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Hypoxic Tumor Microenvironment and Cancer Cell Differentiation

Yuri Kim, Qun Lin, Peter M. Glazer, and Zhong Yun*

Department of Therapeutic Radiology, Yale University School of Medicine, P.O. Box 208040, New Haven, CT 06520-8040, USA

Abstract

Hypoxia or oxygen deficiency is a salient feature of solid tumors. Hypoxic tumors are often resistant to conventional cancer therapies, and tumor hypoxia correlates with advanced stages of malignancy. Hypoxic tumors appear to be poorly differentiated. Increasing evidence suggests that hypoxia has the potential to inhibit tumor cell differentiation and thus plays a direct role in the maintenance of cancer stem cells. Studies have also shown that hypoxia blocks differentiation of mesenchymal stem/progenitor cells, a potential source of tumor-associated stromal cells. It is therefore likely that hypoxia may have a profound impact on the evolution of the tumor stromal microenvironment. These observations have led to the emergence of a novel paradigm for a role of hypoxia in facilitating tumor progression. Hypoxia may help create a microenvironment enriched in poorly differentiated tumor cells and undifferentiated stromal cells. Such an undifferentiated hypoxic microenvironment may provide essential cellular interactions and environmental signals for the preferential maintenance of cancer stem cells. This hypothesis suggests that effectively targeting hypoxic cancer stem cells is a key to successful tumor control.

Keywords

Cancer stem cells; differentiation; hypoxia; oxygen; tumor microenvironment

INTRODUCTION

Solid tumors often contain regions with significantly decreased oxygenation, as compared to normal non-ischemic tissues. As measured *in situ* by the polarographic pO₂ electrode method, a median pO₂ of <15 mmHg has been found in many types of human solid tumors, such as squamous cell carcinoma of the uterine cervix [1], head-and-neck cancers [2], breast cancers [3] and brain tumors [4], in contrast to a median of >35 mmHg in respective adjacent normal tissues. Existence of tumor hypoxia has also been validated using biochemical hypoxia markers, such as EF5 and pimonidazole, or endogenous molecular markers, such as hypoxia-inducible factor 1 α (HIF-1 α) and carbonic anhydrase 9 (reviewed in [5–7]). These studies clearly demonstrate that hypoxia is a salient feature of solid tumors.

Tumor hypoxia is an independent prognostic factor for advanced disease progression and poor clinical outcome [8–12]. Clinical studies have clearly shown that patients with hypoxic tumors (pO₂ $\leq$ 10 mmHg) have significantly lower overall survival or disease-free survival (reviewed in [5,13,14]). This is in part due to enhanced resistance of hypoxic tumors to conventional therapies such as radiotherapy and chemotherapy [8–11]. Over the past decade, there has been explosive progress in the field of hypoxia research. As shown by a series of elegant studies, hypoxia induces a wide range of biological changes, such as decreased cell proliferation [15],

*Address correspondence to this author at the Department of Therapeutic Radiology, Yale University School of Medicine, P.O. Box 208040, New Haven, CT 06520-8040, USA; Tel: 203-737-2183; Fax: 203-785-6309; zhong.yun@yale.edu.

increased expression of drug-resistance genes [16,17], selection of apoptosis-resistant clones [18], facilitation of tumor invasion and metastasis [19,20], reduced expression of DNA repair genes [21–24], and increased genomic instability [25,26]. These mechanisms undoubtedly contribute to the evolution of malignant tumor cells. However, it remains to be fully understood why hypoxic tumor cells tend to be more aggressive in nature and more resistant to treatment than non-hypoxic tumor cells within the same tumor, despite their similar genetic background.

With the recent development in stem cell research and identification of cancer stem cells, a new paradigm starts to emerge that hypoxia may prevent differentiation and thus may maintain tumor cells in an undifferentiated “stem cell-like” state. This hypothesis potentially provides a unifying platform to explain how hypoxia-induced genomic instability, clonal selection and other biological changes may contribute to malignant tumor progression. Because malignant progression is a slow and inefficient process, a stem cell state will allow a tumor cell to have sustained self-renewal power in order to accumulate a battery of genetic and epigenetic changes over a long period of time in order to become fully malignant.

CANCER STEM CELLS AS TUMOR-INITIATING CELLS

In many tumor types, only small numbers of tumor cells are able to proliferate extensively and are capable of tumor initiation [27,28]. These tumor-initiating cells behave much like undifferentiated stem cells. One of the best examples of “cancer stem cells” is teratocarcinoma that contains both undifferentiated and well-differentiated cells [29]. The undifferentiated cell type, known as embryonal carcinoma (EC), is highly malignant and has long been considered the stem cells of teratocarcinomas. One embryonal carcinoma cell suffices to form a complex teratocarcinoma when transferred to a new host [30]. Cancer stem cells have also been found in many commonly diagnosed cancers. The first such example is acute myeloid leukemia (AML) with cancer stem cells identified from AML patients in 1997 [31]. In recent years, putative cancer stem cells have been identified in increasing numbers of solid tumors including breast cancers [32], colon cancers [33–35], cancers of the central nervous system [36–38], head-and-neck cancers [39], and pancreatic cancers [40]. However, the term “cancer stem cell” is only an operational term. “Cancer stem cells” are characterized by the expression of stem cell markers, the ability for self-renewal or clonogenic growth, and the potential to produce a phenotypically diverse tumor cell population upon transplantation. It is not yet clear whether “cancer stem cells” behave exactly like normal stem or progenitor cells.

Studies using the euploid teratocarcinoma model strongly suggest that cancer can be considered a consequence of mis-regulated differentiation [29]. Consistent with this notion, it has been found that increased malignancy of neuroblastoma correlates strongly with a poorly differentiated stem cell phenotype, but seems to be poorly related to amplification of the proto-oncogene *MYCN* or increased expression of N-myc protein [41]. Gene expression profiling of gliomas has revealed that high-grade gliomas express neural stem cell markers, whereas low-grade gliomas possess lineage-specific markers of neuronal differentiation [42]. In a genetic mouse model, it has been shown that loss of imprinting of the insulin-like growth factor 2 (*IGF2*) gene results in increased incidence of intestinal tumors [43]. The increased tumor incidence strongly correlates with a shift toward a phenotype characterized by poorly differentiated intestinal epithelium [43]. Furthermore, it has been shown that cancer stem cells can use many of the same mechanisms that regulate self-renewal of normal stem cells, such as Bmi-1, Notch, Wnt and Sonic Hedgehog [44–46].

Stem cells seem to have enhanced survival potentials. Heterogeneous radiosensitivity has been observed among different subpopulations of hematopoietic stem cells [47,48]. It is interesting to note that the most immature stem cells are more resistant to radiation than partially differentiated stem cells [47]. These immature stem cells seem to have robust repair capability,

which may offer a partial explanation about therapy resistance by stem/progenitor cells. Similarly, as shown recently by Bao *et al.* CD133⁺ tumor stem cells isolated from both human glioma xenografts and primary patient glioblastomas display more robustly activated DNA damage checkpoint in response to ionizing radiation and they repair radiation-induced DNA damage more effectively than CD133⁻ tumor cells isolated from the same tumors [49]. These findings indicate that poorly differentiated tumor cells or cancer stem cells are likely to be the source of resistance to conventional therapies such as radiotherapy and chemotherapy. Therefore, tumor hypoxia could increase resistance to therapy by preventing cancer stem cells from undergoing differentiation.

HYPOXIA AND CANCER CELL DIFFERENTIATION

Hypoxia appears to have a strong impact on tumor cell differentiation. When maintained at a hypoxic condition of 1% O₂, neuroblastoma cells express genes that are typically found in immature neural crest cells, such as *c-kit* and *Notch-1*, whereas expression of genes associated with the differentiated neuronal-neuroendocrine phenotype such as *HASH-1* and *dHAND* is reduced [50,51]. Tacconelli *et al.* have shown that advanced human neuroblastomas express a novel alternatively spliced variant (TrkAIII) of the neurotrophin tyrosine kinase receptor type 1 (TrkA) [52]. In comparison, TrkAIII is primarily expressed in undifferentiated early neural progenitor cells during normal development. Ectopic expression of TrkAIII inhibits neuronal differentiation, increases tumor growth, and protects neuroblastoma cells against doxo-rubicin-induced apoptosis. Interestingly, expression of TrkAIII is increased in the presence of CoCl₂, a hypoxia-mimicking compound [52]. Similarly, increased expression of TrkAIII is also observed in human neural stem cells and neural crest-derived progenitor cells under the same hypoxia-mimicking condition [52]. These observations suggest that hypoxia inhibits differentiation of neuroblastoma cells and potentially induces neuroblastoma cells to acquire stem cell-like phenotypes.

Human ductal breast carcinoma *in situ* (DCIS) cells surrounding necrotic regions show high levels of Hypoxia-Inducible Factor-1 α (HIF-1 α) protein and increased expression of the epithelial breast stem cell marker cytokeratin 19 [53]. These DCIS cells appear to have lost their polarization and acquired an increased nucleus-to-cytoplasm ratio, hallmarks of poor differentiation [53]. When exposed *in vitro* to 1% O₂, breast cancer cells increase expression of cytokeratin 19, suggesting a hypoxia-dependent regulation of cytokeratin 19 transcription [53]. CD34 is a cell surface marker expressed by normal hematopoietic stem cells and is also found in a subpopulation of leukemia cells. Hypoxia has been shown to efficiently preserve CD34 expression in human primary CD34⁺ chronic myeloid leukemia (CML) cells at 1% O₂ [54]. In squamous cell carcinomas of the uterine cervix, expression of involucrin, a marker for the intermediate stage of epithelial differentiation, is inversely correlated with tumor grades, with highest levels of expression found in well-differentiated Grade 1 and lowest levels of expression in poorly differentiated Grade 3 cancers [55,56]. Interestingly, the extent of tumor hypoxia increases from Grade 1 to more advanced Grade 3 cervical cancers, as revealed by immunostaining of pimonidazole, a hypoxia-activated compound [56]. Although immunostaining of involucrin correlates with pimonidazole-positive hypoxic regions in intermediate grade tumors, there is no correlation between hypoxia and involucrin expression in poorly differentiated tumors [56]. It seems likely that expression of involucrin is predominantly determined by status of differentiation because hypoxia-induced expression of involucrin is found in moderately differentiated squamous cell carcinoma cell line SCC9 but not in poorly differentiated SCC4 cells [57]. Although the cause-effect relationship has not been clearly established, these findings, nevertheless, suggest a strong link between tumor hypoxia and poor tumor cell differentiation.

Hypoxia can also affect clonogenicity of tumor cells, a property resembling self-renewal of stem cells. When treated by hypoxia *in vitro*, transformed fibroblasts exhibited higher viability and clonogenicity than those maintained at normoxia [58]. Hypoxia has even been shown to preserve the colony-forming capacity of the CD34⁺ chronic myeloid leukemia (CML) cells [54]. Pretreatment of SK-N-BE(2) neuroblastoma cells *in vitro* at 1% O₂ results in reduced tumor latency and increased growth of subcutaneous xenografts in athymic mice, as compared to SK-N-BE(2) cells pretreated under normoxic tissue culture conditions [50], suggesting that hypoxia increases intrinsic tumorigenic potentials of these tumor cells. Taken together, these observations lead to the notion that hypoxia arrests tumorigenic cells in their undifferentiated state and maintains their clonogenic/tumorigenic potentials.

HYPOXIA-ACTIVATED PATHWAYS AND CANCER CELL DIFFERENTIATION

Hypoxia-induced signaling is primarily mediated by the ubiquitous hypoxia-inducible factor-1 (HIF-1), a master regulator of O₂ homeostasis that consists of O₂-regulated HIF-1 α and O₂-insensitive HIF-1 β [59,60]. HIF-1-induced genes are involved in a wide range of cellular functions such as cell growth, survival, motility, angiogenesis, energy metabolism, and cellular differentiation. Since HIF-1 α was cloned in 1995 [61], there has been significant effort to define the role of HIF-1 in tumor development using a variety of genetic tumor models [62,63]. Although the debate has not been completely resolved, a general consensus is that high levels of HIF-1 promote tumor growth, whereas HIF-1 deficiency leads to much retarded tumor growth [62,63]. HIF-2 α , both structurally and functionally related to HIF-1 α , has also been shown to facilitate tumor growth. Using a genetic “knock-in” approach to replace the endogenous *HIF-1 α* alleles with *HIF-2 α* , Covello *et al.* have found that the subcutaneous teratomas derived from the *HIF-2 α* “knock-in” mouse embryonic stem cells grow at faster rates and have higher microvessel densities than the teratomas derived from control embryonic stem cells [64]. These findings indicate that both HIF-1 and HIF-2 can play a significant role in promoting tumor development.

Increased HIF activity is indeed associated with tumor development. A classical example is the development of renal cell carcinomas (RCC). Loss of the von Hippel-Lindau (*VHL*) tumor suppressor gene results in activation of the HIF pathway under normoxia, which promotes RCC development [65,66]. Elevated levels of HIF-1 α protein are often detected by immunochemical staining in solid tumors, in contrast to normal non-ischemic tissues [14,60, 63]. Clinical studies have shown that elevated levels of HIF-1 α protein [67,68] or HIF-2 α protein [69] show significant statistical correlation with poor patient survival. Hirata *et al.* have shown that ectopic expression of constitutively active HIF-1 α in MDA-MB-231 human breast cancer cells significantly increases metastasis to the bone in athymic mice, whereas dominant-negative HIF-1 α reduces bone metastasis [70]. Furthermore, nuclear accumulation of HIF-1 α protein is correlated with poor tumor differentiation in primary pancreatic cancers [71]. These studies suggest that activation of the HIF pathway may potentially contribute to acquisition or maintenance of aggressive and poorly differentiated tumor phenotypes and thus facilitate malignant tumor progression.

Hypoxia has been shown to activate genes and/or pathways that are involved in maintenance of normal stem cells. The transcription factor Oct3/4, a POU family transcription factor specifically expressed in embryonic stem cells but not in differentiated cells, has been shown to be one of the four to five critical genes that collectively transform adult somatic cells into pluripotent stem cells reminiscent of embryonal stem cells [72–74]. Germ cell cancers and several types of somatic cancers, including human cervical carcinomas, breast carcinomas and pancreatic cancers, express elevated levels of Oct3/4 [75–78]. Using transgenic mice with doxycycline-inducible expression of Oct3/4, Hochedlinger *et al.* have shown that activation of Oct3/4 results in dysplastic growths in epithelial tissues by inhibiting cellular differentiation

in a manner similar to that in embryonic cells [79], illustrating a direct role of Oct3/4 in tumorigenesis. Interestingly, the genetic *HIF-2 α* “knock-in” study has shown that HIF-2 α is directly involved in transcription of Oct3/4 [80], suggesting potential hypoxia-dependent upregulation of Oct3/4 expression.

The erythropoietin (Epo)-erythropoietin receptor (EpoR) pathway plays an important role in regulating differentiation of both hematopoietic and non-hemato-poietic cell types [81–83]. Hypoxia seems to be involved in upregulation of *Epo* and/or *EpoR* expression in many human tumors [82,84,85]. It has been shown that the Epo-EpoR pathway maintains clonogenicity of neuronal progenitor cells and confers them with survival advantage against genotoxic stresses [86–88]. It is likely that hypoxia may enhance clonal survival and/or expansion of poorly differentiated tumor cells via activation of the Epo-EpoR pathway.

Signal transduction via chemokine receptor CXCR4 and its ligand CXCL12 or SDF-1 (stromal cell-derived factor-1) is another key regulatory pathway in stem/progenitor cells. Hypoxia, as well as activation of HIF pathways due to loss of pVHL tumor suppressor protein, strongly induces expression of *CXCR4* and *SDF-1* in both cancer cells and normal stem/progenitor cells [89–93]. CXCR4-mediated signal transduction plays an important role in homing of stem cells to hypoxic regions [89]. It has been shown that metastatic breast cancers and pancreatic cancers express increased amounts of CXCR4 [94]. The CXCR4⁺ subpopulations of the CD133⁺ human pancreatic cancer stem cells are essential for liver metastasis when orthotopically injected in immune-deficient mice [95]. These results strongly suggest that cancer stem cells are the most likely source of tumor metastasis. Hypoxia may therefore promote tumor metastasis by inducing expression of stem cell genes such as CXCR4.

It is also worth noting that loss of the tumor suppressor gene *BRCA1* impairs differentiation of mammary epithelial cells [96,97]. Kubita *et al.* have shown that Brca1 protein expression is increased during mammary epithelial differentiation of HC11 cells *in vitro* [97]. Ectopic expression of *BRCA1* promotes, while down-regulation of endogenous *BRCA1* expression attenuates, mammary epithelial cell differentiation. Using an *in vitro* three-dimensional culture system, Furuta *et al.* have shown *BRCA1* mediates differentiation of mammary epithelial cells for acinus formation whereas reduced expression of *BRCA1* by RNA interference impairs acinus formation but enhances epithelial cell proliferation [96]. Inhibition of *BRCA1* expression results in up-regulation of genes that facilitate proliferation but down-regulation of genes that promote differentiation [96]. Interestingly, Bindra *et al.* have shown that hypoxia represses BRCA1 transcription by differential recruitment of the E2F family transcription factors in a HIF-independent manner [23]. Although both activating and repressive E2Fs simultaneously bind the *BRCA1* promoter at two adjacent E2F sites, hypoxia induces a dynamic redistribution of promoter occupancy by these transcription factors that leads to transcriptional repression of *BRCA1* transcription [23]. Based on the evidence discussed above, it is possible that hypoxia promotes malignant tumor progression by maintaining tumor cells in their undifferentiated state through up-regulation of stem cell genes and down-regulation of differentiation genes. Hypoxia-induced expression of stem cell genes are likely involved in survival, self-renewal and metastasis of cancer stem cells.

HYPOXIA AND DIFFERENTIATION OF MESENCHYMAL STEM/PROGENITOR CELLS

Tumor growth often results in changes of the tumor stromal microenvironment with reduced numbers of well-differentiated stromal cells and loss of normal tissue structures [98–100]. This case in point is best illustrated in breast cancers [98]. Normal mammary tissues have abundant adipocytes and sparse connective tissue. In contrast, the breast carcinoma is overwhelmingly rich in fibroblasts with few mature adipocytes. It is not yet understood what causes the changes

in the stromal microenvironment and what the cellular origin is for tumor-associated fibroblasts, although bone marrow (BM)-derived mesenchymal stem cells and hematopoietic stem cells are among the likely sources of tumor stromal cells [101,102]. Also not clear is the differentiation status of tumor-associated fibroblasts. As discussed below, it is possible that tumor hypoxia regulates the cell fate decisions by mesenchymal stem cells and thus affects the evolution of the tumor stromal microenvironment.

Mesenchymal stem cells are pluripotent stem or progenitor cells capable of differentiating into a variety of mature cell types including adipocytes, myocytes, chondrocytes and neuronal cells [103]. Genetic studies have shown that *HIF-1 α* ^{-/-} mouse embryos die during mid-gestation, around 10 days post coitus, due to loss of mesenchymal cells and impaired cardiovascular development [104–106], suggesting an important role of the HIF pathway in the regulation and maintenance of mesenchymal stem cells. Studies *in vitro* have demonstrated that hypoxia inhibits differentiation of mesenchymal stem/progenitor cells into adipocytes [107,108], chondrocytes [109,110], myocytes [111,112] or osteocyte [109,113]. These observations suggest a role of hypoxia in development and evolution of tumor stroma by regulating the differentiation of mesenchymal stem cells in the tumor microenvironment.

It has been clearly demonstrated that adipogenic differentiation is inhibited under hypoxic conditions [107,108,114,115]. Even at physiologically relevant levels of hypoxia (1–2% O₂), mesenchymal stem and progenitor cells are prevented from undergoing adipogenic differentiation [107,108]. HIF-1 α is expressed in both progenitor cells and differentiated adipocytes, whereas HIF-2 α is only detected in mature adipocytes [107,116]. These data suggest that HIF-1 α is likely to be involved in the regulation of adipogenic progenitor cells whereas HIF-2 α plays a role in mature adipocytes. Lin *et al.* have shown that adipogenic progenitor cells are still capable of adipogenic differentiation under hypoxic conditions when *HIF-1 α* is knocked down by gene-specific siRNA, whereas differentiation of the progenitor cells treated with scrambled siRNA is blocked under the same hypoxic conditions [107]. On the other hand, ectopic expression of constitutively active *HIF-1 α* mutants results in inhibition of adipogenic differentiation under normoxic conditions [107]. These results demonstrate that HIF-1 α plays an essential role in inhibiting adipogenic differentiation.

Mechanistically, hypoxia appears to prevent mesenchymal stem/progenitor cells from committing to terminal adipogenic differentiation. When stimulated by adipogenic hormones under hypoxic conditions, mesenchymal stem/progenitor cells fail to express *PPAR γ 2* and *C/EBP α* , two critical differentiation-determination genes for terminal adipogenic differentiation [107,108]. Although HIF-1 is a transcriptional activator, repression of *PPAR γ 2* expression is mediated by the hypoxia-induced gene *DEC1/Stra13*, a transcriptional target of HIF-1 [108]. *DEC1/Stra13*, also referred to as *BHLHB2*, *SHARP2* and *Clast5*, is a putative transcription repressor and shares homology with the *Hairy* and *Enhancer-of-Split* (HES) family of transcription repressors [117,118]. DEC1/Stra13 protein contains an N-terminal basic helix-loop-helix (bHLH) domain, and the putative repressor domain is located in its C-terminus. Studies have shown that HIF-1 is required for hypoxic induction of *DEC1/Stra13* transcription [108,119,120]. When ectopically expressed in adipogenic progenitor cells, *DEC1/Stra13* inhibits adipogenic differentiation and represses *PPAR γ 2* transcription by directly targeting the *PPAR γ 2* gene promoter [108]. These data demonstrate that signal transduction mediated by HIF-1 plays a critical role in the regulation of adipogenic differentiation.

It seems that HIF-independent signaling pathways may also contribute to the inhibition of adipogenic differentiation by hypoxia. Hypoxia has been shown to activate the Transforming Growth Factor β (TGF β)-Smad pathway by increasing levels of phosphorylated Smad2/3 in human BM-derived mesenchymal stem cells [121]. Consequently, adipogenic differentiation of human BM-derived mesenchymal stem cells is inhibited under hypoxic conditions [121].

However, it is not yet clear whether the canonical HIF-pathway is involved in activation of the TGF β -Smad pathway. Hypoxia has also been shown to strongly increase expression of the stem/progenitor marker *Delta-like 1* or *DLK1* [107], a negative regulator of adipogenic differentiation [122,123]. DLK1, also widely known as preadipocyte factor-1 (pref-1), is a type I transmembrane protein containing six epidermal growth factor (EGF) homology motifs in its extracellular domain and shares homology with the delta/notch/serrate family proteins [124, 125]. However, DLK1 lacks the DSL (delta, Serrate, and Lag-2) domain that is conserved within the delta/notch/serrate family and mediates ligand-receptor interactions [124,125]. Expression of *DLK1* is developmentally regulated, with the highest levels of *DLK1* expression found in early embryonic tissues and decreased expression toward the end of fetal development [126]. Only a few precursor cells in adult tissues show *DLK1* expression [126]. Genetic mouse model studies have shown that *DLK1* negatively regulates adipogenic differentiation [127, 128]. Interestingly, hypoxia-induced expression of *DLK1* in adipogenic precursor cells is independent of HIF-1 [107]. These observations demonstrate that both HIF-dependent and HIF-independent pathways are involved in repression of adipogenic differentiation. Nonetheless, it is worth noting that mesenchymal stem/progenitor cells remain undifferentiated and uncommitted under hypoxic conditions and can still undergo adipogenic differentiation once they return to normoxic conditions [107]. These data suggest that hypoxia has the ability to maintain mesenchymal stem cells in an undifferentiated state and to prevent them from committing to adipogenic differentiation.

Hypoxia also inhibits the differentiation of myogenic differentiation [111,112]. However, inhibition of myofiber formation seems to be dose dependent on the level of hypoxia, with strongest inhibition at nearly anoxic pO₂ level [112]. Expression of the key myogenic transcription factor *MyoD* is strongly inhibited under hypoxic conditions, whereas expression of the transcription coactivator *E2A* is inhibited to a lesser degree by hypoxia [112]. Interestingly, *MyoD* expression is transiently repressed at 0.5–2% O₂ and gradually recovers even when cells are continuously maintained under hypoxic conditions. As a result, myogenic differentiation manages to proceed after adapting to persistent or chronic hypoxia [112]. The mechanisms underlying hypoxia adaptation remain to be fully understood.

In contrast to adipogenesis, the HIF pathway does not seem to play a significant role in the regulation of myogenic differentiation. Ectopic expression of constitutively active HIF-1 α does not affect myogenic differentiation of myogenic progenitor cells under normoxia or hypoxia [112]. Nonetheless, Gustafsson *et al.* have reported that HIF-1 α is involved in inhibition of myogenic differentiation *via* interaction with the Notch intracellular domain (NICD) and subsequently activation of Notch-regulated genes [111]. In contrast, Yun *et al.* have found that expression of *Notch* family genes, *Notch1*, *Notch2* and *Notch3*, is not significantly affected at 0.5–2% O₂, but is rather decreased at <0.01% O₂ [112]. Similarly, levels of endogenous NICD protein are not changed significantly at 1–2% O₂, but again are dramatically reduced at <0.01% O₂. Furthermore, when C2C12 myoblasts are treated with N-[N-(3,5-difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-butyl-ester (DAPT), a specific γ -secretase inhibitor that blocks Notch signaling by preventing NICD formation, there is no significant effect on myogenic differentiation under either normoxic or hypoxic conditions [112]. Due to these inconsistent observations, mechanisms underlying hypoxic regulation of myogenic differentiation remain to be clearly delineated.

A recent study by Sun *et al.* has provided an interesting mechanism linking hypoxia and Notch signal transduction [129]. Mice homozygous-null for *Stra13* (*BHLHB2* or *DECI*) exhibit defects of muscle regeneration characterized by degenerated myotube formation. Primary *Stra13*^{-/-} myoblasts show elevated Notch activity, increased proliferation, and defective differentiation [129]. As shown by co-immunoprecipitation assays, *Stra13* inhibits Notch signal transduction by interacting with NICD. Ectopic expression of *Stra13* rescues the Notch-

dependent inhibition of myogenesis [129]. Because *Stra13* is induced by hypoxia as a HIF target gene [108,119,120], the observations by Sun *et al.* argue against the HIF-dependent activation of Notch reported by Gustafsson *et al.* Based on the reversible inhibition of myogenic differentiation by hypoxia as reported by Yun *et al.* it is intriguing to hypothesize that the transient inhibition of myogenesis is mediated by the HIF-dependent activation of Notch, whereas the recovery of myogenic differentiation under hypoxia is likely mediated by the inhibition of Notch signaling by the hypoxia-induced gene *Stra13* (*BHLHB2* or *DEC1*).

As discussed above, hypoxia clearly has a profound impact on differentiation of mesenchymal stem cells. Tumor hypoxia undoubtedly plays a significant role in the composition of tumor-associated stromal cells and the evolution of tumor stroma. It is conceivable that hypoxic tumor stroma is enriched in undifferentiated stromal cells, which may provide a favorable microenvironment for maintaining tumor cells in a stem cell state.

HYPOXIA AND CANCER STEM CELL NICHES

Stem cell niches provide a unique microenvironment where stem cells are maintained in an undifferentiated state and their commitment to lineage-specific differentiation is tightly regulated [130–133]. A recent genetic study has clearly shown that hematopoietic stem cells are in close contact with reticular cells located either around sinusoids or along the endosteum in the bone marrow [134]. These reticular cells express high levels of SDF-1 or chemokine CXCL12 that can also be induced by hypoxia in a HIF-dependent manner [89,91,135,136]. Interestingly, patterns of SDF-1/CXCL12 expression in the bone marrow overlap with areas of hypoxia, as indicated by immunochemical staining of a hypoxia marker, pimonidazole [89]. Circulating CXCR4⁺ endothelial progenitor cells preferentially home to hypoxic regions or regions with high SDF-1/CXCL12 expression [89]. These data suggest that hypoxia may regulate stem cell maintenance by modulating expression of paracrine factors by niche stromal cells.

Differentiation status of niche stromal cells seems also to play a role in stem cell maintenance. When BM osteoblasts are ablated at early-stage osteoblastogenesis, there is a severe decrease in BM-derived hematopoietic stem cells [137]. In contrast, loss of osteoblasts at later stages of differentiation has little effect on hematopoiesis [138]. Sacchetti *et al.* have further shown that the CD146⁺ osteoprogenitor cells are able to reconstitute the hematopoietic microenvironment in the bone marrow [139]. Undifferentiated CD146⁺ cells, but not differentiated CD146[−] cells, produce significant amounts of angiopoietin-1 that has been shown to play a critical role in maintenance of hematopoietic stem cells [139–141]. These data suggest that immature stromal cells are preferentially suited for maintenance of stem cells [133].

Tumor cells and their microenvironment reciprocally regulate each other. The interactions between tumor cells and their microenvironment exert profound influence upon tumor development and progression toward malignancy [98,101,142]. Although it is not yet clear whether cancer stem cells, like normal stem cells, are localized in specialized niches, it is nonetheless possible that cancer stem cells interact closely with tumor stromal cells, and their maintenance will therefore be subjected to regulation by the tumor stromal microenvironment. There are at least three independent mechanisms by which hypoxia regulates maintenance and differentiation of stem cells. First, hypoxia directly prevents cancer stem cells from undergoing differentiation. Second, hypoxia inhibits differentiation of niche stromal cells and maintains them in an undifferentiated state. Third, hypoxia induces expression of paracrine factors such as SDF-1/CXCL12 in stromal cells that facilitate homing of cancer stem cells and/or interactions between cancer stem cells and stromal cells in the niche.

A recent study by Karnoub *et al.* has elegantly illustrated a role of immature mesenchymal stem cells in tumor progression [101]. BM-derived mesenchymal stem cells can be recruited to tumor stroma [101,143]. Although the cell fate decision of these mesenchymal stem cells is not yet clear, tumor-associated stromal cells are likely to be undifferentiated immature cells. Mouse stromal cells isolated from human breast cancer xenografts have been shown to have the ability to form fibroblastoid colonies (CFU-F) *in vitro*, a hallmark of undifferentiated mesenchymal stem cells [101,103]. In contrast, stromal cells prepared from control Matrigel plugs or from adjacent normal tissues do not form fibroblastoid colonies [101], suggesting that tumor stroma contains higher populations of immature cells than normal stroma does. Interestingly, weakly metastatic human breast carcinoma cells become highly metastatic when injected subcutaneously as a mixture with human BM-derived mesenchymal stem cells [101]. Although it remains to be determined whether mesenchymal stem cells play a role in regulation of cancer cell differentiation, this study clearly demonstrates that immature stromal cells facilitate acquisition of aggressive tumor phenotypes. It will be of great interest to investigate the localization of mesenchymal stem cells within tumor stroma and the role of hypoxia in regulating the recruitment and maintenance of tumor-associated mesenchymal stem cells.

However, a recent report has shown that brain cancer stem cells are located near endothelial cells [144]. This finding seems to argue against the notion that stem cell niches are hypoxic. However, *in vitro* co-culture assays have shown that endothelial cells promote growth or self-renewal of cancer stem cells, at least in part, via endothelial cell-derived paracrine factors [144]. This result suggests that endothelial cells directly regulate cancer stem cells independent of their blood-carrying or vascular functions. As is widely known, tumor-associated blood vessels are often structurally and functionally abnormal, which results in tumor hypoxia [145,146]. It will be interesting to determine whether cancer stem cells are preferentially associated with endothelial cells in hypoxic regions.

SUMMARY

Increasing evidence strongly suggests that hypoxia exerts profound impact on the development and evolution of the tumor microenvironment by regulating differentiation of both tumor and stromal cells. The hypoxic tumor microenvironment will likely be characterized by increased numbers of immature stromal cells and poorly differentiated tumor cells, as illustrated in Fig. (1). Within such undifferentiated hypoxic microenvironment, self-renewal of cancer stem cells is preferred over differentiation as a result of direct and/or indirect interactions with immature stromal cells, as well as hypoxia-dependent signal transduction in cancer stem cells. Because of increased efficiency of DNA repair in cancer stem cells [49], this paradigm also suggests that hypoxia-related maintenance of cancer stem cells constitutes an important alternative mechanism for increased resistance of hypoxic tumors to conventional therapies. A successful cancer therapy will have to include strategies to specifically target cancer stem cells localized in hypoxic regions.

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Fig. 1. Tumor hypoxia and cancer stem cell niches

Because hypoxia inhibits cellular differentiation, cancer stem cells will be preferentially located in hypoxic regions where increased numbers of immature stromal cells are also expected. The self-renewal and maintenance of cancer stem cells will be improved via hypoxia-induced signal transduction, as well as direct and indirect interactions with immature stromal cells. In contrast, the non-hypoxic tumor microenvironment will likely be populated with differentiated stromal cells. Cancer stem cells will be subjected to increasing stresses for differentiation.