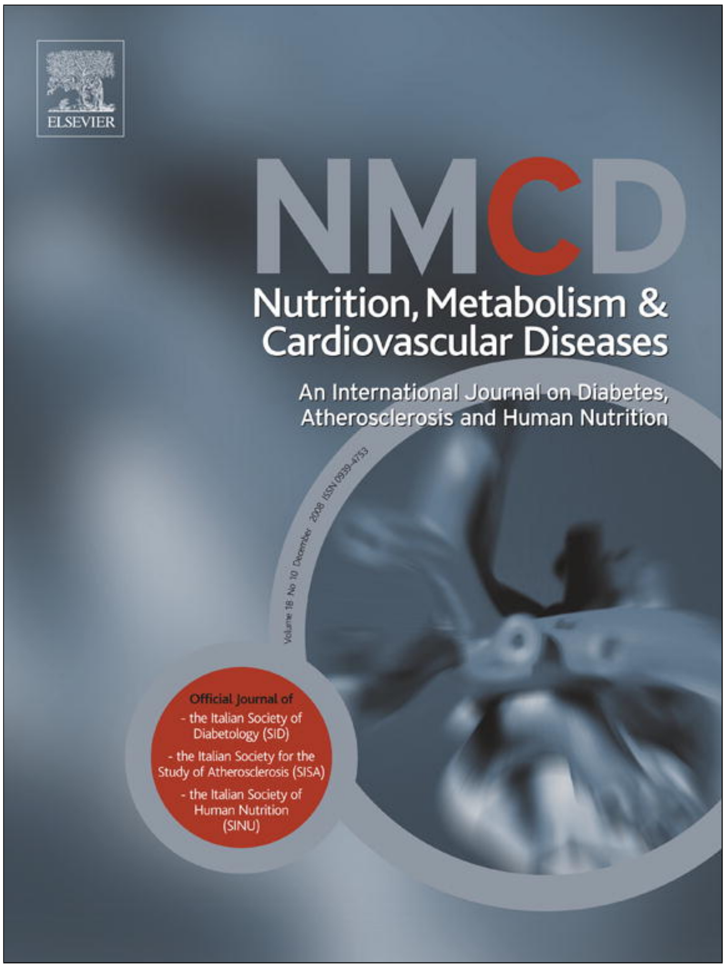
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REVIEW

Homeobox genes in normal and abnormal vasculogenesis☆

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Abstract Homeobox containing genes are a family of transcription factors regulating normal development and controlling primary cellular processes (cell identity, cell division and differentiation) recently enriched by the discovery of their interaction with miRNAs and ncRNAs. Class I human homeobox genes (*HOX* genes) are characterized by a unique genomic network organization: four compact chromosomal loci where 39 sequence corresponding genes can be aligned with each other in 13 antero—posterior paralogous groups. The cardiovascular system is the first mesoderm organ-system to be generated during embryonic development; subsequently it generates the blood and lymphatic vascular systems. Cardiovascular remodelling is involved through homeobox gene regulation and deregulation in adult physiology (menstrual cycle and wound healing) and pathology (atherosclerosis, arterial restenosis, tumour angiogenesis and lymphangiogenesis). Understanding the role played by homeobox genes in endothelial and smooth muscle cell phenotype determination will be crucial in identifying the molecular processes involved in vascular cell differentiation, as well as to support future therapeutic strategies. We report here on the current knowledge of the role played by homeobox genes in normal and abnormal vasculogenesis and postulate a common molecular mechanism accounting for the involvement of homeobox genes in the regulation of the nuclear export of specific transcripts potentially capable of generating endothelial phenotype modification involved in new vessel formation.

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Introduction

The cardiovascular system represents the first organ system to be generated during embryonic development. Hemangioblasts, the pluripotent mesodermal stem cells, generate blood islands, the peripheral part of which differentiate into endothelial cells (EC) responsible for giving rise to de novo vessel formation (vasculogenesis) [1]. The blood flow remodels this early network of capillaries (angiogenesis) [2] to stabilize the developing vessels through the recruitment of vascular smooth muscle cells (VSMC). In parallel, lymphatic unipotent EC, originated from the cardinal vein, generate the lymph sacs that give rise to lymphatic vessels [3]. These processes are consequent to proliferation, differentiation and migration events which contribute to the correct determination of the cardiovascular system.

Remodelling of the vascular system is realized during embryonic development as well as in adult life and is connected to physiological processes such as wound healing and the menstrual cycle, as well as to disease states. Pathological remodelling includes: (i) atherosclerosis where VSMC migrate from the media to the intima progressively occluding, through their proliferation, the arterial lumen and generating hypoxia or even anoxia in downstream tissues [4]; (ii) postangioplasty restenosis with neointimal lesion formation has been recently reported to require ERK1/2 down-regulation [5] and aberrant mir-21 micro-RNA expression [6]. It is worth noting that the propensity to thrombotic occlusion and atherosclerosis is different between blood vessel types used for coronary bypass, corresponding to the gene expression profile of the VSMC in the vessel type used [7]. Furthermore vascular remodelling plays a critical role in tumour angiogenesis where neoplastic cells produce pro-angiogenic factors such as VEGF and l'FGF, responsible for stimulating the growth of new blood vessels from EC cells with an immature phenotype [8]. This process will supply tumour growth through diffusion of oxygen and nutrients.

In spite of the well documented role played by growth factors and cytokines in the activation of receptors and signalling pathways in both EC and VSMC, far less is known about the function of the downstream transcription factors activated by these signalling pathways to regulate tissue-specific gene expression and growth and/or differentiation of these cell types. Transcription factors represent common targets which are regulated by the interaction of multiple signalling pathways and regulate, in turn, transcription of specific gene programmes that are crucial to perform every cell function. They belong to several gene families (winged helix, forkhead, high mobility group proteins, homeobox, zinc fingers) among which homeobox genes play a special role.

Homeobox genes are transcription factors that act during normal development [9] and contain the homeobox, a 183-bp DNA sequence coding for a 61-amino acid domain defined as the homeodomain (HD). Different HD types or classes may be identified through sequence similarities within the homeodomain [10], each characterising a homeobox gene family. Among these, the HD of the homeotic gene of Drosophila Antennapedia (Antp) defines a consensus sequence referred to as class I *HD* or *Hox* genes [11]. In mice (*Hox* genes) and humans (*HOX* genes) there are 39 genes organized into four Hox loci, each localised on a different chromosome (HOX A at 7p15.3, HOX B at 17p21.3, HOX C at 12q13.3 and HOX D at 2q31) [12] and containing from 9 to 11 genes (Fig. 1). On the basis of sequence similarity and position on the locus, corresponding genes in the four clusters can be aligned with each other into 13 paralogous groups [13]. During mammalian development, *Hox* gene expression controls the identity of various regions along the body axis according to the rules of temporal and spatial co-linearity, with 3′ *Hox* genes (retinoic acid responsive) expressed early in development and controlling anterior regions, followed by progressively more 5′ genes (FGF responsive) expressed later and controlling more posterior regions [14]. The *HOX* gene network, the most repeat-poor regions of the human genome [15], is also expressed in normal adult human organs [16]. Homeobox and *Hox* genes appear to regulate normal development, phenotype cell identity [17,18] cell differentiation [19,20] and control primary cellular processes, as proven by the description of congenital [21], somatic [22], metabolic [23] and neoplastic alterations [24,25] involving these genes. In addition to their role as transcriptional regulators, new

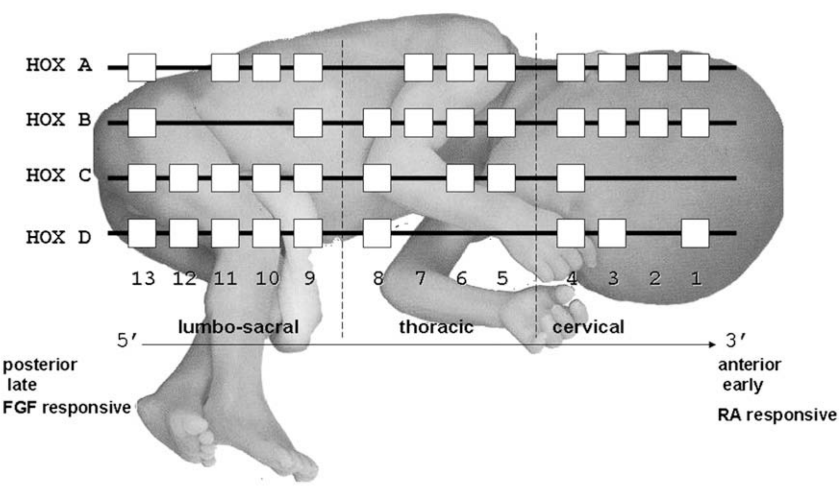

Figure 1 Schematic representation of the HOX gene network (see the text for details).

crucial functions have recently been ascribed to *HOX* genes and homeoproteins mostly related to their interaction with miRNAs and ncRNAs to guarantee transcription and translation of specific RNA transcripts [26,27].

Homeobox and *HOX* gene expression and deregulation of the cardiovascular and lymphatic systems have been reported since the nineties [28]. The increasing identification of multiple physiological roles makes these genes ideal candidates for unravelling the processes involved in cell differentiation and vascular remodelling during normal development, and whose alteration is associated with pathology (Fig. 2). We review here the current knowledge on *HOX* and Homeobox gene involvement during normal and abnormal vasculogenesis.

Hox genes

An initial report has identified Hox A5, Hox A11, Hox B1, Hox B7 and Hox C9 as able to distinguish, through their expression, foetal from adult human smooth muscle cells, thereby modulating vasculature functions [29]. Subsequently, paralogous group 3 *HOX* genes have been involved with the regulation of the angiogenic phenotype: HOX D3 is abundantly expressed in active proliferating EC forming tubes in vitro and its expression is induced by basic fibroblast growth factor (bFGF) [30]. HOX D3 overexpression further generates haemangiomas in vivo. Constitutive HOX B3 expression increases capillary morphogenesis without generating haemangiomas, whereas anti-HOX B3 anti-sense inhibits capillary morphogenesis [31]. In Hox A3$^{-/-}$ knockout mice, the arteries are shortened or absent, cardiogenesis is impaired and neural crest precursors of the carotid artery initially migrate properly but subsequently have a decreased proliferation rate [32]. Taken together, these observations suggest overlapping and complementary roles performed by paralogous genes *Hox A3*, *Hox B3* and *Hox D3* in angiogenesis.

Recently, resulting phenotype of a truncating mutation in HOX A1 has been reported [33] manifesting vascular malformations of the internal carotid arteries and cardiac outflow, besides mental retardation, autism spectrum disorder, facial weakness and hypoventilation. Increased Hox A5 expression is able to block angiogenesis in vivo and cell migration in vitro [34]. Hox A5 is further active in quiescent EC and becomes silent after EC activation by angiogenic stimuli. Interaction between Hox A5 and vascular endothelial growth factor receptor-2 (VEGFR-2) could account for the anti-angiogenic affect [35].

HOX A9 has been involved with endothelial stem cell determination. Hox A9 overexpression increases pro-angiogenic *EphB4* gene expression [36]. Hox A9 null mice display a decreased response to angiogenic stimuli and a low number of EC precursors. Hox A9 is able to bind to endothelial nitric oxide synthase (eNOS) and VEGFR-2 promoters [37]. HOX A9 acts as a proinflammatory factor by mediating cytokine induction of E-selectin [38] and is down-regulated by NF-kB [39]. Thus, Hox A9 appears as a regulator of angiogenesis.

Hox A13 is involved in extra embryonic vascularization, since in Hox A13$^{-/-}$ mice the EC layer of umbilical arteries improperly form resulting in embryonic lethality. Hox A13 interacts with EphA7 and EphA4 in EC as the expression of both Ephrin receptors is decreased in the umbilical arteries of Hox A13 knockout mice [40].

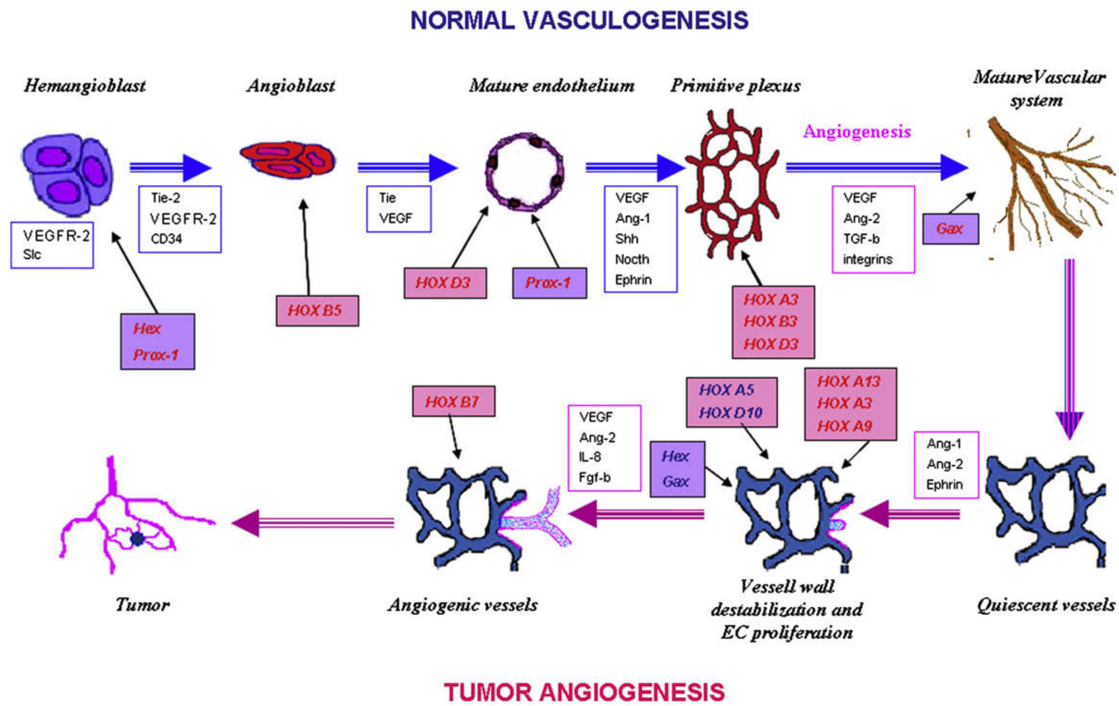

Figure 2 Homeobox and *Hox* genes in normal and abnormal vasculogenesis. Normal vasculogenesis along embryonic development is described at the top. The bottom represents tumour angiogenesis during adulthood. Inside the coloured boxes the homeobox and *Hox* genes and the stage of their involvement in these processes are reported. The genes are indicated in red or blue when acting as proangiogenesis or anti-angiogenic, respectively.

Human umbilical vein endothelial cells (HUVEC) express 8 out of 10 HOX B locus genes suggesting their involvement in the generation of new blood vessels [41]. HOX B2 induces dose-dependent inhibition of in vitro HUVEC proliferation [42]. Hox B5 transactivate the promoter of flk1 (VEGFR), the earliest marker of endothelial precursors. Hox B5 mRNA colocalises with flk1 expression in differentiating embryoid bodies (HBE) and activates the cell-intrinsic events that regulate the differentiation of angioblasts and mature endothelial cells from their mesoderm-derived precursors [43]. Hox B7 is able to transactivate pro-angiogenic factors such as bFGF, VEGF, CXCL1, IL-8, Angiopoietin-2 and MMP-9 [44]. HOX B7 mRNA further appears to be highly expressed in human atherosclerotic plaques than in normal human arterial media [45]. Finally, testing the expression of the whole *HOX* gene network, Chung et al. reported the up regulation of HOX A7 and HOX B3 and the down-regulation of HOX A3 and HOX B13 during endothelial cell differentiation of human bone marrow-derived mesenchymal stem cells [46].

Concerning the HOX C locus, the anti-sense targeting of Hox C5 together with its paralogous genes *Hox A5* and *Hox B5* causes the appearance of an additional pharyngeal arch containing a novel and completely independent aortic arch artery with normal cardiac outflow. Thus, abnormal aortic arch patterning does not necessarily lead to cardiac malformations [47]. HOX C9 is actively expressed both in human smooth muscle cells and the cardiovascular system during embryogenesis [29].

Hox D3, besides the involvement of the whole paralogous group 3 *HOX* genes, appears to be one of the *HOX* genes most involved with angiogenesis. In response to wounding, Hox D3 expression increases in EC inducing the production of collagen A1 and β_3 integrin to improve wound repair [48]. Ectopic Hox D3 expression in the mouse brain induces increased levels of angiogenesis and cerebral blood flow [49]. Basic FGF increases in EC and Hox D3 expression which up-regulates the production of $\alpha v\beta_3$ integrin and urokinase-type plasminogen activator (uPA) necessary for EC adhesion, migration and invasion [30]. Hox D3 is also able to increase transcription of integrin $\alpha v\beta_1$ in EC [50].

Hox D10 appears to be active in resting EC and displays an anti-angiogenic effect blocking angiogenesis and migration both in the endothelium [51] as well as in human breast cancer cells, probably through down-regulation of α_3 integrin expression [52].

Hex (Hhex)

The orphan homeobox gene *Hex* (Haematopoietically expressed homeobox) also termed PRH (Proline Rich Homeodomain) acts as both a transcriptional repressor and activator, and is located on the human chromosome 10 [53]. Hex mRNA and protein are expressed in the developing extra embryonic mesodermal blood islands (where vascular and haematopoietic stem cells are located) immediately after VEGFR-2. Hex is thus required for the initial phases of endothelial differentiation and all along haematopoietic differentiation. Hex is also active in VSMC inducing genes such as plasminogen activator inhibitor 1 (PAI-1), nitric oxide synthase 2A (iNOS), platelet-derived growth factor alpha (PDGF-a), VEGFR-1 and VEGFR-2 [54]. Hex homozygous disruption induces embryonic death in mice at mid gestation due to severe defects in forebrain, thyroid and liver development [55] as well as in B-cell development, vasculogenesis and cardiac morphogenesis [56]. Hex regulate the promoter of Na^+ taurocholate cotransporting polypeptide (ntcp), acts as a transcriptional repressor of the liver, haematopoietic and HUVEC cells and, interacting with GATA transcription factors, reduces the expression of VEGFR-1 and VEGFR-2 in differentiated EC. It has recently been reported that during liver development transition of the hepatic endoderm to a pseudostratified epithelium is dependent upon the activity of Hex to regulate ''interkinetic nuclear migration'' during cell division [57]. Only recently a direct in vivo Hex target has been identified as the endothelial cell-specific molecule 1 (ESM-1) or endocan [58]. ESM-1 protein is primarily expressed in the vascular endothelial cells of the lung, kidney and gut and is induced by VEGF and up-regulated in the endothelial lining of tumour microvessels. A strong correlation has been reported between ESM-1 expression and the degree of tumour vascularity [59]. Hex inhibits EC vessel formation in vitro through the limitation of VEGFR-2 expression and $Hex^{-/-}$ EC display a growth advantage over wild type cells [60]. Although the role of Hex in EC and VSMC differentiation as well as in vasculogenesis and angiogenesis appears to be crucial, it is clearly more complex than suspected. We will postulate at the end a common mechanism accounting for a potential unifying role of *HOX* and *Hex* genes in vasculogenesis and angiogenesis.

Prox1

Prox1 starts to be expressed in the primitive lymph sacs originating from endothelial budding of the cardinal vein to give rise to the lymphatic system. Prox1, homologous to the gene prospero of *Drosophila*, has been connected to the development of the lymphatic system. $Prox1^{-/-}$ mouse embryos do not develop a lymphatic system but undergo normal vasculogenesis and angiogenesis. Other tissues such as the eye lens, central nervous system and liver are compromised leading to embryonic lethality [3]. Prox1 expression in vascular EC induce proliferation (prox1 up-regulate cyclin E1 and E2 expression) as well as expression of lymphatic genes such as VEGFR-3 and desmoplakin I/II [61] and repression of vascular EC genes such as *STAT 6* and *neuropilin I*. A direct target of Prox1 is fibroblast growth factor receptor 3 (FGFR3), required for lymphatic EC proliferation. The *Drosophila* prospero gene is involved in asymmetric cell divisions generating different cell types during embryonic development [62]. Thus, Prox1 plays a role in the control of cell proliferation and in the regulation of the developing lymphatic system.

Gax

Gax (growth arrest-specific homeobox) is a Mesenchyme homeobox 2 (Meox2) transcription factor involved in vascular phenotype determination. It is expressed, during embryonic development, in several tissues and organs including all the three muscle lineages and brain [63]. In adult life, Gax expression concerns the cardiovascular tissues, smooth muscle cells of arteries and lung and mesengial cells of the kidney. In VSMC, Gax is down-regulated by mitogenic stimuli (PDGF, angiotensin II) and up-regulated by growth arrest signals (serum deprivation). Gax negatively regulates the cell cycle in VSMC by up-regulating p21, a cyclin dependent kinase 2 (cdk2) inhibitor [64]. Recently miR-130a has been reported as able to regulate the angiogenic phenotype of vascular endothelial cells modulating the expression of GAX and HOX A5 [65]. Gax is also able to control VSMC migration toward chemotactic growth factors suppressing the β_3 and β_5 subunit of integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [66]. Thus, Gax inhibits the angiogenic phenotype, probably through an NF-KappaB-dependent mechanism [67].

Prx

Upregulation of the paired-related homeobox genes *Prx1* and *Prx2* is detectable in rat pulmonary arteries after pulmonary hypertension, and induces transactivation of Tenascin-C which promotes proliferation of cultured VSMC [68]. Prx1 knockout mice manifest malformations of the aorta and the ductus arteriosus; Prx1/Prx2 double knockout mice display more severe malformations supporting an additive and compensatory role for these two genes [69]. Prx1 and Prx2 regulate embryonic VSCM proliferation as well as VSCM de-differentiation occurring in vascular diseases.

eIF4E, nuclear export and angiogenesis

Eucaryotic translation initiation factor (eIF4E) is actively expressed in human cancers, promoting tumour growth and angiogenesis. Elevated eIF4E levels selectively increase translation of growth factors in malignancy (e.g., VEGF, cyclin D1) and represent potential anticancer therapeutic targets [70]. Recently eIF4E has been found to migrate into the nucleus generating multi-protein nuclear structures (eIF4E nuclear bodies) [71]. eIF4E nuclear bodies promote the selective transport of specific mRNAs, such as cyclin D1 and ornitine decarboxylase (ODC), from nucleus to cytoplasm [72]. In mammalian cells eIF4E nuclear bodies are inhibited by Hex and PML (promyelocytic leukaemia protein), whereas they are modulated by the interaction with HOX A9 through a binding site on the HOX A9 homeoprotein corresponding to the sequence YVDSFLL [73]. The property of HOX A9 to act on RNA export is independent of its role as a transcriptional regulator. Several homeoproteins display consensus sequences to act as eIF4E binding proteins (4EBPs) corresponding to YXXXXLF (where X is any amino acid and F is any hydrophobic amino acid).

It has recently been reported that PML is a critical regulator of angiogenesis in ischaemia and tumours through the inhibition of hypoxia-inducible factor 1 alpha (HIF-1a) translation [74]. We have previously described the role of the homeobox gene *Hex* and *HOX A9* in vasculogenesis and angiogenesis. Thus all the homeoproteins involved in the interaction with eIF4E nuclear bodies in the process of nuclear export of selected transcripts appear to be involved with vasculogenesis and angiogenesis. Alteration in the regulation of nuclear export may well be an upstream event generating endothelial phenotype modification involved in new vessel formation. The reported statistical analysis describing the consensus sequence YXXXXLF as present in 200 out of 800 homeoproteins suggests an increasingly important role for the homeobox genes in normal and abnormal vasculogenesis [73].

Conclusions

Despite homeobox genes being identified 30 years ago, it still appears difficult to define their functions. This is not only due to the lack of effector gene identification but also to the complex and not completely elucidated role that homeobox genes play in physiology and pathology.

From a genetic viewpoint, the *Hox* gene network as a whole represents a complex system responsible for realising specific gene programmes, selected through the cell memory gene mechanisms, to establish phenotype cell identity and cell—cell interactions and to regulate tissue and organ formation whose spatio—temporal organization fits the formal arrangement of the network (Fig. 1). At a molecular level, the gene programmes are realized by the *Hox* genes acting as transcription factors through the coordinate regulation of downstream gene batteries. The biological functions performed by the effector genes span from cell cycle to apoptosis, from the interaction with growth factors to the production of adhesion molecules and to the involvement with cell signalling. Obviously all this cannot be due to a single *Hox* gene but to the coordinate combination of the 39 genes of the network and of their interactions with isolated and divergent homeobox genes in the genome. These processes are consequent to the interactions between *Hox* genes and miRNAs and ncRNAs, crucial players in the regulation of gene expression localised, in some cases, even inside the Hox network.

The *Hox* genes as transcriptional regulators, although crucially involved with gene programme realization, are limited by the generation of mRNAs with an uncertain fate due the complexity of the subsequent processes such as nuclear export and protein synthesis.

In order to secure the correct realization of the gene programmes selected as transcription factors, the Hox proteins (homeoproteins) perform the recently identified different role of interaction with nuclear bodies. Nuclear bodies are protein clumps interacting inside the cell nucleus with specific mRNA sequences (see Hox and eIF4E) involved with RNA maturation and nuclear export. These processes are characterized by the interaction between homeoproteins and nucleoporins, proteins of the nuclear pore able to allow mRNAs to cross the nuclear membrane to reach cytoplasm for protein synthesis. Alteration of these processes is leukaemogenic due to the generation of chimerical proteins between several *HOX* genes and the nucleoporin NUP98 [75]. The interaction between eIF4E nuclear bodies and homeoproteins represents a further potential level of involvement of homeoprotein with translation. Thus, from a molecular viewpoint, *Hox* genes play a crucial role in the regulation of transcription, nuclear export and potentially in the initial steps of protein synthesis.

The general properties of *Hox* and homeobox genes we have described are perfectly superimposable to the role of these genes along vasculogenesis. The expression of the whole *Hox* gene network during EC differentiation concerns the majority of the 39 genes in the network and varies in different body districts according to the spatio temporal rules. The role of homeobox and *Hox* genes acting as a transcriptional regulator, as well as a selector of angiogenic programmes, is supported by several observations mostly related to the interaction with growth factors (Hex and VEGFR-1, Hex and VEGFR-2, Hox A5 and VEGFR-2, Hox B5 and flk1) and with integrins (Hox D3 and avb1, Hox D10 and a3).

The involvement of miRNA in the transcription of angiogenic programmes is proven by the recent discovery of the regulation of angiogenesis through miR-130a that down regulates anti-angiogenic homeobox genes *GAX* and *HOX A5* [65].

The interaction with nuclear bodies is supported by the possibility of homeoproteins to interact with eIF4E in the generation of eIF4E nuclear bodies (this represents the function performed by 68% of eIF4E proteins produced inside a cell). These processes are strictly connected to cell—cycle and angiogenesis. Hyper expression of eIF4E is related to VEGF hyper expression and induces an increase in vasculogenesis.

The interaction between Hox and nucleoporins involved in the nuclear export play an important role with respect to vasculogenesis: it has recently been shown that the fusion protein between Hox A9 and NUP98 increases the expression of PIM-1, a gene able to induce EC migration and vessel reconstruction [76].

Finally, from a practical viewpoint, using small interfering RNA (siRNA) to inhibit genes in vitro and in vivo has improved studies on the mechanism of action of angiogenic genes. The capability of using RNA in vivo to validate angiogenesis factors as drug targets is uniquely important because its pathological impact can only be characterized accurately in animal disease models. siRNA protect mice from fulminant hepatitis [77], viral infection, sepsis and tumour growth. With the emergence of clinically viable delivery vehicles, anti-angiogenesis RNAi agents appear to have a promising and unprecedented role for the treatment of many serious human diseases that result from excessive angiogenesis. Using this systemic delivery of siRNA targeting VEGF pathway factors at sites of neovascularization, anti-angiogenic efficacy has been achieved in a neuroblastoma tumour model [78].

HOX and siRNA interference have only recently, in the last few years, been coupled following the discovery of siRNA functionality in mammalian cells. Deregulation of HOX A9 by RNA interference decreases cell migration and tube formation of endothelial human cells, suggesting that HOX A9 plays a role in endothelial cell migration and may exert its function by regulating the expression of EphB4 [36].

A deeper understanding of the homeobox gene involvement in the regulation of vascular remodelling and angiogenesis will certainly improve the development of therapeutic strategies to contrast tumour angiogenesis, atherosclerosis, restenosis after angioplasty, wound healing and lymphoedema, taking into account that paralogous group *Hox* genes (such as the group 3 *Hox* genes in the cardiovascular system) often interact in performing additive functions to generate the redundancy of the Hox system.

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