BMP-SMAD1/5 Signaling Is Required for Adequate Coupling of Angiogenesis and Osteogenesis in Long Bones

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INTRODUCTION: The bone vasculature is essential for skeletal development, homeostasis, and regeneration. In long bones, specific capillary subtypes couple angiogenesis to osteogenesis, especially at the growth plate (metaphysis) and endosteum. These metaphyseal and endosteal vessels, termed "type H," express high levels of CD31 and endomucin ($CD31^{hi}Emcn^{hi}$), show a columnar structure and are closely related to Osterix (Osx)- and Runt-related transcription factor 2 (Runx2)-expressing osteoblasts and their progenitors [1]. Sinusoidal vessels with low expression of CD31 and Emcn ($CD31^{low}Emcn^{low}$, type L vessels) are mainly found in the diaphysis/bone marrow cavity [1]. The crosstalk between endothelial cells (ECs) of type H vessels and bone-related cells is characterized by osteogenesis-promoting factors provided by ECs and angiogenic mediators secreted by osteoblast-lineage cells. Bone morphogenetic proteins (BMP) are major regulators of vessel formation, however their role within angiogenic and osteogenic coupling and in particular, the involvement of BMP-related intracellular effectors SMAD1 and SMAD5 (SMAD1/5) is unclear. Previously, we demonstrated that during mouse embryonic development, the synergy of Notch and SMAD1/5 is responsible for the balanced selection of tip and stalk cells in vascular sprouting [2]. Furthermore, we reported that endothelium-specific deletion of SMAD1/5 during early postnatal retinal angiogenesis resulted in arteriovenous malformations, a reduced number of tip cells and a hyperdensity in the vascular plexus [3]. Therefore, we hypothesized that functional EC-specific SMAD1/5 signaling is required for (i) formation and maturation of long bone vasculature and (ii) coupling of osteogenesis and angiogenesis, specifically of type H vessel formation in the metaphysis.
METHODS: Cdh5-CreERT2$^{tg/0}$;Smad1$^{fl/fl}$;Smad5$^{fl/fl}$ (dKO^{iEC}, [3]) mice were injected intraperitoneally with tamoxifen (500 µg) at post-natal day 21 (P21), and sacrificed at P28 or P35. All experiments were conducted using littermate Cre-negative controls. All animal procedures were approved by the Ethical Committee of KU Leuven (P039/2017). For contrast-enhanced microCT (CE-CT) analysis, right tibias were collected and stained for 1 week with a Hafnium-substituted Wells-Dawson polyoxometalate (POM) solution (35 mg/ml PBS) at 4°C under constant shaking to visualize and quantify the 3D bone and vascular architecture [4]. A GE NanoTom M was used for scanning at 60 kV and 140 µA, with a 0.2 mm filter of aluminum and a voxel size of 2 µm. During reconstruction (Datosjx, GE Measurement and Control Solutions), a beam hardening correction of 7 and a Gaussian filter of 6 was used and detailed analysis were performed using CTAn and DataViewer (Bruker MicroCT). 300 slices were analyzed both in the metaphysis and diaphysis. For immunofluorescence, femurs and tibias were cryo-embedded and stained using the following primary antibodies: pSMAD1/5 (Cell signaling; 1:100); CD31/PECAM-1 (R&D Systems, 1:100), Emcn (Santa Cruz, 1:100), COLX (Abcam; 1:100). Images were taken with a Keyence microscope (BZ 9000) or a Zeiss LSM880. For RNA analysis, separation of the metaphysis and diaphysis was done by cutting distal from the growth plate in the intact tibia. Afterwards samples were cryo-pulverized and purified with Trizol. RNA was isolated with the RNeasy Mini Kit (Qiagen). cDNA synthesis was performed using the TaqMan Reverse Transcription Reagents Kit followed by qPCR (SYBR green). Ct values were normalized to *Hprt* (Housekeeper); as second control *18s rRNA* was used. GraphPad Prism V.8 was used for statistical analysis applying the non-parametric, unpaired Mann-Whitney-U test. Sample sizes are indicated in the figure legends. Data are displayed with error bars showing mean ± SD or SEM (qPCR).
RESULTS: We observed fewer phosphoSMAD1/5 positive ECs in in the bone marrow of dKO^{iEC} mice compared to control mice (**Fig. 1a**). EC-specific deletion of SMAD1/5 significantly increased vessel volume and size in the diaphysis (primary spongiosa), with a mean vessel diameter nearly twice as large in knockout mice compared to controls (**Fig. 1b,c**). In-depth analysis of vascular diameter distribution indicated a shift towards larger vessel with only a slight reduction in the frequency of smaller vessels in dKO^{iEC} mice (data not shown). The relative bone volume was similar between wild type and knockout mice in the metaphysis, but SMAD1/5 deletion significantly reduced the bone volume in the diaphysis (**Fig. 1d**). Detailed analysis of type H vessels in the metaphysis demonstrated that SMAD1/5 significantly decreased arching vessel density and disrupted the columnar structure (**Fig. 1 e,f**). Since we also observed a modest increase in growth plate thickness at 7 days after induced deletion (data not shown), we analyzed the growth plate after 14 days (P35). EC-specific SMAD1/5 deletion significantly enlarged the hypertrophic zone of the growth plate and disrupted the organization of the adjacent cartilage-degrading capillaries (**Fig. 1g**). EC-specific SMAD1/5 deletion significantly downregulated markers of osteogenic signaling, *Sp7* and *Sost* in the metaphyseal/epiphyseal area (**Fig. 1h**).
DISCUSSION: Previous studies show that the mechanisms that underlie angiogenic and osteogenic coupling during limb development are regulated by multiple pathways, including hypoxia inducible factor (HIF-1) and Notch signaling [5,6]. Here, we identify BMP-related SMAD1/5 signaling in endothelial cells as a critical mediator of osteogenic-angiogenic coupling. SMAD1/5 deletion from ECs caused a striking change in the shape of the long bone vasculature, disturbed type H vessel formation in the proximal metaphysis, and resulted in growth plate enlargement, through a disruption of cartilage-degrading capillary organization and function. Finally, we found that the relative bone volume and bone-related marker expression in the metaphysis were significantly reduced by EC-specific SMAD1/5 deletion, demonstrating a disruption of the angiogenic-to-osteogenic coupling.
SIGNIFICANCE/CLINICAL RELEVANCE: Our findings provides evidence that endothelial-specific SMAD1/5 activity is required for adequate formation and maturation of long bone vasculature as well as coupling of angiogenesis and osteogenesis.
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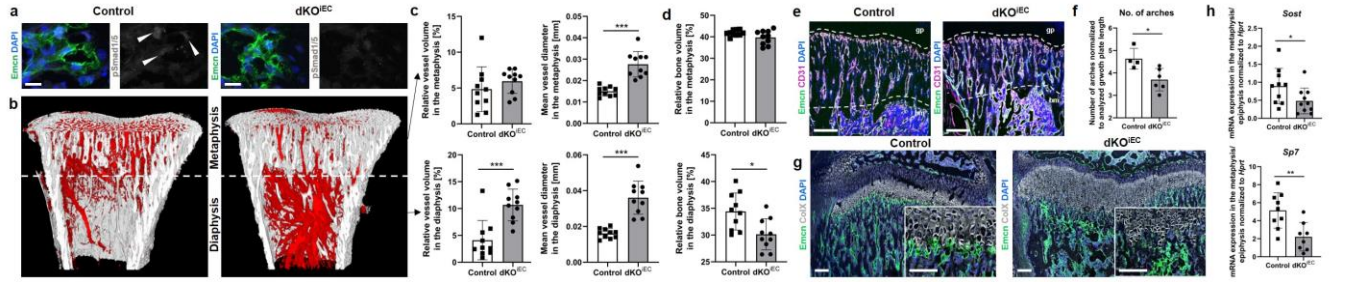

Figure 1: Investigating the role of endothelial SMAD1/5 activity in the long bone vasculature. (a) Vessels within the bone marrow of EC-specific SMAD1/5 deficient mice (dKO^{iEC}) show reduced phosphoSMAD1/5 positive ECs. Scale bar 20 µm. (b) CE-CT based 3D rendering (P28). white = bone; red = vessels. (c,d) Quantitative CE-CT analysis (P28; n=10) of (c) vessels and (d) bone in the meta- or diaphysis. Bar graphs show mean ± SD and individual data points. (e) Type H vessel staining (CD31 Emcn) in the metaphyseal area and (f) quantification of arches number (P28; n= 4-6). Scale bar 200 µm. Bar graphs show mean ± SD and individual data points. (g) Col X and Emcn staining of the metaphyseal area (P35; n= 3). Scale bar 200 µm. (h) mRNA expression in the metaphyseal/epiphyseal area of *Sost* and *Sp7* normalized to *Hprt* (P28; n= 8-10). Bar graphs show mean ± SEM and individual data points. p-values are indicated with *p < 0.05; **p < 0.01; ***p < 0.001.