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Emerging role of RUNX3 in the regulation of tumor microenvironment

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ABSTRACT

A number of genes have been therapeutically targeted to relieve cancer, but cancer relapse is still a growing issue. The concept that the surrounding tumor environment is critical for the progression of cancer may foster an answer to the issue of cancer malignancy. Runt domain transcription factors (RUNX1, 2, and 3) are evolutionarily conserved and have been intensively studied for their roles in normal development and pathological conditions. During tumor growth, a hypoxic microenvironment and infiltration of the tumor by immune cells are common phenomena. In this review, we briefly introduce the consequences of hypoxia and immune cell infiltration into the tumor microenvironment with a focus on RUNX3 as a critical regulator. Furthermore, based on our current knowledge of the functional role of RUNX3 in hypoxia and immune cell maintenance, a probable therapeutic intervention is suggested for the effective management of tumor growth and malignancy.

Keywords: RUNX3, tumor microenvironment, hypoxia, immune cell, HIF-1α

INTRODUCTION

A large number of oncogenes and tumor suppressor genes were identified and therapeutically targeted for effective care of cancer patients in past two decades, which have also extended survival of cancer patients. But the relapse of cancer due to increased resistance to therapeutic intervention has raised another critical question. With this scenario, the new concept is emerging that appreciates the fact that cancer maintenance and expansion is critically regulated by signals from the microenvironment [1, 2]. Tumors are complex tissues i.e, structures comprising not only malignant cells, but also contain genetically stable stromal cells. Cells making up the structural framework of tumors include specialized cells of the connective-tissue family called fibroblasts, endothelial cells and immune cells. Together with products such as the extracellular matrix (ECM), these cellular interactions drive the initiation, growth, and metastasis of tumors [1]. Tumor hypoxia is another critical microenvironmental state that results because of an inadequate oxygen supply in growing solid tumors. Hypoxia compromises biological functions and is associated with malignancy and cancers that are refractory or resistant to treatment [3].

The Runt-related transcription factor (RUNX) family is evolutionarily conserved from simple to complex organisms, suggesting its significant role in developmental and biological processes. It is well described as developmental regulators and its involvement in human neoplasia is also been documented. The Runt domain transcription factors are composed of a larger DNA-binding subunit, α, and a smaller non-DNA-binding subunit, β (known as core binding factor β, CBFβ [4]. RUNX1, RUNX2, and RUNX3, the three members of the *Runx* family genes, encode α subunits. RUNX1 is important for the generation of hematopoietic stem cells, and it is also illustrated to be involved in leukemia [5, 6]. RUNX2 is essential for bone development and has oncogenic potential [7, 8]. RUNX3 is associated with multiple

developmental functions and differentiation of immune cells such as CD8-lineage T cells [5, 9], TrkC-dependent dorsal root ganglion neurons [10], and it also functions as a major tumor suppressor [11-16]. In addition to its role as a tumor suppressor, recent researchers have depicted it as a tumor promoter in various cancers [17-19].

In this review, we discuss our overall knowledge on the emerging role of RUNX3 in the regulation of the tumor microenvironment. The instructions harnessed in the regulatory mechanism and modulation can be instrumental for the development of more effective anticancer therapies.

RUNX3 IN TUMORIGENESIS

RUNX3 is expressed in a wider range of tissues with multiple roles. RUNX3 is a well-recognized tumor suppressor of gastric, colon and many other forms of solid tumors. The RUNX3-deficient phenotype is described as a cause for hyperplasia of the gastric mucosa [16], while severe limb ataxia is also illustrated with a *Runx3* deletion [20]. The tumor suppressive role for RUNX3 is further strengthened by an inactivated RUNX3 on hemizygous deletion, promoter hypermethylation, histone modification and frequent protein mislocalization [14, 21-24]. Recently, RUNX3 is suggested to inhibit migration and invasion of melanoma cells through enhancing the formation of stress fibers and mature focal adhesion and ECM protein production regulating the cell shapes [25].

Recently, in addition to established tumor suppressive role of RUNX3, its oncogenic role is also being revealed in certain cancer subsets. All three RUNX genes were first shown to be associated with MYC proto-oncogene (MYC) thus promoting leukemogenesis [26]. Later its oncogenic behavior was verified in basal cell carcinoma with overexpressed RUNX3 in cancers compared to the normal epidermis [27]. Likewise, RUNX3 overexpression is

observed to increase proliferation and tumorigenesis in ovarian cancer, head, and neck squamous cell carcinoma and Ewing sarcoma [18, 28, 29]. All these observations suggest the cell type and contextual- dependent behavior of RUNX3 as a tumor suppressor or a promoter.

TUMOR MICROENVIRONMENT

Conventional cancer therapies are more focused on eliminating proliferating cancer cells, while recent advances in cancer biology have emphasized the critical importance of the surrounding environment in initiation, progression, and metastasis of cancer [30, 31]. Development of a tumor involves encompassing the proliferating tumor cells, the tumor stroma, blood vessels, infiltrating immune cells and various types of macromolecules comprising the extracellular matrix (ECM) and a large number of secreted cytokines and molecules. The tumor microenvironment (TME) develops during the progression of the tumor as a unique environment, through interaction of tumor cells with the surrounding host cells and their secretions. It plays not only pivotal role in tumor initiation, but also facilitates progress of cancer i.e., metastasis [32, 33]. The microenvironment of the tumor is often hypoxic. As the mass of the tumor increases, the inner side of the tumor grows far beyond the existing blood supply. Angiogenesis may reduce this effect, but at more than 50% of locally advanced solid tumors, the oxygen partial pressure is less than 5 mmHg (oxygen partial pressure of the venous blood (40 mmHg) [34, 35]. The hypoxic microenvironment has been shown to strongly influence the dialogue between tumor cells and nonmalignant stromal cells, thus inducing changes in the proteome of these cells and facilitating tumor propagation by enabling the cells to adapt in a nutrient-deficient hostile microenvironment [36] (Figure 1).

Tumor-associated macrophages (TAMs), abundant in most of the human and mouse tumor microenvironments, have primarily pro-tumorigenic abilities [37]. They are reported as

potent partners for cancer cell migration, invasion, and metastasis [38]. Accumulation of TAMs in hypoxic and necrotic zones of the tumor is facilitated by secreted cytokines, including vascular endothelial growth factor (VEGF) in hypoxic regions [39]. In addition to that fact, infiltrating immune cells, like T cells, myeloid-derived suppressor cells (MDSCs), and dendritic cells (DCs) within a tumor also pose critical roles in the progression of cancer [2] (Figure 1).

RUNX3 MEDIATED REGULATION OF INFLAMMATORY CELLS IN TUMOR MICROENVIRONMENT

Effective immune responses and immunosurveillance of emerging cancer cells are two of the important factors to impede growing tumors. Inflammatory cells have roles at the beginning to prevent tumors through activated innate immune responses, and later a chronic inflammatory state is maintained by infiltrating immune cells, thereby ensuring the survival of tumor cells through intensive crosstalk with tumor cells, leading to its progression [40]. Long-time immunocytes are recognized for antitumor abilities; however, recent research shows the involvement of an imbalanced $CD4^+$ T subset in the tumor immune microenvironment. *Runx* deregulation is associated with both inflammation and carcinogenesis, including inflammation-induced colorectal cancer [41-43]. In a chemical-induced colorectal cancer model, the expression levels of *Runx3*, but not *Runx1* and *Runx2*, showed a much higher increase in dextran sodium sulfate (DSS)-treated/urokinase-type plasminogen activator (uPA)-deficient mice compared to the wild-type/DSS group. DSS treatment produced significant upregulation of immune cells expressing three Runx proteins identified by the -situ investigation. It suggests that immune cells are the main source of

Runx proteins and uPA deficiency may be associated with upregulation of the Runx factor in the natural history of inflammatory colon carcinogenesis [44].

RUNX3 facilitates myeloid differentiation through the retinoic acid receptor signaling pathway [45]. Furthermore, hematopoietic development in zebrafish is also validated to be regulated by RUNX3 [46]. These observations suggest its potential involvement in the regulation of inflammatory cells in immune-tumor microenvironment [2], as well as hematological malignancies. In a large number of tumor types, T regulatory cells (Tregs) are often found in infiltrating tumors. The rapidly elevated Tregs in the tumor microenvironment is associated with a poor clinical outcome due to their effective immunosuppressive nature i.e., limitation of antitumor immunity, thus promoting cancer progression and angiogenesis [47]. Besides, cancer progression, Treg cells are also reported to play a pivotal role in the development and maintenance of terminal immunodeficiency, resulting in serious autoimmune disorders and compartmental defects [48, 49]. The immune suppressive role of RUNX3 has been reported in Tregs in breast tumors [49]. A recent report found that RUNX3, a $CD8^+$ lineage-specific transcription factor, binds to induce transcription of the *Forkhead box p3 (FOXP3)*-promoter and the $CD8^+CD25^+$ $FOXP3^+$ Treg population is increased in the tumor microenvironment with the progression of breast tumors. Infiltration of $CD8^+$ Treg cells in tumor microenvironment produce high-level of immunosuppressive cytokines that ensure repressed effector T cell proliferation and enhanced tumor immune evasion [49, 50]. RUNX3 has crucial role in the cytotoxic T cell maturation. Thymocyte progenitors undergo differentiation through a series of stages and RUNX3 contribute to transcription of CD8 in $CD8^+CD4^-$ T cells by binding to the CD8 enhancer, thus ensuring maturation into CD8 T cell lineages [51]. Natural killer cells (NKC) and cytotoxic lymphocytes infiltrating into solid

tumors have highly expressed levels of RUNX3 and play crucial roles in their proliferation and activation. They regulate markers of the effector cytotoxic T lymphocyte program, including IFN-γ, perforin, and granzyme B suggesting its critical role in the differentiation of NKC and cytotoxic T-lymphocytes [52]. In the report by Li et al. [53], decreased expression of the Th1-associated factor was shown with repressed RUNX3 in gastric carcinoma, as a cause for the cancer progression. IFN-γ, the specific factor secreted by Th1 cells, regulating the function of target T cells and facilitating the killing of the tumor by NKC, was found repressed with a decreased level of RUNX3 expression (Figure 1).

Among the growth factors (including cytokines) secreted by a tumor and surrounding interstitial cells, transforming growth factor-beta (TGF-β) might be the most secreted cytokine [54]. Secreted TGF-β may induce a paradoxical cancer effect by activating an antiproliferative signal or by giving a prooncogenic character to cells, including epithelial-mesenchymal transition (EMT) [54]. A recent report suggests that TGF-β promotes genomic instability in the form of DNA double-strand breaks (DSB) in cancer cells without RUNX3. The down-regulation of the redox modulator, heme oxygenase-1 (HO-1), due to a low concentration of RUNX3, increased oxidative DNA damage and ultimately destroyed genome integrity and triggered cellular senescence accompanied by tumor-promoting inflammatory cytokine expression and acquisition of senescence-related secretory behavior. Tumor-bearing TGF-β gene expression signatures and RUNX3 loss showed higher levels of genomic instabilities [55], suggesting a novel connection between microenvironment-derived extrinsic TGF-β signaling and intrinsic RUNX3 inactivation in genomic instability. In addition, TGF-β– induced EMT is highly noticed in *Runx3* null gastric epithelial lines, emphasizing the role of RUNX3 in repression of TGF-β induced EMT [56].

ECM provides a scaffold for all cells in TME and has a further diverse role in evolution and spreading of cancer. Proteases like matrix metalloproteinases (MMPs) not only degrade ECM but also help in the remodeling of ECM leading to the secretion of chemokines and other angiogenic and growth factors. RUNX3 has substantiated repressive role over MMPs in various cancers [12, 13, 57, 58], and the loss of RUNX3 was found to be correlated with increased secretion of multifunctional glyco-phosphoprotein osteopontin (OPN), promoting gastric cancer metastasis [59], suggesting its critical role over regulation of TME (Figure 1).

Nonmalignant cells of TME, like stromal cells and fibroblasts, can secrete growth factors, such as hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and C-X-C motif chemokine ligand 12 (CXCL12) chemokine. These secreted molecules, not only involve in growth and survival of malignant cells, but also involve in recruitment of other cells into the TME, due to their chemoattractant efficacy [33]. In a report, CXCL12 is shown to regulate the differentiation of the macrophage by an autocrine/paracrine mechanism which is characterized by expression of the angiogenic factors, VEGF and chemokine (C-C motif) ligand 1(CCL1). Interestingly, it is further shown to downregulate RUNX3 expression, which maintains CD4 and CD14 expression in mononuclear phagocytes as a part of the transcriptional program induced by CXCL12 [60]. Besides, immunosuppressive and proangiogenic functions of VEGF, MDSCs induced by VEGF signaling can play important roles in tumor immune evasion in ovarian cancer [61]. In a line VEGF inhibits DC differentiation into mature DC cells [62] and promotes the accumulation of MDSCs, Tregs, and TAMs [61] and RUNX3 can directly inhibit VEGF secretion through transcriptional repression [63] (Figure 1). These reports suggest RUNX3 as a potential candidate in the regulation of the tumor microenvironment, with the ability to suppress activities of tumor

promoting cytokines.

HYPOXIA, EPIGENETICS, AND ANGIOGENESIS

The hypoxic environment is created due to an increase in the tumor size followed by the subsequent inadequate supply of oxygen in a growing tumor and further encompasses metabolic and biological processes, making them more aggressive and irresponsive to therapeutic interventions [64]. Growing body of evidences have shown the role of hypoxia in maintenance of cancer stem cells (CSCs), through repressed tumor cell differentiation and increased therapeutic resistance thus assuring tumor progression [65]. In other report, hypoxia mediated repression of mesenchymal stem and progenitor cell differentiation is substantiated as a critical factor in the evolution of a tumor stromal microenvironment, the putative cancer stem cell niches [66]. In keeping with this fact, hypoxia upregulates hypoxia-inducible factor 1 alpha (HIF-1α) that induces angiogenesis, and it is associated with activation of genes involved in metastasis [13], such as increased cell migration and ECM remodeling [3].

In various cancer tissues, RUNX3 is silenced due to promoter DNA hyper methylation. However, the effect of the hypoxic tumor microenvironment in the regulation of RUNX3 is obscure. Our group has described a mechanism for the first time to silencing of RUNX3 due to histone modification under hypoxic microenvironment. Histone methylation and deacetylation occur under the influence of histone methyltransferase (HMT) G9a and histone deacetylase (HDAC) 1 respectively [67]. HMT G9a is upregulated by hypoxia [68] and is responsible for poor prognosis and metastasis of several human cancers [69-72]. Hypoxia can also activate HDAC1 [73]. Acetylation of RUNX3 by p300 [74] and bromodomain (BRD) [75] is a key to the protein stability and cell-cycle arrest. Thus, RUNX3 inactivation could also be due to the histone deacetylation. In support of this, HDAC inhibitors restored RUNX3

expression and subsequent tumor-suppressive character in cancer cells [67, 76, 77]. Therefore, it can be of critical importance to rescue epigenetic loss of RUNX3 expression to help to prevent cancer cell from facilitated by hypoxic tumor microenvironment.

Small noncoding RNAs, microRNAs (miRNAs), are the important molecule that plays critical roles in post-transcriptional regulation of genes affecting diverse biological processes in a large number of cell types [78], along with their critical roles as tumor suppressors or promoters. Specific groups of miRNA are regulated by hypoxia [79]. Emerging evidences have shown that miRNAs regulated tumor microenvironment influencing tumor immune invasion, tumor angiogenesis and tumor-stromal interaction [80, 81]. However, upregulated RUNX3 expression is identified in epithelial ovarian cancer due to repressed miR130b expression and subsequent promotion of carcinogenic feature [82], in GC, our group has shown silencing of RUNX3 by miR-130a/miR-495 for the early tumorigenic progression due to increased VEGF secretion in hypoxic status [83]. These reports suggest probable manipulation of the tumor microenvironment by targeting RUNX3.

Dynamic shuttling of RUNX3 between nuclear and cytoplasmic compartments is tightly linked with nuclear components, signifying probable importance of targeting RUNX3 to the nuclear matrix for active regulation and biological consequences [84]. In support of this, threonine 209 phosphorylation on RUNX3 by p21 activated kinase 1 (Pak1) translocate it from nucleus to the cytoplasm and subsequently converses its biological functions [85]. In another report, provirus integration site for Moloney murine leukemia virus 1 (pim-1) was shown to phosphorylate RUNX3 and alter its expression by mislocalization in salivary gland adenoid cystic carcinoma (ACC) [86]. Besides mislocalization and inactivation of RUNX3, ubiquitination and subsequent proteasomal degradation are also substantiated for its mode of

inactivation in GC infected by virulent cytotoxin-associated gene A (CagA)-positive *Helicobacter pylori*. CagA can bind to RUNX3, inducing ubiquitination and subsequent degradation of RUNX3 by proteasomal machinery [87]. In response to these reports, we can suggest that blocking mislocalization and ubiquitination of RUNX3 could be of importance in tumors to prevent from advancing to malignancy.

In recent years, a number of reports have shown evidence of hypoxic stress in the tumor microenvironment, playing a prominent role in tumor immune responses. Upregulated HIF-1α in hypoxic environment facilitates the immune-suppressive ability of MDSC and TAM and augment differentiation of MDSC to TAM [88]. Under hypoxic conditions, RUNX3 decreases the half-life of HIF-1α, its stability, transactivation activity and VEGF secretion due to repressed nuclear localization. Furthermore, overexpression of RUNX3 significantly inhibits hypoxia-induced angiogenesis [89]. The critical role played by RUNX3 in inflammatory cells have also been emphasized by some authors, rather than its tumor suppressive role [43]. On this note, we can speculate that forcefully targeting RUNX3 to over express in a hypoxic tumor could be a promising intervention to regulate and activate immune responses, and modulate ECM and CAF in TME to inhibit tumor growth, migration, invasion and angiogenesis (Figure 1).

However, RUNX3 is established as a potent tumor suppressor in GC and some other forms of the tumor; the oncogenic behavior of RUNX3 has also been specified. Patients with oral squamous cell carcinoma (OSCC) are identified for high occurrence and low survival rates. Furthermore, mice subcutaneously inoculated with RUNX3 knockdown OSCC verified for the reduced bone invasion and production of osteolytic factors [19]. In another report RUNX3 restoration in OSCC cells illustrated to suppress cell migration and invasion by

downregulating MMP-9 expression and secretion, thus potentiating antiangiogenic behavior by inhibiting VEGF activity [90]. In a model of skin tumorigenesis, leukocytic loss of RUNX3 suppressed protumorigenic cytokines, interleukin-17a (IL-17a) and OPN substantially decreasing carcinogen-induced skin tumorigenesis [91]. In head and neck squamous cell carcinoma (HNSCC), overexpressed RUNX3 is detected as a critical reason for a malignant phenotype which is caused, in part, due to demethylation during cancer progression [17]. These reports reflect on the organ-specific and contextual based behavior of RUNX3.

IMPLICATIONS FOR CANCER THERAPY

RUNX3 expressed in T cells have an important role in the functioning of the immune system. It has been reported that mice deficient in RUNX3 spontaneously develop immunological abnormalities like airway hypersensitivities, colitis and also gastric hyperplasia [92, 93]. Likewise, T-cell-targeted knockdown in mice spontaneously develop asthma-like symptoms with infiltration of lymphocytes in the lungs [94]. Reduced RUNX3 in gastric carcinoma is positively correlated with repressed Th1-associated factor and decreased Th1 cell-mediated immunity is associated with immune escape of cancer cells [52, 53]. In addition, miRNA-145 could regulate the balance of Th1/Th2 through targeting RUNX3 in asthma patients, and miRNA-145 and RUNX3 can be used as biomarkers or targets in the diagnosis or therapy of asthma [95]. On this note, it can be of great significance to target RUNX3 for the prevention of immunological disease, including the immune escape of cancer cells.

Leukocyte infiltration into tumors of a certain size is interconnected with tumor angiogenesis. Pro-angiogenic factor VEGF and related molecules are effective monocyte attractants leading to the monocytes recruitment into tumors advancing to metastasis

(reviewed in [96]). Thus, the inhibition of leukocyte recruitment could be of therapeutic importance to suppress the tumor angiogenesis. As increased expression of RUNX3 is determined to be needed for VEGF suppression in OSCC [90], it can be speculated that targeting RUNX3 could be a potent molecular tool to prevent tumor growth and angiogenesis through repressing leukocyte infiltration into tumors.

CONCLUSION

Specifically, in GC and many other forms of cancer, RUNX3 suppression through different microenvironmental states, including hypoxia, is highlighted for cancer initiation, invasion and metastasis. While in some cancers, RUNX3 is illustrated oncogene-enhancing cancer cell proliferation and invasion. The involvement of RUNX3 in immune cell maintenance and regulation has forced researchers to address RUNX3 as a tumor immune microenvironmental regulator, rather than focus only on an appreciation of its role as a tumor suppressor. As TME has inevitable importance in the progression of cancer, the role of RUNX3 must be further explored in order to bolster early findings of its potential as a target for the effective treatment of cancer.

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CONFLICT OF INTEREST

None

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Figure Legend

Figure 1. Role of RUNX3 in the tumor microenvironment and its impact on EMT, tumor growth, invasion and metastasis. A growing tumor lacks oxygen (O_2) and becomes hypoxic. The hypoxic tumor modulates the tumor microenvironment for its further progression to malignancy. Extracellular matrix (ECM)-secreted cytokines facilitate epithelial-mesenchymal transition (EMT) and invasion, increased tumor-infiltrating immune cells suppress immunosurveillance and increase angiogenesis, tumor-modified cancer associated fibroblast (CAF) produce chemokines that attract other tumor-growth-favoring cells into tumor. RUNX3 can repress these phenomena by targeting tumor infiltrating cells, ECM and their secretions.

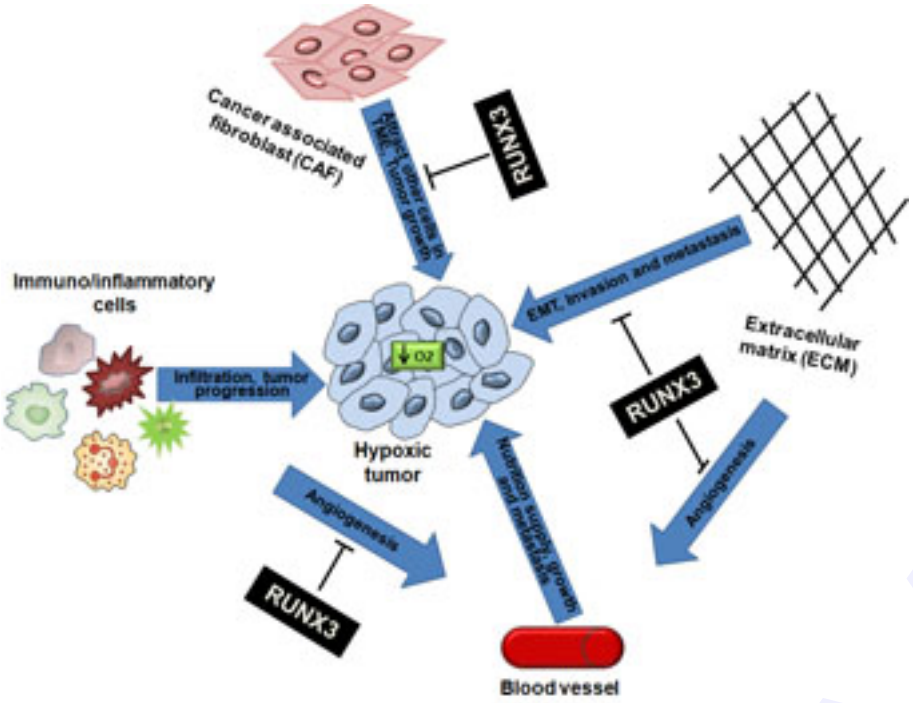

Fig. 1.