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Supporting Information

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Supporting Information

Developmentally Engineered Callus Organoid Bioassemblies Exhibit Predictive In Vivo Long Bone Healing

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Supporting Figures

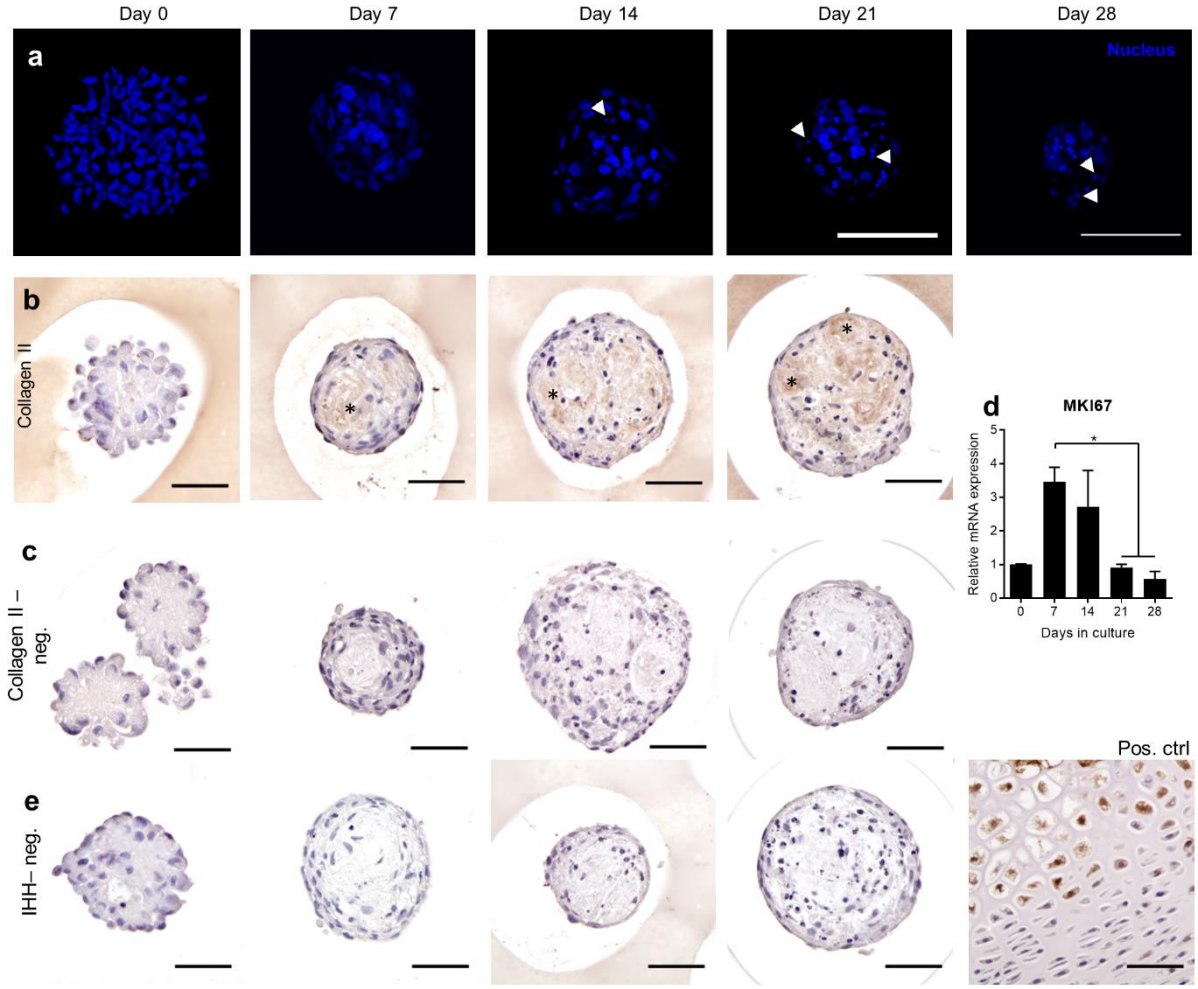

Figure S1. Characterization of cartilage intermediate microtissues. a) 3D renderings of confocal images of micro-spheroids stained with DAPI (nucleus) over time (white arrows represent apoptotic-like nuclei). b) Representative sections of collagen II immunostaining with c) negative control (excluding primary antibody). d) Quantification of MKI67 mRNA transcript (n = 3, mean value ± SEM). *p < 0.05; ANOVA and Tukey's multiple comparisons test. e) Negative control (excluding primary antibody, left panels D0, 7, 14 and 21) and positive control (E14.5 mouse limb, right panel) for IHH immunostaining. Scale bar a: 100 µm; b, d-e: 50 µm.

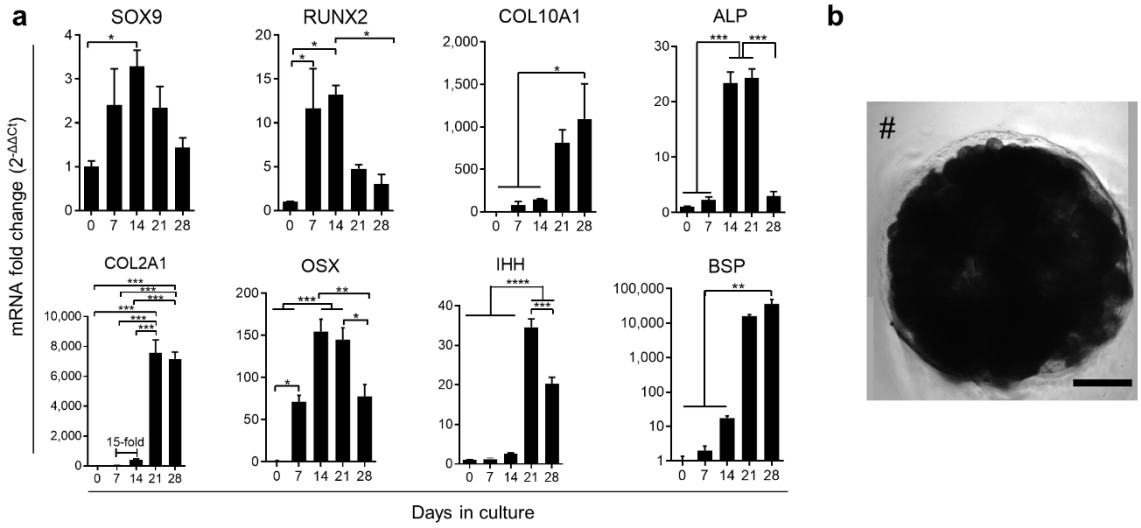

Figure S2. a) Gene expression analysis of spheroids overtime until D28. mRNA transcript quantification normalized to D0 (n = 3). b) Top-view of assembled modules within the in-house designed agarose mold (#: agarose). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ANOVA and Tukey's multiple comparisons test. Scale bar 500 µm.

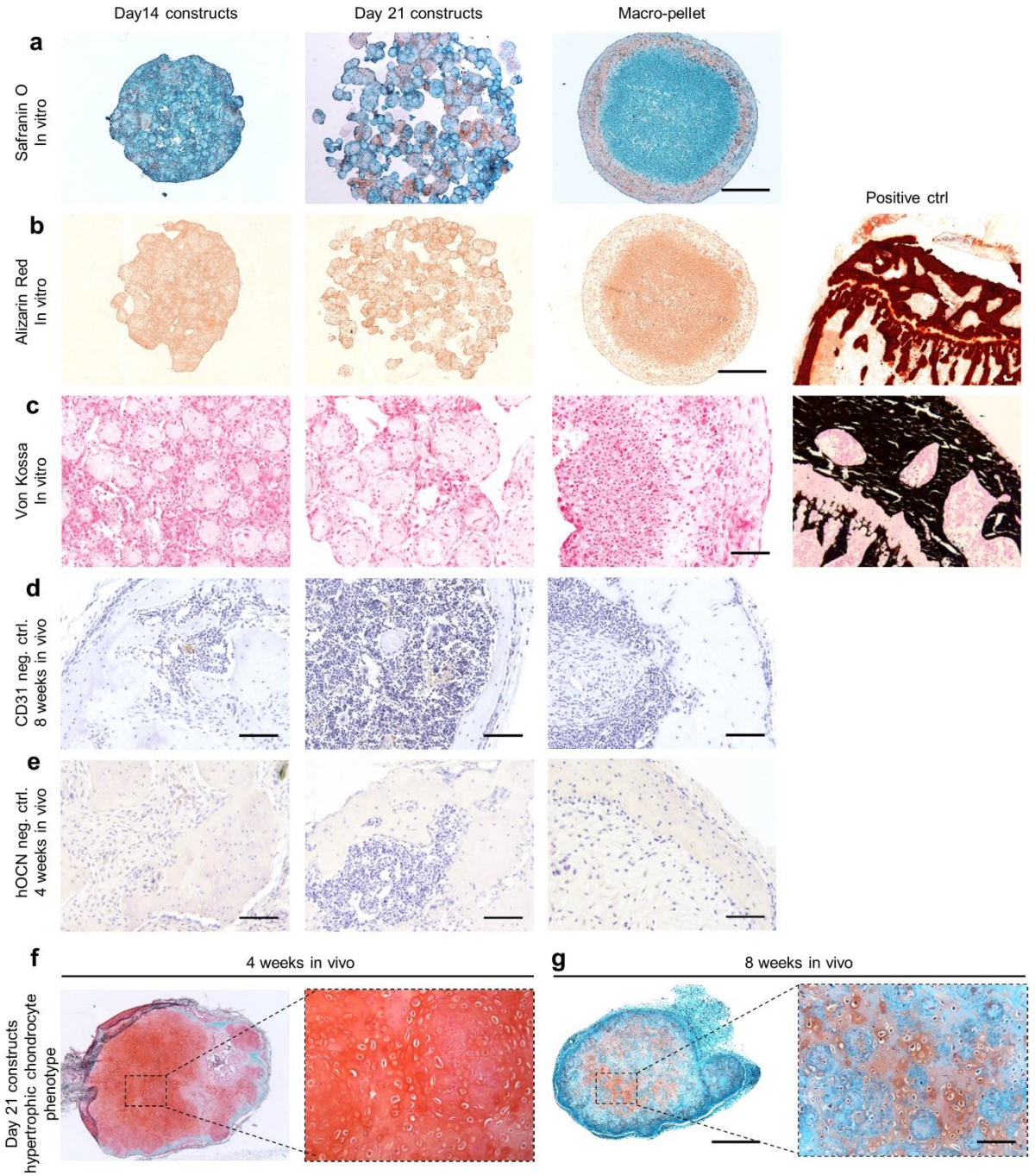

Figure S3. Assembly of callus organoids results in homogenous constructs able to form bone *in vivo*. a) Safranin O staining of constructs analyzed: Day 14, day 21 constructs and Macro-pellet. b) Alizarin Red staining and c) Von Kossa staining with positive control demonstrated lack of mineralization in fused constructs and Macro-pellet *in vitro*. d) Negative control (excluding primary antibody) of CD31 immunostaining after 8 weeks *in vivo* and e) hOCN immunostaining after 4 weeks *in vivo*. f-g) Safranin O staining of day 21 constructs after 4 and 8 weeks *in vivo*, representing a hypertrophic phenotype. Scale bars a-b: 500 µm, c-e: 100 µm, f-g: 500 µm (left) and 100 µm (right).

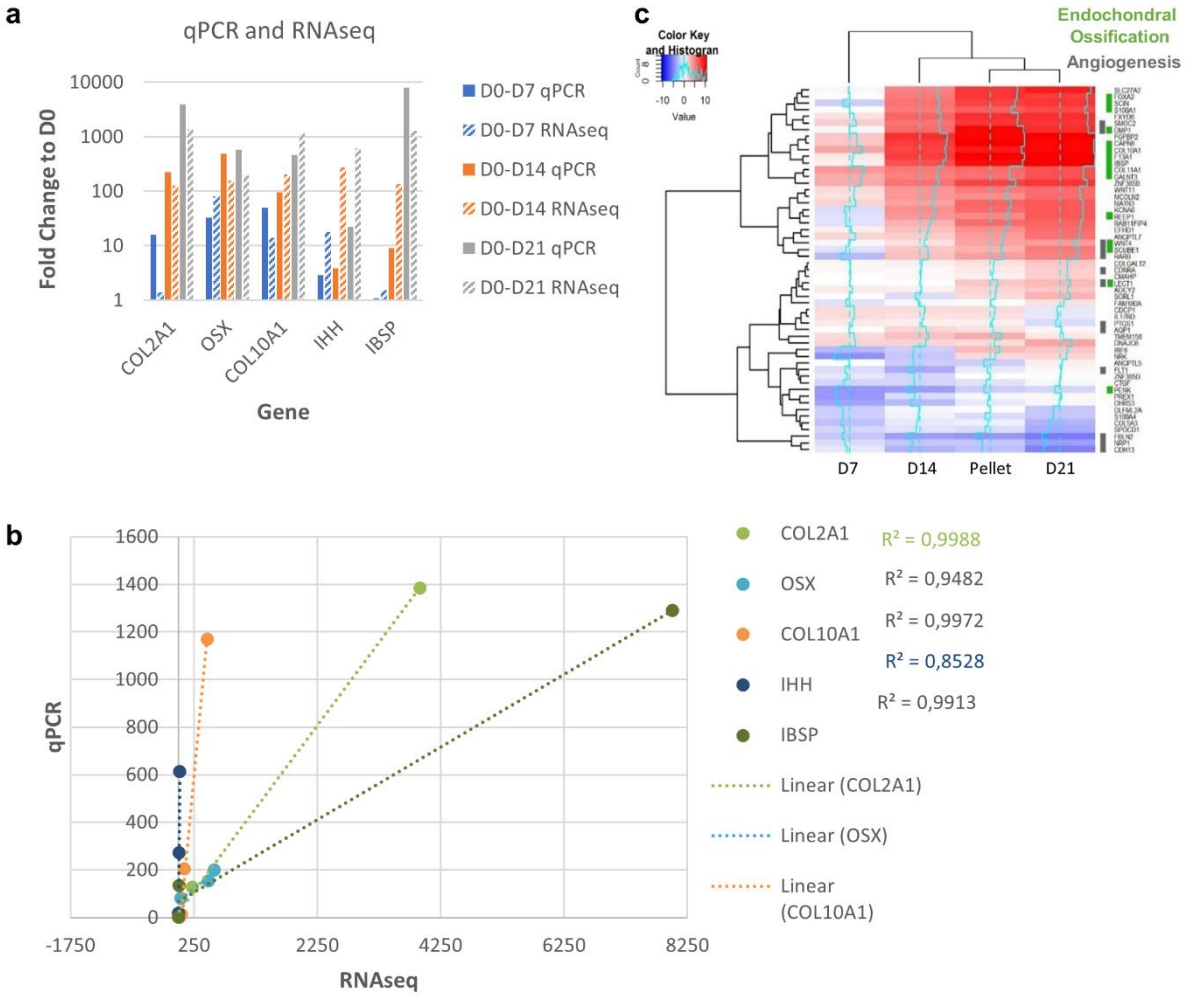

Figure S4. RNA sequencing analysis of callus organoid maturation. a) Comparison of key mRNA transcripts quantified with qPCR and RNA-seq demonstrating b) high linear fit for the two methods. c) Heat map of the 55 differentially expressed genes between D14 and D21 spheroids (green dots, Figure 5a) represented overtime and associated with endochondral ossification (green bar) and/or angiogenesis (grey bar).

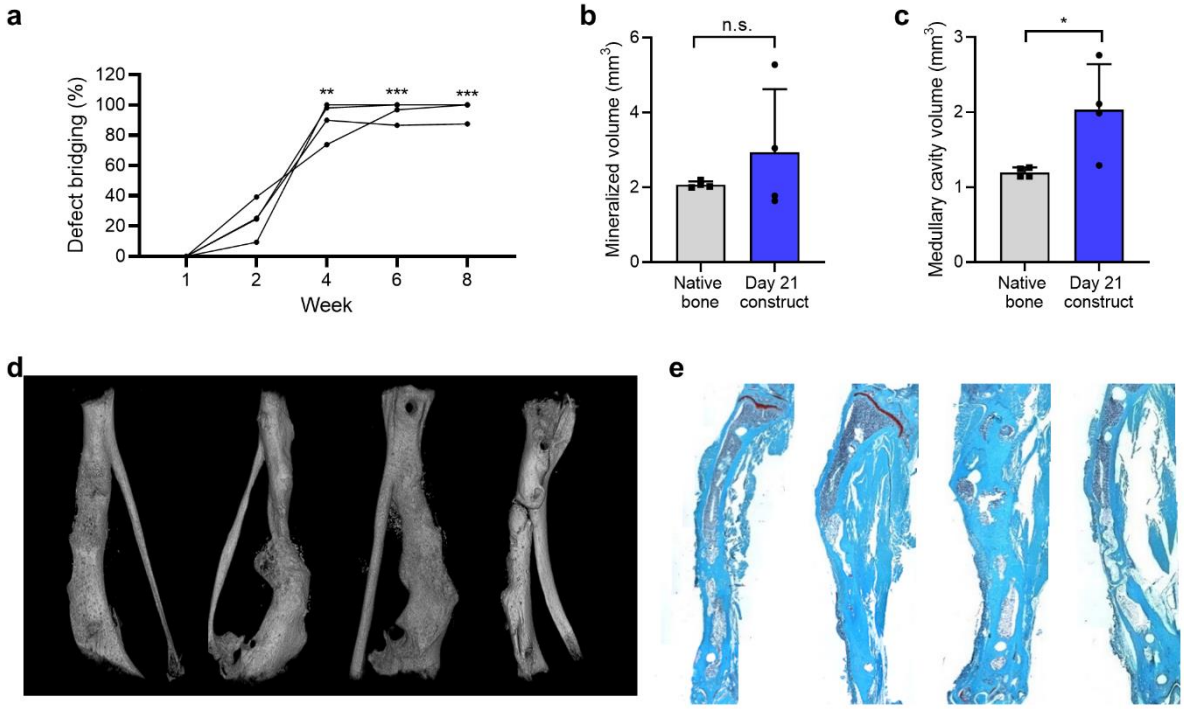

Figure S5. Fused callus organoids (Day 21 construct) heal critical-sized long bone defects in mice (n = 4 animals). a) X-ray analysis of bone bridging over time defined by mineral bridging of the defect on two sides of the defect. ANOVA and Tukey's multiple comparisons test. **p < 0.01; ***p < 0.001 compared to Week 1. b) Comparison between native tibia and healed defect 8 weeks after construct implantation demonstrated by ex vivo nano-CT quantification of b) mineralized volume (mm^3) and c) medullary cavity volume (mm^3); unpaired t-test; *p < 0.05. d) 3D reconstruction of nano-CT scans and e) histological Safranin O staining for all four animals 8 weeks after construct implantation.

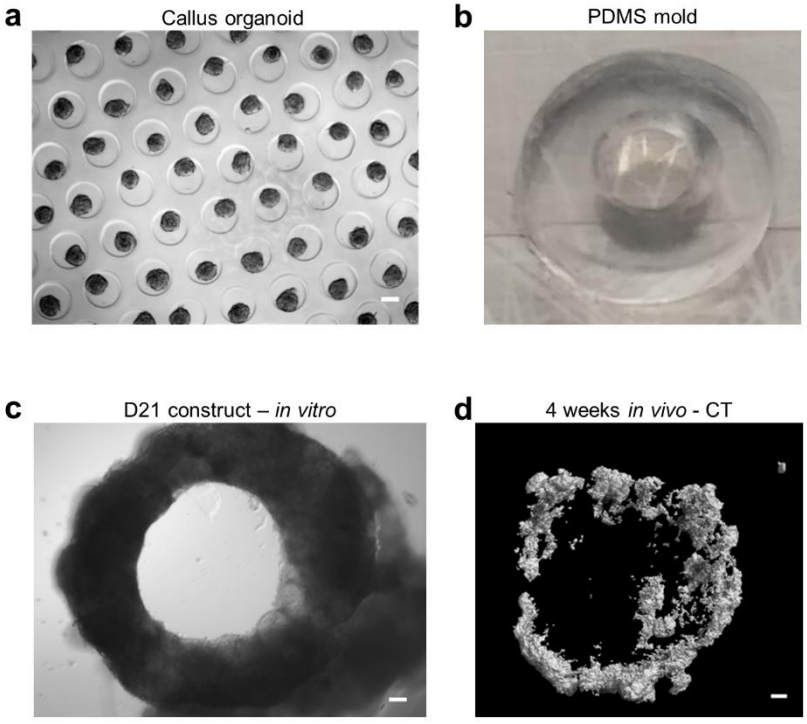

Figure S6. Formation of a ring-shaped mineralized tissue. a) callus organoids cultured in micro-wells for seeding in a ring-shaped agarose well made from b) an in-house designed PDMS mold. c) Mature callus organoids (D21) were assembled in the agarose well and fused for 24h resulting into a ring-shaped construct. d) A mineralized ring was formed after 4 weeks ectopic implantation. Scale bars a, c-d: 100 µm.

Supporting Tables

Table S1. Genes significantly up-regulated D14-21 and associated with endochondral ossification.

Gene	Fold Change	Adj.p-value	Full name	Description	Localization
IBSP	9,64	0,00017	Bone Sialoprotein	Regulates both chondrocyte proliferation and apoptosis as well as transition from cartilage to bone during development of endochondral bone. [1]	Extracellular
LECT1	7,47	0,00015	Chondromodulin (CNMD)	Expressed in the avascular zone of prehypertrophic cartilage and its expression decreases during chondrocyte hypertrophy and vascular invasion. The mature protein likely plays a role in endochondral bone development by permitting cartilaginous anlagen to be vascularized and replaced by bone. [2]	Extracellular
WNT4	6,65	0,03795	Wnt Family Member 4	Wnt4 accelerates chondrocyte differentiation. [3]	Plasma membrane, extracellular
RARB	6,50	0,012	Retinoic Acid Receptor Beta	Retinoid signaling crucial for chondrocyte maturation and endochondral ossification. [4]	Nucleus
FOXA2	6,41	0,00181	Forkhead Box A2	Detected in hypertrophic zone and mice engineered to lack expression of both FoxA2 and FoxA3 in their chondrocytes display defects in chondrocyte hypertrophy. [5]	
REEP1	6,35	0,00002	Receptor Accessory Protein 1	Increased expression in growth plate cartilage as compared to articular cartilage. [6]	endoplasmic reticulum
F13A1	6,23	0,00626	Coagulation Factor XIII A Chain	Increased expression in the hypertrophic zone compared to the proliferative/resting zone of the growth plate. [7]	extracellular
COL10A1	5,81	0,00045	Collagen Type X Alpha 1 Chain	ECM component secreted by hypertrophic chondrocytes. [8]	Extracellular, endoplasmic reticulum
DMP1	5,61	0,00002	Dentin Matrix Acidic Phosphoprotein 1	Critical for proper mineralization of bone. [9,10]	Extracellular, endoplasmic reticulum
SCIN	5,31	0,00017	Scinderin	Restricted to hypertrophic chondrocytes of the embryonic growth plate. Crucial for bone resorption. [11]	Extracellular
PENK	4,89	0,00000	Proenkephalin	Opioid. Expressed in the skeletal tissues, bone, and cartilage. Inhibit ALP expression in osteoblasts. [12]	Extracellular, endoplasmic reticulum
CAPN6	4,48	0,00004	Calpain 6	Calcium-dependent cysteine protease. Involved in proteoglycan degradation during bone development and fracture healing (rat). [13]	Cytoskeleton, cytosol
S100A1	4,10	0,00014	S100 Calcium Binding Protein A1	Transcriptional target of SOX trio and is present in late proliferative and prehypertrophic chondrocytes of mice growth plate. [14]	Extracellular, nucleus, endoplasmic reticulum
GALNT3	3,56	0,00262	Polypeptide N-Acetylgalactosaminyltransferase 3	Runx2 target gene in chondrocytes. Galnt3 is also likely to regulate chondrocyte maturation by modifying the glycosylation of Acan. [15]	Extracellular, golgi apparatus
COL11A1	2,91	0,01385	Collagen Type XI Alpha 1 Chain	A fibrillar collagen gene, critically involved in spatial organization of growth plate chondrocytes. [16]	Extracellular, endoplasmic reticulum

Table S2. Genes significantly up-regulated D14-21 and associated with angiogenesis.

Gene	Fold Change	Adj.p-value	Full name	Description	Localization
IRF6	17,55	0,0065	Interferon regulatory factor 6	Transcription factor that plays a role in late endothelial progenitor cells. [17]	Nucleus, cytoplasm, extracellular
ANGPTL7	13,87	5,16E-16	Angiopoietin Like 7	Stimulate endothelial cell proliferation *in vitro* and vascularization *in vivo.* [18]	Extracellular
FLT1 (VEGFR-1)	12,38	0,00046	Vascular Permeability Factor Receptor	Receptor for vascular endothelial growth factor (VEGF), an essential mediator of angiogenesis. [19]	Plasma membrane, extracellular, cytoskeleton, endosome
SCUBE1	8,76	1,29E-06	Signal Peptide, CUB Domain and EGF Like Domain Containing 1	Cell surface glycoprotein associated to vascular biology. [20]	Plasma membrane, extracellular
LECT1 (CNMD)	7,47	0,00015	Chondromodulin	Expressed in the avascular zone of prehypertrophic cartilage. The mature protein likely plays a role in endochondral bone development by permitting cartilaginous anlagen to be vascularized and replaced by bone. [2]	Extracellular
WNT4	6,65	0,038	Wnt Family Member 4	Promote MSC-mediated angiogenesis. [21]	Plasma membrane, extracellular
RARB	6,50	0,012	Retinoic Acid Receptor Beta		Nucleus
SMOC2	5,76	0,00032	SPARC Related Modular Calcium Binding 2	Angiogenic factor that potentiates angiogenic effects of growth factors. [22]	Extracellular
DMP1	5,61	0,00002	Dentin Matrix Acidic Phosphoprotein 1	Suggested as an inhibitor of VEGF-induced angiogenesis. [23]	Extracellular, endoplasmic reticulum
EDNRA	3,12	0,0081	Endothelin Receptor Type A	EDNRA is involved in the inhibition of angiogenesis in the retina. [24]	Plasma membrane
AQP1	0,36	0,045	Aquaporin 1	Targeted AQP1 gene disruption in mice reduces angiogenesis *in vivo.* [25]	Plasma membrane, extracellular, nucleus
FBLN2	0,34	0,041	Fibulin 2	Suppress angiogenesis in tumors *in vivo.* [26]	Extracellular
NRP1	0,27	0,017	Neuropilin 1	Stimulates angiogenesis in endothelial cells *in vitro.* [27]	Plasma membrane, extracellular
PTGS1 (COX-1)	0,25	0,00047	Prostaglandin-Endoperoxide Synthase 1	Regulates angiogenesis in endothelial cells *in vitro.* [28]	Extracellular, nucleus, ER, golgi
CDH13	0,23	0,00046	Cadherin 13	Code for T-cadherin involved in vascular homeostasis. [29]	Plasma membrane, extracellular

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