.

Myod and Their Inhibitory Molecules

As described above, satellite cells do not express MyoD proteins until activation. Therefore, the molecular regulation of MyoD is considered to be the central pathway to maintaining satellite cells in an undifferentiated state. MyoD dimerizes with E-proteins (E12, E47, and HEB), which are ubiquitously expressed throughout the body. The MyoD/E-protein complex binds to E-box sequences (CANNTG) in the promoters of the target genes, thereby driving the transcription of muscle-related genes. MyoD upregulates not only muscle related genes such as myogenin and myosin heavy chains, but also cell cycle regulators. MyoD promotes the exit from quiescence by binding to the promoter of cdc6, a regulator of cell cycle progression (Zhang et al. 2010). Hence, it might be essential to suppress the expression and functions of MyoD in order to maintain satellite cells not only in an undifferentiated, but also in a quiescent state.

Several molecules that inhibit muscle differentiation in vitro by controlling MyoD have been reported. Inhibitor of DNA binding (Id) protein binds to MyoD and/or E-proteins. Because Id lacks the DNA-binding region, the complexes lack transcriptional activity and act as a repressor of the muscle differentiation program (Benezra et al. 1990). MyoR, a muscle-restricted basic helix-loop-helix transcription factor, forms a complex with E12 and acts as transcriptional repressor that blocks the actions of MyoD and the myogenic program (Lu et al. 1999). MyoD possesses two E-box sequences in its promoter, so MyoD expression is regulated by a positive feedback loop (Zingg et al. 1994). Therefore, these molecules may play roles in the downregulation of MyoD expression.

In addition to these proteins, several signaling pathways that inhibit muscle differentiation have been reported. Transforming growth factor-β (TGF-β) inhibits expressions of MRFs and myotube formation. TGF-β binds its receptor, a

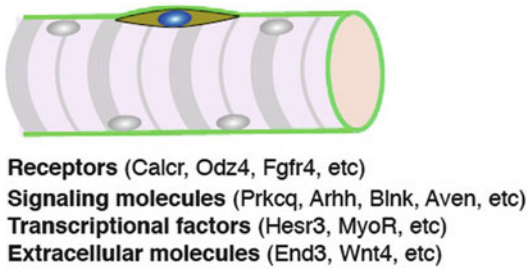

Fig. 9.1 Summary of 'quiescence gene' in muscle satellite cells

heteromeric complex of serine/threonine kinase receptors, and phosphorylates the C-terminals of Smad2 and Smad3. It is demonstrated that Smad3, but not Smad2, inhibits myotube formation (Liu et al. 2001). Smad3 interacts with MyoD in vitro, and this interaction results in the suppression of MyoD transcriptional activity by inhibiting formation of the MyoD/E-protein complex and its binding to E-box DNA sequences. Bone morphogenic protein (BMP) is also known as an inhibitory molecule that suppresses MyoD expression (Murray et al. 1993). Currently, the molecular mechanism by which BMP suppresses MyoD remains unclear.

The Notch signaling pathway controls cell fate determination and differentiation, making it essential for many aspects of embryonic development, as exemplified by a variety of mouse knockout studies. It is well known that Notch signaling inhibits myogenic differentiation and MyoD expression (Kuroda et al. 1999). Therefore, this signaling pathway is also a strong candidate for the suppressor of MyoD in adult satellite cells. In the next section, we introduce our results that identified the 'quiescence genes' by comparing mRNA expressions of quiescent and activate/proliferating satellite cells, and describe the primary target genes of Notch signaling.

Molecular Signature of Muscle Satellite Cells

The discovery of expression-markers of satellite cells has made us easy to identify them by microscopy. However, information about satellite cells has been limited. As in the investigation of other stem cells, development of a cell sorting methodology made it possible to freshly isolate satellite cells and identify the genes expressed by satellite cells using microarray analyses. There have been some isolation methodologies that can isolate muscle satellite cells directly using a cell sorter. We first directly isolated muscle satellite cells with SM/C-2.6, a newly developed monoclonal antibody (Fukada et al. 2004). Using SM/C-2.6 antibody, we sorted muscle satellite cells (quiescent satellite cells) from uninjured muscle, and identified quiescent stage-specific genes by comparison with activated/proliferating satellite cells (myoblasts) and non-myogenic cells in skeletal muscle (Fukada et al. 2007) (Fig. 9.1). In fact, quiescent stage-specific genes are dramatically decreased in satellite cells after their activation and/or proliferation, and they are specifically or highly expressed in satellite cells in skeletal muscle.

We found that MyoR and BMPs are strongly expressed in quiescent satellite cells, but not in proliferating myoblasts. In addition, the Hesr3 gene is highly expressed in quiescent satellite cells. Hesr3 is known the direct target of Notch signaling that inhibits MyoD expression. The expression of Hesr3 is barely detected in activate/proliferating satellite cells, and in skeletal muscle, Hesr3 mRNA and protein are only detected in satellite cells (Fukada et al. 2007). Therefore, Hesr3 is one of the best candidates for maintaining satellite cells in an undifferentiated state by suppression of MyoD expression.

Expression of Notch Effector Genes in Muscle Satellite Cells

As primary targets of Notch signaling, the *Hes* (hairy and enhancer of split) and *Hesr* (hes-related, also known as Hey/Herp/Hrt/Gridlock/Chf) families of basic helix-loop-helix (bHLH) transcriptional repressor genes are well known. Among Hes and Hesr family genes, it has been reported that overexpression of Hes1 or Hesr1 inhibits MyoD expression in myogenic cells. Therefore, Hes1 and/or Hesr1 are considered effectors of MyoD inhibition in Notch signaling.

In our microarray analyses, Hesr1, as well as Hesr3, was expressed in quiescent satellite cells and downregulated in proliferating myoblasts (Fukada et al. 2011). Endothelial cells also express Hesr1, and therefore, Hesr1 was not specific to quiescent satellite cells in skeletal muscle. On the other hand, Hes1 was barely detected in quiescent satellite cells. In addition, in primary myogenic cells, *Hesr1* and *Hesr3*, but not *Hes1* and *Hesr2*, genes were induced by a Notch ligand (Dll1: Delta-like1) (Fukada et al. 2011). It is also reported that *Hesr1* and *Hesr3* were predominantly induced by stimulation of C2C12 cells with Dll4, one of the Notch ligands, while *Hesr2* and *Hes* family gene inductions were not observed (Buas et al. 2009). Therefore, in myogenic cells, *Hesr1* and *Hesr3* seem to be major downstream targets of Notch signaling (Fukada et al. 2011).

Roles of Hesr1 and Hesr3 in Muscle Satellite Cells

Several groups have generated Hesr1 and Hesr3 knockout mice. In contrast to Hes1 and Hesr2 knockout mice, Hesr1 and Hesr3 knockout mice do not show abnormal fertility, life expectancy, or any major developmental defect (Fischer and Gessler 2007). Skeletal muscle development and regeneration potential are normal in Hesr1 and Hesr3 single knockout mice. However, when both Hesr1 and Hesr3 are depleted (dKO), the body size and weight are decreased compared with wild-type and single knockout mice (Fukada et al. 2011). The proportion of skeletal muscle weight to body weight is also decreased in dKO mice. These results implied the functional importance of Hesr1/Hesr3 in muscle lineage cells.

In fact, dKO mice exhibited dramatically decreased numbers of muscle satellite cells, and the number of dKO muscle satellite cells gradually decreased with age. Furthermore, an age-dependent regeneration defect was observed in dKO mice. These results demonstrated the essential role of Hesr1 and Hesr3 in muscle satellite cells. There are several key molecules that affect the satellite cell pool. Pax7 is one of the most widely accepted functional molecules. As described above, Pax7-deficient mice exhibit a severe loss of satellite cells and regeneration potential (Seale et al. 2000). In addition, it is reported that the lack of Pax7 in satellite cells leads to apoptosis during postnatal development (Relaix et al. 2006). However, using Pax7 flox and Pax7-Cre-ERT2 mice, it was demonstrated that Pax7 expression until day 21 after birth is crucial for maintenance of the satellite cell pool (Lepper et al. 2009). In contrast, the absence of Pax7 after P21 does not affect satellite cell number and skeletal muscle regeneration. Thus, Pax7 is essential for satellite cells until P21, but satellite cells do not require Pax7 in adulthood in mice. In addition, Pax7 does not suppress MyoD expression (Zammit et al. 2006).

Sox15 and p38-gamma-null mice also exhibit diminished satellite cell pools (Gillespie et al. 2009; Meeson et al. 2007). However, the relationships between the undifferentiated quiescent state and these molecules in satellite cells are unknown. On the other hand, Hesr1/Hesr3 dKO mice exhibit abnormal expression of myogenic genes and proliferative markers in adult satellite cells. Although MyoD protein is usually not detected in adult muscle satellite cells, approximately 60% of dKO satellite cells express MyoD protein in adult skeletal muscle. Myogenin, known as a downstream target of MyoD, is also absent in adult satellite cells, but its expression is also observed in dKO satellite cells of adult skeletal muscle. Where do these MyoD- or myogenin-positive satellite cells go? Intriguingly, these satellite cells do not exhibit cell death, but are incorporated into myofibers.

Two independent groups demonstrated the roles of Rbp-j (also known as Cbf1) in muscle satellite cells. When Notch is activated, the intracellular domain of Notch is cleaved by γ-secretase and translocates to the nucleus where it activates transcription of target genes through interaction with Rbp-j, and the Hes and Hesr family genes are transcribed. When Rbp-j was deleted in adult satellite cells in Pax7-CreERT2: Rbp-j flox/flox mice, satellite cells start to express MyoD and myogenin proteins (Bjornson et al. 2012; Mourikis

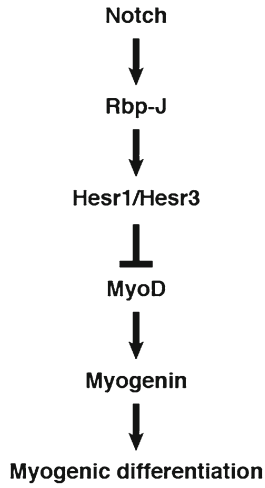

Fig. 9.2 Hypothetical maintenance mechanism for keeping satellite cells in undifferentiated state

et al. 2012). These MRF-expressing satellite cells exhibit premature differentiation, and the pool of satellite cells is diminished. The observation of conditional Rbp-j-null satellite cells was very similar to that of dKO satellite cells. Although it is necessary to investigate Hesr1/Hesr3 conditional null mice, these results strongly suggest that the Notch/Rbp-j/Hesr1/Hesr3 pathway plays central roles in the suppression of MyoD, in other words, the undifferentiated state of muscle satellite cells. In addition, the results of Rbp-j mutated mice also indicate the importance of Hesr1 and Hesr3 as the downstream target of canonical Notch signaling (Fig. 9.2).

Induction of Hesr1 and Hesr3 in Muscle Satellite Cells

As described above, Notch signaling is thought to be active in muscle satellite cells. Satellite cells are located beneath the basal lamina and are adjacent to only myofibers. The canonical Notch signaling pathway requires direct cell-cell interaction; therefore, the myofiber is a candidate cell for expression of Notch ligands. However, such evidence has not been reported. It is reported that DLL1 is shed by ADAM (a disintegrin and metalloproteinase) in undifferentiated myogenic cells in vitro (Sun et al. 2008), and muscle satellite cells locate in proximity to endothelial cells in normal skeletal muscle (Christov et al. 2007). Therefore, there is a possibility that Notch ligand released from endothelial cells or satellite cells might act on satellite cells in a paracrine or autocrine manner.

Intriguingly, the ligand-independent activation of Notch signaling is reported in Drosophila blood cells (Mukherjee et al. 2011). Sima protein, an ortholog of mammalian hypoxia-inducible factor-a (HIF-a), colocalized with Notch in endocytic vesicles and enabled the cleavage of the intracellular domain of Notch. It is also demonstrated that HIF-α interacts with the Notch intracellular domain and promotes the expression of Notch target genes (Gustafsson et al. 2005). The role of HIF-α in muscle satellite cells is still unknown, but these pathways may be used to activate Notch signaling in muscle satellite cells.

Molecular Mechanisms of Hesr1 and Hesr3 in Muscle Satellite Cells

Defects of both Hesr1 and Hesr3 lead to the impairment of the undifferentiated quiescent state of satellite cells in vivo. It has been believed that both Hesr1 and Hesr3 recruit a co-repressor, which is therefore considered a transcriptional repressor. How do Hesr1 and Hesr3 control the undifferentiated state of satellite cells? The simplest mechanism is that Hesr1 and Hesr3 directly suppress MyoD transcription. However, intriguingly, defects of both Hesr1 and Hesr3 do not lead to an increased MyoD mRNA level (unpublished data). Even in Rbp-j-null satellite cells, upregulation of MyoD mRNA was not observed (Mourikis et al. 2012). MyoD protein is not expressed in quiescent satellite cells, but abundant MyoD mRNA is detected even in the quiescent state, in contrast with previous study. Therefore, Hesr1 and Hesr3 may regulate the translation or stability of MyoD protein via an unknown factor in muscle satellite cells. To reveal the importance of MyoD as a target of Hesr1/Hesr3,

the study of Hesr1/Hesr3/MyoD triple-null mice will be necessary. Elucidation of the Hesr1/Hesr3-mediated MyoD suppression mechanism will reveal the mechanism by which satellite cells are maintained in an undifferentiated and quiescent state.

Mice with a single knockout of Hesr1 or Hesr3 have no significant difference in skeletal muscle phenotype, probably due to the redundancy of each gene. When Hesr1 or Hesr3 are expressed in proliferating myoblasts, Hesr1 strongly suppresses MyoD expression and myotube formation, while the effect of Hesr3 is mild. The reason for this discrepancy between in vivo and in vitro results is still unclear. It may simply reflect the fact that the expression of Hesr3 is much higher than that of Hesr1. It is known that Hesr proteins dimerize with one another, and Hes or Hesr family genes bind via their helix-loop-helix motif to E-box or N-box elements within the DNA via their basic domain. Like other bHLH genes, Hesr family proteins require a corepressor to suppress their target genes (Fischer and Gessler 2007). Hesr1 and Hesr2 recruit corepressors such as mSin3A, N-CoR, and histone deacetylase 1 via C-terminal YRPW tetrapeptides. Hesr3 has a further degenerated YXXW sequence, but the cofactors of Hesr3 have not been identified. The co-repressor and/or binding partner for Hesr3 might not be expressed in myoblasts. Therefore, the identification of a co-repressor and/or binding partner of Hesr1 and Hesr3 might be necessary to reveal the Hesr1/3-dependent MyoD suppression mechanism in satellite cells.

Perspective

Our results and those of others strongly suggest that Notch/Rbp-j/Hesr1/Hesr3 is a central axis for maintaining satellite cells in an undifferentiated and quiescent state. This cascade is the first evidence demonstrating a molecular mechanism for maintaining satellite cells. Intriguingly, a decrease of the Notch signaling activator is reported in the serum of aged mice (Conboy et al. 2003). The low Notch signaling leads to a decrease in satellite cell proliferation and results in the loss of regenerative potential. The relationship between this condition and the undifferentiated state of satellite cells is unclear, but there is a possibility that low Notch signaling affects the number or state of aged satellite cells as well as their proliferation. In this case, further investigation of Hesr1/Hesr3 might lead to identification of the molecular target, leading to a treatment for sarcopenia and other muscle diseases.

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The Kinase MIRK/DYRK1B Mediates a Reversible Quiescent State in a Subset of Ovarian, Pancreatic and Colon Cancers

10

Eileen A. Friedman

Contents

Abstract

The serine/threonine kinase Mirk is an active kinase in pancreatic, ovarian and colon cancers, but is not activated by mutation. Mirk was upregulated or amplified in the majority of resected pancreatic or ovarian adenocarcinomas, and may be selected for by enabling cancer cells to enter a reversible quiescent state and thus survive suboptimal conditions. Mirk/dyrk1B levels and activity are highest when cells are quiescent. Some cancer cells can enter a reversible quiescent phase dependent on p130/Rb2 and Mirk/dyrk1B when deprived of growth factors, while others undergo autophagy or apoptosis. Mirk blocks cell cycle progression in G0 by complexing with GSK3ß and destabilizing cyclin D isoforms and by activating by phosphorylation Lin52, which is part of the DREAM complex including p130/Rb2 which sequesters E2F4 and other transcription factors necessary for cells to enter cycle. Mirk transcriptional coactivator activity allows Mirk to decrease ROS levels by increasing expression of a group of antioxidant genes. Since Mirk is activated by oncogenic K-ras/H-ras, its upregulation of antioxidant genes may compensate for the increase in ROS induced by ras oncoproteins. Mirk competes with the SAPK p38 for binding to their common activator MKK3. Thus Mirk is upregulated or amplified in certain pancreatic and ovarian cancers, is an active kinase in these cancers, and under suboptimal

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growth conditions, maintains these cancer cells in a viable, quiescent state.

Keywords
Mirk/dyrk1B • ROS • ras

Introduction

Mirk/dyrk1B is a member of the Minibrain/dyrk family of kinases which mediate survival of differentiating cells in certain normal tissues: skeletal muscle (Mirk/dyrk1B) through blocking cell cycling and aiding the expression of MEF2, neuronal cells (Dyrk1A), erythropoietic cells (Dyrk3) and sperm (Dyrk4). Our group initially cloned Mirk (minibrain-related kinase)/dyrk1B from human colon cancer cells and then by stably overexpressing wild-type Mirk, demonstrated its capacity to mediate colon cancer cell survival under serum limited conditions, whereas kinase-dead mutant Mirk had no survival capacity (Lee et al. 2000). Mirk was an active kinase in colon cancer cells, but was not mutated. Mirk was most abundant and active as a kinase in G0 cells in pancreatic cancer, colon cancer, and ovarian cancer cultures (Friedman 2007).

Mirk Destabilization of Cyclin D

Mirk blocked cycling of tumor cells in a quiescent G0 state by destabilizing the G1 cyclins, cyclin D isoforms, by phosphorylation at a conserved ubiquitination site T288 (Zou et al. 2004). During in vivo phosphorylation experiments in both Mv1Lu and NIH3T3 cells, Mirk potently phosphorylated a construct mutated at the GSK3ß site, cyclin D1-T286A, but not T288A constructs (Zou et al. 2004). Both cyclin D1 residues, T286 and T288, are important for its ubiquitination, and the half-life of cyclin D1-T286A/T288A was three times that of wild-type (Germain et al. 2000). Expression of cyclin D1 mutant at the Mirk phosphorylation site, T288A, enabled serum-starved HD6 colon cancer cells to escape quiescence and move into cycle (Jin et al. 2009). Likewise, depletion of Mirk by RNA interference (Deng et al. 2009) or pharmacological inhibition of Mirk kinase activity (Ewton et al. 2011) allowed pancreatic cancer cells to escape the quiescent state by increasing their expression of cyclin D isoforms. The Mirk site of T288 is directly adjacent to the GSKß site of T286, and Mirk and GSK3ß can be co-immunoprecipitated, indicating that they form a complex. Thus, Mirk may act as a priming kinase for GSK3ß in phosphorylation and destabilization of cyclin D isoforms.

Mirk Elevated in Quiescent Cells

Mirk protein levels rise in cultures enriched in quiescent G0 cells, and are seven to tenfold higher than in cycling cultures by western analysis (Deng et al. 2004). Examination of sectioned pancreatic, colon or ovarian cancers by immunohistochemistry for markers of cycling cells such as Ki67 has revealed that the fraction of cycling cells is low. For example, in a series of 90 ovarian cancers the proliferative fraction had a median value of only 30% (Schindlbeck et al. 2007), while in a series of pancreatic cancers nuclear Ki67 was found in an average of only 28% of cells (Stanton et al. 2003). A subset of the nondividing cells may be in a quiescent state. Mirk reactivity and nuclear Ki67 were assayed on the same human ductal pancreatic adenocarcinomas in serial sections and were mutually exclusive, showing that Mirk was expressed in the nondividing fraction of tumor cells which would include any quiescent cells (Deng et al. 2009).

Mirk Amplified in Cancers and Activated by Oncogenic Ras

Both pancreatic ductal adenocarcinoma and serous ovarian adenocarcinoma lack effective treatment strategies. Pancreatic cancer is the fourth deadliest cancer in the United States, even though it ranks 11th in incidence. Epithelial carcinoma of the ovary is one of the most common gynecologic malignancies and the fifth most frequent cause of cancer death in

women (NIH database). The Mirk/dyrk1B kinase gene is part of the 19q13 amplicon found in pancreatic cancer (Karhu et al. 2006) and ovarian cancer (Thompson et al. 1996). Immunohistocytochemical analysis revealed that the Mirk protein is expressed in about 90% of pancreatic cancers (Deng et al. 2006) and about 75% of ovarian cancers (Hu et al. 2011). Furthermore, the Mirk gene has been localized to the 660 kb core region of the 19q13 amplicon which contains about 20 genes, only four of which might have some role in tumor growth (Mirk/dyrk1B, MED29, PAF1 and PAK4) (Kuuselo et al. 2007). The 660 kb core amplicon is found in 12.2% of all primary pancreatic cancers, but increases to 33.3% of the more advanced T3 and pT4 tumors, as well as in lymph node metastases and distant metastases (Kuuselo et al. 2010). Mirk is activated by signaling from activated Rac1 to MKK3 (Jin et al. 2005) or oncogenic K-ras or H-ras to Rac1 to MKK3 (Jin et al. 2007) and was an active kinase in pancreatic, ovarian or colon cancer cell cultures assayed by the immune complex kinase reaction. Mirk is an active kinase in pancreatic cancers in vivo where it restricts Hedgehog initiated Gli1 activity to the stromal compartment (Lauth et al. 2010). Significantly, Mirk maintains the viability of the most aggressive subset of pancreatic cancer cells that can undergo clonal growth and that should include the tumor stem cells (Jin et al. 2007).

Reactive Oxygen Species (ROS) in Quiescence

Factors that allow the prolonged survival of quiescent tumor cells in vivo are of clinical relevance. These include antioxidant proteins and factors which control their expression (Chen et al. 2008) such as Mirk, which decreases the level of toxic reactive oxygen species (ROS) in tumor cells, increasing their survival (Deng et al. 2009) and their clonogenic growth (Jin et al. 2007). ROS are constantly generated as cells grow, and increased growth rates and oncogenes like H-ras and Bcr-abl raise ROS levels in tumor cells (Trachootham et al. 2006). We hypothesize that tumor cells enter a quiescent state because of damage resulting from oxidative stress, with a few percent entering G0 at each cell cycle. After repair, cells can then re-enter the cell cycle if nutrients are available. Mirk maintains the viability of repaired cells in G0 by reducing ROS levels through activating expression of antioxidant genes, among them ferroxidase and SOD2 in some pancreatic cancers (Deng et al. 2009) and ovarian cancers (Hu and Friedman 2010).

Cycling Ovarian Cancer Cells Enter G0

The presence of quiescent G0 tumor cells has been controversial. Quiescent cells degrade their ribosomes allowing G0 cells to be identified by their lower RNA content than G1 cells, but 2N DNA content. Cellular DNA was stained with Hoechst 33258, Pyronin Y then added to bind to RNA, and both fluorochromes measured by two parameter flow cytometry. The BJ strain of human normal diploid fibroblasts maintained in log phase growth had a low fraction (10%) of cells in G0 while serum-starvation placed the majority of cells (66%) in G0 as noted by others (Coller et al. 2006). Five ovarian cancer cell lines were assayed during log phase growth to determine whether any cells cycled into a G0 state even when the majority of cells were proliferating. Surprisingly, OVCAR3, SKOV3, TOV21G, OVCAR4 and OV90 cultures each contained a fraction of cells in G0. These averaged 17 ± 3% (SD) for SKOV3 cultures, 17 ± 4% for TOV21G cultures, 38 ± 2% for OVCAR3 cultures and 20 ± 2% for OVCAR4 and OV90 cultures (Fig. 10.1a and other data not shown). OVCAR3 cultures with the highest fraction of G0 cells and an amplified Mirk gene (Hu et al. 2011) proliferated the slowest. Reflecting their lower fraction of G0 cells, SKOV3 and TOV21G cultures grew over twice as fast as OVCAR3 cultures (Fig. 10.1b), as did OVCAR4 and OV90 cultures (data not shown). Thus the entry of cells into G0 lowers the fraction of cycling cells within the culture, increasing the average doubling time.

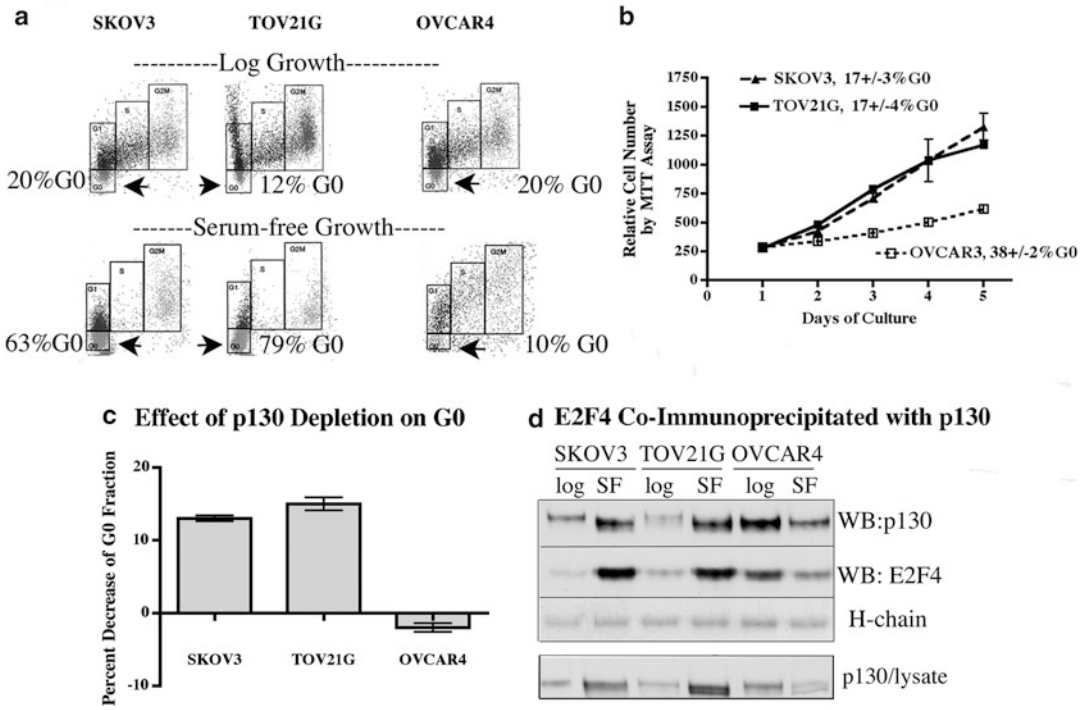

Fig. 10.1 Ovarian cancer cell cultures contain a fraction in G0. **a** Log phase SKOV3, TOV21G, and OVCAR4 ovarian cancer cells, then grown serum-free medium 3 days before cell cycle analysis by two parameter flow cytometry, with G0 cells indicated by arrows. **b** Log phase cells replated at similar cell densities, and cultured in growth medium, with relative cell numbers determined by MTT assay +/− SD (n = 6). **c** Cells depleted of p130, made quiescent, and the fraction of cells in G0 then determined as above, mean +/−SE. **d** Western blot of immunoprecipitated p130/Rb2, E2F4 bound to immunoprecipitated p130/Rb2, and total p130/Rb2 and antibody heavy chain in the respective lysates from cells log phase or serum-starved 3 days

Stress Conditions Increase G0 Cells

TOV21G, SKOV3, OVCAR3, OVCAR4 cells, and OV90 cells were cultured for 3 days either in serum-free DMEM or maintained as log phase cultures in DMEM plus 7% FBS. Culture under serum-free conditions increased the fraction of G0 cells an average of 3.2 (±0.2) fold for SKOV3 cultures, 6.5 (±0.4) fold for TOV21G cultures, and 1.5 (±0.1) for OVCAR3 cultures but did not increase the fraction of G0 cells in OVCAR4 or OV90 cultures (Fig. 10.1a and other data not shown). Similar results were found when cells were cultured to high density.

Were the cells in G0 in the ovarian cancer cell lines terminally arrested, undergoing apoptosis or were they in a transient quiescent state like dormant tumor cells in vivo? SKOV3 cells were accumulated in G0 by serum-free culture for 2 days, then replated at lower density in fresh serum-free growth medium containing the mitotic inhibitor nocodazole. Within 2 days, about 90% of the cells had exited the quiescent G0 state, traversed G1 and S and had been arrested in G2+M by nodocazole, without the appearance of a sub-G1 peak of apoptotic cells (Hu et al. 2011). Thus quiescent SKOV3 cells can eventually re-enter the cell cycle in the presence of fresh nutrients showing that G0 arrest is transient.

Quiescence Markers in G0 Cancer Cells

Possibly a molecular defect in OVCAR4 and OV90 cells prevented their accumulation in G0 under suboptimal culture conditions. The retinoblastoma protein family member p130/Rb2 sequesters the

E2F4 transcription factor, preventing progression of mammalian cells from G0 into G1. Consistent with this function, ectopic expression of the p130/Rb2 gene mediated growth arrest of human ovarian cancer cells (D'Andrilli et al. 2004). The protein level of p130/Rb2 is elevated in quiescent cells where p130/Rb2 functions, but is lower in proliferating cells due to turnover (Smith et al. 1998), so an increase in p130/Rb2 levels should occur to facilitate the entry of ovarian cancer cells into G0. A time-course analysis showed that when TOV21G and SKOV3 cells were cultured in serum-free medium for 3 days and cells entered G0, the protein level of p130/Rb2 increased several-fold, while neither increase in p130/Rb2 nor accumulation in G0 occurred in OVCAR4 cells. This difference led to levels of p130/Rb2 sixfold higher in TOV21G cells and fourfold higher in SKOV3 cells than in OVCAR4 cells (Hu et al. 2011). Levels of p130/Rb2 can be controlled through the Foxo3a transcription factor (Kops et al. 2002), or post-translationally through protein stabilization. One or both of these regulations may be aberrant in OVCAR4 cells. Immunohistochemical analysis by others revealed loss or decrease in expression of p130/Rb2 in about 40% of 45 resected human ovarian cancers assayed (D'Andrilli et al. 2004), so the OVCAR4 line may reflect this large subgroup of ovarian cancers with low p130/Rb2 expression.

Mirk protein levels increased four to sevenfold in SKOV3 and TOV21G cells, but not in OVCAR4 cells, cultured under these suboptimal growth conditions. In time-course studies Mirk levels increased within 24 h of the switch to serum-free culture and then continued to increase. Thus an increase in level of Mirk protein was found only when ovarian cancer cells accumulated in G0. The CDK inhibitor p27kip1 helps to maintain the G0 state by binding to CDK/cyclin complexes. Levels of p27 were increased 20-fold when SU86.86 pancreatic cancer cells were made quiescent in G0 (Deng et al. 2009). Levels of p27 differed dramatically between the OVCAR4, SKOV3 and TOV21G cell lines, but were increased several fold in each line by serum-free culture (Hu et al. 2011). The inability of OVCAR4 cells to arrest in G0 could not be ascribed to alterations in abundance or regulation of p27.

Some Ovarian Cancers Defective in G0 Arrest

SKOV3, TOV21G and OVCAR4 cells were cultured in serum-free conditions for up to 6 days. Measurement of relative cell number by MTT assay showed that both SKOV3 and TOV21G cultures grew to a higher cell density than the OVCAR4 cultures that exhibited declining cell numbers after 3 days of serum-free culture (Fig. 10.2a). Analysis of parallel cultures revealed that OVCAR4 cells underwent more apoptosis than TOV21G or SKOV3 cells (Fig. 10.2b). Critical steps in apoptosis are cleavages of poly (ADP-ribose) polymerase (PARP) and caspase 3, which were prominent only in OVCAR4 cells after 4–6 days of serum-free culture. Measurement of cell viability by dye exclusion showed that about 60% of the OVCAR4 cells were nonviable and unable to exclude dye after 4–6 days of serum-free culture, compared with about 30% of SKOV3 and TOV21G cells (Fig. 10.2c). These nonviable OVCAR4 cells then underwent apoptosis (Fig. 10.2b), reducing total cell numbers (Fig. 10.2a). Other suboptimal culture conditions also led TOV21G and SKOV3 cells to accumulate in G0. Cells were cultured for 3 days either in serum-free DMEM, in normal growth medium to high cell density or in low glucose DMEM without FBS. In each case the fraction of G0 cells increased to 60–80% of the culture, while no such increase was seen with OVCAR4 cultures. Thus ovarian cancer cells that could enter a reversible quiescent arrest in G0 were more protected from suboptimal growth conditions than tumor cells that lacked this capacity. In vivo, such quiescent cells could re-enter the cell cycle under favorable clues from the microenvironment.

Mirk and p130/RB2 Required for G0 Viability

The effect of Mirk on viability of ovarian cancer cells under suboptimal growth conditions was determined by depletion of Mirk by two RNAi duplexes, each added independently to a parallel

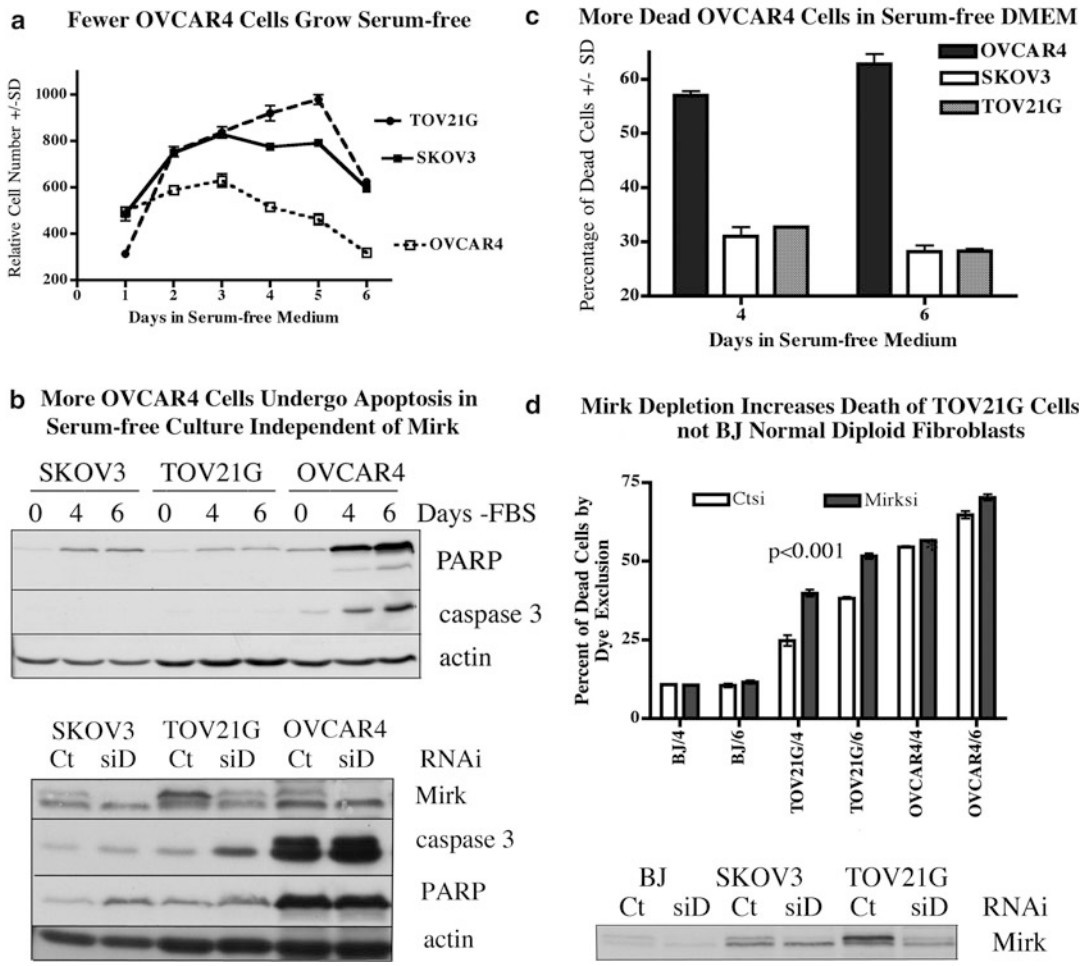

Fig. 10.2 Cultures of OVCAR4 cells with low fractions in G0 under serum-free culture conditions undergo more cell death than cells that can arrest in G0. **a** Cells were plated, and allowed to enter cycle by culture in growth medium for 24 h, then switched to serum-free medium. MTT assay, mean +/−SD shown, n = 2. **b** (*upper panel*) Parallel cultures from panel **a** analyzed by western blotting for markers of apoptosis, cleaved PARP and cleaved caspase 3. (*lower panel*) Cells depleted of Mirk using two different RNAi duplexes independently (siD or siC) or GC-matched controls, then cultured serum-free for 4 days before western blotting for Mirk, actin, cleaved PARP and cleaved caspase 3. Only data with siD shown. **c** The cultures from panel **a** were sampled for the fraction of dead cells incapable of exclusion of trypan blue dye. Mean +/− SD shown, with n = 4 for each point. An average of 488+/−40 cells were assayed per point. **d** TOV21G, OVCAR4 and BJ normal human diploid fibroblasts were depleted of Mirk using two different RNAi duplexes independently (siD or siC) or GC-matched controls as in panel **b**, then cultured serum-free for four and for 6 days before assay of the percentage of dead cells by trypan blue dye exclusion. Mean +/−SD shown (n = 4, with two measurements per RNAi duplex). Student's two-tailed *t* test used to analyze cultures of TOV21G gave $p < 0.001$. (*lower panel*) Western blot shows depletion of Mirk in parallel cultures after 4 days

culture of either TOV21G or OVCAR4 ovarian cancer cells, or the BJ strain of normal human diploid fibroblasts. The Mirk depleted and control-depleted cells were then maintained in serum-free media for 4 or 6 days. About 60% of the OVCAR4 cells, 30% of the TOV21G cells, but only 10% of the normal fibroblasts died under these conditions (average of Ctsi treated cultures, Fig. 10.2d). However, these three cell types differed in their sensitivity to depletion of Mirk.

There was no detectable effect on the viability of normal BJ cells on either day, while Mirk depletion did not further decrease OVCAR4 viability after 4 days of serum-starvation with a marginal effect after 6 days (Fig. 10.2d). In contrast, Mirk depletion led to a mean 50% increase in TOV21G cell death after either 4 or 6 days of serum starvation. In similar studies, Mirk depletion led to increased cleavages of poly(ADP-ribose) polymerase (PARP) and caspase 3 in SKOV3 and in TOV21G cells, not in OVCAR4 cells, indicating that the loss of Mirk led to cell death by apoptosis (Fig. 10.2b, lower panel). Thus, Mirk helps to maintain TOV21G ovarian cells in a viable quiescent state by blocking apoptosis, while having little protective effect for OVCAR4 cells which are not accumulated in G0 by suboptimal growth conditions. In addition, Mirk protein was expressed at much lower levels in normal BJ fibroblasts compared to either SKOV3 or TOV21G cancer cells, and depletion of Mirk in BJ cells led to no detectable increase in cell death (Fig. 10.2d). This data is consistent with earlier studies on Mirk knockout in mice (Leder et al. 2003) and colony formation assays (Jin et al. 2007)that showed a selective sensitivity of cancer cells to Mirk depletion compared with normal diploid cells.

The mechanism for G0 arrest includes the Mirk-mediated reduction of cyclin D1 to prevent escape into G1. Mirk has been shown to slow the exit of SU86.86 pancreatic cancer cells (Deng et al. 2009), and HD6 colon cancer cells (Jin et al. 2009) from G0 quiescence by phosphorylating their cyclin D isoforms at a conserved ubiquitination site that initiates rapid turnover. Mirk was shown to also alter ovarian cancer cell cycling through cyclin D1. Efficient depletion of Mirk in SKOV3, OVCAR3, and TOV21G cells by either of two synthetic RNAi duplexes led to an increase in cyclin D1. Thus Mirk reduced cyclin D1 levels in these cells, restricting their entry into G1 and the cell cycle.

The role of p130/Rb2 in G0 arrest in ovarian cancer cells was examined by depleting p130 in SKOV3, TOV21G and OVCAR4 cells, then placing cells in serum-free medium for 3–5 days in an attempt to induce quiescence. Although the p130 depletion was only partial, fewer p130-depleted SKOV3 and TOV21G cells were found in G0 (Fig. 10.1c). In contrast, depletion of p130 had almost no effect on the G0 fraction of OVCAR4 cells. These data suggest that p130/Rb2 enables TOV21G cells to remain in G0 as part of a stress response, and loss of this capacity for G0 arrest decreases their viability.

When normal diploid cells enter G1 from G0 by addition of mitogens, p130/Rb2 is phosphorylated by G1 cyclin/CDK complexes and other kinases at up to 22 sites, thus freeing the transcription factor E2F4. E2F4 was expressed at similar levels in the three cell lines under different culture conditions (data not shown). However, about fivefold more E2F4 was bound to p130/Rb2 immunoprecipitated from SKOV3 and TOV21G cells arrested in G0 compared with p130/Rb2 from OVCAR4 cells grown under similar conditions (Fig. 10.1d). Thus fewer serum-starved OVCAR4 cells were found in G0 because they did not express enough p130/Rb2 capable of sequestering E2F4 to block entry into G1.

Quiescence is maintained by the DREAM complex (p130/Rb2, E2F4, DP1 and a stable core including the LIN52 protein), which disassembles when cells leave quiescence and enter cycle. In quiescent cells Mirk/dyrk1B and the closely related Dyrk1A phosphorylate the core protein LIN52 at a site necessary for its quiescence function (Litovchick et al. 2011). Mirk was capable of phosphorylating LIN52 in SKOV3 and TOV21G ovarian cancer cells, but its function in OVCAR4 was not determined.

The Stress-Activated Kinase p38 and Mirk Compete

The SAPK p38 regulates a transcription factor network required for tumor cell quiescence (Adam et al. 2009). High levels of p38 coupled with low levels of Erks induce a G0/G1 arrest of HEp3 cells into a dormant state (Sosa et al. 2011). Interestingly, p38 can suppress Mirk function when p38 levels are higher than Mirk levels by competing for their common activator MKK3. The MAPK kinase MKK3 activates Mirk/dyrk1B as a protein kinase (Lim et al. 2002a), implicating Mirk/dyrk1B in the biological response to certain stress agents.

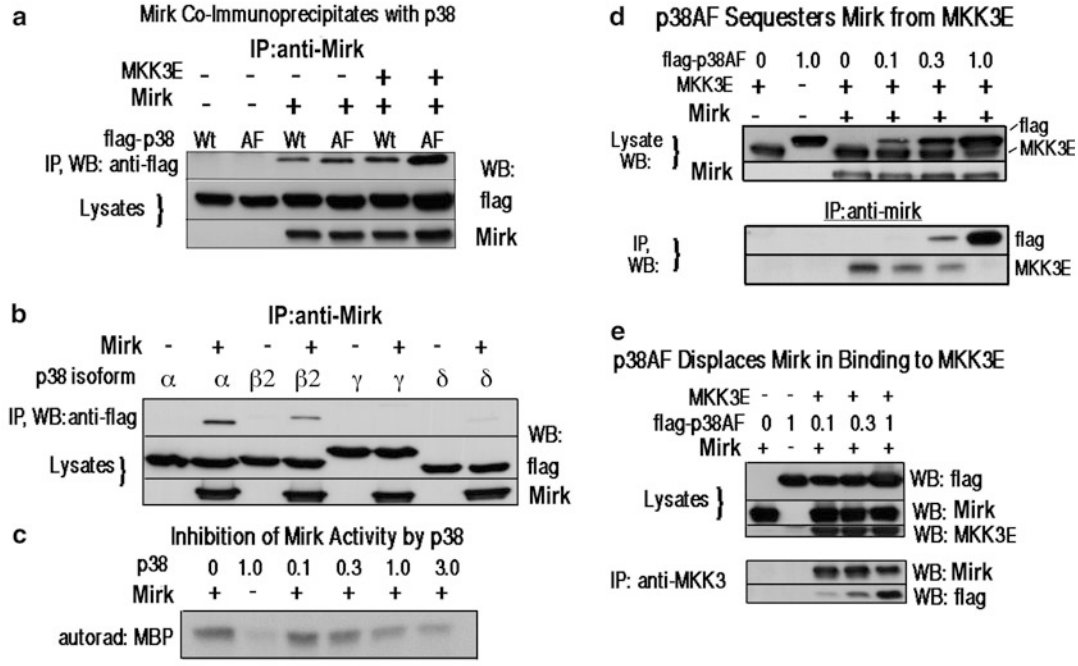

Fig. 10.3 Physical interaction between Mirk and p38 SAPK. **a** 293T cells were co-transfected with either MKK3E, wild-type Mirk, and either flag-p38 wild-type or kinase-inactive flag-p38AF. Mirk and its associated proteins in immunoprecipitates were examined by western blotting for the flag-epitope on the expressed p38 and p38AF proteins. Similar amounts of p38 wild type and p38AF were synthesized in the lysates, and equal amounts of Mirk were found in the appropriate lysates. **b** Physical interaction between Mirk and only the α or ß isoforms of p38 MAP kinase. Equal amounts of p38 SAPK isoforms, α,β2,γ δ, were synthesized in 293T cells following transfection in the presence of co-expressed Mirk or vector DNA (western blots of lysates shown in lower two panels). Immunoprecipitates western blotted for the flag-epitope on the expressed p38 isoforms. **c** Kinase mixtures in vitro contained either recombinant purified Mirk or increasing concentrations of recombinant purified p38, as indicated. Kinases were added to MBP and in vitro kinase assays were performed with [^{32}]P-ATP and analyzed by autoradiography after SDS-PAGE. **d** 293T cells were co-transfected with either MKK3E, wild-type Mirk, and kinase-inactive flag-p38AF. Western blots of total lysates shown in upper two panels. Mirk immunoprecipitates were western blotted for the flag-epitope for complexed p38AF proteins and for complexed MKK3E by antibody to MKK3. **e** 293T cells were co-transfected as above. Western blot of total lysates is shown in upper three panels. The MKK3 immunoprecipitates were western blotted for the flag-epitope for complexed p38AF proteins and for complexed Mirk

Mirk and p38 directly interact in vivo as they co-immunoprecipitate. Flag-epitope-tagged wild-type p38 or dominant negative p38AF were co-transfected into 293T cells with either wild-type Mirk or Mirk plus its activator, constitutively active mutant MKK3E (Fig. 10.3a). Mirk, p38 wild-type and kinase-dead p38AF were synthesized at comparable levels in each experimental mixture, as shown by western blotting of the cell lysates (Fig. 10.3a, lower two lanes) (Lim et al. 2002b). In vivo interaction between p38 and Mirk was demonstrated by their co-immunoprecipitation (Fig. 10.3a). Kinase-inactive p38AF bound Mirk about twice as avidly as wild-type p38. Each of the four isoforms of p38, α,β, γ, δ, were expressed in vivo alone or in the presence of Mirk, and only α and ß were capable of complexing and co-immunoprecipitating with Mirk (Fig. 10.3b).

The complexing of p38 to Mirk blocked Mirk kinase activity. Increasing amounts of purified recombinant non-activated p38 (0.1–3 ug) were mixed together with 1 ug of purified GST-Mirk, and an in vitro kinase assay was performed on myelin basic protein (MBP). Without activation

p38 had little kinase activity on MBP, while Mirk exhibited MBP kinase activity (Fig. 10.3c). Mirk kinase activity was inhibited by roughly equimolar concentrations of inactive p38, consistent with the model that p38 sequesters Mirk. Since p38AF is a potent inhibitor of Mirk activation of HNF1 (Lim et al. 2002b), it is likely that p38 α and ß sequester Mirk, and prevent its activation by MKK3E.

The ability of p38 to sequester Mirk was tested by co-expressing Mirk with MKK3E in the presence of increasing levels of flag-p38AF, and then analyzing the molecules bound to either immunoprecipitated Mirk (Fig. 10.3d) or immunoprecipitated MKK3E by western blotting (Fig. 10.3e). In the absence of p38AF, Mirk bound well to MKK3E (Fig. 10.3d). When the concentration of p38AF increased tenfold, more flag-p38AF and less MKK3E was found associated with Mirk in a dose-dependent manner (Fig. 10.3d, lower panels). Similarly when MKK3E immunocomplexes were analyzed, increasing amounts of p38AF led to the displacement of Mirk (Fig. 10.3e, lower panels). These experiments demonstrated that p38 blocked the association of MKK3E and Mirk in a kinase-independent manner. Synchronization experiments demonstrated that Mirk/dyrk1B levels fluctuate about tenfold within the cell cycle, while p38 levels do not, leading to the speculation that endogenous p38 could only block Mirk function when Mirk levels were low in S phase and not when Mirk levels were elevated in G0/G1. These data suggest a novel cell cycle dependent function for p38, suppression of Mirk functions only when cells are proliferating, and thus limiting Mirk functions to growth arrested cells.

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Part III

Cellular Senescence

Stress-Induced Senescence: Molecular Pathways

11

Peter J. Hornsby

Contents

Abstract

Senescence is an irreversible state of the cell, in which the cell becomes incapable of further cell division. The concept of stress-induced senescence indicates that the major causes of senescence are various types of stresses that act on cells. These stresses act via intracellular pathways, which may be multiple, to a final common state of irreversible cell division. In this state the cell is held in the nondividing state by the combination of cyclin-dependent kinase inhibitor (CDKI) activity, heterochromatin formation, gene expression changes, and other mechanisms. While the mediators of stress-induced senescence are multiple and complex, the p38 MAP kinase pathway has a prominent role in linking stresses to the permanently nondividing state. The means by which it may become activated by stresses and the means by which it acts to cause senescence are being unraveled. While most of the past studies on senescence have used cells in culture, the recent emergence of novel mouse models in which senescence can be studied has opened up exciting avenues toward mechanistic insights and possible therapeutic interventions.

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Keywords

Senescence • Stresses • Cyclin-dependent kinase inhibitors • p38 MAP kinase

Introduction

The defining characteristic of cellular senescence is the inability of the senescent cell to undergo cell division in response to physiological stimuli that normally cause cell proliferation in that cell type. Cellular senescence is one possible end-point as a reaction of cells to various types of stress; it forms one possible outcome of the effects of severe stresses, in addition to various forms of cell death and in addition to full repair of the damage and complete recovery (Kuilman et al. 2010). During the development of the field, and continuing to the present day, senescence has been closely linked with telomere biology: senescence and telomere shortening, the originally identified cause of senescence, have often been considered as a single phenomenon. However, there are many examples of stresses that cause senescence that either have no connection with telomere biology, or at least a nonobvious connection. Additionally, telomere shortening is not invariably associated with senescence. Thus, senescence is now understood to be a general reaction of cells to a wide range of forms of cellular damage. This chapter reviews the basic biology of cellular senescence as it occurs in cell culture and in tissues in vivo, and gives an overview of some of the molecular mechanisms involved.

Cellular Senescence as the End-Point of Cellular Response to Stresses

Almost any stress can potentially cause cells to enter senescence (Fig. 11.1). In fact, many vaguely-characterized phenomena in cultured cells, in which cells have been seen to enter a permanently nondividing state, can now be recognized as examples of cellular senescence. Depending on the nature of the stress, its intensity, and the cell type, the result may be cell death, cellular senescence, or repair and recovery. At least in some cell types, lower levels of damage cause senescence and higher levels cause cell death. Conversely, some cell types are less likely

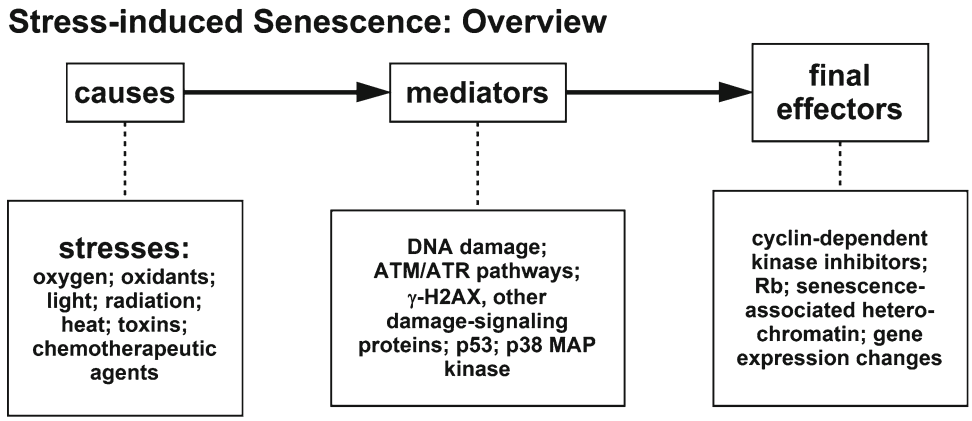

Fig. 11.1 Causes, mediators and effectors of stress-induced senescence. A wide variety of stresses act as initiators of stress-induced senescence, which thereby acts as a common final state of these various forms of damage. These include, but are not limited to, those listed in the diagram. As discussed in this chapter, the mediators by which stresses activate pathways that lead to a state of irreversible cell division are not yet clear. Many act via initiation of DNA damage. These initial actions activate intracellular pathways, including the p53 and p38 MAP kinase pathways, that produce changes leading to an irreversible state of nondivision. The final status of the cells, involving elevated activity of cyclin-dependent kinase inhibitors and heterochromatin changes, are common to senescence that results from stresses, telomere dysfunction, or oncogene activation

to enter senescence under any circumstances and more likely to undergo apoptosis. Even cancer cells, despite multiple abnormalities, can still be forced into senescence under the action of chemotherapeutic drugs (Acosta and Gil 2012). While the defining characteristic of senescence is complete unresponsiveness to physiological stimuli that cause cell proliferation, reinitiation of cell division in senescent cells is possible under some experimental circumstances, other than exposure to appropriate mitogens (Beausejour et al. 2003). Senescence also involves numerous changes in gene expression, but the process is not a form of terminal differentiation as it is normally understood.

The discovery that senescence could be caused by events other than telomere shortening resulted initially from studies of the overexpression of oncogenic Ras in cultured human cells (Serrano et al. 1997). This was the first of many examples in which expression of the activated form of an oncogene was shown to cause senescence, a phenomenon now termed oncogene-induced senescence (Gorgoulis and Halazonetis 2010). Although the range of stresses that are capable of inducing senescence is large, many of them, but possibly not all, involve some type of DNA damage (Fig. 11.1). Activated oncogenes cause DNA damage via replication stress, including perturbation of replication origin firing, rereplication, and delayed fork progression (Ruzankina et al. 2008; Gorgoulis and Halazonetis 2010; Kuilman et al. 2010).

Frequently the term "stress-induced premature senescence" has been used to refer to senescence resulting from cellular events other than telomere dysfunction (e.g., Passos et al. 2010; de Jesus and Blasco 2012; Mirzayans et al. 2012). It would be more appropriate to refer to the process as "rapid" senescence rather than "premature" senescence. Here, the process is simply called stress-induced senescence. While senescence has many causes, the final senescent state always has common molecular features, whether it results from various stresses, telomere dysfunction, or activated oncogenes. However, it is not yet clear that it is valid to place all nontelomere-based mechanisms together into one class. There may be multiple distinctions among senescent cells that have reached that state via different routes. Features of senescence may also vary considerably dependent on the cell type.

Senescence in Tissues In Vivo: Animal Models for Stress-Induced Senescence

As an outcome of the effects of stresses/damage to the cell, senescence is one example of an outcome that does not result in perfect repair; in other words, it does not result in the continued existence of a normal healthy cell, either as a cell with continued division potential or a postmitotic cell. One alternative outcome, recovery from the immediate effects of the damage, but with fixation of the damage in the form of a mutation, is evidently an undesirable outcome, because such a mutated cell may be a precursor to cancer. Both senescence and apoptosis, as outcomes of the effect of damage, have the result of removing the cell from potential contribution to future cell generations, thereby acting to prevent tumorigenesis (Kuilman et al. 2010). Nevertheless, apoptosis removes the cell completely, while senescence leaves it in place, unable to divide, but metabolically active and with the potential to disrupt tissue function (Tchkonia et al. 2010). Additionally, senescence of a stem cell is equivalent to a functional loss of the cell. Defects in stem cell proliferation could be of vital importance in determining the properties of old tissues (Kim and Sharpless 2006; Ruzankina et al. 2008; Waterstrat and Van Zant 2009).

Thus, as a terminal state of cells, distinct from apoptosis and differentiation, the selective value of senescence is much less understood than those other cellular processes. Potentially, future research on senescence may provide evidence that senescence actually has advantages over apoptosis as a way to prevent the potential harm done by stresses that damage cells.

Of course, the validity of this discussion is called into question unless senescence occurs in tissues in vivo as well as in cell culture. During the characterization of cellular senescence as a

cell culture phenomenon, its relevance to in vivo biology was often questioned. Over time, it has become clear that senescence occurs in tissues in vivo, and its importance to the general processes of tissue aging has been increasingly recognized. At the same time, the more recent development of animal models in which the mechanisms and significance of senescence can be studied has the potential to greatly advance the field (Tchkonia et al. 2010; Baker et al. 2011; Tilstra et al. 2012).

Tissue aging could result from damage to macromolecules (DNA, RNA, protein), or by the cellular reaction to such damage, but there is no consensus on this topic. Because damage can cause senescence, the senescent cell could be both a consequence of damage and an amplifier of its effects, by disrupting tissue function. Over the past few years, evidence has accumulated that at least some of the molecular changes that are observed in tissues during aging are consistent with the direct or indirect presence of senescent cells. Three important sets of observations are (1) an increase in $p16^{INK4A}$ mRNA in tissues in aging animals (Kim and Sharpless 2006); (2) increased numbers of nuclear DNA damage foci in tissues (Sedelnikova et al. 2004; Herbig et al. 2006); and (3) the probable involvement of intracellular pathways that are well established to be involved in inflammatory processes, including NF-κB (Tchkonia et al. 2010; Tilstra et al. 2012). DNA damage foci are sites of double strand breaks, together with characteristic proteins such as γ-H2AX and 53BP1 (Polo and Jackson 2011), and are at least one potential cause of the increase in p16, as explained further below (Fig. 11.2). Elevated p16 levels may have a variety of effects via altering the activity of Rb and E2F transcription factors, and, as a consequence, the expression of many types of genes (Kim and Sharpless 2006; Takahashi et al. 2006).

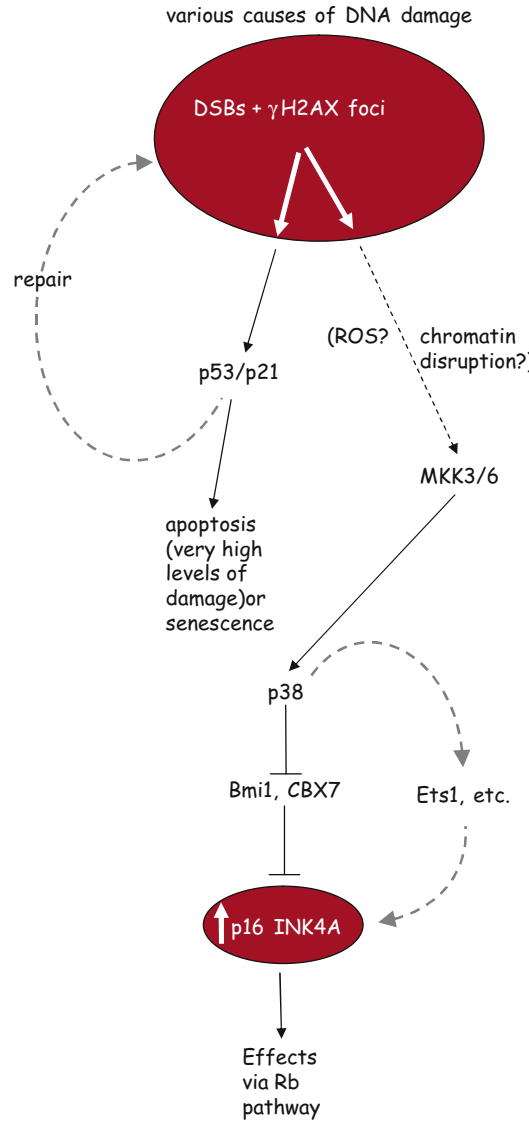

Fig. 11.2 Hypothesized pathway from DNA damage to p16 in cultured cells and in tissues in animals. Potential indirect mediators from DNA damage to p38 are shown as reactive oxygen species (ROS) arising as an indirect consequence of DNA damage and disruption of chromatin via DNA damage that is an indirect cause of cellular stress. Further downstream the pathway from DNA damage to p16 may be via p38 MAPK and factors that regulate p16, including Bmi1 and Ets1

These are examples of an evolving variety of markers that may be employed to detect and measure senescent cells in tissues (de Jesus and Blasco 2012). As these observations have become more extensive it has been realized that some tissues are preferential sites of the accumulation of senescent cells. In particular, one of the more significant sites of senescent cell accumulation in aging is adipose tissue (Tchkonia et al. 2010). In that site, the secreted products of senescent cells

may have adverse consequences for the overall health, contributing to insulin resistance and type 2 diabetes (Tchkonia et al. 2010). In summary, while an age-related increase in the occurrence of senescent cells is an established phenomenon, its causes are likely multiple and its consequences a topic of active investigation, with few firm conclusions as yet.

Possible Causes of DNA Damage in Tissues

If cells with DNA damage accumulate as a result of various stresses which the tissues have encountered, it may be further speculated that among the stresses to which cells are exposed in tissues are endogenously generated reactive oxygen species (ROS), widely thought to be one of the causes of aging and a universal hazard against which cells must be protected (Tchkonia et al. 2010; Shao et al. 2011; Tilstra et al. 2012). ROS generation has an established potential to induce DNA damage foci (Hornsby 2010). Specific examples include: ischemia/reperfusion injury, which is thought to be mediated by oxidative damage, increases nuclear γ-H2AX foci in the heart; treatment of animals with agents that act in part via ROS causes the formation of γ-H2AX foci; chemotherapeutic drugs and irradiation of experimental animals produce γ-H2AX foci in many tissues (Hornsby 2010; Rodier et al. 2011).

ROS are among several potential sources of endogenous and environmental damage in tissues, as well as the action of activated oncogenes (Fig. 11.1). Toxins from the environment and possibly even electromagnetic radiation could be of importance. The implications for stress-induced senescence generally are that, while cells in culture may be exposed to stresses that are not normally encountered by cells in living tissues, tissue cells are exposed to sufficient stresses to undergo stress-induced senescence after sufficiently long periods of exposure. Elucidating these mechanisms will be important for understanding both senescence and aging.

The Critical Role of the Cyclin-Dependent Kinase Inhibitors p21 and p16 in Linking DNA Damage to Stress-Induced Senescence

Although stresses may potentially act via damage to any macromolecules, in many cases DNA damage is the initiating factor; or alternatively, stresses that initially affect some other cell component secondarily cause damage to DNA. A key molecular lesion known to induce senescence is the DNA double strand break (Ruzankina et al. 2008; Polo and Jackson 2011). The induction of senescence by a variety of chemotherapeutic drugs (e.g., bleomycin, adriamycin, mitomycin) provides the most direct evidence that senescence can begin with double strand break damage. Because of the well-understood molecular process by which these agents damage DNA, they form a good model for stress-induced senescence. The senescence-inducing action of short or dysfunctional telomeres is also thought to result from the action of the uncapped telomere as a double strand break (Polo and Jackson 2011; Cao et al. 2011). The response of the cell to such damage is the assembly of large protein complexes around the breaks which stabilize the structure and prepare for potential repair. γ-H2AX and 53BP1 are two proteins present in these foci and have often been used as markers for double strand breaks. They also have been used as indicators of senescence in conjunction with other cellular markers (Sedelnikova et al. 2004; Herbig et al. 2006; de Jesus and Blasco 2012).

DNA damage activates ATM and ATR kinases, thereby initiating a series of signaling events that culminates in the imposition of a cell cycle block via activation of p53 and the cyclin-dependent kinase inhibitor (CDKI) p21 (Ruzankina et al. 2008; de Lange 2010; Polo and Jackson 2011). Early work showed that p21 appeared likely to be at least partially responsible for the nondiving state of senescent cells. These findings led rapidly to studies of whether p21 might be involved in tissue aging in humans and animals. It soon emerged that p21 was generally not elevated in tissues of old animals, but

it did respond in tissues to various kinds of injury, such as ischemia/reperfusion (Hornsby 2010). One of the key issues for both cells in culture and tissues in vivo is the existence of DNA damage foci that represent the site of unrepaired/unrepairable damage, rather than a transient accumulation of proteins that actually results in appropriate repair of the damage (Polo and Jackson 2011). These persistent DNA damage foci (DNA-SCARS; "DNA segments with chromatin alterations reinforcing senescence") are characterized by association with PML nuclear bodies, lack of the DNA repair proteins RPS and RAD51, a lack of DNA synthesis, and accumulation of activated CHK2 and p53 (Rodier et al. 2011). DNA-SCARS were detected in mouse tissues following irradiation. While further study is needed, it appears that DNA-SCARS provide a persistent activation of the DNA repair pathway, which maintains the p53-dependent growth arrest and the gene expression changes characteristic of the senescent state (Rodier et al. 2011).

Following the early discovery of p21, more CDKIs were described, particularly $p16^{INK4A}$, one of two products of the CDKN2A gene (Kim and Sharpless 2006). How p16 is increased as a response to DNA damage is not as well-established as the mode of induction of the p53/p21 pathway, although mechanisms are beginning to be understood (Mirzayans et al. 2012). Agents that cause DNA damage elevate levels of p16; while the p53/p21 pathway is activated as an immediate response to DNA damage, cells that have been senescent for long periods no longer have high p21, but often have high levels of p16 (Takahashi et al. 2006; Mirzayans et al. 2012).

While DNA damage elevates the levels of p16, the molecular pathway is complex (Mirzayans et al. 2012). Some possibilities are indicated in Fig. 11.2 (Hornsby 2010). As most of this work on p16 in the context of senescence has been in cell culture, it is important to consider models in which p16 can be experimentally modulated in tissues in vivo in order to determine the pathophysiologically relevant mechanisms (Kim and Sharpless 2006; Baker et al. 2011) as well as in pathological conditions in human diseases (Lichterfeld et al. 2012).

Telomere Biology in Relation to Stress-Induced Senescence

An area of continuing research activity concerns the role of telomere biology in senescence in aging tissues. While it is well established that telomere shortening in culture causes senescence (the classical "end-replication problem": Kuilman et al. 2010; de Lange 2010; Cao et al. 2011; de Jesus and Blasco 2012), it is much less certain that senescence in vivo is normally the result of shortened telomeres, or that cells that undergo telomere shortening in vivo actually enter a senescent state. Because human cells have short telomeres, and typically very little telomerase activity, telomere shortening leading to senescence is well established in human cells in culture and therefore may potentially occur in vivo (Herbig et al. 2006). However, it has been difficult to unambiguously link telomere shortening in human tissues in vivo to subsequent cellular senescence. Many cell types undergo progressive telomere shortening as a function of age in humans, but in most cases this has not been linked to a resultant increase in senescent cells (Hornsby 2001). One example in which such an association is stronger is provided by CD8 T cells during chronic HIV infection; the excessive proliferation characteristic of HIV disease is associated with telomere dysfunction and the induction of specific senescence features (Lichterfeld et al. 2012). While a complete dissociation of the phenomena of telomere shortening and senescence in human tissues is unlikely, the occasionally encountered opposite assertion—that replication-based telomere shortening is one of the key causes of aging—is equally improbable.

An early concept was that telomere shortening, caused by the "end-replication problem" in telomerase negative cells, was the main cause of senescence. It is now understood that this is one of many causes of senescence; nevertheless, telomere biology plays a prominent role in many forms of stress-induced senescence even when it does not result from progressive telomere shortening (Kuilman et al. 2010; de Lange 2010; de Jesus and Blasco 2012).

What is the evidence that senescent cells in tissues arise via a stress-induced mechanism versus telomere shortening and dysfunction? Although DNA damage is observed at telomeres in cells as well as at nontelomeric sites (Herbig et al. 2006) this could occur via a greater susceptibility of telomeres to DNA damage from causes such as oxidative stress (Passos et al. 2010; de Lange 2010; Hewitt et al. 2012; Fumagalli et al. 2012) rather than shortening as a result of excessive cell proliferation. Although there is little direct evidence, it is significant that dermal fibroblasts, cells in which senescent features are observed, generally have a low rate of cell division in situ; labeling of human skin shows epidermal cells in the cell cycle but typically almost none in the dermis. While there is a lack of direct experimental data, a working hypothesis is that the accumulation of senescent cells and accompanying biochemical changes, such as elevated levels of p16, are the result of stress-induced senescence rather than excessive cell proliferation. Nevertheless, damage may be particularly localized to telomeres, even if this is not related to the effects of continued cell division.

In the mouse, the much longer telomeres (in comparison to humans), combined with higher levels of telomerase in mouse tissues, would seem to make progressive telomere shortening an unlikely cause of senescence in this species. Nevertheless, more recent data show consistent telomere shortening and an increase in the percentage of short telomeres in aging mouse tissues (de Jesus and Blasco 2012). However, because mouse cells do not normally undergo telomere shortening in culture, and therefore do not typically exhibit telomere-based senescence in culture (Hornsby 2003), it seems unlikely that short telomeres in cells in tissues in vivo are the result of progressive, cell division-based telomere shortening. In contrast, telomere shortening does occur in mice as a function of age, even though telomere length varies with strain, and mice generally have much longer telomeres than humans (de Jesus and Blasco 2012). Mouse tissues show age-related senescence changes (Sedelnikova et al. 2004; Baker et al. 2011; Kosar et al. 2011; Tilstra et al. 2012).

Thus, the relationship between telomeres and senescence in tissues is more complex than originally envisioned. While telomerase (hTERT) maintains telomeres and so prevents senescence via telomere shortening, the enzyme has additional effects on senescence beyond its role in telomere maintenance. A variety of extratelomeric effects of hTERT have been reported; such actions include the prevention of apoptosis in neurons, prevention of oxidative damage in many cell types, enhancement of genomic stability by upregulating DNA repair capacity, upregulation of growth-promoting genes, and downregulating genes that promote apoptosis such as TRAIL (reviewed in Chung et al. 2005). In tumorigenicity studies, hTERT allowed Ras/SV40 TAg-expressing human fibroblasts to grow when implanted as a cell suspension under the skin in immunodeficient mice, while they do not need hTERT in the same location when implanted in collagen gel (Hornsby 2010). Moreover, ALT^+ cells (alternative lengthening of telomeres), which are telomerase-negative, need hTERT for growth when implanted under the skin, but not when implanted with better microenvironmental support, i.e., in collagen gel or by subrenal capsule transplantation; moreover, in an unusual animal model of cancer resistance, the naked mole-rat, hTERT overcame the resistance of the cells of this mammal to neoplastic transformation by Ras/SV40 TAg (Liang et al. 2010).

While most of these published studies have added a variety of phenomena to the extratelomeric effects of hTERT, recent data strongly indicate that TERT in both mouse and human tissues stimulates cell proliferation via Wnt signaling (Choi et al. 2008; Shkreli et al. 2012). Conditional telomerase induction causes proliferation of hair follicle stem cells in the mouse. The reverse transcriptase catalytic activity of TERT is not needed for the activation of proliferation of hair follicle stem cells by mTERT, thus clearly demonstrating that this cannot be an effect via classical telomerase activity. These experiments also defined the intracellular pathways that are the main targets of TERT; mTERT stimulation of stem cell proliferation was shown to depending on the activation of the Wnt pathway via β-catenin (Choi et al. 2008).

Evidence that HIV-associated nephropathy in both human and mouse kidneys is accompanied by increased expression of TERT and activation of Wnt signaling indicates that these mechanisms may not require ectopic hTERT expression and may reflect a normal link of TERT with cell division control (Shkreli et al. 2012).

Chromatin Changes in Stress-Induced Senescence

Senescence is a permanent state of nondivision (in response to mitogens that would normally cause cell proliferation) but the reasons for its irreversibility have only partially been elucidated. As with telomere-based senescence and senescence caused by oncogenes, stress-induced senescence involves both CDKIs and changes in chromatin structure (Kuilman et al. 2010). Foci of senescence-associated heterochromatin (SAHF) were first described as a downstream consequence of high-intensity Ras signaling and telomere dysfunction (Serrano et al. 1997). More recently it has been suggested that the formation of SAHF follows the expression of p16 in senescent cells and is dispensable for senescence (Kosar et al. 2011; Jeanblanc et al. 2012).

These chromatin changes in senescent cells are also accompanied by a wide range of changes in gene expression, called the "senescence associated secretory phenotype," SASP, or the "senescence messaging secretome," SMS (Fumagalli and d'Adda di Fagagna 2009; Kuilman et al. 2010). These changes, resembling the acute inflammatory response of fibroblasts in tissues reacting to tissue injury, are very similar in cells that have reached senescence via telomere shortening and those that have undergone stress-induced senescence. In particular, the overexpression of matrix metalloproteinases (MMPs) was recognized in early studies. The SASP may have various adverse effects on tissues, and may be responsible for the deleterious consequences of the presence of senescent cells (Tchkonia et al. 2010). Experimental evidence for the tumor-promoting effects of senescent cells, via MMPs and similar factors, has been obtained in xenograft models in immunodeficient mice. Fibroblasts with senescence induced by bleomycin treatment (stress-induced senescence) behaved in a very similar manner to those made senescent by telomere shortening (Hornsby 2010).

Role of Stress-Induced Kinase, p38 Map Kinase

During the investigation of intracellular pathways that can drive cells into senescence in culture, it became apparent that one common mechanism that may be widely involved is the activation of p38 mitogen-activated protein kinase (MAP kinase; MAPK14). Activation of p38 MAPK is a general reaction to many cellular stresses, including DNA damage and telomere dysfunction; however, this does not require critical shortening of telomeres, thus playing a role in stress-induced senescence (Passos et al. 2010; Gorgoulis and Halazonetis 2010; Shao et al. 2011; Jeanblanc et al. 2012). Agents that cause senescence, such as hydrogen peroxide, activate p38 (Hornsby 2010; Barascu et al. 2012). p38 is attractive as a candidate mediator of stress-induced senescence, because, as a stress-activated kinase, it is responsive to those forms of damage that cause stress-induced senescence, and it is in a pathway that may lead to elevated levels of p16 (Hornsby 2010; Passos et al. 2010; Kim et al. 2011; Kuilman et al. 2010; Shao et al. 2011; Mirzayans et al. 2012; Jeanblanc et al. 2012) (Fig. 11.2).

Some evidence for the role of p38 has come from the use of pharmacological inhibitors of this kinase. Two p38 kinase inhibitors developed by SmithKline Beecham in the early 1990s, SB203580 and SB202190, have been widely used and are highly selective. In some cell culture models, p38 inhibitors oppose senescence (Passos et al. 2010). One interesting example of this is provided by cells from patients with Werner syndrome, a syndrome with features of premature aging. Cultured cells from Werner patients stop dividing prematurely when telomeres are still relatively long when compared to normal fibroblasts. The lack of the Werner RecQ helicase may cause a greater level of replication stress in

cells. Growth of Werner cells in a p38 inhibitor prevents the early telomere-based senescence that is characteristic of this syndrome (reviewed in Hornsby 2010).

While there are other potential candidates for intermediate cellular pathways between stresses and effectors of the permanent senescent state, p38 provides an example of one that fulfills the criteria of appropriate action and appropriate downstream targets. Future experiments in cells in tissues may provide more definitive evidence for the key role of this pathway.

Generation of ROS as a Consequence of DNA Damage

If p38 MAP kinase is a significant mediator of stress-induced senescence then it would be expected that it can be activated by DNA damage or other changes at the chromosomal level. A major potential mediator of the activation of p38 is ROS, generated as result of DNA damage. Although it is well-established that ROS can damage DNA, there is also evidence that DNA damage in itself increases the level of ROS in cells (Hornsby 2010; Mirzayans et al. 2012). Transfection of DNA fragments increases the levels of ROS; human fibroblasts have higher levels of ROS as they approach complete senescence, i.e., during a period when telomeres are shortening to the stage where they function as double strand breaks; inhibition of ROS damage by antioxidants slow the progression of cells to senescence, suggesting that although telomere dysfunction causes cessation of cell division, the indirect effect of telomere dysfunction elevated ROS production has an additional effect on cell proliferation. In some cases antioxidants can prevent both p53-mediated senescence and the activation of p38 MAP kinase (Jung et al. 2004; Barascu et al. 2012). These results are consistent with a model in which DNA damage, including telomere dysfunction, affects two pathways: first, ATM/p53/p21, resulting in a cell cycle block; and second, ROS/p38/p16, also resulting in a cell cycle block.

A key finding is that the generation of ROS downstream of DNA damage in turn acts to create more DNA damage and thereby forms a positive feedback loop (Passos et al. 2010). The essential feature of the loop is that long-term activation of p21 induces mitochondrial dysfunction and ROS generation, via the intermediacy of p38 activation. ROS replenish short-lived DNA damage foci and maintain an ongoing DNA damage response. This loop appears to be both necessary and sufficient for the stability of growth arrest in senescence (Passos et al. 2010). Moreover, DNA damage at telomeres is linked to mitochondrial dysfunction and ROS generation (Sahin and DePinho 2012).

Further evidence is provided by mice with disruption of the ATM gene. They exhibit higher ROS levels and defects in stem cell proliferation in these mice can be corrected by antioxidant administration (Ruzankina et al. 2008; Hornsby 2010). $ATM^{-/-}$ mice have continuously-present unrepaired double strand breaks and increased ROS generation. p38 MAP kinase is activated and pharmacological inhibition of p38 can normalize the defects in stem cell proliferation (Kim et al. 2011). A key mediator of the effects of ROS in ATM-deficient cells is laminin B1, which accumulates via activation of p38 (Barascu et al. 2012). Lamin B1 accumulation causes nuclear shape alterations in stress-induced senescence; lamin B1 overexpression is sufficient to induce nuclear shape alterations and senescence in wild-type cells, while normalizing lamin B1 levels in ATM-deficient cells reduces both nuclear shape alterations and senescence (Barascu et al. 2012).

Other research suggests more direct links between ROS generation and activation of p16/induction of senescence. A mouse model has been generated in which luciferase has been knocked into the INK4A locus, thus providing a real-time readout of the transcriptional activity of the INK4A locus (Ohtani et al. 2010). The authors confirmed a transcriptional increase in p16 as a function of age. They proposed that accumulation of DNA damage activates ROS production, which is linked to increases in $p16^{INK4A}$ mRNA via a block of expression of the

DNA methyltransferase DNMT1 and consequent derepression of the INK4A locus by alterations in the methylation status of the promoter (Ohtani et al. 2010).

Thus, one general model for stress-induced senescence is that DNA damage causes increased ROS, which act as a general stress in the cell, activating the p38 pathway and increasing levels of p16. ROS generation is well-established to activate p38 (Hornsby 2010; Barascu et al. 2012). The facts that antioxidants, which reduce the effects of ROS, can improve cell division and decrease p38 activation in the presence of DNA damage suggests that it may be possible to use antioxidants to intervene in the age-dependent generation of senescent cells in tissues; tests of this hypothesis will likely be a major focus for future research.

Disruption of Chromatin Structure as a Potential Mediator of Stress-Induced Senescence

A second possible pathway for the activation of p38 by DNA damage is via disruption of chromatin structure. Large-scale changes in chromatin structure could provide a form of stress that is transmitted to p38 MAP kinase. Some tentative evidence is provided by the fact that increased histone acetylation, causing gross changes in chromatin structure, activates p38 . Histone deacetylase inhibitors increase γ-H2AX foci. However, DNA double strand breaks, if unrepairable or unrepaired, might lead to chromatin disruption or reorganization and act as a stress, activating p38 (Fig. 11.2) (Hornsby 2010).

Summary

Stress-induced senescence is a very broad concept encompassing a variety of stresses and sources of damage to cells. These stresses act via intracellular pathways, which may be multiple, to a final common state of irreversible cell division. In this state the cell is held in the nondividing state by the combination of CDKI activity, heterochromatin formation, and gene expression changes. Here, a case is made that, although the mediators of stress-induced senescence may be multiple, the p38 MAP kinase pathway stands out as potentially the most important. However, both the means by which it may become activated by stresses and the means by which it acts to cause senescence are both as yet unclear. Future studies should focus on appropriate in vivo models in which stress-induced senescence can be studied under pathophysiologically relevant conditions.

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Accumulation of Reactive Oxygen Species and Induction of Premature Senescence: Role of DDB2

12

Nilotpal Roy and Pradip Raychaudhuri

Contents

Abstract

Oxidative stress results from an imbalance between free radical formation and anti-oxidant defense. Reactive Oxygen Species (ROS) have been implicated in several oxidative stress related pathological disorders such as aging, cancer and neurological abnormalities. Thenucleotide excision repair protein DDB2 plays an important role in the regulation of ROS. DDB2 deficient human or murine cells fail to accumulate ROS following DNA damage. The lack of ROS accumulation in DDB2 deficiency results from high-level expression of the anti-oxidant genes MnSOD and Catalase. DDB2 represses expression of these anti-oxidant genes by recruiting Cul4a and Suv39h and by increasing Histone3K9 tri-methylation in the promoter region. Moreover, expression of DDB2 is induced by ROS. Thus, upon oxidative stress, DDB2 functions in a positive feedback loop by repressing the ROS scavenger genes to cause a persistent accumulation of ROS. DDB2 mediated ROS-accumulation is related to premature senescence and its role in inhibiting skin tumorigenesis. Following UV damage, DDB2 induces ROS accumulation in the skin, triggering a senescence response that contributes to suppression of skin cancer. Thus, in addition to its role in NER, DDB2 plays an important role in the DNA damage induced ROS accumulation and ROS induced premature senescence, which are significant in the suppression of skin cancer.

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Damaged DNA binding protein 2 (DDB2) • Premature senescence • Reactive Oxygen Species (ROS)

Introduction

An Introduction to Reactive Oxygen Species

Reactive oxygen species (ROS) is commonly generated in the living organism as a natural byproduct of the normal metabolism of oxygen, primarily in the mitochondria. Oxygen has two unpaired electrons in the 2p orbitals in its outer shell, which make it susceptible to radical formation. Sequential reduction of molecular oxygen results formation of a group of reactive oxygen species: superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide and others. Mitochondrial electron transport chain is one of the major sources of ROS. In the electron transport chain, electrons move through a series of proteins by oxidation-reduction reactions. Oxygen is the final acceptor of electrons in this chain. However, because of highly reactive nature of oxygen, it undergoes complex chemical reactions to produce reactive oxygen species. ROS level can be augmented also by external stimuli such as ultra violet rays, growth factors, ionizing radiation, heat exposure, tissue injury etc. An imbalance between rate of ROS production and the ability of the cell to detoxify ROS leads to "oxidative stress" (Droge 2002). Oxidative stress is harmful because of its ability to damage important components of the cell: lipids, proteins and DNA. DNA damage induced by ROS causes cells to trigger a variety of responses: apoptosis, senescence and cell cycle arrest (Droge 2002). Oxidative stress has been suggested to be a causal agent of a wide spectrum of pathological disorders including neurodegenerative diseases (Alzheimer's disease, Parkinson's disease), cardiovascular diseases, aging and age related development of cancer (Droge 2002).

Cellular Processes Regulated by Reactive Oxygen Species

ROS also plays an important role as an intracellular signaling molecule. Phosphatases contain -SH groups in their active sites that are inactivated by ROS, leading to activation of many kinases that regulate a variety of cellular processes, including cell cycle, senescence, apoptosis, autophagy and inflammation (Droge 2002).

ROS plays an important role in cell cycle arrest. Oxidative stress results in the induction of p38MAPK activity, which in turn activates expression of p16INK4a, a cyclin dependent kinase inhibitor that establishes stable G1 cell cycle arrest through activating the retinoblastoma (Rb) tumor suppressor protein pRb (Benhar et al. 2002). Also, elevated intracellular level of ROS activates protein kinase C δ (PKC δ) that in turn irreversibly blocks cytokinesis (Takahashi et al. 2006). It is noteworthy that high level of oxidative stress causes DNA damage, which triggers the ATM/ATR pathway that in turn phosphorylates and activates p53 and Chk1/Chk2. Activated p53 can induce either apoptosis or cell cycle arrest depending upon the extent of the damage.

Senescence, which is classically considered an irreversible cell cycle arrest at G1 phase, also is induced by ROS (Campisi and d'Adda di Fagagna 2007). According to the free radical theory of aging, oxidative damage resulted from ROS is a major contributor to the functional decline related to aging such as poor cell membrane permeability, reduced enzyme activity and inhibition of immune function. Senescence function of ROS is majorly attributed to its ability to induce p53 and MAPK family members-P38MAPK, JNK and ERK1/2 (Benhar et al. 2002). These molecules activate downstream cyclin dependent kinase inhibitors such as p16INK4a, p19ARF, p21 and induce permanent cell cycle arrest at G1. It is noteworthy here that ROS-induced premature senescence is involved in tumor suppression, as well as in retarding tumor growth following therapy (Dimri 2005).

ROS are mediators of apoptosis. Both the intrinsic and extrinsic pathway of apoptosis is dependent on ROS. In the intrinsic apoptosis pathway, ROS

has been shown to induce release of cytochrome c in the cytoplasm that leads to activation of caspases and trigger cell death. On the other hand, important role of ROS has been implicated in the Fas/CD95 mediated extrinsic apoptosis pathway as well (Droge 2002). It has also been shown that p53, a central modulator of apoptosis response, induces apoptosis by a pathway that is dependent on ROS production (Johnson et al. 1996).

ROS has recently been implicated in nutrient starvation induced autophagy (Scherz-Shouval and Elazar 2007). Under starvation conditions ROS are found to be essential for autophagosome formation and autophagic degradation. Mechanistically, oxidative stress has been linked to different transcription factors and cell signaling molecules that directly regulates autophagy. For example, oxidative stress activates transcription factors FoxO3, NRF2 and p53, which in turn transcriptionally activates LC3, BNIP3; p62; TIGAR, DRAM, sestrin respectively. All these downstream factors play a significant role in the autophagy process. Moreover, ROS mediated direct up regulation of sestrins induce autophagy by inhibiting mTOR through activation of AMPK (Scherz-Shouval and Elazar 2007).

Reactive Oxygen Species Homeostasis

It was a paradox for quite some time that how ROS, a potentially lethal oxidant, achieve the specificity of signaling process given its highly reactive nature. This apparent discrepancy led to studies that identify signaling pathways that control ROS homeostasis. Studies of these pathways provided evidence that these molecules act as a sensor to measure the intracellular concentration of ROS and proportionally regulate the expression of ROS specific scavengers and maintain the level of ROS at sub-toxic level. There are two major classes of anti-oxidant defense: enzymatic and non-enzymatic. Superoxide dismutase, Catalase, and Glutathione peroxidase are the major anti-oxidant enzymes. The major non-enzymatic anti-oxidant molecules include Glutathione, Thioredoxin, Vitamin A, Vitamin C, and Vitamin E (Droge 2002) For the purpose of this review, we will focus on enzymatic neutralization of ROS.

Superoxide dismutase is a group of enzymes that catalyze the dismutation reaction of superoxide into oxygen and hydrogen peroxide. There are three major families of superoxide dismutase depending upon the metal co-factor: Cu/ZnSOD (co-factor is copper and zinc), MnSOD (co-factor is manganese) and NiSOD (co-factor is Nickel). Eukaryotes use Cu/ZnSOD and MnSOD whereas prokaryotes use MnSOD or NiSOD. In mammals, there are three kinds of SOD present—SOD1, SOD2 and SOD3. SOD1 is present in the cytoplasm; SOD2 is found in mitochondria and SOD3 is extracellular. SOD1 and SOD3 are Cu/Zn SODs whereas SOD2 is MnSOD. The SODs serve as key anti-oxidants in cell. The physiological importance of SOD is evident from the knock out mice models. SOD2−/− mice die within first 10 days of life due to dilated cardiomyopathy, accumulation of lipid in liver and skeletal muscle and metabolic acidosis (Holley et al. 2012). Another group reported neurodegeneration of SOD2−/− mice along with previously mentioned abnormalities (Holley et al. 2012). SOD1−/− and SOD3−/− mice have less severe phenotype. SOD1−/− mice do not show any overt abnormalities during development or early adulthood. However, they develop hepatocellular carcinoma with a short latency (Elchuri et al. 2005). Also, they exhibit elevated oxidative stress that results in rapid acceleration of age related loss of skeletal muscle mass (Muller et al. 2006). Collectively, these mice die prematurely from either of these two pathological responses. SOD3−/− mice exhibit mildest phenotype without any defects in normal lifespan with a minor increase in oxidative stress markers (Sentman et al. 2006). These lines of evidence point to the fact that SOD2 is perhaps the most important player of all the superoxide dismutase enzymes in antioxidant defense mechanism. SOD2 also has been associated with tumor suppression. Increased SOD2 expression was found to suppress breast carcinoma, pancreatic cancer, melanoma, skin carcinoma, lung carcinoma and prostate cancer formation (Holley et al. 2012). It is likely that SOD2 functions as a tumor suppressor by inhibiting mutagenic DNA damages by ROS. Another mechanism by which SOD2 may inhibit tumorigenesis is by altering the expression or activity of tumor suppressor genes (Holley et al. 2012). In contrast, there are reports

suggesting elevated SOD2 expression in the cancer cells compared to the normal tissue such as in colon cancer, gastric cancer, esophageal cancer and lung cancer (Holley et al. 2012). Therefore, it will be important to understand the mechanism by which SOD2 can either promote or inhibit tumorigenesis in order to exploit it for therapeutic purposes.

Catalase is another enzyme critical for anti-oxidant defense. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. Catalase is located in a cell organelle known as peroxisome. Catalase−/− mice develop normally and do not show any gross abnormalities (Ho et al. 2004). Catalase has been linked to cancer suppression as well. For example, catalase overexpression suppresses invasiveness and aggressiveness of breast cancer cells (Goh et al. 2011). In another study loss of catalase expression has been linked to skin tumor progression (Kwei et al. 2004). Nonetheless, deregulation of catalase expression is suggested to be causal agent of cancer formation.

Glutathione peroxidase (Gpx) is an enzyme with peroxidase activity, located in the cytoplasm of the cell. There are several isozymes of glutathione peroxidase—Gpx1-Gpx8 with Gpx1 being the most abundant form. Gpx1−/− and Gpx2−/− mice are phenotypically normal and have a normal life span (Ufer and Wang 2011). However, Gpx4−/− mice were found to be embryonic lethal (Ufer and Wang 2011). Allelic loss of Gpx1 was found to be associated with head and neck squamous cell carcinoma (Hu et al. 2004). Deregulated Gpx activity also has been reported to associate with pancreatic cancer (Liu et al. 2004). In human patients, Gpx Pro198Leu polymorphism associated lower Gpx activity is associated with increased risk of breast cancer and lung cancer (Ravn-Haren et al. 2006). These observations clearly suggest important role of Gpx in carcinoma development.

Collectively, these observations indicate the intricate relationship between the anti-oxidant enzymes and carcinoma development. It is noteworthy that some of the early studies suggest higher antioxidant enzyme expression is associated with suppression of carcinoma progression. Carcinoma cells exhibit higher ROS activity than the normal cells owed to their rapid proliferation rate. Hence, in certain cases increased anti-oxidant enzyme activity may attenuate the ROS level of rapidly proliferating tumor cells and inhibit their expansion at the initial stage. Also, some of these studies indicated reduced invasiveness of tumor cells following overexpression of anti-oxidant enzymes. However, recent evidence suggests higher ROS level of tumor cells might be useful to target them specifically (Trachootham et al. 2006). Instead of reducing the ROS level, increase in the ROS level of transformed cells selectively eliminates them by apoptosis/senescence.

Damaged DNA Binding Protein 2

An Introduction to Damaged DNA Binding Protein 2

DDB2, damaged DNA binding protein 2 is a DNA repair protein. It is the smaller subunit (48 kDa) of the heterodimeric protein complex DDB—damaged DNA binding protein. The larger subunit of the complex is DDB1-damaged DNA binding protein 1 (127kD). Homologs of human DDB2 is found only in mammals. The DDB2 amino acid sequence in human and mice is 73% identical. DDB2 is expressed ubiquitously in human tissue with higher expression in testes, liver, kidney, corneal endothelium and lower expression in brain, skin, lung, muscle and heart. There are several isoforms of DDB2, with D1 isoform being the dominant form (Tang and Chu 2002).

DDB2 plays a pivotal role at the early recognition step of nucleotide excision repair (NER). DDB2 binds to UV damaged DNA with an extremely strong affinity and exhibit a strong preference of binding over undamaged DNA. The most abundant lesions induced by UV are cyclobutane pyrimidine dimmers (CPD) and 6–4 photoproducts. DDB2 binds to both kinds of lesions with a stronger affinity towards 6–4 photoproducts. DDB2 also binds to other forms of damaged DNA including DNA damage induced by Cisplatin, psoralen, abasic sites and single stranded DNA. Mutation in DDB2 gene in human leads to an autosomal recessive disorder-Xeroderma Pigmentosum Group E

(XPE) (Tang and Chu 2002). XPE patients are hypersensitive to UV, predisposed to skin carcinoma formation and have mild neurological abnormalities. It is noteworthy that of all the XP groups, XPE subgroup patients show the mildest phenotype with regard to sun sensitivity, but they show highest risk of skin cancer formation. XPE individuals were found to have mutated form of DDB2 resulting from single amino acid substitution. The commonly found mutations are R273H and K244E. Both these mutation results inactivation of DDB2 (Tang and Chu 2002).

DDB2−/− mice develop normally and do not show any gross abnormalities. Consistent with the phenotype of XPE patients, DDB2−/− mice are hypersensitive to UV and predisposed to UV induced skin carcinoma formation (Itoh et al. 2004). Moreover, DDB2−/− mice were found to develop spontaneous malignant tumor at a high frequency. Tumors were of wide spectrum with a bias towards lymphoid and hematological malignancies (Yoon et al. 2005).

DDB2 is an important substrate receptor for Cul4a-DDB1 ubiquitin ligase. Cul4 employs WD40-like repeat containing proteins as an adapter molecule. Structure analysis suggests that Cul4 uses DDB1 as a linker protein to interact with subset of WD40-like repeats containing proteins, such as DDB2 that serve as a substrate receptor. XPC, p21 and DDB2 itself are some of the well-characterized target of Cul4a-DDB1-DDB2 complex (Stoyanova et al. 2009b). There was some early work that suggested a role of DDB2 in transcription. For example, DDB2 was found to be a co-activator of E2F1 mediated transcriptional activation (Hayes et al. 1998). DDB2 was also found to be associated with p300/CBP histone acetyl transferase (Datta et al. 2001). Another study reported DDB2 to be a component of histone acetylating transcriptional co-activator STAGA complex as well (Martinez et al. 2001).

Damaged DNA Binding Protein 2 in DNA Damage Response

NER is mostly important for UV induced DNA damage such a thymine dimers and 6–4 photoproducts. Mammalian NER consists of two different pathways: transcription coupled NER and global genomic NER. Global genomic NER repairs DNA damage on both transcribed and untranscribed DNA strand, whereas transcription coupled NER works on the transcriptionally active regions. These two processes involve similar but distinct proteins to execute the repair process. DDB2 is one such protein that is involved only in the global genomic pathway of NER (Tang and Chu 2002). Several mechanisms were proposed on how DDB2 participates in DNA repair. Initial observations suggested that DDB2 recruits DDB1 to the nucleus and recognize the UV induced DNA damage. Following the recognition, Cul4a-DDB1-DDB2 complex ubiquitinates XPC and DDB2 gets self-ubiquitinated as well. Ubiquitination of DDB2 leads to its degradation whereas XPC ubiquitination enhances its DNA binding ability. According to this model, DDB2 degradation facilitates XPC binding to the DNA and initiates the repair process (Stoyanova et al. 2009b). Another model linked the DNA repair activity of DDB2 to its protein ubiquitination ability. This model suggested that Cul4a-DDB1-DDB2 complex ubiquitinates histones H2A, H3 and H4at sites of DNA damage and thereby facilitate the removal of histones from the nucleosome and help the repair factors to gain access to the lesion (Stoyanova et al. 2009b). On the other hand, there is a recent report that suggests DDB2 plays role in chromatin decondensation at UV induced DNA lesion independent of the CRL4 ubiquitin ligase complex. DDB2 was found to reduce the density of core histones in the chromatin containing UV induced DNA lesions, which is ATP dependent (Luijsterburg et al. 2012). While the observations are strong, no genetic evidence was provided for these models.

Following DNA damage, Ataxia telangiectasia mutated kinase (ATM) and Ataxia telangiectasia RAD3–related kinase (ATR) gets activated and recruited to the damaged DNA. Activated ATM/ATR phosphorylates p53 at Ser15 in human/Ser18 in mouse. Ser18 phosphorylation in mouse does not increase the stability of p53, but rather makes p53 more transcriptionally active. In low dose UV irradiated cells DDB2

keeps the level of p53 Ser18P low, but does not alter the level of total p53 (Stoyanova et al. 2008). DDB2 induced degradation of p53 Ser18P involves the ubiquitin mediated proteasome pathway. DDB2 imports DDB1 from the cytoplasm to the nucleus following low dose UV. The DDB1-DDB2 complex in association with Cul4a causes proteolysis of p53 Ser18P. By degrading p53Ser18P, DDB2 regulates the level of p21, which is a direct transcriptional target of p53 following DNA damage. DDB2 also regulates p21 at the protein level through its ability to induce proteolysis of p21 (Stoyanova et al. 2009a). p21 has been shown to inhibit the DNA repair function by sequestering proliferating cell nuclear antigen (PCNA), a protein important for repair synthesis. PCNA acts as a DNA clamp to recruit DNA polymerase δ onto the DNA. Thus, by keeping the p21 level relatively low, DDB2 ensures efficient DNA repair activity (Stoyanova et al. 2009b). In the absence of DDB2, high-level p21 sequesters PCNA and inhibit the repair synthesis function. In agreement with that, deletion of p21 in DDB2–/– background eliminated the repair deficiency of DDB2–/– cells. These observations suggest that p21-regulation by DDB2 is important in the global genomic NER pathway.

Role of Damaged DNA Binding Protein 2 in Reactive Oxygen Species Regulation and Premature Senescence

Damaged DNA Binding Protein 2 Is Required for DNA Damage Induced Apoptosis and Premature Senescence

Recently, additional functions of DDB2 in physiological processes other than DNA repair have emerged. As a component of CRL4 ubiquitin ligase complex, DDB2 has been linked to ubiquitin-mediated degradation of proteins. But, this function of DDB2 was mostly linked to its role in DNA repair. However, the tumor prone phenotype of DDB2–/– mice, unlike the XPC–/– or XPA–/–, strongly suggested other tumor suppressor functions of DDB2. In agreement with that, DDB2 was found to be an essential mediator of apoptosis and senescence. DDB2–/– cells are resistant to DNA damage (UV, IR, chemotherapeutic drugs) induced apoptosis (Stoyanova et al. 2009a). Also, keratinocytes from DDB2–/– mice are resistant to UV induced apoptosis response. The apoptosis promoting function of DDB2 is linked to its ability to repress expression of p21. p21 has been shown to exhibit anti-apoptotic effect. High level of p21 inhibits caspases. In DDB2–/– cells there is high-level accumulation of p21, which inhibits the apoptosis response. In agreement with that DDB2–/– p21–/– cells show efficient apoptosis response. Moreover, DDB2–/– p21–/– mice exhibit efficient UV induced apoptosis response (Stoyanova et al. 2012). High-level p21 has been associated with increased senescence response. However, quite intriguingly, despite high level of p21, DDB2–/– cells were found to be deficient in senescence, indicating a role of DDB2 in premature senescence. The senescence function of DDB2 is distinct from its ability to regulate p21.

Damaged DNA Binding Protein 2 Represses the Antioxidant Genes to Induce Persistent Accumulation of Reactive Oxygen Species

MEFs (mouse embryonic fibroblasts) derived from DDB2–/– embryos are resistant to replicative senescence (Roy et al. 2010). WT MEFs undergo senescence after finite number of passages, whereas DDB2–/– MEFs are not only delayed in senescence, but also undergo spontaneous immortalization at a very high frequency. Lack of p19Arf expression, a critical factor for senescence induction, is the reason of senescence deficiency in DDB2–/– MEFs. Overexpression of p19Arf rescues senescence deficiency phenotype of DDB2–/– MEFs. Also, level of DDB2 was found to be positively correlated with the replicative senescence response of the wild type (WT) MEFs, indicating that DDB2 somehow triggers the senescence response in these cells. Human primary cells undergo replicative senescence due to telomere erosion. Unlike human primary cells, MEFs have longer telomere and higher telomerase activity (Campisi and d'Adda

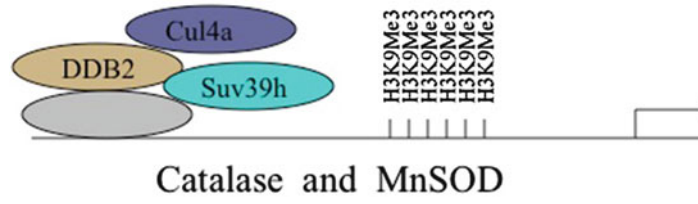

Fig. 12.1 Schematic diagram depicting DDB2 mediated repression of MnSOD and Catalase

di Fagagna 2007). MEFs undergo replicative senescence due to oxidative stress resulted from supra-physiological oxygen concentration (20%) in standard tissue culture condition (Campisi and d'Adda di Fagagna 2007). Therefore, possibly, this oxidative stress increases DDB2 expression in WT MEFs. In agreement with that, when WT MEFs were kept at 3% oxygen, there was no induction in DDB2 expression. Furthermore, exogenous oxidative stress, such as sub-lethal dose of Hydrogen Peroxide also induces expression of DDB2. In the absence of DDB2, both human and murine cells were found to be refractory to premature senescence response such as oxidative stress; Oncogene and DNA damaged induced senescence. These observations demonstrated that DDB2 is an essential mediator of premature senescence response, in vitro and in vivo. Further investigation revealed that DDB2 is required for persistent accumulation of ROS, which is critical for premature senescence.

In both human and mouse, DDB2 deficient cells were found to be deficient in ROS accumulation following DNA damage. Furthermore, DDB2−/− mice accumulated significantly less ROS in the skin following acute UV damage. Further investigation revealed that DDB2 is a transcriptional inhibitor of two important anti-oxidant enzymes, MnSOD and catalase. In fission yeast, schizosachhraomyces pombe, Cul4 was implicated to recruit Clr4, a histone methyltransferase on the promoter of genes and cause their heterochromatinization (Jia et al. 2005). Clr4 induces Histone 3 Lysine 9 tri-methylation (H3K9 Me3), a heterochromatinizing modification. Moreover, Rik1, a fission yeast protein related to mammalian DDB1, was found to be essential for Cul4 mediated recruitment of Clr4 histone methyl transferase. A different study found a cognate binding element for DDB2 on the promoter of MnSOD (Minig et al. 2009). That study further implicated DDB2 as a constitutive repressor of the SOD2 gene. Our study suggested that DDB2 binds to the promoter of both catalase and SOD2 genes. Cul4a-DDB2 complex was found to recruit histone methyl trasnsferase Suv39h on the promoter of these two genes, leading to attenuation of their expression (Fig. 12.1). Thus DDB2 acts as an epigenetic regulator of these anti-oxidant genes. Therefore, by keeping the expression of these anti-oxidant genes low, DDB2 keeps the ROS level high following DNA damage. High-level ROS triggers the premature senescence response. Absence of DDB2 is associated with low level of ROS and therefore deficiency in senescence.

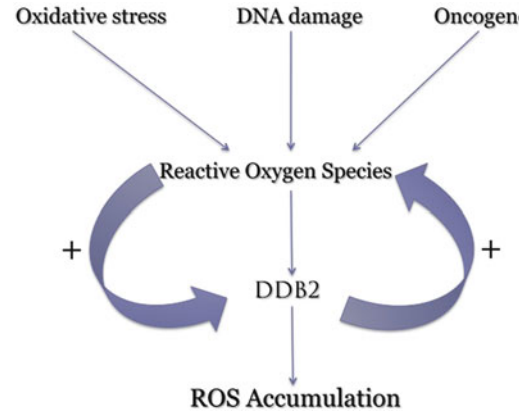

Fig. 12.2 Schematic diagram depicting how DDB2 amplifies ROS accumulation triggering premature senescence

Furthermore, ROS increases expression of DDB, both at the protein and RNA level. This puts DDB2 as a central regulator in a positive feedback loop that following DNA damage, ROS level increases. ROS increases DDB2 expression, which in turn keeps the ROS level high by downregulation of Catalase/MnSOD expression to induce the premature senescence response (Fig. 12.2).

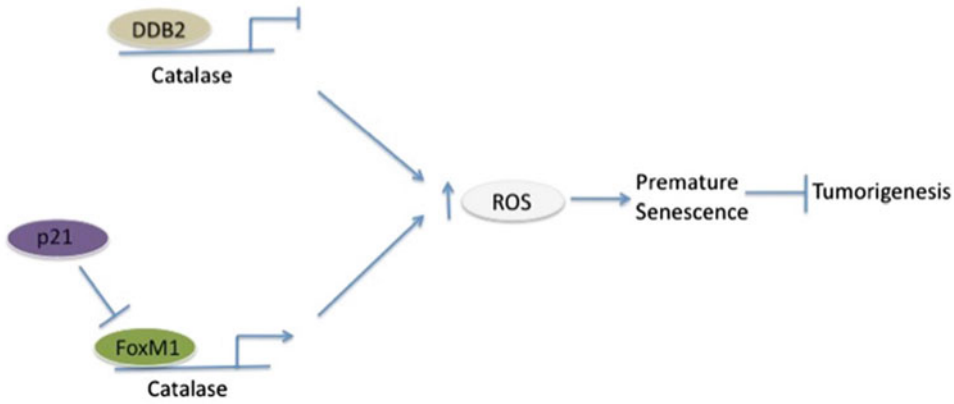

Fig. 12.3 Schematic diagram depicting how DDB2 and p21 cooperate to inhibit UV induced skin carcinogenesis

Physiological Significance of Damaged DNA Binding Protein 2 Mediated Reactive Oxygen Species Accumulation

DDB2 mediated premature senescence plays an important part in its role as an inhibitor of UV induced skin carcinoma formation. DDB2−/− mice were found to be deficient in premature senescence following UV irradiation. This is associated with increased catalase expression in the skin on these mice. DDB2−/− p21−/− mice, which develop skin tumor very aggressively, were found to be further deficient in senescence response. These mice accumulated very little ROS, as there was up-regulation of transcriptional activator FoxM1, expression of which is inhibited by p21 (Barsotti and Prives 2009). Interestingly, in the context of UV induced skin carcinoma progression, DDB2 and p21 cooperate with each other to inhibit tumorigenesis (Fig. 12.3). This finding suggests that the ROS- and senescence induction of DDB2 may be an important pathway through which it acts as a tumor suppressor.

Hepatic stellate cells are the principal contributor of fibrotic response in the liver following chronic liver damage (Krizhanovsky et al. 2008). Upon withdrawal of chronic damage, hepatic stellate cells undergo premature senescence. Senescence of these cells causes their removal, leading to regression of liver fibrosis. Deficiency of hepatic stellate cell senescence keeps these cells active, causing an impaired fibrotic response. DDB2−/− mice were found to be deficient in senescence of liver cells following withdrawal of chronic liver damage, suggesting that DDB2 might play an important role in controlling liver fibrosis.

Interestingly, a recent study found DDB2 to be one of the top ten biomarkers of aging by transcript profiling of whole blood (Nakamura et al. 2012). Authors of this study looked at age reflecting shift in transcript balance in the peripheral blood mononuclear cells of people regardless of gender between age 23 and 77. They identified sixteen transcripts that exhibit age related shift. DDB2 was found to be in the top ten transcripts whose expression levels are positively correlated with age. Authors proposed that the identified genes play a significant role in senescence associated dynamic shift in the immune activity of aged people. However, given the important role DDB2 plays in oxidative stress induced premature senescence, it is very likely that the oxidative stress associated with age increases DDB2 level. The free radical theory of aging states that ROS generated in ones lifetime leads to degenerative diseases associated with aging. Therefore, it will be interesting to examine whether increased ROS production causes DDB2 induction in the aging individuals and whether the levels of DDB2 can be targeted for antioxidant therapy.

Concluding Remarks and Future Perspectives

Various results cited above suggest that regulation of DDB2 gene expression and in turn DDB2 mediated gene expression might be very important in different ROS related pathophysiological conditions including but not limited to cancer. Towards that, DDB2 is found to be down regulated in a wide variety of cancers at the RNA level (Bagchi and Raychaudhuri 2010). Moreover, there is evidence for loss of DDB2 expression at the protein-level in carcinoma patient samples (such as basal cell carcinoma) compared to the normal tissue (Stoyanova et al. 2012). Hence, it is important to recognize how DDB2 expression is lost and the ways it can be increased to trigger the senescence/apoptosis response of cancer cells. DDB2 is a p53-induced gene. Therefore, it is a possibility that with loss of p53 function, DDB2 expression is attenuated. But, there are several instances where p53 mutation gives rise to its gain of function. Also, there are reports showing that regions directly upstream of DDB2 transcription start site are TATA less, G/C rich and consists of Sp1 and NF1 elements (Nichols et al. 2003). These are common properties of house keeping genes and cell cycle regulated genes. These observations clearly indicate that DDB2 is under tight gene expression control and there might be several other factors that contribute to its expression. It is tempting to examine whether there is an increased hypermethylation of CpG islands on the promoter of DDB2 with the carcinoma progression. For several genes, such as p16INK4a, promoter hypermethylation mediated repression has been evidenced in cancer samples (Herman et al. 1995). Therefore, it is very likely that DDB2 is repressed through promoter hypermethylation. It will be important to identify mechanisms to up-regulate expression of DDB2 to induce ROS accumulation that would inhibit tumor progression through senescence/apoptosis. As ROS also increases DDB2 expression, this might be a potential way to elevate DDB2 expression. There are compounds such as PEITC, which elevate ROS level. PEITC is presently in clinical trial for the treatment of prostate cancer (Wang and Chiao 2010). It acts on the transformed cells selectively as transformed cells exhibit elevated ROS generation associated with active metabolism and oncogenic stimulation (Trachootham et al. 2006). PEITC might be an attractive molecule for induction of DDB2 in cancer patients. PEITC induced DDB2 expression will keep the ROS level high by down regulation of MnSOD and catalase and trigger apoptosis/senescence of transformed cells. In conclusion, the discoveries on DDB2's transcriptional function have opened several interesting possibilities that can be exploited in designing novel therapeutics for the treatment of cancer.

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13 p21 Mediates Senescence by a Mechanism Involving Accumulation of Reactive Oxygen Species

Ionica Masgras and Salvador Macip

Contents

Abstract

$p21^{Waf1/Cip1/Sdi1}$ is a potent inhibitor of cyclin-dependent kinases (CDKs) and one of the best characterized p53 direct target genes. Moreover, it is one of the principal inducers of senescence, a permanent arrest phenotype related to ageing and the prevention of cell transformation. It was initially thought that p21 had tumour suppressor properties, due to its ability to stop cell cycle at different stages, either transitorily or permanently. However, recent evidence points to a much more complex picture. It is now well established that p21 itself can trigger apoptosis in certain situations, even independently of p53. On the other hand, p21-mediated cell arrest can actually limit the sensitivity to apoptotic stimuli. To complicate matters even further, p21 has been shown to have other direct pro-survival functions and could even be contributing to tumourigenesis through the secretion of growth factors by arrested cells. At the core of these antagonistic functions of p21 is the generation of Reactive Oxygen Species (ROS), which have been shown to be important both in the induction of apoptosis and the establishment of senescence, and could also participate in the negative effects on tissue homeostasis of the senescent cell secretome. p21 has the ability to stop cancer cell growth and is not mutated in cancer. However, without a better understanding of its pleiotropic functions we will not be able to harness its

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clinical potential. It is therefore important to investigate the mechanisms by which p21 affects cell physiology and its use of ROS as messengers and effectors.

Keywords
p21 • Senescence • Reactive oxygen species • Mitochondria • Tumour suppression

Introduction

The *CDKN1a* gene, localized on chromosome 6p21.2, encodes a 21 kDa protein that was discovered by three independent groups, which named it cyclin/cyclin-dependent kinases (CDKs)-interacting protein 1 (CIP1) (Harper et al. 1993), wild-type p53-activated fragment 1 (WAF1) (el-Deiry et al. 1994) and senescent cell-derived inhibitor 1 (SDI1) (Noda et al. 1994), according to the functions they observed it had. Thus, $p21^{Waf1/Cip1/Sdi1}$ was known from the beginning to be a protein that played a role in the p53 pathway and the cell cycle arrest/senescence response. This placed p21 in a central position in the DNA damage and tumour suppressor mechanisms of the cell. However, in the following years it became clear that p21 functions were far more intricate than previously thought.

p21 is first and foremost a CDK inhibitor. It is a member of the CIP/KIP family, which also includes $p27^{Kip1}$ and $p57^{Kip2}$. They are all capable of inhibiting a broad spectrum of CDKs involved in cell cycle progression and therefore inducing an immediate arrest in different phases of the cell cycle (Sherr and Roberts 1999). In addition, p21 is one of the first direct target genes of tumour suppressor p53 that was characterized, to the point that p21 up-regulation is often used as a marker of activation of this pathway. After being induced by p53 in response to DNA damage and other stimuli, p21 mediates a transient cell cycle arrest. It also binds to proliferating cell nuclear antigen (PCNA), which affects DNA replication and repair processes. Moreover, it has been shown that, depending on the intensity and nature of the stress, this arrest can eventually become permanent as the cell enters a terminal differentiation state known as senescence. The role of p21 as regulator of this process was established when it was discovered that its expression increased in cultured human fibroblasts undergoing replicative senescence (Noda et al. 1994). In recent years, senescence has been identified as a major tumour suppressor mechanism and a determinant process in organismal ageing, and the central role of p21 in triggering it has been confirmed.

A part from its activity as a CDK inhibitor, p21 can also interact with transcription factors, proteins involved in the DNA synthesis and repair processes and apoptotic factors, among others. Because of this, p21 is involved in cell death, DNA repair, cellular differentiation and ageing. It is important to note that p21 can also be induced independently of p53 in response to stimuli such as TGFβ, Histone deacetylase (HDAC) inhibitors or oncogenes such as Ras. Recent studies have shown a completely different side of p21, in which it acts as a promoter of cellular proliferation by preventing apoptosis and inducing growth and pro-survival signals. This implies that p21 could actually be favouring cellular transformation instead of tumour suppression in certain situations. Considering all these aspects, the functions of p21 are still poorly understood. The mechanisms by which it induces senescence are not completely clear and the risk of triggering its potentially carcinogenic effects are a deterrent for exploiting these arresting capabilities in clinical settings.

The Complex Pleiotropy of p21

The Transcriptional Regulation of p21

The p21 promoter contains two highly conserved p53-responsive elements (p53-REs), to which p53 binds with high affinity. The two known p53 homologues, p63 and p73, can also transactivate p21 by binding to these p53-REs. External stress signals, including DNA damage and oxidative stress, activate p53, which in turn transcriptionally up-regulates p21. Similarly, intrinsic and oncogenic stresses activate p53 and subsequently

p21 expression. Because of this downstream position of p21 in the p53 pathway, several proteins that play a role in the activation of p53 also contribute to p21 induction. For instance, BRCA1 functions as a p53-coactivator by recruiting p300/CBP, which in turn acetylates and stabilizes p53. In response to DNA damage, the propyl isomerase Pin1, the tumour suppressor LKB1and GADD34, a member of growth arrest and DNA damage-inducible proteins, facilitate p53 phosphorylation and therefore its transactivation capabilities. Cell division autoantigen 1 (CDA1) stabilizes p53 by inhibiting MDM2, an E3 ligase that ubiquitylates p53 and promotes its degradation. The monocytic leukemia zing finger (MOZ), a MYST-type histone acetyltransferase, induces p21 transcription by interacting with p53 and acetylating histones on the p21 promoter. NORE1A, a pro-apoptotic Ras effector, arrest cells via p21 by promoting the nuclear localization of p53. c-Myc activates p21 transcription through $p19^{ARF}$ which stabilizes p53 by inhibiting MDM2 activity. Interestingly, it has recently been shown that MDM2 not only degrades p53 through a ubiquitin-related proteasome pathway, but it is also able to target p21 to proteasome degradation independently of ubiquitin. All this shows that p21 expression can be modulated by many pathways that impinge on p53.

Other transcription factors induce p21 expression in a p53-independent manner. In the proximal p21 promoter there are several DNA-binding elements, including six Sp1/Sp3 binding sites. They are used to regulate p21 expression in response to various stimuli and stresses, including nerve growth factor (NGF), butyrate, phorbol myristate acetate (PMA) and TGFβ. The retinoblastoma protein, pRB, is also able to induce p21 through the Sp1/Sp3 sites and over-expression of integrin β1 subunit up-regulates p21 transcription by enhancing the recruitment of Sp1 to the p21 promoter. HDAC inhibitors are well-known p21 inducers, and they do so by enhancing histone acetylation around the p21 promoter and the Sp1 sites on the promoter itself, thus releasing the transcriptional repressor HDAC1 from its binding. FOXP3, an X-linked tumour suppressor, induces p21 expression by inhibiting the association of HDAC2 and HDAC4 to a site within the intron 1, and thus by increasing the local histone H3 acetylation. The Ski-interacting protein SKIP/SNW1(SKIP) is a transcription elongation factor critical for both basal and stress-induced p21 expression, confirmed by the fact that depletion of SKIP induces a rapid down-regulation of p21.

Moreover, retinoid X receptor (RXR) activates p21 expression through binding to two consensus RXR-responsive elements in the p21 promoter, and it has recently been shown that androgen receptor induces p21 independently of p53 in order to trigger a senescent response. Tumour suppressor IGFBP-rP1 (Insulin-like growth factor-binding protein related protein 1) is also capable of inducing senescence through p21 and bypassing p53. Intriguingly, caspase 2, which has still poorly characterized functions, is a translational regulator of p21 and participates in its induction after damage. p21 can be also transcriptionally repressed. For instance, c-Myc alleviates G_1 cell cycle arrest via p21 repression by sequestering Sp1 from the p21 promoter. Also, CUT, a homeodomain transcription factor, blocks p21 expression via binding to a sequence that overlaps with the TATA box.

There are eight different p21 splice variants in humans, seven of which (p21V1, p21V2, p21C, p21 alt-a, p21 alt-a′, p21 alt-b, and p21 alt-c) encode for protein p21, while p21B encodes for an entirely different protein (also called p21B). The promoters for p21 and p21B contain common response elements for transcription factors, whereas some response elements are found exclusively in the p21 promoter. A part from the cyclin-cdk interacting region, p21 also has binding domains for procaspase-3 (in the N-terminal region) and PCNA (in the C-terminal region) binding domain, as well as a nuclear localization signal.

The Role of p21 in Cell Cycle Arrest

Upon exposure to growth stimuli, a series of CDKs, including CDK4, CDK6 and CDK2, are sequentially activated during the cell cycle, and this leads to cell proliferation. p21 was originally found in cyclin A, cyclin D1, cyclin E and CDK2 immunoprecipitates (Harper et al. 1993). It was

proposed that p21 arrested cells by affecting the activity of cyclin D-, E- and A-dependent kinases, which regulate progression through the G_1 phase of the cell cycle and initiation of DNA synthesis (Sherr and Roberts 1999). pRB is essential for the G_1-S transition. pRB phosphorylation is initiated by cyclin D-CDK4/6 and phosphorylated pRB is then released from complexes with E2F transcription factors. These are then able to transactivate genes necessary for entry into S phase, including cyclin E. The resulting activation of the cyclin E-CDK2 complex further phosphorylates and completely releases pRB from its interaction with E2Fs. The association of p21 with cyclin D-CDK4/6 inhibits pRB phosphorylation and thus induces cell cycle arrest in G_1. Recent studies suggest that Rb2/p130, a protein that belongs to the same family as Rb, is actually the one that mainly participants in the p53-p21 arrest response after damage (Helmbold et al. 2009).

p21 also associates with and directly inactivates E2F, thus mediating its degradation (Broude et al. 2007b). This is confirmed by the fact that p21 mutants that cannot block cyclin/CDK activity are still capable of inhibiting E2F transactivation. In a similar fashion, it has been proposed that p21 can also reduce p53 stability in some models (Broude et al. 2007a). This would provide a negative feedback loop to limit p53 induction and thus facilitate a recovery of the cell cycle once the transient arrest is resolved. p21 can also arrest cells in the S phase of the cell cycle. Since it forms quaternary complexes with a cyclin, a CDK and PCNA, this binding to PCNA interferes with DNA replication and triggers intra-S phase arrest. This is mainly due to the fact that the p21 binding site in PCNA overlaps with that of the DNA polymerase δ and the replication factor C. p21 may probably control both cyclin-CDK and PCNA activity within the quaternary complex providing a regulatory link between DNA replication and cyclin-CDKs complexes required for the S phase.

Finally, it has also been reported that p21 can arrest cells in G_2 (Roninson 2002). This observation has been linked to the ability of p21 to inhibit both cyclin B-CDK1 and cyclin A-CDK1/2 complexes, which leads to a permanent cell cycle exit in G_2. Furthermore, p21 cooperates with 14-3-3σ, which sequesters cyclin B-CDK1 complexes in the cytoplasm, and thus controls the G_2-M transition. Of note, the ability of p21 to induce a G_2 arrest is not shared by other CDK inhibitors, like p16.

When normal proliferating cells are subjected to DNA damage, the cell cycle temporarily pauses either at G_1, S or G_2 phase, due in great part to p53-dependent p21 up-regulation. Arrest at these checkpoints prevents DNA replication and mitosis in the presence of unrepaired DNA damage and presumably allows time for DNA repair to occur. The proportion of cells that arrest in G_1, S or G_2 after damage depends on cell type, growth conditions, type of damage and the checkpoints operative in the cells. Because of this, p21 is a necessary mediator of p53-induced cell cycle arrest (el-Deiry et al. 1994), as indicated by the fact that p53 cannot arrest cells after DNA damage in p21-null mice. $p21^{-/-}$ cells that fail to arrest in G_1 even after up-regulation of p53 enter mitosis regardless of the presence of DNA damage or chromosome aberrations.

Since p53 function is lost in most human cancers, p21 induction in response to stress is compromised. Because of this, neoplastic cells often have a defective G_1 checkpoint response to DNA damaging agents. Although p21 also participates in the arrest at the G_2 checkpoint, this can also be achieved by the activation of the Chk1 protein kinase, which maintains mitotic cyclin B/Cdc2 complexes in an inactive state. Thus, cancer cells are usually able to stop in G_2 even in the absence of p53-p21 activity (Macip et al. 2006).

The ability of p21 to trigger arrest in different phases of the cell cycle allows p21 to participate in the maintenance of genomic stability. As we will comment below, the role of p21 in the induction of senescence following telomere shortening or certain kinds of stress further corroborates this view. Adding to this, p21 also plays a significant role in modulating DNA repair processes. By temporarily inhibiting cell cycle progression, p21 allows cell survival while DNA repair is completed. Also, p21 can compete for PCNA binding with several proteins that are directly involved in DNA repair processes and modulate PCNA-dependent DNA

repair pathways. Finally, it has recently been shown that p21 regulates the Fanconi Anemia-BRCA1 repair pathway and thus plays an active role in repair processes following exposure to DNA crosslinking agents, which leads to accumulation of chromosomal aberrations in p21 null cells (Rego et al. 2012).

p21 Affects Gene Transcription

Even though p21 is not a transcription factor, some of p21 interactions have an effect on gene expression. In particular, p21 indirectly inhibits E2F1 factors, via pRB dephosphorylation after CDK inhibition, but also directly by binding to them. Moreover, p21 is also able to bind other transcription factors such as STAT3 and Myc, thereby inhibiting their transactivation properties. Thus, p21 may act as a transcriptional cofactor, being able to interact directly with DNA-binding proteins and modulate the activity of the co-activators and co-repressors that associate with them. p21 has also been shown to stimulate NFκB-mediated transcription. This effect is mediated by the activation of transcriptional cofactors p300 and CBP, which enhance not only NFκB but also many other inducible transcription factors (Roninson 2002). p21 can induce the expression of 55 genes, while it represses the expression of 77, including Polo-like kinase 1 (PLK1) and Topoisomerase IIα (TOPO IIα) (Chang et al. 2000b).

To Kill or Not to Kill?

Through its ability to promote cell cycle inhibition, especially after genotoxic insults, p21 is paradoxically preventing cells from undergoing apoptosis. Besides, it has been proposed that p21 could also block apoptosis directly. p21 is implicated in the Nrf2-mediated antioxidant pathway which is considered important for the pro-survival effects of p21 (Chen et al. 2009). Another indication of its anti-apoptotic potential is the observation that p21 is cleaved by caspase 3 at the onset of apoptosis. p21 also blocks the induction of pro-apoptotic genes by Myc and E2F1 through direct binding and inhibition of their transactivation functions. Thus, the ability of p21 to induce cell cycle arrest could be a double-edged sword that could actually act in detriment of the tumour suppressor mechanisms of the cell in certain situations.

Perhaps surprisingly, we and others have shown that p21 can also trigger cell death responses (Inoue et al. 2009; Masgras et al. 2012), although the mechanisms involved in these processes have not been fully elucidated. Tsao et al. (1999) showed that viruses expressing p21 could induce apoptosis as well as cell cycle arrest in cervical cancer cell lines. Consistent with this, p21 can sensitize cells to undergo apoptosis, as shown by the fact that p21 loss in colorectal and ovarian cancer cells or hepatocytes significantly reduced cell death in response to chemotherapy. Also, p21 enhances ceramide-induced apoptosis by increasing Bax expression and antagonizing Bcl2. It has been proposed that p21 can induce pro-apoptotic effectors such as Bax or members of the TNF family (Gartel 2005), as well as p53 (Inoue et al. 2009). However, we have shown that p21 can also induce apoptosis in sensitive cells independently of p53 through a ROS-dependent mechanism (Fig. 13.1). Consistent with this, up-regulation of p21 induces apoptosis in human cervical cell lines independently of p53 and pRB, and retinoic acid triggers senescence via RARβ2-mediated hypomethylation of the p21 promoter.

A part form apoptosis, p21-mediated depletion of proteins that control cell division can lead to abnormal mitosis and genetic destabilization when arrested cells attempt to re-enter cell cycle after p21 down-regulation, causing death by mitotic catastrophe independently of p53 or the apoptotic pathway (Chang et al. 2000a). At the same time, p21 could be inhibiting other pathways, like autophagy, to favour cell death. p21 is known to up-regulate Nrf2 by interfering with Keap1-mediated Nrf2 ubiquitination, and this triggers a series of cytoprotective effects. Moreover, p21-null mice are sensitive to hyperoxic lung injury and to lipopolysaccharide (LPS)-induced endotoxic shock, suggesting that p21 indeed participates in cell survival pathways.

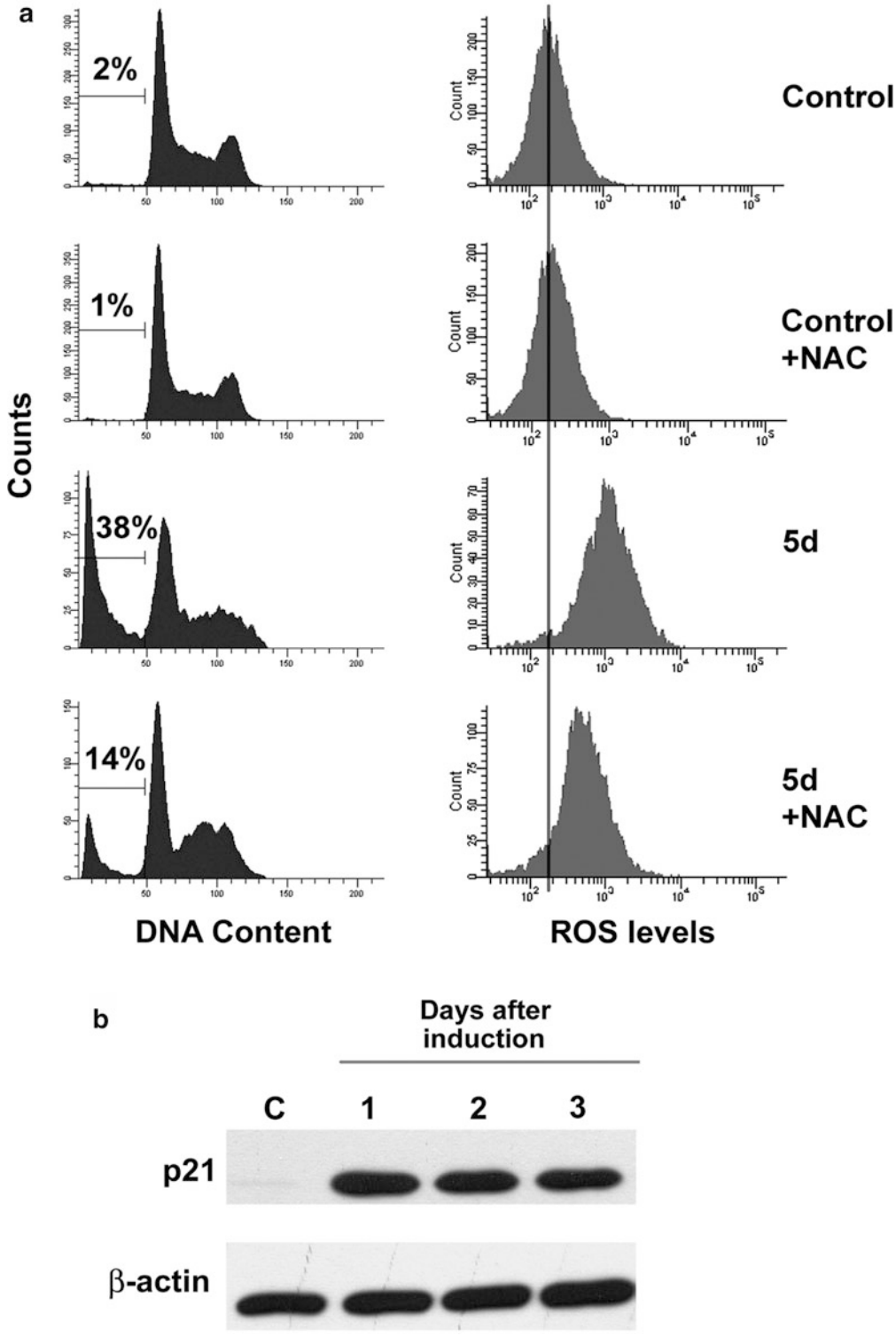

Fig. 13.1 *Increase in intracellular ROS correlates with p21 induction of apoptosis in HT1080p21-9*. (**a**) PI-stained HT1080p21-9, uninduced or 5 days after p21 upregulation, in the presence or absence of 10 mM of the antioxidant NAC. Percentages indicate $SubG_1$ events (dead cells). Right: the same cells stained with DCF (ROS measurement). (**b**) Immunoblot analysis of protein levels in HT1080p21-9 with p21 induced for 0 (**c**) to 3 days (From Masgras et al. 2012)

In view of these seemingly paradoxical results, it is logic to hypothesize that cell fate decisions after p21 induction, ranging from survival to death, largely vary depending on the cellular context. The magnitude of ROS induced by p21 and the involvement of a p21/ROS/p53 feedback signalling pathway will surely play a role in these decisions, as we will comment below (Inoue et al. 2009; Masgras et al. 2012). It has also been proposed that the number of molecules of p21 relative to the cyclin-CDK complex may dictate the ultimate response of a cell to p21.

Post-translational modifications of p21 and its localization may also affect its effects. p21 is post-translationally regulated by various serine and threonine kinases and this determines cell- and context-dependent dissociation of p21 from PCNA, translocation of p21 from nucleus to cytoplasm and stabilization or degradation of the protein. p21 phosphorylation at Thr145 by activated AKT1 or IKKβ signalling prevents the nuclear translocation of p21. Serine and threonine kinase Pim-1 stabilizes p21 protein and instead induces its shift from nucleus to cytoplasm. Whereas the growth-inhibitory functions of p21 are associated with its nuclear localization, the anti-apoptotic activities of p21 are frequently associated with its cytoplasmic accumulation. Cytoplasmic p21 binds to and inhibits the activity of proteins directly involved in the induction of apoptosis, including procaspase 3, caspases 8 and 10, stress-activated protein kinases (SAPKs) and apoptosis signal-regulating kinase 1 (ASK1), while at the same time it promotes cellular proliferation through both the alleviation of CDKs and PCNA inhibition.

The Dark Side of p21: Can It Act as an Oncogene?

As we have discussed, the main function p21 is to stop cell proliferation and, in some cases, even induce apoptosis. Because of this, it seems that its place in the complex network of tumour suppressor mechanisms of the cell is guaranteed. This is supported by the fact that, although p21 is never mutated in tumours, it can be down-regulated by several miRNA that are expressed in cancer cells (Borgdorff et al. 2010). However, while p53 and p16 are two of the most commonly inactivated genes in human cancer, it is usually difficult to correlate the status of p21 to tumour progression or to a prognostic value. In fact, p21 protein levels are often elevated in some cancers without signs of growth inhibition (Abbas and Dutta 2009) and a recent study showed that lack of p21 expression in colorectal tumours could be a good prognostic factor (Lin et al. 2012) . Prolonged p21 induction has been related to events of abnormal mitosis and endoreduplication in recovering cells (Chang et al. 2000a). These events may conceivably lead to genetic destabilization and thus play a role in carcinogenesis and tumour progression of the cells that re-enter into the cell cycle after having had p21 induced.

Several genes induced after p21 up-regulation encode for secreted proteins with paracrine effects on cell growth and apoptosis (Chang et al. 2000b). In particular, it has been reported that conditioned media from p21-induced cells has anti-apoptotic and mitogenic activity and, consequently, the effect of p21 on the cell secretome may contribute to the onset of cancer. Thus, even in the situations where p21 is inducing cell cycle arrest, this may be accompanied by a paracrine growth-stimulatory effect on the surrounding cells. We will further discuss this phenomenon below in the context of senescence.

Because of all this, it has been proposed that p21 can actually favour transformation in certain contexts by inhibiting apoptosis and inducing growth and pro-survival signals, genomic destabilization and expression of secreted mitogenic factors. It is not well understood mechanistically how p21 could exert these radically different functions or even if they reside in separate domains of the protein. Due to the difficulty of selectively activating its tumour suppressor properties without also inducing the potentially oncogenic features, the design of antineoplastic therapies involving p21 regulation has so far been unsuccessful (Abbas and Dutta 2009).

Senescence and Tumourigenesis

The Importance of Senescence in Preventing Cancer

Senescence is a terminal differentiation state in which metabolically active cells are permanently arrested with distinctive morphological changes and phenotypic markers (Dimri et al. 1995). Senescent cells look distinctively flat and elongated under the microscope and are incapable of dividing despite the presence of nutrients and growth factors in the medium (Fig. 13.2a). Once a permanent cell-cycle arrest is established, cells remain viable for long periods of time but never regress to their original state. This phenomenon, named *replicative senescence*, was first observed after serial cell passaging as cells got older, and it was seen that it is a consequence of telomere shortening and/or dysfunction (Campisi et al. 2001).

Shortened and/or damaged chromosome telomeres provided the first molecular explanation for the senescence of cells in culture and it has been shown that they triggered senescence through the p53-p21 pathway. Telomere shortening can be reversed by telomerase, the ribonucleoprotein responsible for telomere lengthening during replication. Telomerase activation is actually one of the major mechanisms through which immortal tumour cells overcome the finite lifespan determined by the barrier of replicative senescence. In vivo, senescent cells increase with age in various tissues and in age-related diseases including atherosclerosis and diabetes.

It was later discovered that senescence can also be prematurely triggered by oncogenes and DNA damage. This senescent phenotype slowly develops over the course of 5–10 days after the stimulus. It has been progressively evident that, apart from its impact on ageing, senescence acts as a tumour suppressor mechanism by controlling the emergence of immortal cells. This alternative type of senescence is mainly due to stress agents and has been called *stress-induced premature senescence* (SIPS). Also, excessive mitogenic signals, like those produced by oncogenes, also induce a premature senescence in normal cells, and this is known as *oncogene-induced senescence* (OIS). These forms of premature senescence have also been observed in vivo. For instance, senescent cells can be observed after chemotherapy and senescent cells have been found in benign nevi but not in the melanomas that arise from them. All this confirms that senescence is a mechanism to prevent malignant progression.

Like apoptosis, senescence is an extreme response to cellular stress and it is important to control damaged cells. It is still unclear what determines whether cells will undergo senescence or apoptosis after damage. Among the determinants that have been characterized there are the cell type and the nature, intensity and duration of the damage. The ability to undergo senescence has been primarily viewed as a property of normal cells, which is lost during neoplastic transformation. However, phenotypic and proliferative changes that resemble terminal stages of replicative senescence can apparently be induced in tumour cells not only through genetic modifications but also by treatment with different classes of chemotherapeutic agents. These findings suggest that certain tumour cells have retained at least some of the components of the senescence-like program of terminal proliferation arrest and that this can be exploited in chemotherapeutic treatments.

The Senescent Phenotype

The principal hallmark of cellular senescence is the inability of cells to progress through the cell cycle. Senescent cells arrest growth normally in G_1 phase, and they fail to initiate DNA replication despite adequate growth conditions. However, they remain metabolically active and they may produce secreted proteins with important effects on neighbouring cells and tissues. In contrast to quiescence, the senescence growth arrest is essentially permanent and irreversible, since senescent cells cannot be stimulated to proliferate by known physiological stimuli. Another characteristic change in the phenotype of senescent cells is the development of resistance to

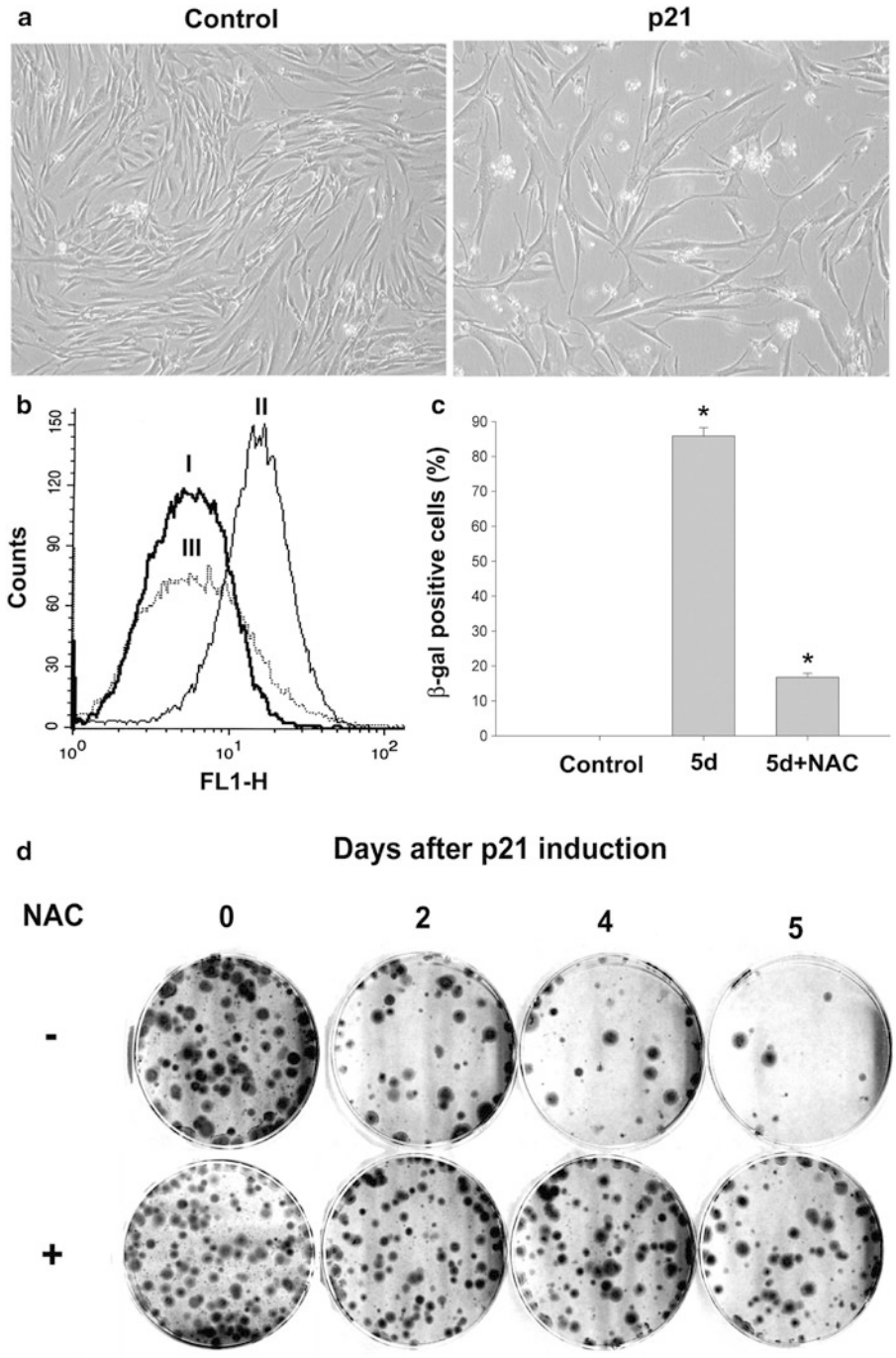

Fig. 13.2 *p21-mediated ROS increases are necessary for maintaining the permanent growth arrest in senescent cells*. (**a**) 501 T human fibroblasts infected with a retrovirus containing p21 and selected with puromycin for 1 week, showing morphology changes and growth inhibition compared to the vector-infected cells. (**b**) ROS levels of EJp21 cells (I) in the absence of exogenous p21 expression, (II) after 5 days of p21 induction and (III) after 5 days of p21 induction with the addition of 10 mM NAC. (**c**) Percentage of SA-β-gal positive cells in EJp21 induced to express p21 for 5 days in the presence or absence of 10 mM NAC. (**d**) Colony formation assay with cells maintained in the presence of induced p21 for 0–5 days before p21 expression was suppressed (From Macip et al. 2002)

apoptosis, although not all cell types display it. Senescence also causes an altered pattern of gene expression, including changes in cell-cycle inhibitors or activators. The two genes that are more often up-regulated in senescent cells are p21 and p16, and they have been used as markers.

Several other markers can identify senescent cells in culture and in vivo. For instance, lack of DNA replication, which can be detected by the incorporation of 5-bromodeoxyuridine or ^{3}H-thymidine, or by immunostaining of proteins such as PCNA and Ki-67. Of note, these detect arrested cells but do not distinguish between senescent, quiescent and differentiated post-mitotic cells. The first marker to be used for the specific identification of senescent cells was the SA-β-gal (Dimri et al. 1995). SA-β-gal activity is thought to be due to the lysosomal β-galactosidase, encoded by the GLB1 gene, and reflects the increased lysosomal biogenesis that commonly occurs in senescent cells. The presence of senescence-associated DNA-damage foci (SDFs) and senescence-associated heterochromatin foci (SAHFs) are other possible markers.

The Side-Effects of Senescence

In spite of the obvious advantages that senescence has in tumour suppressor mechanisms, it has been proposed that it can also have negative effects on the homeostasis of the organism. First, cellular senescence has been linked to tissue and organismal ageing, and thus may play a role in the degenerative and cancerous pathologies related to it. It could also interfere with the effects of chemotherapy by blocking apoptosis.

An altered secretome is emerging as one of the most puzzling aspects of the senescence program. It has a potential wide-ranging impact on a tissue's function, response to damage, and tissue degeneration. Initial studies correlated the senescent phenotype to a paracrine mitogenic and anti-apoptotic effect on the surrounding cells (Chang et al. 2000b). The senescence-associated secretory phenotype (SASP) is complex and includes factors that reinforce senescence-associated proliferation arrest, immune regulators, factors that remodel the extracellular matrix and probably reactive oxygen species that could cause oxidative stress in surrounding cells. Thus, senescent cells might seriously impair tissue function through their secretions. Moreover, although senescent cells cannot themselves form tumours, they may fuel the progression of nearby premalignant cells and facilitate the development of cancer in ageing organisms. A part from these potentially tumourigenic effects, it has been observed that senescent cells also secrete immune regulators that trigger the clearance of incipient cancer cells by the innate immune system. Thus, the real impact of the SASP on the growth of neighbouring cells may be dependent of the context and still needs to be fully elucidated.

How to Induce a Permanent Growth Arrest

Senescence is usually achieved by the activation of the p53-p21 and/or pRB-p16 pathways, which interact with each other but can also independently stop cell cycle progression. Different stimuli and cell-type specific factors may lead to the activation of one pathway rather than the other. For instance, the p53 mediated DNA damage response can induce a temporary arrest through p21 that, if the stimulus persists, can eventually turn into senescence. Similar stresses can also engage the pRB-p16 axis, but this is usually secondary to the activation of the p53. However, other factors, like the expression of oncogenic Ras, preferentially act through pRB-p16. The ability of p21 to trigger senescence was confirmed by the fact that p21 is capable of inducing a permanent arrest in normal and cancer cells in a p53 independent manner.

The importance of the pRB and p53 pathways in senescence is emphasized by the fact that inactivation of both usually abolishes the permanent proliferation arrest, regardless of the initial trigger. Despite this, there are some examples of senescence that appear to be independent of these pathways. The mechanisms involved in these situations have not been yet clearly identified. It has been proposed that genotoxic stresses can induce

senescence in p53-null as well as wt p53-containing cancer cells (for review see Roninson 2003) and that this response plays a role in the suppression of tumour growth by chemo and radiotherapy. However, recent studies have indicated that cancer cells without functional p53 pathways do not undergo a proper senescence response after being treated with a variety of chemotherapeutic agents (te Poele et al. 2002; Macip et al. 2006). Thus, it is not clear whether what has been traditionally considered a G_2-based senescence, observed in p53 null tumour cells after treatment with genotoxic drugs, is really a permanent arrest phenotype comparable to that observed in cells with intact p53-p21 activity.

p21 expression is progressively increased in human fibroblasts in culture, reaching a peak when these cells undergo senescence (Noda et al. 1994). However, this increase is only transient and its levels usually decrease after the establishment of the permanent growth arrest. As p21 level goes down, p16 becomes constitutively upregulated, suggesting that while p21 acts as a trigger, p16 may be responsible for the maintenance of growth arrest in senescent cells. However, a fail-safe mechanism is also induced in the form of a stable increase in intracellular levels of ROS. We have shown that blocking p21-mediated ROS generation prevents the establishment of a permanent arrest and cells resume the cell cycle once p21 levels return to basal levels (Fig. 13.2). Thus, ROS seems to be an important part of the mechanism by which p21 is able to maintain senescence.

The Importance of Relative Oxygen Species (ROS) in Cell Fate Decisions

Reactive Oxygen Species

ROS are generated by normal oxidative processes related to cell metabolism and buffered by antioxidant mechanisms. They are produced initially by the reduction of singlet O_2 to superoxide anion and then H_2O_2 that, if not eliminated, generates the highly reactive hydroxyl free radical that causes DNA damage. Metabolic processes, primarily oxidative metabolism in the mitochondria, and pathological processes, such as inflammation and ischemic reperfusion, are the major endogenous sources of ROS (Bai and Cederbaum 2003). They can also be generated by specific plasma membrane oxidases in response to growth factors and cytokines and serve as secondary messengers in signalling pathways. Both Ras and p53 cause ROS accumulation, which play a role in subsequent cell fate responses (Lee et al. 1999; Macip et al. 2003).

Depending on the level of oxidative stress and the extent of the induced DNA damage, cell fate can vary from temporary arrest to death (Barzilai and Yamamoto 2004). For instance, exposure to H_2O_2 has been shown to induce apoptosis or necrosis depending on concentrations and cellular context (Hampton and Orrenius 1998), whereas low concentrations of oxidants can force normal human fibroblasts to permanently arrest in a senescent-like state (von Zglinicki et al. 1995). The impact of ROS on cellular structures such as DNA and mitochondria depends not only on the magnitude of ROS but also on other physiological factors, for instance the active anti-oxidant mechanisms of the cell. Thus, the threshold of cellular oxidation may vary between different cell types and between normal and cancerous cells.

It is also known that senescent cells have higher levels of ROS than normal cells. Moreover, overexpression of antioxidant genes like Superoxide Dismutase or Catalase causes extension of lifespan in *Drosophila*. This effect can also be observed in cell cultures maintained in low oxygen environments. All of these findings point to a strong relationship between oxidative damage, senescence and ageing. Of note, ROS present at normal physiological levels play a role in regulating signalling pathways and gene expression, and therefore, their production is of vital importance for cellular homeostasis.

Relative Oxygen Species Modulate Cell Fate Decisions

ROS have been implicated as potential modulators of apoptosis that act downstream of p53. Rather

than being just a consequence of the cellular changes associated with apoptosis, p53-generated ROS have been shown to constitute a signal for cell death pathways, presumably mediated by the transcriptional influence of p53 on pro-oxidant genes. One of the classic models for p53-induced apoptosis proposes that p53 triggers cell death through a multistep process that requires transcriptional induction of redox-related genes, formation of reactive oxygen species and oxidative degradation of mitochondrial components.

We have shown that ROS are actually important in determining cell fate after p53 up-regulation, with the redox balance of the cell even being able to turn an initial arrest response into apoptosis (Macip et al. 2003). Moreover, we have reported that p21 can increase ROS levels independently of p53 and that this is required for the permanent arrest observed in senescence. Recent evidence suggests the existence of a threshold of cellular oxidation above which the apoptotic program is initiated (Inoue et al. 2009; Macip et al. 2003). In view of this, a model of p53-p21 activity could be argued, in which cell senescence is triggered via a persistent lower magnitude of ROS accumulation while apoptotic cell death is induced in response to a higher, and sometimes faster, increase in ROS. The intrinsic resistance of the cell to oxidative stress, and particularly that of its mitochondria, may also play a decisive role.

Mitochondria and Relative Oxygen Species in Senescence

Mitochondria are highly dynamic organelles prone to fusion and fission processes. Mitochondrial DNA and proteins can be continuously exchanged and distributed throughout the whole mitochondria population, suggesting that fusion and fission processes may function as a rescue mechanism for damaged mitochondria. The number of mitochondria in dividing cells is thought to be maintained constant through de novo biogenesis and degradation of damaged mitochondria. Some studies have reported an increase in the mitochondrial mass and mitochondrial DNA copy number related to the senescent phenotype. Moreover, mitochondria in senescent cells are elongated due to a decreased expression of Drp1 and Fis1, and it is thought that this protects them against oxidative stress. Increase in mitochondrial mass has also been observed prior to release of cytochrome c from the mitochondria and apoptosis, which suggests that these different mechanisms of tumour suppression could have some common mechanisms of initiation.

These changes in the mitochondrial biogenesis process have been correlated to a mechanism termed *retrograde response*, a nuclear response to mitochondrial dysfunction that has been well characterized in *Saccharomyces cerevisiae* but which is not fully understood in mammalian cells. The retrograde response includes deregulation of Ca^{2+}-dependent signalling, up-regulation of mitochondrial biogenesis and major metabolic and anti-apoptotic adjustments. Calcium appears to play an important role in this process as there is an increase in the cytoplasmic Ca^{2+} levels because of the low Ca^{2+} storage capacity of dysfunctional mitochondria with low membrane potential. Such changes in mitochondrial metabolism are most probably an attempt at adaptation of cells to senescence-associated high levels of ROS.

As cells age, mitochondria increase their superoxide production and it is thought that this contributes to replicative senescence. Also, dysfunctional mitochondria that accumulate around the nucleus are thought to participate in oncogene-induced senescence after mutant Ras expression, with the increase in mitochondria mass and ROS generation being dependent on intact p53 and pRB pathways. Furthermore, the interconnected mitochondria phenotype and the down-regulation of the fission mechanism are accompanied by major resistance to apoptosis of senescent cells. This further demonstrates that mitochondria of senescent cells have evolved mechanisms of protection against the increased levels of ROS.

The pathways leading to the establishment of senescence by p21 and ROS are complex and not fully understood. However, an elegant study by Passos et al. (2010) has defined the implication of a dynamic feedback loop triggered by a DNA damage response, which locks the cell into an

actively maintained state of "deep" cellular senescence. According to this, high levels of ROS may damage mitochondria, leading to the opening of mitochondrial pores and allowing the influx of protons and ions. Thus, increased mitochondrial membrane permeability leads to loss of the proton ion gradient across the mitochondrial membrane and to the consequent decrease in potential which, in turn, may increase ROS production. Since damaged mitochondria generate more ROS, this suggests a self-amplifying feedback cycle. The mechanisms involved in mitochondrial dysfunction are likely to be common to all types of cellular senescence regardless of the trigger, since these changes have been detected in both telomere-dependent replicative senescence and premature senescence due to oncogenic stress. Their results also suggest that during the early establishment phase of senescence, the presence of DNA damage foci, replenished by the increase in ROS, is necessary to maintain growth arrest long enough for the process to become irreversible. All these data together supports the hypothesis that senescence could be a prolonged arrest phenotype sustained by the self-maintained DNA damage caused by a permanent elevation in intracellular ROS. Genes such as p53 or p21, capable of definitely shifting the oxidative balance of the cell towards increased ROS levels, possibly through engagement of mitochondria, could therefore hold the key to the induction and preservation of the phenotype.

Discussion

It is no doubt surprising that 20 years after its discovery, we are still not able to fully describe the basic functions of p21. What initially seemed like a straightforward CDK inhibitor with links to tumour suppressor pathways has become the picture of a complex network of interactions and cell fates, some of them radically opposed. It is even difficult sometimes to place p21 in one of the sides of the pro- and anti-neoplastic balance in the cell. There is obviously a strong dependence on the cellular context when it comes to decide cell fates after p21 induction and we will need more research to understand exactly what p21 is doing in a given model and why. It seems that ROS are important mediators of more than one of p21 functions, and their intracellular balance may hold the key to solve this riddle.

p21 can increase ROS in both normal and tumour cells proportionally to its protein levels, which shows a direct link between these two events (Macip et al. 2002). However, the mechanism implicated is still not clear. It is not just a consequence of cell cycle arrest, since p16 is not able to induce ROS. Prolonged expression of p16 induces a senescence-like arrest in cancer cells, but following p16 down-regulation this was found to be reversible (Macip et al. 2002). These and other findings strengthen the hypothesis that ROS accumulation is necessary for the permanent growth arrest phenotype induced by p21.

p21 blocks cell cycle progression in both G_1 and G_2 through inhibition of several CDKs, as well as PCNA. These functions, which reside in different domains of the protein, are each sufficient to cause growth arrest. In contrast, p16 specifically inhibits CDK 4 and 6 and has no effect on PCNA. Therefore, it was hypothesized that this could be the critical difference that allows p21 but not p16 to induce ROS. However, a p21 mutant that lacks PCNA binding ability retains the ability to induce growth arrest specifically in G_1, implying that PCNA binding is required for maintenance of the G_2 arrest function of p21. This mutant p21 can also induce ROS accumulation and senescence, therefore binding to PCNA and its G_2 arrest function are not required for these responses.

Fibroblasts undergoing replicative senescence exhibit increased ROS, p16 and p21 levels, although p21 up-regulation is only transient. The use of ROS increases as mediator of the permanent growth arrest, which then becomes independent of the presence of p21 or p16, provides a possible explanation for these observations. p21 can also induce a p53-independent cell type-specific and, at least in part, ROS-dependent apoptotic response (Masgras et al. 2012) . This confirms that the elevation of intracellular ROS levels is an important part of the mechanism by which p21 exerts its functions.

The source for p21-mediated ROS is still not clear. Some results point at a mitochondrial origin, maybe through induction of mitochondria-related genes. Since p21 can act as a modulator of transcription, this could explain its effect on the redox balance of the cell. Consistent with this, we observed that p21 induced up-regulation of PIG3, a pro-oxidant gene (Macip et al. 2002). We have also observed that p21 induction of cell death is not immediate and required prolonged expression of p21, which could reflect the necessity to accumulate sufficient intracellular ROS to trigger a certain amount of mitochondrial damage.

According to this model, short-term expression of p21 would induce cell cycle arrest, while apoptosis would only be achieved at a later time point if the stimulus is maintained and a threshold of oxidative stress is surpassed. The difference between inducing senescence or apoptosis could be then determined by the intensity of the stimulus, the levels of p21 induced and, importantly, cell-specific factors. p21 levels could indeed have a dose-dependent effect in cell fate decisions (Inoue et al. 2009). However, protein levels are not necessary determinant, since similar p21 induction can cause different effects depending on cell context (Masgras et al. 2012).

The sensitivity of mitochondria to oxidative stress is likely to be one of these factors. Recent data showed that cancer cells with primed mitochondria respond better to cell death stimuli (Ni Chonghaile et al. 2011). The apoptotic functions of p21 are likely to be preferentially observed in those cancer cells that have accumulated higher mitochondrial damage or defects in the intracellular/mitochondrial ROS buffers. Otherwise, cells would choose to undergo a less drastic response in the form of senescence. Cell fate decisions after p21 up-regulation would no doubt be better understood if we could determine cell sensitivity to oxidative stress and, specifically, which factors make mitochondria resistant to the elevation in intracellular ROS.

Since normal cells usually have intact antioxidant and DNA repair mechanisms, therapies that up-regulate p21 could have the potential to be more toxic for sensitive cancer cells. For instance, compounds that induce p21 independently of p53, like MLN4924, could trigger cell death in p53-null cancer types. The mitochondrial response to ROS could be a predictive marker of cancer cell sensitivity to p21. However, we have to keep in mind that p21 may have several pro-survival effects that could interfere with its tumour suppressor activity. Because of this, there are no antineoplastic therapies specifically directed at up-regulating p21 expression in cancer cells, despite its promising potential. The antitumoural functions of p21 could be enhanced if its abilities to cause cell death were favoured over induction of arrest and senescence, and thus the negative effects of the senescent cell secretome could be averted.

The study of p21 is likely to provide more surprises. We are just beginning to understand the complexity of its functions and further discoveries will determine why and how p21 promotes or suppresses tumourigenesis depending of the context. The ability to induce ROS and the cellular sensitivity to oxidative stress will no doubt take a prominent role in this and could even allow us to modulate p21 activity to selectively induce senescence or apoptosis when needed.

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Role of MicroRNAs and ZEB1 Downmodulation in Oxidative Stress-Induced Apoptosis and Senescence

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Contents

Abstract

It has been recently shown that some microRNAs (miRNAs) are involved in oxidative stress-induced cellular responses. Our studies elucidated a new role for miR-200 family miRNAs and of its target ZEB1 in endothelial cells (EC) respon se to hydrogen peroxide (H_2O_2). Specifically, ZEB1 expression down-modulation was observed upon oxidative stress in human umbilical vein endothelial cells (HUVEC). ZEB1 and miR-200 family miRNAs were previously shown to be involved in the molecular mechanisms underpinning apoptosis and senescence, both in non-transformed cells and in tumor cells. Our study confirmed a prominent role of ZEB1 down-modulation in the induction of growth arrest, apoptosis and senescence in EC, consequently to the oxidative stress increase of miR-200 family expression.

In vivo experiments in a mouse model of acute hindlimb ischemia, an insult that induces an increase in oxidative stress, enhanced miR-200 family expression in wild-type mice skeletal muscle. In contrast in p66ShcA$^{-/-}$ mice, which display lower levels of oxidative stress after ischemia, up-regulation of miR-200 family members was markedly inhibited. Further studies are needed in order to comprehend whether ZEB1 down-modulation following oxidative stress inducing stimuli is also elicited by other miRNAs and which molecular pathways are involved in ZEB1 ability to modulate cell proliferation, apoptosis and senescence.

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In conclusion, miRNAs may represent novel targets to prevent the deleterious consequences of diseases associated with enhanced oxidative stress.

Keywords
Free radicals • Endothelium • Apoptosis • Senescence • Ischemia

Introduction

Reactive oxygen species (ROS) is a collective term that includes a number of reactive and partially reduced oxygen (O_2) metabolites, with some of them being free radicals, such as superoxide anion (O_2^-) and hydroxyl radicals (·OH), that are extremely reactive molecular species with an unpaired electron in their outer orbital. The third most relevant molecule included in ROS is hydrogen peroxide (H_2O_2), that is more properly a prooxidant and nonradical molecule. Formation of ROS is an unavoidable consequence of aerobic metabolism. ROS can be generated within living cells by the following major sources: mitochondria, plasma membrane NADPH oxidase (NOX) and different enzymes involved in redox reactions such as several oxidase, peroxidase, cytochromes, mono- and di-oxigenases (Finkel 2003).

Accumulation of ROS, can inflict damage to DNA, proteins, and fatty acids. It is generally believed that oxidative stress caused by ROS plays a causal role in numerous pathologies, including tissue ischemia and reperfusion, cancer, diabetic vasculopathy, pulmonary fibrosis as well as in aging (Giorgio et al. 2007). To cope with the burden of oxidative damage, a series of enzymatic and non-enzymatic antioxidant defences have evolved to neutralize ROS (Finkel 2003).

In addition, cells also utilize "adaptive responses" aimed at minimizing the damages caused by ROS. Mammalian cells respond to oxidative stress with an increase in expression of antioxidant enzymes and activation of protective genes. To defend themselves against DNA oxidation induced by ROS, proliferating cells arrest their cell cycle, preventing the mutated DNA from being replicated and transferred to daughter cells. This delay in cell growth enables the cells to repair the damaged DNA and to set up an adaptive response to counteract further oxidative damage. According to whether or not damaged DNA is repaired, cells either resume cell growth or enter a status of permanent proliferative arrest known as cellular senescence.

Cellular senescence can result from telomere shortening after multiple rounds of cell division (replicative senescence) or from various stresses such as oncogenes or oxidative stress and occurs independent of a change in telomere length (stress-induced premature senescence, SIPS) (Campisi 2011). While cellular senescence is considered a protective mechanism against cancer, it has also been hypothesized that the progressive accumulation of senescent cells in some tissues may contribute to several age-related diseases and organismal aging (Campisi 2011). Signaling that induces senescence has been extensively studied, and two major tumor suppressor cascades have been unravelled: one involves the retinoblastoma protein (pRb) pathway and the other involves p53; both pathways orchestrate the exit from cell cycle.

The retinoblastoma family of growth-inhibitory proteins is an integral part of the mechanisms that control cell growth under normal conditions and after exposure to genotoxic stimuli. This family includes three members: pRb and the related p107 and p130 (also known as p130Rb2) (Cobrinik 2005). Collectively, these proteins are called "pocket" proteins because they share a common domain, named the "pocket." Pocket proteins control the G1 checkpoint of the cell cycle through their ability to bind and sequester members of the E2F family of transcription factors, which modulate the expression of genes involved in cell cycle progression. The ability of pocket proteins to bind their interactors is abolished through cell cycle-regulated phosphorylation by cyclin-dependent kinases (CDKs).

In early G1, pRb is hypophosphorylated and becomes hyperphosphorylated in late G1 prior to entry into S phase of the cell cycle. pRb phosphorylation increases even further as cells progress through S and G2; p107 and p130 are regulated

in a similar fashion (Cobrinik 2005). Inhibitors of CDKs, Cip/Kip and INK4 families, provide another level of regulation (Pei and Xiong 2005). Indeed, increased levels of CDK inhibitors in response to stress or differentiative cues inhibit pRb phosphorylation and cause growth arrest.

Two cell cycle inhibitors that are often expressed by senescent cells are the CDKIs p21 (also termed CDKN1a, p21Cip1, waf1 or SDI1) and p16 (also termed CDKN2a or p16INK4a) (Campisi 2011). These CDKIs are components of tumor suppressor pathways that are governed by p53 and pRb proteins, respectively. Both p53 and pRb are transcriptional regulators that are frequently deregulated in cancer; both pathways can establish and maintain growth arrest that is typical of senescence. The expression of p21 is induced directly by p53 but the mechanisms that induce p16 are not completely understood. Ultimately, p21 and p16 maintain pRb in a hypophosphorylated and growth arrest active state, but their activities are not equivalent. Cells that senesce solely due to p53–p21 activation can resume growth after inactivation of the p53 pathway. However, although cells that senesce due to oncogenic RAS (which induces p16 expression) can resume limited proliferation, cells that fully engage the p16–pRb pathway for several days usually cannot resume growth even after inactivation of p53, pRb or p16 (Campisi 2011). Certain genotoxic stimuli elicit the activation of p53, which, in turn, transactivates the transcription of the p21 gene. Alternatively, oxidative stress induces p21 protein levels, triggering the stabilization of p21 mRNA in a p53-independent manner (Gorospe et al. 1999).

Another mechanism of induction of growth arrest caused by oxidative stress requires phosphate removal from pRb by phosphatases. We have elucidated the molecular mechanisms underlying pRb dephosphorylation upon oxidative stress by the protein phosphatase 2A (PP2A) (Cicchillitti et al. 2003; Magenta et al. 2008). We demonstrated that pRb dephosphorylation by PP2A was very rapid and did not require p53 or p21 induction, nor CDKs down-modulation. Moreover, we showed that intracellular Ca^{2+} mobilization was necessary and sufficient to trigger pRb dephosphorylation and that PP2A activity was Ca^{2+}-induced (Magenta et al. 2008). Thus, growth arrest and senescence are crucial component of the cellular responses to oxidative stress.

Moreover, ROS can also induce cell death, either by apoptosis or by necrosis, according to the level of oxidative stress experienced by the cell and its genotype (Ray et al. 2012). One mechanism of induction of apoptosis by oxidative stress involves p53 (Borras et al. 2011). A pivotal role in ROS induced apoptosis is also played by the p66 isoform of ShcA protein. The mammalian adaptor protein ShcA has three isoforms, p46, p52 and p66; all isoforms share a common structure, but $p66^{ShcA}$ has an additional domain at the N terminus. This domain contains a serine residue at position 36 (Ser-36) that is phosphorylated in response to several stimuli, including UV irradiation and H_2O_2 (Migliaccio et al. 1999). While p52/p46 are cytoplasmic signal transducers involved in mitogenic signaling from activated tyrosine kinase receptors to Ras, p66 isoform is not involved in Ras activation and regulates ROS metabolism and apoptosis (Giorgio et al. 2007). A fraction of $p66^{ShcA}$ is localized in the mitochondria and functions as a redox enzyme that generates mitochondrial ROS as signalling molecules for apoptosis (Giorgio et al. 2007). According to these data, both $p66^{ShcA}$ ko cells and mice display lower levels of intracellular ROS and are resistant to apoptosis induced by a variety of different stimuli (Giorgio et al. 2007). Likewise, our group demonstrated that $p66^{ShcA}$ ko mice are resistant to ischemia-induced apoptosis and show decreased vascular and muscle damage in response to hindlimb ischemia (Zaccagnini et al. 2004).

Since both ischemia and ischemia/reperfusion induce oxidative stress, we concluded that $p66^{ShcA}$ plays a crucial role in ROS induced cell death following acute ischemia and ischemia/reperfusion, indicating $p66^{ShcA}$ as a potential therapeutic target for prevention and treatment of ischemic tissue damage (Zaccagnini et al. 2004).

MicroRNAs

Oxidative Stress and MicroRNAs

MicroRNAs (miRNAs) are small non coding RNAs, usually 21–23 nucleotides long, which regulate the stability and/or the translational efficiency of target messenger RNAs (mRNAs) (Bartel 2009). A wide range of cell functions are under miRNAs control including regulation of proliferation, differentiation, senescence and death (Chang and Mendell 2007).

Different stimuli that produce ROS are known to induce modulation of miRNAs expression such as UV, H_2O_2, ionizing radiation, and anticancer drugs such as etoposide and anthracyclines. A number of studies have underlined the role of different miRNAs in ROS induced cell responses. For example, UV radiation induces a significant increase of miR-22 expression which may promote cell survival via the repression of tumor suppressor gene phosphatase and tensin homolog (PTEN) expression (Tan et al. 2012). Different reports showed miR-21 up-regulation upon oxidative stress (Lin et al. 2009; Simone et al. 2009). The increase of miR-21 expression following 6 h (hrs) of 200 μM H_2O_2 exposure in rat vascular smooth muscle cells (VSMC) was shown to have an anti-apoptotic function, since programmed cell death protein 4 (PDCD4) was demonstrated to be a miR-21 target (Lin et al. 2009). Moreover, screening of miRNAs in human fibroblasts exposed to radiation, H_2O_2 or etoposide had been performed in order to find common signature in response to genotoxic oxidative stress (Simone et al. 2009).

Our group recently demonstrated that miR-200 family was induced upon oxidative stress (Magenta et al. 2011). This miRNA family consists of five members: miR-200c and miR-141 clustered on chromosome 12, and miR-200a, miR-200b, and miR-429 clustered on chromosome 1. We demonstrated that miR-200c and miR-141 were the most up-regulated miRNAs in human umbilical vein endothelial cells (HUVEC) exposed to 200 μM H_2O_2 for different period of times whereas the other three clustered members were up-regulated to a lower level (Fig. 14.1). Since miR-200c is well expressed in HUVEC, whereas the other family

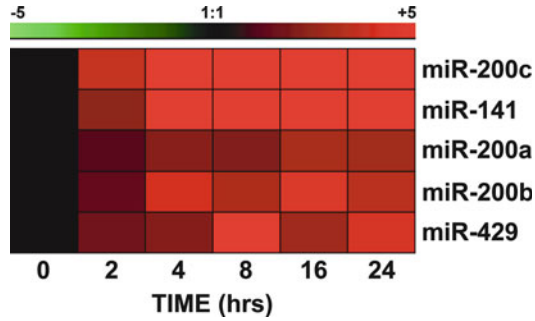

Fig. 14.1 Heat map representing miR-200 family miRNAs modulation in HUVEC exposed to 200 μM H_2O_2 for the indicated times. Modulations are expressed using a log2 scale (−ΔΔCt). *Green* and *red* indicate down- and up-regulation, respectively (n = 4)

miRNAs are barely detectable, miR-200c is likely the main effector of oxidative stress-induced biological responses in endothelial cells (EC). The induction of miR-200c upon H_2O_2 was also confirmed in other cell lines, such as human fibroblasts and C2C12 myoblasts and myotubes (Magenta et al. 2011). In order to demonstrate that miR-200c up-regulation was oxidative stress-dependent we used other interventions that cause red/ox imbalance, i.e. the alkylating agent 1,3-bis(2 chloroethyl)-1-nitrosourea (BCNU) that is a glutathione reductase inhibitor that blocks the conversion of oxidized to reduced glutathione, ultimately leading to an intracellular increase of oxidative stress. Incubation of HUVEC with BCNU enhanced miR-200c and this phenomenon was inhibited by a free-radical scavenger N-acetyl-L-cysteine (NAC). Therefore we concluded that miR-200c induction was oxidative stress dependent.

Our data are also supported by another study showing that miR-200c and miR-141 were up-regulated in a cell model of oxidative stress by treatment of House Ear Institute-Organ of Corti 1 (HEI-OC1) cells with different concentrations of tertbutyl hydroperoxide (t-BHP) (Wang et al. 2010).

MicroRNAs in Apoptosis and Senescence

It is well known the crucial role played by miRNAs in different cellular responses including apoptosis and senescence. Specifically, numerous

miRNAs, referred to as "apoptomirs", have targets involved in apoptotic signaling and both pro-apoptotic and anti-apoptotic miRNAs have been described (Vecchione and Croce 2010). For example, miR-1 has a pro-apoptotic function targeting HSP60 and HSP70, which are primarily anti-apoptotic proteins since they inhibit the mitochondrial death pathway at different points. On the other end miR-133 targets caspase-9 and consequently it has an anti-apoptotic effect (Vecchione and Croce 2010). Similarly, let 7a, one of the let-7 miRNA members, targets caspase-3 and therefore displays an anti-apoptotic function as well (Vecchione and Croce 2010).

As previously described, miR-21 expression is induced by ROS and has an anti-apoptotic function, since its targets molecules are involved in the induction of apoptosis, i.e. phosphatase and tensin homolog (PTEN) and PDCD4 (Vecchione and Croce 2010).

The pro-survival protein Bcl-2 is a crucial molecule that contributes to cell death inhibition, nowadays different miRNAs are known to target Bcl-2, including miR-15a and miR-16-1, miR-1 and miR-34a (Vecchione and Croce 2010).

The miR-34 family plays a crucial role both in cell cycle progression and in apoptosis and senescence. The double positive feedback loop between miR-34 and p53, determines a miR-34 family role in the cellular responses activated by DNA damage such as oxidative stress, radiation and chemotherapeutic drugs (Yamakuchi et al. 2008). This family of miRNA includes three members: miR-34a, miR-34b and miR-34c.

Different targets have been attributed to miR-34 family; specifically, Cyclin E (CCNE2), cyclin dependent kinase 4 (CDK4) and 6 (CDK4), E2F3, E2F4, hepatocyte growth factor receptor (MET) are miR-34a targets and contribute to the arrest of proliferation (Yamakuchi and Lowenstein 2009). Over-expression of miR-34a, in fact, promoted cell cycle arrest, pRb dephosphorylation; apoptosis was also induced, possibly due to Bcl2 targeting (Yamakuchi and Lowenstein 2009).

Another important target of miR-34a is the class III histone deacetylase silent information regulator 1 (SIRT-1), a gene that regulates cellular senescence and limits longevity. SIRT1 plays an important protective role against oxidative stress and DNA damage, deacetylates different proteins such as pRb, FOXO, ku70 NFkB, pGC1 alpha and p53. Deacetylation of p53 diminished its transcriptional activity, inhibiting the expression of p21 and PUMA. The latter is a proapoptotic Bcl-2 homology 3 (BH3)-only Bcl-2 family member, that is considered as one of the main downstream effectors of p53 in apoptosis induction, since it directly binds and antagonizes all known antiapoptotic Bcl-2 family members, inducing mitochondrial dysfunction and caspase activation. Therefore p53 deacetylation mediates cell survival during periods of severe stress inhibiting apoptosis. Yamakuchi et al. (2008) demonstrated that miR-34a over-expression suppresses SIRT1 leading to an increase of deacetylated p53 and of its targets p21 and PUMA expression, ultimately inducing apoptosis (Yamakuchi et al. 2008).

The effects of miR-34a on senescence have been largely demonstrated; miR-34a is known to regulate senescence in several cancer cell lines and elevated level of miR-34a were found in senescent HUVEC as well as in heart and spleen of older mice (Ito et al. 2010). Moreover, miR-34a over-expression induced EC senescence and introduction of miR-34a and b/c in human diploid fibroblasts caused senescence (Ito et al. 2010; Vecchione and Croce 2010). Ito et al. (2010) demonstrated that SIRT1 mediates miR-34a regulation of senescence in HUVEC, given SIRT1 role in cellular senescence, and forced expression of SIRT1 blocked the ability of miR-34a to induce senescence.

Another important miRNA that targets SIRT1 is miR-217. Over-expression of miR-217 induces EC senescence whereas miR-217 inhibition delays senescence. Further, high levels of mir-217 had been found in human atherosclerotic lesion and its expression was associated with reduced level of SIRT1 (Vecchione and Croce 2010).

Somehow, miR-200 family resembles miR-34 gene family in many aspects. First, our study and other reports demonstrated that this miRNA family is under p53 control (Chang et al. 2011; Kim et al. 2011; Magenta et al. 2011) and, secondly, our group demonstrated that miR-200c over-expression in EC is able to induce growth

arrest, apoptosis and senescence (Magenta et al. 2011). We found that over-expression of miR-200c significantly inhibited EC proliferation inducing pRb dephosphorylation and moreover, induced an increase in apoptosis, measured by apoptotic DNA fragmentation and by the percentage of cells displaying subdiploid DNA content. Finally, miR-200c over-expression induced EC senescence, testified by an increase of senescence-associated β-galactosidase (SA-β-gal) activity, characteristic feature of senescence-related growth arrest, and by expression of p21 protein, which increases in response to oxidative stress and, as previously described, plays a major role in permanent growth arrest/senescence. Since all these effects are also induced by oxidative stress we next evaluated whether miR-200c inhibition prevented oxidative stress-induced effects.

Our results demonstrated that H_2O_2 induction of growth arrest, apoptosis and senescence, in EC were all diminished when miR-200c increase was prevented by the means of an anti-miR-200c oligos. Different studies underlined miR-200 family role in apoptosis and senescence also in tumor cells. For example, a pro-apoptotic role of miR-200c was discovered showing that this miRNA targets the apoptosis inhibitor FAP1, thereby sensitizing tumor cells to apoptosis (Brabletz and Brabletz 2010). In keeping with a miR-200c role in senescence, it has been shown that miR-200c is up-regulated by chronic oxidative stress-induced senescence in human fibroblasts and in human trabecular meshwork cells (Li et al. 2009). Notably, SIRT1 was also demonstrated a direct gene target of miR-200a. Eades et al. (2011) showed that loss of miR-200a expression is associated with breast cancer transformation and is responsible, at least in part, for SIRT1 over-expression.

Zinc Finger E-Box Binding Homeobox 1 (ZEB1)

ZEB1 in Normal and Tumor Cells

Zinc finger E-box binding homeobox 1 (ZEB1; also known as Zfhx1A, δEF1, Tcf8 and Zfhep) is a transcriptional repressors that binds a set of E-box-like promoter elements that overlap with those bound by ZEB2 (also known as Sip1) and the Snail family of transcription factors (Brabletz and Brabletz 2010). Each of these E-box-binding proteins can act as a transcriptional repressor through recruitment of the co-repressor, C-terminal binding protein (CtBP; Ctbp1) HDACs and BRGG1. In contrast to Snail proteins, ZEB factors (ZEB1 and ZEB2) are capable of interacting with the transcriptional co-activators p300 and pCAF and can subsequently switch to a transcriptional activator under still poorly defined conditions.

ZEB factors are strong inductors of epithelial-to-mesenchimal transition (EMT) by suppressing the expression of many epithelial genes, including E-cadherin, that is a key event in tumor invasion. Therefore ZEB proteins are increasingly recognized as important contributors to tumor progression and metastatic spread. A crucial activator of ZEB factors is the TGF-β signaling pathway, indicating that they are pivotal players in TGF-β-induced EMT. Forced expression of ZEB factors in epithelial cells results in a rapid EMT characterized by cell polarity impairment, loss of cellular adhesion and induction of cell motility. On the other end, ZEB factors knockdown in undifferentiated cancer cells induces a mesenchymal-to-epithelial transition (MET).

In a number of EMT model systems it has been shown that autocrine TGF-β signaling contributes to the stability of the mesenchymal state, moreover a TGF-β family members, TGF-β2 was shown to be targeted by miR-141/200a in cancer cells (Burk et al. 2008), leading to the hypothesis that repression of miR-200a during EMT may facilitate induction of autocrine TGF-β signaling.

In normal conditions ZEB1 is expressed in proliferating cells in the developing embryo, and in cell culture (Liu et al. 2007). Its expression is under the Rb/E2F cell cycle pathway. Mutation of the Rb or E2F1 genes lead to induction of ZEB1 mRNA, implying that the Rb–E2F1 repressor complex is important for repression of ZEB1. Vice versa, ZEB2 is also regulated by E2Fs, but in contrast with ZEB1 this regulation is Rb-family-independent. In keeping with these notions, ZEB1 mRNA was induced in mouse

embryonic fibroblasts (MEF) from mice where all three Rb family member genes had been mutated; in contrast, expression of ZEB2 mRNA was not induced (Liu et al. 2007).

Moreover, ZEB1 is also a negative regulator of muscle differentiation; ZEB1, in fact, binds to a subset of E boxes in muscle genes and actively represses transcription (Postigo and Dean 1997). The relative affinity of ZEB1 for myogenic basic helix–loop–helix (bHLH) family proteins (i.e. myoD, myf-5, myogenin and MRF-4) varies for E boxes in different genes; as myogenic bHLH proteins accumulate during myogenesis, ZEB1 would be displaced from different genes at distinct times, thus providing a mechanism to modulate the timing of gene expression (Postigo and Dean 1997).

Data from several research groups pointed to the involvement of miR-200 family in EMT (Brabletz and Brabletz 2010); the entire miR-200 family is downmodulated in EMT and the most prominent target factors identified in all studies are ZEB1 and ZEB2. The miR-200 family can be divided in two subgroups according to their seed sequences (a conserved heptametrical miRNA sequence responsible for targets' mRNAs recognition). Subgroup I: miR-141 and miR-200a and subgroup II: miR-200b, -200c and -429. The ZEB1 3′ UTR contains eight miR-200 binding sites (five for subgroup II members and three for subgroup I members), and the ZEB2 3′ UTR contains nine binding sites (six for subgroup II members and three for subgroup I members). Moreover, a double-negative feedback loop between miR-200 family and ZEB factors has been elucidated, ZEB factors, in fact, are transcriptional inhibitors of all miR-200 family members; as a result ZEB factors and miR-200 family miRNAs not only have opposite functions, but also reciprocally control the expression of each other (Brabletz and Brabletz 2010). Since ZEB factors are strong EMT inducers, the consequence of miR-200 over-expression is the reduced expression of ZEB factors and subsequent epithelial differentiation. Different studies demonstrated that in EMT miR-200 family down-modulation enhances cancer aggressiveness and metastases, whereas reintroduction of miR-200 family miRNAs reverted this phenomenon (Brabletz and Brabletz 2010).

ZEB1 in Apoptosis and Senescence

It has been well documented a role of ZEB1 both in apoptosis induction and in the establishment of senescence. In cortical neurons ZEB1 protein is induced by ischemia and activates a prosurvival response involving p73 proteins. ZEB1, in fact, is a transcriptional repressor of the pro-apoptotic isoform of p73, therefore ZEB1 induction is part of neuronal protective response to ischemia (Bui et al. 2009). Moreover, ZEB1 expression has also been shown to protect tumor cells from apoptosis. It is known that the induction of EMT is generally associated with reduced apoptosis and increased cell survival, as a consequence, cells that have undergone EMT are more resistant to toxic stress, including chemotherapy and radiotherapy (Brabletz and Brabletz 2010). In agreement, it has been shown that long-term exposure of breast cancer cells to TGF-β, a highly potent inducer of ZEB1 expression, induces EMT and inhibits apoptosis (Brabletz and Brabletz 2010). Further, knockdown of ZEB1 in pancreatic and colorectal cancer cell lines not only affects their stem-cell properties, but also increases the sensitivity of the cells to chemotherapeutic agents.

Moreover, ZEB1 had been shown to play a role in cellular senescence. Zeb1 mutant MEFs, in fact, revealed proliferation defects and undergo premature replicative senescence through a mechanism involving the up-regulation of p21 and p15INK4b expression, and not the INK4a locus (Liu et al. 2008). In keeping with these results, Liu et al. (2008) demonstrated that ZEB1 binds to p21 and p15INK4b promoters, inhibiting their expression. Notably, both CDK inhibitors take part in TGF-β signaling.

In line with the above described studies we found that ZEB1 knockdown in HUVEC had effects similar to those caused by miR-200c over-expression and by oxidative stress, i.e. induced cell growth arrest apoptosis and senescence (Magenta et al. 2011). Indeed, ZEB1 knockdown resembles the effects described in Zeb1 mutant MEFs, it induced cellular senescence, testified by SA-β-gal-positive cells and by the increase of p21 protein, in contrast we did not find a p16INK4a increase. We also demonstrated a

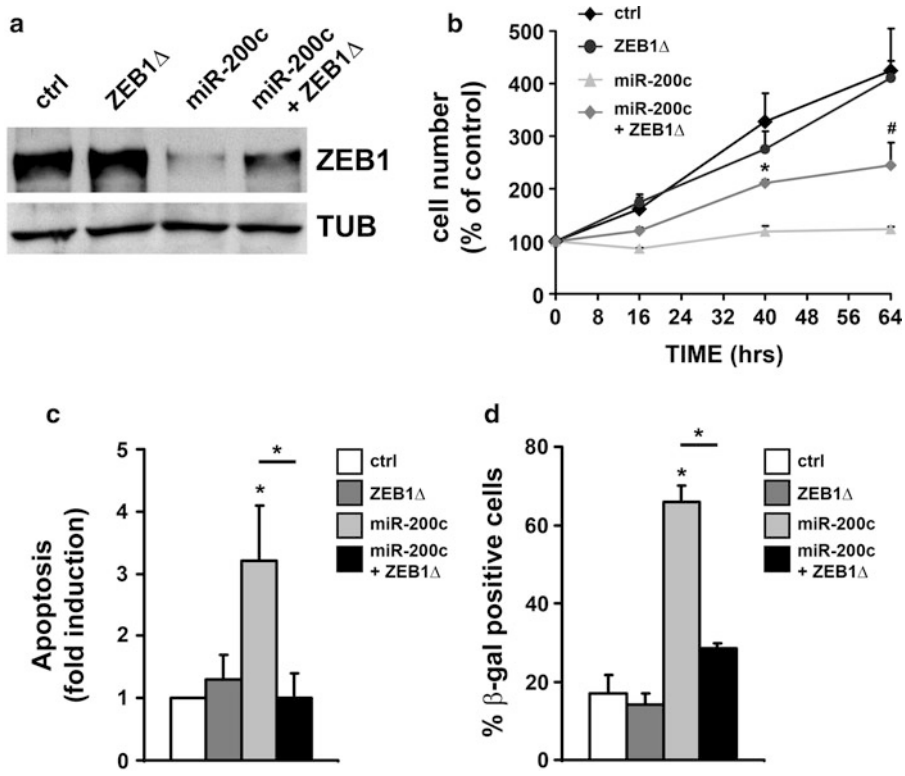

Fig. 14.2 HUVEC were co-infected with lentiviruses encoding miR-200c and a ZEB1 lacking the 3′UTR (ZEB1Δ). Single infection either of miR-200c or ZEB1Δ was performed together with ZEB1Δ backbone vector (vec) or miR-scramble viruses, respectively. Control cells were co-infected with miR-scramble and vec viruses. (**a**) Representative western blot demonstrating the expression level of ZEB1 in cells co-infected with miR-200c and ZEB1Δ. miR-200c forced expression decreased ZEB1, this effect was inhibited in cells expressing ZEB1Δ. Tub, α-tubulin (n = 3) (**b**) HUVEC co-infected with miR-200c and ZEB1Δ showed a higher proliferation rate compared with miR-200c over-expressing cells (n = 5 at each time point;*P < 0.001; #P < 0.05). (**c**) The proapoptotic effect of miR-200c was abolished by the expression of ZEB1Δ (n = 3; *P < 0.05). (**d**) Cells co-infected with miR-200c and ZEB1Δ showed a decrease of the % of SA-b-gal-positive cells compared with miR-200c-infected cells (n = 3; *P < 0.001) (Magenta et al. 2011)

direct implication of ZEB1 down-modulation in the establishment of miR-200c-mediated effects. In fact, the expression of a ZEB1 allele devoid of most-3′UTR sequence, and that therefore cannot be targeted by miR-200 family miRNAs, reverted miR-200c-induced phenotype, enhancing cell growth and inhibiting apoptosis and senescence (Fig. 14.2).

Oxidative Stress and ZEB1 Expression

We also investigated H_2O_2 effects on ZEB factors expression in HUVEC, given the role of miR-200 family in oxidative stress-induced phenotype. We found that upon H_2O_2 exposure both ZEB1 mRNA and protein expression were down-modulated and interestingly, ZEB1 mRNA was inversely related to miR-200c and miR-141 induction. Indeed, the lowest expression level of ZEB1 mRNA (73.7% reduction) was achieved after 16 h exposure to H_2O_2 , that was the time point were miR-200c and miR-141 reached their maximum expression levels compared to control (29.3 and 23.3 fold induction respectively). ZEB1 protein down-modulation was also observed in HUVEC exposed to BCNU treatment and it was prevented by pre-treatment with the ROS scavenger NAC.

The H_2O_2 effect on ZEB2 protein could not be established because its protein level was undetectable in HUVEC. In contrast, ZEB2 mRNA was expressed, but the H_2O_2 effect was relatively minor, and no statistically significant difference from control was achieved.

In agreement with these results, miR-200c-overexpressing HUVEC exhibited a 53% decrease in ZEB1 mRNA, whereas ZEB2 mRNA decrease was minor and did not reach a statistical significance (Magenta et al. 2011).

The oxidative stress effects on miR-200 family expression were reproduced *in vivo*, in a mouse model of hindlimb ischemia. We found that miR-200c, miR-200b and miR-141 increased in ischemic skeletal muscles and the increase in miR-200c and miR-200b was markedly attenuated in p66ShcA$^{-/-}$ mice, which display lower levels of oxidative stress after ischemia, confirming the causal role of oxidative stress in the modulation of these miRNAs also *in vivo* (Magenta et al. 2011).

Discussion

Our work on oxidative stress and miRNAs underlined the role of miR-200 family and in particular of ZEB1 protein in ROS-induced cellular responses. We could establish a direct link between miR-200c induction by oxidative stress, and the establishment of cell growth arrest, apoptosis and senescence through a mechanism involving ZEB1 down-modulation. Indeed, we clarified the complex mechanism of miR-200 family induction. First of all we could establish that miR-200c induction was transcriptionally induced (Magenta et al. 2011). This was achieved demonstrating that the precursor of mature miR-141 and miR-200c, that are cotranscribed in a common precursor, pri-miR-200c-141 was up-regulated upon H_2O_2 exposure in HUVEC. Further, miR-200c and miR-141 common promoter was induced by H_2O_2, supporting the transcriptional regulation of these miRNAs; moreover the promoter mutated in ZEB1 binding sites was not significantly induced upon H_2O_2 exposure, suggesting that H_2O_2 induction of the promoter was largely mediated by ZEB1 down-regulation.

We also demonstrated that pRb and p53 were both involved in H_2O_2-induced miR-200c up-regulation. We showed that miR-200c increase upon H_2O_2 exposure was lower in MEF Rb$^{-/-}$ compared to MEF Rb$^{LoxP/LoxP}$, indicating a role of pRb in this induction. These data are in agreement with Liu et al. (2007) results that showed an increase of ZEB1 mRNA in MEF mutated in all three Rb family genes. It is tempting to speculate that ZEB1 expression could be elicited by the lack of pRb, inhibiting through the negative feedback loop miR-200c induction upon oxidative stress. We also demonstrated that miR-200c forced expression in HUVEC induced pRb dephosphorylation, and p21 expression, in line with the arrest of cell proliferation and establishment of senescence. Moreover, as further confirmation of these data, H_2O_2-induced dephosphorylation of pRb was partially prevented by miR-200c inhibition.

The results of our own studies and of previous reports indicate that in EC, H_2O_2 causes pRb dephosphorylation by different mechanisms: PP2A activity, p53 and p21 increase, and also by a miR-200c-dependent mechanism involving a ZEB1-mediated up-regulation of p21. As a consequence, H_2O_2-dependent pRb dephosphorylation inhibits E2F activity causing the down-modulation of ZEB1 mRNA, contributing to ZEB1/miR-200 double feedback loop reinforcement (Fig. 14.3). Further, we demonstrated a role of p53 in miR-200c induction by H_2O_2, since p53 knockdown markedly inhibited H_2O_2-dependent miR-200c up-regulation, and p53 over-expression induced miR-200c expression in EC, in keeping with other papers indicating a role of p53 in EMT, as p53 it is involved in miR-200c transcription (Chang et al. 2011; Kim et al. 2011). Therefore, H_2O_2-mediated p53 increase and pRb de-phosphorylation are both involved in miR-200c up-regulation, reinforcing ZEB1/miR-200-feedback loop (Fig. 14.3).

We cannot rule out that other targets of miR-200 family are involved in the establishment of oxidative stress induced phenotype. Moreover it is also possible that other miRNAs that target ZEB1 could reinforce this mechanism. Indeed, another miRNA that is induced by p53, miR-192 (Georges et al. 2008) that has ZEB1 protein as a

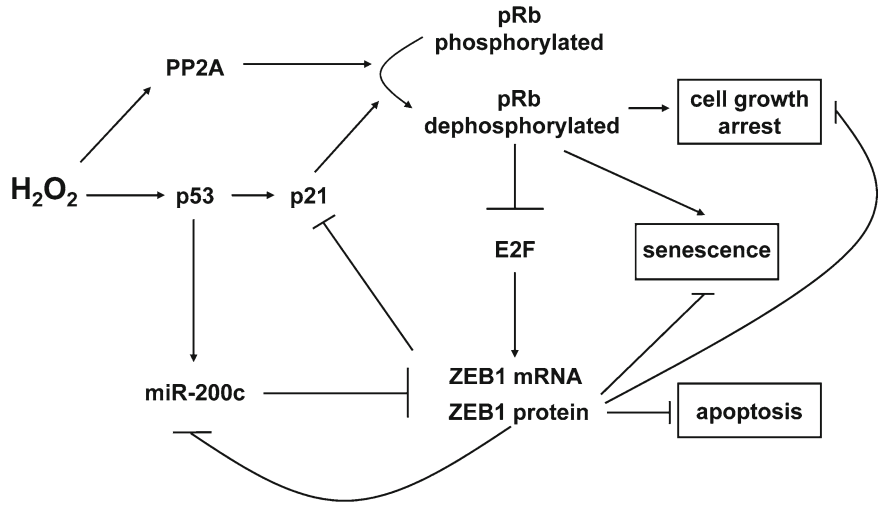

Fig. 14.3 H_2O_2 causes pRb dephosphorylation by a PP2A dependent pathway and by the increase of p53 and p21, which inhibits the cyclin-dependent kinases (CDKs). pRb dephosphorylation, in turn, inhibits E2F activity down-modulating ZEB1 mRNA and consequently ZEB1 protein, allowing p21 transcription. Moreover, ZEB1 down-modulation induces miR-200c up-regulation because of the ZEB1/mir-200 inhibitory feed-back loop. miR-200c up-regulation is also caused by p53. Finally, miR-200c increase caused growth arrest and senescence, by pRb dephosphorylation and ZEB1 down-modulation, and apoptosis by ZEB1 decrease (Magenta et al. 2011)

target, is slightly induced by H_2O_2 in our profiling in HUVEC.

An interesting link between miR-200 family and oxidative stress is also described in a recent article that showed a crosstalk between oxidative stress and miR-200 family (Mateescu et al. 2011). The authors confirmed miR-200 family induction following H_2O_2 exposure in different cell lines i.e., human and mouse immortalized fibroblasts, colon carcinoma (CT26), mammary gland epithelial cells (NMuMG) and human cell lines, melanoma cells (MDA-MB-435S), kidney cells (293T), breast adenocarcinoma (MDA-MB-436 and BT-549) and ovarian adenocarcinoma (SKOV3). Notably, in all these cell lines all miR-200s were upregulated (Mateescu et al. 2011). The authors found similar kinetics in fibroblasts and epithelial cells from mouse and human and, interestingly, miR-200c and miR-141 were the most up-regulated family members, as we found in HUVEC.

Mateescu et al. (2011) found that miR-141 and miR-200a, which display the same seed sequence, targeted p38 α mitogen-activated protein (MAP) kinase, which is a broadly expressed signaling molecule that participates in the regulation of cellular responses to stress, as well as in the control of proliferation and survival of many cell types. Indeed, p38α acts as a sensor of oxidative stress, and its redox-sensing function is essential in the control of tumor development (Mateescu et al. 2011). Enhanced expression of miR-200 family miRNAs mimics p38α deficiency and increases tumor growth in mouse models, but it also improves the response to chemotherapeutic agents. High-grade human ovarian adenocarcinomas, in fact, are associated with an oxidative stress signature and display high levels of miR-200a and low concentrations of p38α (Mateescu et al. 2011). Since p38α inactivation, pharmacologically or genetically, is known to induce ROS accumulation and activation of antioxidant defences, the authors unravel a new interaction between miR-200s and oxidative stress response that affects human ovarian carcinogenesis and prognosis.

In summary, miR-200 family and its targets play an important role in oxidative stress-induced cellular responses and also in the regulation of ROS production.

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Hypoxic Response in Senescent Brain Is Impaired: Possible Contribution to Neurodegeneration

15

Tamer Rabie

Contents

Abstract

Neurodegenerative conditions are characterized by neuronal damage leading to a decline in neuronal functions. As a consequence cognitive and motor functions are impaired. Neurodegenerative conditions can either be chronic/progressive (e.g. Alzheimer's disease or Parkinson's disease) where neuronal damage slowly progresses or it can be acute (e.g. stroke) where the neuronal damage is abrupt. Hypoxia has been shown to contribute to both types of neurodegenerative conditions. Under hypoxic conditions endogenous defense mechanisms are activated to compensate for the reduction in oxygen as well as to increase the self-healing capacity of the organism. The transcription factor hypoxia-inducible factor (HIF) is a master player in initiating the hypoxic defense mechanism by regulating the expression of proteins essential for the adaptation to hypoxia. Prolyl hydroxylase domain proteins (PHD) are negative regulators for HIF. A fine balance between HIF and PHD is thus essential for an appropriate hypoxic defense mechanism. It is thought that this balance is disrupted during the aging process and that this disruption contributes to age-related ailments. In this chapter the possible contribution of a dysfunctional hypoxic response during senescence to neurodegenerative conditions in the brain will be discussed.

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Keywords
Hypoxia • Neurodegeneration • Aging • HIF-1 alpha • PHD

Introduction

Adequate and consistent oxygen supply is essential for the survival of all mammalian organs. Because oxygen acts as the terminal electron acceptor in the electron transport chain within the mitochondria, in absence of oxygen mitochondrial respiration and oxidative phosphorylation is inhibited and cellular energy generation is severely compromised. Whereas atmospheric oxygen levels are 21%, oxygen levels within the body vary depending on the tissue with a mean tissue oxygen levels of 3%. Even within the same tissue, oxygen levels can vary. For instance, in the brain oxygen levels vary from 1 to 5%. Because of its high energy demand and its limited anaerobic capacity, the brain is particularly sensitive to a lack or a reduced supply of oxygen. When oxygen levels are low i.e., hypoxia, different defense mechanisms are activated in order to protect the brain from the possible detrimental effects. For instance, vasodilation may be induced in an attempt to increase cerebral blood flow. Another strategy would be to induce angiogenesis or produce neuroprotective factors. At the cellular level, the metabolism might switch from oxidative phosphorylation towards a less oxygen demanding pathway i.e., glycolysis. For this to be efficient cells may decide to increase their glucose uptake. In order for all these processes to occur, cells need to have molecular oxygen sensors that can detect a low oxygen tension. These sensors must also be able to liaise with executive molecule(s) that initiate the different defense mechanisms.

One of the key executive molecules that are involved in such compensatory mechanisms is the transcription factor hypoxia-inducible factor-1 (HIF-1). When HIF-1 is activated the transcription of a battery of target genes is initiated. Among those target genes are vascular endothelial growth factor (VEGF) which is involved in angiogenesis to improve blood supply to areas of the brain affected by hypoxia as well as in neuroprotection; erythropoietin (Epo) which exerts neuroprotective effects and promotes neuronal survival; glucose transporter 1/3 (GLUT1/3) to increase glucose flux into the cells; in addition to genes involved in glycolysis/glucose metabolism to improve the utilization of glucose under anaerobic conditions. The role of these and other target genes in the endogenous adaptive response to hypoxia will be discussed in detail in later sections. As we grow older the ability of our bodies to adapt to hypoxia diminishes. The focus of this chapter will be on the hypoxic response in the brain, how it is influenced by aging, and any possible implications of aging/hypoxic response interplay on age-related neurodegenerative diseases of the brain.

Hypoxic Response – Key Players

Hypoxia-Inducible Factor-1

Hypoxia-inducible factor-1 (HIF-1) belongs to the basic helix-loop-helix (bHLH) family of transcription factors. HIF-1 is a heterodimer composed of two subunits, an α (120 kDa) and a β (91–94 kDa) subunit. Two other isoforms of HIF-1α exist; HIF-2α, and HIF-3α (Miyata et al. 2011). Although HIF-1α and HIF-2α have the same binding site on their target genes (known as hypoxia-response element (HRE)) they have different tissue distribution and target genes. Interestingly, HIF-3α might act as a negative regulator of the HIF system. Whereas the β subunit (also known as aryl hydrocarbon receptor nuclear translocator; ARNT) is constitutively expressed, cellular levels of HIF-α are determined by several factors. A summary of HIF-α regulation is illustrated in Fig. 15.1.

HIF-α Regulation – Oxygen Tension

Under normoxic conditions HIF-α is hydroxylated on specific proline residues (Pro402 and Pro564) within its oxygen-dependent degradation domain by a family of enzymes called prolyl hydroxylase domain proteins (PHDs) (Miyata et al. 2011). For this hydroxylation reaction to take place, molecular oxygen (O_2), iron (Fe^{2+}) as well as 2-oxoglutarate are needed. This hydroxylation reaction

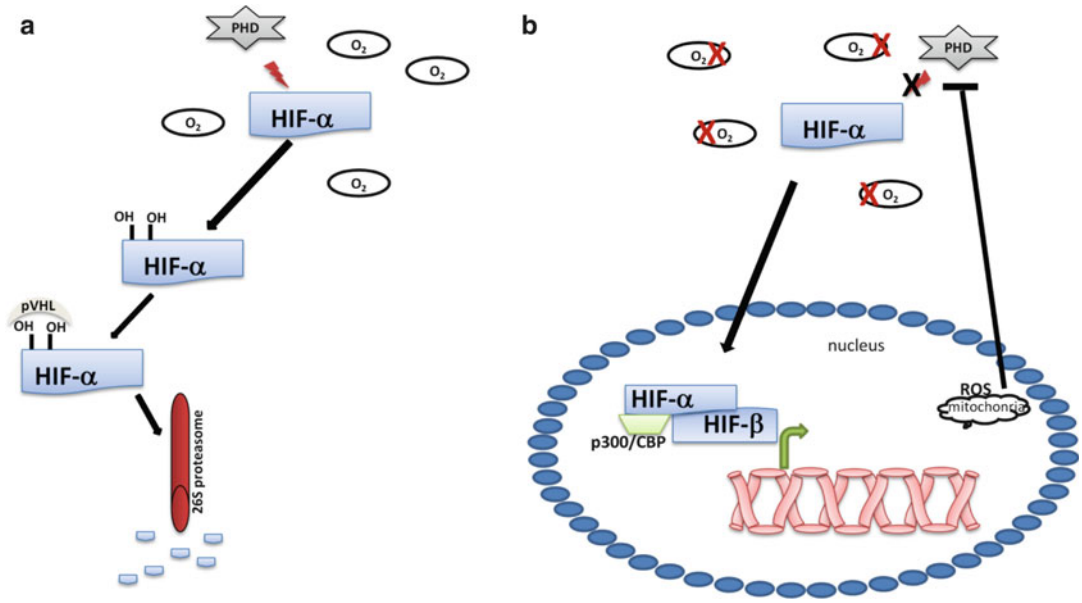

Fig. 15.1 Regulation of HIF-α. (**a**) Under normoxic conditions PHD enzyme is active and hydroxylates HIF-α on two proline residues. This allows the binding of the von Hippel-Lindau (pVHL) protein E3 ubiquitin ligase leading to proteasomal degradation of the α subunit. (**b**) Under hypoxic conditions PHD is inhibited by lack of oxygen and by mitochondrial ROS. As a result, HIF-α is not degraded and translocates into the nucleus where it forms a complex with HIF-β and the transcriptional co-activator p300/CBP. The transcription of target genes is then initiated. *ROS* reactive oxygen species

allows the binding of the von Hippel-Lindau (pVHL) protein E3 ubiquitin ligase leading to the subsequent proteasomal degradation of HIF-α. As PHD requires molecular oxygen for its activity, under conditions where oxygen is scarce (hypoxia) PHD is inactive and thus HIF-α is not degraded and accumulates in the cytoplasm. It then translocates into the nucleus where it binds HIF-β and the transcriptional co-activator p300/cAMP response element-binding protein-binding protein (CBP). HIF complex then binds the HRE elements on the promoter of HIF target genes and their transcription is initiated (Miyata et al. 2011). Another mechanism by which HIF-α activity is regulated takes place in the nucleus by factor-inhibiting HIF-1 (FIH-1). FIH-1 is a hydroxylase that requires Fe^{2+} and 2-oxoglutarate for its activity. Under normoxic conditions FIH-1 hydroxylates a specific asparagine residue (Asn803) within the C-terminal transactivation domain of HIF-1α blocking its binding to p300/CBP and thus the transcription of HIF-1α target genes is repressed (Correia and Moreira 2010).

HIF-α Regulation – Oxidative Stress

It has been shown that oxidants have the ability to induce HIF-1α stabilization. For instance, hydrogen peroxide (H_2O_2) has been shown to stabilize HIF-1α and induce the expression of its target genes under normoxic conditions (Guglielmotto et al. 2009). Paradoxically, reactive oxygen species (ROS) generation has been shown to increase under hypoxic conditions. Mitochondria are the source of ROS generated during hypoxia as mitochondria-depleted cells fail to produce ROS under hypoxic conditions. Genetic and pharmacological studies have shown that ROS generation is mainly attributed to the mitochondrial complex III and cytochrome c. Using these cells it has been reported that mitochondria-generated ROS is essential for hypoxia-induced HIF-1α stabilization. In line with these finding, antioxidants blocked hypoxia-induced HIF-1α stabilization (Guzy et al. 2005). These findings suggest that mitochondria can act as molecular oxygen sensors regulating HIF-1α stability/activity and thus the hypoxic response. Interestingly, functional

mitochondria are important for the stabilization of HIF-1α under hypoxic but not anoxic conditions (Guzy et al. 2005). As the mitochondrial inhibitors rotenone and myxothiazol have been reported to maintain PHD activity under hypoxic conditions, mitochondrial ROS-induced HIF-1α stabilization is most likely mediated by the inhibition of PHD (Guglielmotto et al. 2009).

HIF-α stability/expression can also be influenced by inflammatory mediators and tumor suppressors. However, this is beyond the scope of this chapter and is mentioned in detail elsewhere (Majmundar et al. 2010). Although the levels of HIF-α is governed mainly by posttranslational modifications, HIF-α can also be induced by hypoxia in different organs. For instance, it has been reported that the expression of both HIF-1α and HIF-2α was induced in the brain of rats subjected to systemic hypoxia (Sharp and Bernaudin 2004).

Prolyl Hydroxylase Domain Proteins

As the activity of the PHDs depends on the cellular oxygen tension and PHDs' activity plays a major role in the regulation of HIF-α levels, PHD enzymes are considered cellular oxygen sensors. So far three family members of PHD have been identified (PHD1-3) (Miyata et al. 2011). The expression pattern of these family members differs with PHD2 ubiquitously expressed, PHD1 expressed in the testis, and PHD3 expressed in the heart (Willam et al. 2006). The PHDs differ also in their substrate specificity. Whereas PHD2 shows preference towards HIF-1α, PHD3 seems to be more active towards HIF-2α (Appelhoff et al. 2004). While PHDs negatively regulate HIF-α levels, hypoxia induces the expression of PHD2 and PHD3 in what seems to be a negative feedback loop (Willam et al. 2006). Recent genetic studies delineated the functions of different PHD family members. Conditional knockout of PHD2 induced VEFG expression with a subsequent increase in angiogenesis and new blood vessels formation. Similarly, PHD3 knockout mice demonstrated an improved revascularization after hindlimb ischemia in comparison to wild-type mice (Miyata et al. 2011). In contrast, PHD1 −/− mice showed no change in angiogenesis but a significant change in glucose metabolism (Aragones et al. 2008). The authors reported that absence of PHD1 shifted glucose metabolism towards the anaerobic pathway (glycolysis) by limiting substrate availability for the tricarboxylic acid (TCA) cycle. This effect is mediated through the induction of pyruvate dehydrogenase kinase-4 (PDK-4) expression. When PDK-4 is activated, it phosphorylates pyruvate dehydrogenase leading to its inhibition. As pyruvate dehydrogenase converts pyruvate into acetyl-CoA, the starting compound in the TCA cycle, inhibition of pyruvate dehydrogenase by PDK-4 inhibits the TCA cycle and thus energy generation from oxidative phosphorylation. Interestingly, induction of PDK-4 expression is mediated by HIF-2α and not HIF-1α as is the case for PDK-1 (Aragones et al. 2008).

Pharmacological Inhibition of PHD

It is possible to activate the PHD/HIF-α pathway experimentally to induce the endogenous protective mechanisms. One way to achieve this goal is by the pharmacological inhibition of PHD. As the activity of PHD requires Fe^{2+}, iron chelators such as deferoxamine (DFO) can be used to inhibit PHD activity. Alternatively, cobalt chloride ($CoCl_2$) which depletes intracellular ascorbate, essential for Fe^{2+} activity, is also used as an inhibitor for PHD. These agents are also called hypoxia mimetics. In addition, inhibition of PHD has been sought clinically to increase Epo production for the treatment of anemia. For this, more specific PHD inhibitors, FG-2216 and FG-4592, have been developed and are currently being tested in clinical trials (Bernhardt et al. 2010; Majmundar et al. 2010).

Hypoxic Response During Senescence

As we age the ability of our bodies to adapt to stressful conditions is compromised. Evidence exists that this also applies to the adaptation to hypoxic stress. For instance, it has been reported that DNA binding activity of HIF-1 is

impaired in senescent mice exposed to hypoxia (Frenkel-Denkberg et al. 1999). In addition, HIF-1α protein levels were shown to be reduced in smooth muscles obtained from old rabbits in comparison to young animals (Rivard et al. 2000). Consistent with these findings, it has been reported that ischemic induction of HIF-1α in the hindlimb of old mice is impaired with the consequence of a reduced expression of angiogenic factors and reperfusion (Majmundar et al. 2010). Very recently, we have demonstrated similar findings in the brain of old mice. In that study, hypoxic induction of VEGF, Epo, and GLUT-1 in the brain was reduced in old mice. Moreover, when old mice were subjected to a short period of hypoxia (preconditioning) followed by a strong ischemic insult, no protective effect was observed suggesting that the impaired hypoxic response has functional consequences (Rabie et al. 2011). This effect was attributed, at least in part, to an increase in the levels of PHD1. In addition to HIF-1α, levels of PHD proteins are perturbed during senescence. In particular, PHD1 levels in the brain of old mice (Rabie et al. 2011) and PHD3 in the heart of old mice and humans (Rohrbach et al. 2005) were reported to increase.

Role of the Mitochondria

As mentioned earlier, mitochondria play a key role in hypoxia-induced HIF-1α stabilization. During the aging process mitochondrial function is compromised and the expression of different mitochondrial proteins is reduced. For instance, the expression of complex I, complex III, and complex IV in the brain of 24-old mice has been shown to strongly decrease (Manczak et al. 2005). Since mitochondria-depleted cells or cells treated with a complex III inhibitor fail to stabilize HIF-1α under hypoxic conditions (Guzy et al. 2005), the observed reduction in complex III mRNA in the brain of old mice might lead to a reduced hypoxic induction of HIF-1α and thus to a reduced hypoxic response. Additionally, as inhibition of the mitochondria maintains PHD activity (Guglielmotto et al. 2009) and thus an increased HIF-α degradation, mitochondrial dysfunction might compromise the hypoxic response during aging.

In the following sections the role of the PHD-HIF axis in different neurodegenerative conditions will be discussed. The focus will be on conditions that are more prevalent in the aging population. Thus, possible implications of the perturbed hypoxic response that is observed during senescence will be highlighted. In particular, the implications of any age-related changes in the expression levels or activity (related to hypoxia or not) of HIF and PHD will be discussed.

PHD/HIF Pathway in Neurodegenerative Diseases

Hypoxia has been shown to be involved in the etiology of several neurodegenerative diseases. Genetic and pharmacological studies have shown that activation of PHD/HIF pathway elicits protection against some of these conditions. The effect of PHD/HIF activation in age-related neurodegenerative conditions will now be discussed.

Alzheimer's Disease (AD)

Alzheimer's disease (AD) is a form of dementia characterized mainly by memory loss and impairment in cognitive functions caused by severe neuronal loss. It is a progressive neurodegenerative disease with up to 35 million people affected. As we now live longer and the prevalence of AD increases by age, this figure is likely to increase over the next decades. AD can be familial early onset, caused by gene mutations or can be sporadic affecting persons >65 years old (Guglielmotto et al. 2009). A hallmark of AD pathology is the accumulation of extracellular amyloid-β (Aβ) plaques in the brain as well as intracellular neurofibrillary tangles (NFT) composed of a hyperphosphorylated form of the microtubule-associated protein tau. Aβ is generated from the processing of the amyloid precursor protein (APP). APP is a transmembrane

protein which can be cleaved by an α-secretase to give a neuroprotective peptide called sAPPα. Alternatively, APP can be cleaved by a β-secretase (β-site amyloid precursor protein cleavage enzyme-1, BACE1) followed by a γ-secretase resulting in the formation of two forms of Aβ, Aβ40 and Aβ42, thought to be neurotoxic and to mediate the neuronal damage observed in AD (Guglielmotto et al. 2009; Peers et al. 2009).

Hypoxia and Alzheimer's Disease

There is a strong correlation between exposure to hypoxia and cognitive decline. This is apparent in healthy individuals ascending to high altitudes as extended poor concentration and memory have been observed in otherwise healthy mountaineers (Peers et al. 2009). An association between diseases characterized by systemic hypoxia and dementia/cognitive decline has also been established. For instance, chronic obstructive pulmonary disease (COPD) was directly linked to cognitive decline in a study where 48% of COPD patients showed cognitive deterioration as characterized by a dramatic impairment in verbal memory tasks (Peers et al. 2009). Additionally, stroke survivors are up to tenfold likely to develop AD as compared to age-matched individuals (Peers et al. 2009).

Glucose Metabolism in Alzheimer's Disease

In addition to the accumulation of Aβ and NFT, more recent evidence suggests that glucose metabolism/utilization is dysfunctional in AD and points to a role for glucose metabolism in the pathogenesis of AD (Correia et al. 2012). A few key points in glucose metabolism relevant to AD will now be highlighted. Glucose is first metabolized anaerobically by glycolysis into pyruvate to give two molecules of ATP. When oxygen is absent or is in short supply, pyruvate is converted into lactic acid by lactate dehydrogenase-A (LDH-A) with concomitant formation of NAD^+. On the other hand, in presence of oxygen, glycolysis is followed by the tricarboxylic acid cycle (TCA) and oxidative phosphorylation in the mitochondria leading to the generation of 32 ATP molecules, CO_2 and H_2O. The entry point for the TCA cycle is the breakdown of pyruvate into 2 acetyl-CoA molecules by pyruvate dehydrogenase (PDH). PDH can be inhibited by an enzyme called pyruvate dehydrogenase kinase-1 and -4 (PDK-1/-4), which are targets for HIF-1α and HIF-2α, respectively. In case of AD, it has been reported that the activity of PDH is decreased. Similarly, the activity of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, two major enzymes involved in the TCA cycle, is reduced in brains of AD patients (Correia et al. 2012). These data suggest that the aerobic metabolism of glucose is impaired with a subsequent decease in ATP production. Reduction in ATP generation caused by impaired mitochondrial glucose metabolism was reported to cause ROS formation and apoptosis in mouse embryonic fibroblasts (Majmundar et al. 2010) further supporting the notion that impaired glucose metabolism can be involved in the pathogenesis of AD. Thus in case of AD there is impairment in the aerobic mitochondrial glucose metabolism and not a shift in glucose metabolism towards the anaerobic glycolytic pathway. Consistent with this notion is the finding that brain glucose metabolism in AD patients was reported to be severely reduced as evidenced by positron emission tomography (PET) studies (Correia et al. 2012). A similar finding was observed in a transgenic animal model of AD (Correia et al. 2012).

HIF-1α in Alzheimer's Disease

Genetic and pharmacological studies indicated that activation of HIF-1α pathway has a protective effect in AD. For instance, when PHD was inhibited by DFO a reversion of age-induced memory deficits was observed (De Lima et al. 2008). Similarly, DFO protected cultured cells from Aβ-induced cytotoxicity (Ogunshola and Antoniou 2009). Moreover, expression of a non-degradable form of HIF-1α in an immortalized nerve cell line conferred protection against Aβ toxicity (Soucek et al. 2003). These studies suggest that stabilization of HIF-1α might have a neuroprotective effect in AD. In contrast to the previously mentioned studies, other studies have shown that under hypoxic conditions HIF-1α induces the expression of both BACE-1 and

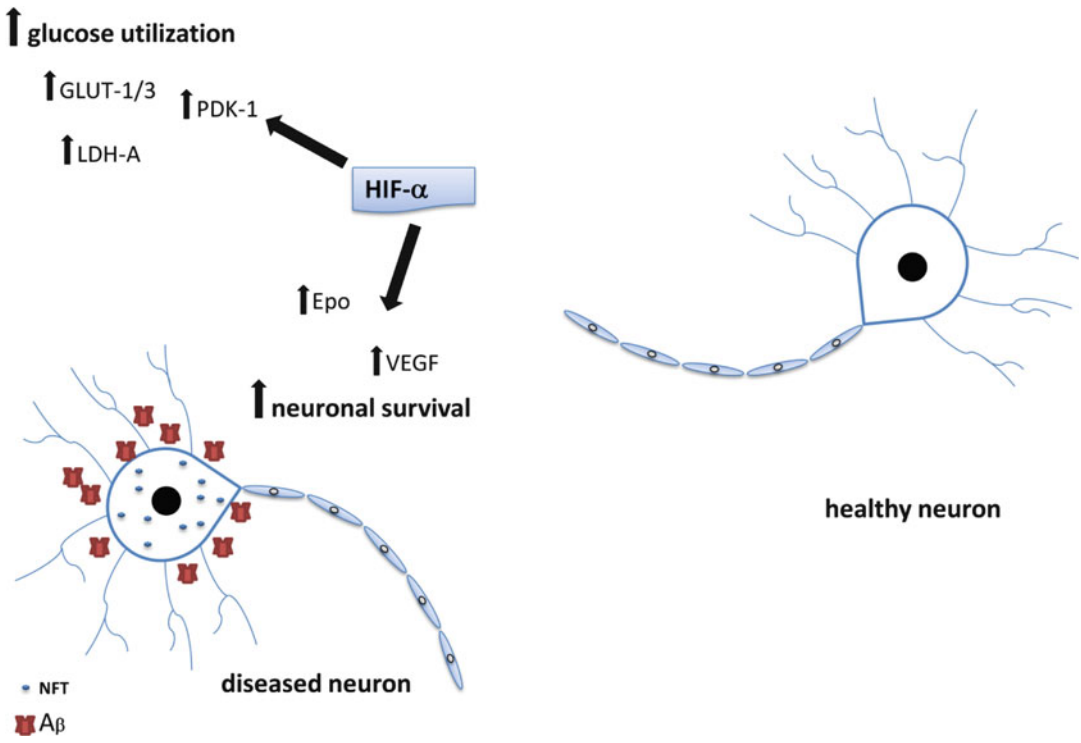

Fig. 15.2 HIF activation protects neurons against Alzheimer's disease. When HIF is activated, the transcription of VEGF and Epo is initiated. These factors promote neuronal survival and protect neurons against Aβ- and NFT-induced toxicity. At the same time, the HIF target genes GLUT1/3, PDK-1, and LDH-A are expressed which improve glucose utilization. *Aβ* amyloid β, *NFT* neurofibrillary tangle protein

APP raising the question as of whether HIF-1α is involved in the production of Aβ and thus in the pathogenesis of AD (Ogunshola and Antoniou 2009). However, it has been shown that in the early phases of AD, Aβ protects neurons against oxidative stress. Therefore, the observed increase in APP levels might be part of an endogenous protective mechanism to produce Aβ in the early phases of the disease. In addition, APP can be cleaved into the soluble sAPPα which is neuroprotective (Peers et al. 2009). Thus, activation of HIF-1α might be protective in AD.

Role of HIF Target Genes in Alzheimer's Disease

Based on evidence described above, the pathogenesis of AD involves neurotoxicity caused by the abnormal accumulation of Aβ and NFT, and impairment in glucose metabolism which ultimately leads to neuronal death. Accordingly, it would be expected that the protection conferred by HIF-1α activation targets the impaired glucose metabolism and neurotoxicity. The role of HIF activation in Alzheimer's disease is summarized in Fig. 15.2.

Effect on Glucose Metabolism

As mentioned above, under conditions where oxygen is in short supply (hypoxia) cells undergo a metabolic switch in glucose utilization from the oxygen demanding oxidative phosphorylation into the anaerobic glycolytic pathway. Recent studies indicate that HIF-1 plays a major role in this metabolic switch. For instance, the expression of PDK-1, a HIF-1 target gene, was shown to be induced under hypoxic conditions, inhibiting the formation of

acetyl-CoA and thus shunting pyruvate away from the TCA cycle. Similarly, LDH-A, another HIF-1 target gene, was also shown to be induced under hypoxic conditions, inducing the formation of lactate and replenishing NAD^+, allowing glycolysis to continue (Majmundar et al. 2010). For an efficient increase in the glycolytic pathway, glucose flux into the cells needs to be improved. This will be discussed below.

Effect on Glucose Transport

During ischemia where the supply of glucose is interrupted or hypoxia where energy generation is inefficient due to impaired oxidative phosphorylation, there is a need to increase glucose influx into the cells to make best use of the available glucose. In such cases activation of HIF enhances glucose transport into the cells by inducing the expression of glucose transporter-1 and -3 (GLUT-1 and -3). GLUT-1 is expressed in neurons, astrocytes, and oligodendrocytes while GLUT-3 is specifically expressed in neurons (Correia et al. 2012). Importantly, it has been shown that the expression of GLUT-1 and -3 is reduced in the brain of AD patients. A link between glucose transport and the other cause of AD, accumulation of abnormal protein aggregates, has been established as the reduced expression of GLUT-1 and -3 was shown to correlate with the hyperphosphorylation of tau and to the density of NFT (Liu et al. 2008; Ogunshola and Antoniou 2009). Interestingly, levels of HIF-1 in the brains of old AD patients were reported to be reduced (Liu et al. 2008; Ogunshola and Antoniou 2009). Thus, by affecting the protein levels of a key player in the hypoxic response, aging seems to have a negative impact on glucose transport in the brain and thus on the pathogenesis of AD.

Effect on Neuronal Survival – Epo

In addition to optimizing glucose metabolism, activation of HIF can be beneficial in AD by inducing the expression of neuroprotective factors such as Epo. Epo is a 30 kDa glycoprotein which belongs to the cytokine type I superfamily. Although Epo was originally identified as a target for HIF-1α, subsequent studies have shown that HIF-2α is the transcription factor regulating Epo expression under hypoxic conditions (Rabie and Marti 2008). Epo is produced by the kidney and to a lesser extent by the liver. Originally it was thought that Epo is only involved in the regulation of erythropoiesis. This effect is attributed to the ability of Epo to inhibit apoptosis in erythroid cells, allowing the maturation of erythrocytes. However, subsequent research showed that Epo and its receptor (EpoR) are expressed in tissues not involved in erythropoiesis which gave the first indication that Epo is involved in other biological processes. These tissues include the brain, the lung, the spleen, and the reproductive tract. Within the brain, Epo expression has been detected in several regions. These include the cortex, hippocampus and amygdale, cerebellum, hypothalamus and caudate nucleus. At the cellular level, in vitro and in vivo studies have shown that neurons and astrocytes are the main source of Epo in the brain (Bernaudin et al. 1999). Subsequent studies have then established that Epo is cytoprotective in several organs. For instance, Epo has been shown to protect the heart, the kidney, and the brain from ischemia-induced injury (Rabie and Marti 2008).

Role of Epo in Alzheimer's Disease

Evidence exists that Epo has a neuroprotective role in AD. For instance, when primary hippocampal neurons were treated with Aβ, addition of Epo prevented Aβ-induced neuronal apoptosis in an NFκB-dependent manner (Ogunshola and Antoniou 2009). A possible therapeutic effect of Epo was also tested in an animal model of AD. In that study, when aged Tg2576 mice, a mouse strain carrying a mutant variant of human APP and is thus used as an animal model for AD, were treated daily with Epo for 5 days, mice showed an improvement in contextual memory. The amount of Aβ and the amyloid plaque in the brain of these mice was also reduced as a result of Epo treatment (Lee et al. 2012). In addition to Epo's effect on neurons, Epo has also been shown to protect microglia from Aβ-induced toxicity in vitro (Shang et al. 2012). Because microglia plays a role in neuronal regeneration and in the prevention of Aβ deposition, this effect would indirectly protect neurons in AD.

Effect on Neuronal Survival – VEGF

Another neuroprotective factor that is a HIF target gene and that can be beneficial in AD is VEGF. VEGF is a growth factor that was originally described to induce angiogenesis and vascular permeability. Several VEGF family members have been identified so far; VEGF-A, placental growth factor (PIGF), VEGF-B, VEGF-C, and VEGF-D. Four different splice variants of VEGF-A exist; $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, where 121, 165, 189, and 206 represent the number of amino acids. The expression of VEGF is regulated by both HIF-1α and HIF-2α. So far five receptors for VEGF have been identified; three tyrosine-kinase receptors: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR3, and two non-tyrosine kinase receptors: neuropilin-1 (NP-1) and neuropilin-2 (NP-2). The main receptor through which VEGF exerts its angiogenic effects is VEGFR-2 (Flk-1/KDR), while VEGFR-3 mediates the lymphangiogenic effects of VEGF and VEGFR-1 (Flt-1) negatively regulates VEGF actions by acting as a decoy receptor. Both neuropilin-1 (NP-1) and neuropilin-2 (NP-2) act as co-receptors for VEGF receptors (Ma et al. 2011).

Role of VEGF in Alzheimer's Disease

Recent studied have shown that VEGF has a protective and a potential therapeutic effect in AD. Spuch et al have shown that implantation of encapsulated VEGF-secreting cells into the brain of APP/PS1 mice (another animal model for AD) reduced brain Aβ plaques, tau hyperphosphorylation, and improved cognitive functions (Spuch et al. 2010). Using the same methodology, this group has further shown that VEGF enhances the proliferation of neuronal precursor cells, induces angiogenesis, and reduces the activity of acetylcholinesterase in the brain of these mice (Antequera et al. 2012). It is noteworthy that inhibition of acetylcholinesterase is the first-line treatment in AD patients.

In summary, HIF-1 activation leads to the expression of genes that (1) enhance the neuronal survival, (2) protect neurons against Aβ toxicity, (3) improve glucose transport, and (4) glucose metabolism. Impaired glucose metabolism and Aβ toxicity are hallmarks of AD pathology. Thus, the observed reduction in HIF-1 levels in the brain of old AD patients (Liu et al. 2008) together with any other age-related changes in the cellular oxygen sensing machinery discussed earlier can have a negative impact on the pathogenesis and progression of AD.

Stroke

Stroke is one of the leading causes of death and disability in the developed world. The most common cause of stroke is cerebral ischemia where blood flow to the brain is interrupted due to a thrombus in a major cerebral artery, in most cases the middle cerebral artery (MCA). The pathogenesis of cerebral ischemia is complex and includes inflammation, ROS, excitotoxicity caused by an increase in glutamate release, lack of glucose and other nutrients, and, importantly, hypoxia. Studies have shown that HIF-1α expression is induced upon experimental focal and global cerebral ischemia suggesting that HIF-1α is part of an endogenous protective response. Indeed, when animals were treated with DFO or $CoCl_2$, prior to or after induction of cerebral ischemia, infarct size was reduced. This protective effect was significantly attenuated in mice genetically engineered to lack HIF-1α in their neurons further suggesting that the observed protective effect is mediated by the activation of HIF-1α. These neuron-specific HIF-1α knockdown mice were also more vulnerable to cerebral ischemia as they had larger infarction as a result of middle cerebral artery occlusion. Furthermore, when rats were treated with 2-methoxyestradiol, an inhibitor for HIF-1α, a reduction in neuronal cell survival after experimental stroke was observed (Correia and Moreira 2010). All these studies point to a protective role of HIF-α activation in cerebral ischemia and thus suggest that impairment of the PHD/HIF-α pathway during aging could have a negative impact on the final outcome of stroke.

Role of Epo in Stroke

The protection conferred by HIF activation in cerebral ischemia could be attributed to the

expression of HIF target genes such as Epo. As mentioned above Epo has neuroprotective properties and is able to promote neuronal survival in case of Alzheimer's disease. A similar neuroprotective role for Epo in cerebral ischemia has also been shown. The first hint that Epo might have a neuroprotective effect in the brain after cerebral ischemia came from the observation that Epo expression in the brain is induced upon cerebral ischemia (Bernaudin et al. 1999). Moreover, intracerebroventricular administration of Epo 24 h before permanent occlusion of the middle cerebral artery reduced infarct size significantly (Bernaudin et al. 1999). Similarly, when Epo was infused in the lateral ventricles in a gerbil global ischemia model, hippocampal CA1 neurons were rescued, the number of synapses formed in the same region increased, and the ischemia-induced learning disability was prevented. In addition, brain-specific Epo overexpression reduced infarct size in mice subjected to transient cerebral ischemia (Rabie and Marti 2008). All these studies suggest that Epo has a neuroprotective effect in cerebral ischemia and might explain how activation of HIF pathway confers neuroprotection in stroke.

Role of VEGF in Stroke

VEGF expression was found to be induced in the brain after cerebral ischemia in the ischemic penumbra, the ischemic core, as well as in remote cortical regions. Specifically, this was observed in neurons of the hippocampus, in astrocytes, in microglia, and in epithelial cells of the choroid plexus (Ma et al. 2011). These findings suggested that VEGF might play a protective role in cerebral ischemia. Indeed, several studies reported that VEGF has a neuroprotective effect in the brain after cerebral ischemia. For instance, neuron-specific overexpression of $VEGF_{165}$ in mice reduced infarct volume after MCA occlusion (Wang et al. 2005). Similarly, intraventricular administration of VEGF in mice subjected to focal cerebral ischemia not only reduced infarct size, but also improved neurological performance and neuronal survival in the subventricular zone and dentate gyrus. These beneficial effects of VEGF could be mediated either by a direct neuroprotective action or by inducing neurogenesis. For instance, VEGF was shown to improve neurogenesis in the subventricular zone as well as in the subgranular zone of the dentate gyrus, areas of the brain where neurogenesis normally takes place in response to cerebral ischemia (Ma et al. 2011). Taken together, the neuroprotective effects conferred by the activation of HIF pathway might be explained, at least in part, by the induction of VEGF expression.

Effect of HIF Activation on Glucose Metabolism

The interruption of blood supply to the brain causes not only a reduction in oxygen supply, but also glucose deprivation leading to inadequate energy production. As mentioned above, activation of HIF induces the expression of neuroprotective factors as well as glucose transporters and proteins involved in glycolysis. Indeed GLUT-1 and LDH-A expression was reported to increase in the ischemic penumbra (Correia and Moreira 2010).

From the data mentioned above, it is clear that activation of the endogenous hypoxic adaptive response, i.e. inhibition of PHD and stabilization of HIF with subsequent transcription of HIF target genes, is paramount for brain recovery after stroke. Thus the state of the hypoxic response, intact or impaired, has a major impact on the final outcome of stroke. As the incidence of stroke increases with age, an impaired hypoxic response in the elderly would worsen the final outcome. Indeed, a strong relationship between age and stroke outcome has been shown (Black-Schaffer and Winston 2004).

Parkinson's Disease (PD)

Parkinson's disease (PD) is a neurodegenerative disease affecting the dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) of the nigrostriatal system. As a result, DA is depleted in the striatum and normal motor function is impaired. The exact cause of the disease is not fully understood. However, studies have shown that abnormal accumulation of iron in

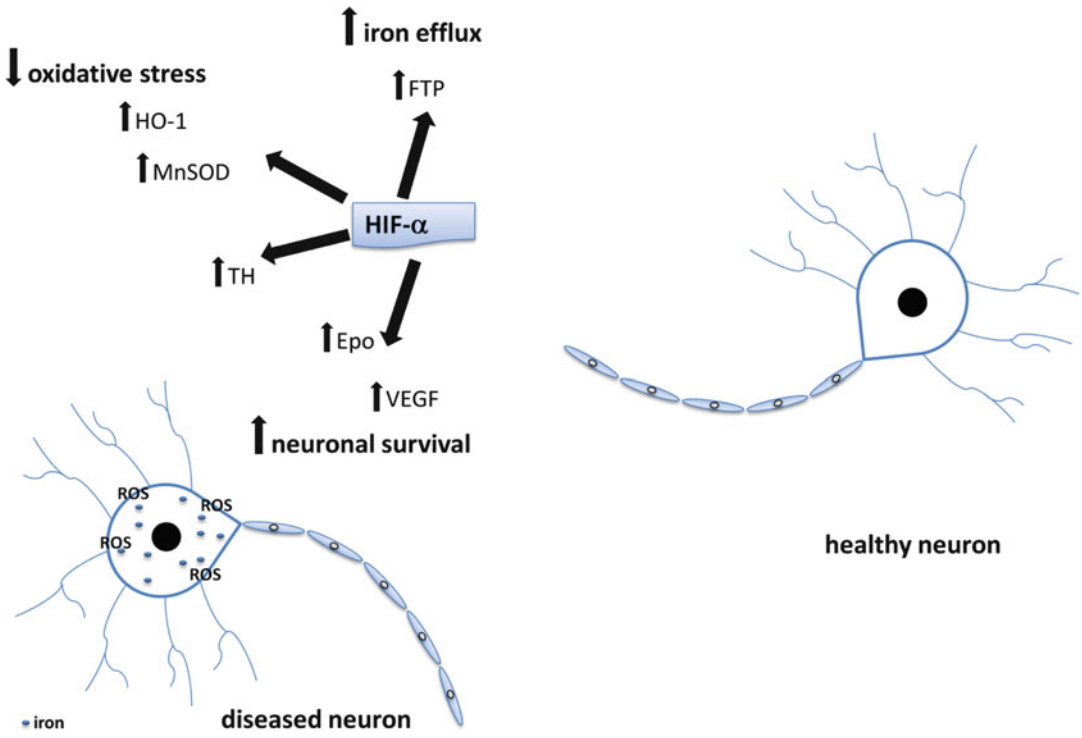

Fig. 15.3 HIF activation protects neurons against Parkinson's disease. When HIF is activated, the transcription of VEGF and Epo is initiated. These factors promote neuronal survival and protect neurons against toxicity induced by ROS and intracellular accumulation of iron. At the same time, HO-1 and MnSOD expression reduces oxidative stress, expression of FTP increases iron efflux, and expression of TH increases dopamine generation. *ROS* reactive oxygen species

SNc, mitochondrial dysfunction, oxidative stress, and neuroinflammation are important contributing factors in the pathogenesis of the disease (Correia and Moreira 2010). So far, hypoxia has not been shown to be implicated in the neurodegeneration observed in PD. However, in a recent study, inhibition of PHD by 3,4-dihydroxybenzoate (DHB) protected against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced toxicity of DA neurons in SNc (Lee et al. 2009). Similarly, intracerebroventricular injection of DFO has been shown to protect rats against 6-hydroxydopamine-induced degeneration of dopaminergic neurons (Correia and Moreira 2010). These data suggest that activation of HIF confers protection in PD. How does HIF activation protect against neurodegeneration in PD? This protection is most likely mediated by modulating the two main pathogenesis contributing factors; abnormal intracellular iron accumulation and oxidative stress. The role of HIF activation in Parkinson's disease is summarized in Fig. 15.3.

HIF and Iron Accumulation

Iron is a trace element, essential for the survival of all cells. However, because iron has the ability to react with oxygen to produce free oxygen radicals and thus has the potential of inducing oxidative stress, a fine balance of intracellular iron concentration must be maintained. This is achieved mainly by regulating iron uptake, storage, and transport. Ferroportin (FPT) is one of the main iron exporters responsible for iron efflux. FPT levels are indirectly controlled by HIF, i.e. high cellular levels of HIF-α maintain high levels of FPT. Thus when PHD is inhibited, levels of HIF-α increase leading to elevated levels of FPT and a reduction

of intracellular iron. Consistent with this, Lee and co-workers reported that the increase in iron levels in the brain of MPTP-treated mice was reversed when mice were pretreated with the PHD inhibitor DHB (Lee et al. 2009). This suggests that if HIF levels in the brain are reduced (as during aging) it is more likely that iron efflux will be reduced, intracellular iron will be increased, leading to an increase in oxidative stress and neuronal damage.

HIF and Oxidative Stress

Because dopaminergic neurons are particularly vulnerable to oxidative stress, strategies to increase the cells' defense mechanisms against oxidative stress would have a positive impact on the final outcome of PD. Heme oxygenase-1 (HO-1) and manganese superoxide dismutase (MnSOD) are two proteins that play an important role in the cellular defense against oxidative stress. Importantly, both these proteins are HIF targets and the expression of both was shown to increase in the brain of DHB-treated mice that were protected against MPTP-induced neurotoxicity (Lee et al. 2009). Other studies have shown that HO-1 plays a protective role in PD. For instance, when the expression of HO-1 was elevated pharmacologically, dopaminergic neurons were protected against 6-hydroxydopamine-induced toxicity (Yamamoto et al. 2010).

Role of Neuroprotective Factors in PD

In addition to the effect of HIF activation on iron homeostasis and oxidative stress in PD, HIF induces the expression of the neuroprotective factors Epo and VEGF that were shown to be protective in PD. Using 6-OHDA, it has been shown that Epo is neuroprotective in immortalized and primary dopaminergic neurons as well as in mice unilaterally lesioned by 6-OHDA. Moreover, using that model, it has been shown that Epo enhances the survival and function of grafted dopamine neurons. Finally, Epo has also been shown to be neuroprotective in the MPTP model of PD (Rabie and Marti 2008).

Similar to AD and stroke, VEGF has been shown to be protective in PD. In the 6-OHDA model, when hVEGF-secreting cells were implanted into the striatum, greater protection of the SNc neurons, greater number of preserved tyrosine-hyrdroxylase positive (TH^+) neurons, and a significant reduction in amphetamine-induced rotational behavior was observed showing that VEGF has a protective effect in PD. The authors attributed this neuroprotection to an indirect effect on neurons mediated by the activation of glial proliferation and promotion of angiogenesis (Yasuhara et al. 2004). In addition, in mice with neuron-specific VEGF overexpression more TH^+ neurons were spared after MTPT challenge (Lee et al. 2009). Interestingly, levels of VEGF in the substantia nigra of old rats were shown to decrease in an age-dependent manner (Villar-Cheda et al. 2009) which might be a result of the changes in the levels HIF-1 and PHD that take place during aging that were discussed earlier.

Another HIF target gene that is relevant to PD is tyrosine hydroxylase, the rate-limiting enzyme for DA formation. Thus, reduced levels of HIF or impairment in HIF/DNA binding in the brain during aging might negatively influence TH levels and DA formation. Indeed, mRNA levels of TH are reduced in the SNc of old rats (Gao et al. 2011).

In conclusion, during the last decades the advancements in medicine meant that people now live longer. Although this is a reason to celebrate, living to an old age increases the risks of acquiring age-related diseases such as cancer or neurodegenerative diseases. A major cause for the increased incidence of these diseases in the elderly is a reduction in the defense mechanisms and in the self-healing capacity of the individual. In other words, the body is less able to fight factors initiating the disease (e.g., hypoxia/ischemia or a small number of transformed cells) and when the damage is done the body is less able to self-heal (e.g. initiate neurogenesis or angiogenesis to compensate for lost neurons or improve blood supply, respectively). Not all neurodegenerative diseases are age-related. For instance, Huntington's disease (HD), multiple sclerosis (MS), or amyotrophic lateral sclerosis (ALS) are all neurological diseases characterized

by progressive neuronal degeneration. However, they can strike at any age. Rather, the focus of this chapter was on the impact of aging on age-related neurodegenerative diseases. Although stroke can also strike at any age, age has a major impact on the progression and the final outcome of the disease (Black-Schaffer and Winston 2004).

The role of the endogenous hypoxic response is to help the body cope with low oxygen levels to prevent/reduce damage as well as to initiate the self-healing process should damage occur. PHD and HIF are major players governing the endogenous hypoxic response. As we age, the expression and activity of these factors are perturbed with an impaired coping/healing mechanism as a consequence. A better understanding of the influence of aging on our brains and other body systems is essential in order to design therapies to treat the ever increasing prevalence of age-related diseases.

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Enhancing Reprogramming to Pluripotency by Controlling Senescence

16

Ana Banito and Jesús Gil

Contents

Abstract

Induced pluripotent stem (iPS) cells can be obtained through the reprogramming of somatic cells by ectopic expression of defined factors. These cells hold immense promise for autologous cell therapy and disease modeling however, understanding the mechanistic of direct reprogramming is also shedding light into important biological processes. Several studies have shown how key tumor suppressors may interfere with reprogramming efficiency by activating cell-intrinsic programmes such as senescence and apoptosis. The repercussions of these findings are profound and reveal a link between tumor suppression and loss of differentiation. Here we analyze the latest findings in the field and discuss their relevance for iPS technology and tumor development.

Keywords

iPS • Senescence • Tumor suppressor • p53 • p16 • Pluripotency

Introduction

Cellular Senescence and Tumor Suppression

It has been almost 50 years since Leonard Hayflick and Paul Moorhead introduced the notion that normal somatic cells cannot be propagated in culture

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indefinitely. Based on their work deriving human diploid strains, they proposed that differentiated normal cells, unlike tumor cells, undergo a limited number of cell divisions after which they entered a state of irreversible growth arrest, which they named cellular senescence (Hayflick 1965). Nowadays we know that replicative senescence of human cells is a consequence of the critical shortening of the DNA molecule at each round of DNA replication a process that can be counteracted in tumor cells by expression of telomerase, a ribonucleoprotein enzyme terminal transferase that extends the 3' end of telomeres and thus elongates them. The work by Hayflick and Moorhead gave birth to the idea that normal somatic cells possess a "mitotic clock" that dictates their maximum lifespan and highlighted the fact that immortality is a key feature of tumor cells. However, throughout the years several studies suggested that senescence can be initiated in human and mouse cells by various other cellular stresses. This has been referred to as premature senescence, as opposed to replicative or telomere-dependent senescence. DNA-damaging agents, improper culture conditions and interestingly, activation or over expression of certain oncogenes can trigger this kind of irreversible growth arrest. In 1997, it was described that activated RAS elicited a proliferative arrest similar to the replicative senescence of human fibroblasts (Serrano et al. 1997). Since this discovery, other oncogenes have been shown to induce a similar response, denominated oncogene-induced senescence (OIS). Importantly senescence has been described in vivo in premalignant lesions underscoring its role as a tumor suppressor mechanism (Campisi and d'Adda di Fagagna 2007).

When cells are exposed to stresses such as telomere uncapping or expression of oncogenes, two tumor suppressor pathways are triggered that converge on modulation of cyclin dependent kinase (CDK) activity and are responsible for senescence onset. The p53-$p21^{Cip1}$ pathway is activated in mouse by the ARF protein, and in both human and mouse cells as a result of a DNA damage response mediated by ATM/ATR. ATM/ATR are protein kinases that phosphorylate and activate p53 while ARF activates p53 by inhibiting MDM2 an ubiquitin E3 ligase that targets p53 for degradation. Active p53 induces a complex transcriptional program including genes such as *CDKN1a* ($p21^{Cip1}$). The other main pathway triggered during senescence is the p16/RB pathway. Both $p16^{INK4a}$ and the p53 target $p21^{Cip1}$ function by inhibiting the activity of CDKs. $p16^{INK4a}$ binds to CDK4 and 6 and inhibits their ability to interact with D-type cyclins that are therefore targeted for degradation while $p21^{Cip1}$ inhibits cell cycle progression through the inhibition of CDK2. Inactive CDKs result in hypophosphorylated RB proteins that sequester E2F transcription factors, avoiding cell cycle progression. As a consequence, senescent cells are arrested in G1 phase of the cell cycle, and importantly are irresponsive to growth signals. Cellular senescence is a broad term often defined by a number of morphological and functional characteristics. These include flat and enlarged morphology, the presence of senescence associated β-galactosidase (SA-β-Gal) activity, senescence associated heterochromatic foci (SAHFs) and the expression of a complex secretory programme. While SA-β-galactosidase activity is thought to reflect an increase in lysosomal activity, SAHFs are associated with a stable transcriptional repression of E2F target genes (reviewed in Campisi and d'Adda di Fagagna 2007).

The Immortality of Embryonic Stem Cells

Embryonic stem (ES) cells, derived from the inner cell mass (ICM) of the mammalian blastocyst, have two main properties, self-renewal and pluripotency; and can be maintained in culture in an undifferentiated state, or differentiate in vitro into derivates of the three germ layers. One of the most remarkable features of ES cells is that they can self-renew in culture indefinitely, and do not undergo senescence, yet they are not transformed. For example, hES cells have been shown to continuously grow in culture for 2 years, which is equivalent to about 500 doubling times. This implies that the pathways responsible for senescence onset must be regulated differently

in ES cells. Indeed, gene expression profiling identified key differences in cell cycle control and DNA repair in hES cells and in mES cells. Additionally, in contrast to most somatic cells, stem, germ and tumor cells have high telomerase activity through *TERT* transcriptional up-regulation. Although some differences exist in the regulation of the cell cycle between human and mouse ES cells both are characterized by a shortened early G1 phase. Mouse ES cells growth in culture is extremely rapid due to a short cell cycle (11–16 h) with a reduced G1 phase. While in somatic cells, including adult stem cells, CDK2 activity is periodic and peaks at the G1 to S transition, mouse ES cells have constitutive CDK2 activity independent of the cell cycle phase. Moreover, mouse ES cells express low levels of the D-type cyclins and lack CDK4 activity (reviewed in Miura et al. 2004).

Therefore, a different regulation of cell cycle checkpoints that normally operate in somatic cells allow for a shorter G1 phase in ES cells. Indeed, cell cycle regulators such as *p53*, *INK4a* (encoding $p16^{INK4a}$), *ARF* and *CDKN1a* (encoding $p21^{Cip1}$), are not expressed or expressed at very low levels in hES cells. This is in contrast to what has been described in mES cells with high levels of *p53*. However, the p53-mediated response to cell cycle arrest in these cells seems disabled (Miura et al. 2004). ES cells may use alternative mechanisms from those present in somatic cells to protect themselves from stress and to maintain genetic stability. In fact, mES cells are highly resistant to oxidative stress, and are very efficient in repairing DNA damage induced by radiation (Miura et al. 2004). Overall, these observations suggest that the absence (hES cells) or the inactivation (mES cells) of the p53 pathway contributes to the absence of senescence in ES cells.

Somatic Cell Reprogramming

The derivation of mouse embryonic stem cells in 1981 ignited an explosion of scientific interest offering unique opportunities for research and disease treatment. Besides the ethical issues associated to the derivation of human ES cells, the ultimate goal of regenerative medicine is to produce isogenic material to circumvent graft rejection of tissues or organ transplantation between genetically distinct individuals. An intermediate step in this process would be achieved through the generation of patient-specific pluripotent stem cell lines. Although several methods have proven that the differentiated state of the somatic nucleus can be reversed by factors present in pluripotent cells it was the work by Yamanaka and colleagues that prove this goal was attainable. Starting from a number of transcription factors either involved in pluripotency or cell cycle regulation they were able to pin down which were essential for this reversion. Expression of four transcription factors: Oct4, Sox2, Klf4 and c-Myc in mouse embryonic fibroblasts was shown sufficient to induce a number of changes that lead to acquisition of ES cell-like characteristics and regression to an undifferentiated state (Takahashi and Yamanaka 2006). The generation of iPS cells from human cells was achieved a year later by using the same combination of four factors, or by substituting Klf4 and c-Myc with Nanog and Lin-28 (Stadtfeld and Hochedlinger 2010).

Although the mechanisms governing this process remained largely unknown, the reprogramming by defined factors and its end product, the induced pluripotent stem (iPS) cells soon captivated the attention of the scientific community. Not only would reprogramming enable the relatively easy generation of patient-specific pluripotent stem cells, but also it could circumvent the ethical issues associated with the use of human embryos as source for derivation of hES cell lines. In addition, iPS would be an useful tool to dissect the mechanisms underlying pluripotency. Induced pluripotent stem cells are practically indistinguishable from ES cells. They have the potential to differentiate into derivates of the three embryonic lineages and can self-renew. Accordingly iPS cells acquire telomerase activity and have elongated telomeres when compared to the parental differentiated cells (Marion et al. 2009b).

Tumour Suppressor Pathways as a Barrier to Reprogramming

Upon expression of the Yamanaka factors, somatic cells undergo a number of changes that ultimately lead to acquisition of pluripotency and self-renewal. However, reprogramming efficiency is extremely low. The majority of the cell population does not acquire these ES cell-like properties suggesting that several barriers hinder this process. Work by several groups has identified roadblocks to reprogramming such as incomplete epigenetic remodeling of lineage-specifying transcription factors and DNA hypermethylation at pluripotency-related loci (Mikkelsen et al. 2008).

Importantly, gain of embryonic stem cell properties during reprogramming to iPS cells involves acquisition of mechanisms to escape replicative senescence. Human embryonic stem cells express low levels of negative regulators of the cell cycle, and display telomerase activity. Given the fact that one of the sequential events during reprogramming is the acquisition of immortality, some groups have interfered with senescence and apoptosis pathways to promote reprogramming. In fact, one of the first studies describing reprogramming of human fibroblasts (usually more difficult to reprogram then mouse cells) required addition of SV40 large T (SV40 LT) and/or the catalytic subunit of telomerase (hTERT) (Park et al. 2008). In another study siRNAs against p53 and Utf1 was shown to increase reprogramming efficiency of human cells by 100-fold (Zhao et al. 2008). Although there was the notion that interfering with the p53 pathway could improve reprogramming by promoting cell survival and inducing rapid proliferation, the precise mechanism by which p53 limits reprogramming was unclear.

p53 Is Activated During Reprogramming

Expanding cells in culture is a known stress inducer. In addition, some reprogramming factors such as c-Myc and Klf4 are well-established oncogenes. Indeed, several research groups have showed that the expression of the four Yamanaka factors, or the combination of Oct4, Sox2 and Klf4 can induce p53 and $p21^{Cip1}$ (Banito et al. 2009; Hong et al. 2009; Kawamura et al. 2009). Interestingly, expression of Oct4 and Sox2 also induces the p53-p21 pathway and expression of these factors individually causes the arrest of human fibroblasts (Banito et al. 2009; Hong et al. 2009). Even though their function during tumorigenesis is not well understood, Oct4 and Sox2 have also been implicated in tumor development (Banito and Gil 2010).

Reprogramming factors have oncogenic functions and that could explain the activation of a p53 response. A clear parallelism can be drawn between senescence triggered by oncogenic RAS and that induced by expression of the reprogramming factors. Similar to oncogenic RAS, expression of four reprogramming factors results in the activation of a DNA damage response (DDR) (Kawamura et al. 2009; Marion et al. 2009a). The triggering factor for this DDR is still uncertain, although it has been suggested that it may be due to aberrant DNA replication (Marion et al. 2009a). In addition, simultaneous expression of the four factors leads to accumulation of 8-oxoguanine adducts (Banito et al. 2009) which are commonly the result of oxidative stress and c-MYC induces DNA damage due to the generation of reactive oxygen species (ROS) (reviewed in Banito and Gil 2010). Accordingly, reprogramming is more efficient under low oxygen conditions (Utikal et al. 2009; Yoshida et al. 2009) or in the presence of vitamin C, a powerful antioxidant (Esteban et al. 2010).

In the light of the results discussed above, several groups showed that knocking down p53 or its transcriptional target *CDKN1a* in human or mouse cells significantly enhance reprogramming (Hong et al. 2009; Kawamura et al. 2009; Marion et al. 2009a; Utikal et al. 2009). Besides *CDKN1a*, miR-34a, is another p53 target induced during reprogramming. Mouse fibroblasts deficient for miR-34a reprogram more efficiently and with accelerated kinetics (Choi et al. 2011). While expression of MDM2, or a dominant-negative mutant of p53 also results in enhanced reprogramming, the activation of p53 reduces

reprogramming efficiency (Kawamura et al. 2009; Marion et al. 2009a; Sarig et al. 2010).

As the regulation and activity of p53 differ in ES and somatic cells, context-dependent mechanisms may explain p53 induction during reprogramming. Recently, it was reported that Δ40p53, an isoform of p53 specifically expressed is ES cells, is essential to maintain pluripotency. The authors suggest that this isoform controls pluripotency by regulating the full-length p53 and promoting IGF signaling (Ungewitter and Scrable 2010). The uncoupling between the ES-specific regulation of cell cycle related pathways and expression of reprogramming factors, could contribute to the activation of anti-proliferative response observed during reprogramming. Inactivation of negative regulators of the cell cycle enables ES cells to continuously self-renew in culture, and this is achieved in part by the expression of ES cell-specific microRNAs that target *CDKN1a* and *RBL2* (Wang et al. 2008). The absence of ESC-specific miRNAS in somatic cells can also contribute to the differential response of reprogramming factors in these cells. Indeed expression of some of these ES-specific microRNAs is able to partially bypass cell cycle arrest induced by the four reprogramming factors (Banito et al. 2009).

The Role of p53 During Reprogramming: Is It a Cell-Intrinsic Mechanism?

Although the connection between the p53 tumor suppressor and reprogramming to iPS is clear there is still controversy on why this pathway is activated and how its inactivation improves reprogramming efficiency. Work by Jaenisch and colleagues suggested that inactivation of p53 influences reprogramming kinetics rather than the overall efficiency of reprogramming. In other words all cells are reprogrammable but in the absence of p53 the process in accelerated due to higher proliferation rates (Hanna et al. 2009). It is still to determine whether this is cell-type specific, as in this case a homogeneous population of B cells obtained from secondary iPS cell-generated mice, was used. It has been described that contrary to what happens with B cells, there is a refractory population of fibroblasts that cannot give rise to iPS cells even after prolonged time in culture (Stadtfeld and Hochedlinger 2010). Additionally, live imaging studies of fibroblasts during reprogramming, revealed that after expression of reprogramming factors in MEFs, several distinct cell types based on broad morphological and proliferative characteristics, arise. As expected, most cells failed to initiate reprogramming and generally resembled the initial somatic fibroblast population, which the authors classified as exhibiting arrested/apoptotic or slow-dividing phenotypes. However early in the process a subpopulation of cells (about 1% of the total population) characterized by high rates of proliferation and small size appears. Tracking experiments showed that this sub-population gives origin to iPS colonies (Smith et al. 2010). The fact that only very small subsets acquire the rapid proliferation rate necessary for successful reprogramming agrees with the activation of anti-proliferative responses. Whether these cells fail to induce the tumor suppressor pathways or whether they escape anti-proliferative mechanisms is still not clear.

Undeniably, rapid proliferation and immortality underlie the in vitro pluripotent state of ES cells. Early studies performed in embryonic carcinoma (EC) cells led to the idea that the length of G1 phase is directly linked to the responsiveness of pluripotent stem cells to differentiation signals (Miura et al. 2004). The short G1 phase of ES cells would limit the time of exposure to differentiation cues. Additionally, DNA replication and mitosis have been proposed to be necessary for acquisition of epigenetic modifications. This would be in agreement with p53 absence influencing the kinetics of reprogramming by accelerating the cell cycle. However reprogramming towards pluripotency in single heterokaryons is efficiently initiated without cell division or DNA replication and depends on activation-induced cytidine deaminase (AID), required for promoter demethylation and induction of *OCT4* and *NANOG* gene expression (Bhutani et al. 2010). Therefore, the relevance of cell cycle progression to initiate the epigenetic changes that underlie de-differentiation is not completely understood.

Interestingly, p53 is also able to increase reprogramming efficiency at lower rates of proliferation. This was demonstrated by comparing the reprogramming efficiency of wild-type MEFs growing in 15% fetal bovine serum, with that of p53 knockout MEFs in 0.5% serum. Although the proliferation rate was lower in the MEFs deficient for p53 growing in low serum conditions, the reprogramming efficiency was still higher when compared to wild-type MEFs (Utikal et al. 2009). The fact that p53 may increase reprogramming efficiency irrespective of the rates of proliferation demonstrates that may it may have a direct role rather than solely regulating proliferation. Several studies have implicated p53 in regulating pluripotency and differentiation. For example p53 suppress the expression of Nanog. Similarly, it has been described that phosphorylation and acetylation of p53 in specific residues increases during differentiation and that p53 transcriptional activity is necessary for proper differentiation (reviewed in Spike and Wahl 2011). This suggests that p53 may also have a role in preventing de-differentiation, and that its activation stabilizes the differentiated state.

Resetting the INK4a/ARF Locus

Inhibition of the *INK4b-ARF-INK4a* locus has also been shown to improve reprogramming efficiency (Banito et al. 2009; Li et al. 2009). This locus encodes for two CDK inhibitors from the same family, $p15^{INK4b}$ and $p16^{INK4a}$ and for *ARF* ($p19^{Arf}$ in mice and $p14^{ARF}$ in humans), an activator of p53. The *INK4*a and *ARF* genes each have their own promoters, which produce different transcripts. *INK4a* consists of exons 1α, 2 and 3, while *ARF* is formed by exons 1β, 2 and 3. Although exons 2 and 3 are common to both transcripts, they are read in different reading frames, lacking any homology in their protein sequence (Quelle et al. 1995).

Like p53, $p16^{INK4a}$ is induced early upon the expression of the four Yamanaka factors in human fibroblasts reminiscent of what happens in response to oncogenic RAS and other stress-inducing signal. Although the mechanism underlying the induction of $p16^{INK4a}$ is not completely understood, it involves chromatin remodeling, including the loss of H3K27me3 marks around the *INK4b-ARF-INK4a* locus (Banito et al. 2009). Individual expression of the reprogramming factors showed that $p16^{INK4A}$ induction is mainly dependent on c-Myc, so it is not clear whether the same response is observed when cells are reprogrammed in the absence of c-Myc or when alternative combinations of reprogramming factors are used. Nevertheless, the *INK4b-ARF-INK4a* locus is kept silenced by Polycomb group proteins in both iPS cells and ESCs (Li et al. 2009), and its adequate epigenetic resetting could be an important barrier during reprogramming. In fact, inhibition of $p16^{Ink4a}$ and $p19^{Arf}$ improves reprogramming efficiency (Li et al. 2009). It is not clear however when and how the locus is repressed during reprogramming. Li et al. suggest that this is an early event during reprogramming but the fact that partially reprogrammed cells express high levels of *INK4a* when compared to fully reprogrammed cells suggests that complete epigenetic repression of the locus occurs late during reprogramming (Mikkelsen et al. 2008).

Different Cell Fates May Limit Reprogramming

The link between reprogramming and tumor suppressor pathways activation emphasize how reprogramming is perceived by cells as a stress. Some of the genes activated during reprogramming are common to anti-proliferative responses, such as apoptosis, senescence or other forms of cell-cycle arrest (Fig. 16.1). Human fibroblasts expressing Oct4, Sox2, Klf4 and c-Myc undergo a cell-cycle arrest that presents multiple characteristics of senescence, such as SA-β-galactosidase activity, increase in the number of nuclei presenting SAHFs and expression of *INK4a*. In addition, young and old cells are not equally amiable to reprogramming. The closer cells are to senescence, and therefore the higher the levels of *INK4a* and *CDKN1a* they express, the more difficult it is to reprogram them (Li et al. 2009). Similarly late generation $Terc^{-/-}$ MEFs which have critically

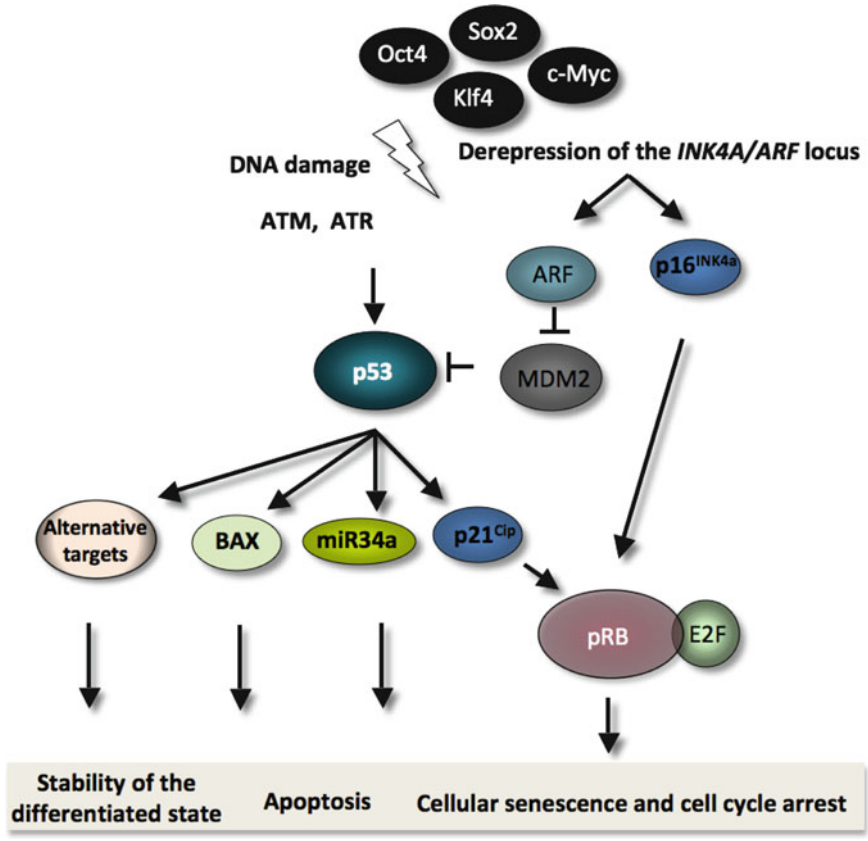

Fig. 16.1 Tumor suppressor pathways limit reprogramming to iPS cells. Expression of the reprogramming factors activates two tumor suppressor pathways; the p53 and the p16/RB pathways. Induction of these pathways triggers anti-proliferative responses and limits survival. Additionally, p53 may have roles in stabilizing the differentiated state, although currently these additional mechanisms are not completely understood. Interfering with components of these pathways such as $p21^{Cip1}$, $p16^{INK4a}$, ARF, miR-34a or expressing the anti-apoptotic protein Bcl2 enhances the efficiency of somatic cell reprogramming

short telomeres, present lower reprogramming efficiencies emphasizing the difficulty of reprogramming cells that are already aged or stressed (Marion et al. 2009b). Therefore senescence seems to limit reprogramming in at least two ways, by functioning as an anti-proliferative/stress response as well as by limiting the number of cells refractory to be reprogrammed due to pre-existent high levels of senescence regulators.

Additionally reprogramming can trigger apoptosis. The pro-apoptotic factor Bax is upregulated during reprogramming, and expression of its antagonist molecule Bcl2 results in enhanced reprogramming efficiency (Kawamura et al. 2009) (Fig. 16.1). Other reports suggest that the expression of the reprogramming factors synergizes with induction of DNA damage to trigger apoptosis, especially in cells with critically short telomeres or which are sensitized by exposure to exogenous DNA-damaging agents, such as ultraviolet or ionizing radiation. In such a scenario, the expression of Bcl2 restores the ability of these cells to be reprogrammed to levels similar to control cells (Marion et al. 2009a). Therefore reprogramming is limited by both anti-proliferative responses, as happens during tumor suppression, in which both senescence and apoptosis are implicated.

Reprogramming and Tumorigenesis

The generation of induced pluripotent stem cells not only opened a vast range of possibilities for stem cell therapy but also provided a new tool to investigate pluripotency and to model diseases. Despite their potential, it is not still unclear whether iPS cells could be part of an effective treatment for human diseases. In fact, even the therapeutic potential of embryo-derived ES cells have only been tested in a few settings, and their effectiveness and safety are matter of debate. For iPS cells to be used in a clinical setting, several issues need to be addressed. Previous work has paved the way to develop methods for the generation of integration-free iPS cells, but probably the most worrying topic is the relationship between reprogramming and tumorigenesis. The intimate relation between tumor suppression and reprogramming highlights this concern.

Cancer Stem Cells: *In Vivo* Reprogramming?

Beyond the technological implications and promise for therapy, reprogramming also highlighted how somatic cells, thought to be trapped in an endpoint of irreversible differentiation, reacquire the cellular plasticity that enables reversion of cell fate and cellular identity. Interestingly, a body of evidence suggest that similar to differentiation observed in normal tissues, tumor growth and maintenance is sustained by cancer stem cells (CSCs), which have both the ability to self-renew and to give rise to an heterogeneous tumor cell population. This view strongly suggests that cancer arises from transformation of stem or progenitor cells within tissues, however the identity of cells undergoing the first pro-tumorigenic events is still obscure. It is therefore tempting to speculate that CSCs could also originate from differentiated cells gaining aberrant self-renewal and pluripotent capabilities. Indeed inactivation of the p53 and $p16^{INK4a}$/RB tumor suppressor pathways, shown to enhance reprogramming efficiency, occurs in most tumors. Loss of p53 during tumorigenesis has been most often associated with resistance to apoptosis, cell cycle arrest and acquisition of genomic instability. However the growing evidence that connects reprogramming, differentiation and the p53 tumor suppressor, suggest that it may also have a role in stabilizing the differentiated state and avoid reversion to a stem cell-like state during tumorigenesis. Accordingly, although p53 mutations are common in cancer progression they seem to be associated with acquisition of cell plasticity and often correlate with poorly differentiated aggressive tumors (Spike and Wahl 2011). This is further supported by the fact that aggressive, poorly differentiated tumors express high levels of ES cell-associated factors (Ben-Porath et al. 2008) and that a robust correlation between ES cell-associated expression signatures and p53 mutational status is observed in high-grade breast tumors (Mizuno et al. 2010). On the other hand, expression of oncogenes such as c-Myc in epithelial tumors reactivates an ES cell-like transcriptional signature suggesting that oncogene activation and p53 inactivation synergize to promote stemness (Wong et al. 2008).

These observations suggest that reprogramming to a more dedifferentiated state occurs during tumor progression and might be favored by loss of tumor suppressors. This has been further supported by several studies. For example p53 deficiency increases the pool of CSCs by promoting the symmetrical versus asymmetrical cell divisions of cancer stem cells (Cicalese et al. 2009). Interestingly, deletion of the three RB family proteins triggers the reprogramming of MEFs to generate CSC-like cells. Cells resembling CSCs can also be generated from MEFs that only lack *RB1* if forced to grow beyond contact inhibition (Liu et al. 2009). Simultaneous inhibition of the RB and p53 pathways (by inhibiting *Arf*) in mice leads to extensive loss of differentiation of post-mitotic myocytes (Pajcini et al. 2010). These results implicate that the *Ink4a/Arf* locus limits regenerative potential in mammals, when compared to lower vertebrates and underscores how tumour protection and regeneration might be intertwined.

If the acquisition of stem-cell-like properties and tumorigenesis go hand-in-hand, it is reasonable

to think that the mechanism triggered by expression of the reprogramming factors, which ultimately leads to reprogramming, elicits the tumor suppressor pathways protecting cells against cancer. In addition, the intricate relationship between pluripotency and tumorigenesis suggests that events leading to reprogramming may overlap with the ones involved in tumorigenesis.

The Consequences of Disabling Tumor Suppressors During Reprogramming

Its function in keeping genetic integrity granted p53 with the prestigious title of "guardian of the genome". In reality, although reprogramming can be improved by interfering with crucial anti-proliferative pathways, the consequence of dismantling cell-intrinsic tumor suppressive mechanisms is too detrimental to consider, as it would affect the safety and functionality of the resulting iPS cells.

The consequences of an inactive p53 are multiple. Not only would cells that have been heavily burdened with DNA damage are still able to acquire pluripotent properties but also it would lead to genomic instability and, consequently, to impaired differentiation in the resulting iPS cells. Indeed, iPS cells derived from *p53*$^{-/-}$ MEFs have increased chromosomal instability (Hong et al. 2009; Marion et al. 2009a). Additionally, teratomas from p53-deficient iPS cells generated with the four factors were mostly formed of undifferentiated tissue suggesting that in the absence of p53, c-Myc blocks differentiation and induce tumor formation. Moreover, single-cell imaging during reprogramming of mouse fibroblasts in the presence of a hairpin against p53 revealed that although p53 inhibition expand the global population responsive to reprogramming, there is a higher number of aberrantly reprogrammed cells (Smith et al. 2010).

Inactivation or deletion of p53 is unfit for clinical use, however it may help to establish useful disease models in which the target somatic cells are particularly difficult to derive, expand and reprogram in vitro. Furthermore, sensible alternatives may exist to benefit from disabling senescence to enhance reprogramming efficiency without compromising the integrity and safety of the resulting iPS cells. This may be achieved by identifying the mediators and triggers of these tumor suppressor responses during reprogramming and by understanding the ES cell-specific mechanisms of cell-cycle regulation. For example, Rem2 GTPase, a suppressor of the p53 pathway implicated in maintenance of hES self-renewal and pluripotency, has been shown to improve reprogramming efficiency (Edel et al. 2010). Rem2 acts by accelerating the cell cycle and protects cells from apoptosis by suppressing p53 and regulating cyclin D1 expression and localization. In this way the authors suggest that imposing cell-cycle features that are specific of hES cells might safely improve reprogramming (Edel et al. 2010). Similarly, iPS obtained from miR34a$^{-/-}$ MEFs are of higher quality than iPS derived from p53$^{-/-}$ MEFs. Providing another example of how interfering indirectly with the p53 pathway may be safer for modulating reprogramming (Choi et al. 2011).

A possible strategy would be to transiently inhibit these tumor suppressors, by using either small interfering RNAs or chemical compounds. Two studies have provided safer methodologies for improving reprogramming by modulating factors contributing to senescence trigger. Reprogramming cells in low oxygen conditions (Utikal et al. 2009; Yoshida et al. 2009) or in the presence of antioxidants such as vitamin C (Esteban et al. 2010) enhances the generation of iPS cells. Pei's laboratory reported that one of the important metabolic activities that occur during the early stages of the reprogramming process is a significant increase in the level of reactive oxygen species (ROS). Addition of vitamin C, results in an increase in cell proliferation and extension of cell lifespan, suggesting that vitamin C can overcome the senescence roadblock during reprogramming by keeping p53 and p21 on check (Esteban et al. 2010). Therefore this study provides a good example of how senescence can be modulated to improve reprogramming efficiency in a safer way. A better understanding of the senescence roadblock during reprogramming will hopefully provide us with similar additional strategies.

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Histone Deacetylase Inhibitor Induces Replicative Senescence of Mesenchymal Stem Cells

17

Seunghee Lee, Ji-Won Jung, and Kyung-Sun Kang

Contents

Abstract

In cancer cells, the expression level of histone deacetylase (HDAC), a well known epigenetic regulator, is abnormally increased resulting in the inhibition of their target tumor suppressor genes. Treatment of HDAC inhibitors to the cancer cells induces rebound of tumor suppressor genes expressions followed by cellular senescence or apoptosis. Based on this mode of action against cancer cells, a number of HDAC inhibitors have been developed as anti-cancer therapeutics. However, considering that HDACs exist ubiquitously in normal cells and have important roles in the maintaining homeostasis and biological activity, the effects of HDAC inhibitors on normal cells need to be evaluated in detail. Here, we discuss the general characteristics of HDACs, HDAC inhibitors and their effects on the mesenchymal stem cell (MSC), an adult stem cell with normal diploidy comparing with the effects on cancer cells.

Keywords

Histone deacetylase inhibitor • Senescence • Mesenchymal stem cells • p16INK4A

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Introduction

The eukaryotic DNA is wrapped around core histone octamer complex which is composed of one H3-H4 tetramer and two H2A-H2B (Chung et al. 1978; Eickbush and Moudrianakis 1978).

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As acetylation neutralizes the positively charged lysine residues at the N termini of the histone, the affinity of histone for DNAdecreases. This allows the termini to be displaced from the nucleosome, unfolding and increasing accessibility for transcription factors (Grunstein 1997). The balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) controls acetylation status of histone followed by adequate regulation of transcriptional activity of the target genes.

Normal diploid cells lose the ability to divide after about 50 cell divisions (Hayflick limit) and the diminution of cell proliferation capability upon repeated division is known as "replicative senescence" or the "Hayflick phenomenon" (Hayflick 1965). One of main reasons of Hayflick limit is shortening of telomeres. In normal cells, telomeres at the end of chromosomes are not able to be copied because of the "end replication problems" of DNA polymerase and low telomerase activity of normal cells (Jiang et al. 2007). The other mechanism which induces replicative cellular senescence is telomere-independent in which increase of cyclin-dependent kinase inhibitors (CDKNs) like $p21^{CIP1/WAF1}$ and $p16^{INK4A}$ play key roles. Eventually, repeated division of a normal cell reaches Hayflick limit and aging or apoptosis (Olovnikov 1996).

However, in general, cancer cells and embryonic stem cells have an unlimited proliferation capacity without confronting "Hayflick limit". One of the reasons of this property is an excessive telomerase activity in cancer cells and embryonic stem cells. The other mechanism by which cancer cells bypass senescence pathway is down-regulated cyclin-dependent kinase inhibitors (CDKNs), like $p21^{CIP1/WAF1}$ and p16INK4A in concordance with augmentation of HDAC activities. In ~40% of diffuse large B-cell lymphomas (DLBCLs), B-cell lymphoma 6 (BCL6) is overexpressed resutling in recruitment of HDAC2 to *CDKN1A* which encodes $p21^{CIP1/WAF1}$ to repress $p21^{CIP1/WAF1}$ expression (Bolden et al. 2006). In addition to aberrant recruitment of HDACs to specific tumor supressor gene loci, the expression levels of HDACs are abnormally increased in several tumour samples. HDAC1 is overexpressed in prostate, gastric, colon and breast carcinomas; HDAC2 is overexpressed in colorectal, cervical and gastric cancer; increased expression of HDAC3 is seen in colon tumours; and overexpression of HDAC6 is observed in breast cancer specimens (Bolden et al. 2006).

$p16^{INK4A}$ is well-known senescence marker in both cellular and organismal aging. Several lines of evidence show that the polycomb group genes (PcGs) and JMJD3 histone lysine demethylase are involved in the regulation of $p16^{INK4A}$ (Barradas et al. 2009; Jung et al. 2010). HDAC inhibitors are reported as down-regulators of polycomb group (PcG) proteins resulting in $p16^{INK4A}$ expression and cellular senescence in cancer cells (Bommi et al. 2010). Based on the relationships between aberrant expression of HDACs and development of various cancers, HDAC inhibitors are considered as potent anticancer therapeutics.

Among the normal cells, adult stem cells share some common features with cancer cells such as undifferentiated and self-renewal ability (Reya et al. 2001). Based on this idea, there is an emerging theory suggesting that cancer cells can be originated from cancer stem cells and that cancer stem cells are started from the accumulation of mutations in adult stem cell populations (Reya et al. 2001). In spite of these similarity and relationships between cancer cells and stem cells, the effects of HDAC inhibitors on the normal adult stem cells have not been studied well than cancer cells. Here, we discuss the effects of HDAC inhibitors to the adult stem cells and their mechanisms to regulate cellular senescence based on our previous studies.

Histone Deacetylase

General Roles of Histone Deacetylase

HDACs are a class of enzymes that remove acetyl groups from lysine amino acids on histone tails. Histone tails are positively charged due to amine groups present on their lysine and arginine amino acids. Positive charges help the histone tails to interact with and to bind to the negatively charged phosphate groups on the DNA backbone. Acetylation neutralizes the charge on the histone

tails by changing amines into amides and releases the histones from DNA backbone. The release of the histones from DNA increases the accessibility of the transcriptional fators onto neighbouring DNA. HDACs remove the acetyl groups from lysine amino acid on a histone tails returning them to the positive charge. The positive charged histone tails bind to DNA backbone and DNA structures become condensed and transcriptionally repressed by reduced accessibility of transcriptional activators such as RNA polymerases and transcription factors (Choudhary et al. 2009).

HDACs are classified in four classes according to their sequence identity and domain organization based on their homology to yeast counterparts (Dokmanovic et al. 2007; Yang and Seto 2008). Class I includes HDAC1, -2, -3 and -8; Class II includes HDAC4, -5, -6, -7, -9 and -10; Class III include SIRT1-7 and Class IV contains only HDAC11. HDAC class I, II and IVcontain zinc and are known as Zn-dependent histone deacetylases. On the other hand, HDAC class III is known as Zn-independent histone deacetylases (Marks and Xu 2009).

Lysine acetylation of hisones and non-histone proteins is controlled by HDACs and HATs. Based on HDACs' and HATs' mechanisms of actions, they play important biological roles both in cellular and in organismal level. Although class I HDACs, which shares high sequence similarities within the group, might anticipate a significant overlap in function, so there are several reports revealing that the class I HDACs have both specificity and redundancy for their functions as (Haberland et al. 2009; Wilting et al. 2010). Wilting et al. (2010) reported that Hdac1 and Hdac2 have overlapping functions in cell cycle regulation and hematopoiesis. On the other hand, Dovey et al. (2010) reported that Hdac1, but not Hdac2, controls embryonic stem cell differentiation. Hdac3 has a critical function in cell cycle regulation and cardiac metabolism (Bhaskara et al. 2008). Hdac8 is a unique member of class I HDACs, as it lacks the conserved C-terminal domain. HDAC8 highly expresses in brain, kidney and prostate, whereas HDAC1, 2 and 3 highly express in heart and placenta. Knockdown of HDAC8 has no effect on histone acetylation, although HDAC1, 2 and 3 are potent regulators of histone acetylation (Yan et al. 2012).

Class II HDACs include six HDAC members; HDAC 4-7 and HDAC 9-10. Comparing with class I HDACs, the expression patterns of class II HDACs are more tissue-specific. Knockout of each Class II HDACs in mice show defects in various tissues; knockout of HDAC4 induces defects in chondrocyte differentiation, knockout of HDAC5 or HDAC9 causes cardiac and HDAC7 has important roles in maintenance of vascular integrity (Dokmanovic et al. 2007).

Non-histone proteins as well are important targets of HATs and HDACs. Acetylation affects the function andstability of the non-histone proteins and influence protein-protein interaction (Glozak et al. 2005). Since the first report of p53 as a non-histone target of a histone acetyltransferase HATs, there has been a number of reports on the non-histone targets of HATs and HDACs. Various transcription factors (p53, YY1, HMG proteins, STAT3, c-MYC), nuclear receptors (GAGA factors, EKLF, MyoD and E2F/Rb) and other cellular proteins (α-Tubulin, Importin- α, Ku70 and Hsp90) have been reported as targets of HATs and HDACs. Protein phosphorylation is one of the most widely studied and understood mechanism of post-translational modification. However, the importance of protein acetylation is emerging as a key regulatory modificiation which effect on the ability of DNA recognition, protein-protein interaction and protein stability (Kouzarides 2000).

Histone Deacetylase Inhibitors

HDAC inhibitors are well known as candidates of anti-cancer therapeutics because of their ability to induce growth arrest, differentiation and apoptosis in cancer cells. A number of HDAC inhibitors with different structural characteristics have been developed, including hydroxamates, cyclic peptides aliphatic acids and benzamides. At present, most of HDAC inhibitors have been developed to target HDAC class I and/or II. Some HDAC inhibitors have broad target against HDACs ranging across classes while others do not.

For example, HDAC inhibitors in hydroxamate group; SAHA, LBH589, PXD101, ITF2357 and PCI-24781 target both class I and II HDACs. On the other hand, MS-275, one of the benzamide group, targets HDAC1 preferentially and has little or no inhibitory effect against HDAC6 and HDAC8. SK7041 and SK7068, novel synthetic inhibitors of HDACs, preferentially target HDAC1 and 2 (Xu et al. 2007).

The biological effects of HDAC inhibitors result from the regulation of gene expression by acetylation of histones or non-histone proteins. Primarily, HDAC inhibitors cause acetylation of histones, which lead to the transcriptional activation by changing chromosomal structures to form open chromatin. However, the targets of HDAC inhibitors are not limited to the histones; HDAC inhibitors are known to target various non-histone proteins as aforementioned.

HDAC inhibitors also affect the expression of other epigenetic modifiers or post-transcriptional regulators. HDAC inhibitors regulate polycomb group proteins (PcGs) expression levels, which in turn modify histone methylation patterns. In addition, through modifying histone methylation patterns of genomic regions of non-coding RNAs, such as micro RNAs (miRNAs), HDAC inhibitors regulate translation of miRNAs, which inhibit translation of target genes (Lee et al. 2011). Based on these broadness of target genes or substrates, HDAC inhibitors involve in a number of biological processes, such as gene expression and regulation of cell proliferation, differentiation and cell death (Xu et al. 2007). On the other hand, the responses to the HDAC inhibitors are highly dependent on the cell type, i.e., cancer cells are more sensitive than normal cells to HDAC inhibitors in general (Kretsovali et al. 2012).

Histonedeacetylase and Senescence

The roles of HDACs in the cellular senescence of cancer cells by up-regulation of CDKNs, such as $p21^{CIP1/WAF1}$ and $p16^{INK4A}$ have been studied with a number of HDAC inhibitors. Regulation of $p21^{CIP1/WAF1}$ expression by HDAC inhibitors is independent of p53 and correlates with an increase in the acetylation of histones associated with the $p21^{CIP1/WAF1}$ promoter region (Dokmanovic et al. 2007). In ARP-1 cells vorinostat, a HDAC inhibitor, causes specific changes in the acetylation and methylation patterns of lysines residues in histones H3 and H4, associated with the proximal promoter of the $p21^{CIP1/WAF1}$ gene (Gui et al. 2004). $p16^{INK4A}$ is known to regulated by PcGs through modulation of histone H3 lysine residues. HDAC inhibitors down-regulates of PcG proteins resulting in an increase of $p16^{INK4A}$ expression and cellular senescence in cancer cells (Bommi et al. 2010).

Valentini et al. (2007) have reported that IC(50) dose VPA (~3 mM) induces G(1) cell cycle arrest (up to 75%) and apoptosis (50%) in M14 melanoma cells by up-regulation of $p16^{INK4A}$, $p21^{CIP1/WAF1}$ and cyclin-D1 related to Rb hypophosphorylation. To date several studies have implicated $p16^{INK4A}$ as a tumor suppressor gene with a major contribution either to development or to inhibition of human melanoma.

The roles of HDACs in cellular senescence have been studied in non-tumorigenic normal cells as well. Zhou et al. (2009) have reported that Lymphoid specific helicase (Lsh) repress $p16^{INK4A}$ via recruitment of HDACs on the promoter regions of $p16^{INK4A}$ and delays senescence in human diploid fibroblasts. Wang et al. have reported that the acetylation of transcription factor HBP1 by p300/CBP induce $p16^{INK4A}$ up-regulation in HEK293T cells and 2BS cells. In the same report they reported that HDAC4 repress HBP1-induced premature senescence through deacetylating HBP1.

Effects of Histone Deacetylase Inhibitors on Mesenchymal Stem Cells

Phenotypic Changes of Mesenchymal Stem Cells by HDAC Inhibitors

Short term (3 day) treatment of HDAC inhibitors, 10 mM valproic acid (VPA) and 5 mM sodium butyrate (SB) to mesenchymal stem cells (MSCs) induce cell cycle arrest at G2/M phase followed by decrease in proliferation potential (Lee et al. 2009). On the other hand, long term (5 or 7 day)

treatment with 2 mM VPA or 1 mM SB increase expression of $p16^{INK4A}$ and senescence associated beta-galactosidase (SA-b-gal) activity in hAD-MSCs or hUCB-MSCs, suggesting that the inhibition of cell proliferation and changes in cell morphology precede cellular senescence (Jung et al. 2010). However, HDAC inhibitors may not induce apoptosis of hMSCs unlikely to their effects on cancer cells.

One of our report have shown that the short term treatment (1 day) of VPA or SB decreased multilineage differentiation capability of hMSCs. In addition, the treatment of VPA or SB decreased the efficiency of adipogenic, chondrogenic, and neurogenic differentiation. In contrast, osteogenic differentiation was elevated by HDAC inhibitor treatment. Several studies by other groups have reported that HDAC plays a pivotal role in osteogenic or adipogenic differentiation. Cho et al. (2005) have shown that VPA treatment enhances calcium deposition and osteogenic gene expression in adipose tissue- and bone marrow-derived mesenchymal stem cells through inhibition of HDAC. Lagace and Nachtigal (2004) have reported that HDAC inhibition by VPA and trichostatin A block adipogenesis of 3T3 L1 cells and human primary preadipocytes, in an HDAC activity-dependent manner.

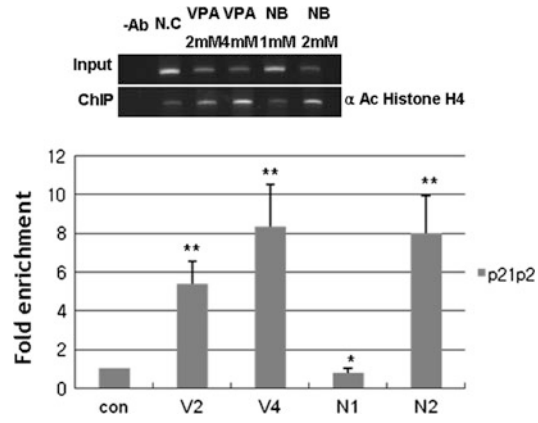

Fig. 17.1 HDAC inhibitor treatment to the hMSCs induces $p21^{CIP1/WAF1}$ expression through increase of acetylated histone H4 on the promoter of $p21^{CIP1/WAF1}$. After treatment of VPA or NB for 3 days as indicated concentrations, Chromoatin immunoprecipitation (ChIP) assay was performed. After immunoprecipitation with acetyl histone H4, enriched DNA fragments were anaylzed by polymerase chain reaction (PCR) with $p21^{CIP1/WAF1}$ promoter primer

Senescence-Associated Down-Stream Targets of HDAC Inhibitors

Up to date, $p16^{INK4A}$ and SA-β-gal activity have been considered as two of major indicators of cellular senescence. Krishnamurthy et al. (2004) have reported that the expression levels of both $p16^{INK4A}$ and $p19^{ARF}$ remarkably increase in aged murine tissues, whereas there is no significant change in the expression levels of other cell cycle inhibitors such as $p15^{INK4b}$, $p18^{INK4c}$, $p21^{CIP1/WAF1}$, and $p27^{KIP}$ up on aging. In the same context, our previous studies have shown that HDAC inhibitors increase the expression level of $p21^{CIP1/WAF1}$ earlier than $p16^{INK4A}$ or SA-b-gal activation. After 5 day treatment of HDAC inhibitors, $p16^{INK4A}$ protein level and SA-b-gal activity increased simultaneously (Lee et al. 2009). Likely to cancer cells, normal hMSCs pass through a simular pathway of $p21^{CIP1/WAF1}$ expression regulation by HDAC inhibitors. VPA or NB treatment induces acetylation of histone H4 and increases exrpession level of $p21^{CIP1/WAF1}$ mRNA as evidenced by chromatin immunoprecipitation and RT-PCR (Fig. 17.1).

The regulatory mechanisms of $p16^{INK4A}$ expression are related with histone modification as mentioned earlier. Bracken et al. have reported that $p16^{INK4A}$ is regulated by histone methyl transferase (HMTase)-induced methylation of H3 core histones on lysine 27 (Bracken et al. 2007). In young fibroblasts, the promoter of $p16^{INK4A}$ is bound by members of the PRC2-initiating complex, such as SUZ12, which in turn recruits EZH2, a member of the PRC2 complex posessing HMTase activity, and specifically trimethylates the H3K27 residue. In cancer cells, HDAC inhibitors inhibit expressions of PcG proteins resulting in increase of $p16^{INK4A}$ expression. In our previous study, we have found that the treatment of HDAC inhibitors decrease the expressions of PcG proteins and increase $p16^{INK4A}$ expressions in hMSCs. In addition, we have also shown that HDAC inhibitors regulate

phosphrylation of Rb protein. After treatment of HDAC inhibitors to the hMSCs, Rb proteins are hypophosphorylated and the binding between Rb proteins and E2F transcription factor increases resulting in repression of transcriptional activity of E2F. PcG proteins, such as SUZ12 and EZH2 which are under the control of transcriptional activity of E2F,are decreased following hypophosphorylation Rb protein. The repression of c-MYC, another target of E2F transcription factor, decreases causing decrease of expression level of BMI1, a down-stream target of c-MYC. Jumonji domain containing D3 (JMJD3), an enzyme of opposite role to EZH2 against histone H3K27 methylation status, is identified as a transcriptional activator of $p16^{INK4A}$ by demethylating H3K27Me3 residues at CDKN2A locus (Agger et al. 2009).We have also reported that HDAC inhibitors increase JMJD3 expression through inducing acetyl histone H3/H4 on the promoter region of JMJD3.

Another pathway which regulates $p16^{INK4A}$ expression is related to high mobility group A2 (HMGA2) protein. Nishino and colleagues have demonstrated that Hmga2 represses the cdkn2a locus in fetal and young mouse neural stem cells and that Ink4a/Arf deletion partially rescues the loss of mouse NSC self-renewal caused by Hmga2 deficiency. At present, there are two major regulatory mechanisms of HMGA2 expression reported. One is let-7 family of miRNAs, which targets HMGA2 mRNA. The 3′-UTR of human HMGA2 contains seven putative let-7 complementary sites. The inhibitory effect of the let-7 family on HMGA2 has been confirmed in various cancer cell lines and mouse NSCs. The other is the transcriptional regulation of HMGA2 via Sp1 and Sp3 by HDAC inhibitor treatment. In our previous study, we elucidated that HDAC inhibitor targets HMGA2 and down-regulates its expression in hUCB-MSCs focusing on the involvement of HMGA2-targeting microRNAs in the regulatory pathway. We have suggested that HMGA2-targeting microRNAs, novel elucidated microRNAs as well as let-7 family are regulated by HDAC inhibitor-induced histone modification (Lee et al. 2011).

Discussion

HDAC inhibition induces different phenotypic changes among the various kind of cells. HDAC inhibition in cancer cells, such as melanoma cells, induces apoptotic signal as well as cell cycle arrest. On the other hand, at the same or higher concentration of HDAC inhibitors, normal cells senesce not apoptose.

These different phenotypic changesby HDAC inhibitor treatment are assumed to arise from the difference of main target genes of HDACs among cell types. In cancer cells, HDACs are abnormally increased and inhibit the expressions of apoptosis or senescence mediators, such as p53, $p21^{CIP1/WAF1}$ and $p16^{INK4A}$. On the other hand, in normal hMSCs, the treatment of HDAC inhibitors induces senescence without activation of apoptotic signals although genes like $p21^{CIP1/WAF1}$ and $p16^{INK4A}$ are up-regulated similary to cancer cells. These suggest that HDACs directly or indirectly repress senescence-related genes, which does not provoke apoptotic signals in normal cells like hMSCs. In this context, elimination of HDAC activity is enough to increase the sensescence-related genes but not the apoptosis-related genes in normal cells partly because their down-stream pathways of $p21^{CIP1/WAF1}$ and $p16^{INK4A}$ may be different among cell types, at least.

Considering that HDACs exist ubiquitously and regulate epigenetic status of chromatin, it is reasonable that HDAC target genes are not limited to senescence related genes. For example, in embryonic stem (ES) cells, a major role of HDAC1/2 corepressor complexes is cell fate determination during differentiation (Dovey et al. 2010). Under this circumstance, inhibition of HDACs may help to maintain the self-renewal potential of stem cells. So, it is likely that HDAC inhibitors are used to increase the efficiency to produce induced pluripotent stem (iPS) cells.

Interestingly, HDAC inhibitors decrease proliferation potential of MSCs by arresting cell cycle at the G2 /Mphase. In addition, a representative target of HDAC inhibitors that affects cell cycle, $p21^{CIP1/WAF1}$, increases by HDAC inhibitor treatment. Previous reports have shown that in cancer

cells, HDAC inhibitors strongly promote cell cycle arrest primarily at the G1 / S checkpoint.p21$^{CIP1/WAF1}$ also plays a pivotal role in G1/S arrest induced by HDAC inhibitors (Richon et al. 2000). Our data suggest that HDAC may share common regulatory mechanisms and similar down-stream target genes between hMSCs and cancer cells; however, the role of the target genes, such as p21$^{CIP1/WAF1}$ and p16^{INK4A}, in MSC cell cycle regulation may differ from that of cancer cells and detailed speculation on the mechanisms underlying these actions is necessary (Lee et al. 2009).

The phenotypes are also different according to the duration of HDAC inhibitor treatment in a single type of normal cells; i.e. hMSCs. A short-term (1–3 days) treatment of HDAC inhibitors, VPA 2 mM or NB 1 mM, induces p21$^{CIP1/WAF1}$ expression resulting in cell cycle arrest without increasing cellular senescence marker, SA-b-gal activity and p16^{INK4A} protein level. On the other hand, extended (5 day) treatment of HDAC inhibitors increased cellular senescence markers. The direct regulation of p21$^{CIP1/WAF1}$ through histon acetylation and indirect regulation of p16^{INK4A} expression mediated by PcG proteins or miRNA-HMGA2 would be one of plausible reasons for the time gap between these genes to increase.

Taken together, HDAC inhibitors induce cellular senescence of hMSCs and the effect depends on the duration of HDAC inhibitors. Considering that the HDAC inhibitors are investigated as cancer therapeutics, their side effect to the adult stem cells including MSCs should be confirmed. Because of the suceptability and phenotypic changes by the HDAC inhibitors are differ among cell types, especially between cancer and normal cell types, adjusting the conditions of HDAC inhibitor treatment might prevent adverse effect on the normal cells, especially for adult stem cells like hMSCs.

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Senescence Arrest of Endopolyploid Cells Renders Senescence into One Mechanism for Positive Tumorigenesis

18

Kirsten H. Walen

Contents

Abstract

The present chapter analyses the cellular genomic status and type of cell divisions of pre-senescent cell populations shortly before the senescent phase. The purpose was to identify cellular mechanisms contributing to genomic unstable escape cells from senescence, in likeness with cancer recurrence from dormancy/remission periods. Primary diploid, human fibroblast cells were passage-extended to dysfunctional telomere-induced senescence (TAS) during which endopolyploidization occurred shortly before senescence. These cells either reduced genomic content to unstable near-diploid cells by irregular whole complement co-segregation, perpendicularly to the cytoskeleton axis or by nuclear fragmentation into multinuclear cells. The endo-division offspring-cells acquired genomic/chromosomal instability (CIN) from inheritance of endo-division traits, and gained proliferative freedom from contact inhibition by the perpendicularity of the endo-division. Other cells during this low proliferative period showed chromosomal aberrations and mitotic restitution, before a general change to senescent cells. The senescent phase showed change to typical cytoplasmic rich, amorphous flat cells, which were beta-galactosidase positive. Several types of escape mechanisms were observed (most frequent: nuclear bud-offs) which were associated with mitotic activity, albeit with limited propagation. These cells

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showed slight cell-shape changes from cell polarity change – a trait caused by endo-division perpendicularity. In experiments designed to simulate tumor therapy-induced genomic damage (to kill cells) endopolyploid cycling activity was increased before change to large amorphous, senescent cells. Young senescent escape-cells used the bud-off escape route, whereas escapes from old cells showed triangular cells in close contact with the senescent cells, which grew into three dimensional (3-D) tumor-like spheres. These observations were discussed as to tumor recurrence after prolonged dormancy/remission periods.

Keywords

Escape cells • 3-D growth • Kalikreins • Contact inhibition • Cell polarity • Skewed cytoskeleton • Meiotic-like

Introduction

Senescence is a physiological and morphological change of cells to a none-replicative state and occurs for both in vitro and in vivo cells. The cells gain large amounts of cytoplasm and change into amorphous flat cells with a nuclear to cytoplasmic ratio forbidding to mitotic activity. Normal proliferating cells in renewal tissues (skin/gut) reach a phase in their replicative ability that is hampered by dysfunctional telomeres (ends of chromosomes) that can no-longer support mitotic fidelity and the cells cease proliferation with change to the senescent cell morphology. This process was first shown for in vitro extended passages of normal diploid cell strains (Hayflick and Moorehead 1961). During the cell expansion-period telomeres progressively become short from loss of their specific repetitive DNA sequences until critically short, which the cell perceives as genomic damage and signals arrest by a senescence response (telomere associated senescence – TAS) (Walen 2007a; Davoli et al. 2010). Lately the senescence status with its secretory phenotype and complex physiology has been linked to several aging diseases, among them, tumorigenesis (Collado et al. 2007; Vergel et al. 2011).

Beta-galactosidase (beta-gal) activity in lysosomal vehicles accompanies the morphological changes to large flat cells, which with staining at pH 6.0 has become a widely used marker of senescent cells, which can also be visualized as small, clear bubbles in phase contrast microscopy of unstained cells (Walen 2011) Other markers have been proposed from nucleolar changes. This long know condition of cytoplasmic digestive vehicles associated with the transition from replicative to non-replicative senescence cells (Hayflick and Moorehead 1961) was recently suggested to involve autophagy (Young et al. 2009). This digestive process apparently breaks down old pro-proliferation cytoplasmic-compounds with "recycling" synthesis to an anti-proliferative condition of senescent cells Other genomic and epigenomic changes involve activation of genes for p53, p21, p16ARF or Rb, p21, p16INK4a, and development of heterochromatic foci (SAHF) with effect of gene-silencing (Collado et al. 2007; Collado and Serrano 2010; Vergel et al. 2011). Many experiments, designed to show reversion of the senescent state, have in general been negative, and when pre-neoplastic cells were shown to enter the senescent state, the conceptual leap to senescence as an anti-tumor, irreversible mechanism became generally accepted (Beausejour et al. 2003; Collado et al. 2005).

Crucial to the present title are a recent review on induced therapeutic genomic damage and its association to endopolyploidy and "leaky" senescence (Mosieniak and Sikora 2010). It cites numerous references many of which supporting present observations/conjectures and will be used as an overall reference in this chapter. Additionally, two works from in vivo/in vitro studies also showed: (1) a link between drug-therapy induced genomic damage (to kill cells) and change to polyploidy with senescence entry, which generally is referred to as premature/accelerated senescence (ACS), and (2) that preserved biopsy materials of neoplasia were positive for the senescent cell phenotype and beta-gal (Roberson et al. 2005; te Poele et al. 2002). Roberson et al. (2005) further showed that the

"reversion" from senescence to genetically changed cells was a very rare (10^{-6}) event, called "escape". The escape clones showed correlated up-regulation of cyclin B1 and Cdk1 (cyclin and kinase) which are indicators of mitotic activity, but routes of escape from senescence were not identified.

Mechanistic Escape Routes from Telomere Associated Senescence

We studied young (1–3 weeks) senescent cells in vitro by extended passages to the senescent phase of two primary, human diploid fibroblast cell strains (Walen 2004, 2005, 2006). Stained and phase contrast photography showed that nuclei can move in the large, flat cells to the cell membrane/envelope and there become wrapped in membrane for exocytosis (i.e., nuclear bud-off). This process most often led to long, thin extensions of cytoplasm away from the mother cell with mitosis often occurring at the tip (Fig. 18.1a–c). When the nuclear tip region was separated from the mother cells, the resulting cells, became flattened from surface attachment and showed one centrosome and a normal mitotic sequence, albeit to very limited population doublings (Walen 2005). Other escape cells returned to the flat cell morphology, indicating senescent-cell genomic heterogeneity. Recently another escape route from thinly seeded lysosomal positive senescent cells gave details of cytoplasmic-reduction aided by lysosomes, not known from earlier works (Fig. 18.2a–j). The difference was due to fixation in absolute methyl alcohol instead of in Carnoy's solution. These cells showed cytoplasmic discard as large, blebs on the surface of mitotic cells (metaphase and anaphase) involving large and small lysosomal vacuoles. The alignment of the lysosomal bodies at the base of the blebs indicates a controlled and selective autophagic process. Figure 18.2e shows a large disconnected bleb which apparently separated from the mother-cell without injury to the cell envelope. The blebs are likely formed by cell envelope puffing-extensions, enclosing various amounts of cytoplasm which was occurring in cells with endopolyploid mitotic activity, suggesting discard of anti-proliferative chemicals (Young et al. 2009). This picture is in contrast to similar non-mitotic flat cells, negative for lysosomal presence undergoing necrosis with bits and pieces of digested cell membrane surrounding the cells (Fig. 18.2h–j).

Other cells, multinucleated cells (MNCs) from fragmentation of giant cells (>8n), in these senescent cell populations, also showed lysosomal "alignment" associated with discard of non-viable necrotic products (Fig. 18.2j), and MNCs furrowing into individual cells, and triangularly shaped cells appeared to tolerate significant amounts of lysosomes (Fig. 18.2k, l). These latter cells different from the striated fibroblasts suggests endopolyploid origin (see below). Other MNCs in pre-senescence showed the nuclear bud-off process which could be increased by a trypsin-digestive reduction of the extra cellular matrix (Walen 2002, 2008). In tumorigenesis, giant cells and MNCs are often present with the extreme example of osteoclast-like cells. Information about radiation-induced MNCs and their potential contribution to tumor genetic variability from their rather unique segregation system is forthcoming (Erenpreisa et al. 2011). Additionally it is known that tumorigenic cells secrete kallikreins (metalloproteinases) that can act like trypsin for potentiating a bud-off process. Various kallikreins have become markers of tumorigenic cells with the best known test being PSA for prostate specific antigen. Nevertheless, all in vitro observations mentioned here, together demonstrate that the nuclear bud-off mechanism is a general phenomenon for attainment of a nuclear to cytoplasmic ratio compatible with mitotic activity. Alternatively, cycling cells stopped in their course (by senescence) are poised for continuation of cycling. This cellular quest to stay cycling active (cells can survive in G0) applies to tumor recurrence and to mitotic slippage of G2 cells into S for endoreplication to endopolyploidy. However, escape cells from senescence must synthesize their own genomic-controlled cytoplasm for mitotic entry, which for MNCs in tumor-cell lines was shown to involve significant increase in

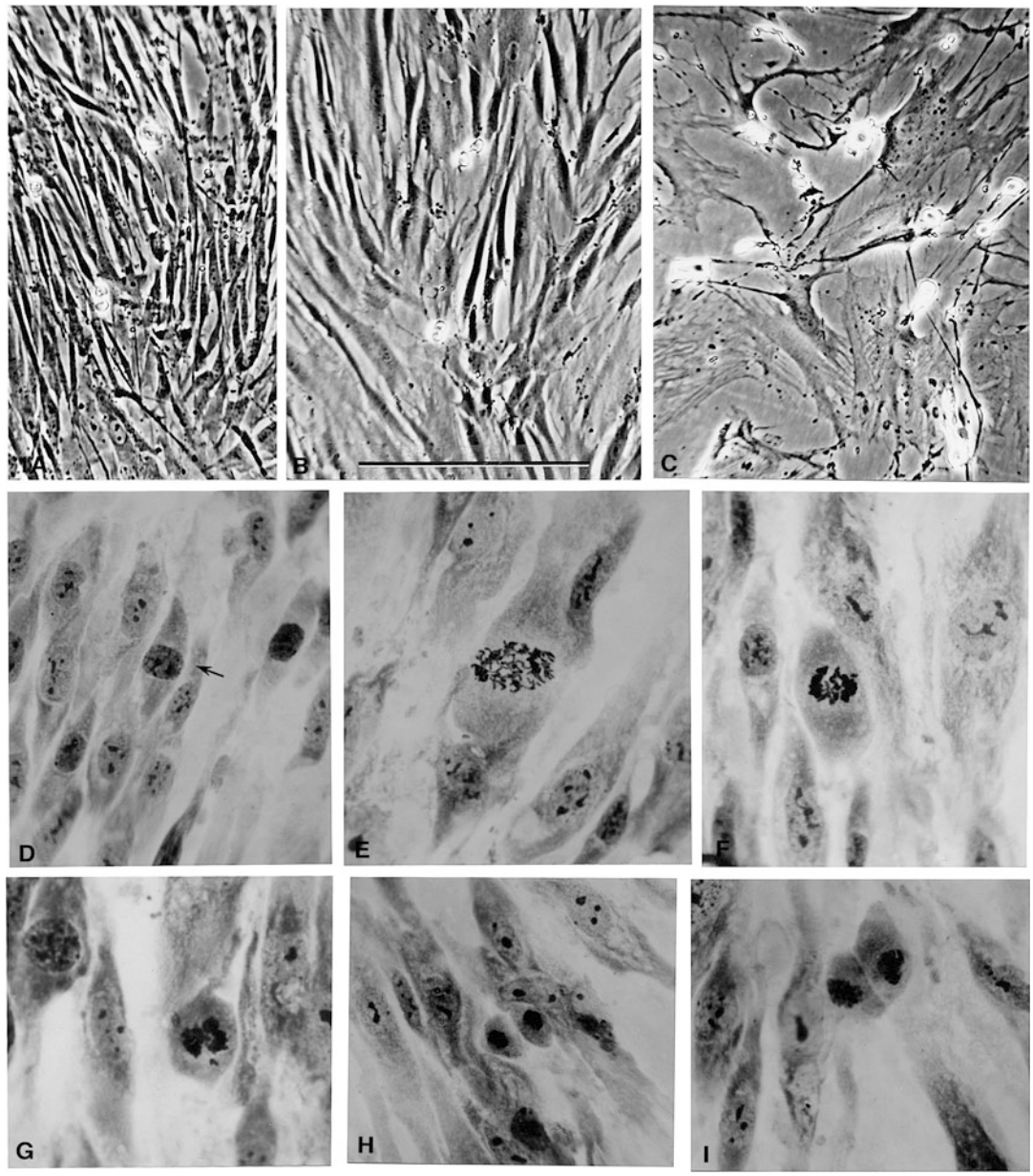

Fig. 18.1 Normal fibroblast (**a**) change to beginning senescence flat cell morphology (**b**) and escape cells with mitotic activity from nuclear bud-offs from senescent cells (**c**). Perpendicular nuclear and endo-divisions relative to the cytoskeleton axis: (**d**) nuclear orientation, (**e**, **f**) endotetraploid prophase and beginning anaphase, (**g**, **h**, **i**) endotetraploid first division products in perpendicular orientation to the surrounding cells. Magnifications: **a**–**c** = 620×; **d**–**i** = 990×

nucleolar ribosomal activity (Erenpreisa et al. 2011). Roberson et al. (2005) showed for lung cancer cells that escape cells were molecularly more similar to the senescence arrested cells than cells of origin.

The next two sections present conflicting observations and suggestions of the cellular status of senescence, and of how endopolyploid cells with irregular, non-mitotic cell division contributes to genomic heterogeneity in senescent cell populations.

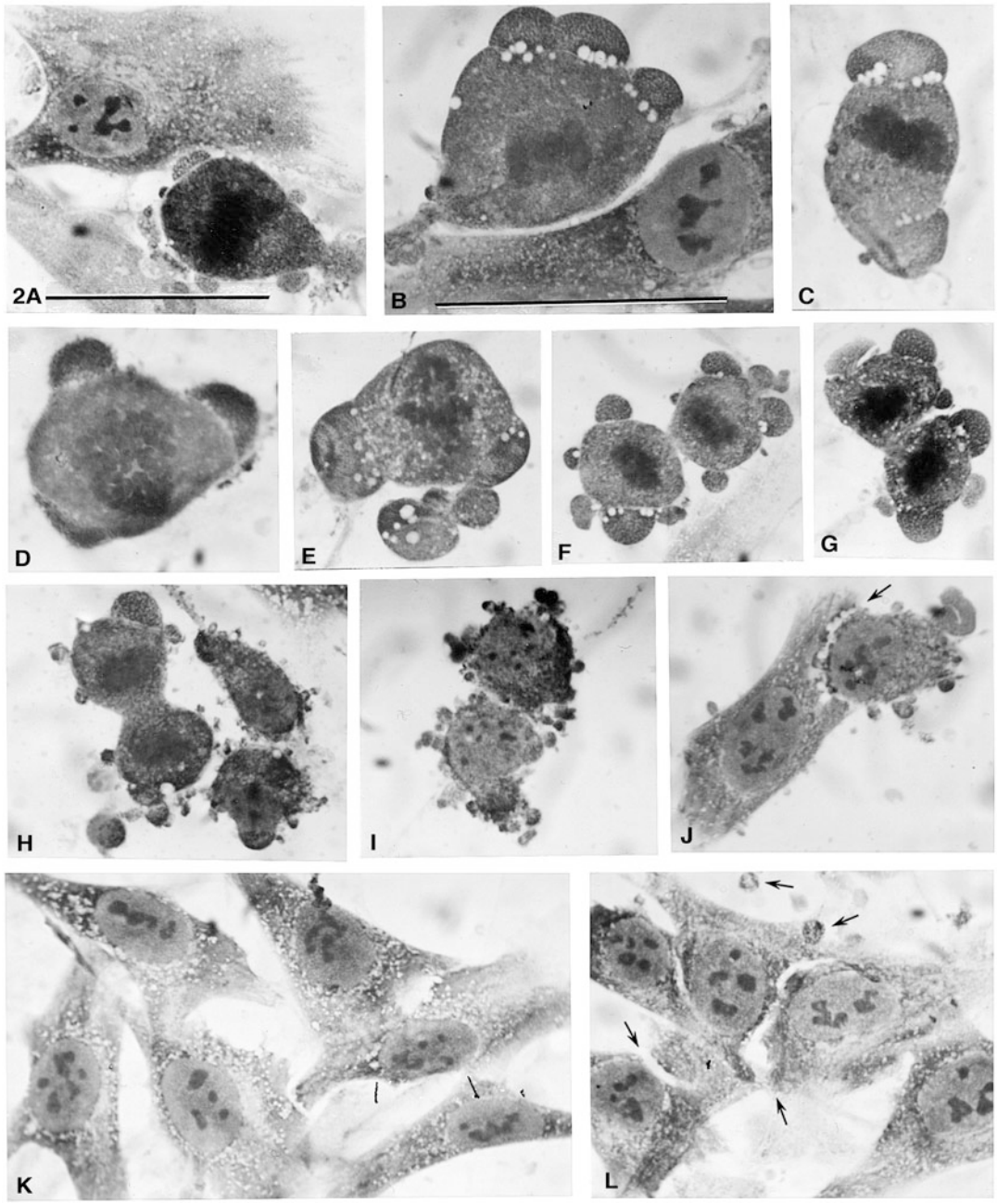

Fig. 18.2 Cytoplasmic reduction by membrane-bound blebs discarded from isolated senescence cells with lysosomal presence. (**a**) A large senescence interphase cell close to a mitotic cell with blebs, (**b**, **c**) mitotic cells with rows of large lysosomes at the base of the blebs, (**d**, **e**, **f**, **g**) mitotic cells in meta- ana- telo-phase to interphase with blebs, of note: the anaphase figure (**e**) is oriented perpendicularly to the cytoskeleton axis, (**h**, **i**) necrosis in the absence of lysosomes, (**j**) lysosomal "aided" discard of a necrotic product from an MNC, (**k**) triangular cells with abundant content of micro-lysosomes without apparent deterioration, (**l**) cytoplasmic furrows between nuclei of multinucleated cells (MNCs), arrows point to discarded blebs. Magnifications: **a**, **c**–**l** = 2,475× and **b** = 3,217×

Escape from Senes cent Cells Preceded by Cell Growth from Increased Endopolyploidy

A rather confusing issue is whether therapy-induced tumor-cell senescence can be compared to replicative senescence (TAS) (Shay and Roninson 2004). There is a tendency to equate beta-gal positivity with senescence induction when in fact such happenings are known from simple cell culturing to be a cell-arrest response. Cytoplasmic granulation, as an indicator of culture decline (Hayflick and Moorehead 1961) was in earlier works during cell culture media-developments ascribed to lysosomal activity. Personal experience with proliferative well-beings of primary cell cultures has always involved a check for presence of granulation, since proliferation-arrest from toxic media components had been encountered. But, with a quick medium replacement the cells regained cytoplasmic-clarity and proliferative power, which demonstrate that lysosomal presence is an indicator of cell-well-being, and not an exclusive marker of senescence (Walen, unpub.). Shay and Roninson (2004) discussed the genetic and epigenetic differences between TAS and STASIS (stress-associated induced senescence) with STASIS being a TAS-like response from "exogenously induced rapid G1 growth arrest". Vergel et al. (2011) stressed the molecular differences in effectors of the senescence program (e.g., p53 and p16ARF is mostly compromised in cancers). Therefore, in the following presentation to avoid confusion, the terms dormancy/remission will be used for other types of senescence.

There are also other great conflicts about the genotypic and phenotypic status and metabolic activities of cells that are inn replicative senescence (TAS): (1) Entry of only diploid cells (2n/2C/G1/G0) versus such cells together with an amount of cells arrested in G2 (either diploid 2n/4C or tetraploid 4n/4C – no cytometry difference). Nuclear size differences support a mixture of G1/G0 and G2 cells (Walen 2006; Matsumura et al. 1979). (2) Questionable DNA synthesis in senescent cells by observation of different cell types labeled with tritiated thymidine (TH3) (Matsumura et al. 1979; Beausejour et al. 2003). (3) Possible physiologic and genomic differences between young (weeks 1–2) and old (5–8 weeks) senescent cells are a neglected subject. This latter possibility was demonstrated by young TAS cells showing DNA repair (γH2AX) and heterochromatic (chromocenters) foci that disappeared in old senescent cells (deep senescence) (Chen and Ozanne 2006). Exposure of the old cells to H2O2 (induced DNA damage) resulted in development of new repair foci together with 53BP1 protein which showed p53 activation. Repaired telomeres were suggested, with likely association with the slow uptake of TH3 as a DNA repair synthesis (Matsumura et al. 1979). If true, aged senescent cells would have greater probability of showing re-growth from escape cells than young cells. The cell-escape from the dormant lung tumor cells mentioned above was a late (3–4 weeks) cell-escape (Roberson et al. 2005), which is also a trend for tumor recurrence from remission/dormancy.

These latter facts prompted an experimental design for senescence induction that would simulate the genome-wide damage often associated with therapy agents and thus, not only be restricted to telomere-associated damage (TAS-treated) (Walen 2011). This induction was achieved by exposure of near-senescent primary human cells to culture medium deficient in glutamine. Earlier studies of amino acid deficiency had shown chromosomal aberrations from breakage and reunions, and high frequencies of endo-tetra-octopolyploidy and giant cells (Freed and Schatz 1969). The shortly (2–3 days) treated cells were propagated (two to three passages) in recovery medium (glutamine positive) to senescence, which again was rather sudden with change to large, amorphous flat cells, containing high levels of lysosomal vehicles. These TAS-treated cells, on their way to senescence showed increased endo-divisions as compared to TAS alone, and expressed a tendency for cells to be on top of each other (Fig. 18.3f), and also shoved small dysplastic foci of cells with cell-shape changes from cell polarity change. The senescent cultures were not sub-cultured, only

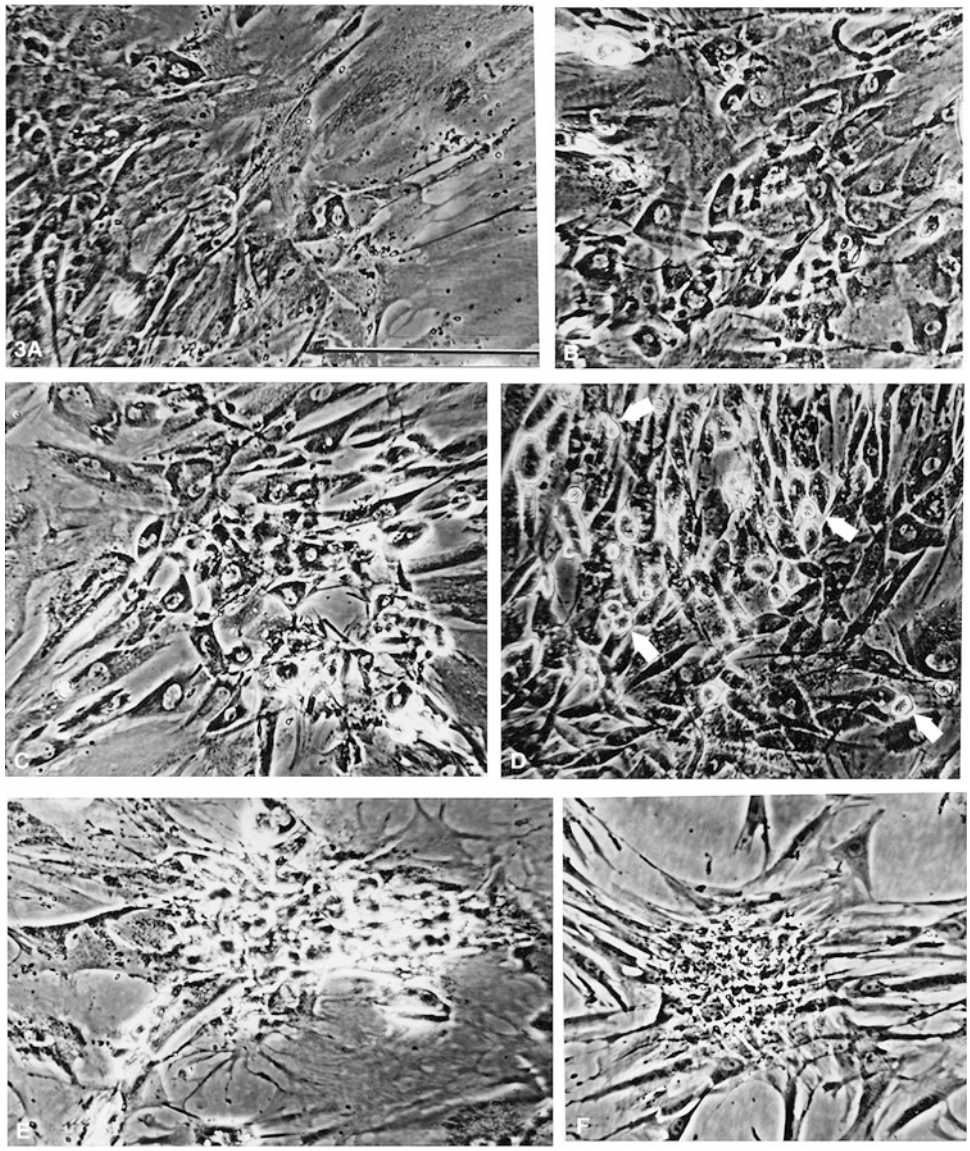

Fig. 18.3 Escape cell proliferation of cell polarity changed cells associated with three dimensional (3-D) tumor-like growths. (**a**–**e**) Different growth foci in close association with the large amorphous old, flat cells, all showing similar cell-shape-changes and a small condensed nucleus, (**d**) contains many mitoses (*arrows*) and (**e**) shows beginning to a 3-D structure morphologically very different from an early 3-D senescence-escape growth (**f**), seemingly with largely retained fibroblastic phenotype. Magnification: All illustrations 2,475×

exposed to frequent media changes and pH adjustments in order to counteract the cell-destructive effects from beta-gal-lysosomes, which was discarded into the medium (Walen 2011).

Young senescent cells demonstrated the nuclear bud-off process, showed gamma-H2AX repair- and senescent associated heterochromatic foci (SAHF – gene silencing) (Mosieniak and Sikora 2010). But with increasing maintenance-time to

more than a month, the two types of foci had gradually disappeared and amounts of lysosomes were significantly reduced. For a fraction of cells, lysosomes were concentrated in a band around the nucleus/nuclei (MNCs), also shown by others as a distinguishing feature of senescent cells. In these TAS-treated 5–8 week old cultures, several different sized clumps of cells appeared floating in the medium, and also surface-attached with three dimensional (3-D) spherical tumor-like growths (Walen 2011). The proliferating cells showed a triangular shape and small condensed nuclei, very different from cells of origin, strongly striated/fibroblastic (Fig. 18.1a versus Fig. 18.3a–d). The surface attached 3-D growths (Fig. 18.3e) appeared to be situated on top of the senescent cells, implicating a cell-central nuclear bud-off mechanism (Walen 2002). These late occurring 3-D growths had a possibility of origin from two types of cells, either with or without added induced genomic damage to that from dysfunctional telomeres. Both conditions however, implicated biological events of unknown nature in the protracted senescence phase. Interestingly, the gamma-H2AX- and SAH-Foci also disappeared with extended dormancy period for preneoplastic adenomas from which escape cells grew into full blown adenocarcinoma cancers (Collado et al. 2005). Both types of escape cells (in vitro/in vivo) had gained different genetic characteristics, and most importantly, regain of proliferative capacity by telomere "repair DNA synthesis" (Chen and Ozanne 2006) and/or by telomerase activation.

Escape Cells Can Harbor Genomic Status for Positive Tumorigenesis

The above statement is based on 3-D growths in liquid media as opposed to colony growth in semi-solid medium commonly used as a cell-transformation assay. These growths with cell-shape changes from change/loss of cell polarity can be a consequence from the irregular (non-mitotic) endopolyploid division-system. There are many studies that link tumorigenesis to polyploidy, but few discriminate between regular polyploidy and endo-polyploidy. These two types have entirely different division systems, – regular with mitosis, and endo-polyploidy – with a two-step meiotic-like division. Moreover, endo-division chromosomes have four chromatids as compared to metaphase chromosomes with two chromatids (Levan and Hauschka 1953; D'Amato 1989; Walen 2006, 2007a, b; Davoli et al. 2010). The 4-chromatid condition was a consequence of re-replication of G2 chromosomes (bichromatid chromosomes) having escaped arrest, caused by genomic damage from short telomeres in pre-senescence (Walen 2007b; Davoli et al. 2010). The first division of the meiotic-like segregation separated the 4-chromatid complexes into two groups of bichromatid chromosomes (e.g., 4n/8C into two 4n/4C products). This was accomplished by co-segregating whole complements in a perpendicular orientation relative to the cell-polarity axis, the cytoskeleton. Unlike mitosis, this division was independent of the spindle apparatus which without an intervening S-period further divided the 2-chromatid chromosomal groups into four single chromatid diploid-sized nuclei (two 4n/4C divisions to four 2n/2C nuclei) (Walen 2007b). The endo-division is thus a genome reductional division by reverting back to near-diploid cells.

Before becoming senescent cells the reverted offspring cells with mild cell polarity changes divided with modified mitosis and cell cycles due to genomic instability caused by inherited endo-division traits, merged with the innate mitotic machinery (Walen 2012). Senescence with entry of endopolyploid cells, and endo-division products, together with diploid cells, aberrant or not, is a genetically heterogeneous phase. Escape cells will have probability of giving rise to for example, cancer-cell like proliferation (3-D growth) as observed in the old in vitro senescent cell cultures. Data from in vitro/in vivo treatment of cells/tumors with chemo-agents are increasingly showing or suggesting, (endo)-polyploid senescent cells with reversion to lesser ploidy cells as escape cells in tumor relapse (Puig et al. 2008; Mosieniak and Sikora 2010). Thus, the conjecture that senescence can be a positive contributor

to oncogenesis is based on endopoly-ploidy as a genomically unstable cell status in senescence, and on escape-cells showing 3-D tumor-like growths composed of genetically changed cells with cell polarity change/loss.

Cell Polarity, a Control System Against Unscheduled Cell Growth

Over a century ago progressive cell-shape changes from cell polarity loss in anaplasia during cancer progression all the way to round cells was interpreted to lead to "greater capacity for independent living" (Bignold et al. 2007). Numerous investigations have shown that cancer progression, especially the transition to metastatic cells, is associated with loss of adhesion between cells. In cancer-cell pathology dedifferentiation-associated cell-shape changes plays a major role in classifications of mild, moderate or severe dysplasia. Mechanisms for these early cellular indications of potential cancer-development have not been forthcoming. In normal cell-growth, cell-to-cell adhesion acts as a cell-proliferative control, as, for example, expressed by contact inhibition of fibroblast cells. On the molecular level the normal adhesion (e.g., tight junction) is regulated by diverse adhesion proteins of which E-cadherin is a major inter-cellular molecule. In cancerous growths the level of this protein goes from mildly reduced to complete loss which is correlated with increasing pathological grade to metastatic cancer cells, often expressing round cell-shapes.

In normal skin development the nucleus orients from a parallel basement membrane position to a vertical orientation with associated reorientation of the cytoskeleton proteins (actin, fibrils etc.). This latter re-orientation does not happen in endo-division-associated perpendicularity relative to the cytoskeleton axis (Walen 2011). The first meiotic-like division-products become perpendicularly oriented relative to surrounding cells (Fig. 18.1d–i; out of sequence from space limitation). Notably, the co-segregating genomes move from each other in a parallel direction in agreement with the basolateral cell cortex. The endo-cell becomes stretched in the mid-zone with gradual flattening of the apical and basal regions. The net effect is collapse/destruction of the cytoskeleton which does not lead to cell arrest or apoptosis, but to continuation of the second meiotic-like division, and to mitosis of the genome reduced, cell-shape changed offspring cells. These observations offer a physical mechanism for disruption of cell-to-cell contact. For example, assuming an E-cadherin "gluing" system in contact-inhibited fibroblasts in pre-senescence, this protein has an external-cell part between cells (the glue) and another short part that goes through the cell envelope and into the cytoplasm where it is tied to the cytoskeleton by bridging catenin-molecules. The endo-division system with genomic movements perpendicularly to the cytoskeleton axis which flattens the mother cell will have destructive effect on the E-cadherin-catenin complex and on the glue between cells. The adenomatous polyposis coli (APC) gene in vivo when mutated (lost) produce tetraploidy-associated with catenin dysfunction, suggesting endotetraploid-divisions to unstable diploid cells in colon cancer (Ceol et al. 2007). Furthermore, foci of dysplastic cells (loss of normal cell orientation) associated with endopolyploid cell cycling in pre-senescence (Walen 2011) suggest a similar route from endopolyploidy for early clinical dysplastic lesions (e.g., dysplastic nevi contain endoreplicated nuclei). Such lesions can increase in severity (pathological grade) by repeated endo-divisions.

Discussion

It must be emphasized that this summary exclusively deals with experimental results from normal, diploid, primary cell strains (L645 & WI-38) for the purpose of identifying *normal* cell changes relevant to a tumorigenic potential. Use of cancer-cell lines are first of all informative to therapy-related issues, but has also been crucial in providing of cancer-related hallmarks (e.g., genomic instability and aneuploidy) – genomic and epigenomic changes which the normal cell must acquire.

The present evaluation of proliferative cells in pre-senescence over three to four passages (with dilution factor 1:2) before senescence-entry clearly demonstrated that the change of diploid cells to endopolyploid cycling cells showed versatility for the creation of some salient tumorigenic features, namely genomic instability and proliferative ability of senescence escape cells (free from contact inhibition). Six main features set endopolyploidy apart from other suggested tumorigenic mechanisms, as most likely to function in cancer initiation and progression: (1) it occurs as a genome damage response in normal aging cells from dysfunctional telomeres, when cancer is at the highest incidence (Collado et al. 2007); (2) the two-step meiotic-like endo-reduction division is independent of the spindle apparatus which nullifies arrest from checkpoint controls; (3) the endo-division perpendicularity disrupts cell-to-cell contact from cytoskeleton defects and releases offspring cells from contact inhibited growth; (4) low level endopolyploidy (4n & 8n) revert back to mitotic near-diploid and -tetra-ploid cells by genome reductional division; (5) genome reduced endo-offspring cells acquire CIN from mergence of inherited endo-division traits with the mitotic machinery, which leads to modified mitosis and cell cycles; and (6) endopolyploid cycling is a mitotic-meiosis for primitive organisms (e.g., Aulachantha; Walen 2011) and is an inherent evolutionarily preserved property of mammalian cells (e.g., tropho-blastic tissue). Numerous cellular mechanisms have been proposed for the creation of genetic variability, all extracted from their occurrence in cancer cells, with breakage-fusion-bridge cycle (B-F-B) and multiple centrosomes for chromosomal instability and multipolar mitosis being the most frequent suggestions. None of these mechanisms have however, been shown to result in cells with acquired inherent, genomic instability (#5 above), a most crucial characteristic of cancer cells (Mosieniak and Sikora 2010).

The pivotal feature of endopolyploid cell cycling as a mechanism in tumorigenesis is the change to 4-chromatid chromosomes which "demands" orderly segregation (mitotic-meiosis #6 above) – a division system that does not involve regular mitosis. Currently there is only one study showing a link between endopolyploidization and change to 4-chromatid chromosomes (Davoli et al. 2010) in addition to some pioneering works (Levan and Hauschka 1953; Walen 1965; D'Amato 1989) and our own recent studies. Of note, is that similar to the present normal-cell endopolyploidization process, a "G_2^{endo}"-cell sequence to endopolyploidy from re-replication of therapy-related genomic damage was shown for human tumor cells (Smith et al. 2007). The further step to observations of 4-chromatid chromosomes was not mentioned, and consequently endopolyploid divisions were treated with regular mitosis – interpretations which unfortunately are a general occurrence today (Ceol et al. 2007).

Perpendicular endo-division relative to the cytoskeleton axis produced offspring cells with cell-shape changes and with proliferative freedom from loss of cell contact inhibition. These events occurred in pre-senescence, and for 3-D growths from old senescence escape-cells the cell-shape changes were augmented. This increase was suggested to arise from escape-cell retention to some degree of flat cell "amorphous-physiology" which does not express a polarity system (Walen 2011). Cancer-associated cell dedifferentiation to complete loss of cell polarity, expressed as "round cells", currently has no definitive origin(s), implying high probability of endopolyploid cycling in tumorigenesis. This was recently shown from technologically advanced methodology (Puig et al. 2008) which is an important positive addition to the classical report on 4-chromatid chromosomal cycling in lymphomas (Levan and Hauschka 1953). Importantly, repeated endopolyploidizing events from presence of genomic damage (e.g., oncogene Myc activation) will increase cell-dedifferentiation from the innate process of endo-perpendicularity. For Drosophila-cells, perpendicular orientation in stem cell-division led to cancer-cell phenotypes of progenitor cells. For human cancers only one study of aggressive oral cancers hints at a similar relationship by descriptions of skewed divisions from "cytoskeleton defects" (Saunders et al. 2000). Genes for cytoskeleton

organization were found up-regulated (e.g., Rho-ATPases) at different stages of tumorigenic progression, but without associated mutational changes, supporting events of repeated endo-perpendicular divisions.

By-pass of dormancy periods is a general suggestion for recurrence of cancers following therapy, based on the in vitro immortalization-model (Vergel et al. 2011), but can equally well be suggested to arise from any of the described escape-cell mechanisms to mitotic events. A fair comparison to dormancy is TAS-treated-associated "senescence" with its development of 3-D tumor-like spheres from old escape-cells. This dramatic cellular change from primary, diploid cells inflicted by telomere dysfunction and genome-wide damage has no in vitro likeness (immortalization does not involve senescence escape). But, contrary to the 3-D growth's origin from old senescence escape cells, increased tumorigenic potential of dormancy-escape cells is more recently thought to be effected from adjacent senescent, stromal secretory cells (Vergel et al. 2011). An additional route is the recent emphasis on autophagy with the dual ability to suppress or promote tumorigenesis (Young et al. 2009; Erenpreisa et al. 2011). Cells with a band of lysosomes around the nuclei (see above) may indicate that the "quality" of the senescent state might differ among the old senescent cells (Young et al. 2009).

The claim here that the senescent phase can lead to positive tumorigenesis from cells with endopolyploid division characteristics is firstly supported by endopolyploid chromosomal cycling of escape cells (Fig. 18.2a–g; Walen 2008), and secondly by the finding that endo-polyploid-reduction division was a source of tumor re-growth-cells (Puig et al. 2008). Moreover, chemo-agents against cancer cells were shown to induce endo-polyploidization (giant cells) associated with induced senescence-like (TAS-like) morphology (Mosieniak and Sikora 2010). From these giant cells "late" genome reduced, escape cells propagated, showing mild aneuploid changes which is a recent disclosure associated with endopolyploid reductional division (Walen 2012). Fragmentation of giant cells (>8n) into genome reduced euploid cells occurs in trophoblasts of the human placenta and also for normal diploid endopolyploid (>8n) cells in pre-senescence which showed mitotic capacity (Walen 2010). In agreement with Mosieniak and Sikora (2010) such genome progressions from endopolyploidy indicate a risk of the senescent state of promoting tumorigenesis, which is a potential challenge to recent suggestions of induced senescence as an anti-cancer therapy condition (Shay and Roninson 2004; Haugstetter et al. 2010; Collado and Serrano 2010; Vergel et al. 2011). There are warnings against prolonged induced dormancy periods unless, as observed, the immune system eliminates the senescent cells (Collado and Serrano 2010). The gap in our knowledge of the dormancy/remission period for reduction/curtailment of tumor recurrence has probability for rectification by molecular studies of the TAS-treated induced senescence. Such gained information can be applied to preserved biopsy materials for potential disclosures of markers relevant to targeted therapy (te Poele et al. 2002). But in the mean time "dormant tumor cells" – "represent a dangerous potential for tumor relapse" from escape cells (Collado and Serrano 2010).

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The Two Faces of Senescence-Associated Epigenetic Alterations: Tumor Suppressors and Oncogenic Drivers

19

Anabelle Decottignies

Contents

Abstract

Cancer is primarily a disease of the elderly. During aging, senescent cells displaying altered epigenetic profiles accumulate in tissues. Although these epigenetic alterations are responsible for establishing the cell cycle arrest characteristic of senescent cells, tumor suppressive barriers can be bypassed through loss of cellular checkpoints, leading to the emergence of cancer cells that display epigenetic alterations closely resembling the ones of senescent cells. Strikingly, evidences are now accumulating in favor of potent tumor-promoting effects of these senescence-associated epigenetic alterations. Notably, retention of senescence-associated heterochromatic foci (SAHF) may protect cancer cells against unwanted DNA damage response activation and apoptosis. Here, I review the alterations of epigenetic landscapes in senescent and cancer cells and put forward the idea that cellular senescence may epigenetically contribute to tumor formation.

Keywords

Senescence • DNA methylation • Heterochromatin • Cancer • Aging

Introduction

Cancer incidence increases with age. Organismal aging is thought to result from the accumulation of senescent cells that reduce the regenerative

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potential of the stem cell pool and impair organ function. Cellular senescence in aging tissues is most likely the consequence of accumulated telomere defects possibly triggered by oxidative stress (Herbig et al. 2006). In addition to cellular senescence driven by telomere defects, activated oncogenes can induce premature entry into senescence, a process referred to as oncogene-induced senescence or OIS (Decottignies and d'Adda di Fagagna 2011). Hence, whether due to aging or to oncogene activation, cancer probably develops in a context of cellular senescence. Early research works showed that cellular senescence is a powerful tumor-suppressing mechanism characterized by stable cell cycle arrest. However, recent evidences pointed towards the ability of cells to escape from senescence through inactivation of cellular checkpoint mechanisms (Beauséjour et al. 2003; Halazonetis et al. 2008; Di Micco et al. 2011).

Although seemingly distinct processes, aging and oncogene activation induce similar alterations of epigenetic profiles that, altogether, constitute the driving force behind the senescent phenotype (DiMauro and David 2009; Tsurumi and Li 2012). These include alterations of histone modification profiles, chromatin-associated protein distribution and DNA methylation. Overall, senescent cells are characterized by changes of the epigenetic profiles into two opposite directions in agreement with the current "redistribution of chromatin modifiers" hypothesis that suggests a relocalization of chromatin modifiers from their normal loci to other chromosomal sites as cells age (Imai and Kitano 1998; DiMauro and David 2009). Evidences accumulated over the last decade suggest that a subset of the epigenetic alterations acquired during senescence are likely to contribute to tumorigenesis through changes in gene expression, genomic stability and/or sensitivity to DNA damage response activation. Thus, it appears of major importance to acquire a good knowledge of these senescence-associated epigenome alterations and to investigate how these might impact on cancer progression. Here, I review the epigenetic landscape alterations of senescent cells, compare them with the ones detected in cancer cells and discuss about the potential role senescence may play in tumor progression.

Alterations of Histone Modification Profiles and of Heterochromatin-Associated Factor Distribution in Senescent and Cancer Cells

Histone Modifications and Heterochromatin-Associated Proteins: A Brief Overview

Eukaryotic genomes are organized into highly ordered chromatin structures with nucleosomes being the most fundamental units. Nucleosomes consist of 146-7 base pairs of DNA wrapped around octamers of H2A, H2B, H3 and H4 major core histones. In specific chromosomal contexts however, minor histone variants replace the major core histones. Chromatin conformation is regulated by post-translational modifications of histones consisting of phosphorylation, methylation, acetylation, sumoylation and ubiquitylation. Altogether, these covalent modifications of histones are referred to as the "histone code" and dictate the condensation state of chromatin, establishing a distribution between loose euchromatin and tightly packaged heterochromatin (HC). This, in turn, regulates DNA accessibility and specifies transcriptional outcome, thereby tremendously expanding the information carried out by the DNA sequence only. Comprehensive reviews of histone modifications have been published recently (Bannister and Kouzarides 2011) and interested readers should therefore refer to these.

Main hallmarks of HC include trimethylation of H3 on either lysine 9 (H3K9me3) or lysine 27 (H3K27me3) and trimethylation of H4 on lysine 20 (H4K20me3). These modifications are catalyzed by, respectively, SUV39H1/2, EZH2 and SUV420H1/2 histone methyltransferases and can be actively removed by histone demethylases. In some instances, covalent modifications of histone tails act as docking sites for HC-binding proteins. Namely, H3K9me3 is an anchorage site for HP1α, HP1β and HP1γ isoforms, the main HC-associated proteins. When bound to H3K9me3, HP1 is able to, in turn, recruit SUV39H1, thereby ensuring a self-feeding loop

further contributing to heterochromatinization of the locus (Maison and Almouzni 2004). In normal human cells, H3K9me3, HP1 and H4K20me3 are typically enriched within constitutive HC at pericentromeric and telomeric regions of chromosomes. Conversely, acetylation of lysine 9 residue of histone H3 (H3K9ac) is one of the most prominent activating marks detected within euchromatic regions of chromosomes. Similarly to the removal of methyl groups, histone tails can be actively deacetylated by various specialized histone deacetylase enzymes, including the conserved Sirtuin family of proteins (North and Verdin 2004). Hence, when coordinated with the action of SUV or EZH2 histone methyltransferases, deacetylases contribute to chromatin condensation increase. Acetylation is however not the only modification associated with open chromatin as H3K4me3, catalyzed by MLL1 histone methyltransferase, represents another mark frequently detected at active gene promoters.

The sole lecture of the histone code is however not always sufficient to predict the outcome of gene transcription. Indeed, both active (H3K4me3) and repressive (H3K27me3) marks can coexist at a subset of gene promoters, namely developmentally-regulated genes (Decottignies and d'Adda di Fagagna 2011). This mechanism is believed to ensure finely tuned regulation of gene expression during differentiation, when H3K27me3 marks are lost from these so-called bivalent promoters, leaving H3K4me3 as only activating mark. Hence, histone code is not fully deciphered yet and is likely to be far more complicated than previously anticipated.

Alterations of Histone Modifications, Heterochromatin-Associated Protein Distribution and Formation of Senescence-Associated Heterochromatic Foci (SAHF)

In aging cells, two opposite types of chromatin modifications are observed, with an overall net decrease in HC accompanied by local HC gains. Evidences for aging-associated global losses of HC marks, namely reduction of H3K9me3 and HP1γ, were initially provided by the analysis of cells isolated from patients suffering from progeria syndromes, like the Hutchinson-Gilford Progeria Syndrome (HGPS) (Shumaker et al. 2006). Similar conclusions were subsequently drawn from the investigation of physiological aging (Scaffidi and Misteli 2006). It was then proposed that reduction of H3K9me3 and, subsequently, of HP1, may result from reduced levels of several components of NURD, a multisubunit complex containing histone deacetylase activity, as cells age (Pegoraro et al. 2009). Senescence is also characterized by down-regulation of EZH2, the histone methyl transferase of the PRC2 complex responsible for trimethylation of H3 on lysine 27. In concert with the removal of H3K27me3 methyl groups by JMJD3 histone demethylase, this leads to progressive reduction of trimethylated H3K27 levels in senescent cells (Cruickshanks and Adams 2011). Hence, genes normally repressed by H3K27me3, like the INK4-ARF locus (Bracken et al. 2007) or the BMP2 Bone Morphogenetic Factor 2 gene (Kaneda et al. 2011), become upregulated in senescence. These observations point towards an overall decrease of H3K9me3, HP1 and H3K27me3 during aging although, on the contrary, an overall increase in the H4K20me3 constitutive heterochromatic marker was detected in senescent cells (Tsurumi and Li 2012).

The loss of HC model of aging is, at first glance, incompatible with the formation of senescence-associated heterochromatic foci (SAHF) characterized by high levels of H3K9me3 and HP1β (Narita 2007). To account for this apparent discrepancy, Sedivy et al. (2008) proposed the "heterochromatin redistribution" mechanism based on the model established 10 years earlier by Imai and Kitano (1998) in which chromatin modifiers were proposed to be delocalized from their normal target loci to other sites as cells age. Supporting a model of HC mark redistribution are kinetic studies monitoring SAHF formation. These studies indeed suggested that SAHF form sequentially, starting first with chromosome decondensation, followed by H3K9 methylation, binding of HP1 proteins and, finally, replacement of H2A with macroH2A (Tsurumi and Li 2012). Hence, HC

loss is likely to precede SAHF formation in aging cells. Note however that, although SAHF appear to be clearly induced in cells experiencing OIS (Di Micco et al. 2011), it remains unclear whether genuine SAHF actually form in vivo in aging human tissues. It is likely however that SAHF-like structures develop in aging cells as cells positive for HC and SAHF components (HP1β, HIRA, H3K9me2/3) accumulate in the skin of aging baboons (Herbig et al. 2006). Similarly, SAHF formation in cells experiencing replicative senescence in vitro remains controversial, possibly reflecting distinct responses of fibroblast strains to culture stress. Notably, when cultured, various cell strains may differ in their propensity to induce $p16^{INK4A}$, a master regulator of SAHF formation. Nevertheless, when formed, SAHF constitute potent tumor suppressor structures in which embedded E2F target gene promoters, like MCM6 or PCNA, display enrichment in repressive H3K9me3 marks (Tsurumi and Li 2012). Remarkably, SAHF-like structures appear to be retained in various human tumors at different stages of cancer progression, despite the concomitant expression of cellular proliferation markers (Di Micco et al. 2011). In vitro data revealed that retention of oncogene-induced HC in E2F gene-expressing cells that bypassed senescence was made possible through inactivation of DNA damage response (DDR) genes like ATM or p53. The role played by these SAHF-like structures in oncogene-expressing cells is likely to be important as HC appears to restrain DDR signaling and prevent induction of apoptosis (Di Micco et al. 2011).

Although presence of SAHF (or SAHF-like) structures may be a common feature of senescent and cancer cells, other histone modifications appear to be differentially regulated in aging and cancer. Notably, although H3K27me3 levels decrease in senescent cells, EZH2 is often upregulated in cancer and, conversely, H4K20me3 is upregulated in aging and downregulated in cancer (Cruickshanks and Adams 2011). It was proposed that the counter-regulation of these marks in aging and cancer may reflect an age-associated tumor suppression barrier that is breached in cancer cells (Cruickshanks and Adams 2011).

Alterations of DNA Methylation Profiles in Senescent and Cancer Cells

DNA Methylation Profile and Maintenance in Normal Cells

DNA methylation provides a stable gene silencing mechanism and occurs on cytosine residues of CpG dinucleotide motifs, leading to the formation of ^{me}CpG. The mechanisms of DNA methylation have been extensively reviewed by others (Suzuki and Bird 2008; Law and Jacobsen 2010) and will only be briefly overviewed here.

The overall frequency of CpG in the vertebrate genome is lower than expected but there are small regions of generally hundreds of nucleotides displaying an expected, or slightly higher, CpG frequency. In normal cells, these small stretches of CpG-rich DNA, called CpG islands, are usually either unmethylated or hypomethylated when located within promoter regions. There are a few noticeable exceptions to this general scheme as a subset of promoter-associated CpG islands, notably promoters of the so-called cancer-germline genes, undergo methylation during early development and the genes are therefore silenced in normal somatic cells. Repetitive DNA sequences present in pericentromeric regions of chromosomes and in retrotransposon elements represent additional examples of CpG-rich sequences being heavily methylated in normal cells. Methylation of repetitive DNA is believed to ensure silencing of transposable elements and to prevent genomic instability as lowering ^{me}CpG content of pericentromeric DNA favors rearrangements of these chromosomal loci (Ehrlich et al. 2003).

In dividing cells, DNA methylation is transmitted to the daughter cells by DNMT1 DNA methyltransferase, an enzyme acting on hemimethylated DNA to ensure faithful transmission of methylation patterns during replication. DNMT1 is therefore referred to as the DNA methylation maintenance enzyme. On the other hand, DNMT3a and DNMT3b enzymes are referred to as the de novo DNA methyltransferase enzymes, being required for the establishment of genomic imprinting in concert with DNMT3L. Unlike

histone modifications, once established, DNA methylation is considered a stable mark. However, transient failures of the DNA methylation maintenance mechanisms happen under pathological conditions or during aging, and these are associated with waves of DNA demethylation. Conversely, local DNA hypermethylation events arise as another default of the DNA methylation maintenance mechanism, and both demethylated and hypermethylated nucleotide sequences have been reported to co-exist within the same nucleus of both senescent and cancer cells.

DNA Hypomethylation in Senescence and Cancer

In normal cells, repetitive DNA sequences account for the majority of ^{me}CpG content. Accordingly, the bulk of DNA methylation decrease observed during senescence and in most cancer cells is attributed to repetitive DNA hypomethylation (Decottignies and d'Adda di Fagagna 2011).

Global decrease of ^{me}CpG content was first described in mammalian cells experiencing in vitro replicative senescence and later reported in tissues isolated from aging individuals. Overall, senescence-associated DNA demethylation mostly occurs at pericentromeric sequences and at interspersed Alu and LINE-1 sequences. Loss of repetitive DNA methylation appears to be linked to cellular senescence as human fibroblasts undergoing hydrogen peroxide-induced premature senescence display similar profiles of hypomethylated DNA sequences. Similarly to what is observed during senescence, most cancer cells display repetitive DNA hypomethylation (Decottignies and d'Adda di Fagagna 2011). We recently showed that in vitro activation of RasV12 oncogene is able to induce demethylation of specific CpG residues within heat shock factor 1 (HSF1) binding sites of human pericentromeric Satellite 2 DNA (Tilman et al. 2012). Provided that we detected similar demethylation events within the HSF1 binding sites of Sat2 DNA in cancer cell lines displaying moderate genome-wide hypomethylation, we hypothesized that oncogene activation may contribute to tumorigenesis-associated Sat2 demethylation. In light of the well-described binding of HSF1 to other pericentromeric regions of human chromosomes under conditions activating the heat shock response, it is likely that such DNA demethylation events may similarly occur at additional pericentromeric loci (Tilman et al. 2012). Although the reduction of ^{me}CpG content observed in senescent or cancer cells is mainly attributed to demethylation of repetitive DNA, various single-copy gene promoters similarly lose methylation in aging tissues. Examples of such promoters include c-Myc, NR2B (NMDA receptor 2B), CD11a (integrin αL) and Usp9x (Decottignies and d'Adda di Fagagna 2011). The so-called cancer-germline (CG) genes represent additional well-described examples of genes frequently activated in tumor cells following demethylation of their CpG-island containing promoters. However, hypomethylation of CG gene promoters is associated with profound genome-wide hypomethylation (Decottignies and d'Adda di Fagagna 2011) that is observed neither in fibroblasts experiencing replicative senescence, nor in aging cells, nor during in vitro OIS (Tilman et al. 2012). Hence, it is possible that profound genome-wide DNA demethylation results from the passage of cells through a distinct senescent state during tumorigenesis. Although not proven yet, it appears rather unlikely that this results from in vivo OIS as, given the intricate links between repressive histone marks and DNA methylation and the proposed self-reinforcing repressive mechanisms between H3K9me3 and DNA methylation (Fuks et al. 2003), drastic heterochromatinisation of cells undergoing OIS appears to be incompatible with global genome-wide DNA demethylation. Alternatively, the drastic DNA demethylation events observed in a fraction of cancer cells – but not all – may be the consequence of distinct mechanisms that are activated independently of cellular senescence.

The mechanisms responsible for genome-wide DNA demethylation have been under intense investigation over the past few decades but it remains unclear whether these involve passive or active mechanisms, or both. Several non-specific

mechanisms were proposed to be causally involved in passive demethylation of genomic DNA (Decottignies and d'Adda di Fagagna 2011). The first one proposes that the progressive accumulation of $p21^{WAF1}$ during senescence may disrupt PCNA-DNMT1 interaction at replication forks and result in the failure to maintain methylation of the newly synthesized DNA strands as long as the intracellular $p21^{WAF1}$ levels have not reached the critical threshold signaling cell cycle exit. Alternatively, genome-wide DNA demethylation may be related to the reported rise in reactive oxygen species (ROS) concentrations in pre- and senescent cells as ROS were reported to interfere with the capacity of DNA to act as a substrate of DNMTs and to alter DNMT activity by oxidizing cysteine residues in the catalytic sites. Induction of a non-specific DNA demethylase activity in senescent cells represents another possible mechanism, although strong evidences in favor of active genome-wide DNA demethylation processes are still lacking. Additional DNA demethylation events, acting more locally and more specifically, are likely to contribute to repetitive DNA demethylation. One of them is related to alterations in the Piwi-piRNA pathway as inactivation of Mili, a key player of the piRNA pathway, results in demethylation of LINE-1 transposable elements, an event frequently detected in cancer cells (Decottignies and d'Adda di Fagagna 2011). It is however still unknown whether alterations of the piRNA pathway occur during senescence. We also recently showed that in vitro activation of the heat shock response following oncogene activation induces localized demethylation of pericentromeric Satellite 2 DNA (Tilman et al. 2012). We similarly detected heat shock response activation and localized Sat2 demethylation in fibroblasts undergoing in vitro replicative senescence, although to a lesser extent (our unpublished data). Interestingly, HSF1 acts as a powerful driver of oncogenic transformation, notably by ensuring escape from oncogene-induced senescence and we therefore hypothesized that heat shock response activation may contribute to tumorigenesis-associated demethylation of Sat2 and, possibly, of other pericentromeric repetitive DNA sequences bound by HSF1 (Tilman et al. 2012). The mechanisms underlying HSF1-dependent DNA demethylation have not been elucidated yet but may be linked to the chromatin remodeling events associated with HSF1 binding to its target sequences. Hyperactivation of HSF1 was indeed shown to induce extensive chromatin remodeling of *HSP70* gene promoter through recruitment of the SWI/SNF complex (Corey et al. 2003) and, in a distinct experimental context, local recruitment of SWI/SNF complex at $p16^{INK4a}$ promoter was associated with dissociation of DNMT3b from the promoter, followed by demethylation of the locus (Kia et al. 2008).

Hypermethylation of Gene Promoters in Senescence and Cancer

Despite an overall loss of ^{me}CpG content in aging cells, there are also a subset of CpG islands displaying gains of methylation associated with the formation of repressive chromatin domains around transcription start sites. Such hypermethylation events are also detected in cancer cells. The best-known example of gene promoter subjected to DNA hypermethylation is undoubtedly $p16^{INK4A}$. Age-related hypermethylation events of $p16^{INK4A}$ gene promoter were detected in fibroblasts and non-neoplastic gastric epithelia (Decottignies and d'Adda di Fagagna 2011). $p16^{INK4A}$ promoter hypermethylation is also clearly linked to cancer, being frequently detected in colon carcinomas, oesophageal squamous cell carcinoma, lung tumors and melanoma. At first glance, transcriptional repression of $p16^{INK4A}$ through promoter hypermethylation in a subset of aging cells may be counter-intuitive as senescent cells are generally characterized by transcriptional induction of $p16^{INK4A}$ following EZH2 down-regulation and H3K27me3 loss. How can we therefore reconcile the senescence-associated up-regulation of $p16^{INK4A}$ with the observed acquisition of repressive DNA methylation at the promoter level? In $p16^{INK4A}$-expressing cells, the INK4A-ARF locus is flanked by chromatin boundary domains enriched in the histone variant H2A.Z (Cruickshanks and Adams 2011). The CTCF insulator protein participates in chromatin boundary

function to prevent spreading of the nearby HC characterized by high H3K9me3 content (Witcher and Emerson 2009). For reasons that are still unclear, these boundaries may become leaky in aging cells, resulting in the spreading of H3K9me3 marks into the INK4A-ARF locus. According to the previously reported connection between enzymes responsible for DNA methylation on one hand and for histone methylation on the other hand (Fuks et al. 2003), increased H3K9me3 density may then recruit DNMT1 and/or DNMT3A to the INK4A-ARF locus to induce DNA hypermethylation. Hypermethylation events at the INK4A-ARF locus are likely to be positively selected, leading to the emergence of $p16^{INK4A}$-silenced cells. In this context, it is interesting to mention that, although $p16^{INK4A}$ is strictly required for SAHF formation, it is not essential for the maintenance of these specialized HC structures (Narita 2007). Hence, inactivating $p16^{INK4A}$ through promoter hypermethylation is not incompatible with the reported maintenance of SAHF in tumors (Di Micco et al. 2011).

Importantly, several other gene promoters were found to be hypermethylated in cancer cells and gene promoter hypermethylation is currently considered an important and early step of the tumorigenesis process (Baylin and Ohm 2006). Recent evidences provided by genome-wide studies suggested that aging-associated DNA hypermethylation may occur in a non-random fashion within specific chromatin contexts (Rakyan et al. 2010; Teschendorff et al. 2010). The authors of these two studies indeed discovered that aberrant promoter hypermethylation in aging cells occurs predominantly within the so-called bivalent chromatin domain promoters. Interestingly, the same class of gene promoters is also subjected to hypermethylation in tumors. Bivalent promoters are characterized by the simultaneous presence of repressive H3K27me3 mark and of activating H3K4me3 mark and are associated with developmentally-regulated genes. These genes are silent, but "transcription-ready", in embryonic stem cells. Upon differentiation, H3K27me3 is lost from promoters and additional H3K4me3 marks are acquired. As aberrant hypermethylation of bivalent promoters is associated with reduced differentiation abilities and increased proliferation potential in vitro, hypermethylation may contribute to cancer development by providing "stemness" properties to aging cells (Decottignies and d'Adda di Fagagna 2011). The mechanisms underlying the increased vulnerability of bivalent promoters to hypermethylation are however still elusive. Because of the reportedly down-regulation of EZH2 in aging cells, susceptibility to hypermethylation is unlikely to result from increased H3K27me3 density at bivalent promoters. Alternatively, in line with the recent observation that DNA methylation and H3K27me3 marks are mutually exclusive at the promoter of mouse imprinted Rasgrf1 gene, decreased H3K27me3 density may possibly favor DNA methylation (Lindroth et al. 2008). Although in apparent contradiction with the demonstration that the EZH2 polycomb group protein that catalyzes H3K27me3 formation appears to directly control DNA methylation (Viré et al. 2006), this hypothesis is supported by the recent observation that H3K9me3 marks compensate for H3K27me3 loss at bivalent promoters of hematopoietic stem cells (Weishaupt et al. 2010). If this chromatin modification switch (from H3K27me3 to H3K9me3) is generalized to other bivalent gene promoters, it is possible that, as previously suggested (Fuks et al. 2003), acquisition of H3K9me3 may result in recruitment of DNMT1 and/or DNMT3A to the loci. Acquisition of localized DNA hypermethylation at gene promoters may also result from oncogene activation as supported by a recent report establishing that Ras oncogene is able to induce epigenetic silencing of the pro-apoptotic Fas gene through activation of an elaborate pathway involving recruitment of DNMT1 to the CpG-rich promoter (Gazin et al. 2007).

Conclusion

Whether in the context of organismal aging or in response to oncogene activation, evidences are accumulating in favor of cellular senescence being a general step on the way to cancer. Senescence is associated with a panel of chromatin

Table 19.1 Epigenetic alterations in senescence and cancer

	Senescence	Cancer	Senescence-associated epigenetic alterations as oncogenic drivers?
Global H3K27me3	↓	↑	NO: $p16^{INK4A}$ activation
Global H4K20me3	↑	↓	?
H3K9me3/HP1 enrichment in SAHF (or SAHF-like) structures	YES	YES	NO: repression of E2F target genes YES: barrier against DDR activation
Repetitive DNA hypomethylation	YES	YES	YES: genomic instability
Promoter-associated CpG island hypermethylation	YES	YES	YES: tumor suppressor gene silencing, possible acquisition of "stemness" properties through bivalent gene promoter hypermethylation

Comparison between main alterations in the profiles of histone modifications and DNA methylation in senescent and cancer cells. The possible impacts of senescence-associated epigenetic alterations on tumor progression are indicated in the last column

alterations that, in some circumstances, like in OIS, can be very drastic. Although initially intended to block cellular proliferation, these senescence-associated chromatin alterations can be beneficial to cancer cells that managed to exit from cell cycle arrest by inactivating cellular checkpoints. These mostly include SAHF-like structures reported to protect cancer cells from DDR activation, hypomethylation of repetitive DNA, a possible driver of genomic instability, and hypermethylation of tumor suppressor genes (Table 19.1). Hence, it is now generally believed that epigenetic alterations associated with cellular senescence contribute to tumorigenesis and probably underlie the increased risk of cancer in aging individuals. Conversely, tumor suppression barriers imposed by some other senescence-associated epigenetic alterations, including EZH2 down-regulation, are usually breached in cancer.

In summary, as far as chromatin is concerned, cancer cells confirm their extraordinary ability to keep advantageous alterations while circumventing the tumor suppressive ones, in an amazing pro-survival mode of living. In the future, it would be interesting, using genome-wide approaches, to go deeper into the characterization of epigenetic alteration profiles in senescent and cancer cells. This may help finding ways to prevent cancer progression during aging and to target histone modifications in cancer therapy. In this regard, Di Micco et al. (2011) showed that, in vivo, histone deacetylase inhibitors induce chromatin relaxation, DDR activation, apoptosis and tumor regression.

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20 Chemotherapy- and Radiation-Induced Accelerated Senescence: Implications for Treatment Response, Tumor Progression and Cancer Survivorship

Molly L. Bristol and Lynne W. Elmore

Contents

Abstract

Standard chemotherapy and radiotherapy are designed to eradicate malignancies by depriving tumor cells of their reproductive potential. Traditionally, it has been assumed that the loss of proliferative potential by cancer cells predominantly involves the triggering of cell death via apoptosis. However, it is becoming clear that cancer cells derived from solid tumors often undergo rapid and widespread induction of senescence following exposure to DNA damaging therapeutic agents. Tumor cells undergoing this accelerated senescence response share several cellular and molecular features with replicatively aged cells, including activation of DNA damage response pathways and a similar senescence-associated secretory pattern. Here, we discuss accelerated senescence in response to chemotherapy and radiation focusing on the potential implications for treatment response, tumor progression, and cancer survivorship. One emerging theme is that the persistence of metabolically active, senescent cells plays an active and diverse role in shaping the tumor microenvironment. There is hope that a better understanding of the molecular mechanisms (1) initiating and maintaining senescence in cancer cells and (2) underlying the senescence-associated bystander effects in tumor and non-tumor cells will lead to the future development of more efficacious and less toxic cancer treatments.

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Keywords
Senescence • Telomeres • DNA damage • Treatment response • Cancer survivorship

Introduction

It was first proposed by Hayflick and Moorhead (Hayflick and Moorhead 1961) that somatic cells have a limited replicative capacity. "The Hayflick Limit" refers to the finite number of mitoses a normal cell is capable of undergoing in tissue culture before it stops dividing. Due to enzymatic limitations during semi-conservative replication of DNA, telomeres gradually shorten in somatic cells because they lack a telomere maintenance mechanism. The cellular consequence is replicative senescence. In contrast, the vast majority of cancer cells during the early stages of malignant transformation activate telomerase, a ribonuclear protein that maintains telomere length. Consequently, cancer cells, despite having relatively short telomeres, have an infinite lifespan (thus, commonly described as 'immortal'). Although, this certainly does not mean that cancer cells are refractory to aging. In fact, as presented here, rapid and widespread induction of senescence is a frequent fate of cancer cells lines derived from solid tumors following exposure to chemotherapeutic agents or irradiation, with evidence mounting to implicate accelerated senescence as a mediator of therapeutic response. In this chapter, we also consider recent data suggesting that DNA damaging modalities used in the clinic trigger accelerated senescence in normal cells, leading us to postulate that such off-target, rapid cellular aging may contribute to the myriad of chronic conditions manifesting during cancer survivorship (Rowland 2008).

Triggers of Accelerated Cellular Senescence

Telomere attrition, due to the "end replication problem", has been investigated in numerous somatic cell types and strongly implicated as an internal biological clock limiting proliferative potential of normal cells. Once telomeres reach a critically short length, the protective telomeric cap structure is compromised. When the ends of linear chromosomes are exposed, a cell appears to 'sense' this damage leading to the activation of DNA damage response (DDR) pathways. Induction of replicative senescence is mediated in part through ataxia telangiectasia mutated (ATM) kinase, with subsequent activation of p53 as well as multiple downstream effectors of p53, most notably $p21^{WAF1}$ (Li et al. 2003). Typically, induction of accelerated senescence is also associated with activation of DDR pathways, however, the onset is more rapid than replicative senescence and the inciting stimuli are more diverse (Fig. 20.1). When growth promoting genes (such as c-myc, Ha-ras, STAT5A) are overexpressed or activated in normal cells, they are perceived as inappropriate proliferative signals resulting in senescence or apoptosis (Hemann and Narita 2007). It is generally accepted that when a cell senses such mixed cellular signals, replication origins are misfired, contributing to DNA damage in addition to causing perturbations in other cellular processes. Recent data have been generated to indicate that treatment of cells with a histone deacetylase inhibitor, which favors chromatin adopting a more relaxed, euchromatic state, activates the DNA damage response pathways prior to induction of senescence with no evidence of DNA damage. But most triggers of accelerated senescence, whether in normal or cancerous cells, involve *bona fide* DNA damage activating DDR pathways. This damage can result from a number of diverse triggers including chemotherapeutics agents, irradiation, reactive oxygen species (ROS), and/or telomere dysfunction. While Fig. 20.1 depicts a simplistic schematic, several triggers of accelerated senescence have overlapping cellular targets with subsequent activation of common signaling pathways. Telomeres are one such target that plays a central role in inducing a rapid senescence response. Since telomeric sequences are guanosine-rich, they are particularly susceptible to ROS, which are generated in cells as byproducts of normal metabolic activities as

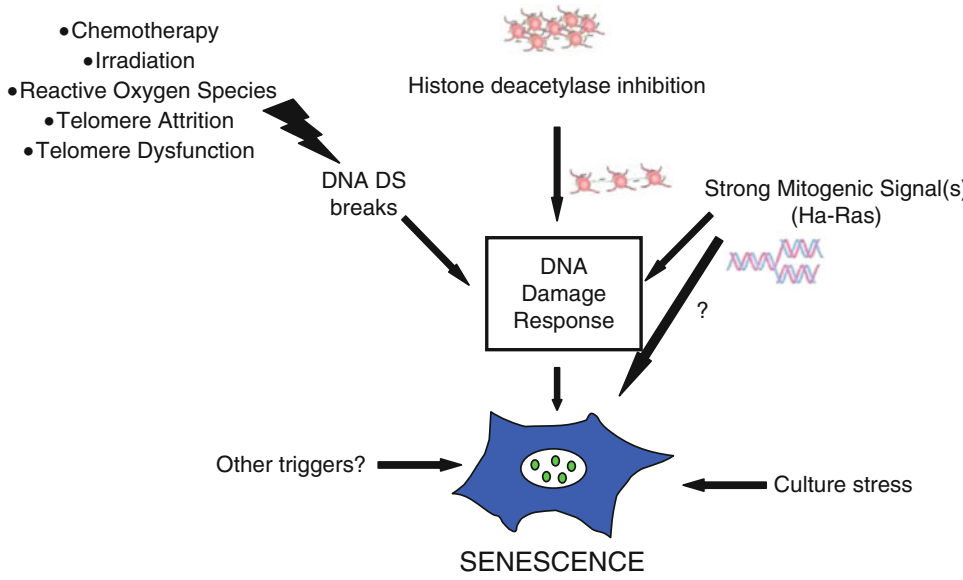

Fig. 20.1 Illustration of the numerous triggers of accelerated cellular senescence. Accelerated senescence can be triggered in cells by a number of stimuli, most of which involve activation of DNA damage response pathways. Strong mitogenic signals, such as expression of the Ha-ras oncogene, can cause DNA damage by triggering errors in the firing of replication forks. Inhibition of histone deacetylase activity causes relaxation of chromatin resulting in the activation of DNA damage pathways without DNA damage per se. However, the majority of triggers of accelerated senescence, including exposure mainstay chemotherapeutic agents and irradiation, activate DNA damage response pathways prior to induction of senescence

well as following irradiation and exposure to many DNA damaging, chemotherapeutic agents. Accelerated senescence caused by sub-optimal standard tissue culture conditions (for example extrinsic factors relating to incubator oxygen levels, oxygen tension, media formulations and attachment surfaces) is the apparent outlier with a scarcity of data implicating DNA damage and/or activation of DDR pathways. While the nature of this tissue culture-associated, stress-induced senescence is poorly understood, upregulation of p16 and the subsequent activation of p16-retinoblastoma (pRB) pathways are hallmarks (Campisi and d'Adda di Fagagna 2007).

Mechanisms of Accelerated Cellular Senescence in Tumor Cells

Shortly after the discovery of telomerase and the cloning of its catalytic component, hTERT, compelling experimental data were generated to implicate gradual telomere shortening as a primary mechanism for replicative senescence. Specifically, the ability to prevent cellular aging in human somatic cells by reconstituting telomerase activity via ectopic expression of hTERT provided important proof-of-principle for the telomere hypothesis of aging (Bodnar et al. 1998). A similar strategy has been used to directly test whether cancer cells undergoing accelerated senescence in response to DNA damaging treatments is also a telomere-length dependent phenomenon. In the vast majority of cancer cells, telomeres are maintained at a relatively short length despite high levels of telomerase activity. Since cancer cells acutely exposed to Adriamycin, a mainstay chemotherapeutic agent in the treatment of breast cancer, exhibit a precipitous decline in telomerase activity within the first week after drug treatment, it has been hypothesized that telomeres would quickly reach a critically short length triggering widespread, accelerated senescence. This, however, proved

not to be the case; breast cancer cells with artificially elongated telomeres due to ectopic telomerase expression still senesced at the same frequency and kinetic as empty vector controls and parental cells (Elmore et al. 2002). Cytogenetic analyzes revealed that within 1 day of Adriamycin or irradiation treatment, breast cancer cells exhibit telomere dysfunction as defined by a disproportionate frequency of chromosome end-associated cytogenetic aberrations (i.e., rings, radials and end-to-end fusions) in addition to evidence of telomeric and sub-telomeric breaks (Elmore et al. 2002; Jones et al. 2005). Collectively these data make a strong case for accelerated senescence, that develops in cancer cells in response to DNA damaging modalities, is mechanistically distinct from replicative aging in that it is not a telomere length-dependent process per se but instead triggered by telomere dysfunction. In some cases, this dysfunction may result from telomere shortening due to a sudden break within the telomere or proximal in the subtelomeric region, as experimental data suggest (Elmore et al. 2002; Jones et al. 2005; Yoon et al. 1998). But, clearly, this senescence-triggering event is unique from gradual telomere attrition.

Another theme that has emerged relating to the mechanistic basis of accelerated senescence is the central role of the p53 tumor suppressor gene. As mentioned above, cancer cell lines derived from solid tumors are prone to undergo accelerated senescence in response to standard chemotherapeutic agents, but typically only in the context of functional p53 (Gewirtz et al. 2008; Roninson 2003). In response to make DNA damage, p53 is activated and induces transcription of $p21^{WAF1}$, an inhibitor of cyclin dependant kinases (CDK). $p21^{WAF1}$ activation, with subsequent dephosphorylation and activation of pRb, allows pRb to bind to the E2F family of transcription factors. Activated pRb then sequesters and inactivates the E2F activity that is necessary for cell proliferation, thus initiating G_1 cell cycle arrest. However, this induction of $p21^{WAF1}$ is only transient, with levels declining after growth arrest is established. In some experimental systems, as $p21^{WAF1}$ levels decrease, another CDK-inhibitor, p16, becomes constitutively active, suggesting that p16 may be responsible for the maintenance of senescent growth arrest (Roninson 2003); the basis of this mechanism has yet to be fully understood. There are multiple examples in established cancer cell lines where p16 is epigenetically silenced, yet these cells can readily undergo accelerated senescence in response to DNA damaging therapeutic agents (Gewirtz et al. 2008), revealing that p16 is not always essential. Another fundamental, but still partially understood mediator of accelerated senescence is cdc2, which is inactivated via inhibition of cdc25C by Chk1/Chk2 or by p53 acting through 14-3-3. While a more complete understanding of critical downstream targets of p53 is still needed, there is overwhelming data generated from in vitro and in vivo experimental models indicating that when p53 function is compromised (whether due to a natural occurring point mutation or experimental manipulation), cells no longer senesce. Instead they undergo delayed apoptosis and/or another cellular fate that compromises reproductive capacity, such as autophagy or mitotic catastrophe (Gewirtz et al. 2008; Portugal et al. 2010).

Cellular and Molecular Features of Senescence

Senescent cells, whether arising gradually due to replicative age or with rapid onset following an extrinsic stressor, can be identified by their enlarged and flattened appearance, positive β-galactosidase activity, senescence-associated heterochromatic foci (SAHF), and their inability to enter the cell cycle in response to a mitogen (Gewirtz et al. 2008). This is in stark contrast to a quiescent cell, which typically maintains a normal appearing morphology in monolayer culture, displays unappreciable β-galactosidase activity, and readily resumes cell cycling following mitogenic stimulation. As the signaling pathways mechanistically linked to induction of senescence has been rather than are better understood, the number of distinguishing features (and potential markers) of senescence has greatly expanded. This has best been illustrated in the areas of chromatin remodeling, DDR, and the secretory profiles of senescent cells.

In senescent cells, proliferation-promoting genes are incorporated into transcriptionally silent heterochromatin (i.e., SAHF), leading to their stable repression. A number of proteins have been identified that facilitate and/or maintain SAHF (i.e., High-Mobility Group A [HMGA] proteins, macroH2A and HP1), many of which are distinct from chromatin-associated proteins in quiescent or terminally differentiated cells (Narita 2007). Promyelocytic leukemia (PML) nuclear bodies are 0.2–1.0 μM diameter structures that contain the PML protein and numerous other proteins that have been implicated in the induction of senescence. Two such proteins, HIRA (a histone chaperone) and HP1, transiently localize to PML bodies prior to associating with SAHF (Narita 2007), suggesting a dynamic interplay between PML bodies and the formation of heterochromatin, potentially resulting in the induction and/or maintenance of senescence. This is an active area of investigation that, to date, has predominantly focused on the induction of senescence in normal cells with their cell cycle check points and DDR pathways intact.

Senescence is also associated with persistent nuclear foci that contain DDR-related proteins. If DNA damage foci are used as a marker of senescence, care must be taken to distinguish persistent senescence-associated DNA damage foci from those that are transient due to the repair of damaged DNA. For experimental systems, a kinetic analysis of transient versus persistent DNA damage foci can be conducted with relative ease in cells and tissues using immunolabeling approaches in conjunction with antibodies that specifically represent key DDR proteins (such as 53BP1 or phosphorylated γ-H2AX). However, this experimental design is usually impractical when assessing senescence in clinical specimens where subsequent tissue sampling often presents challenges. Incorporating dual immunolabeling of a stable telomere binding protein (i.e., telomere repeat factor 1; TRF1) or fluorescent in situ hybridization (FISH) using a pan-telomeric probe permits assessment of the percentage of damage sites that localize at telomeres. But both telomeric and non-telomeric DNA damage can be determinants of cellular senescence and both have also been reported in transient DNA damage foci. However, recent data indicate that persistent DNA damage foci may represent distinct nuclear structures, termed 'DNA segments with chromatin alterations reinforcing senescence' (DNA-SCARS) by Campisi and colleagues (Rodier et al. 2011). Unlike transient foci, DNA-SCARS lack the DNA repair proteins, RAD51 and RPA, associate with PML bodies, lack single-strand DNA and DNA synthesis, and accumulate activated forms of p53 and CHK2. It is important to emphasize that DNA-SCARS associate with, but are not exclusive to, senescent cells. This is a continuing theme for many features of senescence (i.e., non-telomeric and telomeric DNA damage foci, accumulation of p53, upregulation of $p21^{WAF1}$, absence of proliferation markers such as PCNA or Ki-67, etc.). Hence, it remains essential to assay numerous markers of senescence in order to make a compelling case that this is indeed the cellular outcome.

The cellular aging features that may have the most far-reaching implications is that senescent cells persist for long periods in a metabolically active state. Senescent cells are typically resistance to numerous apoptotic stimuli, which may contribute to their stability in vitro and tendency to accumulate with age in vivo. They also exhibit an altered pattern of gene expression, most notably upregulation of a wide array of soluble factors. The term 'senescence-associated secretory phenotype' (SASP) (Campisi and d'Adda di Fagagna 2007) has been coined to describe this unique profile so characteristic of senescent cells, whether triggered by "The Hayflick Limit" or an external stressor. The term "senescence messaging secretome" has also been used, but less frequently. SASP is characterized by the expression of numerous extracellular matrix-degrading proteases, growth and angiogenic factors, and pro-inflammatory cytokines. As described below, the persistence of senescent cells with this distinctive secretory profile appears to affect the behavior of neighboring cells within the tumor microenvironment and perhaps cells in more distal tissues as well.

Accelerated Senescence in Cancer Cells: Implications for Tumor Progression and Treatment Response

As we gain a better understanding of the molecular pathways mechanistically linked to the induction of accelerated senescence and critical mediators that can alter cell fate, it is natural to ask, "Is induction of accelerated senescence in cancer cells a desirable outcome?" Intuitively, one may think that killing a cancer cells is far better than having it persist, especially in light of experimental data that suggests senescence cells may have the potential to resume cell cycling (Wang et al. 2004). If a therapeutic regimen could selectively kill all malignant cells without causing significant damage to normal cells, this is the ideal scenario for the host. However, tumors are highly heterogeneous in many respects, including in their response to treatments. Subpopulations of tumor cells (most notably cancer stem cells) exist that are refractory to therapies, providing potential for disease recurrence. Mechanisms contributing to a 'partial response', 'stable disease' or 'progressive disease' are multiple, but include cancer cells evading apoptosis and/or senescence (Elmore et al. 2005). Recognizing that invasive cancer cases described by clinicians as 'complete responders' likely still harbor residual cancer cells, having persistent senescence cells-if they impart an inhibitory effect on cancer growth, invasion, angiogenesis and/or metastasis- could keep these refractory cancers cells at bay perhaps without the need to subject an individual to further cancer treatment.

Experimental data have been generated, which are consistent with the possibility that senescent cancer cells may help stave off disease recurrence. As illustrated in Fig. 20.2, acute Adriamycin exposure has a direct senescence-inducing effect in MCF-7 human breast cancer cells, which is telomere-length independent and associated with the formation of numerous DNA damage foci, transient accumulation of p53, stable upregulation of $p21^{WAF1}$, and telomere dysfunction. In response to a single, clinically relevant dose of Adriamycin, senescent MCF-7 cells secrete a soluble factor(s) that is sufficient to trigger senescence in naïve cancer cells (Di et al. 2008). Hence the term "senescence bystander effect" has been used to describe this novel finding in order to distinguish it from the more frequently documented irradiation-induced bystander effects involving DNA damaging and/or death signals being transferred to neighboring cancer cells. A recent study (Elzi et al. 2012) implicates insulin-like growth factor binding protein 3 (IGFBP3) as a secreted mediator of this breast cancer senescence bystander effect, which acts through suppression of AKT and is dependent on both p53 and pRB proteins. Topoisomerase inhibitors, including Adriamycin, are commonly used anti-cancer drugs that rely on their ability to induce DNA damage in cancer cells.

Often 'stable disease' is the response of breast cancers after chemotherapeutic treatments, which is interpreted by many as treatment failure (Bartsch et al. 2006). The biological basis for a partial response rather than tumor regression may be due to the prominent senescence response of breast cancer cells following exposure to DNA-damaging cytotoxins, which has been confirmed in breast cancer clinical specimens (te Poele et al. 2002). Even if not all breast tumor cells are rendered senescent as a result of chemotherapeutic treatment, such cells may provide a reservoir of senescence-inducing factors. Future studies are now necessary to identify additional mediators of this senescence amplifying effect as well as extend investigation beyond in vitro analyses recognizing that there are many cell types and soluble mediators within the tumor microenvironment that could collectively influence the response of cancer cells initially refractory to a chemotherapeutic agent(s).

In vivo data generated from murine models suggest that induction of senescence is associated with a favorable therapeutic response, thus, further supporting the concept that induction of accelerated senescence in cancer cells can be a desirable cellular fate. Specifically, studies using the Eμ-myc transgenic mouse prone to Burkitt's-like B-cell lymphomas, in which apoptosis has been compromised via bcl-2 over-expression, have demonstrated that these lymphomas retain the ability to undergo senescence in response to cyclophosphamide with a complete cessation of

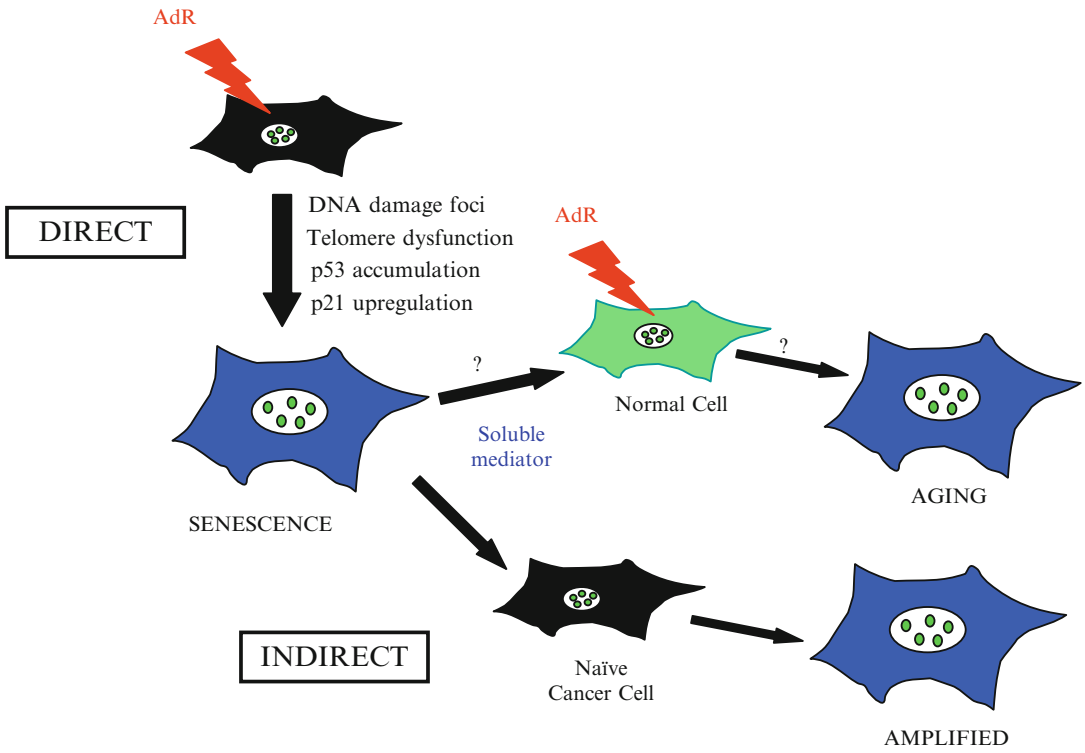

Fig. 20.2 Cascade of hypothesized biological changes leading to induction of accelerated senescence in breast cancer cells in response to Adriamycin. Acute (AdR) exposure of breast cancer cells (MCF-7; wild-type p53) causes DNA damage, telomere dysfunction, transient stabilization of p53 protein, and stable upregulation of $p21^{WAF1}$ (a downstream effector of p53), culminating in widespread senescence (Elmore et al. 2002). Induction of senescence is also achieved in naïve breast cancer cells via an indirect mechanism involving soluble mediator(s) derived from senescent cancer cells (Di et al. 2008). We hypothesize that DNA damaging therapeutic agents (including Adr) also cause cellular AdR aging in normal cells via direct and indirect/bystander effects. Adapted from a figure published by Di et al. (2008)

cellular proliferation (Schmitt et al. 2002). Mice bearing senescent lymphomas survived for an extended period of time despite a large tumor burden, suggesting that the capacity of a senescence response may impede tumor progression. Reversible RNAi was employed to control p53 expression in a chimeric liver cancer mouse model to definitively show that brief reactivation of p53 in p53-deficient tumors can produce complete tumor regression due predominantly to induction of senescence (Xue et al. 2007). In complementary studies, restoration of endogenous p53 expression led to tumor regression in sarcomas through the promotion of senescence (Ventura et al. 2007). Additionally, using two distinct murine models, it was shown that cellular senescence can limit tumor growth and may contribute to improved long-term survival of the host (Ventura et al. 2007; Xue et al. 2007). The studies by Xue et al. (2007), clearly implicate senescent cells and the innate immune system [most notable natural killer (NK) cells] acting in tandem to limit tumor growth. Additional evidence supporting the role of NK cells in the control and clearance of senescent cells has been found in the progression of multiple myeloma (Soriani et al. 2009).

Collectively, these experimental data make a compelling case that induction of accelerated senescence is a frequent response to chemotherapy and that this cellular fate is associated with tumor regression and improved survival. But, it is important to point out that the vast majority of cancers developing in humans are derived from epithelial cells (carcinomas), while the experimental

data implicating induction of accelerated senescence in cancer cells as a beneficial response have largely relied on in vivo tumor models of mesenchymal origin (i.e., lymphomas and sarcomas). However, as described below, one recent study hints that therapeutic response may be hindered in malignant epithelial cells that have undergone accelerated senescence, or at least this is the case for a mouse mammary tumor virus (MMTV)-driven transgenic model (Jackson et al. 2012).

Induction of senescence also appears to be a desirable outcome in the case of pre-malignant cells. It is generally accepted that senescence is a tumor suppressive mechanism, which removes damaged cells from the proliferative pool (Campisi 2011). Support for this concept comes from the presence of senescent cells in a number of pre-malignant tissues (including common nevi, benign prostatic hyperplasia, and colonic polyps), but not their malignant counterparts. The fact that cancer cells during malignant transformation adopt mechanisms to evade the senescence barrier is also consistent with senescence representing an intrinsic tumor suppressor pathway. Recent epidemiologic data have lended further credence to this concept. Specifically, individuals with type 2 diabetes receiving metformin demonstrate a lower risk and improved outcomes for most common cancers (Libby et al. 2009). Interestingly, metformin activates a DDR pathway resulting in lowering the threshold of stress-induced senescence. As a consequence, cells (including stem-like cells) are more prone to undergo senescence in response to the DNA damaging agent, Adriamycin (Cufi et al. 2012). Clearly, eliminating the risk of neoplastic transformation is beneficial to an organism, and senescence appears to play a critical role in protecting against cancer. Further investigations are now needed to better understand how to manipulate the threshold for accelerated senescence in order to encourage pre-malignant cells to senesce earlier in response to external stress stimuli, as this could offer exciting opportunities in the area of chemoprevention.

Despite data supporting induction of senescence in pre-malignant and malignant cells as a beneficial cellular fate, one cannot ignore the fact that senescent cells secrete: (1) proteases (multiple MMPs) that have been implicated in cancer cell invasion; (2) growth factors that could stimulate proliferation of cancer cells; (3) angiogenic factors (i.e., VEGF and bFGF) that could facilitate tumor vascularity; and (4) cytokines (IL-1α/β, IL-6, IL-8, MCP-2, MIP-1α, nitric oxide), that could recruit inflammatory cells to the tumor microenvironment, which could then further stimulate tumor cell growth, survival, angiogenesis, etc. (Salminen et al. 2012). Of note, the SASP is highly reminiscent of the expression profile of carcinoma-associated fibroblasts, which have been strongly implicated in cancer progression (Franco et al. 2010). Consistent with the notion that induction of accelerated senescence may be associated with a detrimental outcome, Jackson et al. (2012) recently report that p53-mediated senescence impairs the apoptotic response to chemotherapy and clinical outcome in breast cancer. This investigation involved breeding MMTV-Wnt-1 transgenic mice with p53 +/+, a dominant negative mutant of p53, or p53−/− in order to generated mammary carcinomas with different p53 backgrounds. When tumors developed, animals were treated with Adriamycin to compare treatment response and biologic fate of the malignant cells. As documented previously, a senescence-like phenotype was observed in p53 wild-type tumors, whereas tumors null or mutant for p53 instead exhibited aberrant mitosis, apoptosis, and a superior clinical response. In contrast, breast cancers with wild-type p53 generally underwent minimal tumor regression, stabilized for a few days, and then quickly relapsed. Moreover, senescent breast cancer cells in this transgenic model secreted multiple cytokines exhibiting autocrine/paracrine activity and mitogenic potential.

Accelerated Senescence in Normal Cells: Implication for Adverse Outcomes During Cancer Survivorship

DNA damaging chemotherapies and irradiation target rapidly diving cells, with no apparent discernment between those that are cancerous and

normal. The fast proliferating normal cells most readily affected are blood cells forming in bone marrow, and cells of the reproductive tract, digestive tract, and hair follicles. Consequently, common treatment-related side effects include nausea, diarrhea, anemia, and hair loss, which are all typically short-lived. Data are accumulating to indicate that cancer survivors have long-term challenges that extend beyond the risk of disease recurrence. Aging of the United States population has resulted in an increase in the number of cancer diagnoses. This, together with earlier cancer detection and treatment, is resulting in the number of cancer survivors steadily increasing. A trend is emerging in adult survivors, including young adults who are survivors of a childhood cancer: these individuals are at greater risk of developing a second cancer, cardiac and vascular abnormalities, infertility, endocrinopathies, and other chronic conditions (Diller et al. 2009; Oeffinger et al. 2006).

As described above, cultures of normal human cells undergo widespread accelerated senescence following exposure to DNA damaging chemotherapeutic agents or irradiation. In fact, normal cells tend to undergo accelerated senescence with more consistently than cancer cells lines since tumor-derived cells often harbor p53 mutations making them refractory to senescence while more susceptible to apoptosis (Li et al. 2012). Moreover, genotoxic stress, induced by replicative aging or ionizing radiation, has been shown to induce SASP in normal human fibroblasts and epithelial cells (Salminen et al. 2012). This begs the question: Does the induction of treatment-associated accelerated senescence in normal cells contribute to the more rapid onset of degenerative/chronic conditions in cancer survivors? While an attractive working model, there is a paucity of data relating to accelerated senescence in normal cells of individuals with cancer following DNA damaging therapies. There is evidence to suggest that telomere lengths are shorter in peripheral blood lymphocytes of individuals with cancer following chemotherapy (Lee et al. 2003; Schroder et al. 2001). One could hypothesize this telomere shortening may be due to breakage as a direct consequence of the therapy, indirectly from the generation of treatment-related ROS, and/or the stress of cancer (i.e., cancer diagnosis, treatment, life style changes, etc.). Certainly there is a wealth of data associated with the acquisition of chronic diseases in adults with accelerated telomere shortening (Epel 2009; von Figura et al. 2009). But detectable telomere shortening is not synonymous with senescence and, in the case of accelerated senescence, the trigger can be telomere dysfunction without measurable changes in telomere length. Certainly, this is an area worthy of further study.

Aging is associated with bone and fat loss as well as reduced collagen production and elasticity of the skin. Therefore, studying the effects of chemotherapy and irradiation on the biology of mesenchymal stem cells is of particular interest. From a practical perspective, studies involving the use of adipose-derived MSCs are desirable because these cells are relatively easy to obtain in large numbers and to propagate in vitro (Francis et al. 2010). Studies utilizing breast adipose-derived MSCs offers the added advantage of studying stromal cells derived from the breast tumor microenvironment. We have recently demonstrated that a single clinically relevant dose of Adriamycin causes widespread senescence (β-galactosidase positivity, enlarged and flattened morphology, and the presence of 53BP1 DNA damage foci) in breast adipose-derived MSCs (unpublished data). These preliminary data are consistent with a recent study reporting bone marrow-derived MSCs exhibiting telomere shortening and activation of a DDR pathway in response to sublethal doses of Adriamycin or etoposide (Buttiglieri et al. 2011) Moreover, this chemotherapy-induced telomere shortening was associated with reduced clonogenic survival and accelerated adipogenic differentiation, which is also the differentiation pattern for replicatively aged MSCs (Buttiglieri et al. 2011).

In order to begin to test whether senescent breast cancer cells may impart a bystander effect on resident MSC, we have exposed naïve breast adipose MSCs for three consecutive days with conditioned media from senescent MCF-7 breast cancer cells, as previously described for treatment of naïve breast and colon cancer cells

(Di et al. 2008; Elzi et al. 2012). Our findings preliminarily support senescent breast cancer cells activating a DDR pathway and triggering senescence in resident MSCs via a paracrine mechanism (unpublished data). There is a need to better understand molecular mechanisms (both genetic and epigenetic) underlying the accelerated development of treatment-related aging in cancer survivors. Advances in this area of cancer survivorship research will require the use of in vitro and in vivo experimental models as well as patient-based studies with integration of a longitudinal design and the incorporation of multiple markers of senescence.

Conclusion

The senescence field has come a long way since the serendipitous finding that lysosomal β-galactosidase activity at pH 6.0 identifies cells that are permanently growth arrested (Dimri et al. 1995). Concerns that senescence merely represents a tissue culture artifact are rarely voiced. Instead, basic and translational researchers are now asking "Can we manipulate a treatment-related senescence response in order to ultimately maximize therapeutic response while minimizing risks of recurrence and adverse outcomes during cancer survivorship?" There are now a number of functionally-associated markers that, when assayed concurrently, allows one to identify senescent cells in vitro and in vivo with confidence. Researchers now have a better appreciation for the dynamic changes in chromatin structure that accompany induction of senescence and the complex nature of the SASP. It is also becoming clear that the biological effects of the myriad factors comprising the senescent secretory profile will influence and be influenced by the microenvironment. Adding to the complexity, a tumor microenvironment will likely be in a state of fluctuation as additional stromal cells (i.e., inflammatory and endothelial cells, MSCs, and fibroblasts) are recruited in response to the production of cytokines and chemokines. While murine models have been instrumental in validating accelerated senescence as a frequent cellular fate of cancer cells following exposure to chemotherapeutic agents and assessing the influences of a particular cells fate on treatment response, it is necessary to now conduct longitudinal, patient-based studies. This is particular important to begin to understand the cellular and molecular basis for the accelerated development of chronic conditions in cancer survivors. We feel strong consideration should be given to investigations relying on adipose-derived MSCs (rather than or in addition to the more standard sampling of peripheral blood leukocytes) as treatment-associated senescence of these adult stem cells could be the root of several degenerative conditions manifesting during survivorship.

Acknowledgements This work was supported by a National Institutes of Health KO1 CA105050-01A131, a Department of Defense BCRP Concept Award (BC085416), and the Department of Pathology at Virginia Commonwealth University (to LWE). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH or DOD. We regret that we were unable to cite all relevant studies in the literature. Due to the fact that, in several circumstances, no appropriate reviews were available, it was necessary to select one or two primary papers while other equally relevant papers were omitted.

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21 Suppression of Cellular Senescence in Glioblastoma: Role of Src Homology Domain-Containing Phosphatase 2

Lisa-Marie Sturla, Pascal Zinn, and Ekkehard Kasper

Contents

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Abstract

Resisting cellular senescence and becoming immortal is a pre-requisite step in the tumorigenic transformation of a cell. In this chapter we describe a novel mechanism by which inhibition of a key phosphatase can induce cellular senescence in the aggressive brain tumor, Glioblastoma Multiforme (GBM). We also provide an overview of recently published data suggesting additional mechanisms of senescence observed in this tumor type. Epidermal Growth Factor Receptor (EGFR) signaling is frequently altered during glioblastoma pathogenesis. An important downstream modulator of this signal cascade is Src Homology domain-containing Phosphatase 2 (SHP2/PTPN11). The Cancer Genome Atlas (TCGA) data demonstrates SHP2 to be mutated in 2% of the GBM's studied. Both mutations identified are likely to be activating mutations. We found that the four subgroups of GBM as defined by TCGA differed significantly with regard to expression level of specific phosphatases by comparative marker analysis. Surprisingly, the four subgroups can be defined solely on the basis of phosphatase expression by principle component analysis. This result suggests that critical phosphatases are responsible for modulation of specific molecular pathways within each subgroup. SHP2 constitutes one of the 12 phosphatases that define the classical subgroup. We confirmed the biological

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significance of this phosphatase by siRNA knockdown. The loss of cell viability induced by SHP2 silencing could not be explained by a significant increase in apoptosis alone, as demonstrated by TUNEL and propidium iodide staining. SHP2 silencing, however, did induce an increase in β-galactosidase staining and significant morphological changes. Propidium iodide staining also showed SHP2 silencing to reduce the population of cells in G2/M and S-phase. Since G1 arrest is also a marker of cellular senescence these data suggest that the inhibitory effect of SHP2 silencing is largely due to increased senescence rather than necrosis or apoptosis. Our data suggests that SHP2 may in part promote the growth of glioblastoma cells by suppression of cellular senescence, a phenomenon not previously described. Since it is becoming clear that both accelerated senescence and conventional growth arrest are likely to represent alternative options to apoptosis in GBM cells, it is feasible that as more selective inhibitors of SHP2 become commercially available they should be considered as a therapeutic strategy for glioblastoma.

Keywords
Glioblastoma multiforme • Phosphatases • SHP2 • Cellular senescence

Introduction

Glioblastoma Multiforme (GBM) is the most common type of malignant primary brain tumor in adults. Around 30,000 new cases are diagnosed every year in the United States and Europe (CBTRUS 2008). The prognosis is poor and despite the standard of care, surgery, radiation and chemotherapy median survival remains below 15 months (Stupp et al. 2005) with a clear need for improved therapeutic approaches. There has however been substantial progress in the understanding of molecular cancer subgroups (Verhaak et al. 2010), pathways involved in gliomagenesis, and disease progression (Furnari et al. 2007; Parsons et al. 2008; Yan et al. 2009; Verhaak et al. 2010). These efforts to understand the underlying molecular biology of the disease is now paving the way for the development of targeted therapeutics.

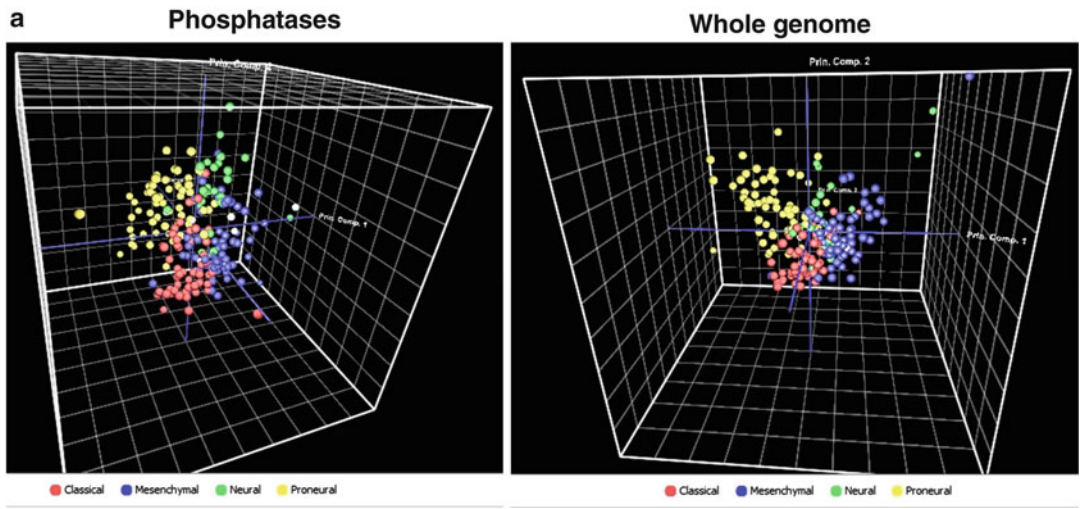
Importance of Phosphatases in Glioblastoma Multiforme (GBM): The Cancer Genome Atlas (TCGA) Data Analysis

Our analysis showed that the four subgroups of GBM, as defined by TCGA using a whole genome-derived 840 gene identifier (Verhaak et al. 2010), differ significantly with regard to expression of phosphatases (Sturla et al. 2011). This suggests that phosphatases play an important role in determining the underlying biology of GBM.

The four subgroups can be defined solely on the basis of phosphatase expression by principle component analysis (PCA; Fig. 21.1a). Gene pattern software was used to perform K nearest neighbor (KNN) analysis using leave one out cross validation. This class prediction analysis was used to determine how accurately the samples could be grouped into their various classes using only phosphatase expression as compared to the combined phosphatase/kinase or whole genome. KNN cross-validation using only phosphatase gene expression accurately predicted the sample class for 64.5 ± 6.5% of the samples when analysis was performed on both the original and validation data sets. This compares to 75 ± 0% when using the whole genome. When the accuracy of prediction is broken down by class, however, the phosphatase-only-gene-set accurately predicts the sample class for 74 ± 7% of the classical samples compared to 73 ± 5% using the whole genome. There was no significant difference between percentage correctly assigned samples determined using phosphatase expression only and that determined using the whole genome (unpaired t-test, $p = 0.17–0.92$).

Studies of receptor tyrosine kinases (RTK), including EGFR, demonstrate that the overall phosphorylation state is a net result of RTK and protein tyrosine phosphatase (PTP) activities (Reynolds et al. 2003). Since the catalytic activity

a



b

Position in Comparative Marker list	Phosphatase genes only	Phosphatase and Kinase genes combined	Whole genome
1	PTPRA [PTPRA]	FGFR3	GALNT17
2	PTPN21 [DUSP6]	PTPRA	SLC3A1
3	PTPRZ1 [PNKP]	EGFR	OTUD6A
4	MTMR3 [PTPRZ1]	STK33	CYP2B
5	PTPN14 [MTMR3]	PTPN21	LDLRAD2
6	**PTPN11 [PTPN11]**	CAMK2D	CYP26C1
10		CKB	RHOXF2B
20		CDK6	TUBA3E
40		**PTPN11**	ORM1
3163			**PTPN11***

$p<0.0001$, $^{*}p<0.032$ (T-test with Bonferonni correction). [Validation data]

Fig. 21.1 Analysis of TCGA profiling data reveals potential role of SHP2 in defining the classical subgroup of GBM (Figure 2; Sturla et al. 2011). (**a**) Principle component analysis (PCA) of TCGA GBM data using 191 phosphatase genes or the whole genome. (**b**) Comparative Marker analysis (CMA) identified most significant genes that define the classical subgroup of GBM using phosphatase genes only, phosphatase and kinase genes combined or the whole gene expression profile. Validation data results are shown in square brackets []

of PTP's can be 1,000-fold greater than that of kinases (Wang et al. 2010; Haque et al. 1995), perturbation of activity may have a significantly more profound effect on signal propagation than that of kinases. Most targeting strategies for RTKs emphasize the kinase activity (Shimizu et al. 2008). In autocrine regulated tumour cells, the Tyr kinase activity is always 'on' and thus net RTK activity will be mostly regulated by PTP activity. This suggests that the greatest therapeutic gain may be achieved by targeting the counteracting PTP. Since kinases are also far more numerous than phosphatases it is quite safe to say that one phosphatase must target multiple kinases.

As such, targeting phoshatases may prove more effective in tumors, such as GBM, with co-activation of multiple RTK pathways, which respond poorly to kinase inhibitor monotherapy.

SHP2 Is a Comparative Marker of "Classical" GBM

Comparative marker analysis (CMA) identified SHP2 as one of the 12 phosphatases that define the classical subgroup (Fig. 21.1b). Twelve phosphatases were found to be significantly associated with classical GBM with SHP2 being sixth on the list with a ($p < 0.0001$). When CMA was used with the phosphatase and kinase genes combined, SHP2 fell to 40th in the list but retained significance ($p < 0.0001$). Finally when CMA was carried out with the entire gene expression profile of over 20,000 genes, SHP2 falls to the 3163th position with a ($p = 0.032$). SHP2 therefore holds its significance as a classical subgroup-defining phosphatase even in the context of the entire gene set. When this analysis was repeated using a validation data set available from TCGA SHP2 remained a significant marker of the classical subgroup.

SHP2

SHP2 is a non-receptor protein tyrosine phosphatase which regulates several of the RTK pathways known to be over-expressed in glioblastoma, including EGFR, Fibroblast Growth Factor Receptor (FGFR) and Platelet Derived Growth Factor Receptor (PDGFR) (Grossmann et al. 2010). Perhaps the most well studied role of SHP2 is that in the modulation of EGFR phosphorylation, the RTK most widely over-expressed in GBM (Bredel et al. 2010). EGFR is over-expressed in a variety of human tumors where it has been linked to radiation resistance and poor prognosis. Several groups, including ourselves, have been able to show that activation of EGFR results in cytoprotective and proliferative downstream signaling (Schmidt-Ullrich et al. 2003; Sturla et al. 2005).

In addition, SHP2 has been shown to both antagonize and potentiate the action of its target PTKs and was the first phosphatase described as oncogenic (Bentires-Alj et al. 2004). More than 58 different SHP2 mutations have been identified in various tumors and 18 mutations in Noonan and Leopard syndromes, where patients exhibit disruption of normal cell proliferation and migration during development (Bentires-Alj et al. 2004). In its basal state, SHP2 activity is suppressed by intra-molecular interactions between residues in the "backside loop" of the N-terminal SH2 domain and the catalytic surface of the PTP domain (Hof et al. 1998). The mutations have been found to cluster mostly in the N-SH2 and PTP domain interface of the protein and are therefore predicted to be activating mutations, suggesting a positive role for SHP2 in tumorigenesis.

Considering the well documented role for SHP2 in the regulation of EGFR phosphorylation it is not too surprising that the classical subgroup of glioblastoma, as defined by TCGA, was also the group found to have deregulation of EGFR signaling. High level EGFR amplification was observed in 97% of this subtype along with 70% of the EGFRvIII mutations (Verhaak et al. 2010). Platelet Derived Growth Factor α (PDGFA), also over-expressed in this group, has been shown to enhance EGFR signaling through heterodimerization of PDGFRA and EGFR (Milenkovic et al. 2003). SHP2 is well known to extend the half-life of active RAS and increase ERK1/2 signaling downstream of a variety of receptor tyrosine kinases, including EGFR, PDGFR and FGFR all of which have been found to be over-expressed in GBM (Giannini et al. 2005).

Activating Mutations of SHP2 Are Present in GBM

We identified activating mutations of the putative oncogenic phosphatase, SHP2, in approximately 2% of the tumors analyzed by TCGA. Both of the mutations that passed validation in the TCGA data set could be considered activating mutations based on their location within the

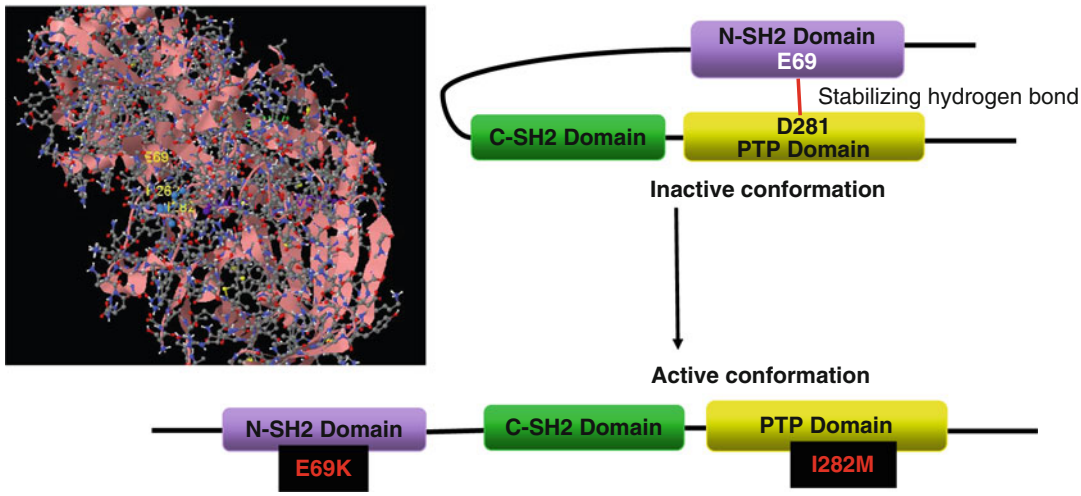


Fig. 21.2 TCGA mutation data reveal potential activating mutations in GBM (Figure 1; Sturla et al. 2011) Structural representation of SHP2 showing mutations found in GBM and a schematic representation of SHP2 depicting active and inactive conformations

N-SH2/PTP-domain interface. The first mutation, E69K has been well studied and is known to have 16-fold higher phosphatase activity than wild type SHP2 (Bentires-Alj et al. 2004), although a more recent study by the same group determined that the tumorigenic potential of SHP2 mutation does not necessarily correlate with PTP activity (Keilhack et al. 2005). With respect to the second mutation, I282M, we also predict this to be an activating mutation due to its location adjacent to a hydrogen bond known to stabilize the inactive conformation (Fig. 21.2).

Induction of Cellular Senescence in Glioblastoma Multiforme by SHP2 Knockdown

SHP2 siRNA Decreases GBM Cell Viability Without a Significant Increase in Apoptosis

All five siRNAs tested reduced SHP2 expression by 70–100% as compared to the non-targeting siRNA or a mock transfected control from 36 h post-transfection. Equality of protein loading was confirmed by actin staining. Profiling the phosphorylation status of established molecular targets of SHP2 (ERK1/2 and STAT3) (Agazie and Hayman 2003; Zhang et al. 2009) confirmed specificity of these siRNAs. SHP2 has previously been shown to enhance Ras activation by dephosphorylating EGFR tyrosine 992, responsible for translocation of RAS GAP to the plasma membrane where it inhibits RAS activity (Agazie and Hayman 2003). Knockdown of SHP2 by SHP2-specific siRNA resulted in a decrease in ERK1/2 phosphorylation without a significant change in total ERK1/2 levels as shown by Western blot. In contrast, SHP2 has been shown to have an inhibitory effect on JAK-STAT signaling (Zhang et al. 2009). In accordance with the established effect of SHP2 on STAT3, knockdown of SHP2 siRNA resulted in an increase in phospho-STAT3 without a significant increase in total STAT3 levels.

The viability of the human GBM cell lines, U87 and A172 transfected with non-targeting siRNA, was not significantly different to that of untransfected or mock transfected cells. Viability of U87 and A172 cells transfected with SHP2-specific siRNA reduced glioblastoma cell line growth by up to 80%, as shown using an alamar blue assay (Fig. 21.3a). Five commercially available SHP2-specific siRNAs were tested and viability of U87 cells was reduced by 60–75% ($p<0.0001$) and A172 by up to 60% ($p<0.005$).

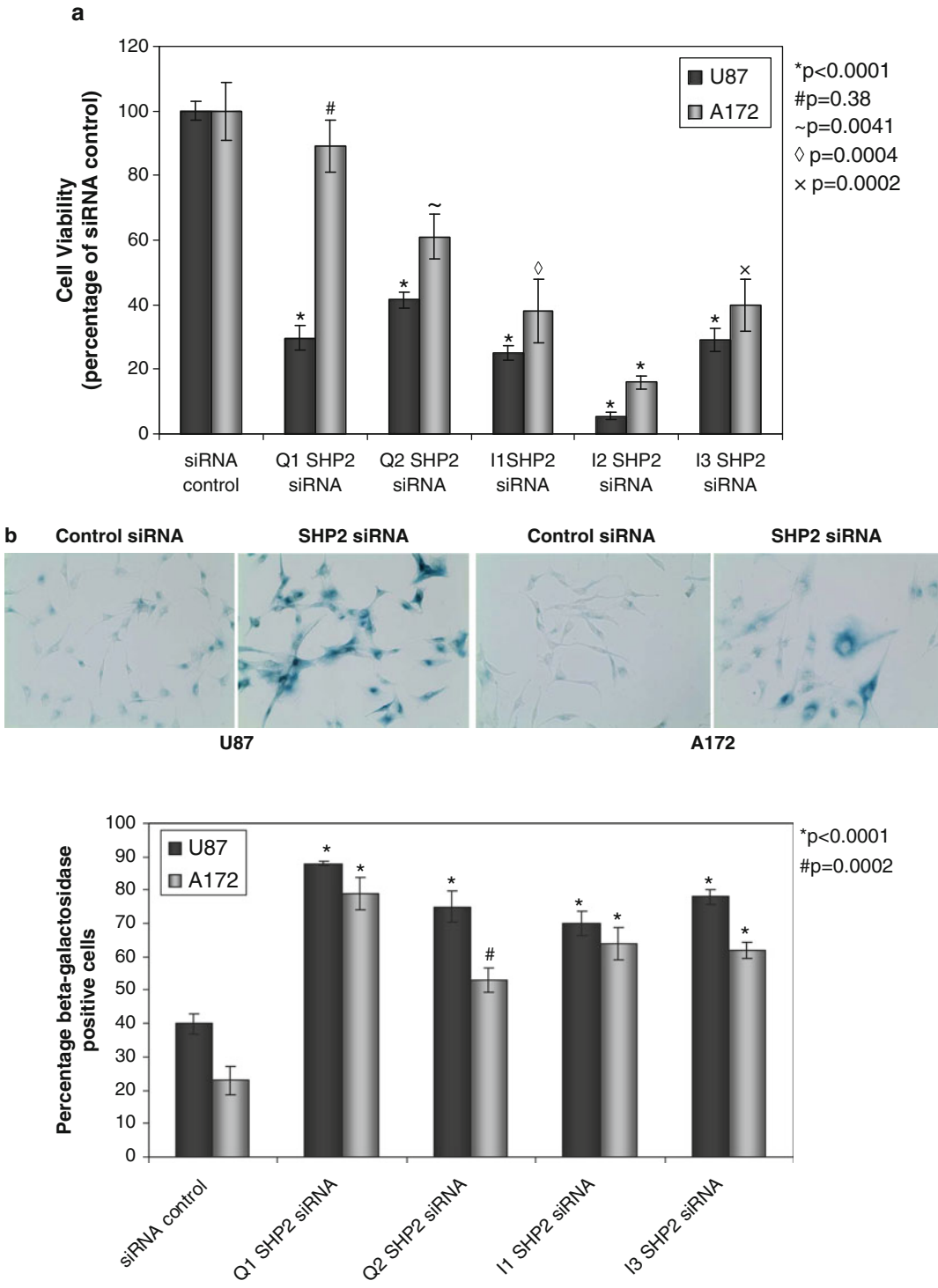


Fig. 21.3 siRNA knockdown of SHP2 reduces viability and induces senescence in U87 and A172 glioblastoma cell lines (Figures 4 and 6; Sturla et al. 2011). (**a**) U87 and A172 cells were transfected with siRNA and cell viability was assessed using an alamar blue assay. (**b**) Senescence was determined by the classic morphological changes including enlarged and flattened or elongated morphology and β-galactosidase staining in U87 and A172 cells transfected with SHP2-specific siRNA

The classical DNA degradation associated with apoptosis was assessed by TUNEL in non-transfected, non-targeting siRNA-transfected and SHP2-specific siRNA transfected cells. Less than 1% of the control cell populations were shown to be apoptotic as determined by TUNEL positivity. U87 cells transfected with SHP2-specific siRNA exhibited TUNEL positivity in up to 5%. Although the percentage of apoptotic cells was higher in SHP2 siRNA transfected cells as compared to the non-transfected and non-targeting siRNA controls, this was not high enough to account for the loss of cell viability observed. Similarly in A172 cells, only 1% of the cell population was TUNEL positive. Whilst this percentage increased up to 13% in SHP2-specific siRNA transfected cells respectively, this was not high enough to explain reduced cell viability.

SHP2 siRNA Induces Cellular Senescence

Both U87 and A172 cells transfected with SHP2-specific siRNA showed significant morphological changes. Cells showed enlarged and flattened or elongated morphology consistent with that seen in senescent cells (Fig. 21.3b). Although similar morphological changes were noted in U87 cells (Zhan et al. 2009), cellular senescence was neither studied or suggested as a mechanism. In this study SHP2 was shown to be essential for oncogenic transformation by EGFRvIII. Mouse embryonic fibroblasts lacking SHP2 (SHP2 +/– or –/–) could not be transformed by EGFRvIII. They also showed that expression of a catalytically inactive SHP2 mutant (C459S) in EGFRvIII-expressing U87 glioblastoma cells resulted in slower cell growth and a more flattened cellular morphology very similar to that seen in our own study. They attributed this to an increase in actin stress fiber organization and did not pursue the possibility of senescence.

Since we saw a loss in cell viability without a significant increase in cellular apoptosis alongside morphological changes consistent with senescence, we further analyzed the effects of SHP2 knockdown on cellular senescence. Cells with knockdown of SHP2 exhibited cellular enlargement and elongation classically associated with senescence. β-galactosidase staining at pH6.0 is also a classical marker of cellular senescence. SHP2 knockdown induced a significant increase in both intensity of β-galactosidase staining and number of β-galactosidase positive cells in both U87 and A172 cells (Fig. 21.3b) from 4 days post-transfection. Cells with greater than 15% positivity were considered positive for quantitation. Knockdown of SHP2 induced a highly significant increase in the percentage of β-galactosidase positive cells in both U87 and A172 cells as compared to control siRNA or mock transfected control. Cell populations transfected with SHP2 siRNA demonstrated 60–90% β-galactosidase positivity as compared to 20–40% β-galactosidase positivity in cells transfected with the negative control siRNA ($p < 0.0001$). An increase in p53 protein level was also observed in the majority of SHP2 knock down cells. This is consistent with the increase in p53 known to precede senescence (Quick and Gewirtz, 2006).

Propidium iodide staining was also used to observe cell populations in the various phases of the cell cycle. Senescent cells have previously been shown to arrest in late G1 phase of the cell cycle and consistent with this phenomenon we found an increase in the percentage of cells in G1 phase of the cell cycle in SHP2 knock down cells. These data suggest that the inhibitory effect of SHP2 silencing is largely due to increased cellular senescence rather than necrosis or apoptosis.

Human Telomerase Reverse Transcriptase (hTERT) and Senescence of GBM

SHP2 and hTERT

The exact mechanism of SHP2-knockdown induced senescence is unknown at present but two strong possibilities exist. SHP2 is well known to enhance the half-life of active RAS and as such it is possible that the knockdown of SHP2 activity

and its consequent effects on RAS activity are responsible for the senescence that we observe in GBM cell lines. We confirmed a reduction in the MAPKs, ERK1 and 2, downstream of RAS in cells with reduced SHP2 protein levels. Although Ras is an important mediator of glioblastoma tumorigenesis, its loss is not typically associated with the induction of cellular senescence. In fact, there are several reports of induction of senescence by over-expression of active RAS, a phenomenon known as oncogene induced senescence (Kosar et al. 2011; Rai et al. 2010). Previous studies have also shown SHP2 activating mutations to be mutually exclusive to mutations such as NF1 loss which leads to an increase in active RAS (Holzel et al. 2010).

Interestingly, SHP2 has been shown to regulate hTERT localization (Jakob et al. 2008). The human telomerase catalytic subunit, when localized to the nucleus, protects cells against cellular senescence. hTERT alone has been found to immortalize normal human astrocytes (Sonoda et al. 2001) with the addition of SV40 T-Ag and active Ras (H-ras) allowing maximum tumorigenicity as determined by anchorage independent growth and formation of tumors in nude mice (Rich et al. 2001). This is consistent with the finding that most grade III gliomas express telomerase. Its reactivation is associated with non-malignant grade II to malignant grade III conversion (Kim et al. 1994; Sano et al. 1998). The study by Jakob and colleagues showed over-expression of SHP2 to block oxidative stress-induced nuclear export of hTERT. As a consequence hTERT is retained in the nucleus resulting in resistance to cellular senescence and apoptosis. This study demonstrated SHP2 to be associated with hTERT under basal conditions and dissociates from nuclear TERT prior to its export and down-regulation. It is thought that SHP2 forms a complex with hTERT to prevent its nuclear export. The data from this study also suggests that SHP2 is responsible for the de-phosphorylation of tyrosine 707 in hTERT. SHP2 does not affect the phosphorylation of the Y707F mutant hTERT but negatively regulates the wt-hTERT. It remains to be seen if this is the case in GBM cells but the localization and interaction of hTERT and SHP2 in these cells clearly merits further study.

Neural Cancer Stem Cells and Telomerase

It is clear that telomerase is an important mechanism by which cancers escape replicative senescence. It has now been shown that the cancer stem cells present in neural tumors, an important contributor to recurrence and therapeutic resistance, also have aberrant expression and regulation of telomerase and telomeres.

A study by Castelo-Branco et al. (2011) studied telomerase activity, telomere maintenance and stem cell maturation in tumor sub-populations taken from freshly resected gliomas. In this study telomerase was undetectable in the majority of tumor cells but present in tumor initiating cells which also possessed critically short telomeres. In contrast normal tissue stem cells had longer telomeres, undetectable telomerase and were insensitive to telomerase inhibition. This is an observation critical to the therapeutic approach of telomerase inhibition since normal neural stem cells should remain unaffected. In this study a xenograft model demonstrated irreversible loss of self renewal and stem cell capabilities in the tumor initiation cells even after cessation of telomerase inhibition treatment in vitro and in vivo.

Alternative Routes to Cellular Senescence of GBM

Although telomerase and hTERT figure prominently in the resistance of GBM to cellular senescence there have been sporadic reports involving other key signalling pathways known to play a role in GBM tumorigenesis.

NF_KB

NF_KB has been found to be activated in glioblastoma tumor initiating cells undergoing differentiation and that blockade of this activation promotes senescence of differentiating cells (Nogueira et al. 2011). This activation of NF_KB could be a result of exposure to pro-inflammatory mediators in the tumor microenvironment or

upregulation of upstream signalling. NF_KB has been implicated in many of the mechanisms contributing to tumor development including growth factor-independent proliferation, apoptotic resistance, replicative potential and tissue invasion and metastasis (Naugler and Karin 2008). Nogeuira et al. (2011) showed NF_KB activation to be up-regulated during differentiation of glioma initiating cells (GICs). The study revealed upregulation of multiple cytokines, chemokines and cell cycle regulators known to be NF_KB targets. The group used various genetic strategies and small molecule inhibitors in a xenograft model to block NF_KB in differentiating GICs and found induction of senescence of tumor cells without disruption of the normal brain parenchyma. Similar results were observed with siRNA knockdown of the NF_KB target cyclin D1. Data suggests that NF_KB may prohibit differentiating GICs from acquiring a mature phenotype and support the rationale for therapeutic strategies aimed to promote premature senescence.

Wnt Inhibitory Factor 1 (WIF1)

WIF1 encodes a secreted Wnt antagonist, identified as a tumor suppressor gene following gene-expression based prediction of genomic copy number aberrations in glioblastoma (Lambiv et al. 2011). WIF1 was found to be strongly down-regulated in glioblastoma a phenomenon mediated by deletion or silencing by promoter hypermethylation. Ectopic expression of WIF1 in glioblastoma cells was, not surprisingly, found to decrease Wnt pathway activity in a dose-dependent manner. The consequence of Wnt signalling inhibition was reduced cell proliferation in vitro, reduced anchorage-independent growth in soft agar assays and completely abolished tumorigenicity in vivo. A senescence-like phenotype was also observed in vitro, again in a dose-dependent manner. WIF1 down-regulation was observed in approximately 75% of the glioblastomas studied indicating frequent involvement of aberrant Wnt signalling. Inhibition of Wnt signalling therefore provides an alternative therapeutic strategy by diverting tumor cells into a senescence-like state.

Aurora A

Most recently Aurora A inhibition has been shown to be a potential drug target in gliomas. It is critical for mitosis and its over-expression has been shown to transform cultured cells. Both the over-expression and knock-down of this critical protein causes genomic instability (Lehman et al. 2012). Aurora A expression has been found to increase with increasing grade and hypoxia in glial tumors and also with hypoxia in cultured glioma cells. Inhibition of Aurora with MLN8237 is potently cytotoxic to glioblastoma cells, a phenomenon potentiated by ionizing radiation. Lehman et al. (2012) also noticed features of senescence and differentiation in glioblastoma cells treated with MLN8237.

Discussion

Much more work is required to determine the exact mechanism by which SHP2 suppresses cellular senescence in glioblastoma but it is clear that it plays an important role in the viability of these cells. As the selectivity of commercially available SHP2 inhibitors is improved they should be considered a potential strategy for glioblastoma therapy.

To date the gold standard has been to induce apoptosis in brain tumors but due to mechanisms such as signalling pathway compensation, inhibition of key growth regulatory pathways alone is not always successful. It is therefore becoming clear that both accelerated senescence and conventional growth arrest are likely to represent alternative options to apoptosis in GBM cells.

The one concern with induction of senescence rather than apoptosis/cell death is that the senescent state may not be irreversible. The few in vivo studies where this phenomenon was observed showed that senescence was sufficient to render tumor cells incapable of producing tumors in

mice (Lambiv et al. 2011). This would suggest that the induction of senescence could be a viable therapeutic endpoint.

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Chemotherapy of Malignant Pleural Mesothelioma Induces Both Senescence and Apoptosis

22

Emanuela Felley-Bosco and Rolf A. Stahel

Contents

Abstract

It has recently been appreciated that several mechanisms, including apoptosis, senescence and autophagy control the growth of tumors exposed to cytotoxic chemotherapy. The knowledge of processes involved in the response to chemotherapy is important to understand mechanisms of resistance and identify means to enhance tumor response. Clinical research into this issue is hampered by the need for serial tumor biopsies which is often not feasible. Our current trial for patients with malignant pleural mesothelioma (MPM) includes neoadjuvant chemotherapy followed by surgery rendering it possible to evaluate in situ the effect of chemotherapy. For some patients tissue was available before chemotherapy so that we could investigate apoptosis and accelerated senescence induced by chemotherapy and could determine changes at individual level.

We observed that both apoptosis and senescence to be induced by chemotherapy in malignant pleural mesothelioma. In addition, our results demonstrated that induction in a proportion of patients with MPM of the senescence marker plasminogen-activator inhibitor-1 by neo-adjuvant chemotherapy has potential association with a poor outcome.

Keywords

Malignant pleural mesothelioma • Chemotherapy • Senescence markers

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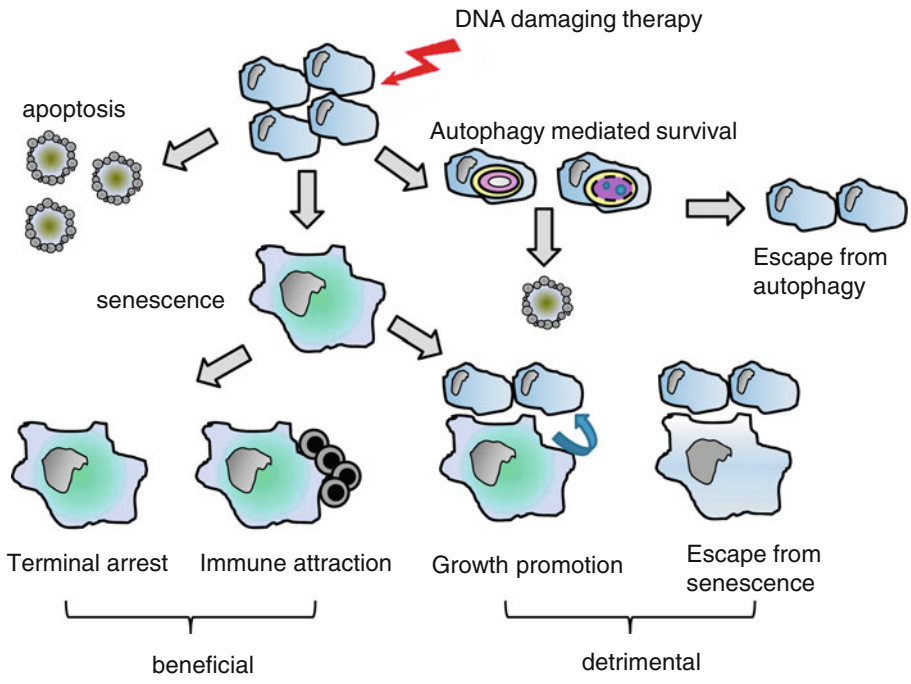

Fig. 22.1 In response to DNA-damaging agents, cancer cells can rapidly undergo apoptosis, may enter premature senescence or can undergo autophagy. A terminal arrest of the entire cancer cell population, possibly augmented through increased immunogenicity of senescent cells, is beneficial for the host. In contrast, feeder-like growth that reflects paracrine activity of senescent cells on their non-senescent neighbors, or escape from senescence based on acquired or preexisting mutations, is considered a detrimental outcome. Autophagy is a survival mechanism that delays apoptosis. Upon removal of stress, surviving cells may resume proliferation that can potentially lead to regeneration and tumor relapse

Introduction

DNA-damaging chemotherapy was initially thought to exert its effect through the induction of apoptosis, which is a regulated mechanism of cell death activated downstream DNA-damage response. More recently the molecular description of other mechanisms of cell death (reviewed in Yuan and Kroemer 2010) has challenged the concept of apoptosis being the only effector of tumor shrinkage. For example autophagy, a mechanism of cellular self-consumption, is also activated by DNA damaging agents (reviewed in Mathew and White 2011). On the other hand tumor shrinkage is not the only response achieved with chemotherapy, since stable disease is also, to a certain extent, beneficial. Therefore, growth arrest may also represent a desired end point. In this context it is noteworthy that the activation of DNA-damage response can result in induction of cellular senescence, also called accelerated senescence. The latter is a state of stable cell cycle arrest with active metabolism. In contrast to apoptosis where the cytotoxic signals converge in a common mechanism, senescence is typically a delayed stress response involving multiple effectors mechanisms including epigenetic regulation (Narita et al. 2006), DNA damage response (Bartkova et al. 2006) and senescence-associated secretion phenotype (Kortlever et al. 2006). The relative contribution of these effectors varies depending on the trigger and cell type. As it has been recently described by Khanna and collaborators there are practically no clinical studies in which these different pathways (represented in Fig. 22.1) responsible for tumor responses to

anticancer treatment have been investigated concurrently (Al-Ejeh et al. 2010). In our study (Sidi et al. 2011) we sought to partially fill this gap by investigating apoptosis and accelerated senescence induced by chemotherapy in malignant pleural mesothelioma (MPM).

MPM is a rapidly lethal pleural cancer which arises from the parietal pleural mesothelium (Fennell and Rudd 2004). It is characterized by multifocal tumor growth originating in the parietal pleura and often accompanied by pleural effusion. The tumor spreads to involve visceral pleura producing a rind that constricts the lung, heart and mediastinum. It is supposed that, due to the tumor location and rapid growth, death usually results from compression of vital mediastinal structures rather than from metastatic spread. Common initial symptoms are dyspnea and chest wall discomfort, often related to pleural effusion. Without treatment the expected median survival is 4–12 months.

More than 80% of cases of MPM are linked to exposure to asbestos. Asbestos refers to a family of silicate minerals divided into two major groups; the serpentine form of asbestos-chrysotile and the amphibole forms (crocidolite, anthophylite, anconoliteamosite and tremolite). Erionite is a naturally occurring fibrous mineral belonging to a group of minerals called zeolites. Asbestos has been shown to cause asbestosis, lung and laryngeal cancer and mesothelioma. However, erionite specifically causes mesothelioma and is more potent than asbestos in causing mesothelioma (Carbone and Yang 2012). Importation of crocidolite and amosite was banned in the U.K in 1985. Importation of chrysolite was banned in the E.U from 2005 (Fennell and Rudd 2004). It is estimated that the 15-year cumulative frequency of MPM during 1994–2008 in the 56 countries reporting MPM to be 174,300 (Park et al. 2011) however, the real incidence of MPM is unknown since there are countries in which MPM mortality is not reported, including asbestos producing countries such as Russia, Kazachstan, China and India (Park et al. 2011). Since overall MPM incidence is increasing worldwide, it is urgent to improve the treatment of these patients.

Systemic Therapy of Malignant Pleural Mesothelioma

Recent reviews (Campbell and Kindler 2011; van Meerbeeck et al. 2011) summarize milestones that resulted in the current standard first line systemic therapy, with the antifolate pemetrexed and cisplatin. For potentially operable disease, combined modality therapy including extrapleural pneumonectomy is an option (reviewed in Felley-Bosco and Opitz 2012). We and others have examined the use of neoadjuvant therapy followed by extrapleural pneumectomy, initially with cisplatin and gemcitabine and more recently with cisplatin and pemetrexed (Weder et al. 2007). In a proportion of our patients tumor tissue at the time of diagnosis and after chemotherapy is available. This allows us to directly explore the effect of cytotoxic chemotherapy on the tumor.

Mechanism of Cell Death and Clinical Response in Malignant Pleural Mesothelioma

In order to examine mechanism of cell death a translational study was performed on tissue samples obtained from patients treated for MPM at the Department of Medical Oncology and the Department of Thoracic Surgery at the University Hospital of Zurich during the time period from May 1999 until the 31st January 2009. Neoadjuvant chemotherapy consisted of three cycles of cisplatin 80 mg/m^2 on day 1 and gemcitabine 1,000 mg/m^2 on days 1, 8 and 15 administered every 28 days or, since March 2003, of cisplatin 80 mg/m^2 on day 1 and pemetrexed 500 mg/m^2 on day 1 administered every 21 days with vitamin supplementation. Response to chemotherapy was assessed by CT scan after completion of the third cycle of chemotherapy. Clinical response was evaluated according to modified RECIST criteria (Byrne and Nowak 2004). Surgery was performed within 6 weeks after completion of the last administration of chemotherapy. Follow-up was performed in our outpatient clinics. We identified 31 patients

with tumor material available before and after chemotherapy. There were 29 males and two females, with a median age of 59 (range 44–72). The histotype was epithelioid in 21 patients (68%), biphasic in 7 (23%) and sarcomatoid MPM in 3 (10%).

Clinical stage according to TNM-classification was I in 15 patients (49%), II in 5 (16%), and III in 11 (35%). Neoadjuvant chemotherapy was administered to all patients and consisted of three cycles of cisplatin and gemcitabine in 5 (16%) or three cycles of cisplatin and pemetrexed in 26 patients (84%). Thirty patients underwent thoracotomy, which was explorative in three, pleurectomy/decortication in 6 and extrapleural pneumonectomy in 21 patients. From one patient tumor material was obtained by lymph node biopsy via mediastinoscopy after chemotherapy. He did not undergo thoracotomy.

For all these patients, paraffin-embedded tumor samples before and chemotherapy were available for immunohistochemistry. For a proportion of them RNA or OCT-embedded material was also available. In order to assess that RNA was representing clinical samples containing at least 50% tumor content, a preliminary study was performed comparing different genes expression in normal pleura and tumors. As a second quality control, tumor content assessed on H&E stained sections was correlated with the quantitative expression of MPM markers calretinin (Doglioni et al. 1996), podoplanin (Chu et al. 2005) and mesothelin (Chang and Pastan 1996). The comparison of morphological vs. MPM markers was used to set up a threshold defining samples containing at least 50% tumor cells. First, a score was calculated as average of centered relative expression levels of calretinin, podoplanin and mesothelin for each sample. When this score was compared to morphology we found that a relative score of >10% corresponded to more than 50% of tumor cells. Therefore only samples satisfying this criterion were further analyzed.

Detection of Accelerated Senescence

One obstacle to identify senescent cells in vivo has been the lack of reliable markers of senescence. The widely recognized gold standard of senescence detection, senescence associated β-galactosidase assay (SAβ-gal) relies on enzymatic activity in cells and thus it requires fresh frozen tissue sections. Available cryo sections were stained for SAβ-gal activity as described by Dimri et al. (1995). Positive control consisted of samples incubated in the same reaction mixture but pH 4, while negative controls were at pH 7.5. We observed an increase in senescence-associated β-galactosidase activity indicating that in some patients there is a strong induction of accelerated senescence after platinum-based chemotherapy. However, this observation does not provide mechanistical insight, since SAβ-gal activity is not an effector of senescence but rather just a byproduct. To gain further knowledge, we assessed the expression of several genes that had been previously described as being involved in senescence. The ratio of senescence markers (p21, plasminogen activator inhibitor-1 (PAI-1), aldehyde dehydrogenase one family member A3 (ALDH1A3), insulin-like growth factor-binding protein 7 (IGFBP7) (Fridman and Tainsky 2008)), measured after as compared before chemotherapy, was evaluated in nine patients where RNA from "bona fide" tumor samples was available. The expression of p21 and PAI-1 after chemotherapy was significantly increased ($p < 0.05$ and <0.01, respectively, paired t test), while there were no changes in ALDH1A3 and IGFBP7. Furthermore, the increase of PAI-1 was significant ($p < 0.05$, paired t test) in patients with stable disease, but not in patients with partial response. These data were corroborated at the protein level for p21 and PAI-1 by immunohistochemistry confirming, at least for p21, previous studies showing strict correlation between p21 protein and mRNA expression (Marchetti et al. 1996).

Detection of Apoptosis

Apoptosis can be activated after DNA damage by mechanisms involving the activation of p53 (Reinhardt and Schumacher 2012). Although p53 is rarely mutated in MPM, increased expression levels have been observed (reviewed in Lechner et al. 1997). Nevertheless, in the absence of p53

mutations it would be expected that chemotherapy would trigger apoptosis via p53-mediated activation of the intrinsic or extrinsic signaling pathways, which converge at the level of caspase activation but differ in upstream stimuli. The intrinsic apoptotic pathway is regulated by the ratio of pro-apoptotic to pro-survival Bcl-2 family members. All family members share homology in one or more BCL-2 homologydomains (called BH domains, numbered BH1–H4) that are essential for their function. Pro-apoptotic Bcl-2 effector proteins such as Bax and Bak oligomerize at the mitochondrial outer membrane, resulting in mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c, eventually activating effector caspases. The pro-survival Bcl-2 family members, including Bcl-2, Bcl-XL, and Mcl-1, bind directly to Bax and Bak, inhibiting MOMP. BH3-only pro-apoptotic Bcl-2 family members, including Puma and Noxa, perturb these interactions, liberating Bax and Bak to promote apoptosis. Therefore, BH3-only protein are the master regulator of MOMP and their expression has been associated with the concept that cells are "primed for death" (Del Gaizo Moore and Letai 2012). p53 can engage the intrinsic cell death pathway through the induction of Bax, Puma and Noxa. By contrast, the extrinsic apoptotic pathway is activated by engagement of transmembrane death-domain proteins at the cell surface, and p53 participates in this pathway by inducing the transcription of genes encoding death receptors and ligands, such as Fas, and Killer/DR5. Caspase activation results in endonuclease which cleaves DNA and this phenomenon can be visualized in paraffin-embedded tissues by TUNEL assay, a method based on the in situ labeling of nuclear DNA breaks through the binding of terminal deoxynucleotidyltransferase (TdT) to 3′-OH ends of DNA. We applied this method to detect apoptosis before and after chemotherapy in six of the nine patients where senescence markers had been investigated by gene expression analysis. Apoptosis could be detected in 1% (range 0–4) and 8% (range 1.5–15) of the cells before and after chemotherapy, respectively representing a significant ($p<0.01$, Mann-Whitney test) increase. Looking at individual results of these six patients, objective radiological responses were only seen in patients with induction of apoptosis and no change in PAI-1 senescence marker.

Expression of "Senescence Marker" PAI-1 and Clinical Outcome

For twenty six patients paired TMA material was available from before and after chemotherapy and was used to examine p21 and PAI-1 protein expression. This approach was necessary since mRNA was not available for all patients at the two time points. A linear relationship between p21 mRNA and IHC staining was observed ($r^2=0.71$, $p<0.001$), while for PAI-1 the relationship between mRNA and immunohistochemistry was significant ($p<0.05$) but less stringent ($r^2=0.33$) as it has been previously described for secreted proteins (Gronborg et al. 2006). The best relationship between mRNA and PAI-1 IHC was found when staining of stromal cells was taken into account; hence PAI-1 score corresponded to the sum of tumor and stromal cells protein expression. Data on response assessment were available for 23 patients; 14 were classified as stable disease and nine as partial response according to modified RECIST criteria. Patients with stable disease tended to have more often an increase of PAI-1 expression as compared to patients with objective response while for p21 no trend was evident. In an exploratory analysis we looked for a possible association of increase of PAI-1 expression with overall survival and progression-free survival. There was a significant association of increased PAI-1 expression with both poor survival and shorter progression-free survival ($p=0.0098$ and $p=0.022$, respectively).

Discussion

As shown by our study, combined cisplatin and pemetrexed chemotherapy not only induces apoptosis, but also senescence of MPM (Fig. 22.2). A recent study using cell culture has confirmed our observation by demonstrating that pemetrexed treated MPM cells undergo senescence.

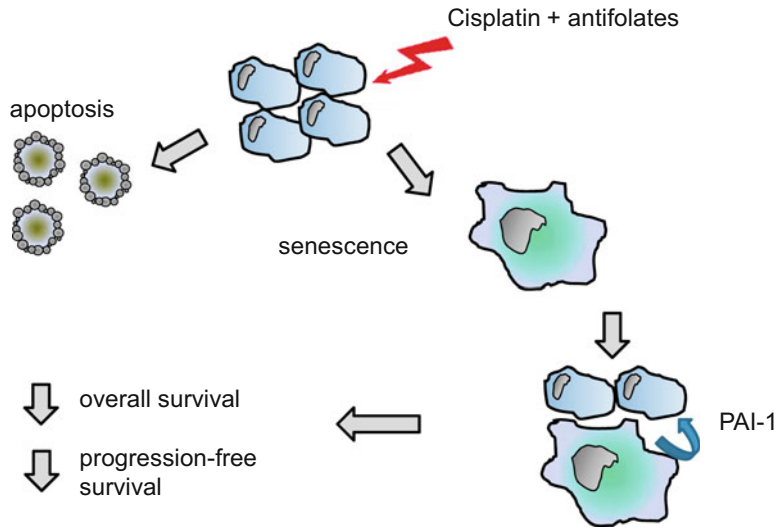

Fig. 22.2 Molecular mechanism of response to chemotherapy was evaluated in situ in malignant pleural mesothelioma (MPM) patients. Tissue samples were obtained at the time of diagnosis and at the time of surgery performed within 6 weeks after completion of the last administration of chemotherapy. In response to DNA-damaging agents, MPM undergo apoptosis but showed also increased senescence markers (senescence-associated β-galactosidase activity, p21 and PAI-1). Patients with increased PAI-1 had the worst progression-free survival and overall survival

Conditioned medium from these cells contained cytokines/chemokines typically associated with senescence-associated secretion phenotype (Kortlever et al. 2006) and triggered epithelial-mesenchymal transition accompanied by chemoresistance (Canino et al. 2012). Other experimental evidence has also suggested that senescence might be responsible for poor chemotherapy response in mesothelioma (Nguyen et al. 2004; Lazzarini et al. 2008; Vandermeers et al. 2009). Compared to tumors undergoing apoptosis, there are several problems with tumors becoming senescent. The first more obvious one is that the tumor arrests and does not regress. Second, a cell-cycle-targeting chemotherapy will lose efficiency in growth arrested cells. This has been very recently illustrated in an animal experimental model of breast cancer where p53-mediated senescence after doxorubicin treatment lead to relapse earlier compared to mice bearing mutated p53 where an increase in apoptosis triggered by mitotic catastrophe was observed (Jackson et al. 2012). Third, subpopulations of chemotherapy-induced senescent cancer cells have already been shown capable of escape leading to relapse (Elmore et al. 2005; Roberson et al. 2005). In one of these studies, tumor cells escaping senescence had re-acquired cyclin-dependent kinase CDC2/CDK1 expression which had been lost upon senescence, and escape from senescence could be blocked using CDC2/CDK1 inhibitors (Elmore et al. 2005; Roberson et al. 2005) indicating that strategies can be found to avoid tumor relapse. Fourth, senescent tumour cells are metabolically active and can secrete cytokines and other paracrine factors which feed and stimulate the growth of nearby cells, (Kahlem et al. 2004). This could have some implications in treatment outcome, especially in terms of time to progression. Although these data point toward a detrimental effect of senescence, it should be kept in mind that there might be different types of senescence. For example it has been recently shown that combination of PARP inhibition and irradiation induced senescence in tumor cells, leading to a secretory profile that was not pro-tumoral but was instead activating the immune response in an experimental animal model (Meng et al. 2012). Therefore, the question is not only whether a certain therapy induces senescence or not but which type of senescence is induced. Simultaneous evaluation of senescence markers

and immune response may help answering to this question in the future.

In an exploratory analysis we have identified increased PAI-1 as being associated with lack of objective response. PAI-1 is a secreted protein, component of the plasminogen activation system, that has been investigated in several cancers and elevated levels of PAI-1 are correlated with shortened overall and/or disease-free survival in renal, ovarian and breast cancer (Duffy and Duggan 2004). Although expression of PAI-1 has been observed in mesothelial cells in vitro after exposure to TGFβ (Shetty et al. 2010) it was the first time that PAI-1 expression is investigated in malignant pleural mesothelioma tissues in vivo. In our study the relative change after chemotherapy was determinant for the response, suggesting that it plays a role in tumor evolution. PAI-1 is considered as a senescence marker (Fridman and Tainsky 2008), is essential for p53-induced senescence (Kortlever et al. 2006) and has tumor promoter functions (Duffy et al. 2008). Since too few frozen samples were available, a definitive association between increase in PAI-1 levels and induction of senescence-associated β-galactosidase activity could not be determined in our study and it will be necessary to clarify this issue in further studies.

In our study only a very small percentage of cells were positive for TUNEL immunostaining, used as apoptosis biomarker, which is not unusual in solid tumors (Al-Ejeh et al. 2010). It is possible that apoptosis is not a major cell death mechanism in MPM because mitochondria are not "primed for death" (Green 2007) or that apoptotic cells are rapidly cleared by macrophages or mesothelial cells themselves (Wagner et al. 2011).

In conclusion, both apoptosis and senescence can be observed after chemotherapy in malignant pleural mesothelioma and increased expression of senescence marker PAI-1 was associated with lack of response to chemotherapy. Because DNA damage can also induce autophagy (Notte et al. 2011) future studies will need to address whether chemotherapy induces autophagy in MPM and how this contributes to outcome. A better understanding of mechanisms controlling the balance between the all these processes may benefit the patients by identifying factors that should be targeted.

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MicroRNA as a Modulator of Cell Proliferation and Senescence: Role in Lung Cancer Cells

23

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Contents

Abstract

MicroRNA (miRNA), which is a type of non-coding RNA, has attracted attention in the field of cancer research because of its involvement in the suppression of gene expression regulation, and this suppressive effect may be one explanation for carcinogenesis. So far, a subset of miRNAs has been characterized as oncogenic miRNAs or tumor-suppressive miRNAs based on their expression changes in certain cancers and the repertories of their target genes. Using bioinformatics, we aimed to identify the target genes of five selected miRNAs, the expression levels of which are likely affected by the deregulated oncogenic expression of HRAS, JUN and E2F family members in a human lung cancer cell line (A549). We also attempted to validate the identified target genes functionally, particularly the genes responsible for cell fate determination, for selected miRNAs using conventional experimental approaches to uncover the functional aspects of miRNAs in the proliferation and senescence of A549 cells. Among the five miRNAs, we performed a functional analysis of the miR-345 candidate targets using a luciferase reporter assay. We demonstrated that miR-345 is likely to be involved in the regulation of A549 cells by targeting AKT2, EPS8, E2F3 and E2F6. Next, we performed an expression analysis of cancer-critical genes in A549 cells stably expressing miR-483-5p and found that the mRNA expression levels of IGFBP2,

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ID2, MCM5 and MCM6 in a miR-483-5p-stably-expressing clone were significantly decreased, compared with those obtained in a control clone. Taken together, these results are expected to contribute to *in silico* analyses and *in vitro* experiments performed to validate the candidate target genes of miRNA in the context of lung carcinogenesis.

Keywords
MicroRNA • Cancer • Gene expression • Bioinformatics

Introduction

There is no longer any doubt that the molecular etiology underlying cellular carcinogenesis is mainly attributable to the activation of oncogenes and the functional loss of tumor suppressor genes. Therefore, understanding the signal transduction pathway and the transcriptional mechanism regulated by oncogenes and tumor suppressor genes is essential. The traditional approach to obtaining mechanistic insights into gene expression control has primary relied on analyses of mRNA levels. Now, however, noncoding RNA has begun to attract attention; most notably, microRNA (miRNA), which is comprised of a short, approximately 22-nucleotide RNA molecule, has been extensively investigated in the field of cancer research because of its involvement in the suppression of gene expression regulation, and this suppressive effect may be one explanation for carcinogenesis (Garzon et al. 2009). So far, miRNA has been characterized as either oncogenic miRNA or tumor suppressor miRNA. For example, as an oncogenic miRNA, the overexpression of miR-17-92 leads to the suppression of tumor suppressor genes, while let-7 has been frequently mentioned as a tumor suppressor miRNA (Zhang et al. 2007). The miR-17-92 cluster, comprised of seven miRNAs, has also been shown to be highly expressed in lung cancer, especially in small-cell lung cancer (SCLC) (Hayashita et al. 2005).

In the beginning, miRNA was originally described as a fluctuating RNA detected in a development stage-specific manner in *Caenorhabditis elegans*. Since that time, miRNA has been found in various organism species, from *Drosophila* to humans, and the conservation of miRNA beyond organism species has strongly indicated that miRNA is likely responsible for crucial biological functions. Indeed, in humans, accumulating evidence has revealed that miRNA is involved in the regulation of the pathogenesis of serious diseases such as cancer and adult-onset diseases (Esteller 2011).

In the nucleus, miRNA is transcribed either alone or cluster and forms a stem-loop structure, which is called pri-miRNA. The pri-miRNA is then cleaved to form pre-miRNA by the ribonuclease III Drosha and is exported from the nucleus by exportin5. In the cytoplasm, pre-miRNA is further processed by ribonuclease III Dicer and then incorporated into an RNA-induced silencing complex (RISC). This complex binds to the 3′-untranslated region (UTR) of mRNA, leading to mRNA cleavage or translational suppression from mRNA (He and Hannon 2004). The seed region is generally regarded as two to eight bases of the 5′-terminal of miRNA, and this region is considered to be important for the binding of the miRNA to the target mRNA. Currently, more than 1,000 kinds of human miRNA have been estimated to exist, and one miRNA can target approximately 200 kinds of mRNA because miRNA can recognize target mRNA even if a partial mismatch exists, although miRNA preferably binds the 3′-UTR of its target mRNA in a complementary style and a sequence-specific manner. This enables miRNA to regulate more than approximately 60% of all genes encoding proteins (Krek et al. 2005). For a long time, whether miRNA cleaves the target mRNA, making it unstable, or suppresses translation from the target mRNA remained a mystery; however, Guo et al. (2010) clearly demonstrated that the gene expression control mediated by miRNA can be attributed to the instability of the mRNA after cleavage by miRNA in mammalian cells.

We recently performed global expression analyses using an miRNA microarray in the human lung cancer cell line A549 to search for miRNA whose expression levels are controlled by pivotal transcriptional regulators associated with cell growth and proliferation, such as HRAS (v-Ha-ras Harvey rat sarcoma viral oncogene homolog), JUN (jun proto-oncogene), and E2F family members (E2F1-E2F6). Consequently, we identified several miRNAs (miR-494, miR-483-5p, miR-345, miR-139-3p and miR-370) that are commonly and uniquely regulated by HRAS, JUN and E2F1-E2F6 (Ohdaira et al. 2010). In particular, HRAS, JUN, E2F1 and E2F3 increased the expression levels of miR-494 and miR-345 (Fig. 23.1a). On the other hand, activator E2Fs (E2F1-E2F3) elevated the miR-483-5p expression level, whereas E2F5 and E2F6 decreased the miR-483-5p expression level (Fig. 23.1a). The expression levels of miR-345, miR-139-3p and miR-370 were commonly reduced by E2F5 and E2F6 ectopic expression (Fig. 23.1a) (Ohdaira et al. 2010).

Notably, these miRNAs have been associated with some cancers. For instance, miR-494, which maps to human chromosome 14q32.31, is highly expressed in retinoblastoma (Zhao et al. 2009). The elevation of miR-483-5p expression in malignant adrenocortical tumors, compared with benign adrenocortical tumor, has also been reported and is probably involved in the poor patient survival rate (Soon et al. 2009). The expression level of miR-345 is reportedly restored in 5-aza-2′-deoxycitidine-treated colorectal cancer, based on the results of an miRNA microarray, in response to a decline in promoter-region methylation and also reportedly suppresses colorectal cancer cell growth and invasiveness through the negative regulation of BAG3 (BCL2-associated athanogene 3) (Tang et al. 2011). The expression level of miR-139-3p is relatively high in liver metastases of colon cancer (Lin et al. 2011). The upregulation of miR-370 has been identified in stomach cancer and has been shown to suppress the expression of TGFβ-RII (transforming growth factor-β receptor II) (Lo et al. 2012). Each miRNA is expected to show a characteristic expression pattern in cancer cell types and to select a unique set of target mRNAs, depending on the stimulus sensed by cells and the cellular signalling pathways that are mobilized under the given circumstances. Accordingly, understanding miRNA function by investigating the target genes of miRNA as well as the transcriptional regulators of miRNA is quite important. Actually, a number of reports have discussed the formation of a feedback loop between miRNA and its target genes, especially miRNA that target transcription factors, which in turn regulate miRNA expression. For example, miR-200 and the zinc-finger enhancer binding (ZEB) transcription factor are mutually regulated, and this feedback loop regulation is assumed to promote cancer proliferation and metastasis, probably because of the influence on cancer stem cells (Brabletz and Brabletz 2010).

Fairly recently, we demonstrated that miR-494 reduced the proliferation rate of A549 cells and induced senescence (Ohdaira et al. 2012). By performing an *in silico* search for target genes of miR-494 along with a series of *in vitro* experiments, we clearly showed that miR-494 suppressed the mRNA expression levels of IGF2BP1 (insulin-like growth factor 2 mRNA binding protein 1), JUND (jun D proto-oncogene) and PTEN (phosphatase and tensin homolog) (Ohdaira et al. 2012). In the present chapter, we describe our subsequent search for target genes that are potentially regulated by five miRNAs, including miR-494, using a representative miRNA target gene database in a systematic manner.

Database Search for MicroRNA Targets

For the miRNA database, we referred to the Sanger miRBase (http://mirbase.org/). To search for miRNA target genes, we used the following five algorithms.

- TargetRank (http://hollywood.mit.edu/targetrank/) (Nielsen et al. 2007).
- TargetScan Release 5.1 (http://www.targetscan.org/) (April 2009) (Grimson et al. 2007).

- MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) (Rehmsmeier et al. 2004).
- miRDB Version 3.0 (http://mirdb.org/miRDB) (April 2009, microRNA source miRBase V13.0) (Wang 2008).
- PicTar (http://pictar.mdc-berlin.de/) (Last update March 26, 2007) (Krek et al. 2005).

First, we identified target genes for each of the five miRNAs using individual algorithms (Fig. 23.1b). Next, we made a short list of target genes that overlapped more than one (Fig. 23.1c). The most important findings are listed here. As miR-494 targets, 21 genes were commonly listed in TargetRank, TargetScan and miRDB, and these targets included IGF2BP1, CDK6 (cyclin-dependent kinase 6) and PTEN (Fig. 23.1c). In addition, 29 genes were commonly listed in TargetScan, MicroCosm Targets and miRDB, and these targets included JUND (Fig. 23.1c). As we mentioned above and in our previously published report, we have confirmed that IGF2BP1, PTEN and JUND are potential miR-494 targets (Ohdaira et al. 2012). As miR-483-5p targets, four genes were commonly listed in TargetRank, MicroCosm Targets and miRDB; in particular, MAPK3 (mitogen-activated protein kinase 3) was an ideal target in the context of cell fate determination (Fig. 23.1c). Surprisingly, MAPK3 was reported as a target gene for miR-483-5p in gliomas during the preparation of this chapter (Wang et al. 2012). As miR-345 targets, 20 genes were commonly listed in TargetRank, TargetScan, MicroCosm Targets and miRDB, and RPA1 (replication protein A1, 70 kDa) was likely to be important because RPA1 has been shown to play a role in the initiation of DNA replication (Fig. 23.1c). Similarly, we identified the overlapping of AKT2 (v-akt murine thymoma viral oncogene homolog 2), EPS8 (epidermal growth factor receptor pathway substrate 8), E2F3 and E2F6 in three databases, and the results of functional analyses will be described in greater detail in the next paragraph. Finally, as miR-139-3p targets, few targets were commonly listed in any of the databases, and only SH3BP2 (SH3-domain binding protein 2) was commonly listed in four databases as an miR-370 target gene (Fig. 23.1c).

MicroRNA-345 Candidate Target Genes

We further checked whether AKT2, EPS8, E2F3 and E2F6, which were commonly retrieved from three databases, are actual miR-345 candidate targets using a luciferase reporter assay in A549 cells. These genes are known to play fundamental roles in the control of cell growth and proliferation. For instance, AKT2 is an oncogene that belongs to the serine/threonine kinases subfamily and is considered to be involved in the cell growth, survival, and metastasis of cancer cells through RAS and phosphatidyl inositol 3-kinase (Cheng et al. 2008). As a substrate for EGFR (epidermal growth factor receptor) and multifunctional proteins, EPS8 is involved in the endocytosis of EGFR and the activation of ERK (extracellular signal-regulated kinase)-regulated and AKT-regulated signaling pathways that are central to cell growth and survival (Di Fiore et al. 2002). E2F3 and E2F6 are members of the E2F transcription factor family, and E2F3 primarily promotes the cell cycle (especially the G1 to S phase transition), while E2F6 is considered to function as a suppressor of the cell cycle in a dominant-negative manner (Chen et al. 2009).

Besides AKT2, EPS8, E2F3 and E2F6, we subjected CDKN1A (cyclin-dependent kinase inhibitor 1A (alias, p21 or Cip1) to a luciferase reporter assay as a positive control, since CDKN1A has been previously reported to be a miR-345 target (Wu et al. 2010). TargetScan predicted the sequences in the 3'-UTR of miR-345 candidate target mRNAs that form a complementary strand with miR-345, and these sequences are listed below (also refer to Fig. 23.2a).

- AKT2 (NM_001626)

A: 2,287–2,309-bp (poorly conserved, 8-mer, 59% match)
B: 3,290–3,312-bp (poorly conserved, 8-mer, 45% match)
C: 4,057–4,078-bp (poorly conserved, 7-mer-1A, 27% match)

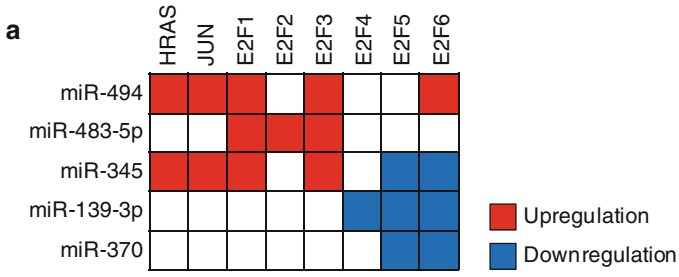
- EPS8 (NM_004447)

A: 4,003–4,024-bp (conserved, 7-mer-m8, 32% match)

- E2F3 (NM_001949)

A: 2,742–2,763-bp (poorly conserved, 7-mer-m8, 50% match)

a



b

microRNA	< Database > TargetRank	TargetScan	Microcosm Targets	miRDB	Pictar
miR-494	59	360	814	779	0
miR-483-5p	41	4	41	33	0
miR-345	405	500	973	158	109
miR-139-3p	55	7	1073	20	173
miR-370	97	239	947	252	195

c

microRNA	< Database > AB	AC	AD	AE	BC	BD	BE	CD	CE	DE
miR-494	25	4	45	0	48	153	0	119	0	0
miR-483-5p	0	9	8	0	0	0	0	5	0	0
miR-345	254	52	85	6	86	121	2	30	5	2
miR-139-3p	2	14	8	0	1	2	0	5	4	0
miR-370	7	1	8	5	25	7	43	2	17	16

microRNA	< Database > ABC	ABD	ABE	ACD	ACE	ADE	BCD	BCE	BDE	CDE
miR-494	2	21	0	3	0	0	29	0	0	0
miR-483-5p	0	0	0	4	0	0	5	0	0	0
miR-345	46	79	1	20	0	1	25	0	0	0
miR-139-3p	0	1	0	3	0	0	0	0	0	0
miR-370	1	4	3	0	0	0	0	4	0	2

microRNA	< Database > ABCD	BCDE
miR-494	2	0
miR-483-5p	0	0
miR-345	20	0
miR-139-3p	0	0
miR-370	0	1

< Database >
A. TargetRank
B. TargetScan
C. MicroCosm Targets
D. miRDB
E. Pictar

Fig. 23.1 Five miRNAs commonly regulated by transcription regulators in the human lung cancer cell line A549 at the transcriptional level. (**a**) miR-494, miR-483-5p, miR-345, miR-139-3p and miR-370 were identified as miRNAs that were upregulated 1.5 times (red square) or downregulated 1.5 times (blue square) by two or more different transcription regulators in A549 cells in which HRAS, JUN or E2F family members (E2F1-E2F6) were ectopically overexpressed. (**b**) Number of miRNA target genes explored using five databases. The candidate miR-345 target genes predicted by TargetScan were limited to the top 500 genes out of 2,053 genes. (**c**) Number of overlapping target genes retrieved by two (*upper table*), three (*middle table*), or four (*lower table*) databases. The databases that were used were TargetRank (**a**), TargetScan (**b**), MicroCosm Targets (**c**), miRDB (**d**) and Pictar (**e**)

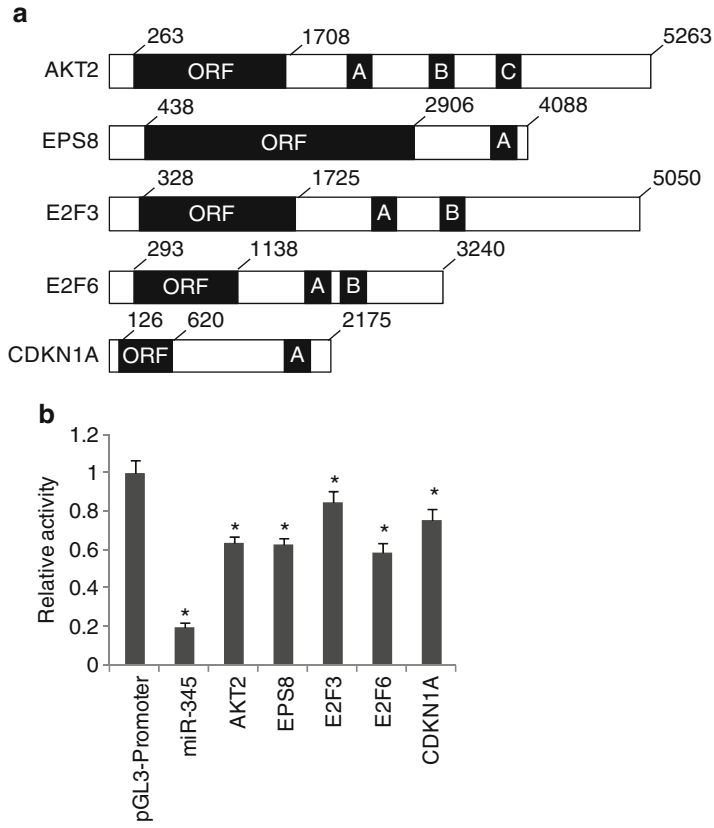


Fig. 23.2 Candidate genes under the control of miR-345. (**a**) Schematic view of predicted and previously reported miR-345-target mRNA. The GenBank accession numbers were AKT2 (NM_001626), EPS8 (NM_004447), E2F3 (NM_001949), E2F6 (NM_198256) and CDKN1A (NM_000389). ORF indicates the open reading frame. In the 3′-UTR, A, B and C represent the locations of TargetScan-predicted sequences that were partially complementary to miR-345. (**b**) miR-345 or negative control RNA were coexpressed with pGL3-Promoter (or the reporter plasmids shown in A) in A549 cells; 24 h later, the luciferase activity was measured. The numbers '345' represent the reporter plasmid with a complementary sequence to miR-345 in the 3'-UTR of the luciferase gene in the pGL3-Promoter. Values were calculated by dividing the luciferase activity of miR-345 by the luciferase activity of the negative control RNA, and the calculated value obtained for pGL3-Promoter was set as 1. The relative activity was shown as the mean ± standard deviation. $*P<0.01$ (n = 3, versus pGL3-Promoter)

B: 3,305–3,327-bp (poorly conserved, 8-mer, 45% match)

- E2F6 (NM_198256)

A: 1,837–1,858-bp (poorly conserved, 8-mer, 32% match)

B: 2,052–2,073-bp (poorly conserved, 7-mer-m8, 32% match)

- CDKN1A (NM_000389)

A: 1,778–1,800-bp (poor conserved, 8-mer, 43% match)

The GenBank accession numbers are indicated in parentheses, and '8-mer' indicates alignment with one to eight nt of the seed region, '7-mer-1A' indicates alignment with one to seven nt of the seed region with the first nt being adenine, and '7-mer-m8' indicates alignment with two to eight nt of the seed region. Within the 3′-UTR of each target gene, an miR-345-recognizable sequence was predicted at three sites (a–c) for AKT2 and at two sites (a and b) for E2F3 and E2F6. We selected the site with the least mismatches. To construct reporter plasmids, we inserted A of AKT2, EPS8, E2F6 and CDKN1A and B of E2F3 into the 3'-UTR of the luciferase gene reporter of a pGL3-Promoter plasmid (Promega, Madison, WI). We also constructed a

reporter plasmid bearing a completely matched complementary sequence to miR-345 downstream of the luciferase reporter gene of the pGL3-Promoter (hereafter referred to as pGL3-Promoter-miR-345). The primers used for each reporter plasmid construct are shown below.

pGL3-Promoter-miR-345
5′-GCTCTAGAGAATTCGAGCCCT
GGACTAGGAGTCAGCTCTAGAGC-3′
5′-GCTCTAGAGCTGACTCCTAGTC
CAGGGCTCGAATTCTCTAGAGC-3′
pGL3-Promoter-AKT2
5′-GCTCTAGAGAATTCGCTCCCTG
GTGAATGGAGTCAGATCTAGAGC-3′
5′-GCTCTAGATCTGACTCCATTCA
CCAGGGAGCGAATTCTCTAGAGC-3′
pGL3-Promoter-EPS8
5′-GCTCTAGAGAATTCTGCCTTTT
TCACATGAGTCAGCTCTAGAGC-3′
5′-GCTCTAGAGCTGACTCATGTG
AAAAAGGCAGAATTCTCTAGAGC-3′
pGL3-Promoter-E2F3
5′-GCTCTAGAGAATTCACTGCGG
GAATGAGGGAGTCAGATCTAGAGC-3
5′-GCTCTAGATCTGACTCCCTCAT
TCCCGCAGTGAATTCTCTAGAGC-3
pGL3-Promoter-E2F6
5′-GCTCTAGAGAATTCCAGTGCTA
AAATTGGAGTCAGATCTAGAGC-3
5′-GCTCTAGATCTGACTCCAATTT
TAGCACTGGAATTCTCTAGAGC-3
pGL3-Promoter-CDKN1A
5′-GCTCTAGAGAATTCTAGCAGCG
GAACAAGGAGTCAGATCTAGAGC-3
5′-GCTCTAGATCTGACTCCTTGTT
CCGCTGCTAGAATTCTCTAGAGC-3

The sense and antisense oligonucleotides listed above were annealed with each other and digested with XbaI (underlined), then inserted into the XbaI site of the pGL3-Promoter. A luciferase assay was performed in accordance with a previously reported protocol (Ohdaira et al. 2010). Briefly, RNA (mature miR-345 or negative control RNA) along with each firefly luciferase reporter plasmid were coexpressed in A549 cells using Attractene reagent (Qiagen, Venlo, Netherlands). First, 0.4 μg of firefly luciferase reporter plasmid and 0.6 ng of *Renilla* luciferase reporter plasmid plus 6 pmol of RNA were incubated with 2 μL of transfection reagent in 60 μL of Earle's modified Eagle's medium (MEM) (Invitrogen, Carlsbad, CA) for 15 min; then, the mixture was directly added to 500 μL of MEM containing 10% fetal bovine serum, with 5×10^4 cells inoculated in each well of a 24-well plate. After 24 h of transfection, 100 μL of passive lysis buffer was added directly to each well and a luciferase assay was performed using the Dual Luciferase Reporter Assay kit (Promega). The light intensity was measured using a GloMax 20/20nLuminometer (Promega). To normalize the transfection efficiency, the firefly luciferase activity was divided by the *Renilla* luciferase activity. The luciferase activity obtained by miR-345 was divided by the luciferase activity obtained using negative control RNA, and the activity relative to a defined luciferase activity of one for the pGL3-Promoter was then used to construct a bar graph (Fig. 23.2b). Consequently, miR-345 reduced the luciferase activity of the pGL3-Promoter-miR-345 to about 20%, compared with that obtained using the pGL3-Promoter (Fig. 23.2b). Remarkably, the luciferase activity obtained using pGL3-Promoter-AKT2, -EPS8, -E2F3 and -E2F6 as well as pGL3-Promoter-CDKN1A were significantly ($*P < 0.01$, $n = 3$) repressed by miR-345, compared with the activity of the pGL3-Promoter (Fig. 23.2b).

Taken together, these results indicate that some miR-345 candidate targets selected by database mining are highly likely to be capable of meeting experimental validation. Therefore, miR-345 is probably involved in the regulation of growth and the induction of senescence in A549 cells through mechanisms targeting AKT2, EPS8, E2F3 and E2F6.

MicroRNA-483-5p Candidate Target Genes

To begin constructing the miR-483-5p expression plasmid (pmR-ZsGreen1-miR483-5p), the genomic region encompassing the miR-483-5p sequence was amplified using nested PCR (polymerase chain reaction) from human genomic

DNA (Promega). The primer sequences are listed below.

First-round PCR:
5′-CCTGTGCCCTCTCTCTTGCC-3′
5′-CTTGGTCCCACAACCAGAGG-3′
Second-round PCR:
5′-GAAGATCTGATGGCACCTGCCCTTTGG-3′
5′-GGGGTACCAGCTTGGACTCTGGCCTGGC-3′

In the second-round PCR primer, the BglII and KpnI recognition site is underlined. The PCR products were digested with BglII and KpnI and inserted downstream of green fluorescent protein (ZsGreen) derived from *Zoanthus* sp. of pmR-ZsGreen1 (TaKaRa Bio, Otsu, Japan). Prior to the establishment of A549 cells that stably express miR-483-5p, we coexpressed pmR-ZsGreen1-miR483-5p and the luciferase reporter plasmid pGL3-Promoter-miR-483-5p, which bears a 100%-match complementary sequence against miR-483-5p in the 3′-UTR of the luciferase gene in the pGL3-Promoter, into A549 cells to ensure that miR483-5p expressed from pmR-ZsGreen1-miR483-5p was functional. pGL3-Promoter-miR-483-5p was constructed in accordance with the method described above and the following primers (XbaI sites are underlined).

5′-GCTCTAGAGAATTCCTCCCTTCTTTCCTCCCGTCTTTCTAGAGC-3′

5′-GCTCTAGAAAGACGGGAGGAAAGAAGGGAGGAATTCTCTAGAGC-3′

Subsequently, we confirmed that miR483-5p expressed from pmR-ZsGreen1-miR483-5p reduced the luciferase activity of pGL3-Promoter-miR-483-5p, compared with that of the pGL3-Promoter (data not shown). To establish A549 cells that stably express miR-483-5p, pmR-ZsGreen1-miR483-5p was transfected using Lipofectamine Plus (Invitrogen) in A549 cells, and 24 h later, 800 μg/mL of G418 (Invitrogen) was added to the culture medium. At the same time, pmR-ZsGreen1 was introduced into the A549 cells to make a control clone. As shown in Fig. 23.3a, we obtained an A549 clone bearing pmR-ZsGreen1-miR483-5p or pmR-ZsGreen1. Subsequently, we performed a WST-1 assay to monitor the cell proliferation rate. The A549 cells that stably expressed pmR-ZsGreen1-miR483-5p showed a decreased rate of cell proliferation, compared with a control clone (data not shown), and this phenotype obtained by miR483-5p expression was consistent with that obtained in glioma cells (Wang et al. 2012). A senescence assay was also performed in accordance with a previously reported method (Ohdaira et al. 2012). As a result, one clone of the A549 cells stably expressing miR-483-5p at the highest level was dominated by β-galactosidase, compared with control cells (Fig. 23.3b). Collectively, analogous to miR-494, miR-483-5p was thought to suppress cell proliferation and to induce senescence in A549 cells. Actually, miR-483-5p induced G0/G1 arrest in glioma cells (Wang et al. 2012).

To further clarify the functional aspect of miR-483-5p, we checked the mRNA expression levels of cancer-associated genes in an miR-483-5p-stably-expressing A549 clone, which was tested using a senescence assay, and a control clone. In the miR-483-5p-stably-expressing A549 clone, the expression level of miR-483-5p was approximately 150-fold higher than that of the control clone, as was quantitatively determined using TaqMan real-time RT-PCR (Applied Biosystems, Foster City, CA). The TaqMan assay was performed according to a previously reported protocol (Ohdaira et al. 2012). Subsequently, we extracted the total RNA and synthesized cDNA, then performed SYBR green real-time RT-PCR (n = 4) using a SmartChip Human Oncology Panel (WaferGen Biosystems, Fremont, CA), which contains approximately 1,000 cancer-associated genes. The mRNA expression values were calculated using GAPDH as an endogenous control, following the comparative *Ct* (ΔΔ*Ct*) method. This approach is based on the concept that miRNA affects gene expression chiefly through mRNA degradation (Guo et al. 2010). For filtration, "not informative" and "primer dimers detected" were omitted, and only mRNA expression changes not exceeding × 0.4 were listed (Fig. 23.3c). To check the reliability of the genes listed by the SmartChip Human Oncology Panel, we confirmed some of the mRNA expression levels using TaqMan real-time RT-PCR. Consequently, the mRNA expres-

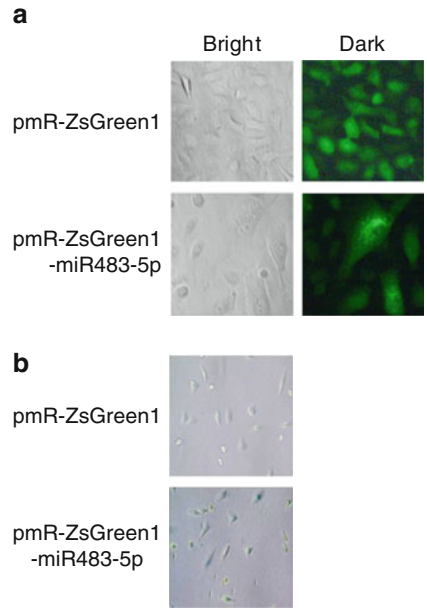


Fig. 23.3 Establishment of A549 cells constitutively expressing miR-483-5p and an expression analysis. (**a**) GFP (green fluorescent protein) was observed using fluorescence microscopy in A549 cells stably bearing pmR-Zs-Green1 and pmR-Zs-Green1-miR-483-5p. The images were photographed (×200) using bright and dark viewing fields. (**b**) β-galactosidase staining to detect senescence in A549 cells stably bearing pmR-Zs-Green1 and pmR-Zs-Green1-miR-483-5p. (**c**) Decreased mRNA in A549 cells stably bearing pmR-Zs-Green1-miR-483-5p, compared with A549 cells stably bearing pmR-Zs-Green1, among 986 cancer-critical genes on a SmartChip. Expression levels less than ×0.4 are shown (n = 4)

c

Gene	Fold change
IGFBP2	0.15
MSH6	0.17
ID2	0.17
ETV4	0.18
RALB	0.21
HSPD1	0.21
MCM6	0.22
CDC25A	0.23
RBL1	0.23
MCM5	0.24
MKI67	0.25
CCND3	0.25
PTMA	0.26
EDN1	0.27
E2F1	0.30
PPP1CA-1	0.30
CCT4	0.31
ALOX12B	0.31
HMGB1	0.31
E2F6	0.31
TGFB3	0.32
HMGB3	0.32
CCNF	0.33
LIG1	0.33
BCCIP	0.35
GNA13	0.35
KPNA2	0.36
FOSL1	0.37
HBEGF	0.37
AR	0.37
CCNA2	0.38
COL7A1	0.38
MAD2L1	0.38
ANGPTL4	0.39

sion level of IGFBP2 (insulin-like growth factor binding protein 2, 36 kDa; TaqMan Assay ID: Hs01040719_m1), ID2 (inhibitor of DNA binding 2, dominant negative helix-loop-helix protein; TaqMan Assay ID: Hs00747379_m1), MCM6 (minichromosome maintenance complex component 6; TaqMan Assay ID: Hs00195504_m1) and MCM5 (TaqMan Assay ID: Hs01052142_m1) in an miR-483-5p-stably-expressing clone were decreased to ×0.036, ×0.137, ×0.188 and ×0.276, respectively, compared with those obtained in a control clone (n = 2). In this experiment, GAPDH was used as an internal control. On the other hand, we detected mRNA expression changes between a stably-miR-494-expressing A549 clone and a control clone using SmartChip. The miR-494-expressing clone has been previously described (Ohdaira et al. 2012). Contrary to the results obtained with the miR-483-5p-expressing A549 clone, only ABR (active BCR-related gene, ×0.092), IL6 (interleukin 6, ×0.225), FOSL1 (FOS-like antigen 1, ×0.306) and HBEGF (heparin-binding EGF-like growth factor, ×0.399) exhibited an expression level less than ×0.4 of the control clone. Taken together, the results obtained by mRNA expression analyses in the miR-483-5p-expressing clone seem to be reliable but should be validated by a functional analysis in the near future.

Finally, a matching operation was performed between the candidate targets predicted by the *in silico* analysis and the gene list identified using

the SmartChip analysis. Remarkably, PTMA (prothymosin, alpha), ALOX12B (arachidonate 12-lipoxygenase, 12R type) and LIG1 (ligase I, DNA, ATP-dependent) predicted by MicroCosm Targets were included in the list obtained by the SmartChip analysis. Whether these genes actually function in the context of miR-483-5p-regulated cell fate determination in A549 cells will be a subject of future examination.

Discussion

Revealing how miRNA is transcriptionally regulated by signal transduction pathways and transcription factors, along with the identification of target genes regulated by miRNA, is a significantly challenging task. Indeed, miRNA expression levels have been extensively analyzed, and some miRNAs have been classified as oncogenic miRNA or antitumor miRNAs (Zhang et al. 2007). We recently identified several miRNAs (miR-494, miR-483-5p, miR-345, miR-139-3p and miR-370), the expression levels of which were uniquely regulated in HRAS, JUN or E2F family member-transfected A549 cells (Ohdaira et al. 2010). HRAS, particularly the V12 mutant, has been shown to play a pivotal role in carcinogenesis (Malumbres and Barbacid 2003), and JUN has been shown to be involved in a variety of functions such as cell proliferation and growth (Dunn et al. 2002). E2F1-E2F6 are known to be strongly associated with the transcriptional regulation of cell cycle regulators, namely E2F1-E2F3, as activator transcription factors, participating in the regulation of the G1 to S phase of the cell cycle to promote cell proliferation, while E2F4-E2F6 principally function as transcriptional suppressors during the G0 and G1 phases (Chen et al. 2009).

Recently, Lee et al. (2011) investigated the role of seven miRNAs, including tumor repressor miR-34a, known to be under the regulatory control of p53, by performing expression analyses for 31 SCLC tumors, 14 SCLC cell lines, and 26 NSCLC (non-small-cell lung cancer) cell lines to reveal the role of these miRNAs in SCLC; they concluded that these miRNAs were not related to SCLC either diagnostically or therapeutically. Additionally, the expressions of the miR-34a target genes cMET and Axl were inhibited in NSCLC cell lines, where miR-34a was overexpressed, but the expression levels of cMET and Axl were unaffected in SCLC cell lines because the endogenous expression levels of cMET and Axl were relatively low (Lee et al. 2011). Other evidence of a common pitfall in miRNA research is that the overexpression of miR-30a and miR-191 had no significant effect on cellular proliferation and the cell cycle in A549 cells, although alterations in the expression levels of miR-30a and miR-191 have been detected in lung cancer using an miRNA microarray (Patnaik et al. 2010). Therefore, any results obtained using expression profiling should be considered with caution; for instance, miRNA identified by expression profiling should be checked in more detail in other cell types and tissues and should be simultaneously tested using a functional analysis to confirm whether miRNA actually exerts a tumor-promoting effect or a tumor-suppressing effect.

Arora et al. (2011) identified miR-328 and reported that the presence of brain metastasis can be detected by performing an miRNA microarray analysis, based on a comparison of analysis results for NSCLC with brain metastasis and NSCLC without brain metastasis. Moreover, they identified an elevation in the gene expression of PRKCA (protein kinase C, alpha) when A549 cells and A549 cells constitutively expressing miR-328 were compared, indicating that PRKCA has a key role in the promotion of cell migration seen in A549 cells constitutively expressing miR-328 (Arora et al. 2011). Consequently, attention should be paid to miRNA target genes with expression levels that are upregulated, probably through an indirect effect of the signal transduction pathway, as well as downregulated directly by miRNA-induced inhibition. In this context, if the miRNA target is a suppressor in a certain signal transduction pathway, a downstream gene of the signal transduction pathway has a high probability of being upregulated. Collectively, these results suggest that a search for signal transduction pathways regulated by miRNA is urgently needed, rather than merely uncovering relationships

between miRNA and its target gene. Currently, numerous miRNA target genes have been identified, even in lung cancer. By coupling bioinformatics tools with these reported individual miRNA targets, signal transduction pathways regulated by miRNA could be predicted, and this would lead to a better understanding of the miRNA-centered gene regulation underlying carcinogenesis. Thus, investigations of the effects of miRNA on signal transduction pathways are is likely to be beneficial to widespread clinical applications.

In this chapter, we demonstrated that the miR-483-5p target genes predicted by five different algorithms were at least partially matched with genes whose expression levels were significantly suppressed in A549 cells constituently expressing miR-483-5p. We also confirmed that some of the miR-345 target genes predicted by the database search and that were likely to be involved in the regulation of cellular proliferation were actually downregulated by miR-345 in A549 cells, based on results obtained using a luciferase reporter assay. The further combination of *in silico* analyses and *in vitro* experiments is expected to be useful for validating the biological activities of miRNA in various cell types and tissues.

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Role of Senescence Induction in Cancer Therapy

24

Hiroshi Kondoh, Takumi Mikawa, and Matilde E. LLeonart

Contents

Abstract

Cellular immortalization is a crucial and early step during the development of cancer, while normal primary mammalian cells reach replicative limitation after several passages in vitro, called replicative senescence. Senescent cells have altered cell morphology and gene expression patterns with preserved metabolic activity, which are quite distinct from others. Interestingly, senescent cells have also been detected in vivo, particularly in benign lesions of human tumors. Senescence would constitute a protective barrier against cancerous immortalization. In other words, during tumorigenesis, cancer cells acquire genetic alterations to override senescence. By using high throughput genetic screening to search for genes involved in senescence, several candidates for oncogenes and putative tumor suppressor genes have been recently isolated, including subtypes of microRNAs. These findings offer new perspectives in the senescence biology and open new avenues for cancer therapy.

Keywords

Senescence induction • Cancer therapy • Cellular immortalization • Tumorigenesis • Telomere • Telomerase • Oxidative stress • Oncogene-induced senescence (OIS) • CDK inhibitors • miRNA

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Introduction

It is well known that about one-fourth of populations in the developed countries experience cancer during their life time. It would be useful to understand cancer biology from various aspects for any diagnostic or therapeutic development against it. Indeed, cancer cells display several biological hallmarks that can be distinguished from those of normal counterparts. These properties include cellular immortalization, evasion of apoptosis, growth factor independence, metabolic adaptation to hypoxia and low nutrients, anchorage-independence, resistance to contact inhibition, tumor-angiogenesis, degradation of matrix components, invasion, migration, and so on. Each of these properties might be a possible good candidate as therapeutic target for anti-cancer drugs. For example, DNA damaging agents might work as apoptotic inducer, while anti-angiogenic compound might be useful to block the growth of feeding vasculature in the solid tumors.

One thing which we should notice very carefully is the fact that malignancy would be established through multi-step genetic events. Once established, or rather earlier than its establishment, cancer cells might start to display mutator phenotype, which would contribute to the occurrence of additional genetic alterations in cancer. Moreover, recent advance in understanding cancer stem cells, suggests that even in solitary tumor, cancer cells might involve different populations, stem-like cells and others. These complexity both in their genetic background and cytological populations might confer the resistance against single anti-cancer therapy on cancer cells. In this sense, it would be of great importance to innovate a novel approach for cancer therapy or to explore the combination among conventional and newly-developed ones.

One of the possible new targets for this purpose is cellular senescence. The recent findings in senescence biology implicates senescence-induction might be alternative choice for cancer therapy, which would be overviewed here.

Physiological Importance of Senescence in Tumorigenesis

It has been interminably speculated that incipient cancer cells must breach senescence barrier to acquire their infinite proliferative potential. In this sense, the cellular senescence observed in tissue culture might have been expected to constitute a good model for understanding the significance of this barrier in vivo. Under normal tissue culture condition, primary mammalian cells reach proliferative exhaustion after serial passages, resulting in a permanent and irreversible cell-cycle arrest with metabolically active state (Hayflick and Moorhead 1961). This process, called replicative senescence, is characterized by a drastic phenotypic change in the cells, compared to their proliferating counterparts at early passages. Senescent cells are characterized by following properties; expression of β-galactosidase, over expression of PAI-1 (plasminogen activator protein 1), altered cell morphology with giant cell size, increased cytoplasmic granularity and a single large nucleus, and characteristic transcriptional prolifes that distinguish them from quiescent cells (Shelton et al. 1999). Thus it was clear that senescent cells display the distinct phenotype from others.

But it has been not clear how much senescence in vivo would be relevant to tumor biology and cancer until recent breakthrough. First, recent attempts of genetic screening to isolate senescence-bypassing clones have identified several putative novel oncogenes and tumor suppressor genes. Genes whose ectopic expression would immortalize primary cells are considered as potential oncogenes and are over expressed in some types of cancer. On the other hand, genes whose inactivation results in cellular immortalization are potential tumor suppressor genes(Berns et al. 2004) and are found to be down regulated in tumor-resident tissues (LLeonart et al. 2006). Second, senescent cells have recently been detected during tumorigenesis in mouse models and in human tumors, particularly in benign lesions (Collado et al. 2005)

and their appearance is rather relevant to tumor progression. There are examples of senescent cells in vivo that may reside for years in tissue, such as the senescent melanocytes of moles of nervi. Other senescent cells can be rapidly removed as tumors progress. As senescence is now considered to be essential physiological barrier against tumorigenesis, the emerging question is whether senescence-induction in cancer might be possible or not as therapeutic approach.

Telomere and Telomerase

Telomere regulation would be the first topic to discuss as a target of senescence induction. The length of telomeric DNA at the end of chromosomes decreases progressively in primary cells as they replicate. Human telomeres consist of tandem repetitive arrays of the hexameric sequence TTAGGG, while overall telomere sizes range from 15 kb at birth to less than 5 kb in old individuals. The ends of telomeres are protected and regulated by telomere-binding proteins and form a special lariat-like structure, called the T-loop. Telomerase, a cellular ribonucleoprotein enzyme, is composed of a number of distinct subunits responsible for adding telomeric DNA repeats to the 3′ ends of chromosomes. It has two major components, a transcriptase catalytic subunit hTERT, and its RNA component hTERC. Telomerase uses its integral RNA component as a template in order to synthesize telomeric DNA directly onto the ends of chromosomes. It was observed that normal primary human cells ectopically expressing telomerase could divide indefinitely, providing direct evidence that telomere shortening has a causal effect on replicative senescence (Bodnar et al. 1998). The telomerase enzyme is normally expressed in very few primary cells, such as embryonic stem cells, adult male germ line cells and proliferative stem cells in renewal tissues.

Telomerase activity is detected in approximately 90% of all malignant tumors in comparison with their normal counterparts. Interestingly, some viral oncoproteins are able to modulate telomerase expression, while inhibition of telomerase limits the growth of human cancer cells. This suggests that telomerase may be a good target for anti-cancer drugs. It was demonstrated that siRNA against telomerase or over expression of a dominant-negative mutant of telomerase abolished telomerase activity and resulted in entry of cells into crisis (Hahn et al. 1999). Several clinical trials targeting telomerase are ongoing in cases of advanced cancer patients. These include immunotherapy (a vaccine against telomerase), inhibitory compounds against telomerase activity (a telomerase template antagonist),and the modulation of telomeric structure (telomestatin).

The expected outcome of telomerase inhibition would be cellular-context dependent. As the tumor suppressor p53 is presumed to sense dysfunctional telomeres as damaged DNA, accelerated telomeric shortening by telomerase inhibitors could activate the p53 pathway, followed by initiation of massive apoptotic death in p53-intact cells. Or possibly in p53-inactivated cells, telomerase inhibition might slow tumor growth or induce senescence-like phenotype.

Moreover telomerase activity is a useful prognostic indicator of some cancers, for example, neuroblastomas that are usually encountered in very young children. Telomerase activity should be incorporated into the clinical investigation of each individual neuroblastoma at the time of diagnosis because its mere presence is sufficient basis for predicting disease outcome (Poremba et al. 2000). Therefore, neuroblastomas would be a good target for the above-mentioned telomerase inhibitors.

Oxidative Stress and Glycolytic Inhibitor

The second topic would be focusing on senescence-inducible stress. Recent reports suggest that cellular senescence is triggered not only by telomeric erosion but also by other forms of stress. Several factors provoke premature senescence in a telomere-independent manner, which is designated as stress-induced senescence (SIS). SIS can be induced by several stress, including DNA damage, ionizing radiation such as Xraysor UV, oxidative stress, culture conditions, histone

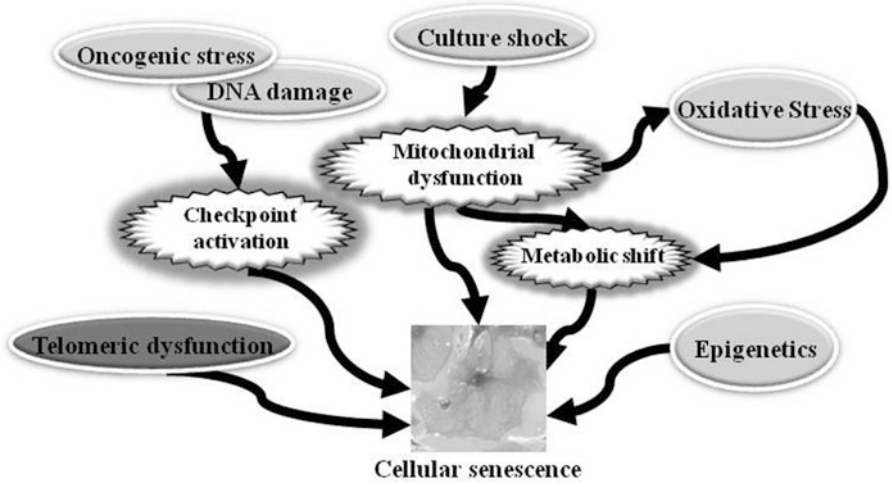

Fig. 24.1 Stress-induced senescence (SIS). In addition to telomeric dysfunction, cellular senescence is induced, even at early passages, by several stress including onco-gene-expressions, oxidative stress, DNA damage, culture chock, epigenetic change, and so on

deacetylase inhibitor, oncogenic insults and so on (Fig. 24.1). Senescence-inducible stress could provoke senescence even in telomerase-expressing cells, implicating the possibility that these stress could be applicable to anti-cancer therapy.

Among others, oxidative stress is noted as a key of ageing, since the proposal of free radical theory by Harman (1956). Cumulative oxidative damage causes or accelerates senescence, while immortalized cells are resistant to the senescence effect of oxidative damage. The most striking observations on senescence effect of oxidative damage have been reported when cells have been exposed to different oxygen concentrations in tissue culture. When oxygen is reduced from 20% to 1–5%, human diploid fibroblasts increase their replicative life-span between 20 and 50% (Chen et al. 1995). In this sense, the modulation of oxidative stress could be another target of anti-cancer drug.

Now the rising issue is how oxidative stress would be modulated as cancer therapy. It has been reported that enhanced glycolysis via ectopic expression of a glycolytic enzyme (phosphoglycerate mutase; PGM) immortalizes primary MEFs, protecting them from oxidative damage (Kondoh et al. 2005). Enhanced glycolysis is a well-known property of most cancerous cells and tissues, commonly referred to as the Warburg effect. This property is well utilized in clinical practice for the detection of metastatic tumor mass through positron-emission scanning of 2-[18F]fluoro-2-deoxy-D-glucose. Thus, inhibition of glycolysis may represent an alternative therapy for cancer treatment. PGM inhibition in primary MEFs induced premature senescence. Indeed, the PGM inhibitor MJE3 has been identified as the most potent anti-proliferative reagent via chemical screening in breast cancer cells (Evans et al. 2005). Currently several glycolytic inhibitors with promising results in cultured cells and animal models are being tested in clinical trials. For example, studies on 3-Bromopyruvate (3-BrPA) is under clinical trials (I/II)0.3-BrPA inhibits hexokinase causing a depletion in ATP leading to massive cell death in treated cells with respiration defects. Another compound is 2-Deoxyglucose(2-DG), now under clinical trials (II/III). 2-DG is an agent that is phosphorylated by hexokinase, but cannot be further metabolized via glycolytic pathway, resulting in blockade of glucose metabolism. 2-DG has shown promising results in combination with adriamycin and paclitaxel in mice bearing human osteosarcoma and no small-cell lung cancer xenographs (Maschek et al. 2004). Collectively, glycolytic inhibition with modulation of oxidative stress would be a good tool for cancer treatment in the future.

Oncogene-Induced Senescence (OIS) and p53

Another well-characterized example of stress-induced senescence is the response of normal primary fibroblasts to expression of H-ras with activated allele (H-rasV12), called oncogene-induced senescence (OIS). RAS protein is mutated into constitutively active forms among approximately 20% of human cancers. While these activated alleles contribute to transformation in human cancer by increasing proliferation and invasiveness of tumors, as well as desensitizing cells to apoptosis, human normal primary cells enter into senescence in response to oncogenic RAS. Several other oncogenes are also known to induce such a senescence response upon over expression: raf, MEK,Akt, E2F1/3, mos, PTEN, NF1, Stat5, KLF-4, Runx and so on.

It is of note that SIS and OIS in primary cells can be bypassed partially by inactivation of tumor suppressors, including the p53 orRb axis, implicating the involvement of several tumor suppressor genes in these processes. Thus the contrast behavior against oncogenic stimuli between cancer and normal primary cells could be partly explained by the different status in tumor suppressor genes. In cancerous cells or tissues, tumor suppressor genes may be inactivated by any means; deletion of one or both alleles, promoter methylation, splice-site mutations, nonsense mutations, or a combination of these. Alternatively, mutations in tumor suppressor genes can provide a dominant negative protein that interferes with the wild-type protein produced by the other allele, as is the case for several p53 mutants. Such genetic alterations result in a complete absence or partial reduction of the tumor suppressor protein and their functions. Although the careful evaluation for the status of tumor suppressor genes would be required in clinical patients, tumor suppressor activation can be another target of cancer therapy in vivo.

Promising results have been recently reported in a murine model where complete tumor regression could be provoked by p53 activation (Martins et al. 2006). In vitro restoration of p53 function triggers dramatic and rapid induction of p53 target genes, as well as apoptosis. While several groups have reported similar effects, it is not just p53 status itself that determines therapeutic efficacy of p53 restoration, but also the status of p53-activatingsignals that pre-exist, or can be induced, in tumor cells. Although this situation would not be exactly the same as that which occurs in human cancer (where p53 is affected or the p53 pathway is corrupted), regression of these tumors was closely correlated with the presence of senescent cells. Thus, these studies provide the first evidence that senescence induction in vivo can be a crucial mechanism of tumor clearance. It would be worthy to further investigate whether p53 activating chemicals, such asquiacrine, might be beneficial as a tool of senescence-induction for cancer patients.

Other Tumor Suppressor Pathways and CDK Inhibitors

The ARF/INK4a (Alternative Reading Frame) locus encodes two distinct tumor suppressors, ARF (p14ARF in humans and p19ARF in mice) and p16INK4A. p16INK4A and ARF respectively regulate the RB and p53 pathways of senescence and tumor suppression. p16INK4A binds and inhibits cyclin-D-dependent kinases CDK4 andCDK6. Such kinases have oncogenic potential because they phosphorylate the retinoblastoma family of tumor suppressors Rb, p107 and p130, which are negative regulators of the cell cycle (Fig. 24.2). ARF is an antagonist forMdm2, which regulates p53 stability through its ubiquitin ligase activity. ARF sequesters Mdm2 through its translocation to the nucleolus and the final consequence is p53 activation and stabilization. During replicative senescence in vitro, the accumulation of these tumor suppressors (p53, ARF, INK4A) or their downstream targets (p21Cip1) is observed (Adams 2007). Oncogenic insults also upregulate these genes. It is noteworthy that the ectopic expression of these tumor suppressors can provoke senescence-like phenotype both in primary and immortalized cells.

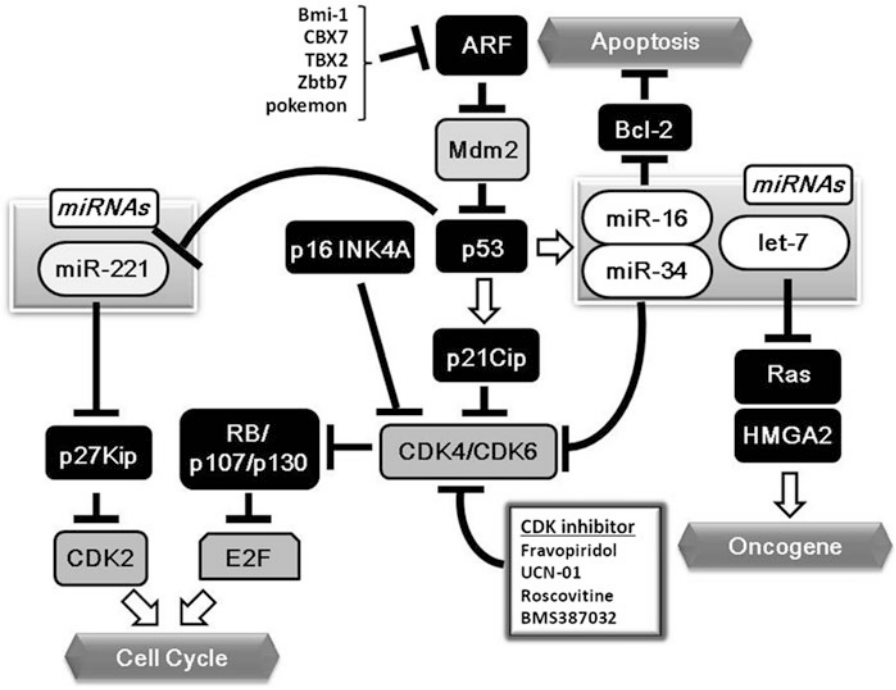

Fig. 24.2 Tumor suppressor pathways related to senescence. There are two major tumor suppressor pathway, p53 and RB axis. Several other tumor suppressors are also implicated in these pathways. Both pathways partly target cycline-dependent kinase (CDK4 /6). In addition, p53 regulates several mi-RNAs. miR-34, miR-16 and let-7,induce apoptosis, cell cycle arrest and oncogene. miR-221 releases cell from cell cycle arrest via down regulation of p27. See the text in details how these tumor suppressors affect senescence process

Thus all of these senescence-inducing tumor suppressor genes may also be targets of anti-cancer drugs.

Several ARF transcriptional repressors have been identified, includingBmi-1 (Jacobs et al. 1999), CBX7 (Gil et al. 2004), TBX2 (Jacobs et al. 2000), Zbtb7 or pokemon (Maeda et al. 2005). INK4 is known to be regulated by the Ets transcriptional family. These regulators for ARF/INK4 might be good candidates for anti-cancer drugs through modulation of their senescence-bypassing effect. However, as is often the case with p53, the inactivation of the ARF/INK4a locus by complete deletion or by aberrant promoter methylation is very common, being present in about 30% of all known types of malignancies. Presumably, in cancer cells harboring genetic inactivation of this locus, the modulation of ARF/INK4 function would be less effective as cancer treatment. Alternatively, a CDK inhibitor may be effective, as both the ARF/p53 and INK4/Rb axis exert their tumor suppressing function partially via inactivation of CDK4. Several small chemical modulators for CDK kinases are currently under investigation in clinical trials. So far, these are notCDK4-specific inhibitors, but rather pan-CDK inhibitors, such as Fravopiridol, UCN-01, Roscovitine, and-BMS387032. The anti-cancer effects of these compounds mainly result in induction of apoptosis rather than provocationof senescence. This is possible because CDKs playadditional and essential roles beyond cell cycle control, including transcription, DNA repair, migration, and secretion.

Micro-RNAs with Senescence-Bypassing Ability

Micro-RNAs (miRNA) are a novel class of non-encoding genes that have appeared to play an

important role in the post-transcriptional regulation of gene expression. Mature miRNAs are double-stranded RNA products of several processing steps inhibited by PolII-transcription. Finally one of the mature RNA strands would be loaded into the RNA-induced silencing complex (RISC) that subsequently binds to a specific mRNA with a certain degree of complementarity, often in the 3'UTR of the target mRNA. Depending on the degree of complementarity, miRNAs are involved in sequence-specific degradation of mRNAs or inhibition of protein translation (He and Hannon 2004). One miRNA can control the expression of many different genes, and it is speculated that 20–30% of total gene expression may be regulated by miRNAs. Bioinformatics approaches have identified 300human miRNA genes, but more recent work has predicted the number to be closer to 1,000 (Berezikov et al. 2005).

Recent studies have shown that profiles of miRNA expression are different between normal and tumor tissues (He and Hannon 2004; Berezikov et al. 2005). Interestingly, down regulation of subsets of miRNAs is common finding in some tumors. The discovery that miRNA silencing could revert the tumorigenic phenotype of the colon cancer cell line HCT116 unveils its novel regulatory mechanism in cancer proliferation (Lujambio et al. 2007). Recently, several laboratories have reported that members of the miR-34 family are direct targets of p53, which induces apoptosis, cell cycle arrest and senescence (He et al. 2007). miR-16 and let-7 are significantly induced by p53,with reduction of the oncogene bcl-2,ras and HMGA2. Among the mi-RNAs repressed by p53, miR-221 downregulatesp27 (Fig. 24.2).

In a genetic screen to identify miRNAs characterized by their ability for bypassing oncogenic ras-induced senescence (OIS), miR-372 and miR-373 were cloned (Voorhoeve et al. 2006). This study was performed in partially immortalizedIMR90 fibroblast. These miRNAs are considered as novel-oncogenes participating in the development of human testicular germ cell tumors by numbing the p53 pathway and promoting tumorigenic growth in the presence of wild-type p53. Importantly, the fact that miR-373 expressing cells could form foci in soft-agar assays shows its transforming capability. On the contrary, introduction of miR-34a andmiR-34b/c into primary human diploid fibroblasts induced premature senescence. Tumor cells also showed senescent phenotypes after introduction of ectopic miR-34a (He et al. 2007). Down regulation of miR-138 is associated with over expression of telomerase and the acquisition of malignant behavior in human anaplastic thyroid carcinoma cell lines (Mitomo et al. 2008). Thereby, it is expected that targeting miRNAs would-be useful as a diagnostic tool or might contribute to the development of new strategic treatments for specific kinds of carcinomas.

Epigenetic Cancer Therapy

Epigenetic changes in wild-type mammalian cells are a dynamic feature forth modulation of gene expression. Examples of epigenetic changes would include the methylation of DNA, the deacetylation, the ubiquitination, the phosphorylation of histone and so on. DNA methylation is considered to play an essential role in cellular senescence and aging. It has been demonstrated that the methylation level in the genome decrease gradually during SIS as well as replicative senescence. This has been associated with the reduction in the expression of the methylation enzymeDNMT1. Such changes reflect global hypomethylation as a distinct feature of senescent cells (Zhang et al. 2008), while there are slight differences in the expression profile of methylation-associated enzymes between SIS and replicative senescence.

As aging is also accompanied by changes in DNA methylation in tumor suppressor genes, the pattern of altered gene expression or epigenetic changes is of major importance in common malignancies. DNA methylation patterns are severely affected in cancer.p16INK4Ais one of the most important tumor suppressor gene altered in human cancer. Methylation of the p16INK4A promoter implies p16INK4A silencing and subsequently the loss of the p16INK4A protein. For example p16INK4A inactivation by promoter methylation is one early

event that contribute to HMEC immortalization (Hinshelwood et al. 2007). p16INK4A promoter methylation occurs in specific cancers, such as breast and hepatocellular carcinoma. Importantly, promoter hypermethylation of the p16INK4A gene is associated with poor prognosis in recurrent early-stage hepatocellular carcinoma. Interestingly, although a great number of tumor suppressor genes are hypermethylated in regions rich in CpG island in cancer, the general pattern of the cancer genome is a global DNA hypomethylation.

It has been recently identified that in a loss-of-function genetic screening, inactivation of the methylation enzyme S-adenosylhomocysteine hydrolase (SAHH) immortalizes primary murine cells affecting both p53 and pRb pathways (Leal et al. 2008). In addition, it was observed that SAHH was altered in human tumors at mRNA and protein levels, suggesting that it could be a putative novel tumor suppressor gene (Leal et al. 2008). The fact that SAHH could modulate senescence reinforces the importance of methylation enzymes in immortalization and cancer development. In order to stimulate the expression of those tumor suppressor genes silenced in cancer, much attention has recently been focused on developing small molecule inhibitors of DNA methyltransferases that could be used as anti-cancer drugs, such as 5-Azacytidine and 5′-Aza-2′-deoxycytidine (decitabine). Surprisingly, they have been quite effective in case of leukemias, but not so successful in case of solid tumors. It was observed that p53 function was restored by treatment with 5-Aza-2′-deoxycytidine, suggesting that epigenetic cancer therapy is possible for some cancers in combination withp53 activation (Yagi et al. 2008).

Discussion

Cellular senescence has become an attractive therapeutic concept. Importantly, the therapeutic potential of senescence-induction strongly relies on the irreversibility of this process. As an intact apoptotic machinery is unavailable in most established malignancies, a senescence-induction mechanism emerges as a back-up program to substitute for or to reinforce an insufficient apoptotic response. It would be tempting to speculate that the combination of a senescence-induction and a conventional therapy might cooperate to abolish cancer.

The rising future question is how cancer could escape from senescent state. Do cancer eliminate senescent cells? Or do senescent cells transform into immortalised state? These are quite difficult to answer so far. It is possible that senescent cells will ultimately be cleared by phagocytosis. For example, senescent neutrophils might face phagocytosis through an unknown mechanism (Murphy et al. 1998), while aged human fibroblast arrested in replicative senescence might eliminate themselves by autophagy. But almost nothing is known about how much this senescence clearance is relevant to cancer progression.

If senescent cells are able to reside for years in tissue, an epigenetic change or others might help cells to escape from senescence. Therefore future research should also focus on discovering senescence markers that can be used for monitoring the presence of senescent cells in vivo. As described here, recent progress in the biology of cellular senescence provides another clue to understanding the mechanism of cancer progression, as well as to development of new anti-cancer drugs. This fact suggests that senescence-inducing mechanisms might be applicable as cancer therapies in the future.

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Cellular Senescence Limits the Extent of Fibrosis Following Liver Damage

25

Valery Krizhanovsky

Contents

Abstract

Cellular senescence is emerging as a mechanism that limits tissue damage. In most tissues of the body, following short-term damage this response aims to ensure the return to the pre-damaged state. This novel physiological role of cellular senescence is additional to its well-established function as a tumor suppressor. In a mouse model of liver fibrosis, cellular senescence limits fibrosis progression by several mechanisms. Cell cycle arrest limits expansion of activated hepatic stellate cells, senescent cells produce less extracellular matrix and more enzymes that degrade it, and the immune system specifically recognizes and eliminates senescent cells, returning the liver tissue to its normal state. The mechanisms responsible for interaction with the immune system are part of the senescence phenotype and are needed to prevent the long-term destructive effects of senescent cells. Initially cellular senescence is beneficial, as it limits tumorigenesis and tissue damage, but when senescent cells persist in tissues they contribute to tissue ageing and potentially promote tumorigenesis in their microenvironments. The coordinated molecular pathways governing the induction of senescence followed by elimination of senescent cells seems to be a product of evolutionary selection as a program that protects the organism from a variety of internal and external threats, while also preserving the organism's integrity. Understanding

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of these molecular pathways might eventually be exploited therapeutically by elimination of senescent cells in order to treat a variety of fibrosis-related conditions, prevent cancer, and delay tissue ageing.

Keywords
Cellular senescence • Fibrosis • NK cells • Immune surveillance

Introduction

Cellular senescence, a program of terminal cell cycle arrest, is a mechanism that limits tissue damage after short-term injury. When tissue is injured, a rapid initial proliferation of cells is needed to replace the damaged tissue. Following this stage however, cell proliferation should be terminated and excessive cells removed to ensure integrity of the repaired tissue and its return to its pre-injury structure. Cellular senescence stops the unfavorable continuation of cell proliferation, thereby providing a way to limit cell number.

For many years, since its discovery by Leonard Hayflick (Hayflick and Moorhead 1961), cellular senescence was considered an in-vitro phenomenon and its relevance in vivo to human disease and aging was not fully recognized. Evidence accumulated over the last decade has shown, however, that cellular senescence prevents tumorigenesis, limits short-term tissue damage, and promotes tissue aging. The most fully described function of senescence in vivo is its tumor suppressor function. Senescence limits the oncogenic transformation of cells in response to various triggers with cancer-promoting potential, such as activated oncogenes, DNA damage, and oxidative stress. These triggers activate pathways regulated by p53 and p16-Ink4a (formally CDKN2A) proteins, the main molecular pathways of senescence (Collado et al. 2007; Adams 2009; Kuilman et al. 2010). The role of cellular senescence in limiting tumorigenesis in response to these stimuli has been described in numerous types of tumors in human patients and in mouse models. Senescence thus appears to be a major mechanism for tumor suppression.

Such tumor suppression, however, comes at a price. While the senescent state prevents tumorigenesis, it is also associated with the age-related limitations in tissue regeneration and stem-cell proliferative capacity. Moreover, senescent cells that accumulate in tissues can promote damage to neighboring cells and induce inflammation in their microenvironment as a result of their increased secretion of various cytokines and chemokines. This increase in secretion, producing a senescence-associated secretory phenotype (SASP), is an integral part of the phenotype of senescent cells (Campisi and d'Adda di Fagagna 2007) and can contribute to disease development and ageing (Burton 2009). If we assume that cellular senescence is a robust program, the question of its evolutionary selection arises. Tumors are late events and therefore their effect on reproduction would not be sufficient to influence selection. Senescence can therefore be assumed to play a wider role in organisms, justifying its evolutionary selection.

This more general role of cellular senescence has to do with limiting the tissue damage that occurs in response to a short-term injury, and ensuring repair of the tissue and its return to the pre-injury state. Four years ago we introduced the novel concept of this functional role of senescence and demonstrated it in a model of liver fibrosis (Krizhanovsky et al. 2008), a syndrome caused by hepatitis viruses B and C, nonalcoholic steatohepatitis (fatty liver disease), chronic alcohol abuse, or exposure to aflatoxins (Bataller and Brenner 2005). Fibrosis represents the initial stage of the liver disease that may develop in response to these stimuli. If the damaging stimulus—such as viral infection—persists, the disease becomes more severe and may progress to cirrhosis, which is a significant health problem worldwide (Bataller and Brenner 2005). Cirrhosis, one of the most common diseases of the liver, is characterized by changes in liver architecture and a significant decrease in liver function. However, if the damaging stimulus is not permanent the fibrosis is likely to resolve without developing into cirrhosis.

How do viruses and toxins cause fibrosis? Hepatocytes, the most abundant cell type in the

liver and responsible for performing all of the liver's functions, are their main targets. Direct damage to these cells causes their death. Molecules released as a result of hepatocyte death cause activation of Kupffer cells, the resident macrophages of the liver. Molecules released from the dying hepatocytes and activated Kupffer cells signal to activate hepatic stellate cells (HSCs). In the normal liver, HSCs, also known as Ito cells, are quiescent. They are located in the areas adjacent to blood vessels, contain numerous fat droplets, and represent the main sites of vitamin A storage in the organism (Bataller and Brenner 2005; Friedman 2008). The embryonic origin of the HSCs is not completely understood. These cells express mesenchymal genes such as alpha smooth muscle actin, desmin, ICAM-1 and vimentin, as well as neuroectodermalgenes such as glial fibrillary acidic protein (GFAP), synaptophysin and nestin (Friedman 2008). It is possible that the HSC population is heterogeneous and versatile.

During acute liver damage, signals from dying hepatocytes and activated Kupffer cells activate the HSCs, causing them to differentiate into myofibroblasts. The activated HSCs proliferate extensively, migrating through the liver to form fibrotic scars and depositing extracellular matrix (ECM) in the scars. If the damage is chronic or repetitive, expansion of activated HSCs causes scarring of the liver tissue due to the excessive ECM produced. The production and deposition of ECM is the hallmark of fibrosis and cirrhosis in the liver. The scars are used to evaluate the extent of fibrosis in mouse models and in human patients (Bataller and Brenner 2005; Friedman 2008). The presence of numerous fibrotic scars supports inflammation and interferes with the liver's normal function. This might eventually lead to liver cirrhosis, with is severe destruction of the liver cytoarchitecture. This in turn could eventually lead to liver failure, for which the only therapeutic option is liver transplantation (Bataller and Brenner 2005).

Liver fibrosis and cirrhosis are not only deleterious pathophysiological conditions in themselves, but are also among the main risk factors for developing hepatocellular carcinoma (HCC). This is the most frequent type of liver cancer, and the sixth leading cause of cancer-related deaths in the United States. Recent studies indicate that about 90 % of patients with HCC have a history of severe fibrosis, cirrhosis, and unresolved inflammation, irrespective of the underlying cause of liver disease (Elsharkawy and Mann 2007). Therefore, limiting fibrosis is helpful not only in protecting and sustaining proper liver function but also in preventing the development of liver cancer. This points to the likely establishment, in the course of evolution, of a mechanism that would protect the liver from uncontrolled fibrosis after short-term insults and ensure its return to the normal, pre-damage state. Cellular senescence of activated HSCs is just such a mechanism. It is important, therefore, to understand how cellular senescence limits the progression of fibrosis so that new strategies can be developed to treat this condition and prevent its long-term deleterious effects.

Senescence of Activated Stellate Cells Limits Liver Fibrosis

Our health is constantly in danger from the frequent invasion of our bodies by noxious substances that are secreted by microorganisms in food and drinking water or discharged into the air as a result of environmental pollution. The liver is the organ that protects the body from these potentially harmful compounds (Bataller and Brenner 2005), which accumulate in the liver and are degraded in the hepatocytes. Some hepatocytes might die as a result of this process, and their death serves as a signal for activation of HSCs in their environment. Because such activation might initiate liver fibrosis, proper control of the expansion of activated HSCs is necessary to sustain liver function.

The activated HSCs formed as a result of short-term damage need to be under surveillance to ensure liver integrity and functionality. Here molecular mechanisms of senescence come into play. Intensive proliferation is likely to be followed by an accumulation of damaged DNA in the activated HSCs. Such damage might also

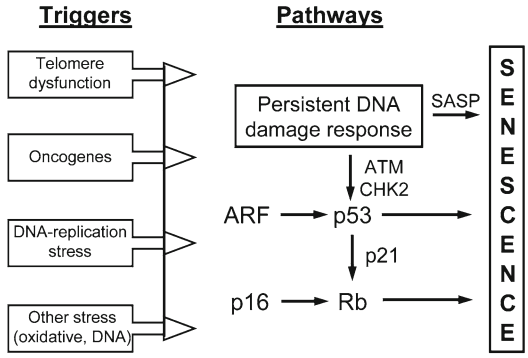

Fig. 25.1 Triggers and pathways of cellular senescence

result from the signals that initiate HSC activation. For example, transforming growth factor-β or AKT signaling and reactive oxygen species (ROS), all of which contribute to HSC activation, can cause cellular senescence under certain circumstances. Therefore, activation and intensive proliferation of HSCs eventually leads to activation of the molecular machinery of senescence.

Cellular senescence can be induced by several types of damage or stress, for example by dysfunctional telomeres, activated oncogenes, or chemotherapeutic agents, and is characterized by an irreversible blockade of proliferation, which is maintained in either the G1 or the G2/M stage of the cell cycle (Collado et al. 2007). A major regulator of senescence is the machinery of DNA damage, which generates a persistent DNA damage response (d'Adda di Fagagna 2008). Along with the DNA damage two tumor suppressor pathways, controlled by p53 (TP53) and p16, regulate the senescence response (Fig. 25.1). Inhibitors of cyclin-dependent kinases 2 and 4, p16 and p21 (which is the target of p53), prevent Rb phosphorylation and promote formation of repressive heterochromatin to silence proliferation-associated genes (Narita et al. 2006; Collado et al. 2007). Interestingly, the alternative reading frame from the CDKN2A locus, termed ARF, regulates the stability of the p53 protein by inhibiting MDM2,an ubiquitin ligase that ubiquitinates p53 and thus leads to its degradation. Therefore, the regulation of two main pathways of senescence can be ascribed to a single genomic locus. The two pathways regulated by p53 and p16 act concomitantly, and their relative contributions might differ in a cell-type-dependent manner.

Disabling the Molecular Machinery of Senescence Augments Fibrosis Progression

To understand how the intact senescence machinery contributes functionally to the progression of fibrosis, a mouse model was developed (Krizhanovsky et al. 2008). Fibrosis in this model was induced chemically by genetic ablation of central elements of the molecular machinery of senescence. Initially, two different models were used to disable the senescence machinery. Fibrosis was developed both in mice harboring knock-out alleles of p53 or in mice engineered for knockout of the entire genomic locus around CDKN2A gene, including both p16 and ARF. Experiments showed that compared with wild-type controls, mice with knockout of either p53 or p16/ARF exhibit increased accumulation of ECM in liver fibrotic scars and therefore more extensive scarring, indicating advanced disease progression. It thus seems important that in order for an organism to limit the progression of fibrosis, the senescence machinery should be intact.

The p53 and p16 pathways can be activated in cells under several different conditions. However, the combinatorial contribution of these pathways is more characteristic of cellular senescence than of any other cellular state. It was therefore necessary to disable both pathways in order to evaluate their combined contribution to the progression of fibrosis. To achieve this, p53 knockout and p16/ARF knockout mice were crossed, resulting in a mouse model in which both were knocked out, meaning that three main players of the senescence machinery were absent (Fig. 25.1). HSCs derived from these mice were unable to senesce in culture, and instead proliferated without limitation. The most striking outcome of this cross was the mouse phenotype observed after fibrosis induction. Fibrosis in these mice developed much more strongly, compared not only with the wild type but also with mice lacking only p53 or only p16/ARF. Their fibrosis was so severe that the

fibrotic scars were interconnected, a sign of progression from fibrosis to cirrhosis. Therefore, inactivation of both of the central molecular pathways of senescence in vivo led to a synergistic increase in the severity of fibrosis, confirming that the progression of fibrosis is indeed limited by senescence.

The next important question is whether the effect of the knockdown in the whole body of p53 or p16/ARF or both on fibrosis is mediated by the activated HSCs. This question was addressed by inducing knockdown of the relevant genes only in HSCs in the liver. This cell-specific knockdown was achieved by combining two mouse models. In one, HSC-specific expression of the tetracycline transactivator protein tTA ('Tet-Off') was achieved from the GFAP promoter (GFAP-tTA mice). These mice were crossed with mice expressing short hairpin RNA (shRNA), which efficiently targets p53 (shRNA-p53) from the tet-responsive element promoter (Dickins et al. 2007). The tTA protein binds the tet-responsive promoter in the absence of tetracycline and induces expression of the transgene. In mice of the combined genotype, shRNA-p53 was expressed in the liver only in activated HSCs, and resulted in p53 knockdown in these cells. When fibrosis was induced in these mice the amount of ECM that accumulated in the fibrotic scars in the liver was significantly increased compared with control mice, confirming that senescence of activated stellate cells limits progression of liver fibrosis. Recent studies have shown that induction of senescence in HSCs by interleukin-22 can restrict fibrosis progression (Kong et al. 2012). We can therefore reasonably assume that it will eventually be possible to exploit senescence induction for the treatment of liver fibrosis.

How Senescence of Activated Hepatic Stellate Cells Limits Liver Fibrosis

Before we can begin to focus on the therapeutic induction of senescence, we need to acquire a much deeper understanding of the mechanism of action of senescence during liver fibrosis. One likely mechanism might employ cell cycle arrest of the senescent cells. If activated HSCs do not proliferate their number will be limited, resulting in a decrease in the amount of fibrotic tissue they produce. This simple explanation, however, is far from being complete.

The transcriptional profile of senescent cells has been extensively studied. In addition to the down regulation of growth-promoting genes and upregulation of cell-cycle inhibitors, senescent cells exhibit changes in the expression of secreted molecules that participate in ECM production and degradation (Campisi and d'Adda di Fagagna 2007). These molecules are associated with the marked change in secretion from senescent cells, the SASP. It was suggested that SASP components participate both in supporting senescence in neighbouring normal cells and in promoting inflammation in the microenvironments of senescent cells, in some circumstances even leading to tumor promotion (Campisi and d'Adda di Fagagna 2007). Matrix metalloproteases, which are capable of degrading components of the ECM, are among the main constituents of SASPs (Campisi and d'Adda di Fagagna 2007). An increase in the expression and secretion of these enzymes from senescent cells might not only limit the progression of fibrosis, but also contribute to its resolution.

The anti-fibrotic function of senescent cells is restricted not only by the production of ECM-degrading enzymes. Expression of the ECM components themselves is strongly decreased in senescent cells (Krizhanovsky et al. 2008; Jun and Lau 2010), which in addition to producing fewer ECM components also degrade existing ones. The ECM itself can also have an impact on senescence. During wound healing an ECM protein, CCN1, participates in senescence induction in skin fibroblasts (Jun and Lau 2010). Similar mechanisms might be implicated in the induction of senescence in pancreatic stellate cells during pancreatitis when there is fibrosis in the pancreas (Fitzner et al. 2012). It follows, therefore, that the influence of senescent cells on ECM components plays a role both in limitation of the unrestricted progression of fibrosis and in fibrosis resolution.

Clearance of Senescent Cells by Natural Killer Cells Facilitates Reversion of Fibrosis

Senescent cells can affect their microenvironment in multiple ways. The influence of SASP components is not restricted to the ECM or to cells in their immediate microenvironment. Several of these components are potent pro-inflammatory cytokines that might attract cells from the immune system to the site of their location. This might serve as a signal from the damaged cells, warning the organism to take the necessary care so that these cells cause it no further damage. To prevent such harm, components of the immune system recognize and eliminate the damaged cells.

In the fibrotic liver the level of inflammation is high and cells from both the innate and the adaptive immune systems are abundant (Bataller and Brenner 2005; Friedman 2008). Immune cells reside in the proximity of activated HSCs, including senescent ones (Krizhanovsky et al. 2008). Among the cell types that are strongly implicated in the interaction with activated HSCs are the natural killer (NK) cells (Kong et al. 2012). NK cells belong to the innate immune system and their function is to eliminate dangerous cells, such as damaged or virus-infected ones, from the organism. Activated senescent HSCs exhibit increased expression of several ligands of activating NK-cell receptors and adhesion molecules that can potentially mediate the interaction between these cells and NK cells (Krizhanovsky et al. 2008). Therefore, NK cells can recognize activated senescent HSCs in the fibrotic liver.

Several studies indicate that the specific recognition of senescent cells by NK cells leads to elimination of the senescent cells. For example, the NK-cell-mediated clearance of senescent cells leads to tumor regression after a senescence program is activated in tumors (Xue et al. 2007), and might help to eliminate senescent cells after chemotherapy (Soriani et al. 2009). In the fibrotic liver, depletion of NK cells results in increased accumulation of senescent cells during reversion of fibrosis, suggesting that NK cells are necessary for the efficient elimination of senescent cells from the liver (Krizhanovsky et al. 2008). Furthermore, compared with control cells growing in tissue culture, cultured NK cells preferentially recognize and efficiently eliminate senescent cells induced by a variety of stimuli and conditions, thereby providing an efficient in-vitro model for studying this process (Krizhanovsky et al. 2008).

Reversion of fibrosis is possible if the stimulus leading to fibrosis is efficiently eliminated. It was only recently, however, that the role of elimination of senescent cells in this process was evaluated. Recent reports have described success in antiviral therapies for viral hepatitis in humans, where continuous infection is prevented and fibrosis can be reversed if the treatment is started early enough (Bataller and Brenner 2005). Similarly, in the mouse model of fibrosis, if the chemical induction of fibrosis is stopped its reversion begins. During the reversion process, senescent cells are eliminated from the liver. When NK-cell function is boosted by an augmented interferon-gamma response, both the elimination of senescent cells and the reversion of fibrosis are enhanced (Krizhanovsky et al. 2008). That report was the first to demonstrate that the presence of senescent cells in the organism can be altered therapeutically by manipulation of the immune system. A similar approach to the removal of senescent cells located in other sites might eventually be evaluated as a therapeutic option.

Natural Killer Cells Preferentially Kill Senescent Cells Through Granule Exocytosis

NK cells recognize and eliminate senescent cells to protect against liver fibrosis and to cause tumor regression after p53 reactivation or chemotherapy (Xue et al. 2007; Krizhanovsky et al. 2008; Soriani et al. 2009). To kill their target cells, NK cells use two mechanisms in parallel -granule exocytosis and activation of death receptors (Fig. 25.2). To activate the death receptors, the death-receptor ligands TRAIL and FASL are brought to the surfaces of NK

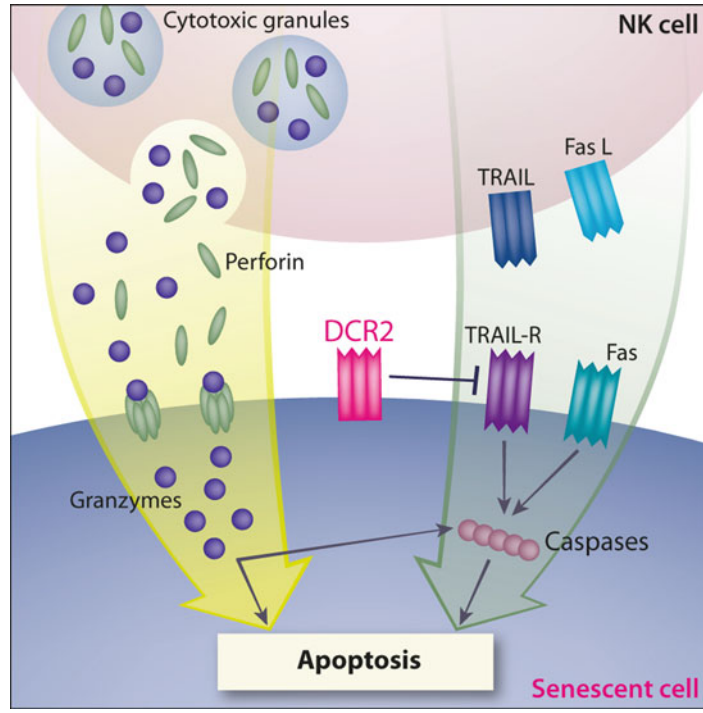

Fig. 25.2 Killing of senescent cells by NK cells is regulated by DCR2. NK cells use two pathways to kill their target cells: exocytosis of cytotoxic granules containing perforin and granzymes or activation of the death receptors (TRAIL-R and FAS) by their ligands. In senescent cells DCR2 inhibits killing through the death receptor pathway. Therefore, granule exocytosis pathway is prevalent in killing of senescent cells by NK cells

cells. Binding with the receptors on the target cells induces activation of caspase 8, which in turn activates the caspase cascade to induce death of the target cells (Johnstone et al. 2008). In granule exocytosis, granules containing the proteins perforin and granzyme A or B are secreted from the NK cell at the surface of its interaction with the target cell. The perforin enables the granzyme to penetrate target cells, where it induces both caspase-dependent and caspase-independent apoptosis. Granzyme B can efficiently induce death of the target cells by triggering mitochondrial permeabilization even when the caspase pathway is blocked as a result of mutations or pharmacologically (Cullen and Martin 2008).

We recently discovered that inhibition of granule exocytosis or inhibition of granzyme activity prevents specific killing of the senescent cells by the NK cells in vitro (Sagiv et al. 2012). Moreover, in the mouse model of liver fibrosis, perforin knockout leads to more severe fibrosis and accumulation of senescent activated stellate cells in the liver. Therefore, perforin-mediated exocytosis is evidently necessary for NK-cell-mediated immune surveillance of senescent cells both in vitro and in vivo.

The mechanism that regulates the balance between killing of senescent cells by granule exocytosis and by death receptors is of particular interest. One reason why senescent cells are resistant to death induced by death-receptor ligands is up regulation of the decoy receptor DCR2 in senescent cells. DCR2 was proposed as a biomarker of oncogene-induced senescence (Collado et al. 2007). Our experiments showed that DCR2 is upregulated also in damage-induced senescence in fibroblasts and activated HSCs. This decoy receptor binds the death receptor ligands, preventing downstream signaling through competitive inhibition of the death-receptor pathway. Therefore, up regulation of DCR2 in senescent cells is responsible for the preferential killing of senescent cells through the granule exocytosis pathway (Fig. 25.2). High levels of DCR2 on the membranes of senescent cells also protect senescent cells from killing by soluble death-receptor ligands. This mechanism represents the first demonstration of regulatory machinery that allows specific killing of senescent cells.

Therapeutic exploitation of the NK-cell-mediated recognition and killing of senescent cells will necessitate a thoroughgoing understanding of the mechanisms of these processes. Recognition of senescent cells might rely on the NK-cell-activating receptors NKG2D and DNAM1 (Xue et al. 2007; Krizhanovsky et al. 2008; Soriani et al. 2009). Ligands of these receptors are upregulated on senescent tumor cells and on activated senescent HSCs, and might provide the signal needed to activate NK cells for killing. However, the interaction of senescent cells with NK cells is not limited to these receptors. Senescent cells upregulate expression of the adhesion molecule ICAM-1 (Xue et al. 2007; Krizhanovsky et al. 2008; Chien et al. 2011), which binds to its receptor CD58 on the NK cells. Interestingly, the combined action of NKG2D ligands and ICAM-1 on NK cells induces more efficient killing by the NK cells of target cells that express both of these components (Hayakawa and Smyth 2006). Multiple molecules on senescent cells mediate the interaction between these cells and NK cells. The functional contribution of these molecules requires further study.

Discussion

Our work in a mouse model of fibrosis demonstrated that senescence of activated HSCs limits the progression of fibrosis and facilitates its reversion by several mechanisms (Krizhanovsky et al. 2008). First, the cell cycle arrest of activated HSCs limits their number, leading to a reduction in the amount of fibrogenic cells. Secondly, the senescent cells that result from this process not only generate less ECM but also produce ECM-degrading enzymes, thereby supporting the resolution of fibrotic scars. Finally, NK cells recognize and eliminate senescent cells, halting inflammation and tissue damage. This last step is the mechanism that enables the liver to return to the healthy undamaged condition. Once damage to the tissue is arrested, the most beneficial outcome for the organism is restoration of the tissue to its pre-damage functional state as quickly as possible. Here senescence comes into play, ensuring that those cells which responded to the initial damage but are not needed in the post-damage condition stop proliferating and are eventually eliminated.

Since our initial discovery (Krizhanovsky et al. 2008), cellular senescence has been shown to limit tissue damage in other pathophysiological conditions in addition to the liver fibrosis that we described. During wound healing in the skin, senescence of fibroblasts limits fibrosis in a CCN1-dependent manner (Jun and Lau 2010). In patients with oral submucous fibrosis, senescent mesenchymal cells limit fibrosis progression by producing enzymes that degrade the ECM and are then cleared by the immune system (Pitiyage et al. 2011). Senescence of activated stellate cells in the pancreas limits pancreatic fibrosis and pancreatitis in way similar to their liver counterparts (Fitzner et al. 2012). These reports, together with our reported findings on liver fibrosis, have established that the physiological role of senescence is to limit the tissue-damage response after short-term insults.

When tissue damage is prolonged and repetitive, however, the outcome is likely to be different. Long-term damage to the liver is characterized by repetitive rounds of hepatocyte death and proliferation, accompanied by constant activation of myofibroblasts derived from both intra- and extrahepatic sources, as part of the pathophysiology of cirrhosis (Bataller and Brenner 2005). In this situation, unlike in the case of short-term damage, the replicative capacity of hepatocytes might become exhausted, limiting liver regeneration (Wiemann et al. 2002). Senescent hepatocytes might therefore be present in the liver at the later stages of liver disease.

The combination of apoptosis and senescence as a fail-safe program in response to a variety of damaging stimuli is not limited to liver fibrosis. Over the last several decades, apoptosis has been studied as a major response of mammalian cells to a damage signal (Johnstone et al. 2008). Many of these studies showed that in cancer cells and cell lines, even moderate damage leads to apoptosis. Recent studies in normal human cells in culture and in mouse models suggest, however, that in normal mammalian cells senescence is preferred to apoptosis as a first response to a damage

stimulus (Collado et al. 2007). This situation is beneficial in complex organisms such as humans, whose capacity to regenerate is limited. Such organisms, unlike lower organisms in which damaged tissues and organs can regenerate easily, cannot afford to have large numbers of cells succumbing to apoptotic death in a short period of time. This would cause irreparable damage to the organism and would therefore be an evolutionary disadvantage. It thus seems that in the evolution of complex mammalian organisms natural selection favored the alternative mechanism, namely cellular senescence, as a first line of damage response.

Unlike apoptosis, cellular senescence is a cytostatic mechanism, not a cytotoxic one. To prevent uncontrolled proliferation it limits the replicative capacity of damaged cells that might lead to tissue damage and cancer on the one hand, while on the other hand it does not immediately compromise the structural integrity of the affected organ. Arrest of the cells poses no immediate danger to the organism, while allowing the system time to reevaluate the extent of damage. In response to moderate damage, normal cells senesce. If the damage is strong enough to compromise the normal cell function, the cells will die later. In that case there is a time window during which undamaged cells can proliferate to sustain organ integrity. Cellular senescence thus serves as the first line of defense of the mammalian organism against internal threats (e.g., oncogenic mutations) as well as against external damage.

Senescent cells are formed to protect the tissue. These cells are damaged, however, and their persisting presence in a tissue might have devastating consequences. SASP components secreted from senescent cells can cell-nonautonomously promote inflammation, tissue destruction, and even carcinogenesis in the senescent cell microenvironment (Campisi and d'Adda di Fagagna 2007). The persistence of senescent cells in tissues might contribute to tumorigenesis in a cell-autonomous manner as well (Kang et al. 2011). To prevent negative long-term consequences, the organism needs to eliminate senescent cells after they have completed their protective function.

This last need is addressed by immune system control of senescent cell presence in the organism. Interaction between senescent cells and the immune system was first demonstrated in a model of transplanted liver carcinoma induced by tumor suppressor reactivation leading to senescence (Xue et al. 2007). Once senescence was induced, the tumor shrank in a way that depended on innate-immune-system components, namely NK cells, neutrophils, and macrophages. NK cells eliminate senescent multiple myeloma cells after chemotherapy, and senescent HSCs facilitate the reversion of fibrosis and return of the liver to its pre-damage state (Krizhanovsky et al. 2008; Soriani et al. 2009). Mutant N-ras-driven senescent hepatocytes are eliminated by combinatorial action of the adaptive and innate immune systems (Kang et al. 2011). These senescent cells are recognized in the liver by CD4 T cells, which drive the elimination of senescent cells by macrophages. Immune surveillance of senescent cells might be relevant not only in the liver, but may play a role also in other fibrosis-related pathological conditions such as lung fibrosis, atopic dermatitis, and atherosclerosis. Altogether, senescent cells are eliminated by various components of the both innate and the adaptive immune systems in a plethora of diseases, and their elimination is necessary for the limitation of disease progression.

Variability in the immune surveillance of senescent cells might contribute to differences in the presence of these cells in normal tissues all over the human body. The first location at which senescent cells were shown to accumulate in the human organism was at the aging skin (Dimri et al. 1995). Senescent cells were later shown to accumulate in aging tissues of humans, monkeys and, most recently, in mice (Campisi 2011). It is not clear, however, if the accumulation of senescent cells with age results from increased formation of senescent cells in aged tissues, decreased immune surveillance of senescent cells owing to an age-related decline in the functions of the innate and adaptive immune systems, or a combination of the two.

Senescent cells in aged tissues not only accumulate, but also contribute to the decrease in tissue

function. Elimination of senescent cells from tissues of aged animals increased tissue fitness in a genetically engineered mouse model of accelerated aging (Baker et al. 2011).It thus seems likely that a decrease in immune surveillance of senescent cells is one of causes of decline in tissue function with age. The increase in clearance of senescent cells observed in fibrosis provided the first evidence that forced elimination of senescent cells might contribute to the restoration of tissue function and structure (Krizhanovsky et al. 2008). Better understanding of the mechanisms by which the viability of senescent cells is regulated both cell autonomously and cell non-autonomously will eventually lead to the ability to manipulate the presence of senescent cells therapeutically. Elimination of senescent cells in certain pathological conditions can be expected to contribute to the treatment of such conditions by boosting tissue rejuvenation, delaying aging, and even prolonging lifespan.

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Formation of Secretory Senescent Cells in Prostate Tumors: The Role of Androgen Receptor Activity and Cell Cycle Regulation

26

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Contents

Abstract

The induction of senescence in cancer cells is believed to be a potent mechanism of tumor suppression; however, senescent cells remain metabolically active and secrete a broad spectrum of factors, modulate the tissue microenvironment, and potentially promote tumorigenicity in neighboring malignant cells. Another important subpopulation of secretory cells modulating the prostate tissue microenvironment is represented by neuroendocrine cells. Interestingly, androgen deprivation therapy, a widely used treatment for advanced prostate cancer, induces both the emergence of neuroendocrine-like prostate cancer cells and the senescence-associated secretory phenotype in prostate cancer epithelial cells.

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The induction of the senescence-associated secretory phenotype by androgen depletion is tightly connected with the regulation of the cell cycle machinery through the downregulation of S-phase kinase-associated protein 2, whereas the emergence of neuroendocrine-like cancer cells (through the process of neuroendocrine differentiation) is under separate control. In this chapter, we summarize possible mechanisms and consequences of the formation of the aforementioned secretory phenotypes in prostate tumors and the role of the androgen receptor and cell cycle regulation in these processes.

Keywords
Prostate cancer • Senescence • Neuroendocrine differentiation • Senescence-associated secretory phenotype • Androgen receptor • Skp2

Introduction

The prostate gland changes substantively in aging men, resulting in the disruption of tissue homeostasis and various clinical symptoms, such as lower urinary tract syndrome, urethral constriction, or the potential development of malignant phenotypes. The anatomical and histological descriptions of the aging prostate were reported decades ago, and they reflected morphological changes at the organ and tissue level rather than deciphered biological processes linked to senescence. Cellular senescence is considered a protective trait that is effective against immortal phenotypes, and many cancers override the induction of senescence by deregulating principal cell regulators such as p53, retinoblastoma (Rb), or cell cycle inhibitors. Despite their permanent growth arrest, senescent cells retain biological activity and can substantially modify the local tissue microenvironment. Various cells that undergo senescence also acquire similar a senescence-associated secretory phenotype (SASP) and secrete a complex set of cytokines and other biomolecules. Soluble growth factors and signaling molecules produced by senescent prostate fibroblasts and epithelial cells contribute to the enhanced proliferation of neighboring preneoplastic cells and induction of the epithelial-to-mesenchymal transition (EMT) and tumor spreading. In addition, SASP is involved in proinflammatory signaling and immune response modulation. A better understanding of normal prostate development and aging will therefore open new possibilities to therapeutically target various pathologies including prostate cancer.

Prostate Histology and Development

The prostate is an exocrine tubuloalveolar gland surrounding the urethra that develops from the pelvic portion of the urogenital sinus (UGS). Development of the prostate is driven by embryonic androgens that stimulate the mesenchyme of the UGS surrounding the presumptive prostatic urethra. Then, the androgen receptors (AR) located in the UGS mesenchyme induce by so far undefined factors the epithelial outgrowth, acinar development and prostate duct canalization (Chung and Cunha 1983). Before the appearance of prostatic buds, the UGS cells of the developing embryonic prostate contain cytokeratin markers of both basal and luminal cell lineages. Differentiation to distinct epithelial cell types occurs concurrently with the development and branching of canalized ducts during prostate morphogenesis, and the pattern of molecular markers of individual cell types defining the secretory phenotype becomes prominent during definitive androgen-dependent maturation of the prostate in puberty.

Histologically, the prostate is composed of two distinct compartments — the fibromuscular stroma and the glandular epithelium — organized into secretory acini and ducts. The stroma consists of fibroblasts/myofibroblasts and smooth muscle cells, with the participation of endothelial cells, nerve cells, and infiltrating inflammatory cells constituting the supportive and regulative architecture for the glandular epithelium. The prostatic epithelium is heterogeneous, largely pseudostratified, tall, and columnar with patches of cuboidal or squamous cells. It consists of two histologically different layers, the morphology of which is essential for clinicopathologic assessment in routine diagnostics. The basal epithelial

Table 26.1 Molecular phenotype of prostate epithelial cells

Cell type in the adult prostate epithelium	Molecular pattern
Prostate-regenerating (stem) cells	Sca-1, Bcl-2, integrin α6, α2β1, CD133 (prominin-1), CD44, CK5, CK14, low AR, GSTπ, pp32
Basal cells	CK5/6, CK14, p63, calcyclin, Bcl-2, GSTπ, pp32
	Low AR
Amplifying (intermediate) cells	Basal and luminal cytokeratins, absent p27, α2β1, GSTπ, pp32, CD44
Luminal secretory cells	CK8, CK18, CK19, Nkx3.1, p27, Bcl-2 absent, low epidermal growth factor receptor
	High AR, prostate-specific antigen (PSA), prostate-specific acid phosphatase (PSAP)
NE cells	Survivin, synaptophysin, CgA, NSE, tubulin β-III, neuropeptides, CK18

layer separates the luminal layer of glandular cells from the stroma and serves as a source of newly differentiating functional cells. The morphology of the basal cells ranges from flattened to cuboidal according to the level of chromatin condensation and amount of cytoplasm. Basal cells express specific high-molecular cytokeratins such as cytokeratins 5/14, p63, and generally low levels of AR (see Table 26.1). The basal layer contains a subpopulation of stem cell-like cells contributing to epithelium renewal. The basal layer exhibits low sensitivity to apoptosis due to Bcl-2 expression and high proliferative capacity and is generally considered androgen-independent (for review see Schalken and van Leenders 2003). The luminal glandular layer is rather heterogeneous and contains several cell types, including secretory cells, intermediate progenitors differentiating from basal cells, and neuroendocrine (NE) cells. The secretory columnar cells arise from cells of the basal layer. These cells are oriented into the lumen of the secretory acinus and are characterized by the prevailing expression of low-molecular-weight cytokeratins (such as 8 and 18) and transcription regulators (such as Nkx3.1). Secretory acinar cells also abundantly produce AR and prostate-specific antigen (PSA). The cells of the luminal epithelium are functionally differentiated and androgen-dependent, having a low proliferative capacity and high apoptotic index. The NE cells are rarely interspersed between secretory cells. They are androgen-independent and produce a variety of biologically active substances including synaptophysin, chromogranin A (CgA), neuron-specific enolase (NSE), and tubulin β-III, as well as neuropeptides including calcitonin, somatostatin, bombesin, and serotonin (for review see Kasper 2008). The cells constituting the prostatic epithelium possess a different proliferation capacity and sensitivity to androgens or proapoptotic stimuli. Pioneering experiments performed in the 1980s indicated the presence of cells capable of repopulating the glandular epithelium even after androgen removal or castration and re-administration (English et al. 1987). These experiments suggested the localization of these repopulating cells in the basal layer, as this cell population remained intact after castration, whereas the luminal cells underwent programmed cell death. However, fluorescent-based sorting for surface stem cells markers such as CD133, integrin α2β1, and CD44 distinguished several populations with different stem cells characteristics, including the capability of asymmetric division, proliferation, and tumorigenicity in immunodeficient mice (for review see Kasper 2008).

Intermediate, transiently amplifying cells are located within the multilayered epithelium and are possible common precursors for differentiated cell types constituting the functional epithelium (for review see Goldstein et al. 2010). The cell types of the definitive prostate epithelium are defined by particular sets of molecular markers (Table. 26.1).

Secretory Phenotypes in the Prostate

Senescence-Associated Secretory Phenotype (SASP)

The concept of SASP was firstly reported in the work of Krtolica and colleagues in 2001 (Krtolica et al. 2001), in which they described that senescent fibroblasts can support the proliferation and tumorigenesis of preneoplastic and neoplastic cells, but not normal cells. Soluble factors secreted by senescent fibroblast were proposed to be responsible for this protumorigenic effect. This was confirmed also in xenograft models, in which the secretion of matrix metalloproteinases (MMPs) was identified as a factor involved in the promotion of tumor growth and microenvironment changes (Liu and Hornsby 2007). Importantly, the protumorigenic effect of senescent fibroblasts was confirmed by experiments in which senescent fibroblasts supported the invasion and EMT of relatively low-aggressive cancer cells. All of these effects were associated with the secretory function of senescent cells; this secretory function of senescent cells is denoted SASP. SASP development is characteristic of both senescent fibroblasts and senescent epithelial cells (Coppe et al. 2010). A striking similarity in the SASP patterns between senescent cells from different donors, tissues, and different cell types was described. Over 40 factors have been identified to participate in SASP. Factors secreted by senescent cells include inflammatory cytokines and chemokines (interleukin [IL]-6, IL-8, interferon-γ, granulocyte-macrophage colony-stimulating factor, C-X-C chemokine receptor type 2 [CXCR2]), growth factors and regulators (transforming growth factor [TGF]-β, epidermal growth factor [EGF], vascular endothelial growth factor [VEGF], and insulin-like growth factor binding proteins [IGFBPs]), proteases and regulators (tissue inhibitor of metalloproteinases, MMPs, and plasminogen activator inhibitor-I), shed cell surface molecules (tumor necrosis factor-related apoptosis-inducing ligand receptor 3 and Fas), and other factors including collagen, fibronectin and nitric oxide (for review see Coppe et al. 2010). Because senescence cells secrete a broad spectrum of factors with different functions, SASP influences many biological processes in surrounding cells.

The SASP factors can affect tumor cells and stimulate tumor progression. Senescent fibroblasts were demonstrated to promote the proliferation and tumor formation of premalignant and malignant breast cancer cells, and this growth stimulation was independent of the inducer of senescence (Krtolica et al. 2001). The proliferation-stimulating effect of senescent fibroblasts was confirmed also in prostate and ovarian cancers (Coppe et al. 2010).

The most important proinflammatory cytokine connected with SASP is IL-6, the secretion of which was detected upon oncogene- or DNA damage-induced senescence (Coppe et al. 2010). In addition to senescent fibroblasts, tumor cells also undergo senescence in response to drug treatment and DNA damage due to the secretion of a broad spectrum of inflammatory cytokines (IL-6, IL-8, IL-24, and TGF-α) (Novakova et al. 2010). Further, SASP factors can influence the motility and invasiveness of cancer cells. Namely, it was found that senescent fibroblasts stimulate the EMT of cancer cells. Further, senescent fibroblasts can affect epithelial differentiation via secreted SASP factors (Coppe et al. 2010).

Taken together, the aforementioned pro- and antitumorigenic effects of senescence induction lead to the hypothesis of so-called antagonistic pleiotropy. Briefly, although senescence can have cancer-preventive effects early in life, it may promote aging later in life because the regenerative capacity of cells and tissues is reduced when increased numbers of senescent cells are present in the body (for review see Giaimo and d' Adda di Fagagna 2012). Conversely, the authors of that review note that using senescence as an example of antagonistic pleiotropy is not unequivocal. Factors secreted by senescent cells support tumor cell proliferation, but on the contrary, these factors can trigger innate immune responses, support wound healing, and prevent organ degradation, thus acting in a positive manner. Secondly, there is no evidence that the beneficial effects of senescence prevail in young age and the detrimental effects prevail in older age, which is one of the characteristics of antagonistic pleiotropy.

Therefore, more studies of this phenomenon are needed, especially in humans.

Senescence, SASP, and Prostate Cancer

Senescence (aging) plays an important role in the development of both benign prostate hyperplasia (BPH) and prostate cancer. Both prostate epithelial cells and prostate stroma can undergo senescence. The histological changes in the senescent prostate include focal atrophy of a small number of secretory acini, the development of epithelial atypia, increased stromal disorganization, disordered orientation of smooth muscle cells, and increased numbers of foci of inflammatory infiltrate (Bianchi-Frias et al. 2010). Array-based transcriptional profiling of fibromuscular stroma adjacent to the glandular epithelium revealed the enhanced expression of genes associated with inflammation or genotoxic and oxidative stress (Bianchi-Frias et al. 2010).

Aging and Senescence in Prostate Stroma

It was confirmed that senescent fibroblasts can change the structure and function of normal epithelium and support the proliferation of premalignant and malignant, but not normal, epithelial cells (Krtolica et al. 2001). Three main groups of genes deregulated in senescent fibroblasts were identified: (a) secreted autocrine- and paracrine-acting growth factors (VEGF, GDF-15, BMP-1, IGFBPs, and others), (b) chemokines and cytokines (CXCL12, CCL11, IL-6, IL-8, and others), and (c) extracellular matrix proteins, proteases, and protease inhibitors (several types of collagen, integrin αV, β1, β4, fibronectin, lamimin β2, and others) (for review see Dean and Nelson 2008).

The molecular profile of the prostate microenvironment significantly changes with aging, and several genes with altered expression during aging were also upregulated in the SASP of fibroblasts induced to senesce by different approaches (Bianchi-Frias et al. 2010). Further, components of the extracellular matrix were downregulated and inflammation-associated genes were upregulated in aged stroma. In another study, the transcription profile of senescent prostate fibroblasts was evaluated. Among others, genes encoding the senescent factors fibroblasts growth factor 7 (FGF-7), MMP2, and IGFBP-2, -3, and -5, all associated with SASP, were upregulated in senescent fibroblasts (Bavik et al. 2006). Another factor, CCL5, was identified as the factor that stimulated the proliferation of BPH-1 immortalized primary human prostate cells grown in conditioned medium from fibroblasts derived from elderly donors (Eyman et al. 2009). Furthermore, CCL5 was found to enhance the expression of genes involved in angiogenesis.

Therefore, based on these facts, senescent fibroblasts must be considered potential targets for cancer prevention and therapy.

Senescence in Nonmalignant Prostate Epithelium

There is an evident connection between senescence and BPH development because senescent cells accumulate in tissues with aging and the incidence of BPH also increases with increasing age. Generally, the presence of senescent cells in premalignant state is considered a cancer-preventive mechanism that blocks neoplastic transformation. When this senescent state is broken or bypassed, premalignant lesions can become malignant. Using SA-β-gal staining, it was revealed that there were significantly more senescent prostate epithelial cells in tissue from patients with enlarged prostates than from patients without this enlargement. Importantly, senescence was evident only in epithelial cells, not in prostate stroma (Choi et al. 2000). Moreover, it was confirmed that the presence of senescent cells drives BPH in older men. These senescent prostate epithelial cells express IL-1α, which activates FGF-7 secretion, leading to the proliferation of nonsenescent epithelial cells (Castro et al. 2003). Taken together, BPH is associated with increased numbers of senescent cells, and beneficial effects of senescence induction are desired in this case because senescence halts the progression of BPH to prostate cancer.

A similar effect of senescence was observed in connection with prostatic intraepithelial neoplasia (PIN), a precursor of invasive prostate cancer. Using transgenic mice, in which Akt expression led to PIN development, the relationship between PIN and senescence was investigated. In this model, high levels of the cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$ were reported (Majumder et al. 2008). This overexpression was associated with the induction of a senescence checkpoint, whereas $p27^{Kip1}$ inhibition rescued the senescent phenotype, concomitantly increasing the proliferation of epithelial cells and promoting the development of invasive prostate cancer.

Senescence in Prostate Cancer Progression

As mentioned previously, senescence is primarily considered a tumor-suppressing mechanism that is present in pretumorigenic states and halts malignant transformation. Therefore, the induction of senescence in malignant cells is one possible mechanism of treating different cancers. Although senescent cells do not undergo apoptosis, they do not proliferate, which halts tumor growth. Therefore, treatment with senescence inducers together with other therapeutics may be beneficial for patients. However, because we know that senescent cells acquire SASP and we know the factors involved in SASP, we must account for the negative effects of senescence induction, which can support tumor promotion.

The detailed role of senescence in prostate cancer progression in relationship to AR status and the role of selected signaling pathways will be discussed later in this chapter.

Neuroendocrine Phenotype (NEP)

NE Cells

NE cells, the minor cell type scattered throughout the prostate epithelium, were identified originally by Prettl in 1944 as cells with the dual properties of endocrine cells and neurons, acting in both secretory and autocrine/paracrine manner (for review see Komiya et al. 2009). There are two types of NE cells: open and closed. The open NE cells have an apical cytoplasmic process through which they extend to the glandular lumen, and the closed NE cells do not express this process; both types have thin branching dendritic-like processes, which they extend between adjacent epithelial cells. NE cells are postmitotic and terminally differentiated and lack evidence of the presence of AR in the nucleus. Moreover, NE cells are characterized by the presence of many dense core granules, which correlates with their important secretory function (Komiya et al. 2009). NE cells secrete a broad spectrum of substances and biological active factors, such as calcitonin gene products, CgA, gastrin-releasing peptide (GRP), histamine, NSE, serotonin, and VEGF. Via these factors, NE cells regulate prostatic growth, differentiation, and secretion.

Neuroendocrine Differentiation (NED) of Prostate Cancer Cells

As mentioned previously, NE cells are postmitotic. However, it is known that the number of cells with NE properties increases in prostate cancer. We can find pure NE prostate tumors only rarely, but we can find foci of cells with NE characteristics in almost every prostate tumor.

The increasing number of cells with NE characteristics is thought to arise via the NED (transdifferentiation) of prostate cancer cells (Yuan et al. 2007). NED in tumors is often described as scattered clusters of cells with NE properties among a predominant population of non-NE malignant cells (for review see Komiya et al. 2009). Cancer cells undergoing NED acquire properties similar to NE cells, and thus they have been termed NE-like prostate cancer cells. They are characterized by the expression of markers distinct from those of basal or luminal cells (Huang et al. 2006). Importantly, NE-like cancer cells are negative for AR staining and therefore are androgen-independent and resistant to hormonal

(androgen ablation) therapy. Moreover, they overexpress the antiapoptotic protein survivin, making them resistant to apoptosis (Gong et al. 2007).

Many mechanisms of NED induction in prostate cancer cells have been identified, in particular via *in vitro* models of NED. NED was induced in prostate cancer cells by androgen depletion, increased levels of IL-6, ionizing radiation, and activation of several signaling pathways, such as the Wnt and cyclic adenosine 3′, 5′-monophosphate (cAMP) signaling pathways (for review see Komiya et al. 2009).

Recently, Germann et al. investigated the role of NE cells in tumor recurrence in an androgen-dependent BM18 xenograft model after castration (Germann et al. 2012). They revealed that only two populations of cells, stem cell-like cells and NE cells survive the castration process. However, they simultaneously demonstrated that NE cells are nonproliferative both before and after castration and therefore are not responsible for initiating the recurrence of tumor growth after castration. Whether this holds true for other models remains for further investigation.

Secretory Phenotype Connected with the NED of Prostate Cancer Cells

One of the important characteristics connected with NED in prostate cancer cells is their secretory function. The transdifferentiation of cancer cells into NE-like prostate cancer cells is associated with the acquisition of the ability to express and secrete a broad spectrum of NED markers and biologically active factors, similar to normal NE cells. It is hypothesized that through these factors, NE-like cancer cells can influence surrounding non-NE-like cancer cells, support their survival during androgen deprivation therapy (ADT), and thus contribute to disease progression.

Some of these NED markers have been used to detect NED, and their levels are correlated with the disease stage and cancer progression. Immunohistochemical detection of CgA is correlated with the clinical stage of the disease, whereas NSE and synaptophysin are correlated with the grade (Ather et al. 2008). Circulating CgA appears to be a useful marker for obtaining detailed information about the disease stage and progression (Berruti et al. 2001). In addition to potentially being a useful marker, the biological properties of CgA were also elucidated. CgA can induce the phosphorylation of Akt, and this activation of Akt leads to the increased expression of survivin and protection from apoptosis (Gong et al. 2007).

Another factor secreted by NE cells is serotonin, which activates mitogen-activated protein kinase and the phosphoinositide 3-kinase (PI3K)/Akt pathway in prostate cancer cells and supports their migration (Dizeyi et al. 2011). Together with bombesin and GRP, serotonin influences angiogenesis in patients with prostate cancer (Heinrich et al. 2011). Additionally, bombesin was found to stimulate the expression of the proangiogenic factors VEGF and IL-8 through nuclear factor-κB activation (Levine et al. 2003). This correlates with the fact that the increased presence of NED correlates with higher neovascularization in prostate cancer (Grobholz et al. 2000).

Chemokines also play an important role in prostate cancer progression. Both benign NE cells and NE-like cancer cells produce IL-8 (Huang et al. 2005). There are two receptors for IL-8: CXCR1 and CXCR2. CXCR1 is overexpressed by malignant prostate cells, whereas CXCR2 is overexpressed by NE-like cancer cells. This suggests that NED induced in response to androgen withdrawal leads to the induction of IL-8, and IL-8 can influence the androgen-independent growth of prostate cancer cells through a paracrine mechanism; simultaneously, IL-8 can regulate NED and the function of NE-like cancer cells in an autocrine manner.

In summary, the induction of NED by different stimuli leads to the transdifferentiation of cancer cells into NE-like cancer cells. These cells acquire the ability to secrete many factors, several of which are useful markers of NED, whereas other factors have important biological functions. NE-like cancer cells can influence surrounding non-NE cells in a paracrine manner, whereas simultaneously they are regulated by other factors in an autocrine manner.

Changes of Secretory Phenotypes During Cancer Progression and Cancer Therapy

Role of Androgen Receptor in the NEP and SASP of Prostate Cancer Cells

Androgens (male steroid sex hormones) are important for the terminal differentiation of luminal epithelial cells in the prostate gland. Androgens act through their binding to AR. AR is a ligand-dependent transcription factor and a member of the nuclear receptor superfamily. After ligand binding, AR sheds inhibitory chaperons, undergoes homodimerization, and translocates to the nucleus, where it binds to DNA at specific sequences (androgen-responsive elements). By regulating the transcription of many different genes, AR signaling affects secretory function, cell survival, and cell cycle initiation in prostate cells (for review see Knudsen and Scher 2009).

Generally, AR and AR signaling are critical regulators of the G1 to S phase transition in prostate cancer; AR regulates the translation of the family of cyclin D proteins through the mTOR pathway and therefore enables cyclin D/CDK4 complex assembly; further, AR downregulates the CDK inhibitor $p27^{Kip1}$, which enables the activation of CDK2 (for review see Balk and Knudsen 2008). This activation of cyclin/CDK complexes leads to the phosphorylation and inactivation of Rb, which enables the G1 to S transition.

ADT is a standard treatment for advanced stages of prostate cancer and is usually achieved by medical or surgical castration. This leads to the depletion of gonadal testosterone, which is the main source of circulating androgens. Despite the primary response of prostate tumors to ADT, metastatic disease almost always progresses to currently incurable castration-resistant prostate cancer (CRPC) connected with re-activated AR. This re-activation may occur via direct AR modulation (deregulation, mutation, or alternative splicing; posttranslational modifications of AR), alterations in AR cofactors (coactivator enhancement, loss of function of corepressors), and intratumoral androgen synthesis mediated by autocrine or paracrine mechanisms (for review see Knudsen and Scher 2009). Therefore, in the following section, we will focus on the role of androgen withdrawal and AR signaling in the induction of both secretory phenotypes of prostate cancer cells (NEP and SASP).

Role of Androgen Receptor in NEP

NED is hypothesized as one of the mechanisms responsible for the progression of CRPC. The presence of NED is significantly more common in patients with hormone-refractory prostate cancer following long-term treatment with different hormone-deprivation therapies and chemotherapy (Hirano et al. 2004). In accordance with these results, AR silencing using RNA interference *in vitro* led to induction of NED in both androgen-dependent and androgen-independent prostate cancer cell lines, which implies that AR may actively repress NED in prostate cancer cells (Wright et al. 2003). Importantly, NE-like cancer cells in both primary and recurrent disease lack AR expression (Bonkhoff 2001). Thus, NE-like cancer cells are insensitive to ADT, and possibly through factors that they secrete, they can influence surrounding non-NE-like cancer cells in a paracrine manner and stimulate the proliferation of these non-NE-like cancer cells during ADT.

With regard to the activation of AR by ligands other than androgens, IL-6 can stimulate both AR expression and activity in the absence of androgens in LNCaP prostate cancer cells (Lin et al. 2001). Further, treatment of prostate cancer cells with IL-8 resulted in increased proliferation of these cells in androgen-depleted conditions accompanied by increased AR expression and activation (Seaton et al. 2008). Both IL-6 and IL-8 are associated with prostate cancer progression; IL-8 is secreted by NE cells and NE-like cancer cells (Huang et al. 2005), and importantly, both IL-6 and IL-8 are associated with the SASP. The ability to stimulate the growth of cancer cells in the absence of androgens was also confirmed for other neuropeptides secreted by NE-like cells, namely GRP and parathyroid hormone-related protein.

The important role of NE cells in supporting the growth of cancer cells through AR activation was confirmed in the work of Jin and colleagues, in which they implanted LNCaP cells into one flank and NE-prostate tumors into the opposite flank of castrated immunodeficient mice (Jin et al. 2004). Interestingly, NE tumors supported the proliferation of LNCaP prostate cancer cells; this was mediated by the increased sensitivity of LNCaP cells to androgens, increased AR expression, and NE tumor-secreted factors. Overall, NE tumor cells increased the activity of functional AR in LNCaP prostate cancer cells. Therefore, targeting the signaling of factors secreted by NE cells may be beneficial for prostate cancer treatment.

Role of Androgen Receptor in SASP

One of the hallmarks of senescence is cell cycle arrest. It is known that in the prostate epithelium, AR drives cell cycle progression, and therefore, primarily the protumorigenic role of AR is well documented. Contradictory to this, a recent publication by Mirochnik and colleagues indicated that persistent AR activity drives senescence in both normal and cancer prostate epithelial cells; this AR-driven senescence was associated with decreased tumorigenicity (Mirochnik et al. 2012). They demonstrated that via increased ROS production, AR decreases Rb phosphorylation, which leads to cell cycle arrest. Subsequently, AR causes the accumulation of $p21^{Cip1/Waf}$, leading to the attenuation of p63 expression.

As previously mentioned, the senescent phenotype is accompanied by the secretion of a broad spectrum of biological factors. Some of these factors can interact with AR and affect its activity. Both IL-6 and IL-8, factors associated with both the NEP and SASP of prostate cancer cells, can activate AR signaling. Moreover, keratinocyte growth factor and EGF treatment of prostate cancer cells induced AR transactivation (Culig et al. 1994). Thus, SASP factors can transactivate AR in androgen-depleted conditions in the same manner as NED factors.

Role of the PTEN—Skp2 Pathway in the Induction of SASP by Inhibition of AR Activity

Skp2 is an important component of the SCF multisubunit complex (Skp1, Cullins, F-box proteins), which acts as an important E3-ubiquitin ligase. Skp2 recognizes and subsequently degrades many substrates, such as $p21^{Cip1/Waf}$, $p27^{Kip1}$, p57, cyclin E, cyclin D1, cyclin A, E2F-1, Orc-1, TOB1, FOXO1, c-Myc, B-MYB, Cdt1, CDK9, and Smad-4 (for review see Frescas and Pagano 2008). Many of these substrates are known tumor suppressors, and thus, Skp2 is classified as an oncogene that is crucial for the regulation of cell cycle progression, proliferation, differentiation, apoptosis, and cell survival. Skp2 overexpression was documented in several types of cancer, including colorectal, prostate, breast, gastric, and lung cancers (for review see Hershko 2008). This overexpression usually correlates with poor overall survival.

One of the important targets of Skp2 is the CDK inhibitor $p27^{Kip1}$. Skp2 physically interacts with phosphorylated $p27^{Kip1}$, and this interaction targets $p27^{Kip1}$ for ubiquitin-mediated degradation (Carrano et al. 1999). In prostate cancer samples, Skp2 expression was increased in PIN lesions and prostate cancer, compared to its very low expression in normal epithelium (Yang et al. 2002). Moreover, Skp2 overexpression is inversely correlated with the expression of its downstream target $p27^{Kip1}$ and its regulator PTEN (Phosphatase and tensin homolog). It was confirmed that PTEN regulates $p27^{Kip1}$ levels through Skp2 (Mamillapalli et al. 2001).

PTEN is an important tumor suppressor gene that is commonly mutated in cancer, and its expression is decreased to various degrees in several cancers including prostate cancer. In the prostate, PTEN drives cancer progression; deletion of one PTEN allele causes hyperplasia, dysplasia, and low-grade PIN, and further downregulation of PTEN using a hypomorphic allele leads to the development of high-grade PIN that developed into carcinoma in 25% of mice (Trotman et al. 2003). Moreover, homozygous loss of PTEN induces cellular senescence mediated

by the p53 signaling pathway and suppresses tumorigenesis, whereas the loss of both PTEN and p53 accelerates prostate cancer (Chen et al. 2005). A novel type of cellular senescence, PTEN-loss-induced cellular senescence, was recently described (Alimonti et al. 2010). In that study, PTEN-loss-induced cellular senescence occurred in the absence of massive proliferation and the DNA damage response.

Interestingly, the reciprocal feedback regulation between AR and the PTEN-PI3K/Akt pathway was elucidated. The loss of PTEN causes activation of the PI3K pathway, which is connected with decreased AR expression. Inhibition of the PI3K pathway led to the partial rescue of AR protein expression and activity (Carver et al. 2011). Further, this crosstalk was confirmed in the work of Mulholland and colleagues, in which PTEN-null prostate cancer cells were less dependent on AR signaling; prostate cancer cells with AR loss were more dependent on the PI3K/Akt/mTOR pathway (Mulholland et al. 2011). Therefore, the combined inhibition of AR/androgen signaling and the PI3K/Akt/mTOR pathway may be more effective for treating CRPC with PTEN loss.

The role of Skp2 in cellular senescence was elucidated in experiments in which the loss of Skp2 alone did not induce senescence, but concomitant Skp2 loss together with PTEN inactivation or Arf deficiency led to the acquisition of a senescence phenotype in mouse embryonic fibroblasts (Lin et al. 2010). This Skp2 loss-mediated induction of senescence impaired tumorigenesis independent of the $p19^{Arf}$-p53 pathway. Currently, it is therefore desirable to target Skp2 or some other components of the SCF E3 ubiquitin ligase complex because targeting these components by different approaches leads to senescence, apoptosis, and autophagy, processes that impair cancer cell growth (for review see Jia and Sun 2011). As mentioned previously, Skp2 is a part of the SCF complex involved in ubiquitin-proteasome-mediated degradation. One of the components of this complex is cullin-1, which must be posttranslationally modified by neddylation to support Skp2-SCF complex formation (for review see Chan et al. 2010). Although there are no direct inhibitors of Skp2, one potential mechanism of Skp2 inhibition is to inhibit the entire complex by inhibiting neddylation. NEDD-8 is carried to cullin-1 by a specific E1 ligase called the NEDD-8 activating enzyme. Recently, MLN4924, a small molecule inhibitor of NEDD-8 activating enzyme, was identified (Soucy et al. 2009). This inhibitor inhibited neddylation in cancer cells, resulting in defects in S phase of the cell cycle, induction of apoptosis, and importantly inhibition of tumor xenograft growth. Interestingly, MNL4924 treatment induced both partial apoptosis and irreversible senescence that was mediated by the $p21^{Cip1/Kip}$ pathway, leading to tumor growth suppression (Jia et al. 2011).

In our recent publication, we revealed that long-term androgen depletion leads to cell cycle arrest associated with the irreversible induction of senescence (Pernicova et al. 2011). This was mediated by the downregulation of Skp2 and upregulation of its downstream target $p27^{Kip1}$. In our model, this effect was independent of PTEN because we observed similar effects in cell lines expressing (LAPC-4) and lacking PTEN (LNCaP). Moreover, the induction of senescence by androgen depletion was paralleled by the acquisition of both aforementioned secretory phenotypes: SASP and NED. However, only the induction of senescence was dependent on the PTEN/Skp2/$p27^{Kip1}$ pathway, whereas modulating the levels of Skp2 did not lead to the modulation of NEP. Additionally, we determined that both androgen depletion and a high cellular density in the presence of androgens can induce NED, but not senescence, in prostate cancer cells (Pernicova et al., manuscript in preparation). A high cellular density increased AR activity and induced NED only transiently; this NED induction was not paralleled with the induction of senescence. Both a high cellular density and androgen depletion caused cell cycle arrest, but the induction of cell cycle arrest was not crucial for the induction of NED. Additional investigation revealed the involvement of cAMP signaling in high density-induced NED.

We suggest that androgen depletion leads to the formation of highly secretory senescent NE-like cancer cells possessing the characteristics of both NE-like cancer cells and senescent

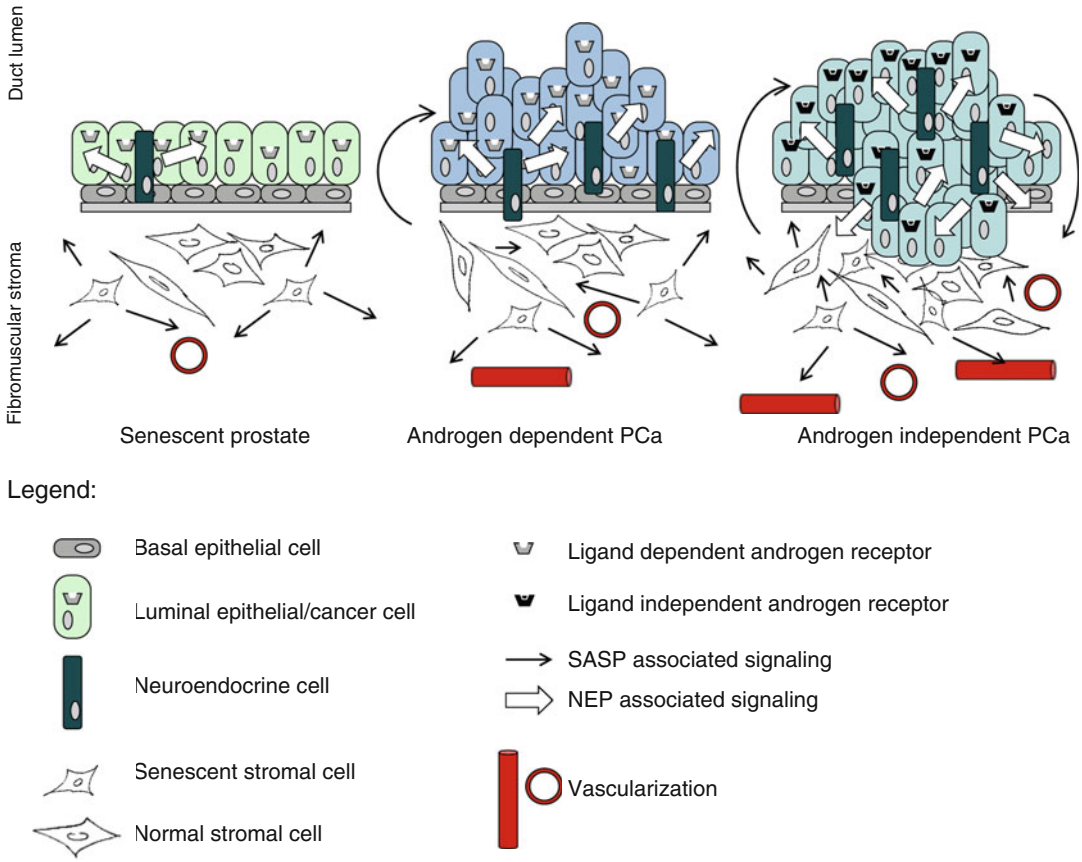

Fig. 26.1 Senescent stromal as well as neroendocrine cells modify prostate microenvironment by autocrine and/or paracrine soluble molecules (see the text for details). In PCa, cancer cells acquire both SASP and NEP that promote formation of epithelial neoplasia as well development of the androgen independent phenotype

cells. As mentioned previously, some of the secreted factors may transactivate AR in the absence of androgens, and consequently, acquisition of this highly secretory phenotype may contribute to the acquisition of androgen independence and development of CRPC. These results are in accordance with published observations, which revealed that androgens regulate Skp2 expression; in particular, AR was found to stabilize Skp2 (Wang et al. 2008). Moreover, the depletion of AR or Skp2 alone lead to cell cycle arrest, but only AR depletion to the induction of NED. Using an AR-positive castration resistant subline of LNCaP cells, the connection between AR and Skp2 was confirmed; treatment of this subline with androgens led to the suppression of proliferation through AR, Skp2, and c-Myc (Chuu et al. 2011). Skp2 is regulated by androgens in a biphasic manner, primarily at the mRNA level. Subphysiological concentrations of androgens slightly increase Skp2 levels, and physiological levels decrease Skp2 levels in LNCaP cells. Moreover, the pocket protein p107 was identified as an important molecule that mediates the androgenic repression of Skp2 in LNCaP cells, but not the only such molecule (Jiang et al. 2012).

Summary

In summary, based on the literature and our own work, we assume that there is a clear connection among androgen depletion, senescence, and NED

in prostate cancer cells. Androgen depletion induces complete changes in the phenotype of cancer cells; these cells display the characteristics of both NE and senescent cells. Importantly, both of these phenotypes are accompanied by massive secretory function; many of these secreted factors associated with NEP, SASP, or both were confirmed to support proliferation, differentiation, and other biological processes in neighboring cancer cells (Fig. 26.1). These factors may contribute at least in part to the survival of cancer cells during ADT because several of these secreted factors can transactivate AR. Interestingly, it was demonstrated that antagonists of bombesin/GRP inhibited the growth of androgen-independent tumors in mouse xenograft models (Stangelberger et al. 2005). Moreover, the neuropeptides endothelin-1 and bombesin stimulated the proliferation of prostate cancer cells and expression of IL-8 and VEGF, and this effect was abrogated by neuropeptide inhibition using bortezomib (Tsapakidis et al. 2012). Therefore, these promising results indicate that targeting these secretory factors could improve the efficiency of anticancer therapy.

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