Context, tissue plasticity, and cancer: Are tumor stem cells also regulated by the microenvironment?

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Introduction

Lest we forget, within every higher organism, there are literally billions of cells with identical genetic information that serve as constituents of the different tissues and organs. Given that genetic information is the same in all cells, including the stem cells, by definition, cells in higher organisms do not possess a sense of place or purpose by themselves. Therefore, in order for each organ to operate successfully within the context of the organism, all cells must be integrated into an architectural and signaling framework such that each cell knows exactly which commands to execute at any given time. Success at this daunting task leads to homeostasis, while failure results in a spectrum of dysfunctions, including cancer. How do organisms achieve this remarkable feat, and how does each cell in return know what to do within the tissues?

The microenvironment exerts control over the genome in both normal and malignant cells

If the genome of differentiated cells had complete autonomy, there would be no tissue specificity, and isolated cells would continue to function in cell culture as they would in the organ. This clearly is not the case: isolated cells are known to lose most functional differentiation when separated and placed in traditional cell cultures (Bissell, 1981). However, the cellular identity is not lost permanently, as we have learned that by controlling the microenvironment of the cells in culture, we can make them "remember" many of their original tissue specific traits (for review, see Bissell et al., 2003).

It is also known that over the lifespan of an organism, individual cells incur multiple harmful genetic lesions due to environmental exposure and physiologically induced reactive oxygen species. If cancer were exclusively due to genetic mutations, then we should expect every organ to eventually become cancerous. Moreover, heritable cancer syndromes almost exclusively affect just a single tissue type, even though every cell contains the mutation. Therefore, in addition to known defense mechanisms such as DNA repair, factors from the tissue microenvironment must play key roles in cellular decision-making and maintenance of homeostasis.

Experiments performed in chimeric mice and in chickens have provided evidence of the delicate equilibrium maintained by the normal microenvironment in spite of cells that might otherwise be predisposed to neoplasia. One of the most dramatic examples is that of studies by Mintz and Illmensee in which embryonal carcinoma cells injected subcutaneously in mice formed teratocarcinomas, whereas the same cells injected into a blastocyst gave rise to normal chimeric mice instead of tumors (Mintz and Illmensee, 1975). This experiment posed a conundrum: Can a tumor cell beget a normal offspring? If cancer is only a genetic disease, were there cancer-causing mutations in the teratoma cells, and if so why were the mice phenotypically normal? If there were no mutations in the teratoma, how did it make tumors in the first place when injected subcutaneously? These and a number of other perplexing questions were left unanswered as the field entered the exciting era following the discovery of oncogenes, suppressor genes, and the role of genetic mutations in cancers. Thirty years earlier, the same fate had befallen a series of experiments performed by Duran-Reynals and his colleagues using Rous sarcoma virus (RSV). In Nobel Prize-winning work, Rous had shown that RSV, which contains one of the most potent oncogenes, $pp60^{src}$ (for review, see Martin, 2001, 2004. and references therein), can cause aggressive tumors when injected in the wing of a chicken; in doing so, he proved Koch's postulate for the first tumor-causing virus (Rous, 1979). However, experiments by Milford and Duran-Reynals showed that RSV injected in the chick embryos did not form tumors (Milford and Duran-Reynals, 1943). Subsequent discoveries that RSV caused transformation of chick embryo fibroblasts in culture (Martin, 2004) rendered findings in the embryo suspect, as virologists believed at the time that the findings could be explained by lack of virus integration and/or gene expression in the chick embryo, and consequently, these data were ignored for decades.

Many years later, we performed experiments in virus-free chicken embryos infected in ovo with tagged $pp60^{src}$, and showed that the virus was expressed in most organs of the infected embryos, but was not tumorigenic (Stoker et al., 1990). However, if the infected embryos were dissociated and placed in culture, there was mass transformation within 24 hr (Dolberg and Bissell, 1984). Interestingly, even the tumors that formed in adult chickens were shown to be dependent on the puncture wound at the injection site or at distally wounded sites (Dolberg et al., 1985). In one of the first demonstrations of the contradictory roles of TGFβ in regulation of normal and malignant cells, the molecule responsible was shown to be the wound-induced TGFβ (Sieweke et al., 1990). There are many more examples of the importance of context and the microenvironment in attenuation or induction of tumors (Kenny and Bissell, 2003), and it is interesting to note that there are now more than 900 entries in a Google search on the relation between wounding, inflammation, and oncogenes, confirming the connection observed by physicians and researchers as early as the mid-19^{th} century (for review, see Sieweke and Bissell, 1994). Such observations support the concept of cancer as a disease that must simultaneously subvert the microenvironmental controls as well as the genetic program.

The examples discussed above were published decades ago, but it is encouraging that current technologies are making it possible to reexamine these findings at the molecular level. That a single oncogene is not sufficient to induce tumors was con-

firmed definitively by the first engineered oncomouse: tumors were formed only in occasional cells rather than in all cells, were heterogeneous, and had the appropriate latency (Stewart et al., 1984). There are now numerous examples of other engineered mice with different oncogenes, both with and without conditional expression, that attest to the need for additional mutations in the cancer cells and/or additional promotion from the microenvironment. In a striking example of this principle, inappropriate expression of different metalloproteinases (MMPs) leads to loss of integrity of tissue microenvironment and eventually tumors; here, an extracellular signal was shown to effectively act as an oncogene (Sternlicht et al., 1999; for revies, see Wiseman and Werb, 2002). Most recently, in a compelling and elegant permutation of the Mintz and Illmensee experiments, nuclei from malignant cancer cells were introduced into enucleated oocytes, which were then used to generate embryonic stem cells that were subsequently used to make chimeric mice. Although the chimeras had a predisposition for malignancy, the vast majority of their tissues were normal, apparently because the malignant phenotype was held in check by the normal microenvironment (Hochedlinger et al., 2004).

That genetic manipulation of stromal cells can result in tumor formation strongly suggests that in some cases, the source of mutation, and thus the original cause of the tumor, may be the neighboring cells. A recent example is the interaction between fibroblasts and epithelial cells in intraepithelial neoplasia in prostate and invasive squamous cell carcinoma of the forestomach. Experiments demonstrated that making stromal fibroblasts unresponsive to TGFβ leads to unrestrained epithelial cell growth and invasion. In this case, it was proposed that the mutated fibroblasts generated HGF and thus an abnormal paracrine signaling leading to epithelial tumors (Bhowmick et al., 2004a; Radisky and Bissell, 2004), and this may be a general principle, as some tumors appear to influence the development of their own supportive environments. Neurofibromatosis, e.g., affects 1 in 4000 people who are born heterozygous for the tumor suppressor, neurofibromin (NF1); loss of both alleles in Schwann cells is required to form neurofibromas. In addition to loss of heterozygosity (LOH) in Schwann cells, the other surrounding cells of the tumor must also become NF1 heterozygotes for tumors to form, because the mast cells and fibroblasts then produce factors to support the NF1 null Schwann cells (Zhu et al., 2002).

Stromal cells are responsible for producing both many of the ECM-degrading enzymes and most of the connective tissue ECM. In some mammary carcinomas, stromal cells were shown to have acquired unique chromosomal rearrangements relative to the tumorigenic epithelium (Moinfar et al., 2000), and some heritable diseases that afflict carriers with higher incidence of cancer have been shown to be due to stromal defects (Howe et al., 1998; Jacoby et al., 1997). Taken together, these examples provide evidence to support the notion that the microenvironment can function both as a powerful tumor suppressor even in the presence of strong oncogene expression, and as a tumor promoter for precancerous, or even apparently normal, cells (Sternlicht et al., 1999).

Do tumors originate from mutated stem cells?

A frequent observation by pathologists has been that while some malignancies resemble the organ of origin morphologically and biochemically, many appear less differentiated and even embryonic, as is the case of Wilm's tumors and neuroblastomas (Sell, 2004). There are a number of conclusions that may be derived from these observations: (1) if the somatic cells are severely damaged, they may be able to "dedifferentiate" to a progenitor-like state; (2) tumor cells may encounter the right microenvironment with appropriate biochemical signals as they migrate out of the original tissue that would allow them to redifferentiate, analogous to the "tumor reversion" scenario observed in 3D cultures of breast tumor cells (Weaver et al., 1997, and discussion below); and (3) some tumors may originate from "cancer stem cells" which could get reactivated in specific sites to recreate the original tissue phenotype. The latter possibility is supported by observation of a phenomenon coined "maturation arrest" due to the fact that within a tumor there are cells that appear to be tissue-specific stem cells (TSSC), arrested at a progenitor stage of development (Cozzio et al., 2003). Whether TSSCs represent the cellular origin of cancer is a distinct area of discussion, but the experiments that support the concept have demonstrated that maturation arrest is possible, and it is clear that the source of the signals that cause the arrest would have to be from the local microenvironment. By combining observations made in the human population with more recent experimental evidence, the cancer stem cell hypothesis can be extended to include the microenvironment as a key player in both keeping cancer in check and in releasing anticancer restraints.

It has been hypothesized and tested (though not yet proven) that mutated mammary stem cells are the origin of breast cancer (Al-Hajj et al., 2003; Smith and Boulanger, 2002), and in this case, the structure of the organ provides insight into how this may occur. The mammary gland consists of a bilayered epithelium (the inner luminal epithelium and the outer myoepithelium) that is ramified into a tree-like structure, in which each branch is capped by a terminal end bud in the developing gland, becoming lobules in the mature gland. By examining separate branching structures, Deng et al. found unique signatures of genomic loss of heterozygosity (LOH) in individual branches (Deng et al., 1996), suggesting that mutations in single stem cells during gland development in puberty may initiate genomic instability in progeny cells, and ultimately, breast cancer.

All else being equal, the TSSCs that are thought to mediate repair, replenishment, and regeneration of their respective tissues must be maintained in a quiescent state until they are called upon to act on behalf of the tissue. Their normal behavior is strictly governed by the stem cell microenvironment, or niche, which is composed of other cells, ECM, and other secreted factors (reviewed in Ohlstein et al., 2004). A well-studied example of this phenomenon involves hematopoietic stem cells, which are modulated by BMP-related signaling and the Notch pathway due to their association with osteoblasts in trabecular bone (Calvi et al., 2003; Zhang et al., 2003). Similarly, self-renewal and differentiation of neural stem cells is controlled by their association with endothelial cells that signal through the Notch pathway, among others (Shen et al., 2004). Though the niche may act to maintain stem cell quiescence for decades, these cells are highly dynamic once activated: an embryo develops from a single cell in 9 months, the intestine regenerates rapidly and constantly, the liver recreates itself in a few days after partial hepatectomy, and the mammary gland involutes completely after lactation but regenerates only after a few weeks. In the same fashion, the microenvironment of the niche apparently also controls damaged stem cells that may act as cancer progenitors, since

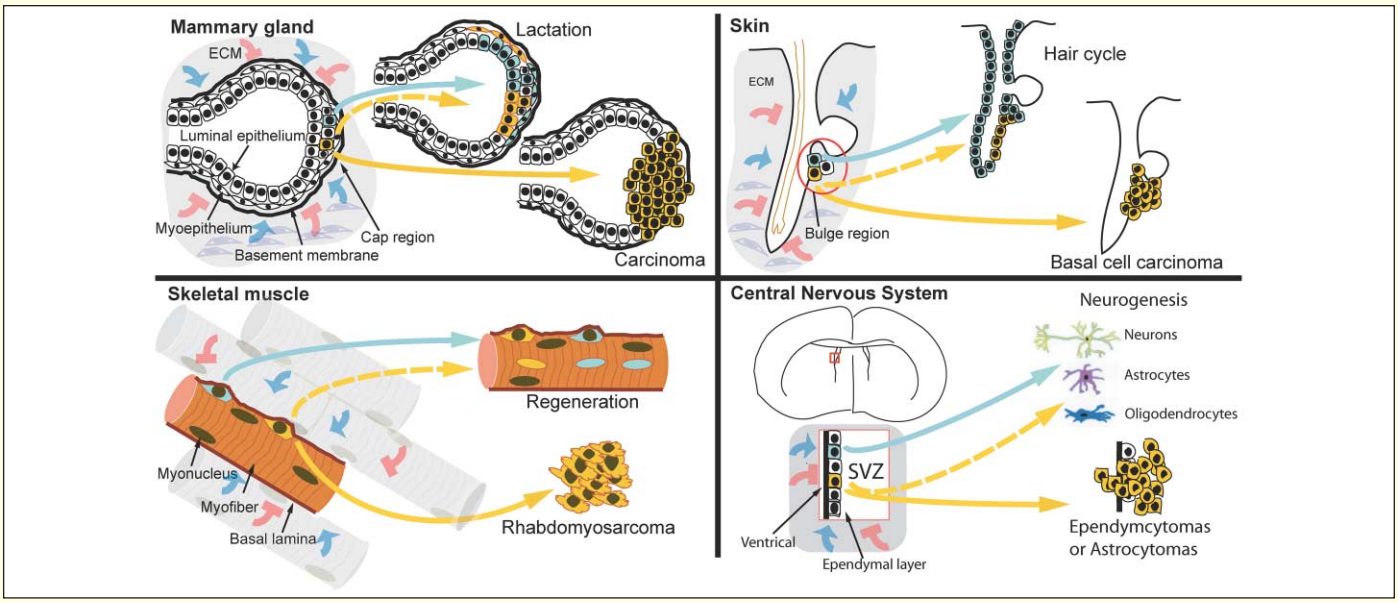

Figure 1. A normal microenvironment can preserve the tissue architecture even in the presence of predisposed cells, and an aberrant microenvironment can promote the mutated cells to form tumors

Normal (blue) and genetically damaged (yellow) tissue-specific stem cells are shown in their niche surrounded by an instructive microenvironment that is composed of cellular (stroma), structural (ECM molecules), and soluble components (growth factors, cytokines, proteases, and hormones, among others). Normal and damaged TSSC can regenerate and replenish their respective tissues of origin in response to normal microenvironmental cues (blue solid and yellow dashed arrows). However, when balanced control over the TSSCs is altered, they can also give rise to tumors (yellow solid arrows). Cells in the cap region of the virgin rodent mammary gland, and those in upper basal region of the ducts and possibly acini, thought by some to be the physical location of the mammary stem cells, can give rise to both luminal and myoepithelial cells during development and lactation (Williams and Daniel, 1983); others assert that the TSSCs are located throughout the gland (Smith and Boulanger, 2002). The same signals that promote normal stem cell activity could push a predisposed stem cell to initiate carcinoma of the breast. The skeletal muscle stem cell is shown in its characteristic satellite cell position; however, reports have also shown that they may reside in interstitial spaces. Normally, during times of muscle regeneration, they fuse with muscle fibers to provide new myonuclei, but under rare circumstances, they are thought to give rise to rhabdomyosarcomas (Keller et al., 2004; Tiffin et al., 2003). Skin stem cells residing in the bulge region give rise to the root sheathe during the hair cycle, but in some cases are thought to be the origin of basal cell carcinomas (Owens and Watt, 2003). The ependymal cells of the subventricular zone (SVZ) in the CNS normally give rise to astrocytes, neurons, and oligodendrocytes. Under some conditions, however, they are known as the cause of ependycytomas or astrocytomas (Maher et al., 2001).

many tumors can lie dormant or develop slowly for decades before manifesting as a clinical outcome.

That tumors take a long time to develop is illustrated clearly by the following examples. A large number of female patients, including girls at puberty, living in Massachusetts in the United States circa 1920–1950, underwent screening for tuberculosis by use of an X-ray fluoroscope. Repeated exposure to fluoroscopes, as was generally done at the time, generated toxic doses of irradiation, and retrospective analysis revealed a significantly higher incidence of breast cancer in women who were young at the time of irradiation than would have been predicted by the frequency of breast cancer in the population at large. The cancer occurred 25–30 years later in their lives (Hrubec et al., 1989). Similar patterns of breast cancer are reported for survivors of the atomic bombs that were used in Japan, and the incidence again was skewed to younger women (Carmichael et al., 2003). It is fascinating that the patients described in both studies were young at the time and developed breast cancer only many years after their initial exposure, especially since we now know that the microenvironment of the irradiated tissue also becomes abnormal, as discussed below. How could a mammary stem cell that had incurred deleterious genetic lesions have been held in check for so long? Whether the reason is the ability of the niche to be repaired more quickly in young girls, which could then hold the mutated stem cells dormant, or whether the stem cells had to acquire additional mutations over the years, is an important area of research that could help and guide decisions on chemoprevention and therapy in the future.

How microenvironmental signals override genetic infidelity

Physical damage of DNA is a long-known consequence of exposure to excessive ionizing radiation. Pertinent to the above hypothesis are the questions of how many deleterious genetic lesions a cell can incur before the microenvironment can no longer control its growth, and which signaling pathways are likely to be most important for maintaining microenvironmental control over a damaged cell. Based on recent experiments in culture and in vivo, we can elaborate a set of rules that define which microenvironmental constituents and which cellular sensors appear to be required to restrain a TSSC predisposed to cancer.

It appears that cells can incur a great deal of damage to their genetic programming and still remain phenotypically dormant (Chin et al., 2004). In fact, even after the cells become tumorigenic, they still can retain the aberrant genome, but for all practical purposes still revert to a normal phenotype if tissue polarity is restored. The HMT-3522 luminal epithelial cell line was isolated from a reduction mammoplasty (Briand et al., 1987). These cells were used to derive S1 cells that have a number of mutations, but when injected into NOD/SCID mice

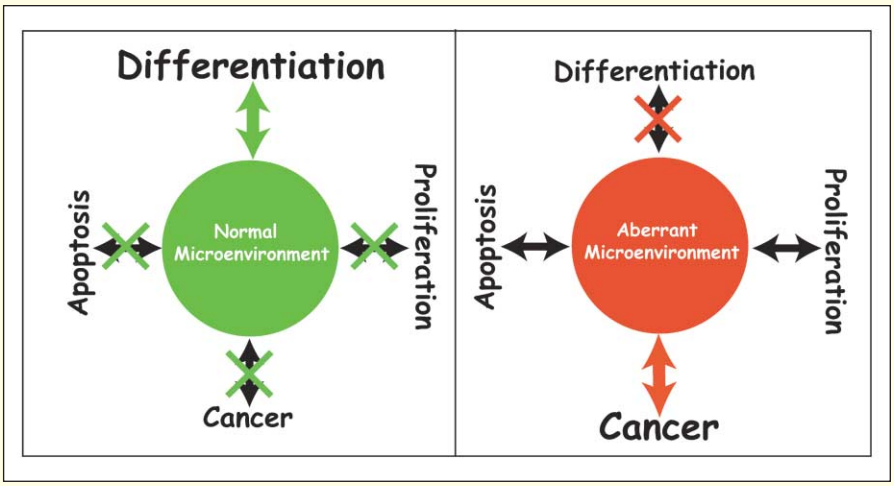

Figure 2. Integration of signaling pathways by the microenvironment

The important element to consider here is that the normal microenvironment must integrate what is correct (tissue-specific functions, differentiation) by giving the pathways involved the green light, but also prevent what is wrong (pathways for inappropriate growth, apoptosis, and cancer). When the microenvironment becomes aberrant, the opposite will happen: the normal differentiated state is blocked and those pathways that were blocked previously now become operative.

are nonmalignant; when cultured in 3D, laminin-rich basement membrane gels that recapitulate the normal niche, they growth-arrest and form structural mimics of mammary acini found in vivo (Petersen et al., 1992). Following extensive passaging of S1 cells in the absence of EGF, a cell population was derived (T4-2) that could form tumors in mice (Briand et al., 1996). These cells form nonpolarized, disorganized masses in 3D cultures that do not growth-arrest (see Bissell et al., 2003 for review). T4-2 cells do not express p53 (Briand et al., 1996) and have acquired a number of additional genetic lesions, determined by array CGH (J.W. Gray and M.J.B., unpublished data). S1 cells are arguably predisposed cells that can still be held in check by their microenvironment, whereas T4-2 cells have lost these controls. Therefore, the system presents an opportunity to dissect the elements of the microenvironmental regulation that T4-2 cells have lost.

Extensive analysis of T4-2 and S1 cells has shown that T4-2 cells have a number of altered signaling pathways. For instance, EGFR, MAPK, PI3 kinase, and β1-integrin are highly active, but PTEN and intact dystroglycan are downmodulated. These changes in signaling mediators reveal how T4-2 cells have lost the ability to properly transduce the microenvironmental signals that would otherwise allow differentiation, and suppress proliferation and apoptosis (Liu et al., 2004; Wang et al., 1998; Weaver et al., 1997). Treating T4-2 cells in laminin-rich 3D gels with blocking antibodies or with pharmacologic reagents that reduce signaling through these key pathways, causes formation of phenotypically normal acinus-like structures. Remarkably, the cells also become significantly less tumorigenic (Liu et al., 2004; Wang et al., 1998, 2002; Weaver et al., 1997), and this phenomenon of phenotypic reversion can be extended to other breast cancer cell lines, including metastatic ones (Wang et al., 2002). A similar "reversion" phenomenon has been described and exploited for therapy in acute myelogenous leukemia (AML) patients. Niitsu and colleagues demonstrated that bone marrow-residual disease, which results in AML relapses following chemotherapy, is caused by VLA-4-mediated adhesion of AML cells to bone marrow stromal cells (Matsunaga et al., 2003). VLA-4-positive AML cells were resistant to apoptosis and persisted in an essentially reverted, dormant state. In T4-2 cells, reestablishment of tissue polarity also renders the acini-like structures resistant to chemoptherapeutic agents (Weaver et al., 2002). (Other studies have shown that coculture of neoplastic and normal cells renders the former phenotypically normal [see, e.g., Javaherian et al., 1998].) These studies demonstrate how modulating and correcting a genetically damaged cell's ability to communicate with the microenvironment could result in the acquisition of a polarized and phenotypically normal state, which in turn would make them essentially resistant to chemotherapy.

It is useful to consider whether the same mechanisms unraveled in 3D cultures for the maintenance of polarity and tissue structure are also operative in vivo. Given the importance of laminin-1 in these processes in culture (Weaver et al., 2002), we asked what cell types in breast produce laminin-1. In vivo, it is myoepithelial cells that produce laminin-1, and in culture, myoepithelial cells can replace a laminin-rich gel in conferring acinus polarity to luminal epithelial cells. The surprising finding was that a majority of myoepithelial cells isolated from breast cancers appeared to have lost the ability to produce laminin-1 and could no longer confer polarity to luminal cells (Gudjonsson et al., 2002). Although production of laminin-1 may not be the only mechanism by which myoepithelial cells impose tissue polarity (Runswick et al., 2001), the hypothesis that myoepithelial cells behave as "structural tumor suppressors" for luminal epithelial cells in the breast (Gomm et al., 1997; Gudjonsson et al., 2002; Sternlicht et al., 1996) appears to be correct. Since regulation of adhesion, polarity, and proliferation is of utmost importance in tissue homeostasis, agents that disrupt the integration of these pathways could lead to signaling imbalance and eventually cancer (Sternlicht et al., 1999; Wiseman and Werb, 2002). Moreover, if it is true that cancers represent arrested or improper TSSC development, then pathways such as Notch and Wnt, which operate both in stem cell maturation and in cancer development, must also be tightly regulated by microenvironmental cues (Beachy et al., 2004).

Stem cells and their microenvironment

In addition to genetic damage to the TSSC, the stroma is also susceptible to damage from ionizing radiation, and here, epigenetic changes appear to be even more important (Barcellos-Hoff and Brooks, 2001). If there were lasting changes to the composition of the mammary microenvironment, the aberrant signals would be additional contributing factors to tumor onset in the irradiated women in the examples cited above. While the application of γ irradiation to organisms is generally thought to kill the cells with proliferative potential by inflicting irreparable genetic lesions, there is also global damage to organs due to production and/or activation of proteases and growth factors that can cause systemic alterations to cellular microenvironments (Ferrara, 1993; Wiseman and Werb, 2002). For instance, in skeletal muscle, the terminally postmitotic multinucleate

myofibers are cells that incur little or no detectable damage, as judged by their apparent normal function and morphology, following high doses of γ irradiation (Goyer and Yin, 1967; Warren, 1943), while the resident muscle stem cells are susceptible (LaBarge and Blau, 2004; Schultz and McCormick, 1994). It is clear that γ irradiation causes potentially deleterious effects also to the skeletal muscle microenvironment. Under normal circumstances, muscle stem cells can be isolated and passaged in tissue culture, then injected back into skeletal muscle where they fuse with the existing muscle fibers during myogenesis. Interestingly, when the skeletal muscle was γ irradiated prior to injection of normal muscle stem cells, the TSSCs were shown to give rise to tumors (Morgan et al., 2002; Murtuza et al., 2002). In an analogous fashion, mammary epithelial cells harboring a Trp53 mutation in both alleles were nontumorigenic when implanted into normal cleared fat pads. When the recipient mouse stroma was γ irradiated prior to the implantation of unirradiated epithelial cells, tumors were formed at a high percentage in the injection sites (Barcellos-Hoff and Ravani, 2000). That the tumors could be the result of stem cell subpopulations in these studies was implied, but remains to be proven. Nevertheless, it was made clear by these reports that an otherwise normal microenvironment was modified globally by the application of γ irradiation, which in the long run was sufficient to initiate tumor growth.

What changes might have occurred in the microenvironments of the skeletal muscle and mammary epithelial cells following irradiation? Application of γ irradiation to mammary tissue was shown to cause the release of TGF-β, among other molecules, which is a postulated and proven participant in many of the tumorigenesis models discussed above (Barcellos-Hoff, 1993; Ferrara, 1993; Kenny and Bissell, 2003). Similarly, it is possible that γ irradiation causes deleterious changes in gene expression in the resident fibroblasts (Boerma et al., 2003), which in turn could promote tumorigenesis (Bhowmick et al., 2004b). Similar changes may occur with age, as quiescent fibroblasts were shown to produce secreted proteins that were proproliferative to predisposed breast epithelial cells (Krtolica et al., 2001). Finally, processes such as wound repair and tissue regeneration, in which a mitogenic microenvironment is favorable to the tissue, may in fact facilitate TSSC-derived tumors. Muscle and prostate regeneration and wound healing are associated with induction of molecules such as hedgehog that are known to be involved also in tumorigenesis. Gorin's syndrome, which results in a high frequency of sporadic tumors, occurs due to the loss of negative regulation of the hedgehog signaling apparatus (Beachy et al., 2004). Moreover, hedgehog overexpression is known to be sufficient to cause basal cell carcinomas that are thought to originate from bulge cells of the skin, where the skin stem cells reside (Oro et al., 1997; Tumbar et al., 2004), and there are also examples in intestinal development and cancer (Sancho et al., 2004). Taken together, these scenarios suggest mechanisms by which a normal environmental response to irradiation, aging, or other damages could push a predisposed TSSC toward frank cancer (Figure 1).

Summary view

There is now much evidence that the microenvironment regulates tissue specificity and contributes significantly to tumorigenesis. With respect to "cancer stem cells" as potential originators of cancers, here we propose mechanisms for at least two important elements in the tumorigenic process that were predicated on observations in the human population. First, a genetically damaged TSSC can be held in check for long periods of time, which can explain the long delay between environmental exposure and/or germ line suppressor mutations and cancer onset. Second, ionizing radiation, or physical and chemical insults generally, can result in changes to the microenvironmental composition that can by themselves trigger mutations in the stem cells and eventually cancer. We hypothesize that a link between the two mechanisms exists as a dynamic and reciprocal relationship between genetically damaged cells in a tissue and their microenvironment, whereby damaged cells modify their environment, which, in turn, in a vicious cycle, brings about more pathological behavior from the cells, analogous to the mechanism postulated for normal homeostasis (Bissell et al., 1982) (Figure 2). Ultimately, it is this relationship that precipitates a deleterious change over many years, resulting in cancer.

Not even a cancer cell is an island. No cell can perform without instruction from its microenvironment, and the latter of course would not exist without its initial genetic instructions. Thus, the cellular origin of cancer, while critically important, is insufficient to explain the process of tumorigenesis. If we are to consider the tissue-specific stem cell view of cancer, then we need to learn how to prevent tumor onset without also completely blocking normal tissue regeneration. Understanding which cues stimulate a stem cell to get activated may lead to prophylactic approaches for therapy and possible prevention.

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