Editorial

Achieving tissue specific levels of angiogenesis: Not(ch) a big deal!

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Our understanding of the molecular mechanisms regulating endothelial cell behavior in bone *vs.* other organs has been challenging to date given the inaccessibility of blood vessels deep within the bone mineral and the difficulties associated with bone endothelial cell isolation. A recent study published by Ramasamy *et al.* (1), combined postnatal modification of endothelial cell specific Notch signals with improved immunohistochemical approaches and bone endothelial cell isolation, to highlight for the first time the presence of clear bone endothelial cell heterogeneity.

To date, the Notch pathway has been shown to be a highly conserved mechanism of cell fate determination and differentiation, regulating a wide variety of developmental processes. Notch signaling involves interactions between adjacent ligand-and receptor-expressing cells and allows for stimulation of different processes in neighboring cells. The role of Notch signaling in blood vessel sprouting provides an example of how Notch signals can determine cell fate, and was first described in the study of angiogenesis of the mouse retina (2). The process of angiogenesis or blood vessel sprouting from pre-existing vessels involves the continued interaction between "tip" and "stalk" cells and Notch signaling has been shown to be central to the formation of these distinct endothelial cell identities. Vascular endothelial growth factor (VEGF) binding to VEGF receptor 2 (VEGFR2) causes an up-regulation of the Notch ligand Delta-like 4 (Dll4) which interacts with Notch1 receptors on adjacent endothelial cells. This leads to the down-regulation of VEGFR2 in those cells, whereby only one leading tip cell expresses high VEGFR2 levels. Therefore, the Notch signaling pathway can limit the angiogenic behavior of endothelial cells within developing blood vessel sprouts, in part through the modulation of the VEGF signaling pathway. This suppression of tip-cell features in stalk cells by Dll4/Notch and lateral inhibition of VEGFR2 has similarly been described during development and in tumor growth (3,4).

Bone is highly vascularized and the blood supply is critical during periods of rapid growth, remodeling, and repair. Extensive published data demonstrates the depth and complexity of the reciprocal co-dependency which exists between the vascular and skeletal tissues in which each tissue provides morphogenetic signals or environmental cues crucial for the other's development. To effectively couple bone formation with angiogenesis, bone forming osteoblast cells produce pro-angiogenic factors such as VEGF in response to a range of factors including mechanical strain, hypoxia, estrogen and prostaglandins [reviewed in (5)]; and conditional loss of early osteoblast VEGF expression *in vivo* results in reduced endothelial cell number in bone and an osteoporotic phenotype of reduced bone mass and increased marrow fat (6). Given the important roles for VEGF in bone and the involvement of Notch in the negative regulation of VEGF signaling in other tissues Ramasamy and colleagues (1) investigated the function of Notch signaling in coupling angiogenesis with osteogenesis.

The study describes the influence of *in vivo* endothelial cell specific deletion of Rbpj, which encodes an essential mediator of Notch-induced gene transcription. In contrast to the positive effects of Notch deletion on retinal angiogenesis, these mutant animals showed a decrease in the total number of proliferating endothelial cells in bone with the patterning of the vessels disrupted and filopodia from mutant vessels appearing disorganized. This defective angiogenesis was accompanied with a bone phenotype including shortened long bones, disorganization of the metaphysis with large irregular lacunae, and a lack of clearly

separated trabeculae. Micro-computed tomography analyses also showed significant loss in bone mass, but there were no differences in osteoclast numbers. In the growth plate hypertrophic zones were enlarged and VEGF expression by mature chondrocytes was reduced.

Mesenchymal Notch expression has been shown to promote proliferation and suppress osteoblast differentiation (7,8) and to test whether Dll4 may signal through osteoblasts Rbpj was also deleted in Collagen 1 expressing cells and the same experimental schedule undertaken, with no apparent defects in either metaphyseal vasculature or osteoprogenitor cells present in these mutant animals. Therefore, Notch signaling effects appear to be endothelial cell specific and cell autonomous, coupling angiogenesis to osteogenesis. To investigate the factors acting on osteoblasts via Notch, endothelial cells were isolated from bones of Notch gain of function mutant mice and control animals with a candidate approach utilized to identify gene expression changes. Noggin, a secreted antagonist of bone morphogenic proteins (BMPs) was found to be 40-fold higher in the gain of function mutant mice than in controls. Osteoblast specific Noggin mutants developed a skeletal phenotype similar to Rbjb endothelial cell knock outs, and daily injection of recombinant Noggin to Rbjb mice improved bone formation and restored metaphysis organization.

These results have demonstrated for the first time, the presence of a distinct population of endothelial cells which exist and function within the bone microenvironment and utilize Dll4/Notch signaling in a positive manner. In addition to the underlying mechanistic differences described in bone endothelial behavior a unique phenotype of the blood vessels in bone was also shown. The metaphyseal vessels in the long bones were found to point towards the chondrocytes of the growth plate (which produce VEGF), with columns linked by tubular arches with blind-ended, bulb shape protrusions. These arch vessels did have extended filopodia pointing towards the growth plate but perfusion experiments showed that they were fully lumenized. Therefore, the leading endothelial cell structures in bone were dissimilar to the angiogenic sprouts present in tissues which utilize Dll4/Notch-mediated lateral inhibition of VEGFR2 signaling.

The utilization of negative or positive regulation of angiogenesis by Dll4/Notch in different tissues is intriguing and may be explained in part by environmental influences controlling the expression and availability of tissue derived factors. We know from tissues where spatial interactions with vasculature are clearly defined, that Dll4/Notch signaling is stimulated in the first instance by tissue-derived VEGF and its cellular actions will depend on matrix binding and the spatial distribution and extracellular gradients of VEGF, which acts as a chemoattractant to direct capillary growth (9,10).

Whether distinct VEGF gradients are utilized within the bone microenvironment remains unclear but the production and localization of bone-derived VEGF could contribute to the differences in bone endothelial cell phenotype and Dll4/Notch signaling described by Ramasamy and colleagues (1). Osteoblasts are known to be an important source of VEGF which can exert paracrine, autocrine (11) and intracrine (6) effects on both endothelial and bone cell behavior, coupling angiogenesis to bone remodeling. Bones mechanical responsiveness drives the remodeling process and has been shown to regulate blood vessel growth by the production of VEGF and regulation of VEGFR2 signaling (12) in osteoblasts. Interestingly, altered mechanical signals in bone can modulate alternative splicing of VEGF mRNA in osteoblasts which results in the production of VEGF isoforms which together can form VEGF gradients. Expression of short freely diffusible VEGF isoforms 121,165 for example, have been shown to be expressed under low mechanical frequency while matrix-bound larger VEGF isoforms 206,189,165,145 are expressed under high mechanical frequency in human osteoblasts (13). The spatial distribution of VEGF has been shown to regulate the cell shape of sprouting vessels in mouse retina (9). Whether a VEGF gradient established in response to mechanical influences and remodeling requirements does exist in bone and can influence the morphology and Notch signaling of blood vessels is an interesting question for future interrogation.

Reduced VEGF has been linked to osteoporosis (6,14,15) and irregular vascular supply to bone is associated with age and other bone pathologies. Changes in bone endothelial cell expression of DII4/Notch/Noggin and their control of osteogenesis during disease, aging or fracture repair provide a novel and exciting area for further research. Identification of unique endothelial cell phenotypes and signaling responses which can influence osteogenesis could now bring the vasculature to the forefront as an innovative means to therapeutically promote bone anabolism.

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None.

Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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