Supplement to Maes et al.

Supplemental methods

Murine model of semi-stabilized fracture repair

A transverse bone fracture was induced in the left tibia of 11-week-old mice, under pentobarbital anesthesia (100 mg/kg i.p.). First, a longitudinal incision of approximately 8 mm was made in the skin, on the anterior side of the lower leg. The proximal tibia was exposed by carefully inserting a small rod transversally underneath the tibia, thereby gently placing the surrounding muscles aside. Then, a small transversal bore-injury was created in the tibia at approximately 0.5 cm distal from the knee using a small-diameter (26 G) needle, in order to weaken the bone to facilitate localized fracture induction and avoid splintering of the cortex. A steel pin (0.4 mm diameter) was inserted into the tibia at the proximal articular surface and localized as to longitudinally span the bone through the marrow cavity (Figure S1A). Then, the pin was retracted and subsequently, micro-pincers were used to induce the fracture, taking care not to injure the surrounding tissues (Figure S1B). The full thickness of the break (through both cortical surfaces) was assured. The pin was re-entered to function as an intramedullary fixating pin semi-stabilizing the fracture, and cut at the articular surface of the proximal tibia (Figure S1C). To avoid displacement of the tibia halves, the fibula was cut at mid-diaphysis, as also seen on the radiographic images further illustrating the fracture model (Figure S1D-F).

Histology, immunohistochemistry and in situ hybridization

Mice at PFD3 were injected with 150 µg of BrdU (Sigma-Aldrich) in saline per g body weight 2.5 h prior to sacrifice. For histological examination, mice were sacrificed at PFD3, 8, 13 and PFW3 and the fractured tibia was carefully isolated. Bones were fixed in 2% paraformaldehyde overnight

and decalcified in 0.5 M EDTA (pH 7.4)/PBS for 10 days at 4°C prior to dehydration, paraffin embedding and sectioning (4 μm). Sections were stained with H&E, SO combined with Fast green stain to visualize cartilage proteoglycans (stained red), or the collagen dye Sirius red. Additional sections were reacted for TRAP activity, or used for CD31, CD45, BrdU, MMP-9, collagen 2 or osteocalcin immunohistochemistry. In situ hybridization was performed using complementary ^{35}S-labeled riboprobes for collagen 10 and Runx2/Cbfa1. Staining methods were as described before (6,7). Additional tibias at PFD13 were fixed in Burckhardt's solution, embedded undecalcified in methyl-methacrylate, sectioned at 4 μm, and stained with Von Kossa's stain and toluidine blue.

Supplemental table 1. Oligonucleotide sequences used in real-time (quantitative, q) RT-PCR

Gene	(A)	Sequence
Primers and probes used for qRT-PCR on tissue samples		
Collagen 2	F	5'-AGAACATCACCTACCACTGTAAGAACA-3'
	R	5'-TGACGGTCTTGCCCCACTT-3'
	P	5'-FAM-CCTTGCTCATCCAGGGCTCCAATG-TAMRA-3'
Collagen 10	F	5'-CCTTTCTGCTGCTAATGTTCTTGA-3'
	R	5'-ATGCCTTGTTCTCCTCTTACTGGA-3'
	P	5'-FAM-TAGCCCCAAGACACAATACTTCATCCCATACG-TAMRA-3'
Cathepsin K	F	5'-TGTGGACTGTGTGACTGAGAATTATG-3'
	R	5'-CCTTTGCCGTGGCGTTAT-3'
	P	5'-FAM-CCTCCGTTCTGCTGCACGTATTGGAAG-TAMRA-3'
Ihh	F,R,P	(S1) [B]
MMP-13	F,R,P	(S1) [B]
MT1-MMP	F,R,P	(S1) [B]
VEGF	F,R,P	(S2) [B]
PlGF	F,R,P	(S2) [B]
MMP-9	F,R,P	(S2) [B]
VEGFR-1	F,R,P	(S3) [B]
VEGFR-2	F,R,P	(S3) [B]

NRP1	F,R,P	(S3) [B]
HPRT	F,R,P	(S3) [B]

Primers used for (q)RT-PCR on MAPC samples

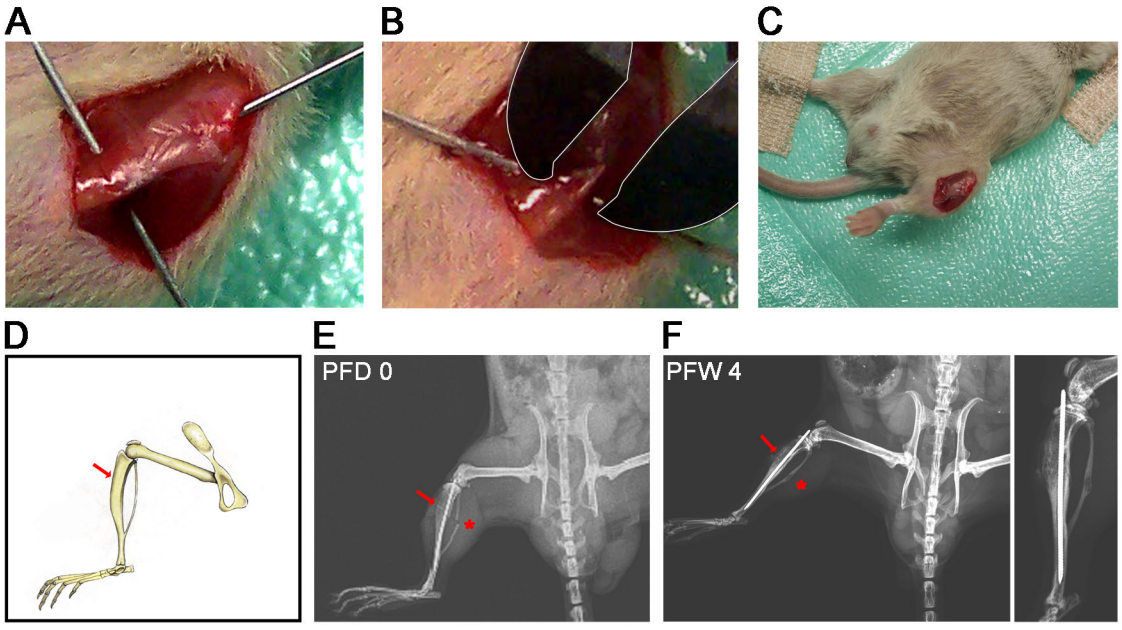
VEGFR-1	F	5'-CTGTGCGGAAATCTTCAAGTCA-3'
	R	5'-CCTTGATCTCCTCTGTGGAGTTG-3'
	P	SybrGreen
Osterix	F	5'-CGGGGAAGAAGAAGCCAATC-3'
	R	5'-GAAGAAAAAGTTGAGGAGGTCGG-3'
Osteocalcin	F	5'-TCTGCTGACCCTGGCTGCG-3'
	R	5'-GCTGCTGTGACATCCATACTTGC-3'
Runx2	F,R	(S4) [B]
ALP [A]	F,R	(S5) [B]
BSP [A]	F,R	(S5) [B]

(A) Abbreviations: F, Forward primer; R, Reverse primer; P, Probe.

(B) References: (**S1**) Maes, C., Stockmans, I., Moermans, K., Van Looveren, R., Smets, N., Carmeliet, P., Bouillon, R., and Carmeliet, G. 2004. Soluble VEGF isoforms are essential for establishing epiphyseal vascularization and regulating chondrocyte development and survival. *J. Clin. Invest.* **113**: 188-199. (**S2**) Maes, C., Carmeliet, P., Moermans, K., Stockmans, I., Smets, N., Collen, D., Bouillon, R., and Carmeliet, G. 2002. Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mech. Dev.* **111**: 61-73. (**S3**) Carmeliet, P., Ng, Y.-S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V.V., Stalmans, I., Mattot, V., et al. 1999. Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular

endothelial growth factor isoforms VEGF164 and VEGF188. *Nat. Med.* **5**: 495-502. (**S4**) Fujita, T., Fukuyama, R., Izumo, N., Hirai, T., Meguro, T., Nakamuta, H., and Koida, M. 2001. Transactivation of core binding factor alpha1 as a basic mechanism to trigger parathyroid hormone-induced osteogenesis. *Jpn. J. Pharmacol.* **86**: 405-416. (**S5**) Tropel, P., Noel, D., Platet, N., Legrand, P., Benabid, A.L., and Berger, F. 2004. Isolation and characterisation of mesenchymal stem cells from adult mouse bone marrow. *Exp. Cell Res.* **295**: 395-406.

Figure S1



Supplemental figure legend

Figure S1. Murine model of semi-stabilized tibia fracture healing.

(**A-C**) Illustration of the surgical procedure employed to induce the fracture. (**A**) The tibia was first exposed by carefully inserting a transversal rod under the tibia. An intramedullary pin was inserted to facilitate its correct positioning when re-entered after fracture induction. (**B**) The pin was retracted, a transversal bore-injury was created at the fracture target site, and the fracture was induced using the sharp tips of the micro-pincers shown in the picture (lined for reasons of clarity). (**C**) Next, the pin was re-entered to semi-stabilize the tibia halves, and cut at the proximal articular surface of the tibia. The transversal rod was removed and the wound was closed using surgical sutures. (**D**) Schematic view localizing the site of fracture induction in the proximal tibia (arrow). (**E,F**) Radiographic images illustrating the tibia fracture model in WT mice. (**E**) Radiograph at PFD0 showing the full thickness (bi-cortical) break and alignment of the tibia halves semi-stabilized by the intramedullary fixating pin. The site of the fracture is indicated (arrow). Also note the break induced in the fibula (asterisk). (**F**) This semi-stabilized fracture model supports aligned healing of the tibia, as seen at PFW4. Also note the callus formation in the fibula (asterisk). The right panel shows a magnified view of the hind limb.