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The dual roles of homeobox genes in vascularization and wound healing

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Homeobox genes represent a family of highly conserved transcription factors originally discovered to regulate organ patterning during development. More recently, several homeobox genes were shown to affect processes in adult tissue, including angiogenesis and wound healing. Whereas a subset of members of the Hox-family of homeobox genes activate growth and migration to promote angiogenesis or wound healing, other Hox genes function to restore or maintain quiescent, differentiated tissue function. Pathological tissue remodeling is linked to differential expression of activating or stabilizing Hox genes and dysregulation of Hox expression can contribute to disease progression. Studies aimed at understanding the role and regulation of Hox genes have provided insight into how these potent morphoregulatory genes can be applied to enhance tissue engineering or limit cancer progression.

Introduction

Delivery of nutrients and the removal of anabolites by the vascular network are critical for the viability of biological tissues. For this reason, nearly every cell in the human body lies at a distance no further than 100–200 μm from a blood vessel, which correlates with the mathematically-calculated limit at which oxygen tension reaches a critical level for cell survival.[1,2] Not surprisingly, regulation of vascular development is closely tied to tissue architecture, and changes in tissue homeostasis during development and in pathological conditions trigger changes in the supporting vasculature.

Vascularization typically occurs via two processes: vasculogenesis and angiogenesis. Vasculogenesis is the de novo formation of blood vessels that arises from the coalescence of angioblasts to form the primary capillary plexus.[3] Vasculogenesis occurs largely during embryonic development; however, the presence of a population of circulating endothelial progenitor cells (EPCs) originating from the bone marrow, suggests that it may also occur in the postnatal stages of life.[4,5] Conversely, angiogenesis is the sprouting of vessels from existing vasculature and occurs during development and throughout life. This process involves endothelial cells (ECs) degrading the basement membrane of the parent vessel, collectively migrating through the provisional extracellular matrix (ECM) and subsequently establishing mature neovessels stabilized by pericytes.[6] This requires the coordinated expression of cytokines, proteases, adhesion molecules and other cell cycle regulatory factors. During embryonic development, angiogenesis is the means by which vessels expand from the primary capillary plexus into an extensive, closed circulatory network. Excluding corpus luteum formation and endometrium redevelopment, angiogenesis generally does not occur in adults, but is initiated during wound healing or in response to tumor growth.

Activation of angiogenesis under pathological conditions is controlled by what Judah Folkman termed the "angiogenic switch,"[7,8] which was originally coined to describe how tumors generate an angiogenic stimulus to support their growth and viability. This concept states that at homeostasis, there exists a balance between pro-angiogenic and anti-angiogenic factors, but under pathological conditions, expression of pro-angiogenic factors tilts the balance toward vessel formation, and conversely, when the process is complete the anti-angiogenic factors prevail, resulting in vessel pruning and regression. Tumors were shown to mimic features of healing wounds by producing copious amounts of angiogenic factors (e.g., VEGFA and FGF2) and macrophage chemoattractants (e.g., CCL2 and CSF1).[8-10] Macrophage infiltration at the tumor site augments the pro-angiogenic environment by increasing the local availability of pro-angiogenic cytokines.[11-17] However, exogenous addition of anti-angiogenic factors, such as thrombospondin 1, causes vessel regression by reversing the tilt of the angiogenic switch.[18] Restoration of tumor suppressor genes that limit tumor growth and subsequent metabolic demand can also shift the balance toward an anti-angiogenic environment.[19,20]

Understanding the mechanisms that modulate the angiogenic switch during normal wound healing and pathological vascularization has been the subject of intense investigation by researchers with the aim of developing novel approaches to either drive therapeutic angiogenesis or impair this process as a means of limiting tumor growth. Much attention has focused on a few well-established factors, particularly VEGFA, in driving angiogenesis, but inconsistent results achieved when applying anti-VEGFA agents to different tumor types have underscored the need to investigate the contribution of numerous other factors that drive

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pathological angiogenesis.[21] In this review we will focus on the role of homeobox genes during wound and tumor angiogenesis. These morphoregulatory genes, which are intimately linked to tissue architecture and homeostasis, have shown tremendous therapeutic potential to activate angiogenesis in poorly healing wounds or limit neovascularization in aggressive tumors.

Homeobox Genes

Homeobox genes encode a family of highly conserved transcription factors that were originally discovered to be essential for segmental identity in Drosophila.[22] Further research showed that homeobox genes were highly conserved throughout the animal kingdom and acted as master regulators of tissue and organ patterning.[23]

Homeobox genes are divided into families and subfamilies based on the similarity of their DNA-binding homeodomains. Most families contain between two to nine genes; these include the Msx, En, Nkx, Pax and Dlx families. However, the largest, and most extensively studied, family of homeobox genes is the class I Hox family, which includes 39 members in mammals. The Hox genes are arranged in clusters—with eight genes clustered on a single chromosome in Drosophila, while mammals have 39 genes grouped in clusters of 9–13 genes on each of four chromosomes. During vertebrate development, there exists a strict temporospatial control of Hox gene expression in a manner such that 3' Hox genes are expressed prior to 5' genes within a given cluster.[24] This expression pattern is responsible for controlling segmental identity and morphology on the anteroposterior axis; 3' Hox genes are associated with the development of anterior or rostral tissues and 5' genes are associated with posterior or caudal tissues. Mutations or deletions that lead to misexpression of Hox genes have shown to result in homeotic transformations in Drosophila and mammals, in which anterior features develop in posterior regions and vice versa.[22,25,26] The finding that Hox genes are arranged into highly conserved chromosomal clusters, with the lowest density of interspersed repeats in the entire human genome,[27] is most likely due to the need to preserve *cis*-regulatory elements to maintain this temporospatial control.

In humans, the 39 mammalian Hox genes are arranged into four chromosomal clusters; designated HOXA, HOXB, HOXC and HOXD, located on chromosomes 7p14, 17q21, 12q13 and 2q31, respectively. Each of these 39 genes is assigned to one of 13 paralog groups, with no single cluster containing a gene from each group (**Fig. 1**). Hox paralogs, defined by their homeodomain sequence, occupy the same relative positions on a given chromosomal cluster (e.g., *HOXA3*, *HOXB3* and *HOXD3*).[28] Paralogous group members have been shown to exhibit some level of functional redundancy, as they are able to perform compensatory functions during development upon mutation or deletion of a fellow group member.[29-34] In these experiments, mice that are double or triple mutants for paralogous Hox group members display more complete and severe phenotypes than single mutants of any of these paralogs. Functional redundancy between non-paralogous, neighboring Hox genes,[35-37] as well as non-paralogous genes in separate clusters has also been documented.[38]

Pro-Angiogenic Homeobox Genes

Studies over the last decade have clearly defined a role for the homeobox genes in regulating neovascularization during both embryonic vasculogenesis and pathological angiogenesis in adults.

The initial phases of embryonic vascular development consist of the formation of blood islands through the proliferation and differentiation of mesodermal hemangioblasts into angioblasts and hematopoietic progenitor cells.[3] Fusion of these blood islands results in the formation of the primary capillary plexus and the primitive heart tube.

Early studies by Chisaka and Capecchi linked Hox function to embryonic vascular development in vertebrates.[39] They observed that *Hoxa3* (originally referred to as *Hox1.5*)-knockout mice died at or shortly after birth, with abnormalities including an absent right carotid artery and irregularly sized heart compartments. These effects could be ameliorated by inserting *Hoxd3* within the *Hoxa3* locus, suggesting that the paralogs are somewhat interchangeable if expressed "properly" in space and time.[34]

In addition, the non-Hox homeobox genes *Prx1* and *Prx2* are normally expressed in the vessel wall during development. *Prx1*$^{-/-}$ mice displayed an irregularly curved aortic arch along with a misdirected and elongated ductus arteriosus.[40] In contrast, *Prx2*$^{-/-}$ mice did not display any vascular deformities. However, *Prx1*$^{-/-}$ *Prx2*$^{-/-}$ double mutant mice presented similar, but more pronounced, abnormalities as the *Prx1*$^{-/-}$ mice. Interestingly, although the great arteries were malformed in the double mutants, the heart developed normally compared with wild-type mice. Additionally, vascular smooth muscle cells (VSMCs), which represent the major cellular component of the vessel wall, were found to express *HOXA5*, *HOXA11*, *HOXB1*, *HOXB7* and *HOXC9* in fetal tissue, while Hox gene expression was absent in their adult counterparts, suggesting their importance during early vascular development.[41]

Subsequent studies revealed that homeobox genes, at least partially, direct the differentiation of EPCs into ECs. Transactivation of *Vegfr2* occurs via *cis*-acting *Hoxb5* in embryoid bodies, resulting in their differentiation into angioblasts. These angioblasts expand and further differentiate into an endothelial phenotype.[42] We have also profiled the dynamic expression of several Hox genes during the differentiation of embryonic stem cells into ECs in culture.[43] Early expression of *HOXA3* and *HOXD3* was concomitant with the emergence of an EC-phenotype and upregulation of pro-angiogenic factors such as *CCL2*, *MMP14* and the β3 integrin subunit. Furthermore, *Hoxa3* is critical in maintaining the endothelial phenotype in embryonic vasculature and preventing its hematopoietic differentiation.[44] At later time points, *HOXA3* and *HOXD3* are downregulated while the anti-angiogenic *HOXA5* and *HOXD10* are upregulated. This corresponds with the dowregulation of the aforementioned pro-angiogenic factors and the expression of the potent angiogenesis inhibitor thrombospondin 2. A separate study identified *HOXA9*

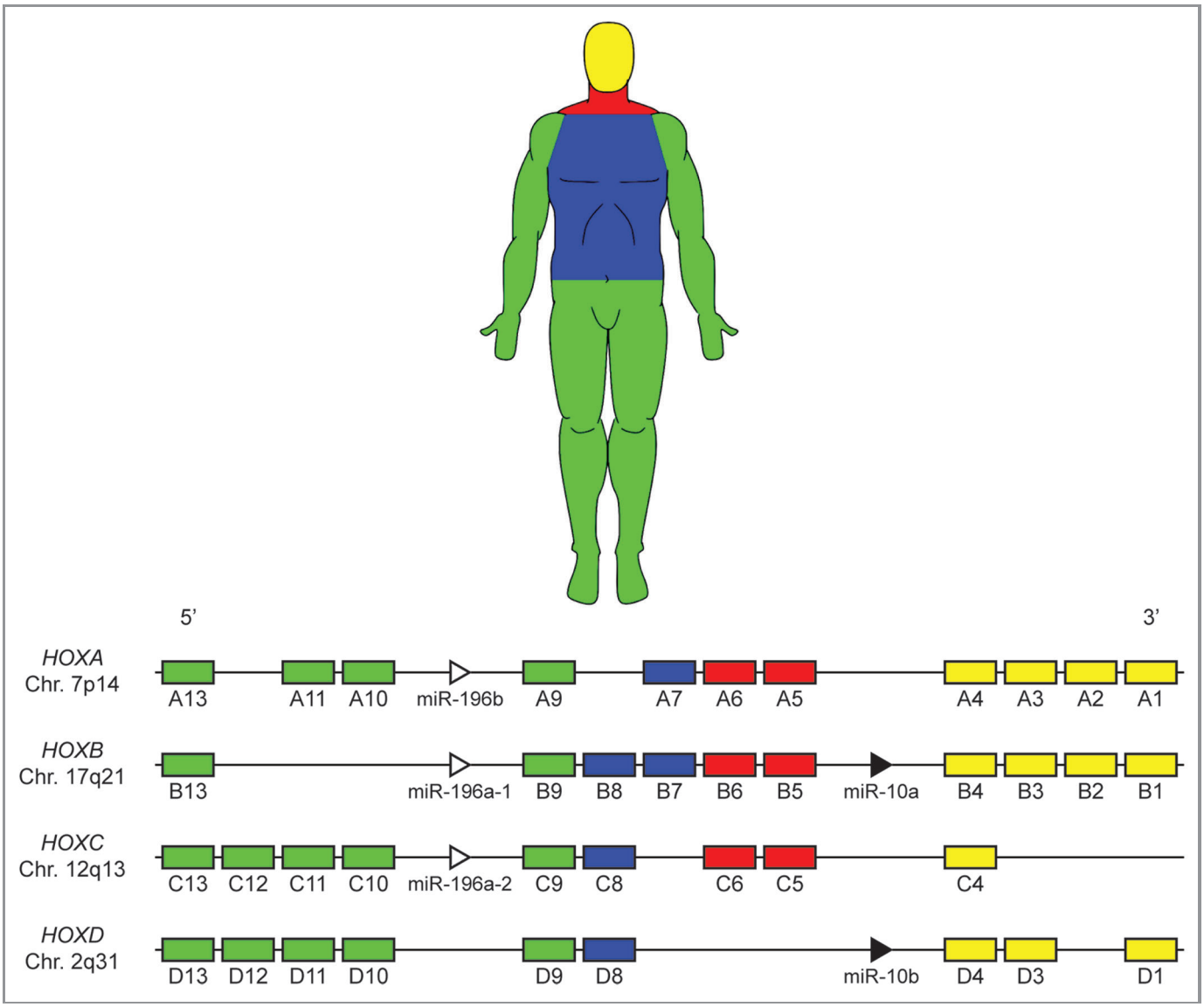

Figure 1. Mammalian Hox clusters. Thirty-nine Hox genes are arranged into four distinct chromosomal loci. During human development Hox genes are activated in a 3′ to 5′ manner within each cluster, concomitant with the development of the anteroposterior axis; Hox gene activation is color-coded based approximately on anatomical location during development. Note the presence of two miRNA families (*MIR10* and *MIR196*) within the Hox clusters, which target many Hox genes and may contribute to the posterior prevalence phenomenon.

as a master regulator of EC differentiation from progenitor cells by directly enhancing transcription of *VEGFR2* and e*NOS*.[45]

The non-Hox homeobox gene *Hex* plays a complex role in vascularization. During embryonic vasculogenesis, *Hex* is expressed in the blood islands, and is required for endothelial and hematopoietic differentiation.[46] *Hex*-knockout mice displayed embryonic lethality typified with several defects, including impaired vasculogenesis.[47] However, *Hex* inhibits EC tube formation in culture through the downregulation of angiogenic factors such as VEGFR1, VEGFR2, TIE1, TIE2 and the α_v integrin subunit,[48,49] suggesting that *Hex* promotes vasculogenesis rather than angiogenesis, or that other factors are integral in determining the outcome of *Hex* signaling (the anti-angiogenic effects of homeobox genes will be subsequently detailed in this review). The precise hierarchy and interactions between each of these Hox factors during EC differentiation remains to be established, but suggests a complex network of factors required for EC development.

Hox genes also serve important roles in vascularization post-development in response to injury or other pathological conditions. HOXD3, through binding of cofactor PBX1, directly enhances EC expression of the α_5 and β_3 integrin subunits in response to FGF2.[50-52] Heterodimerization of these subunits with separate subunits form the fibronectin receptors $\alpha_5\beta_1$ and $\alpha_v\beta_3$, which allow adhesion and migration through fibronectin-rich matrices found in wound healing and tumor microenvironments. HOXD1 was recently shown to provide a synergistic effect to this by directly enhancing the EC expression of the β_1 integrin

subunit.[53] Disruption of *HOXD3* expression inhibited the expression of the α_5 and β_3 integrin subunits, and was sufficient to inhibit angiogenesis, consistent with previous findings that these integrins are essential for angiogenesis.[54,55] Additionally, HOXD3 upregulated the expression of the serine protease urokinase plasminogen activator (uPA), which locally activates plasmin during the degradation of the provisional matrix,[50] suggesting that EC *HOXD3* is associated with the invasive phases of angiogenesis. Subsequent studies revealed that the paralogous Hox members *Hoxa3* and *Hoxb3* also display pro-angiogenic capabilities, albeit through distinct pathways, consistent with the notion of functional redundancy among Hox paralogs.[34] EC expression of *Hoxa3* correlated with increased EC migration and expression of the proteases uPA receptor (uPAR) and *Mmp14*,[56] while *HOXB3* increased the expression of the angiogenic ligand ephrin A1 and induced capillary morphogenesis in endothelial sprouts.[57] Because adult angiogenesis is generally limited to pathological conditions, it is not surprising that these pro-angiogenic *HOX3* paralogs are not normally expressed in quiescent vasculature, but are induced in response to cytokines linked to tissue injury or tumors.[50,51,56,58]

Originally thought to be a potential inhibitor of angiogenesis, *HOXA9* was actually found to promote it. Early studies found that post-transcriptional modification in ECs generated a *HoxA9* isoform that was unique from that found in fetal tissue.[59] This isoform was found to be downregulated in response to the pro-angiogenic TNFα, and therefore, thought to possibly suppress angiogenesis. Subsequent studies however, found that *HOXA9*, in fact, promotes EC migration and tube formation in culture through direct upregulation of the Ephrin receptor B4 (*EPHB4*),[60] along with direct or indirect upergulation of *eNOS* and *VEGFR2*.[45]

Anti-Angiogenic Homeobox Genes

Angiogenesis is a highly regulated process that consists of many control mechanisms that temporally initiate and terminate the progression through its different phases. In contrast to the pro-angiogenic genes described above, a subset of homeobox genes normally acts to terminate the angiogenic response and/or maintain the vasculature in a quiescent state.

The 5' Hox gene *HOXD10* was shown to be expressed in normal, quiescent vascular endothelium, but not in highly angiogenic, tumor-associated vasculature.[20] Overexpression of *HOXD10* in ECs mitigated their angiogenic response to FGF2 and VEGFA in two and three-dimensional cultures. Furthermore, *HOXD10*-expressing ECs showed a marked decrease in the angiogenic factors *RHOC*, β_4 and α_3 integrins, *MMP14*, *uPAR* and cyclin D1. The expressions of the pro-angiogenic *HOXA3, HOXB3* and *HOXD3* were not altered, suggesting that HOXD10 functions to mute the downstream effects of those genes, rather than attenuate their expression.

In addition to its direct anti-angiogenic effect on ECs, HOXD10 also attenuates tumor angiogenesis by acting within tumorigenic epithelial cells to reduce expression of angiogenic factors including VEGFA.[61] Restoring expression of *HOXD10* in mammary tumor cells, from which it is frequently lost, reestablished polarity and the formation of acinar structures in three-dimensional cultures and reduced EC recruitment.[61] It is also worth noting that the ability of HOXD10 to reduce VEGFA expression was not due to a direct inhibition of *VEGFA* transcription, or interference with binding of the transcriptional activator HIF1A, but arose indirectly through the re-establishment of tissue polarity. When *HOXD10* was expressed, but tissues were prevented from adopting basolateral polarity, expression of *VEGFA* and other angiogenic factors remained high. Thus, the ability of Hox genes to modulate the angiogenic capacity of tissues can arise directly through transcriptional modulation of angiogenic effector genes, or indirectly via the Hox mediated changes in tissue architecture.

The findings that *HOXD10*, whether expressed in epithelial or endothelial cells has a similar inhibitory effect on angiogenesis, also highlights the fact that Hox genes have the capacity to coordinate the responses of different cell populations within a tissue. These coordinated actions represent one means by which these morhpregulatory genes can work to collectively maintain differentiated tissue function.

HOXA5 is another gene expressed in quiescent, but not activated, endothelium.[62] Similar to *HOXD10*, *HOXA5*-expressing ECs did not respond to the angiogenic stimuli provided by either VEGFA or FGF2. Among the angiogenic genes that were downregulated in ECs were *VEGFR2*, ephrin A1, *HIF1A* and *COX2*. Correspondingly, these cells also upregulated the expression of the anti-angiogenic thrombospondin 2. Further evidence demonstrated that EC HOXA5 stabilized adherens junctions through β-catenin retention.[63] Presumably, this prevents endothelium dissociation in the parent vessel—the first step in angiogenesis. Interestingly, paralogous *Hoxb5* in ECs was shown to promote both vasculogenesis[42] and angiogenesis,[64] counterintuitive to the paralog redundancy theory.

The homeobox gene *GAX (MEOX2)* is also largely confined to quiescent cardiovascular tissue in adults. Originally found to induce G_0/G_1 cell cycle arrest in VSMCs,[65,66] *GAX*, and its related gene *MEOX1*, were also found to be expressed in the endothelium and induced similar growth arrest in ECs.[67,68] Additionally, *GAX*-expressing ECs, through NFκB inhibition, exhibited an impaired angiogenic response to stimuli by VEGFA, FGF2 and TNFα.[69] Translation of both GAX and HOXA5 is directly regulated by *MIR130A* (located outside of the *Hox* clusters).[70] EC expression of *MIR130A* downregulates these homeobox genes and effectively restores EC activation by angiogenic stimuli.

Thus, the ability of tissues to activate the angiogenic switch relies upon upregulation of pro-angiogenc genes and suppression of anti-angiogenic or vascular-stabilizing genes (**Fig. 2**).

Homeobox Genes in Wound Healing

Wound healing is the coordinated process by which the body repairs damaged tissue and is generally divided into three overlapping phases: inflammation, proliferation and remodeling.[71] In the initial inflammatory phase, the body functions to prevent

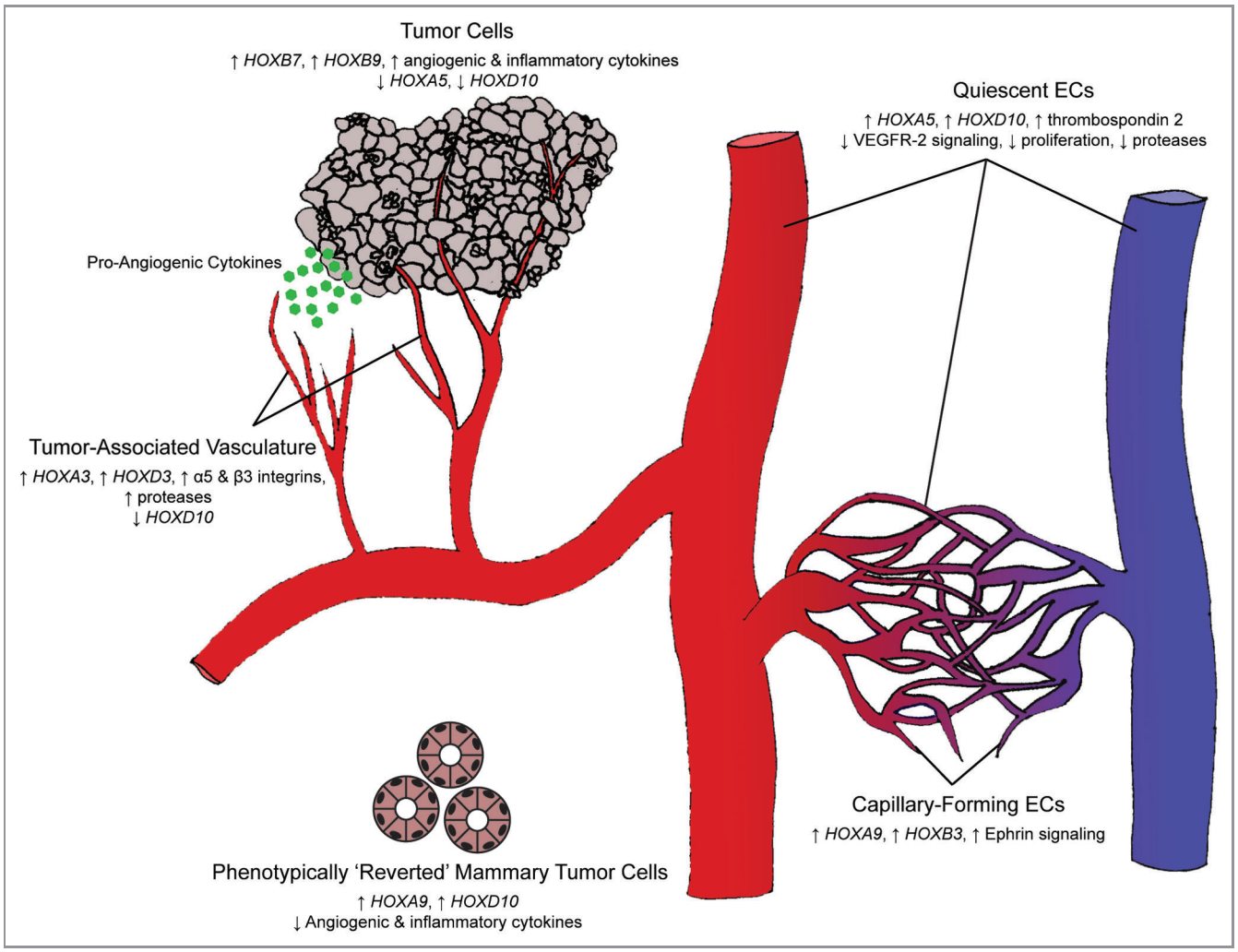

Figure 2. Hox gene control of the angiogenic switch. Differential expression of Hox genes is key in transitioning between homeostasis and angiogenesis. During homeostasis, HOXA5 and HOXD10 upregulate anti-angiogenic cytokines and inhibit pro-angiogenic signals, thereby maintaining a quiescent endothelial phenotype. Tumor cells can initiate an angiogenic response as a result of a change in Hox gene expression. Loss of *HOXA5* and *HOXD10* expression (along with the upregulation of *HOXB7* and *HOXB9*) leads to the secretion of pro-angiogenic and pro-inflammatory cytokines. Responsive ECs subsequently downregulate the expression of *HOXD10* and upregulate the expression of *HOXA3* and *HOXD3*, thereby assuming an invasive phenotype. Tumor-associated endothelium maintains this invasive phenotype, whereas normal angiogenesis is an ordered process that concludes with a closed capillary network. ECs undergoing capillary morphogenesis express *HOXA9* and *HOXB3* and are phenotypically distinct from their invasive counterparts.

blood loss through vasoconstriction, platelet aggregation and fibrin clot formation—a process known as hemostasis. Platelet secretion of inflammatory cytokines recruits neutrophils and macrophages, which function to remove foreign debris from the wound site. Macrophages also initiate the transition to the proliferative phase by secreting factors that recruit fibroblasts, which in turn secrete collagen and fibronectin at the wound site. Concurrently, ECs undergo angiogenesis within this freshly-laid granulation tissue. Differentiated myofibroblasts contract the wound margins and epithelial cells migrate across the vascularized granulation site to close the wound. Epithelialization of the wound bed does not occur over necrotic tissue;[72] therefore, angiogenesis is critical in this phase and poorly healing wounds have been linked to impaired angiogenic responses. The remodeling phase, which can last years, is characterized by myofibroblasts depositing and reorganizing collagen fibers so as to increase tissue tensile strength.

Given the essential role of angiogenesis in wound healing, it is not surprising that several homeobox genes contribute to effective wound repair. Gene delivery of the pro-angiogenic *Hoxd3* accelerated wound closure in poorly healing diabetic mice and was accompanied by a robust induction of angiogenesis.[58] As transgene uptake is evident in ECs, as well as keratinocytes and fibroblasts, wound closure was accompanied by increased collagen (*Col1a1*) production mediated by fibroblasts. Similar to *Hoxd3*, the paralogous *Hoxa3* was found to be upregulated in ECs in a wound healing mouse model, whereas diabetic mice were unable to induce its expression;[56] *Hoxa3* transfer into the wounds of these diabetic mice significantly improved angiogenesis and wound closure. Mechanistically, in addition to the angiogenic response by

ECs detailed previously, HOXA3 promoted uPAR-dependent keratinocyte migration and subsequent epithelialization—a feature that was not directly enhanced by HOXD3. Furthermore, *HOXA3* expression within wound tissue recruited bone marrow-derived progenitor cells,[73] and promoted their differentiation into pro-angiogenic, $CD11b^+$ $Gr\text{-}1^+$ myeloid cells.[74] The synergistic functions of HOXA3 and HOXD3 in wound healing mimic their complementary roles in angiogenesis, and further support the functional redundancy concept conceived from double *Hoxa3*/*Hoxd3*-knockout mice.[29]

While wound contraction is a normal part of wound healing, excessive or prolonged contraction leads to diminished tissue function and appearance. Enhanced angiogenesis within the wound bed reduces the contractile activity of myofibroblasts and scarring.[75] Building from this concept, we have engineered composite skin grafts consisting of a dermal fibroblast layer, which produces a secretable form of HOXA3, overlaid with an epidermal keratinocyte layer. Following wounding and engraftment onto mice, the secretable HOXA3 is taken up by keratinocytes and ECs within the wound tissue, and these grafts displayed increased angiogenesis and reduced contraction compared with controls (unpublished observations, manuscript submitted). Additionally, cells within the graft produced less inflammatory cytokines, consistent with the reduced inflammatory response in wounds treated with the *HOXA3* transgene.

In contrast to *HOXA3* and *HOXD3*, the 5' *Hoxb13* was found to impair wound healing.[76,77] *Hoxb13* transgenic mice displayed increased fibrin clot and immune cell retention at the wound site, leading to a prolonged inflammatory response.[77] Additionally, increased expression of VEGFA and TNFα in the wounds of these transgenic mice may be responsible for the formation of irregular, enlarged vasculature. Forced expression of anti-angiogenic *HOXA5* or *HOXD10* in wounds also impairs angiogenesis and healing (unpublished observations), but whether these genes are normally sustained in poorly healing or chronic wounds that are deficient in angiogenesis, remains to be seen.

Angiogenic Hox Genes and Pathological Angiogenesis

Whereas endogenous HOXA3 and HOXD3 can contribute to wound-induced angiogenesis, diseases characterized by the formation of excessive vasculature, including endotheliomas and tumor vascularization, are often the result of the sustained expression of pro-angiogenic Hox genes.

One of the cornerstones of physiological angiogenesis is the transition from vessel invasion to vessel maturation. ECs in the maturation phase show decreased proliferation relative to their invasive counterparts, and also intimately associate with supporting pericytes, which function to regulate vessel tone and leakage. As mentioned, EC *HOXD3* expression correlates with the invasive angiogenic phenotype, and correspondingly, *Hoxd3* overexpressing vessels formed in the chick chorioallantoic membrane (CAM) assay did not mature, but formed large endothelima-like masses.[50] This result is in agreement with the finding that *HOXD3* is expressed in proliferating hemangiomas, but not involuting ones.[78] Similarly, tumor-associated vasculature, which is typically comprised of immature, leaky vessels, also highly expressed *HOXD3*.[51] It is also worth noting that *HOXA5*, which can stabilize vessels and reduce permeability, is absent in both endothelioma tissues and tumor vessels,[62,63] suggesting that the unopposed actions of HOXD3 contribute to aberrant vascular morphology. Indeed, ectopic expression of *HOXA5* in endotheliomas leads to a reduction in cavernous cyst formation and restores the phenotype of normal, differentiated vessels.[79]

In addition, to promoting an angiogenic phenotype within the EC compartment, Hox gene expression within tumor cells can further sustain a pro-angiogenic response. An early study found that *HOXB7* was expressed in each of 25 melanoma cell lines, as well as in primary melanoma tumors and their metastases.[80] Furthermore, *HOXB7* was found to increase expression of the angiogenic factor FGF2. This observation was also evident in other glioblastoma, carcinoma and leukemia cell lines; introduction of *HOXB7* to the non-*HOXB7*-expressing SkBr3 breast cancer cell line also lead to increased FGF2 expression.[81] A subsequent study by this group found that the *HOXB7*-transduced SkBr3 cells also upregulated the expression of additional pro-angiogenic factors including VEGFA, IL-8 and CXCL1.[82] ANG2 is also elevated in these cells, which, in conjunction with sustained VEGFA expression, produces a robust angiogenic response.[83] Correspondingly, tumor explants of SkBr3-*HOXB7* xenografts revealed greater levels of vascularization than their control counterparts.[82] Another group found that *HOXB9* expression was elevated in several breast carcinoma biopsies.[84] Culture studies with *HOXB9*-transduced MCF10A cells revealed increased expression of VEGFA, FGF2, IL8 and ANGPTL2—nearly identical to the effect of the non-paralogous *HOXB7*. Functionally, these cells, with an additional *HRAS* mutation, rapidly gave rise to large, vascularized tumors compared with MCF10A cells displaying the *HRAS* mutation alone. In addition, the *HOXB9*-expressing cells developed several lung metastases, whereas non-expressing cells developed none—a likely consequence of the enhanced angiogenesis induced by HOXB9.

Homeobox Genes and Regulation of Inflammatory Responses

During the inflammatory phase of wound healing, recruited leukocytes can initiate or augment an angiogenic response through the secretion of several pro-angiogenic factors.[85] In addition to directly stimulating ECs to undergo angiogenesis, tumors also frequently induce an indirect angiogenic response via leukocyte recruitment. Macrophages are recruited to the tumor microenvironment via tumor secretion of chemotactic factors such as CCL2 and VEGFA.[10] Once within the microenvironment, macrophages increase the local concentration of VEGFA through de novo secretion[13] and by catalyzing the release of the matrix-bound form through protease secretion.[86] Additionally, a different subset of leukocytes, responding to primary tumor secretion of VEGFA, TNFα and TGFβ, was found to colonize lung tissue and prime it for the seeding of metastases.[87-89] As detailed throughout

this review, homeobox genes regulate the expression of several key leukocyte chemoattractants.

Tumor expression of *HOXB7* or *HOXB9* has correlated with an increase in VEGFA, and the neutrophil attractants CXCL1 and IL8.[80-82,84] HOXB13-mediated inflammation within wound tissue is characterized by neutrophil infiltration and the development of irregular vasculature.[77] Additionally, HOXA9 was found to bind the CCL2 promoter and enhance its transcription in individuals displaying a single nucleotide polymorphism within the promoter region.[90] Conversely, we have found the anti-angiogenic HOXD10 reduces tumor cell production of CCL2 and CXCL12, and abates leukocyte recruitment in culture (unpublished data). Interestingly, while HOXA3 also attenuates recruitment of $CD45^+$ cells in wound tissues, it selectively promotes recruitment/differentiation of endothelial precursors and pro-angiogenic myeloid cells.[73,74]

Given that homeobox genes play a fundamental role in regulating tissue remodeling and organogenesis, it is not surprising that they can profoundly alter the immune microenvironment to facilitate tissue remodeling or maintain normal homeostasis. Interestingly, Hox genes have also been implicated in directly regulating the phenotype of immune cells exposed to activating stimuli,[91] thereby suggesting a broader role for Hox genes in regulating the inflammatory milieu in remodeling tissues (for comprehensive reviews on the role of Hox genes on immune cell function, see refs. 92 and 93).

Regulation of Homeobox Genes

Considering the powerful role of Hox genes in normal and pathological tissue responses, it follows that strict regulatory mechanisms have evolved to maintain the correct spatiotemporal expression pattern of Hox genes. Hox genes are commonly regulated epigenetically via histone methylation and acetylation. Chromatin trimethylation by trithorax-group (TrxG) proteins are associated with activating and maintaining Hox gene expression, while trimethylation by polycomb-group (PcG) proteins are known to repress it.[94-97] Whereas pluripotent stem cells do not display any significant Hox gene expression, cell lineage commitment is linked to changes in PcG and TrxG genes leading to tissue specific patterns of Hox gene expression.[98,99] Several studies have also identified significant roles for TrxG and PcG proteins in regulating expression of Hox proteins in adult cells in the context of angiogenesis and wound healing.

Expression of *HOXA9* during EPC differentiation and angiogenesis is reliant on histone deacetylase (HDAC) activity,[45] while upregulation of both *HOXA9* and *HOXD3* has been shown to occur via histone trimethylation by the TrxG protein MLL.[100] The expression of the PcG gene *MEL18 (PCGF2)* is linked to an upregulation in *VEGFR2* and *ANGPTL2*, with a corresponding increase in EC migration and tube formation;[101] however, its downstream effectors and global function is still debated, as another group was able to show that MEL18 exhibits anti-angiogenic activity.[102]

The PcG genes *Eed*, *Ezh2* and *Suz12* are downregulated in wound epidermis, corresponding with a decrease in target histone trimethylation.[103] Although this study did not explore the effects of this downregulation on Hox gene expression, other reports have demonstrated that protein products of these three PcG genes assemble to form the polycomb repressor complex 2 (PRC2) and attenuate the expression of *HOXC8* and *HOXA9*.[104-106] In separate studies, *HOXC8* expression has been associated with the promotion of wound epithelialization by keratinocytes,[107] while decreased expression of *HOXA9* was found in keloid fibroblasts that are associated with aberrant wound healing.[108]

Deregulation of Hox Genes and Oncogenesis

Although mutations in Hox genes, per se, are relatively rare, the regulatory mechanisms that control Hox gene expression are common targets of mutagenesis, and the subsequent misexpression or deregulation of Hox genes is associated with the formation or progression of several tumors.[109-115]

An exhaustive study of Hox gene expression in several esophageal carcinomas revealed abnormally high expression of several 5' Hox genes in a tissue that normally displays low levels of 5' Hox genes and high levels of 3' Hox genes.[112] Similarly, expression of the 5' *HOXB13* is lost in colorectal tumor cells, leading to increased cell proliferation,[116,117] The 3' *HOXB7* was shown to directly upregulate EGFR and confer tamoxifen resistance to estrogen receptor-positive breast cancer cells,[118] while loss of the 5' *HOXA9* and *HOXD10* are frequently associated with breast cancer progression.[109,119] Contrastingly, overexpression of *HOXA9* in several leukemias correlates with chemotherapy resistance and poor patient prognosis. Transgenic studies with $Hoxa9^{-/-}$ mice demonstrated an inhibition of leukemogenesis, but also impaired normal hematopoiesis in the process;[120] $Hoxa9^{+/-}$ mice developed normally with no leukemogenesis, demonstrating a dose-dependent response of Hox gene expression mediating the shift between organogenesis and oncogenesis.[121] Thus, the potential of HoxA9 to act as either a tumor suppressor in breast tissue or an oncogene in leukocytes underscores the tissue-specific and context-dependent function of Hox proteins. Whether tissue-specific repertoires of Hox cofactors and/or differences in tissue architecture (adherent vs. circulating cells) contribute to the differential responses has not been directly addressed. It is worth noting again, that the ability of HOXD10 to reduce VEGFA expression and revert the tumorigenic phenotype of breast epithelial cells is highly context-dependent, as it requires cell adhesion to the basement membranes and acquisition of apical-basal polarity.[61]

As mentioned in the previous section, the expression of several Hox genes is regulated by histone trimethylation by TrxG or PcG proteins. Changes in chromatin conformation allow CpG motifs in Hox promoter regions to be further methylated or demethylated. Epigenetic deregulation occurs when members of this regulatory process become mutated or misexpressed. The TrxG gene *MLL* is mutated in some leukemias, causing irregular histone methylation and increased expression of *HOXA4*, *HOXA5*, *HOXA7* and *HOXA9*.[114,115] The inactivation or loss of the tumor suppressor gene *P16* was shown to upregulate the PRC2 proteins

and consequently repress *HOXA9* in mammary epithelial cells, thereby perpetuating tumorigenesis.[106]

Additionally, miRNAs that regulate Hox expression may also have altered expression patterns in tumor cells through methylation or other means.[122,123] For instance, upregulation of *MIR10B* in mammary tumor cells inhibits translation of *HOXD10* mRNA and produces robust invasion and metastasis.[124]

Hox gene dysregulation arises through a variety of mechanisms and produces a wide array of aberrant responses in several tumor types; it is still unclear whether Hox dysregulation is a cause or consequence of tumor progression. However, in many instances, acquisition of the tumorigenic phenotype by Hox misexpression results in the upregulation of angiogenic factors that act on adjacent ECs and alter expression of endogenous Hox genes, leading to the formation of new vessels (for a comprehensive review of Hox genes and cancer, see ref. 125).

It is not currently known whether any of the previously mentioned regulatory mechanisms are linked to Hox gene misexpression in non-tumorigenic tissues such as endotheliomas, which exhibit constitutive *HOXD3*, or in poorly healing wounds, which fail to induce *Hoxd3* or *Hoxa3*.[50,56,58]

Determinants of Functional Dominance among Hox Genes

During embryogenesis, Hox genes are expressed in a sequentially overlapping pattern with 3' Hox genes activated prior to 5' genes, corresponding with anteroposterior axis development. In anatomical regions where the expression of non-paralogous Hox genes overlap, the more 5' paralogs generally tend to exert dominance over the more 3' paralogs. This occurrence is thought to be the reason why Hox gene mutations commonly result in homeotic defects found in locations anterior to the normal expression domain of the mutant Hox genes, but not posterior to it.[25,126,127] These mechanisms remain active in adult tissue, as observed in ECs expressing *HOXA3* and *HOXD10*. Sustained expression of the 5' *HOXD10* inhibits the expression of angiogenic factors such as uPAR and MMP14,[20] both of which are upregulated by the 3' *HOXA3*.[56]

This phenomenon, termed "phenotypic suppression" in invertebrates[126,128] and "posterior prevalence" in vertebrates,[129] is believed to be part of an ancient, poorly understood, cross-regulatory mechanism, in which posterior Hox transcription factors can inactivate anterior Hox proteins possibly through mechanisms that do not involve transcriptional repression (i.e., even if both genes are expressed, the posterior one is functionally dominant).[130] Alternatively, two groups of miRNA sequences, *MIR10* and *MIR196*, which downregulate, several 3' Hox genes have been identified within the Hox chromosomal clusters, and possibly contribute toward this phenomenon (**Fig. 1**).[124,131,132]

However, regardless of the mechanistic basis, the posterior prevalence theory has its share of shortcomings, as several groups have reported instances where the 3' Hox gene exerts dominance,[133] 3' and 5' Hox genes act cooperatively,[134] or the functional response is dose-dependent.[135] In all of these models, however, the prevailing theme is that repression of an effector gene product takes precedence over its activation, as is the case for other transcriptional control pathways.[136]

One plausible model for repression involves the requirement of cofactors for Hox proteins to efficiently bind DNA. Most 3' Hox proteins contain a hexapeptide motif that allows them to bind members of the PBX family of transcription factors, while most 5' Hox proteins (members of paralog groups 10–13) lack this motif.[137,138] 3' Hox-PBX-DNA complexes exhibited dramatically increased stability compared with 3' Hox-DNA complexes alone. Many of the Hox genes discussed in this review have been characterized to complex with PBX proteins, including HOXA9,[139] HOXB3, HOXB5, HOXB7, HOXB9[138] and HOXD3.[52] The common theme among these factors is that they are all activators of angiogenesis, wound healing or both (**Table 1**). In contrast, anti-angiogenic HOXD10, and related paralogs, can bind target DNA with much higher affinity in the absence of PBX proteins. Thus, by requiring additional factors for transcriptional activation by the pro-angiogenic Hox genes, it is evident how repressor Hox proteins could exert functional dominance over activators. Interestingly, ectopic expression of *Hoxd10* in Drosophila antennal imaginal discs inhibited nuclear translocation of EXD (Drosophila homolog of PBX1 and PBX2) and rendered it unavailable for cooperative DNA binding with 3' Hox genes.[140] It remains to be seen if this mechanism is recapitulated in mammalian cells, but it does suggest a possible means by which the anti-angiogenic HOXD10 can dominantly counteract Hox3-mediated pathological vascularization.

Phosphorylation of certain residues may also be required for Hox protein activation or nuclear translocation, and its absence may trigger transcriptional repression. While Hox phosphorylation has not yet been reported in mammals, consensus phosphorylation sites have been identified in Drosophila Hox homologs, and mutation of these sites have led to the repression of limb development.[141,142]

Future Directions

Many of the studies covered in this review have demonstrated that spatial or temporal misexpression of homeobox genes often results in malformations or oncogenesis; therefore, therapies intent on modulating cellular homeobox gene expression must consider the extent and duration of such a change.

For instance, overexpression of *HOXD3* in healthy endothelium may trigger an unwanted inflammatory response or generate large cavernous vessels reminiscent of endotheliomas. To address this issue, our group commonly uses Hox DNA expression plasmids embedded within methylcellulose pellets to limit gene uptake to cells within wound tissue and their duration of expression.[56,58]

While topical application presents a straightforward approach for cutaneous tissues, targeted delivery strategies must be developed for tumors in internal organs. The mammary tumor suppressing properties of HOXA5 and HOXD10 have been documented,[109,143] but a clinically viable treatment for breast cancer would need to target delivery of these Hox genes/proteins to tumors and their metastases in an effective manner. Like most

Table 1. Homeobox activators/repressors of vascularization/wound healing

Gene/ protein	Pro/Anti-vascularization/ wound healing	Observation (expressed in)	Reference(s)
		Hox family genes	
HOXA3	Pro-Vasc.	• Knockout mice developed abnormally sized heart chambers • Expression concomitant with *CCL2*, *MMP14* and β3 integrin expression in ESC → EC differentiation (ESCs) • Expression concomitant with EC migration along with uPAR and MMP14 expression (ECs)	39, 43 and 56
	Pro-WH	• Natively upregulated in keratinocytes within wound tissue. Enhances uPAR-mediated keratinocyte migration and accelerates wound closure in diabetic mice (dermal wound tissue) • Promotes differentiation of bone marrow progenitor cells into an M2-phenotype myeloid-like cell (dermal wound tissue)	56 73 and 74
HOXA5	Anti-Vasc.	• Expressed in quiescent endothelium. Dowregulates *VEGFR2*, ephrin A1, *HIF1A* and *COX2*. Increases thrombospondin 2 (ECs) • Stabilizes adherens junctions via β-catenin retention (ECs)	62 and 63
HOXA9	Pro-Vasc.	• Promotes EC migration and tube formation in culture through direct upregulation of *EPHB4* (ECs) • Upregulates eNOS, VEGFR2 and VE-cadherin during EPC → EC differentiation (EPCs)	45 and 60
	Pro-WH	• Decreased expression in keloid fibroblasts associated with abnormal wound healing (dermal fibroblasts)	108
HOXB3	Pro-Vasc.	• Increases expression of ephrin A1 and induces capillary morphogenesis in endothelial sprouts (ECs)	57
HOXB5	Pro-Vasc.	• Triggers differentiation of embryoid bodies into angioblasts through VEGFR2 transactivation (ESCs) • Upregulates ANG2 and promotes vessel sprouting (ECs)	42 and 64
HOXB7	Pro-Vasc.	• Directly enhances expression of FGF2, VEGFA, IL8, CXCL1 and ANG2 (many tumor lines)	80–82
HOXB9	Pro-Vasc.	• Increased expression of VEGFA, FGF2, IL8 and ANGPTL2. Produced highly vascularized tumors which developed metastases (MCF10A breast epithelial cells)	84
HOXB13	Anti-WH	• Longer retention of fibrin clot, sustained inflammatory response and formation of large, irregular vasculature (dermal wound tissue) • Downregulated following wounding of fetal dermis (fetal wound tissue)	77 and 144
HOXC8	Pro-WH	• Expression correlates with keratinocyte proliferation and migration, leading to wound epithelialization (keratinocytes)	107
HOXD1	Pro-Vasc.	• Directly increases β1 integrin expression, allowing formation of pro-angiogenic α5β1 integrin (ECs)	53
HOXD3	Pro-Vasc.	• Correlates with invasive phase of angiogenesis. Directly enhances expression of the α5 and β3. Indirectly increases uPA. Expressed in immature tumor vasculature (ECs) • Expressed in proliferating hemangiomas (hemangioma tissue)	50, 51 and 78
	Pro-WH	• Increases angiogenesis and COL1A1 production, leading to accelerated wound closure (ECs, fibroblasts, keratinocytes) • Upregulated in wounded fetal skin (fetal wound tissue)	58 and 145
HOXD8	Pro-WH	• Upregulated in wounded fetal skin (fetal wound tissue)	145
HOXD10	Anti-Vasc.	• Expressed in quiescent endothelium. Overexpression inhibits angiogenic respone to FGF2 and VEGFA. Decreases expression of *RHOC*, β4 and α3 integrins, *MMP14*, *uPAR* and cyclin D1 (ECs) • Overexpression in mammary tumor cells decreases VEGFA expression and EC recruitment (mammary tumor cells)	20 and 61
		Non-Hox homeobox genes	
GAX (MEOX2)	Anti-Vasc.	• Induces cell cycle arrest (VSMCs and ECs) • Impairs angiogenic response to VEGFA, FGF2 and TNFα through NFκB inhibition. (ECs)	66, 67 and 69

Table 1. Homeobox activators/repressors of vascularization/wound healing (continued)

Non-Hox homeobox genes			
HEX	Pro-Vasc.	• Required for EC and hematopoietic cell differentiation during vasculogenesis (blood islands) • Knockout mice develop several cardiovascular defects.	46 and 47
	Anti-Vasc.	• Inhibits EC tube formation in culture and dowregulates VEGFR1, VEGFR2, TIE1, TIE2 and α_V integrin (ECs)	48 and 49
MEOX1	Anti-Vasc.	• Induces cell cycle arrest (ECs)	68
	Pro-WH	• Expressed in fetal epidermis and dermis, but not in adult skin	146
MSX1	Pro-WH	• Expressed in fetal epidermis and dermis, but only in adult epidermis	146
MSX2	Pro-WH	• Expressed in fetal epidermis and dermis, but only in adult epidermis	146
PRX1	Pro-Vasc.	• Knockout mice develop malformations of arteries	40
	Pro-WH	• Increased following wounding of fetal dermis (fetal wound tissue)	144
PRX2	Pro-Vasc.	• Double knockout with *PRX1* results in severe vessel abnormalities	40
Trithorax and polycomb-group genes			
EED	Anti-WH	• Complexes with EZH2 and SUZ12 to form PRC2. Downregulated in wound epidermis. • Inhibits expression of HOXA9 and HOXC8 (HeLa cells and leukemia cells)	103–105
EZH2	Anti-WH	• Complexes with EED2 and SUZ12 to form PRC2. Downregulated in wound epidermis. • Inhibits expression of HOXA9 and HOXC8 (HeLa cells and leukemia cells)	103–105
MEL18 (PCGF2)	Pro-Vasc.	• Overexpression causes EC migration and tube formation in culture. Upregulation of VEGFR2 and ANGPTL2 (ECs)	101
	Anti-Vasc.	• Another study contradicts above. Overexpression inhibits EC tube formation in culture and decreases VEGFA production (ECs)	102
MLL	Pro-Vasc.	• Upregulates HOXA9 and HOXD3 via trimethylation of lysine 4 of histone H3 (ECs)	100
SUZ12	Anti-WH	• Complexes with EED and EZH2 to form PRC2. Downregulated in wound epidermis. • Inhibits expression of HOXA9 and HOXC8 (HeLa cells and leukemia cells)	103–105
Hox Cofactors			
PBX1	Pro-Vasc.	• Complexes with HOXD3 to more efficiently elicit its angiogenic functions (ECs)	52

systemic chemotherapies, systemic Hox delivery would likely produce unwanted side effects; therefore, engineering targeted-gene delivery vehicles can reduce off-target effects, but will require identifying appropriate tumor biomarkers for targeting. Moreover, while localized delivery to large tumors may be effective in reducing primary tumor angiogenesis, additional approaches must be considered to limit further progression linked to metastases.

Homeobox gene therapy can also be leveraged in the field of regenerative medicine to induce vascularization within engineered tissue; however, in these cases it will be important to temporally regulate expression levels to effectively transition through the different phases of angiogenesis. In situations such as these, designing inducible homeobox gene expression systems, placing homeobox expression cassettes downstream of phase-specific promoters, or delivering Hox proteins rather than genes may prove beneficial.

In addition, continuing research should also investigate if and how Hox genes regulate the activity of TrxG and PcG genes/proteins and Hox-regulating miRNAs. This will help shed light on Hox cross-regulatory biology and functional dominance and allow researchers to more efficiently manipulate Hox expression for therapeutic purposes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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