

p53, Stem Cells, and Reprogramming: Tumor Suppression beyond Guarding the Genome

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Abstract

p53 is well recognized as a potent tumor suppressor. In its classic role, p53 responds to genotoxic insults by inducing cell cycle exit or programmed cell death to limit the propagation of cells with corrupted genomes. p53 is also implicated in a variety of other cellular processes in which its involvement is less well understood including self-renewal, differentiation, and reprogramming. These activities represent an emerging area of intense interest for cancer biologists, as they provide potential mechanistic links between p53 loss and the stem cell-like cellular plasticity that has been suggested to contribute to tumor cell heterogeneity and to drive tumor progression. Despite accumulating evidence linking p53 loss to stem-like phenotypes in cancer, it is not yet understood how p53 contributes to acquisition of “stemness” at the molecular level. Whether and how stem-like cells confer survival advantages to propagate the tumor also remain to be resolved. Furthermore, although it seems reasonable that the combination of p53 deficiency and the stem-like state could contribute to the genesis of cancers that are refractory to treatment, direct linkages and mechanistic underpinnings remain under investigation. Here, we discuss recent findings supporting the connection between p53 loss and the emergence of tumor cells bearing functional and molecular similarities to stem cells. We address several potential molecular and cellular mechanisms that may contribute to this link, and we discuss implications of these findings for the way we think about cancer progression.

Keywords: p53, stem cell, cancer, dedifferentiation, plasticity, reprogramming

Introduction

p53 (the product of the human TP53 and mouse TRP53 genes) is best known and most extensively studied as a pivotal signaling node that converts diverse upstream stress signals into downstream responses including cell cycle arrest, senescence, DNA repair, and programmed cell death. These activities limit the contribution of corrupted genomes to tissue and have earned p53 the august designation “guardian of the genome”.¹ However, the tumor-suppressive effects of p53 likely extend beyond its ability to limit the perpetuation of cells undergoing genome destabilization, given the myriad cellular processes reported to both elicit and respond to p53 activation. Indeed, among tens of thousands of publications on p53, one finds it implicated in diverse aspects of cellular biology including metabolism, meiosis, cell migration, differentiation, embryo implantation, response to teratogens, autophagy, and reprogramming, among others.^{2–5} While in some cases these “nonclassic” p53 functions may be

integrated with classic DNA damage signaling,⁶ it is possible that they can also act independently to impact tumor susceptibility, tumor progression, and cellular sensitivity to therapy.⁷ Importantly, elucidation of these relatively understudied p53 functions and their molecular mechanisms has the potential to uncover additional targets for pharmacological intervention in cancer.

An emerging role for p53 in regulating cellular differentiation, self-renewal, and plasticity has generated intense interest, particularly among cancer researchers. Enforced differentiation is a powerful tumor-suppressive mechanism because normal development and differentiation are antithetical to the abnormal development and incomplete differentiation that hallmark cancer. p53 has been implicated as an enforcer of differentiation by virtue of its ability to limit the cardinal stem cell characteristic of self-renewal in several systems.^{8–11} This, together with the demonstration by Yamanaka *et al.* that differentiated cells can be reprogrammed to a dedifferentiated state,¹² and the demonstration that p53 is a potent

reprogramming barrier^{13–19} have caused a resurgence of interest in the idea that loss of differentiation²⁰ may be linked to p53 pathway disruption in tumors. Recent studies provide additional evidence for the link between p53 and the emergence of dedifferentiated, stem-like phenotypes.^{9,21}

The implications of these findings are far reaching and will cause us to rethink the role that p53 inactivation plays in tumor pathophysiology and the relationship between stem cells and cancer more generally. They call into question existing models for the genesis of cells that perpetuate tumors because plasticity would permit phenotype to change during cancer progression. They also challenge the idea that a static, uniform cancer stem cell pool may be selectively targeted to eradicate the disease. Clearly, optimal

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therapies will be those that eliminate all malignant cancer cells including both the putative cancer stem cell compartment and cells able to reacquire stem-like characteristics. A better understanding is now needed of the molecular and cellular mechanisms underlying the developmental plasticity and stem-like characteristics that surface when p53 function is corrupted or lost. These mechanisms could well represent targets for the mitigation of critical tumor cell behaviors including chemoresistance, dormancy, and metastasis.

Here, we critically examine the literature to uncover relationships between p53 and the normal or neoplastic stem cell state. Our objectives are to raise questions about the commonality of stem-like cells in cancer and to re-examine the “cancer stem cell” concept in light of the possibility that the large number of cancers in which p53 is inactivated may be susceptible to tumor cell reprogramming “on the fly” to generate multifarious stem-like cells.

p53: Finely Tuned Tumor Suppressor

p53: Tumor Suppressor

First identified as a target for the transforming viral oncogene, large T,^{22,23} p53 was subsequently found to be mutated in a wide variety of human and murine cancers.²⁴⁻²⁸ Germline transmission of one mutant allele in humans (e.g., Li-Fraumeni syndrome²⁹) or in engineered mice leads to broad-spectrum tumor formation with high penetrance.^{30,31} p53-null mice exhibit even more rapid onset of primitive lymphoma and other tumors including various sarcomas and stem-like germ cell teratomas.³⁰⁻³² Tumors in other tissues including epithelial cancers can be elicited if principal lesions that would otherwise occur are bypassed using conditional alleles, transplant settings, alternative strains, or in the presence of cooperating lesions.³³⁻³⁶ p53 is also found to be mutated in over 50% of sporadic human cancers,³⁷ and it is thought to be functionally inactivated through disruption of other pathway members in a

significant proportion of the remainder (Fig. 1).³⁸ The broad spectrum of cancers harboring mutated or inactivated p53 or p53 pathway components indicates that it is a general, rather than tissue-specific, tumor suppressor.

In somatic cells, an intact p53 pathway responds to a variety of stimuli ranging from nucleotide depletion⁶ to oncogene overexpression³⁹ to DNA clastogenic treatments.^{40,41} p53 activation is accomplished by its rapid nuclear accumulation and subsequent transcriptional activation of numerous genes involved in cell cycle arrest (e.g., p21) apoptosis (e.g., Puma) and other pathways. Additionally, over the past few years, p53 has been shown to directly activate an increasing number of miRNAs able to broadly modulate the function of diverse cellular processes (Fig. 1).⁴²⁻⁴⁴ The responses that p53 activation generates are highly stress and tissue specific. For example, in thymocytes, gamma irradiation elicits a robust p53-dependent apoptotic response,⁴¹ while the same stimulus in mouse embryo and human fibroblasts induces prolonged cell cycle arrests resembling senescence.^{40,45} Either response can provide a potent cell-autonomous, tumor-suppressive mechanism. Recently, p53 has been shown to exert profound cell-nonautonomous effects on the stroma and immune system that can be either growth stimulatory or antagonistic.^{46,47} The cellular responses of apoptosis and cell cycle arrest or senescence have been the principal focus of most p53 tumor suppressor studies to date and provide the most common *in vitro* readout for chemotherapeutic screens and tumor therapy studies. It has become clear that the nature of the p53 response depends on many factors including the cell type, nature, duration, and intensity of the stress and the status of other prosurvival and cell cycle modulators,^{7,48,49} and the effects of paracrine factors secreted by the stroma.^{47,50}

p53 Regulation in Brief

p53 function is orchestrated in normal cells by a number of upstream signaling

mediators responding to damage, metabolic, proliferative, or other stresses. Most of these pathway components have well-established roles as oncogenes or tumor suppressors (Fig. 1). Stress signals converge principally on 2 related, interacting proteins, Mdm2 and Mdmx, that appear to function cooperatively to promote p53 degradation and thereby inhibit p53 activity.^{48,49} The levels of p53, Mdm2, and Mdmx are tightly regulated in a coordinated network to provide a sensitive rheostat for stress sensing and response.⁵¹ Disruption of this network leads to profound developmental defects, enhanced tumor suppression, or significant protection against oncogenesis. For example, Mdm2 (or Mdmx) knockout in mice leads to early embryonic lethality that can be rescued by p53 ablation.⁵²⁻⁵⁴ Conditional knockout of either Mdm2 or Mdmx leads to cell death or reduced proliferation in a variety of different adult tissues and tissue stem/progenitor cells.^{49,55} Conversely, overexpression of Mdm2 or Mdmx is observed in a variety of cancers in which they overwhelm the stress sensing system by constitutively inactivating p53.⁵⁶

The polycomb complex component Bmi1 is another p53 pathway regulator with profound consequences in both development and cancer. Bmi1 regulates chromatin to promote stem cell self-renewal.^{57,58} Its deletion in mice results in hematopoietic and neuronal stem cell loss⁵⁹ and affects proliferation and differentiation of the mammary epithelium.⁶⁰ In lymphoma, Bmi1 overexpression can result in silencing of the Ink4a/ARF locus⁶¹⁻⁶³ that encodes p14/ARF (p19/ARF in mice). ARF promotes p53 activation by antagonizing Mdm2-mediated p53 degradation in response to oncogene overexpression.⁶⁴ Thus, Bmi1 overexpression, or p14/ARF loss of function by epigenetic and other mechanisms, attenuates p53 activity by increasing Mdm2's ability to antagonize p53.^{65,66} These mechanisms of p53 inhibition are also observed in bladder, skin, prostate, breast, ovarian, and colorectal cancers and in mantle cell lymphoma.⁶⁷ Taken in the

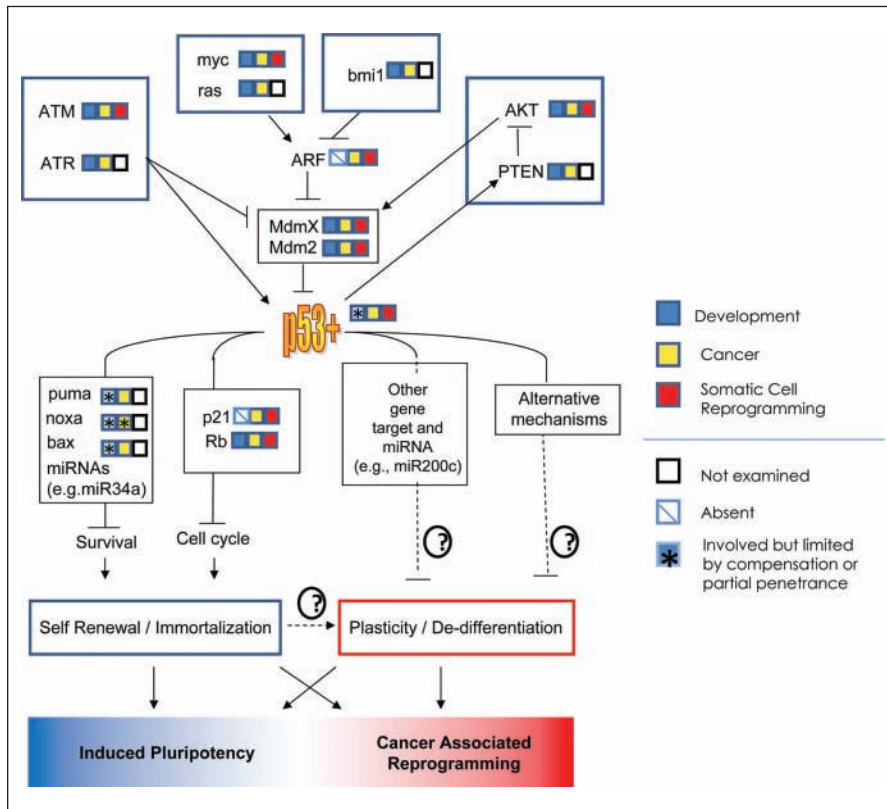


Figure 1. The p53 pathway in development, cancer, and reprogramming. A select set of p53 upstream regulators and downstream effectors are shown in the context of p53-dependent plasticity and self-renewal. The involvement of pathway members in development, cancer, or reprogramming is indicated by their adjacent color-coded squares. Whether induced plasticity leads to induced pluripotent stem cells or stem-like cancer cells may depend on the extent or reversibility of oncogene activation, p53 inactivation, or microenvironmental factor.

aggregate, genes that either regulate p53, or that are regulated by p53, comprise networks intimately tied to cellular physiology (e.g., DNA damage sensing and repair, metabolism, epigenetic regulation, etc.) (Fig. 1). In this light, it is not surprising that components of the p53 regulatory pathway can contribute to both tumorigenesis and developmental dysfunction when disrupted (see below).

Emerging Roles for p53 in Stem Cells and Differentiation

p53 in Adult Stem Cells

Recent studies suggest roles for p53 in adult stem cells, but such studies are complicated by several factors. Adult tissue stem cells are very rare, and it is very

difficult to identify them unambiguously and to then isolate and purify them.^{68,69} Indeed, almost all stem cell isolates actually comprise cell mixtures. (For this reason, most stem cell preparations should be referred to as “stem/progenitor populations,” but for simplicity, we will refer to them as “stem cells” below.) Adult stem cells have also proven difficult to maintain in culture without limiting their capacity for self-renewal or inducing differentiation. However, recent improvements offer hope in this area.^{70,71} In spite of these challenges, studies on adult stem cells, and implications for the functional relevance of p53, continue to appear with increasing frequency.

Small changes in p53 activity profoundly affect hematopoietic stem cell

(HSC) abundance and function. HSCs can be quantified by limiting dilution transplantation analyses and by flow cytometry using a variety of surface markers that enable their isolation to a purity of up to 50%.⁷²⁻⁷⁴ Impressively, transplantation of as few as one purified HSC rescues the lethality caused by myeloablation.⁷⁵ Competitive transplantation experiments provide a powerful means of determining the relative abilities of HSCs encoding particular mutations to compete with wild-type HSCs for engraftment of lethally irradiated hosts.^{72,73} Subsequent to engraftment, the size of the stem cell-containing population defined by flow cytometry can be measured to indicate stem cell expansion/self-renewal. Using such methods, HSCs in genetically engineered models reveal that dysfunctions in p53, ATM, Rb, and p21, among others, significantly affect HSC/progenitor abundance and function at baseline and in response to DNA damage or transplantation-associated stresses.⁷⁶⁻⁸⁰ For example, even the subtle change in baseline and inducible p53 activity caused by deletion of a single Mdm2 allele dramatically reduces the ability of the mutant to compete with wild-type cells during transplantation.^{81,82} Interestingly, inactivation of the *Ink4a/ARF* locus along with p53 enables conversion of multipotent progenitors into engrafting stem cells.⁸³ This suggests that p53 function may be required to stabilize the differentiated phenotype of such cells or that its loss enables reprogramming at high efficiency in this cell type.

p53 has also been implicated in regulating mammary stem cell number *in vitro* and *in vivo*. Here, stem cells comprise a far lower fraction of cell populations obtained by flow cytometry using cell surface markers, although use of the membrane dye, PKH26, to enrich for quiescent or slowly dividing putative mammary stem cells has been suggested to yield impressively pure populations.⁸⁴ Mammary stem cells are typically quantified *in vitro* through generation of mammospheres from single initiating

cells and *in vivo* by limiting dilution transplantation assays measuring the capacity of the cells to fill a de-epithelialized fat pad with a functional mammary gland.^{84,85} In comparison to wild-type littermates, p53 knockout mice appear to contain a higher concentration of cells able to form mammospheres in culture or to repopulate the gland *in vivo*.^{84,85} Furthermore, whereas wild-type sphere-forming cells decline upon serial replating of dissociated mammospheres, the p53-null sphere-forming cells can be expanded.⁸⁴ Conversely, stem cell depletion accompanies the serial transplantation of mammary cells from mice carrying an overactive p53 gene,⁸⁶ suggesting that the level of p53 activity dictates the percentage of graftable stem cells in the tissue. However, such interpretations must be moderated because *in vitro* propagation of mouse cells can activate p53, so the positive growth effects seen in cells lacking p53 may result from the absence of p53-mediated growth arrest responses. Similarly, transplantation may induce stresses that activate p53, as observed in the hematopoietic system. Finally, the identity of the cell(s) that initiate mammosphere formation remains obscure, as does their relationship to the stem cells that underlie gland formation in a transplantation assay.

p53 has also been implicated in regulating stem cell/progenitor compartment dynamics in the epidermis,⁸⁷ prostate,⁸⁸ and central nervous system.^{11,89-91} While these studies generally point to p53 loss in increasing the stem cell pool and p53 activation in blocking stem cell self-renewal, this remains to be demonstrated in a physiological setting using methods able to unambiguously distinguish stem cells from other cells.

Thus, while the existing data make tantalizing links between p53 and stem cell abundance and function, the important role of p53 in the response to diverse stresses complicates unambiguous conclusions. The existence of a substantial number of other cell types in the stem-enriched populations also precludes

attribution of differences in inferred stem cell number to the stem cells themselves. In order to make such conclusions, uncovering methods and markers to identify, localize, and quantify stem cells more specifically remains an important challenge to the field.

p53 in Embryonic Stem Cells

Stem cell genomes must be rigorously “guarded” from embryogenesis through adulthood because such cells expand periodically to enable tissue repair and replacement. Thus, faithful genome duplication over a lifetime is required to minimize accumulation of oncogenic lesions during such expansions. Inadequate genomic stability control would be especially deleterious in embryonic stem cells (ESCs), as they are the progenitors of all adult organ systems. However, there are conflicting reports regarding p53 activity in ESCs. We and others found that wild-type mouse embryonic stem cells (mESCs) proliferate rapidly, with cell cycles mainly consisting of S and M phases without the obvious gap phases (G1 and G2) in which p53 normally acts in differentiated cells.^{92,93} Although mESCs express high levels of p53 protein,^{93,94} it appears to be preferentially cytoplasmic.^{92,95,96} Surprisingly, p53 activation by certain types of DNA damage, subsequent nuclear accumulation of p53 protein, and transactivation of specific target genes appear to be inefficient in mESCs.^{93,95,97,98} On the other hand, mESCs possess robust repair mechanisms that may compensate for p53 to preserve genomic stability and potentially dampen damage responses that would cause cell cycle slowing.⁹⁷ While high levels of damage induce ESC death or differentiation,^{10,93,94,99-102} these responses do not appear to be strictly p53 dependent.^{93,103} In human ESCs (hESCs), p53 has been implicated in damage-induced cell death, but its role inducing apoptosis was again independent of its classic nuclear accumulation and transactivation activity, involving

instead a noncanonical function at the mitochondria.¹⁰⁴

Mechanisms that inhibit the high levels of mESC p53 from being activated may be required to permit rapid ESC cycling in the presence of strong proliferation signals. For instance, downregulation of cdk2, which is highly expressed in stem cells, lengthened G1 and allowed p53-dependent activation of p21.¹⁰⁵⁻¹⁰⁷ Thus, a brief G1 may make it difficult to activate p53, but mechanisms that slow the cycle by lengthening G1 may enable p53 activation. Conversely, the prolonged G1 of most differentiated cells might limit reversion to rapid ESC cycling kinetics by affording an ample opportunity for p53 to act.

Two independent molecular mechanisms may counter the high level of p53 protein in ESCs. p53 deacetylation by SIRT1 prevents its nuclear accumulation at high reactive oxygen species (ROS) levels.⁹⁶ Thus, increasing ROS level in mESC cultures growing at ambient (~21%) oxygen by removal of reducing reagents caused mESCs lacking SIRT1 to differentiate.⁹⁶ As ROS levels are affected by metabolism, proliferation, tissue culture stress, oxygen tension, oncogene overexpression,¹⁰⁸⁻¹¹² and many forms of DNA damage,¹¹³ effective mechanisms for modulating cellular responses to ROS are critical. The second p53 inhibitory mechanism involves the truncated p53 isoform $\Delta 40$ -p53 (also referred to as ΔN -p53 or p47 in humans and p44 in the mouse) produced from an internal translational start site in the p53 gene. $\Delta 40$ -p53 is profoundly defective for p53 transactivation function.¹¹⁴ It is highly expressed in ESCs, and mutant ESCs generated by homologous recombination to lack the internal start site, but that still produce full-length p53, exhibit loss of self-renewal, increased differentiation, and establishment of an obvious G0/G1 population.¹¹⁵ These data are consistent with $\Delta 40$ -p53 antagonizing wild-type p53 function in ESCs. Interestingly, $\Delta 40$ -p53 expression was recently identified as a common tumor-associated lesion in a chemically induced bladder cancer

model.¹¹⁶ The identification of other p53 isoforms, including several splice variants, adds new opportunities to fine tune p53 activity in specific cell types.¹¹⁷ Whether these other isoforms contribute to development, stem cell function, and genomic stability control remains to be investigated.

While the above functional and mechanistic studies provide evidence of p53 attenuation in ESCs, other studies indicate that p53 is active in mouse and human ESC cultures.^{10,94,100,101,118} These analyses used reporter constructs to detect active p53 and functional studies to examine p53-mediated responses.^{94,119} p53 activation was observed in ESC populations following treatment with actinomycin D¹²⁰ or the Mdm2:p53 interaction disruptor Nutlin 3a.¹²¹ Importantly, the effect was reversible by p53 knockdown using shRNA.¹¹⁹ Notably, the reporter analyses revealed high basal expression that was augmented in differentiating cultures in the absence of additional stress.¹¹⁹ As high baseline activity attributed to basal p53 activity in undifferentiated cultures was not appreciably reduced by p53 knockdown, it is likely that p53-independent factors contribute. Earlier functional studies reporting activation of endogenous Mdm2, cyclin G, and Bax in response to DNA damage or induced differentiation with retinoic acid^{94,100} revealed a correlation between p53 activation and induction of programmed cell death that was reduced or absent in p53 knockout mESC cultures.^{94,100} Interestingly, time course experiments analyzing p53 localization and target gene activation showed transient p53 activation in a subset of cells.⁹⁵ The controversy over p53 pathway activation in ESCs extends to hESCs that, although different in important ways from mESCs,¹²² also have an accelerated cell cycle with abrogated G1 checkpoints.¹²³ Different studies report that hESCs possess or lack a stress-activated p21 response,^{124,125} undergo p53-dependent apoptosis,¹²⁶ or execute p53/p21-independent checkpoints in response to genotoxic stresses such as ultraviolet radiation.¹²⁷ However, persistent p53 activation using

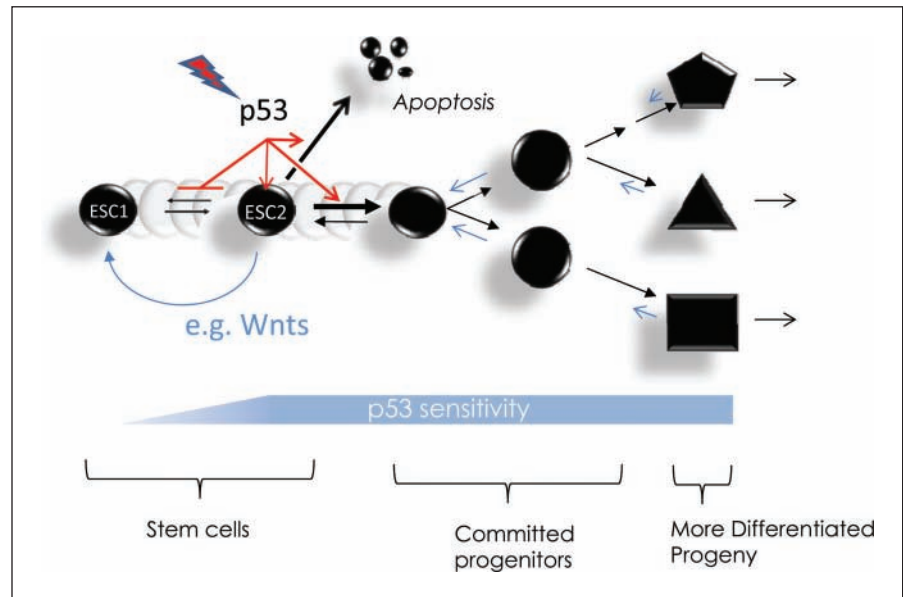


Figure 2. Impact of stem cell state on p53 activation and differentiation. In this model, stem cell populations can be heterogeneous, with some being more responsive to p53 activation. Various stresses can activate p53 to induce differentiation or apoptosis in the sensitive stem, while insensitive cells with dampened p53 responses survive and may proliferate in response to paracrine signals such as Wnt. This model is compatible with recent reports of multiple pluripotent stem cell states in embryonic stem cell cultures^{130,227} and that multipotent progenitors in the hematopoietic compartment act as stem cells if p53 activation is abrogated in conjunction with Ink4a/ARF deletion.⁸³

continuous Nutlin exposure induces differentiation of mouse and human ESCs.^{101,128} One interpretation of such results is that ESCs have an altered stress response threshold for p53 activation, and Nutlin is able to consistently meet the activation criteria for these cells.

We offer the following suggestions to resolve the apparent discrepancies in the literature concerning p53 functionality in ESCs. Given the role played by p53 in stress sensing, differences in culture conditions, growth factors,¹²⁹ and oxygen tension or the level, duration, and type of DNA-damaging insults are likely contributors to differential p53 activation. However, another variable is suggested by the recent identification of 2 distinct ESC/iPS cell states distinguishable based on their miRNA expression profile and by their expression of p53 pathway genes.¹³⁰ These 2 pluripotent states are interconvertible by expression of appropriate miRNAs or over the course of *in vitro* differentiation.¹³⁰ Another potentially related possibility is

the existence of distinct pluripotent populations derived from cells with temporally distinct developmental origins.^{131,132} In this regard, hESCs that are more similar to mouse epiblast-derived stem cells (mEpiSCs) can be reprogrammed to a metastable mESC-like state.¹³³ Given such data, it is reasonable to consider ESC cultures being inherently heterogeneous, with one cell type lacking canonical p53 stress responses but being interconvertible with a second type able to undergo p53 activation (Fig. 2). The latter could lead to differentiation or apoptosis. Thus, the different results reported in the literature might relate to the relative contributions of each cell type to the culture.

Impact of p53 on Development and Differentiation

As p53 responds to many conditions that impact cell growth, and it appears to contribute to stem cell self-renewal and differentiation capacity, one might expect p53 pathway function to also

impact development. However, p53-null mice appear superficially normal. This may be due in part to the presence of 2 related proteins, p63 and p73, evolutionary antecedents of p53¹³⁴ that play key roles in epithelial stem cell self-renewal and neural cell differentiation, respectively.¹³⁵⁻¹³⁷ As these p53 family members can regulate some of the same genes that p53 regulates as they have related DNA-binding domains, it is possible that they can at least partially complement developmental aberrations engendered by p53 deficiency.

p63 also plays a key role in maintaining the integrity of the female germline.¹³⁸ Note, however, that p53 also contributes to maintaining genomic fidelity during embryogenesis, as indicated by the profound increase in aberrant embryos in p53-null mice exposed to teratogens.^{5,139-141} In light of p53's likely descent from a more ancient relative, its response to DNA damage may be viewed as an extension of the role of the ancestral proteins in ensuring germline genomic integrity.^{142,143} The roles of somatic genome guardian and mediator of responses to diverse stresses that impinge on the soma and that could impact genome stability have mainly been relegated to p53 in mammals, although p73 can serve as a backup when p53 is compromised.¹⁴⁴

A closer evaluation of the literature does reveal that p53 plays modulatory, but significant, roles in development. It is commonly observed that intercrosses of p53 heterozygotes generate a lower than Mendelian yield of p53 offspring, and at least 25% of all p53-null mice die from nontumor-related maladies.^{31,139,141,145} Furthermore, fertility of p53-null mice is compromised by defects in spermatogenesis and embryo implantation,¹⁴⁶⁻¹⁴⁸ and their numbers are further reduced by developmental defects *in utero*¹⁴¹ such as elevated incidence of exencephaly in females.^{147,149} Broad effects of p53 deficiency on development might be expected based on its activation in multiple tissues during normal fetal development.¹⁵⁰⁻¹⁵² This may explain the eye and tooth

defects and polydactyly occasionally observed in p53-deficient mice.¹⁴⁹ p53 has also been implicated in regulating normal differentiation of muscle, keratinocytes, neurons, thyroid, and macrophages.^{153,154} It thus remains possible that closer examination of these and other tissues may uncover additional differentiation abnormalities associated with p53 deficiency. It is also possible that manifestations of p53 loss may only be evident in combination with alterations in other genes and then only in specific tissues. For instance, through mechanisms associated with the retinoblastoma gene (Rb), p53 controls neuron cell cycle exit,^{155,156} promotes myogenic differentiation *in vivo*,¹⁵⁷ and may inhibit differentiation of adipocytes.^{158,159}

Subtle, nonlethal p53-dependent developmental defects may also impact long-term health. For instance, as cancer is a developmental aberration that manifests over decades in humans, the understated defects caused by p53 malfunction may have profound effects when integrated over time and many cell divisions. Reduced fidelity in lineage commitment may be accompanied by the favorable epigenetic landscapes and expanded stem and/or progenitor cell pools for subsequent transformation. Tumor progression could be further accelerated by loss of p53-mediated genomic stability control. Consistent with this, increased stem/progenitor pools in mammary, neural, and hematopoietic tissues have been reported in p53-null mice.^{84,85,90,160}

Mechanisms by which p53 Modulates Differentiation

The mechanisms by which p53 affects cellular differentiation remain to be enumerated. While numerous possibilities have been suggested,^{10,161-163} we will focus on 3 to exemplify the range of possibilities.

The first mechanism involves regulation of self-renewal through the Wnt pathway. p53 has been reported to impact on expression of Wnt genes.^{101,164,165} As noted above, ESCs are heterogeneous,

and some of the cells appear more prone to differentiation than others. DNA damage can activate p53 to induce differentiation of a subset of cells within ESC cultures.^{93,95,101} ESC heterogeneity in response to damage might, therefore, partially derive from the induction of secreted Wnt proteins¹⁰¹ that act in a paracrine fashion to support the self-renewal of nearby undamaged stem cells, enabling them to remain stem like. p53 also induces the E3 ligase SIAH, which reduces β -catenin levels, leading to reduced Wnt pathway signaling^{164,165} within a cell, which should either lead to a lower probability of cell cycle entry or increased probability of differentiation. Together, these p53-mediated effects on the Wnt pathway enable it to regulate both cell-autonomous and cell-nonautonomous effects. This may provide an elegant mechanism for p53 activation to enable less damaged stem cells to undergo self-renewing divisions to re-establish homeostasis within the stem cell pool under potentially genotoxic conditions.

A second mechanism involves the regulation of symmetric versus asymmetric division. According to this mechanism of differentiation control, symmetric stem cell division generates 2 identical daughter stem cells and thus enables expansion of the stem cell pool. By contrast, asymmetric division produces one stem cell and one proliferative progenitor. Expansion of the latter enables development to proceed but reduces net stem cell concentration within the organ. Recently, p53 loss in the mammary gland was shown to increase the probability of symmetric divisions occurring in an *in vitro* cell culture system using enriched adult mammary stem cells.⁸⁴ Asymmetric division was quantified by measuring the distribution of Numb,^{84,166} a protein linked to asymmetric stem cell division in other systems.^{167,168} Interestingly, Numb directly interacts with Mdm2 and p53 and has been suggested to activate p53 by preventing Mdm2-mediated ubiquitination.¹⁶⁹ While Numb provides a potential direct mechanistic

link between p53 and propensity to differentiate via increasing asymmetric cell divisions, the activation and colocalization of p53 with Numb were not analyzed.

A third mechanism involves p53's ability to limit stem cell self-renewal by inhibiting its ability to cycle rapidly through activation of CDK inhibitors such as p21.^{170,171} Rapid cell cycles are characteristic of ESCs and are required as an early step in the reprogramming of differentiated cells to iPS cells (see below). Inactivating p53 appears to enable oncogenic lesions to induce such rapid cycles. Conversely, activating p53 induces the lengthened cell cycles that not only typify differentiated cells but may also allow the molecular machinery that implements differentiation programs to function. Overexpression of cyclin/cdk antagonists such as p15, p16, and p21 imposes longer cell cycles and limits reprogramming. Conversely, inactivating Rb, a critical cell cycle regulator downstream of p53, by deleting it, by reducing p21 expression (which enables Rb inactivation through increased activity of G1 cyclin-CDKs), or by overexpressing cyclin D-cdk4 all enable more efficient reprogramming and conversion to S/M cell cycles. As reprogramming represents acquisition of a less differentiated state from differentiated cells, the results suggest that factors such as p53 may impact on the balance between differentiation and stemness by regulating G1 progression.

Beyond Genome Guardian: p53 Deficiency and Stem-Like States

p53 Loss Is Associated with Primitive Tumors, While Activation Induces Differentiation

Although p53 mutation and pathway inactivation are found in the majority of tumors, they appear to be especially concentrated among tumors showing plasticity and loss of differentiation characteristics. p53 loss was almost exclusively associated with poorly differentiated thyroid cancers.¹⁷² In breast

cancer, p53 mutations are most frequently found within the poorly differentiated basal-like, metaplastic, and medullary types.^{21,173-175} Collateral mutations of p53 and Pten are the most common tumor suppressor aberrations in glioblastomas, which are poorly differentiated, developmentally plastic brain tumors derived from the neuronal stem/progenitor cells. Here, p53 was observed to limit neurosphere self-renewal.¹⁷⁶ A similar association between p53 loss and loss of differentiation characteristics has been observed in lung cancer,¹⁷⁷ and recent work in a murine lung cancer model indicates that p53 reactivation suppresses malignant adenocarcinoma progression without affecting less aggressive adenomas.¹⁷⁸ Although the mechanism of this suppression remains uncertain, one interpretation is that p53 loss permits tumor plasticity and malignancy in a reversible manner. Likewise, in the classic model of tumor progression from neoplasia to colorectal carcinoma, p53 mutation demarcates tumors progressing from adenomas to less differentiated and more aggressive carcinomas.²⁷ Recently, p53 loss was reported to specifically endow progenitors in AML with self-renewal capacity and correlated with a block to progenitor differentiation.⁹ Interestingly, undifferentiated stem cell tumors, such as embryonal carcinomas (ECs), infrequently contain mutated p53 (e.g., NCCIT, S2¹⁷⁹) but usually express wild-type p53 (such as the F9 line analyzed by Levine *et al.*¹⁸⁰) that is presumably functionally inactivated by one of the many mechanisms summarized in previous sections. The association between p53 loss and the compromised enforcement of differentiation characteristics has also been observed in other settings including mouse models of chemically induced skin carcinogenesis¹⁸¹ and in an Rb model of mammary cancer.¹⁸²

p53 activation promotes differentiation in various cancers. Evidence from the hematopoietic system suggests that p53 enforces differentiation and prevents

ectopic self-renewal at various stages of lineage commitment.¹⁸³⁻¹⁸⁵ AML blasts could be induced to mature by p53 activation mediated by Nutlin.¹⁸⁶ This may also carry over to solid malignancies, as p53 promotes the differentiation of pancreatic carcinoma¹⁸⁷ and osteosarcoma cells¹⁸⁸ *in vivo*.

p53 Loss and Cellular Reprogramming

Selection for p53 functional inactivation during cancer progression has typically been attributed to survival benefits accruing due to reduced apoptosis, cell cycle arrest, and increased opportunities for cellular evolution afforded by genomic instability. However, in light of the above discussion, it is also possible that p53 loss might destabilize the differentiated state and enable reversion to a more stem-like state. Recent *in vitro* model systems, and analyses of tumors *in vivo*, support this additional role for p53 in the control of developmental plasticity.

Noting the relatively undifferentiated phenotype of carcinomas and some papillomas from p53-null and heterozygous mice relative to corresponding tumors from wild-type mice, Kemp *et al.* speculated nearly 2 decades ago that p53 loss might drive loss of differentiation and increase malignancy.¹⁸¹ Similarly, over a decade ago, Bond *et al.* suggested that the apparent “dedifferentiation” accompanying malignant progression in thyroid cancer “. . . may play a causal rather than a passive role in this critical switch in tumour behaviour.”¹⁸⁹ Prescient as these interpretations may have been, it seems they were largely forgotten by the mainstream of cancer research. Rather, an alternative model prevailed in which tumors adhere to essentially irreversible hierarchies of cellular differentiation parallel to those proposed for normal tissues. Early experiments reconstituting complex differentiated tumors following transplantation of stem cells from teratomas appeared to confirm this model.¹⁹⁰ However, teratomas may be a very peculiar case, as they often encode wild-type

p53 that may help enforce differentiation of most daughter cells, and they can be formed from untransformed ESCs.¹⁹¹ Nevertheless, and despite examples from amphibians and other organisms,¹⁹² there has been little compelling experimental evidence to directly support the possibility of cellular dedifferentiation in mammalian cells until recently.

This all changed in 2006 when Takahashi and Yamanaka demonstrated that the enforced expression of 4 transcription factors, (Oct4, Sox2, Klf4, and c-Myc) could induce mouse fibroblasts to adopt pluripotent cell fates resembling stem cells.¹⁹³ These and subsequent studies demonstrated that all cell types can generate induced pluripotent stem (iPS) cells with the appropriate reprogramming gene set. This provided the missing evidence that most, if not all, somatic mammalian cells possess dedifferentiation potential.¹⁹⁴ However, the frequency of reprogramming appeared to be extremely low, raising the possibility of cellular barriers against induced reprogramming.

Several genes in the original Yamanaka iPS cocktail, such as c-Myc, generate oncogenic stresses that activate the p53 pathway to induce cell cycle arrest or death.¹⁹⁵ Consequently, Myc expression, along with general tissue culture stresses, would be expected to activate p53 during iPS cell generation to reduce reprogramming frequency or rate. Several groups tested the role of the p53 pathway in iPS cell formation and found that inhibiting the p53 pathway dramatically increases the apparent efficiency of iPS cell generation (Fig. 1).¹⁴⁻¹⁹ Reducing expression of genes contributing to cell cycle arrest or apoptosis also increased reprogramming. Importantly, a mutation in Mdmx that reduces p53 activity only 2-fold at baseline also dramatically increased reprogramming efficiency.¹⁴ These results have several important implications. First, subtle changes in p53 activity are all that is needed to increase the probability of reprogramming. Thus, even subtle elevations in oncogene signaling that are insufficient to activate p53, but sufficient to

enable cell cycle progression, might increase reprogramming efficiency. Second, reprogramming is limited by a variety of p53-induced protective pathways, including but not limited to those involved in cell cycle arrest, senescence, and apoptosis. Third, in the absence of p53, fewer factors were needed for reprogramming. Finally, through its ability to inhibit cell cycle progression, p53 provides a potent barrier to the acquisition of the epigenetic changes that underlie the dedifferentiation involved in iPS cell formation.

Understanding the mechanisms by which p53 limits reprogramming is complicated by the various methods used for introduction of the reprogramming factors as well as by the expression levels of these factors. However, generation of mice encoding an inducible set of the reprogramming factors provides a powerful, controllable system for analyzing reprogramming kinetics. Such an analysis indicated that p53 inhibition enhances iPS cell generation probabilistically through cell cycle acceleration,¹⁹⁶ although the data left open the possible involvement of cell cycle-independent contributions. Another analysis employing single-cell time-lapse photography of iPS cell formation revealed that a very early step in reprogramming involved oncogene-induced establishment of the very rapid cell cycles that typify ESCs. Here, the effects of p53 loss were cell cycle related, immediate, and restricted to the early phase of the reprogramming process.¹⁹⁷ The authors suggested that early cell cycle changes in a subset of fibroblasts are followed by a multistep sequential process that resets the epigenetic architecture of the cell to resemble that of a stem cell, although not perfectly.¹⁹⁸

p53 Loss, Cellular Dedifferentiation, and Tumorigenesis

The success of induced pluripotency protocols reveals an inherent reversibility of the steps of cellular differentiation. Given the prominent role of p53 loss in this induced pluripotency, the frequent

occurrence of p53 mutations in cancer, and the common occurrence of oncogenic lesions that activate c-Myc or that might phenocopy the effects of other reprogramming factors, it is reasonable to ask whether functional loss of p53 during cancer progression correlates with acquisition of a stem-like state. Recently, several groups have assessed the relationship between cancer and stem cells using comparative gene expression profiling. These comparative studies were facilitated by the archiving of a large number of experimental and disease-associated microarray data sets into publicly accessible databases. Collecting differentially expressed gene lists from a broad set of published studies, Assou *et al.* derived an ESC expression signature comprised of several hundred genes that are consistently upregulated in ESC culture.¹⁹⁹ These signatures included the common reprogramming factors Lin28, Oct3/4, Sox2, and Nanog and many other genes reproducibly identified in ESCs. Subsequently, enrichment analyses using this ESC signature along with other signatures indicative of the ESC state found them to correspond to high-grade tumors in diverse tissues.²⁰⁰ When we applied these signatures to breast and lung tumor data sets in which p53 mutational status was determined by DNA sequencing, we identified a robust correlation between the ESC signatures and p53 mutation.²¹ The correlation could be extended to functional inactivation of p53 inferred from the broader transcriptional profile or deregulation of upstream p53 pathway genes and to rare tumor subtypes known to bear frequent p53 mutation. An iPS cell signature based on archived iPS cell microarray data using the method of Assou *et al.* was similarly correlated to p53 status, suggesting significant similarities between p53-mutated cancers and cells that have undergone intentional reprogramming *in vitro*.

Concurrent work analyzing bladder carcinoma suggested that an ESC-like tumor signature can be driven to a considerable extent by an oncogene such as

c-Myc, but these effects appear to be tissue and context dependent, as the signature they employed did not significantly contribute to the stem-like profiles we observed in breast cancers.²⁰¹ On the other hand, forced c-Myc expression in mammary epithelial cells *in vitro* has been reported to confer a stem-like phenotype.²⁰² Additionally, given the ability of c-Myc to activate p53, it seems likely that p53 must have been functionally inactivated to enable the strong proliferative signature these authors reported to correlate with tumorigenicity. Interestingly, recent evidence shows that chemically induced tumors in the bladder do harbor the aforementioned dominant-negative embryonic Δ N-p53 isoform.¹¹⁶

The above studies did not identify the classic pluripotency genes Oct3/4, Sox2, and Nanog as significant or consistent contributors to stem-like tumor profiles,^{21,200,201} suggesting that cancer-associated stem cell states are not identical to ESC states. In this regard, it may be telling that the identification of stem-like states using expression data has thus far required the use of multi-gene signatures collected from diverse stem cell or iPS cell studies.^{21,200-203} These signatures are comprised of genes that are frequently, but by no means universally, identified as differentially expressed in stem cells relative to differentiated cells. In fact, Assou *et al.* found that among 20 studies, only one gene, Oct3/4, was identified in all studies.¹⁹⁹ This lack of consistency in the stem cell expression profile may reflect natural variability in the cell types present in the stem cell cultures or the use of different culture conditions. Additionally, other technical and statistical differences likely contribute. Nevertheless, the genes comprising these signatures are inherently crossvalidated in that they are found frequently among independent stem cell studies. Furthermore, the statistical tools to evaluate gene expression similarities do not require that all genes of the signature be equally present in the tumor but rather only that a significant subset is present. Importantly, this subset could vary from one stem-like tumor

to another and could indicate diverse and potentially flexible mechanisms for achieving core stem cell functional properties under divergent *in vivo* pressures.

Tumor Heterogeneity and Cellular Plasticity

A Mixed Stochastic/Cancer Stem Cell Hypothesis

The mechanisms by which cancers initiate and expand into masses exhibiting cellular and genetic heterogeneity at diagnosis have long been debated. Two predominant models have emerged to explain phenotypic and functional heterogeneity in tumors (see recent review²¹⁵). The first, called the “stochastic” or “clonal evolution” model, is based on classic theories of selection of mutants most fit to survive in particular environments. In this model, stochastic mutations in an appropriate cell type will be selected if they afford that cell a survival or proliferative advantage. This cell then grows, and subsequent mutations in its descendants ultimately yield a tumor with numerous mutations, only some of which were selected for the advantages they provide, while the others are “passengers.” Recent deep sequencing analyses reveal that different types of tumors differ significantly with regard to their mutation load,^{204,205} and they further reveal the existence of multiple clones within each tumor mass.²⁰⁶ In this model, tumor heterogeneity derives from the constellation of mutations introduced and the phenotypes they engender.

A second model is based on the principles of stem cell biology. In this model, cancers represent an aberration of the molecular and cellular mechanisms that govern development of the corresponding tissue. In normal organs, the final structure derives from a developmental hierarchy in which the undifferentiated stem cell resides at the top, followed by proliferative but relatively undifferentiated descendants, and then (generally) nondividing, terminally differentiated functionally committed cells (Fig. 3). Cellular heterogeneity in tumors is

envisioned to result from a “cancer stem cell” that undergoes aberrant differentiation to form the disorganized mass recognized pathologically. Consistent with such a model, some tumors have mixtures of very immature-appearing cells mixed with more differentiated cells,^{20,190} and specific cells within the tumor can regenerate tumors with the cellular heterogeneity of the original mass in xenografts.²⁰⁷ From this, many researchers have inferred a parent-progeny relationship between tumor-derived cells that initiate xenografts and those that do not^{208,209} and have defined “cancer stem cell markers” capable of isolating the xenograft-initiating population.^{207,209}

Cancer stem cells in the hierarchical model may arise from mutations in a multitude of target cells. For example, transforming mutations may occur in normal tissue stem cells, which would confer obvious advantages deriving from the presumed capacity of such cells to self-renew (Fig. 3A-ii).²¹⁰ Alternatively, a more limited progenitor may acquire mutations and/or epigenetic changes conferring the ectopic capacity for perpetual self-renewal (Fig. 3A-iii).²¹⁰ Several reports now document genetic lesions conferring self-renewal to progenitors,^{211,212} including p53 loss.^{9,211,212} Importantly, in the epidermis and small intestine, the target cell within which such lesions occur impacts disease phenotype and outcome.^{213,214} In such cases, aberrant differentiation of the mutated stem-like cell generates the nontumorigenic cells that can constitute the bulk of the tumor mass. However, the mutations that initiate the disease are insufficient for tumor progression, implying that mutation accumulation according to the stochastic model must also occur to generate aggressive tumor clones.²¹⁵

Reprogramming the Cancer Stem Cell Hypothesis

Neither of the traditional models incorporates the possibility of tumor-associated cellular reprogramming and plasticity associated with loss of p53 function. Given the impact of p53

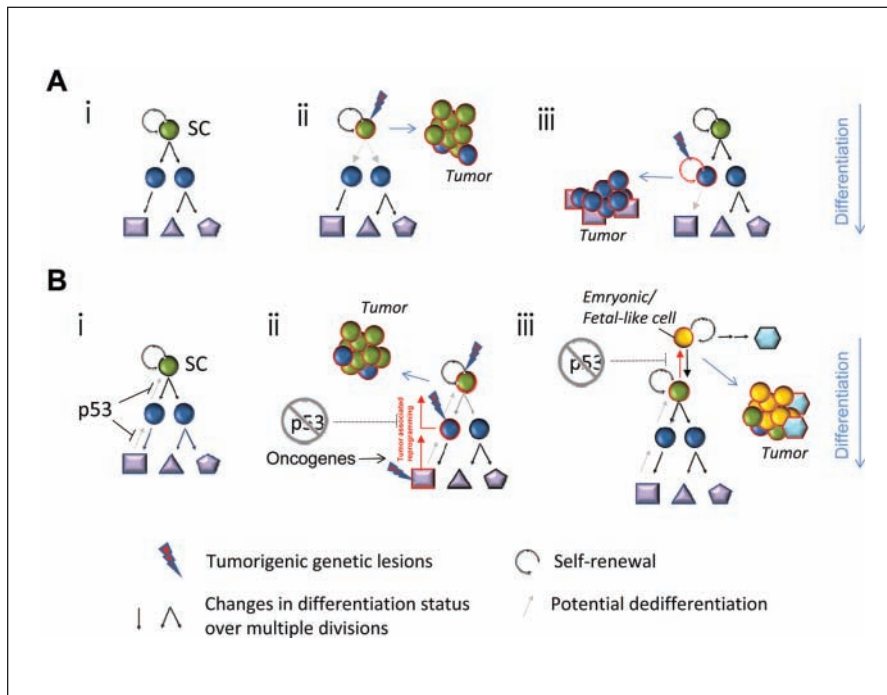


Figure 3. Models of stem cell state acquisition in cancer. **(A)** A model of the classic differentiation hierarchy initiated by a self-renewing stem cell (**A-i**). Tumorigenic transformation occurs within a self-renewing stem cell (**A-ii**). Mutations engender self-renewal competence in a progenitor cell (**A-iii**). **(B)** In normal tissues, p53 function limits the possibility of reprogramming to a stem-like state (**B-i**). Cancer-associated reprogramming following p53 inactivation would permit the evolution of a stem-like cancer from stem or non-stem cell antecedents (**B-ii**). It is also possible that reprogramming could drive cells toward even more primitive embryonic stem cell states, as occurs in the reprogramming of differentiated cells to induced pluripotent cells (**B-iii**). SC = stem

inactivation on cellular dedifferentiation in the presence of appropriate oncogenic lesions, and the commonality of p53 defects in all human cancers, it is reasonable to consider induction of developmental plasticity as an important correlate of tumor progression. In breast cancer, where p53 mutations are inferred to occur late^{173,216} and are associated with stem-like states in basal-like cancers,²¹ we infer that cells that acquire this property have survival advantages that enable their accumulation. Acquisition of developmental plasticity by p53 inactivation may explain why targeting Brca1 deficiency to either basal or luminal cells in the mammary gland generated tumors that could not be distinguished by gene expression but did show morphological differences.^{217,218}

We suggest that a more accurate description of the complex events occurring during tumor progression requires

incorporating the potential for cellular reprogramming with the stochastic and cancer stem cell models. Here, cells with stem-like properties may be formed at any time during cancer progression, so long as p53 (or other factors that phenocopy its function) is disabled and appropriate oncogenic lesions that can drive proliferation and enable epigenetic reprogramming to a stem-like state are present (Fig. 3B-ii and 3B-iii). This model could help explain why it has been difficult to apply hierarchical models to some tumors.²¹⁹ In fact, plasticity has been demonstrated with regard to numerous putative cancer stem cell markers,²¹⁹⁻²²² and it remains to be determined if there are markers that enable general identification of “stemness” in a given tumor. However, one interesting candidate is CD44, a putative cancer stem cell marker frequently reported to segregate with the capacity for xenograft initiation (e.g., in

estrogen receptor–negative breast cancer²²²). CD44 is repressed by p53 directly and through p53-mediated induction of miRNA34a.^{223,224} Thus, upregulated CD44 expression may be a surrogate marker for p53 inactivation and associated plasticity.

A corollary of this composite model is that tumor-associated reprogramming may generate aberrant stem-like states. For example, reprogramming p53-deficient, oncogene-expressing cells could generate phenocopies resembling fetal stem cells or iPS cells (Fig. 3B-iii). Additionally, the aberrant microenvironments created within tumors might influence the type of stem-like cells that arise and may explain why the expression signatures of such cells in tumors are similar to, but distinctly different from, other adult or ESCs. Depending on the probability of reprogramming within tumors, it is conceivable that multiple independently derived and molecularly distinct stem-like clones could evolve (Fig. 4). This might explain the appearance of multiple clonal lineages within tumors identified by single-cell sequencing.²⁰⁶ Finally, it is not inconceivable that unique stem-like states could be created within tumors as a consequence of the influence of microenvironments on cells with p53 deficiency and oncogenic changes. Such states might explain the ability of some tumor cells to transdifferentiate into functional vascular-endothelial cells that resist antiangiogenic therapy,²²⁵ to exhibit remarkable plasticity regarding chemoresistance,²²⁶ and to migrate and metastasize.

Going Forward

Despite differences resulting from their specific genetic lesions and microenvironmental contexts, stem-like cancer cells are unlikely to arise by “inventing” completely novel biology. Rather, it is more likely that their genesis reflects the corruption of a reactivated normal stem cell repertoire. Thus, to gain greater insight into the mechanisms underlying

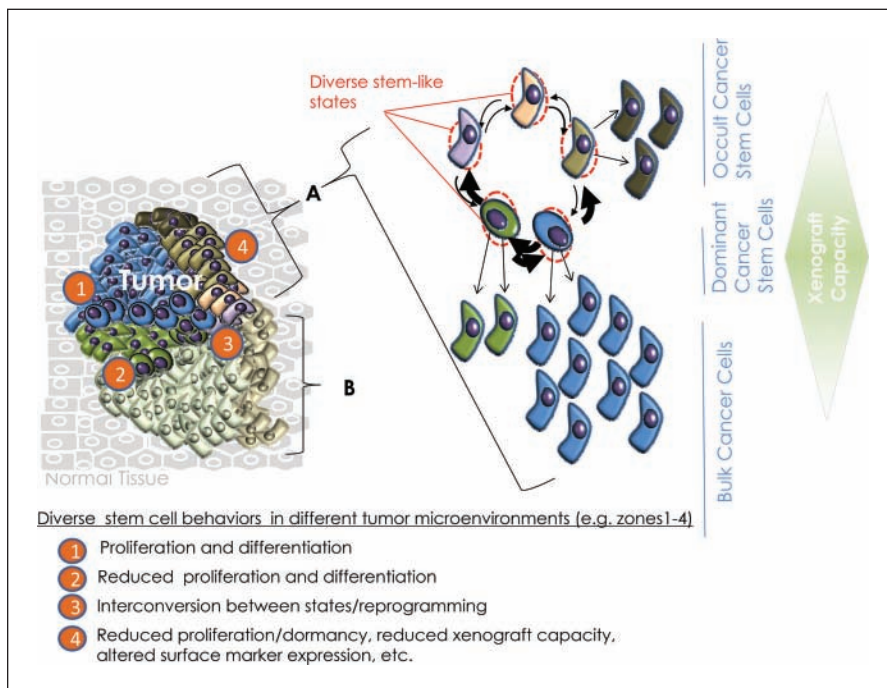


Figure 4. Induced plasticity in cancer and the potential for multiple cancer stem-like cells to coexist. A tumor mass (left) may contain multiple related but genetically distinct and independently propagating clones (**A** and **B**).²⁰⁸ Each clone may be sustained by a genetically and epigenetically unstable pool of stem-like cells (right), whose behavior (e.g., proliferation v. dormancy) can be influenced by the level and nature of oncogenic stimuli and the dissimilar local microenvironment in which they are situated. While some stem cells may initiate or perpetuate clonal growth in response to local microenvironments, other stem-like cells may remain indolent until appropriate signals are received. The resulting heterogeneity may be manifested as diverse stem-like states that vary in terms of their proliferative, biomarker, and chemosensitivity profiles as well as their ability to xenograft but not in their net tumorigenicity. Furthermore, disaggregation of the tumor mass for analysis obscures the local heterogeneity initially present. Together, inherent plasticity as well as additional acquired genetic traits may endow these cells with differential capacities for interconversion to more aggressive stem-like states during tumor evolution or recurrence.

acquisition of stem-like states in cancer, we are compelled to investigate the spectrum of both normal and neoplastic stem cell states. Achieving this goal imposes significant challenges. The uncontrolled stress effects associated with *in vitro* sphere and *in vivo* transplantation assays can activate p53, which may prevent normal stem cell function. The heterogeneity of all stem cell-enriched populations prevents one from assigning a definitive gene signature to the actual stem cell. To overcome these challenges, the field will need to develop methods that minimally perturb the natural tissue or tumor setting, potentially by combining *in vivo* lineage

tracing with the robust but physiologically less relevant stem cell assays that are currently most often employed. Solving the problem of population heterogeneity will require that we devise genome-wide, single-cell analyses and then reintegrate the information obtained into systems level understanding of the stem cell state.

Finally, given the link between p53 and dedifferentiation and the implications this association has for cancer stem cell models, it may be useful to focus not only on the processes underlying “stemness” but also the plasticity that allows stemness to emerge and diversify in the tumorigenic context. In this regard,

perhaps targeting plasticity, rather than selected “stem-like cells,” will afford more robust strategies for tumor management. Furthermore, the apparent commonality of metastable genomes and differentiation states in cancer suggests that developing therapies that enable conversion of cancers to chronic but manageable diseases is a commendable goal in addition to searching for “cures.” Dissecting the role played by p53 in preventing dedifferentiation may provide valuable inroads into such therapeutic strategies.

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