Cell–matrix signals specify bone endothelial cells during developmental osteogenesis

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Blood vessels in the mammalian skeletal system control bone formation and support haematopoiesis by generating local niche environments. While a specialized capillary subtype, termed type H, has been recently shown to couple angiogenesis and osteogenesis in adolescent, adult and ageing mice, little is known about the formation of specific endothelial cell populations during early developmental endochondral bone formation. Here, we report that embryonic and early postnatal long bone contains a specialized endothelial cell subtype, termed type E, which strongly supports osteoblast lineage cells and later gives rise to other endothelial cell subpopulations. The differentiation and functional properties of bone endothelial cells require cell–matrix signalling interactions. Loss of endothelial integrin β1 leads to endothelial cell differentiation defects and impaired postnatal bone growth, which, in part, phenocopied by endothelial cell-specific laminin α5 mutants. Our work outlines fundamental principles of vessel formation and endothelial cell differentiation in the developing skeletal system.

The skeletal system grows rapidly in embryonic and postnatal life, which requires tightly coordinated cell proliferation, differentiation and mineralization processes together with a substantial expansion of the local vasculature. Chondrocytes and bone-forming osteoblasts release vascular endothelial growth factor (VEGF) and stimulate angiogenesis through the activation of VEGF receptors in endothelial cells (ECs). Likewise, bone repair involves angiogenesis and osteoblast precursors enter fracture lesions along with invading blood vessels. In addition to their essential transport function, vascular ECs release paracrine-acting signalling factors that control growth and regeneration in various organs. In the skeletal system, osteogenesis has been associated with a specific capillary EC subtype, termed type H, which shows high expression of the markers CD31/PECAM1 and endomucin (CD31hi Emcnhi) and is found in the metaphysis and endostem of postnatal long bone. In addition to mediating angiogenic growth, type H ECs provide molecular signals acting on osteoprogenitor cells and thereby couple angiogenesis and osteogenesis. By contrast, type L (CD31lo Emcnlo) ECs, characterized by relatively low CD31 and Emcn expression, form the bone marrow sinusoidal vessel network and are not associated with osteoprogenitors expressing the transcription factor osterix (Osx). Interestingly, impairment of the function of bone-degrading osteoclasts by cathepsin K (CTSK) inhibitors, a treatment that might help to prevent bone loss in osteoporosis and other disease conditions, led to an increase of CD31hi Emcnhi capillaries in mice, arguing that type H vessels might have therapeutic relevance.

Extracellular matrix (ECM) molecules promote mineralization and regulate the behaviour of osteoblasts and of bone-degrading osteoclasts. Cell–matrix interactions are frequently mediated by integrin receptors, composed of α and β subunits, which can bind a wide range of ECM proteins but also soluble factors and cell surface proteins. Integrin β1, a subunit that can partner with 12 different α chains, is an important regulator of EC function. EC-specific inactivation of Itgb1, the gene encoding β1 integrin, is incompatible with embryonic angiogenesis and survival beyond midgestation. Inducible genetic strategies have shown that integrin β1 controls arteriolar lumen formation as well as postnatal vessel growth and endothelial barrier function.

While these previous studies have focused on well-established models of embryonic and postnatal angiogenesis, insight into endothelial cell–matrix interactions in the developing skeletal system is lacking. In addition, it is not known how different EC subtypes are

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Received 6 November 2015; accepted 18 January 2017; published online 20 February 2017; DOI: 10.1038/ncb3476
specification during bone morphogenesis. By combining high-resolution confocal imaging, RNA sequencing and mouse genetics, we provide insight into these important questions and outline fundamental principles of vessel growth and EC specialization.

RESULTS
Developmental vascularization of long bone

As reported previously, vascularization of murine femur is initiated at embryonic day (E) 14.5. In a process resembling sprouting angiogenesis (Fig. 1a and Supplementary Fig. 1a), blood vessels entered the initially avascular cartilage containing hypertrophic chondrocytes and by E15.5 the primary ossification centre contained a highly branched vessel plexus (Fig. 1a and Supplementary Fig. 1a). Later, long bones expanded both longitudinally and radially, which was accompanied by expansion of the local vasculature (Fig. 1b). From postnatal day (P) 6 onwards, morphologically distinct capillary populations became visible in the metaphysis and diaphysis (Fig. 1b and Supplementary Fig. 1b). Metaphyseal capillaries displayed a column-like structure, while the diaphysis contained a dense sinusoidal network surrounded by haematopoietic cells (Fig. 1c and...
Supplementary Fig. 1b). Metaphyseal and diaphyseal capillary beds were interconnected by a transition zone, which contained sprouts and thin endothelial connections indicative of vascular remodelling (Supplementary Fig. 1b). As previously shown for 4-week-old long bone,13 metaphyseal but not diaphyseal capillary ECs showed high expression of CD31 and Emcn at P0, P6 and P14 (Fig. 1c).
Previous work has indicated that type H EC abundance declines during adult life\(^{13}\). Flow cytometry analysis of long bone showed that the Emcn\(^{hi}\) CD31\(^{lo}\) EC fraction was highest around birth and gradually decreased during the first 4 weeks postnatally (Fig. 1d). During the same period, Oxs\(^{+}\) cells expanded rapidly indicating bone growth (Fig. 1e and Supplementary Fig. 1c).

**Characterization of a third bone EC subpopulation**

Flow cytometry analyses using the same settings as established for P21 (Fig. 1d) revealed the existence of a third, previously not described subpopulation of bone ECs (Fig. 2a). Emcn\(^{hi}\) CD31\(^{hi}\) clusters from E16.5, P6 and P14 femur were found in two distinct locations in two-dimensional plots and argued for a separate EC subset in addition to type H endothelium (Fig. 2a). We termed this population type E because of its high abundance in embryonic long bone at E16.5, while type H cells were comparably rare at this stage (Fig. 2b). Type E EC abundance decreased postnatally to 3.8% at P14 and 2.2% at P28, respectively. The fraction of type H ECs initially increased during early postnatal life, peaked at P6 and was already in decline at P14. In accordance with the high abundance of sinusoidal (type L) endothelium in adult bone\(^{13}\), Emcn\(^{lo}\) CD31\(^{lo}\) ECs increased continuously throughout all postnatal stages analysed and represented the major population after P6 (Fig. 2b).

To gain insight into their molecular properties, type H, E and L ECs were sorted by flow cytometry from P6 bones in triplicate. Principal component analysis of RNA-sequencing samples showed low variation within each sample group, while sample clustering indicated distinct profiles of individual EC populations (Fig. 2c). Expression profiles of type E and H ECs were more similar to each other than to type L. Differential gene expression analysis for every possible pairwise combination of samples, with a false discovery rate (FDR)-adjusted \(P\) value <0.01 and an absolute log \(2\) values of 0.98 for type H versus L, 4.602 for type E versus L, and 1.619 for type E versus H (Fig. 2d and Supplementary Fig. 2a). A high proportion of genes (1,645) was upregulated in both E and H subtypes relative to type L cells, while 828 and 350 genes were specifically higher in type E and H, respectively (Fig. 2e). RNA-sequencing results were confirmed by quantitative PCR with reverse transcription (RT–qPCR) for a panel of 14 genes. \(R^2\) values of 0.98 for type H versus L, 0.93 for type E versus L, and 0.90 for type E versus H indicate high correlation of results (Supplementary Fig. 2b). RNA-seq results for Emcn and Pecam1 confirmed enrichment of these markers in type H and E ECs relative to type L (Fig. 2f). Consistent with the flow cytometry data, type E cells showed higher Pecam1 and lower Emcn transcription than type H ECs (Fig. 2a,f).

Analysis of selected markers showed high expression of Vegfr2/Kdr and Vegfr3/Flik1 in type H and E ECs relative to type L endothelium (Fig. 2g). Both genes encode VEGF receptors and regulate angiogenesis in numerous settings\(^{26–30}\). Surprisingly, anti-VEGFR2 immunostaining labelled column-shaped type H vessels less than the adjacent type L network (Supplementary Fig. 2c). VEGFR3 protein was also low on type H columns, whereas the most distal type H vessels next to the growth plate and the sinusoidal network were strongly stained (Supplementary Fig. 2d). As VEGF receptors undergo rapid turnover in the growing retinal vasculature\(^{31}\), mice were injected with the proteasome inhibitor MG132, which limits the availability of ubiquitin and interferes with protein degradation\(^{32}\). MG132 led to elevated VEGFR2 and VEGFR3 staining of type H vessel columns, whereas changes in diaphyseal vessels were comparably modest (Fig. 2h,i and Supplementary Fig. 2e,f). This validated the corresponding RNA-seq and RT–qPCR results and indicated high turnover and low steady-state levels of VEGF receptors in type H vessels.

**Functional properties of type E ECs**

Further analysis and validation of RNA-seq results enabled the identification of markers that were expressed at high levels in type E endothelium relative to other EC subtypes, including Esml, Kitl, Unc5b, Bcam, Cav1 and Apln (Supplementary Fig. 3a). In P6 femur, the type E-enriched markers cavelolin 1 and BCAM/CD239/Lutheran blood group glycoprotein, a specific receptor for laminin 511 in blood cells\(^{33}\), showed expression in Emcn-negative arterioles, which therefore did not correspond to type E ECs, as well as in Emcn\(^{+}\) capillaries in compact bone and endostem (Fig. 3a–c). In E16.5 femur, most capillaries were CD31\(^{+}\) (Fig. 1c) and a fraction also showed expression of cavelolin 1 (Supplementary Fig. 3b). Cross-sections through P6 femur showed cavelolin 1\(^{+}\) or BCAM\(^{+}\) vessels in the endostem and the adjacent compact bone (Fig. 3b,c and Supplementary Fig. 3c). Interestingly, type E vessels were even more strongly associated with Oxs\(^{+}\) osteoprogenitors than type H capillaries (Fig. 3c,d), which we attribute to high expression of bone morphogenetic proteins (BMPs) and of other factors controlling angiogenesis and osteogenesis (Fig. 3e). When co-cultured with murine mesenchymal C3H10T1/2 cells\(^{34}\), freshly isolated type H or type E ECs induced remodelling of cell spheroids and expression of osteoblast lineage markers (Fig. 3f,g and Supplementary Fig. 3d,e). In contrast, spheroids consisting of C3H10T1/2 cells and type L ECs lacked osteoblast lineage differentiation (Fig. 3f,g and Supplementary Fig. 3d,e).

As apelin (encoded by the Apln gene) was one of the markers enriched in type E ECs, we performed in vivo fate mapping with Apln-CreER knock-in mice\(^{35}\). Following a single administration of 4-hydroxy tamoxifen (4-OHT) at E15.5, Apln-CreER-induced expression of green fluorescent protein (GFP) under control of the R26-mT/mG Cre reporter\(^{36}\) labelled ECs throughout the E16.5 and E18.5 femur, which predominantly represented type E endothelium (Fig. 4a,b and Supplementary Fig. 4a). At P6, GFP\(^{+}\) ECs were highly abundant and had contributed to metaphyseal type H capillaries, type L sinusoids in the diaphysis and arteries (Fig. 4b and Supplementary Fig. 4a). Following 4-OHT administration to Apln-CreER R26-mT/mG newborn animals (P0), GFP\(^{+}\) ECs were observed at P1 in type H capillaries near the growth plate and in endostem and compact bone, that is, in sites enriched in type E endothelium (Fig. 4c and Supplementary Fig. 4b). At P6, GFP\(^{+}\) ECs were highly abundant in arteries and all capillary subtypes (Fig. 4c and Supplementary Fig. 4b). Induction of Apln-CreER R26-mT/mG animals with 4-OHT at P6 showed that labelled ECs were initially concentrated in the distal metaphysis, endostem and compact bone at P7, whereas a substantial expansion of the GFP\(^{+}\) endothelium into all capillary subtypes and arteries was seen at P11, P15 and P21 (Fig. 4d and Supplementary Fig. 4c). Flow cytometry of P6-injected Apln-CreER R26-mT/mG bone ECs at P7 showed enrichment of type E ECs in the GFP\(^{+}\) population, but in contrast to E16.5, the fraction of type H ECs was substantially increased (Fig. 4a,e). Analysis of P6-injected Apln-CreER R26-mT/mG
mice at P7 and P21 indicated a significant increase in GFP+ type L ECs (Fig. 4f).

Together, these data show that type E vessels represent a spatially and temporally confined subpopulation of capillaries in developing bone. Characteristic features of type E ECs are their strong association with Osx+ cells and capacity to promote osteogenesis. Moreover, type E ECs can give rise to type H endothelium and both subtypes can differentiate into type L and arterial ECs during postnatal development.
**Enrichment of cell–matrix adhesion molecules in type H and E ECs**

To unravel the biological relevance of the RNA-seq data set, we performed Generally Applicable Gene-set Enrichment for Pathway Analysis (GAGE)\textsuperscript{37}. Altogether 78 gene sets displayed significant levels of upregulation in type E versus L, while 76 gene sets were upregulated in type H versus L ECs (q value <0.0001 and Supplementary Tables 1,2). Representation of the top 15 upregulated gene sets in type subpopulations (including type L, white arrowheads) and arteries (yellow arrowheads) at the respective later stages. (e) Flow cytometric analysis of GFP\textsuperscript{+} cells in P7 Apln-CreER R26-mT/mG long bone at 24 h after 4-OHT induction at P6. Note the significant increase of labelled type L ECs. Data represent mean ± s.e.m. (n=7 mice for P7 and n=8 mice for P21), (P = 0.008, two-tailed unpaired t-test). (g) Gene-set enrichment analysis for significantly differentially regulated genes in type E or type H ECs relative to type L ECs. Cell–matrix adhesion-related gene sets are highlighted in yellow.
Figure 5 Altered bone vasculature in EC-specific \textit{Itgb1} mutant mice. (a) Confocal images of 3-week-old \textit{Itgb1} \textsuperscript{1LEC} and littermate control femurs stained for Emcn (red), Nuclei, Hoechst (blue). (b) High-magnification images showing Emcn-immunostained vessel columns (marked by dashed lines) in the \textit{Itgb1} \textsuperscript{1LEC} and control femoral metaphysis. (c) Number of branch points in Emcn-stained metaphyseal vessels. Data were normalized to 100% in control littersmates and represent mean ± s.e.m. (\textit{n}=5 individual femurs per group), (\textit{P}=0.02, two-tailed unpaired t-test). (d) Quantitation of length of vessel area with low VEGFR3 immunostaining relative to total vessel area in \textit{Itgb1} \textsuperscript{1LEC} and control femoral sections. Dashed lines indicate upper and lower border of metaphysis. (e) Confocal images of VEGFR3 (white) immunostaining in \textit{Itgb1} \textsuperscript{1LEC} and control femoral sections. (f) Flow cytometry analysis for EdU\textsuperscript{+} cells among total CD31\textsuperscript{hi} Emcn\textsuperscript{hi} cells expressed as percentage of total bone ECs. Data represent mean ± s.e.m. (\textit{n}=16 mice per group), (\textit{P}=0.008, two-tailed unpaired t-test). (g) Flow cytometry analysis of CD31\textsuperscript{hi} Emcn\textsuperscript{hi} cells expressed as percentage of total bone ECs. Data represent mean ± s.e.m. (\textit{n}=16 mice per group), (\textit{P}=0.006, two-tailed unpaired t-test). (h) Flow cytometry analysis of CD31\textsuperscript{hi} Emcn\textsuperscript{hi} cells expressed as percentage of total bone ECs. Data represent mean ± s.e.m. (\textit{n}=11 mice per group), (\textit{P}=0.003, two-tailed unpaired t-test). (i) Representative flow cytometry analyses of \textit{Itgb1} \textsuperscript{1LEC} and \textit{Cre}-littermate control bone marrow cells stained for endomucin and CD31. Gates indicating type L (green), type H (orange) and type E (purple) ECs. Charts show quantification of type E ECs per total ECs in P21 \textit{Itgb1} \textsuperscript{1LEC} relative to control bone. Data represent mean ± s.e.m. (\textit{n}=11 mice per group), (two-tailed unpaired t-test). (j) Flow cytometry analysis for EdU\textsuperscript{+} cells among total CD31\textsuperscript{hi} Emcn\textsuperscript{hi} Emcn\textsuperscript{hi} cells expressed as percentage of total bone ECs. Data represent mean ± s.e.m. (\textit{n}=7 independent samples), (\textit{P}=0.03, two-tailed unpaired t-test). (k) Close-up views of [\textit{Itgb1} \textsuperscript{1LEC}] and [\textit{Cre}-littermate] control bone marrow vessels in 3-week-old \textit{Itgb1} \textsuperscript{1LEC} and [\textit{Cre}-littermate] control bone. Note the strong CD31 staining of \textit{Itgb1} \textsuperscript{1LEC} diaphyseal vessels relative to control (arrowheads). (l) Close-up views of [\textit{Itgb1} \textsuperscript{1LEC}] and [\textit{Cre}-littermate] control bone marrow vessels in 3-week-old \textit{Itgb1} \textsuperscript{1LEC} and [\textit{Cre}-littermate] control bone. Note the strong CD31 staining of \textit{Itgb1} \textsuperscript{1LEC} diaphyseal vessels relative to control (arrowheads).
E versus L and type H versus L ECs showed a striking enrichment of ECM, basement membrane and cell adhesion components (Fig. 4g). In particular, RNA-seq and RT–qPCR data indicated high expression of Lama4 and, to a lower extent, Lama5 in type E and H ECs (Supplementary Fig. 4d). Both encode alpha subunits of heterotrimeric laminins that are central constituents of the endothelial basement membrane and control cell behaviour. By contrast, Ftn1 transcripts (encoding fibronectin) were comparable in all three EC subtypes (Supplementary Fig. 4d). Transcripts for integrins were also enriched in type E and H ECs relative to type L (Supplementary Fig. 4e–g).

Accordingly, strong vascular staining was seen for lamin α4, laminin α5, fibronectin, and the integrin β1 subunit in P6 or P21 femoral metaphysis (Supplementary Fig. 4h–j). Anti-integrin β1 staining also decorated the type L endothelium of the diaphysis, whereas the interstitial matrix proteins collagen type I and osteopontin labelled trabecular bone in the metaphysis (Supplementary Fig. 4i,j).

The phenotype of bone ECs is controlled by endothelial integrin β1

To analyse integrin function in the endothelium of developing bone, Cdhs5(PAC)-CreERT2 transgenic animals42 were interbred with mice carrying loxP-flanked alleles of Itgb140. Cdhs5(PAC)-CreERT2 activity is strictly EC-specific at P21 (Supplementary Fig. 5a,b) and all other stages investigated previously13. As tamoxifen administration during embryonic development led to rapid lethality of the resulting Itgb1ΔEC mutants24, only postnatal mutants generated by tamoxifen injection from P10–P12 were analysed. Efficient Itgb1 inactivation was confirmed by RT–qPCR of freshly isolated bone ECs at P21 (Supplementary Fig. 5c). Three-week-old Itgb1ΔEC mutants were smaller and had significantly lower body weight than control littermates (Supplementary Fig. 5d). freshly isolated mutant femurs were 9% shorter than controls, displayed a dark red colour and contained a disorganized vasculature (Fig. 5a and Supplementary Fig. 5e,f). Type H vessel columns were much shorter, whereas the rest of the metaphyseal capillaries were highly branched and lacked a straight columnar organization (Fig. 5b,c). VEGFR3 immunostaining, with the distinct low steady-state levels of the protein in type H EC columns (Supplementary Fig. 2d), highlighted the loss of Itgb1ΔEC columnar vessels (Fig. 5d,e). Reflecting the reduced expansion of Itgb1ΔEC long bone, the number of ECs was slightly but significantly increased despite reduced EC proliferation (Fig. 5f,g). Arteries, which connect to type H capillaries in metaphysis and endosteum13, were present both in Itgb1ΔEC and control femurs (Supplementary Fig. 5g).

Flow cytometry analysis of Itgb1ΔEC femoral ECs showed an unexpected increase in CD31hi Emcnhi cells (Fig. 5h) and immunohistochemistry showed strong CD31 signal in diaphyseal sinusoidal vessels (Fig. 5i). Moreover, proliferation of mutant CD31hi Emcnhi ECs was strongly reduced, whereas no significant change was seen in the type L subset (Fig. 5j and Supplementary Fig. 5h,i). Flow cytometry analysis of Itgb1ΔEC bone ECs indicated that the increase in CD31hi Emcnhi cells was also in part attributable to expansion of the type E subset (Fig. 5k). Together, this indicated that the loss of endothelial integrin β1 leads to substantial phenotypic changes in bone ECs. Significantly more ECs showed elevated expression of the markers CD31 and Emcn but lacked the normal morphological and functional properties of type E or H endothelium. These changes were accompanied by alterations in regional metabolic properties. The non-hypoxic zone in the distal metaphysis13 was shortened, whereas phosphorylated mitogen-activated protein kinase (phospho-Erk1/2), a marker of high metabolic activity, was decreased (Supplementary Fig. 5j,k).

Regulation of osteogenesis by endothelial integrin β1

Bone angiogenesis and CD31hi Emcnhi ECs are tightly coupled to osteogenesis12,13. Micro-computed tomography (μCT) analysis of the P21 Itgb1ΔEC tibial metaphysis uncovered profound structural defects in bone formation (Fig. 6a). Mutants displayed significantly reduced bone volume, trabeculae number and trabecular thickness, whereas trabecular separation was increased. Connectivity density of trabecular bone was slightly but not significantly decreased (Fig. 6a). Accordingly, the abundance of Osx+ cells was strongly reduced in EC-specific Itgb1 mutants (Fig. 6b,c). Osteocalcin, a marker for mature osteocytes, was also profoundly decreased in Itgb1ΔEC mice (Fig. 6d). Analysis of collagen type I and osteopontin, which are potential binding partners of integrin β1, revealed reduction of these proteins. Mutants displayed lower staining intensities than control samples and the length of trabecular segments was reduced, which was confirmed by visualization of matrix-rich structures by second-harmonic generation imaging using multi-photon microscopy (Fig. 6e and Supplementary Fig. 6a–c). Furthermore, immunostaining for the proteoglycan NG2/Cspg4 and the transcription factor Runx2, markers for primitive mesenchymal cells and preosteoblasts, respectively, was enhanced in Itgb1ΔEC femurs relative to littermate controls, suggesting defective maturation of osteoblast progenitors (Fig. 6f,g).

As shown above, the initially labelled EC population expanded and, to a lower extent, CD31hi Emcnhi cells with only minor changes in the type L subpopulation (Fig. 7a–d). In addition to these vascular alterations, Itgb1ΔAPlnhi mutants also phenocopied the reduction in Osx+ cells and osteopontin deposition, the increase in Runx2 preosteoblasts, and the
Figure 6 Bone defects in EC-specific Itgb1 mutant mice. (a) Representative three-dimensional reconstruction from μCT measurements of tibial metaphysis of 3-week-old Itgb1<sup>fl<sup>EC</sup></sup> and littermate control mice. Diagrams represent bone parameters measured in μCT analyses: bone volume/total volume (BV/TV) in percentage, trabecular number in 1 per millimetre, trabecular thickness in millimetres, trabecular separation in millimetres, and connectivity density in 1 per cubic millimetre. Data represent mean ± s.e.m. (n = 6 mice), (P values determined by two-tailed unpaired t-test). (b) Quantitation of metaphyseal Osterix<sup>+</sup> cells in Itgb1<sup>fl<sup>EC</sup></sup> mutant and Cre-negative littermate bone sections. Data represent mean ± s.e.m. (n = 4 individual femurs), (P = 0.01, two-tailed unpaired t-test). Statistics source data are shown in Supplementary Table 6. (c) Tile scan and high-magnification confocal images of Itgb1<sup>fl<sup>EC</sup></sup> and littermate control femurs stained for osteocalcin (green). Dashed lines indicate borders of metaphysis to growth plate and marrow cavity, respectively. (d) Itgb1<sup>fl<sup>EC</sup></sup> and littermate control femurs stained for osteocalcin (red) and counterstained with Hoechst (blue). (e) High magnification of two-photon second-harmonic generation signals (white) of sections of P21 Itgb1<sup>fl<sup>EC</sup></sup> and control femurs. Dashed line indicates adjacent growth plate (top). (f, g) Maximum-intensity projection of femoral sections from Itgb1<sup>fl<sup>EC</sup></sup> mutants and littermate controls stained for NG2 (f, green) or Runx2 (g, red). Nuclei in g, Hoechst (blue). (h) RT-qPCR analysis of growth factor transcripts in freshly sorted Itgb1<sup>fl<sup>EC</sup></sup>CD31<sup>hi</sup>Emcn<sup>hi</sup> (orange) and control ECs (blue). Data represent mean ± s.e.m. (n = 9 mice per group), (P values, two-tailed unpaired t-test). NS, not significant.
Figure 7  Defects in Apln-CreER-generated Itgb1 mutants. (a) Confocal images of 3-week-old Itgb1<sup>ΔApln</sup> and littermate control femurs stained for Emcn (red). Nuclei, Hoechst (blue). Dashed line marks growth plate. (b) High-magnification images showing Emcn-immunostained Itgb1<sup>ΔApln</sup> and control femoral metaphyseal vessels. Dashed line marks growth plate. (c) Charts of flow cytometry analysis of total ECs relative to 100% in control littermates, CD31<sup>hi</sup> Emcn<sup>hi</sup> cells per total ECs and type L cells per total ECs in P21 Itgb1<sup>ΔApln</sup> and Cre-littermate controls. Data represent mean ± s.e.m. (n = 6 mice per group), (P = 0.13 for total ECs, P = 0.04 for CD31<sup>hi</sup> Emcn<sup>hi</sup> cells and P = 0.04 for type L ECs, two-tailed unpaired t-test). (d) Confocal images of VEGFR3 (white) immunostaining in Itgb1<sup>ΔApln</sup> and control femoral sections. Dashed lines indicate upper and lower border of metaphysis. (e) Overview and high-magnification confocal images of Itgb1<sup>ΔApln</sup> and littermate control femurs stained for osterix (green). Dashed lines indicate growth plate. (f,g) Confocal images of P21 Itgb1<sup>ΔApln</sup> and control femoral sections stained for osteopontin (f, white) and Runx2 (g, red). Nuclei, Hoechst (blue). Dashed line marks growth plate. (h) Maximum-intensity projections of tile scan confocal images showing Apln-CreER R26-mT/mG-generated GFP signal (green) in Itgb1<sup>ΔApln</sup> and littermate control femurs. (i) Bar charts showing ratio of CD31<sup>hi</sup> Emcn<sup>hi</sup> cells in total GFP<sup>+</sup> (Apln-CreER R26-mT/mG) cells in Itgb1<sup>ΔApln</sup> littermate control bone (n = 6 mice per group), (P = 0.08 for CD31<sup>hi</sup> Emcn<sup>hi</sup> ECs and P = 0.04 for type L ECs, two-tailed unpaired t-test). Plots represent mean ± s.e.m.

reductions in body weight, femur length, mineralized bone and TRAP staining seen in pan-endothelial Itgb1<sup>ΔEC</sup> animals (Fig. 7c–g and Supplementary Fig. 7c–i). GFP<sup>+</sup> cells, generated by the activation of the R26-mT/mG reporter in the Itgb1<sup>ΔApln</sup> background, were confined to the metaphysis and showed defective expansion into the diaphysis (Fig. 7h). Moreover, the abundance of type L ECs in the GFP-labelled
Figure 8 Phenotypes of extracellular matrix mutants. (a) Overview and high-magnification confocal images of Emcn-immunostained (red) femoral sections of 3-week-old Lama4KO and control mice. Dashed lines indicate upper/lower border of column-like vessels in proximity of the growth plate (top). (b) Flow cytometry quantitation of total and fraction of CD31hi Emcnhi ECs in 3-week-old Lama4KO and control bone. Data for total ECs were normalized to 100% (control) and represent mean ± s.e.m. (n=4, P=0.49). CD31hi Emcnhi data represent mean ± s.e.m. (n=4, P=0.60; two-tailed unpaired t-test; n represents number of mice per group). Statistics source data are shown in Supplementary Table 6. (c) Emcn staining of 3-week-old Spp1KO and control mice. (d) Osterix (green)-stained sections of Spp1KO or control femur. (e) Flow cytometry quantitation of total and CD31hi Emcnhi ECs in 3-week-old Spp1KO and control bone (n=3, P=0.25 and n=3, P=0.01, respectively; two-tailed unpaired t-test; n represents number of mice per group). Statistics source data are shown in Supplementary Table 6. (f,g) Emcn (f, red) and osterix (g, green) immunostaining of Lama5KO or control femur sections. Dashed lines indicate upper and lower borders of trabecular region. (h) High magnification of two-photon second-harmonic generation signals (white) of thick sections (100 μm) of P21 Lama5KO and control femurs. Dashed line indicates adjacent growth plate. (i) Flow cytometry quantitation of total and fraction of CD31hi Emcnhi ECs in 3-week-old Lama5KO and littermate control mice. Data for total ECs were normalized to 100% (control) and represent mean ± s.e.m. (n=7, P=0.72). CD31hi Emcnhi data represent mean ± s.e.m. (n=7, P=0.04; two-tailed unpaired t-test; n represents number of mice per group). (j) RT-qPCR analysis of growth factor transcripts in freshly sorted Lama5KO CD31hi Emcnhi (green) and control ECs (blue). Data represent mean ± s.e.m. (n=10 mice per group), (P values, two-tailed unpaired t-test). NS, not significant.
population was significantly reduced in Igfb1-/-Apln-/- mutants (Fig. 7i) arguing that integrin β1 is required for the expansion of CD31hi Emcnhi ECs and their conversion into type L ECs.

Role of matrix molecules in the specification of bone ECs

Given the numerous important roles of matrix proteins in bone formation,35-37, we wanted to identify ECM components interacting with endothelial integrin β1. Global deletion of Lama4 did not result in any overt phenotype in P21 femur. Vascular organization was normal, the length of metaphyseal columnar vessels and Osx immunostaining were comparable to controls, and total and CD31hi Emcnhi bone ECs were not altered (Fig. 8a,b and Supplementary Fig. 8a-c).

Osteopontin-deficient (Spp1lox/lox) mice had weak defects resembling certain aspects of the Igfb1-/-AEC phenotype. Metaphyseal vessel columns were shortened and branching between columns was increased, and Oxs+ cells and the VEGFR3-low area within the metaphyseal vessel columns were slightly reduced (Fig. 8c,d; Supplementary Fig. 8d). However, the total number of ECs was not significantly changed and the CD31hi Emcnhi subpopulation was decreased in Spp1lox/lox long bone (Fig. 8e).

As global Lama5 knockout mice are embryonic lethal,41 bone from constitutive EC-specific Lama5 mutants42 (Lama5AEC) was analysed, which showed defects resembling major aspects of Igfb1-/-AEC mutants even though the overall phenotype was comparably milder. Vessel columns near the growth plate were shortened, branching within the metaphyseal vasculature was increased, and the VEGFR3-low vessel area, Oxs+ cells and femur length were reduced (Fig. 8f-h and Supplementary Fig. 8e-h). Mineralized bone, however, was not reduced significantly (Supplementary Fig. 8j). While the total number of Lama5AEC bone ECs was not substantially altered, the Emcnhi CD31hi fraction was significantly increased (Fig. 8i). The analysis of selected transcripts in freshly isolated ECs by RT-qPCR indicated strongly reduced expression of Lama5 and Igfb1 in the Lama5AEC bone endothelium (Fig. 8j). Expression of Lama4 and Fgfl1 was increased, while levels of Bmp2 and Dll4 were slightly but significantly decreased.

These results indicate that disrupted cell–matrix interactions contribute, at least in part, to the defects in Igfb1 mutant bone. While it is likely that several ECM molecules are relevant in this context, our data argue for roles of osteopontin and the laminin α5 subunit-containing laminin 511 or 521 isoforms, which are produced by ECs43 and deposited in the subendothelial basement membrane.

DISCUSSION

ECs are highly heterogeneous in terms of gene expression, morphology and functional specialization.44,45 While differences between arterial and venous or lymphatic and blood vessel ECs are widely recognized, much less is known about the existence and specification of distinct capillary subsets. In kidney, glomerular capillaries are part of the renal ultrafiltration system, whereas peritubular capillaries surrounding nephron tubules participate in ion and mineral reabsorption.46,47 In adult brain, neurogenesis occurs in the adult subventricular zone and the hippocampal subgranule zone, which contain neural stem cells and vessels that are morphologically and functionally distinct from other brain capillaries.48,49 Specific properties of distinct capillary subtypes are perhaps best documented for the skeletal system. Type H capillary ECs support osteogenesis, whereas type L ECs form the sinusoidal vessel network of the bone marrow cavity.50 However, the molecular signatures of the different capillary EC subtypes and the processes controlling their specification have remained unknown. Our current study not only provides unbiased insight into the molecular properties of bone ECs but also establishes the existence of a third capillary EC subpopulation, termed type E, with high capacity to support perivascular Oxs+ osteoprogenitors in embryonic and early postnatal bone. While type E cells predominate in early long bone, genetic lineage tracing supports the existence of type E-to-H and type H-to-L conversion processes, which accompany the rapid developmental expansion of bone and bone marrow. On the basis of the sum of the findings presented here, we propose a developmental and functional hierarchy of bone ECs where type E cells occur upstream of type H and type L endothelium. Similar principles are likely to apply to other organ systems where, however, the identity, localization and functional roles of specific capillary EC subsets remain to be uncovered.

ECM molecules and cell–matrix receptors were particularly enriched in the type E and type H gene expression profiles. Integrin family cell–matrix receptors are well known for their important roles in a wide range of cell differentiation processes.50,51 Our data establish that the β1 subunit is essential for the normal specification and function of bone EC subpopulations. In addition to matrix, integrins can bind and signal in response to growth factors and other secreted molecules.52-55 However, Igfb1-/-AEC defects were partially phenocopied in Lama5AEC and, to a lesser extent, in Spp1 global knockout mice, which argues for a role of cell–matrix signalling in bone EC specification. A caveat of the genetic approaches in our study is that they affect ECs throughout the body and might therefore potentially influence processes in the skeletal system indirectly. Nevertheless, the data presented here argue that integrin β1 and laminin α5 are required in bone ECs in a cell-autonomous and direct fashion. Thus, our findings raise the possibility that changes in the matrix environment of bone, which are the result of congenital defects and other pathological conditions in human patients,56-58 might potentially lead to disease-promoting alterations in the vasculature. Our identification and characterization of distinct, functionally specialized capillary EC subsets might facilitate the characterization of disease-associated changes in the future.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

Note: Supplementary Information is available in the online version of this paper.

ACKNOWLEDGEMENTS

We thank H.-W. Jeong for his support in RNA-seq experiments, K. Kato for experimental guidance, and M. Stehling for expert advice in flow cytometry experiments. The Max Planck Society, the University of Muenster, the Cells in Motion (CiM) graduate school, the DFG cluster of excellence ‘Cells in Motion’ (L.S., J.M.V. and R.H.A), and the European Research Council (AdG 339409 AngioBone) have supported this study.

AUTHOR CONTRIBUTIONS

U.H.L., M.E.P., J.M.V. and R.H.A. designed the study. R.E.-G., A.S. and J.M.V. performed bioinformatics analyses, M.G.B. the two-photon microscopy, K.K.S. the accession codes and references, are available in the online version of this paper.
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METHODS

Mouse models. C57BL/6j mice were used for the analysis of wild-type bones. For embryonic stages, females were checked for the presence of a vaginal plug in the morning.

Lineage tracing experiments were performed by mating Apln-CreER mice and R26-mG/mT reporter animals. Cre activity and GFP expression were induced by intraperitoneal injection of 4-hydroxy tamoxifen (4-OH) (H7904, Sigma). Induction was performed at E15.5 (750 µg 4-OH per female), P0 (15 µg 4-OH per pup) and P6 (25 µg 4-OH per pup). For postnatal analysis after embryonic induction, cesarean sections were performed at E19.5 and pups were raised by foster mothers. As the Apln-CreER knock-in allele disrupts the function of the X-linked Apn gene, only females were analyzed.

For inducible and EC-specific genetic experiments, Cdh5(PAC)-CreERT2 transgenics were interbred with mice carrying loxP-flanked Iggb1 (Iggb1flox) and Cre- negative Iggb1flox/littermate controls. The specificity of Cdh5(PAC)-CreERT2 transgenic mice for ECs in long bone was determined by crossing Cdh5(PAC)-CreERT2 mice with R26-mT/mG reporter mice. Cre activation was induced from P10 to P12 and mice were analyzed at P21. Furthermore, the Apln-CreER was introduced into the R26-mT/mG reporter background for tracking of Apln+ cells with the same induction and analysis scheme. For gene inactivation in Apln- ECs, Apln-CreER was bred into the Iggb1flox/littermate background. Gene inactivation was induced by tamoxifen administration from P10 to P12 (200 µg per pup per day). Long bones were collected at the age of 3 weeks from Iggb1flox/Apln-CreERflox/+ mice and Cre-negative Iggb1flox/littermate controls. Furthermore, Iggb1flox/mT/mG reporter mice were interbred with R26-mT/mG reporter mice. The resulting Iggb1flox/R26-mT/mGflox/Apln-CreER+/+ and Iggb1flox/+R26-mT/mGflox/Apln-CreER+/+ mice were analyzed at 3 weeks of age after tamoxifen administration from P10 to P12.

The role of endothelial Lama5 was investigated by crossing mice carrying loxP-flanked Lamas1 alleles with Cre- transgenics. In addition, global Apln-CreERT2 mice were interbred with mice carrying loxP-flanked Lama4 and Spp1-/- knockouts. Genotypes of mice were determined by PCR.

All animal experiments were performed in compliance with the relevant laws and institutional guidelines, were approved by local animal ethics committees and were conducted at the University of Münster and the MPI for Molecular Biomedicine with permissions granted by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) of North Rhine-Westphalia. Animals were combined in groups for experiments irrespective of their sex. Apln-CreER knock-in mice were excluded from analysis because these mice lack expression of the X-linked Apn gene. The investigators were not blinded to allocation during experiments and outcome assessment. Experiments were not randomized and no statistical method was used to predetermined sample size, which was, instead, chosen according to previous experiments.

Proteasome inhibition, EdU labelling and hypoxia analysis. Three-week-old wild-type mice were injected intraperitoneally with 50 µg·g⁻¹ MG132 (Millipore, no. 474790) in dimethylsulfoxide (DMSO; Sigma, D8418) or with DMSO alone as a vehicle control. Three hours after injection, femurs were dissected and processed for immunostaining as described above.

Proliferating cells in vivo were labelled by intraperitoneal injection of 500 µg of EdU (Invitrogen) 2 h before the analysis. Detection of proliferating cells in fixed bone sections and in flow cytometry analysis was achieved with staining according to the manufacturer's instructions (Invitrogen).

Hypoxic cells were detected with the hypoxia probe pimonidazole (Pimo, HypoxyProbe Inc) according to the manufacturer's instructions. Mice were intraperitoneally injected with 60 mg kg⁻¹ Pimo 2 h before analysis.

Bone immunohistochemistry. Murine femurs and tibiae were dissected, cleaned from adjacent muscles, immediately transferred into cold 2% paraformaldehyde solution and fixed overnight at 4 °C under rotation. Next, bones were transferred into 0.5 M EDTA solution and kept overnight at 4 °C under rotation for decalcification. On the following day, EDTA was exchanged for 20% sucrose and 1% polyvinylpyrrolidone (PVP) solution. After 24 h at 4 °C under rotation, bones were embedded and frozen in 8% gelatin (porcine) with 15% sucrose and 1.5% PVP. Finally, samples were cut into 100 µm sections using low-profile blades on a Leica CM3050 cryostat.

For immunofluorescence staining, sections were air-dried at room temperature, rehydrated in PBS and permeabilized with 0.3% Triton X-100 for 15 min at room temperature. After washing with PBS, blocking solution (5% donkey serum +0.1% Triton X-100) was applied for 20 min at room temperature. Following another PBS wash, sections were treated with primary antibodies overnight at 4 °C. The next day, sections were washed four times with PBS and incubated with secondary antibodies for 1.5 h at room temperature. After five wash steps with PBS, sections were mounted using FluoroMount-G (Southern Biotech). Finally, coverslips were sealed with nail polish.

RNA sequencing and data analysis. Femurs and tibiae of 20 wild-type P6 mice were pooled and sorted by flow cytometry for type H, type E and type L ECs. RNA from 120,000 cells from each group was isolated using the RNeasy Plus Micro Kit (QIAGEN) according to the manufacturer's instructions. RNA quality was checked using a 2100 BioAnalyzer (Agilent). One hundred nanograms of RNA was used for
Quality assessment. The quality assessment of raw sequence data of endothelial cell subtypes H, E and L was performed using FastQC (Version: FastQC 0.11.3, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). No samples were discarded from the analysis.

Alignment to reference sequence. Paired-end sequence reads were mapped to the mm10 mouse genome assembly (GRChm38) using TopHat-2 with the following settings (Version: tophat-2.0.13; [tophat –g 2])27. The mouse genome was downloaded from the iGenome portal (https://supportillumina.com/sequencing/sequencing_software/igenome.html). HTSeq was used to count the aligned reads on a per gene basis using the following settings (Version: HTSeq-0.6.1; [–count-mode=intersection-nonempty=nonempty-strict=reverse-stranded]). RNA-sequencing data were uploaded to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with the accession number E-MTAB-4066.

Differential gene expression analysis. The count data were normalized using the Variance Stabilizing Transformation (VST) function from the DESeq2 package (ref. 11). Principal component analysis was performed on transformed read counts using the top 500 most variable genes to assess the overall similarity between the samples28. Differential gene expression analysis between the H versus L, E versus L and E versus H groups was performed using DESeq2. Differentially expressed genes were selected using an FDR-adjusted P value cutoff <0.01 and an absolute log, fold change >1. Gene symbols were annotated using biomart (Biocductor version 3.1). Lists of differentially regulated genes for the different contrasts are provided in Supplementary Tables 3–5.

Functional analysis. Gene set enrichment analysis was performed using Generally Applicable Gene-set Enrichment (GAGE; Biocductor version 3.1)29. For functional annotation, we used gene sets from org.Mm.eg.db, a genome-wide annotation package for mouse (Bioconductor, version 3.1). The analysis was performed on the basis of one-on-one comparisons between H versus L, E versus L and H versus E subtypes where L was always used as a control data set. For this analysis, read counts were normalized as described in the GAGE vignette (Bioconductor, version 3.1). Significant GO terms were selected using an FDR-adjusted P value <0.0001. Lists of enriched GO terms in upregulated gene sets are provided in Supplementary Tables 1 and 2.

Quantitative RT-PCR. RNA was isolated from flow cytometrically sorted bone marrow endothelial cells using the NReasy Plus Micro Kit (QiAGEN) according to the manufacturer’s instructions. The iScript cDNA Synthesis Kit (BIO-RAD) was used to transcribe 100 ng RNA per reaction into cDNA. The quantitative PCR (qPCR) was performed on an ABI PRISM 7900HT Fast Real Time PCR System (Applied Biosystems). The following FAM-conjugated Taqman gene expression probes were used in combination with Taqman Gene Expression Master Mix (Life Technologies): Flt4 (Mm00433337_m1), Pecam1 (Mm01242584_m1), Ebfn2 (Mm01215897_m1), Notch1 (Mm00434254_m1), Notch3 (Mm01345646_m1), Bcam (Mm01321451_g1), Igfb1 (Mm01233233_m1), Esml (Mm00469593_M1), Kitl (Mm00442972_m1), Unc5b (Mm00500540_m1), Car1 (Mm00480357_m1), Igsa5 (Mm00439797_m1), Igva (Mm00434506_m1), Lamd4 (Mm01193660_m1), Lamd5 (Mm01122051_g1), Pdgfa (Mm01205760_m1), Pdgfb (Mm01985781_m1), Tgfbr2 (Mm00117820_m1), Tgfb3 (Mm00436960_m1), Bmp2 (Mm01340178_m1), Bmp4 (Mm00432087_m1), Fgfl (Mm00438906_m1), Dll4 (Mm00444619_m1), Noggin (Mm01297833_s1), Sox9 (Mm00448840_m1), Runx2 (Mm00501584_m1), Sp7 (Mm00420986_m1), Hspb (Mm00492555_m1), Bglap (Mm03413826_m1), VIC-conjugated Actb (ACTG14324141). Taqman probe was used to normalize gene expression. Per group at least six mice from three independent litters were analysed to obtain the relative expression differences.

ELISAs. Serum levels of parathyroid hormone and calcitonin were measured by quantitative enzyme-linked immunosorbent assay (ELISA) kits (CEA866M and CEA72M, Cloud-Clone Corp.) in blood samples from mutant and control mice.

Second-harmonic generation (SHG) imaging using multi-photon microscopy. A TriM Scope II multi-photon system from LaVision BioTec was used for SHG imaging to visualize anisotropic structures, such as collagen fibres. The microscope set-up is a single-beam instrument with an upright Olympus BX51 WI microscope stand equipped with high-sensitivity non-descanned detectors close to the objective lens. The TriM Scope II is fitted with a Coherent Scientific Chameleon Ultra II Ti:Sapphire laser (tuning range 680–1,080 nm) and a Coherent Chameleon Compact OPO (automated wavelength extension from 1,000 nm to 1,600 nm). A 20× IR objective lens (Olympus XLMPlanFL 20×/0.1 W) with a working distance of 2.0 mm was used. SHG signals were detected using Tisa light at 850 nm, a 420/40 band-pass filter and a blue-sensitive photomultiplier (Hamamatsu H67080-01). Three-dimensional images were acquired and processed with LaVision BioTec ImSpector Software under Microsoft Windows. Overview images were reconstructed from a sequence of tiled three-dimensional images with Image J.

Cell culture experiments. Mice CH10T1/2 cells (no. CCL-226, ATCC) were cultured in Dulbecco’s modified Eagle’s medium (Sigma), supplemented with 10% FBS (PAA Laboratories), 2 mM L-glutamine, 100 U ml–1 penicillin, and 100 mg ml–1 streptomycin (Invitrogen). Cultured cells were maintained at 37 °C in a humidified 5% CO2 atmosphere.

For co-culture spheroids, freshly sorted EC subpopulations were combined with CH10T1/2 cells by the hanging-drop method. In brief, equal numbers of suspended CH10T1/2 cells were plated and sorted into well plates. To sort ECs or an equal number of only CH10T1/2 cells (5,000 cells per spheroid) were suspended in spheroid-forming media consisting of DMEM containing 1% FBS, 0.05% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich), N2 supplement and 0.25% (w/v) methylcellulose (Sigma-Aldrich; 400 cP). Drops of 17 µl cell suspension were placed onto each position of a 96-well plate lid. The lids were flipped on and placed on non-adherent round-bottom 96-well plates, which were kept at 37 °C in a humidified 5% CO2 incubator overnight to generate spheroids. Forming spheroids were seeded into each well with a plate spin, and then cultured for 7 days with spheroid culture media of DMEM containing 1% FBS, 0.05% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich), N2 supplement.

For immunostaining, spheroids were embedded into 2% low melting agarose gel. For easy detection of ECs in spheroids, each endothelial population was labelled with the lipophilic fluorescent dye CM-Di I (1 µM; Invitrogen) for 10 min at 37 °C, and then for an additional 40 min at 4 °C. Cells were then washed with complete culture medium twice. Dil-labelled ECs were combined with CH10T1/2 cells as described above. ALP staining was performed after 7 days of culture in spheroid culture media. ALP activity was detected with Leucocyte Alkaline Phosphatase kit (Sigma 86R) following the manufacturer’s instructions.

Cell lines used in this study were tested for mycoplasma contamination, were not authenticated and are not listed in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample.

Statistics and reproducibility. Statistical analysis was performed using GraphPad Prism software or the R statistical environment (http://r-project.org). All data are presented as mean ± s.e.m. unless indicated otherwise. Unpaired two-tailed Student’s t-tests were used to determine significant phenomena. P < 0.05 was considered significant unless stated otherwise. All experiments were performed independently at least three times (unless noted otherwise in the respective figure legend) and respective data were used for statistical analyses. Sample sizes for each experiment are described in the respective figure legends, and source data from independent replicates are provided in Supplementary Table 6. No animals were excluded from analysis.

Data availability. RNA-sequencing data are available through the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-4066.

Source data for Figs 1e, 2g, 3e, 5d, 6b and 8f and Supplementary Figs 2b, 3a, 4d, 5g, 7b and 8j have been provided as Supplementary Table 6. All other data supporting the findings of this study are available from the corresponding author on request.
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Supplementary Figure 1 Long bone development and vascularization. 

a, Emcn (red) immunostained embryonic hindlimb sections at the indicated developmental stages. Nuclei, Hoechst (blue). b, Overview (left) and high magnification images of P10 wild-type femur stained for Emcn (red). Nuclei, Hoechst (blue). Lines mark metaphysis (mp, orange) with columnar vessels, diaphysis (dp, green) containing highly branched sinusoidal vessels, and the transition zone (tz, blue) interconnecting metaphyseal and diaphyseal vessels. c, Tile scan confocal images of Osterix-immunostained (green) femoral sections at the indicated developmental stages.
Supplementary Figure 2 Gene expression analysis of the bone vasculature.

a, MA-plots of differentially regulated genes in P6 bone EC subpopulations. The x-axis represents the mean normalized counts and the y-axis shows the log2 fold change between EC subtypes. Differentially regulated genes are represented by red colored points (FDR-adjusted p-value <0.01 and absolute log2 fold change <1). Data points outside of the range of the y-axis are represented as triangles.

b, Experimental validation of differentially regulated genes by RT-qPCR. Pairwise comparison of gene expression by RT-qPCR and RNA-seq in DESeq2 between different conditions (E vs. L, H vs. L and E vs. H) The correlation coefficients are ~0.98, ~0.93 and ~0.90. Data represents mean± s.e.m. (n=3 for RNA-seq and n= 3 for qPCRs; n represents individual experiments). Statistics source data are shown in Supplementary Table 6.

c, d, Sections of wild-type femur at the indicated developmental stages stained for VEGFR2 (c, white) or VEGFR3 (d, white). Dashed lines mark area of high/low staining.

e, f, Immunostaining for VEGFR2 (e, green) or VEGFR3 (f, green) on femoral sections of 3-week-old wild-type mice after 3 hours of vehicle (DMSO) or proteasome inhibitor (MG132) treatment. Nuclei, DAPI (blue). Note strongly increased VEGFR2 and VEGFR3 signals in metaphyseal ECs but not in the diaphysis after MG132.
Supplementary Figure 3 Bone vessel subtypes and their osteogenic potential. a, Graph illustrating expression of selected genes in type E endothelium relative to type L or type H ECs, respectively. Data based on RPKM values obtained from RNA-seq of sorted cells at P6. Data represents mean± s.e.m. (n=3). Statistics source data are shown in Supplementary Table 6. b, Longitudinal section of E16.5 femur stained for Emcn (red) and Caveolin 1 (green). White arrowheads indicate double positive vessels. c, Transverse section of P6 mouse femur stained for Caveolin 1 (green). Nuclei, Hoechst (blue). White arrowheads mark Caveolin 1-positive vessels in endosteum and compact bone. d, Light microscopic appearance of spheroids at day 1 and day 7 of culture. Note disaggregation of cells in the presence of type L ECs. e, Visualization of Dil-labeled bone ECs (arrowheads) in C3H10T1/2 spheroids day 1 and day 7 of culture. Remodelling of C3H10T1/2 spheroids (formation of internal cavities) was induced by type H and E ECs but not by type L ECs or in absence of ECs. Nuclei, DAPI (blue).
Supplementary Figure 4  Hierarchy of EC subtypes and cell-matrix interactions. a-c, Overview pictures of femoral sections from Apln-CreER R26-mT/mG mice treated with 4-OHT at E15.5 (a), P0 (b) or P6 (c) and analysis at the indicated stages. Note colocalization of GFP+ (green) cells and Emcn+ (red) capillaries. Insets in the rightmost panel of (a) show higher magnifications of metaphysis (top) and diaphysis (bottom) at P6. Arrows in (c) indicate expansion of GFP+ vessels into the marrow cavity. d, Expression of selected matrix molecule transcripts identified by RNA sequencing of sorted P6 type L (green), type H (orange) and type E (blue) ECs. Data represents mean± s.e.m. (n=3 independent experiments). e, Expression (RNA-seq) of transcripts encoding integrin α subunits in type L, type H and type E ECs. Data represents mean± s.e.m. (n=3 independent experiments). f, RNA-seq results for Itgb1 transcript expression in bone ECs at P6. Data represents mean± s.e.m. (n=3 independent experiments). g, RT-qPCR analysis of Itgb1 expression in P21 type L (green) and type H (orange) ECs. Data represents mean± s.e.m. (n=3 independent experiments). Statistics source data are shown in Supplementary Table 6. h, Confocal images showing Emcn (red) and integrin β1 (white) protein expression in the P21 femoral metaphysis. Arrowheads mark endothelial integrin β1 signal. i, Confocal images of integrin β1 (white), Osterix (green) and CD45 (red) staining in wild-type P21 metaphysis and diaphysis. j, Maximum intensity projection confocal images of 3 week-old femoral metaphysis immunostained for Emcn (red) and Laminin α4, Laminin α5, Fibronectin, Collagen 1 or Osteopontin (white). Arrowheads mark endothelial expression of matrix proteins.
Supplementary Figure 5. Bone phenotype of EC-specific Itgb1 mutant mice. 

a, Scheme showing the time points of tamoxifen administration and analysis for the Itgb1<sup>ΔEC</sup> mutant mice. 

b, Tile scan overview and high magnification images of femur from P21 Cdh5-CreERT2 R26-mT/mG mice treated with tamoxifen from P10 to P12. GFP signal is strictly confined to endothelium. ECs, Emcn (red). 

c, Quantitative RT-qPCR analysis of Itgb1 expression in sorted bone ECs from P21 Itgb1<sup>ΔEC</sup> mutants and Cre-negative littermate controls (p<0.001, two-tailed unpaired t-test). Data represents means± s.e.m. (n=6 mice per group). 

d, Picture of 3 week-old Itgb1<sup>ΔEC</sup> mutant and control littermates. Chart shows reduction of Itgb1<sup>ΔEC</sup> mutant body weight relative to control (p<0.001, two-tailed unpaired t-test). Data represents means± s.e.m. (n=10 mice per group). 

e, Freshly dissected P21 Itgb1<sup>ΔEC</sup> and littermate control femurs. Ruler indicates length in centimeters. Chart shows length of femurs in millimeters (p=0.028, two-tailed unpaired t-test). Data represents means± s.e.m. (n=6 mice per group). 

f, Tile scan confocal images of 3 week-old Itgb1<sup>ΔEC</sup> and control femurs stained for Emcn (red). 

g, Confocal images of αSMA (red) staining in Itgb1<sup>ΔEC</sup> mice and littermate controls. Dashed line indicates border to adjacent growth plate (top). 

h, Flow cytometry analysis of type L ECs per total ECs. Data represents means± s.e.m. (n=16), (p<0.001, two-tailed unpaired t-test). 

i, Quantitative analysis of proliferating (EdU+) type L ECs per total type L ECs (p=0.558, two-tailed unpaired t-test). Data represents means± s.e.m. (n=7 mice per group). 

j, Maximum intensity projections of Itgb1<sup>ΔEC</sup> and Cre- littermate controls femoral sections stained for Pimonidazole (green) (j) or phospho-ERK1/2 (pERK1/2, green) (k). Nuclei, Hoechst (blue). Note reduction of the hypoxia-free zone (arrows) and of pERK1/2 signal in the Itgb1<sup>ΔEC</sup> metaphysis.
Supplementary Figure 6 Analysis of EC-specific $\alpha$EC mutant bone. a, b, Maximum intensity projections of Collagen 1 (a, white) and Osteopontin (b, white) immunostainings in 3 week-old $\alpha$EC and control femur. c, 2-photon-generated second harmonic generation signal (white) in femoral sections of 3 week-old $\alpha$EC mice and littermate controls. d, e, Immunostaining for Calcitonin receptor (d, green) or TRAP (e, red) in 3 week-old $\alpha$EC and control femur. Nuclei, Hoechst (blue). f, Analysis of serum parathyroid hormone (PTH) and calcitonin levels in $\alpha$EC mutants and Cre- littermate controls. Data represent mean±s.e.m (n=5 mice per group) (p=0.80 for PTH and p=0.53 for calcitonin, two-tailed unpaired t-test).
Supplementary Figure 7 Apln-CreER-controlled Itgb1 mutants. a, Scheme showing the time points of tamoxifen administration and analysis for Apln-CreER R26-mT/mG and Itgb1ΔApln mice. b, Overview of P10 femur of Apln-CreER R26-mT/mG mice without tamoxifen-induced recombination. Note absence of GFP+ cells (green). Nuclei, Hoechst (blue). c, d, Overview and high magnification confocal images of GFP signal (green) and Emcn staining (red) in femur, metaphysis and endosteum of Apln-CreER R26-mT/mG mice P13 (c) and P21 (d). Nuclei, Hoechst (blue). e, Body weight of Itgb1ΔApln mutants relative to Cre- littermate controls. Data represents mean± s.e.m. (n=9) (p<0.001, unpaired two-tailed t-test). f, Quantitation of Itgb1ΔApln and Cre- littermate control femoral length. Data represents mean± s.e.m. (n=9) (p<0.001, unpaired two-tailed t-test). g, Representative 3D reconstruction from µCT measurements of 3 week-old Itgb1ΔApln and littermate control tibial metaphysis. h, Diagrams represent bone parameters measured in µCT analyses: bone volume/total volume (BV/TV) in percentage, trabecular number in 1 per millimeter, trabecular thickness in millimeters, and trabecular separation in millimeters. Data represent mean± s.e.m. (n=6 controls and 4 mutants), (p-values determined by two-tailed unpaired t-test). Statistics source data are shown in Supplementary Table 6. i, Confocal images of TRAP immunosignal (green) in 3 week-old control or Itgb1ΔApln femur. Nuclei, Hoechst (blue).
Supplementary Figure 8 Characterization of matrix molecule mutants.

a, High magnification confocal images of Emcn (red) staining in femoral metaphysis of Lama4KO and wild-type control mice. Dashed line indicates border to adjacent growth plate. b, d, e, Confocal images of VEGFR3 immunosignal (white) in 3 week-old control and Lama4KO (b), Spp1KO (d) or Lama5ΔEC (e) femur. Dashed lines indicate upper/lower borders of metaphysis containing columnar vessels with low VEGFR3 signal.

c, Osterix (green) stained sections of 3 week-old wild-type control and Lama4KO femur. Dashed lines indicate upper/lower borders of trabecular region.

d, 2-photon-generated second harmonic generation signal (white) in femoral sections of 3 week-old Lama5ΔEC mice and littermate controls.

g, Quantitation of body weight of Lama5ΔEC mutant mice relative to Cre- littermate controls. Data represents mean± s.e.m. (n=13) (p=0.46, unpaired two-tailed t-test).

h, Bar chart illustrating femoral length of Lama5ΔEC mutant mice and Cre- littermate controls. Data represents mean± s.e.m. (n=13) (p=0.04, unpaired two-tailed t-test).

i, Representative 3D reconstruction from µCT measurements of 3 week-old Lama5ΔEC and littermate control tibial metaphysis.

j, Bone parameters measured in µCT analyses: bone volume/total volume (BV/TV) in percentage, trabeculae number in 1 per millimeter, trabecular thickness in millimeters, and trabecular separation in millimeters. Data represent mean± s.e.m. (n=4 controls and 3 mutants), (p-values determined by two-tailed unpaired t-test). Statistics source data are shown in Supplementary Table 6.
Supplementary Table Legends

Supplementary Table 1 Full list of enriched gene sets for significantly differentially regulated genes in type H relative to type L ECs.

Supplementary Table 2 Full list of enriched gene sets for significantly differentially regulated genes in type E relative to type L ECs.

Supplementary Table 3 List of differentially regulated genes for the comparison of type H vs. type L ECs (FRD-adjusted p-value cut-off < 0.01 and an absolute log2 fold change > 1).

Supplementary Table 4 List of differentially regulated genes for the comparison of type E vs. type L ECs (FRD-adjusted p-value cut-off < 0.01 and an absolute log2 fold-change > 1).

Supplementary Table 5 List of differentially regulated genes for the comparison of type E vs. type H ECs (FRD-adjusted p-value cut-off < 0.01 and an absolute log2 fold-change > 1).

Supplementary Table 6 Statistical source data for Fig. 1e, 2f, 2g, 3e, 5d, 6b, 8b, 8f and Supplementary Fig. 2b, 3a, 4d, 4e, 4f, 4g, 7h, 8j.