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TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Spontaneous In Vivo Chondrogenesis of Bone Marrow-Derived Mesenchymal Progenitor Cells by Blocking Vascular Endothelial Growth Factor Signaling

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Key Words. Mesenchymal stromal/stem cells • Chondrogenesis • Hypoxia • Vascular endothelial growth factor blockade

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ABSTRACT

Chondrogenic differentiation of bone marrow-derived mesenchymal stromal/stem cells (MSCs) can be induced by presenting morphogenetic factors or soluble signals but typically suffers from limited efficiency, reproducibility across primary batches, and maintenance of phenotypic stability. Considering the avascular and hypoxic milieu of articular cartilage, we hypothesized that sole inhibition of angiogenesis can provide physiological cues to direct in vivo differentiation of uncommitted MSCs to stable cartilage formation. Human MSCs were retrovirally transduced to express a decoy soluble vascular endothelial growth factor (VEGF) receptor-2 (sFlk1), which efficiently sequesters endogenous VEGF in vivo, seeded on collagen sponges and immediately implanted ectopically in nude mice. Although naïve cells formed vascularized fibrous tissue, sFlk1-MSCs abolished vascular ingrowth into engineered constructs, which efficiently and reproducibly developed into hyaline cartilage. The generated cartilage was phenotypically stable and showed no sign of hypertrophic evolution up to 12 weeks. In vitro analyses indicated that spontaneous chondrogenic differentiation by blockade of angiogenesis was related to the generation of a hypoxic environment, in turn activating the transforming growth factor-β pathway. These findings suggest that VEGF blockade is a robust strategy to enhance cartilage repair by endogenous or grafted mesenchymal progenitors. This article outlines the general paradigm of controlling the fate of implanted stem/progenitor cells by engineering their ability to establish specific microenvironmental conditions rather than directly providing individual morphogenic cues. STEM CELLS TRANSLATIONAL MEDICINE *2016;5:1730–1738*

SIGNIFICANCE

Chondrogenic differentiation of mesenchymal stromal/stem cells (MSCs) is typically targeted by morphogen delivery, which is often associated with limited efficiency, stability, and robustness. This article proposes a strategy to engineer MSCs with the capacity to establish specific microenvironmental conditions, supporting their own targeted differentiation program. Sole blockade of angiogenesis mediated by transduction for sFlk-1, without delivery of additional morphogens, is sufficient for inducing MSC chondrogenic differentiation. The findings represent a relevant step forward in the field because the method allowed reducing interdonor variability in MSC differentiation efficiency and, importantly, onset of a stable, nonhypertrophic chondrocyte phenotype.

INTRODUCTION

Bone marrow-derived mesenchymal stromal/stem cells (MSCs) have been proposed for cell-based cartilage regeneration because of their capacity to differentiate into chondrocytes and their availability in an autologous setting. The chondrogenic differentiation of MSCs critically requires the exposure to potent morphogens [1] and is associated with a large degree of donor-to-donor variability. In addition, the preculture of MSCs in the presence of chondroinductive molecules is typically associated with the onset of a hypertrophic chondrocyte phenotype, leading to vessel ingrowth, mineralization, and remodeling into bone tissue upon in vivo implantation [2, 3].

During limb development, chondrogenesis of condensating mesenchymal cells occurs in the absence of vasculature. Moreover, the stability of the chondrocytic phenotype at the articulating

surfaces of long bones, as opposed to progression of differentiation toward endochondral ossification, is associated with the maintenance of an avascular environment [4]. Consistent with these observations, seminal experiments of marrow stromal cells implanted subcutaneously in diffusion chambers, not allowing blood vessel ingrowth, found the spontaneous formation of cartilage matrix, along with bone-like and fibrous tissue [5].

Vascular endothelial growth factor (VEGF) is the master regulator of angiogenesis during skeletal growth [6, 7]. VEGF expression critically regulates cartilage ossification [8] because it is produced by hypertrophic chondrocytes at the start of endochondral bone formation, but not by quiescent and proliferating chondrocytes at earlier stages. VEGF inhibition prevented vascular infiltration of hypertrophic cartilage and ossification of the growth plate during bone elongation [8]. Moreover, systemic VEGF blockade was proposed to promote chondrogenesis at the expenses of osteogenesis upon ectopic grafting of fetal bones or murine skeletal stem cells [9, 10]. The soluble variants of VEGF receptors (VEGF-Rs), namely soluble Flt-1 and Flk-1 (which are the cleaved extracellular domains of VEGF-R1 and VEGF-R2, respectively [11, 12]) are capable of acting as decoy receptors, efficiently sequestering VEGF in the microenvironment of producing cells and leading to blockade of VEGF signaling [13]. Previous work indeed showed that the cartilage repair capacity of skeletal muscle-derived stem cells genetically modified to express bone morphogenetic protein (BMP)-4, a potent chondrogenic inducer, could be improved by concomitant overexpression of soluble Flt-1 in osteochondral defect [14] or osteoarthritis [15] models.

In the present study, we addressed whether the simple inhibition of angiogenesis by VEGF blockade is sufficient to direct in vivo chondrogenic differentiation of implanted human MSCs. The hypothesis was tested in an ectopic environment that is permissive, but not inductive of chondrogenesis [16, 17], and in the absence of any additional delivered chondrogenic morphogens. We demonstrated that MSCs transduced to express sFlk-1 and directly implanted subcutaneously in nude mice without in vitro preculture spontaneously differentiated into the chondrocytic lineage. The resulting cartilage was hyaline in nature and remained phenotypically stable during the observation time of the study, up to 12 weeks. In vitro studies revealed that the hypoxic environment, resulting from the blockade of angiogenesis, could directly cause chondrogenic induction by stimulating activation of endogenous transforming growth factor (TGF) β signaling.

Materials and Methods

Cell Isolation and Transduction

Bone marrow aspirates were harvested from the iliac crest of five healthy donors (four men and one woman; average age, 31 years [range, 20–43 years]) during orthopedic surgical procedures, in accordance with the local ethical committee and after provision of informed consent. Freshly isolated bone marrow [18] was plated at a density of 10^5 nucleated cells per cm^2 and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; complete medium) and 5 ng/ml fibroblast growth factor-2 to maintain MSC differentiation capacity [19, 20]. Six days after the first plating, primary MSCs were transduced with previously described retroviral vectors, carrying the gene for the mouse VEGF-R2 extracellular domain (sFlk-1) fused to the IgG2α Fc domain (provided by C.J. Kuo, Stanford University) [21], linked through an internal ribosomal entry site to a mouse truncated CD8a version [22, 23]. Naïve cells or MSCs transduced only with the truncated murine CD8a were used as controls.

In Vitro Pellet Culture Model

MSCs transduced for sFlk-1 and control cells were cultured for 2 weeks in pellets, as previously described [24], with serum-free medium supplemented with 10 ng/ml of TGFβ3 or without TGFβ3. Unless otherwise stated, all cultures were performed at 20% oxygen tension.

Cell-Based Construct Preparation and Ectopic Implantation

A type I collagen sponge (Ultrafoam, Bard, Davol Inc., Warwick, RI, https://www.davol.com/) of 4-mm thickness was punched into 6-mm diameter disks, each of which was statically seeded with 1.7×10^6 MSCs (density of 1.5×10^7 cells per cm^3). The constructs were cultured overnight with DMEM supplemented with 10% FBS and 1% penicillin-streptomycin before implantation.

Four cell-based constructs were implanted subcutaneously for each nude mouse (CD-1 nude/nude, 6–8 weeks old; Charles River, Germany,). The mice were sacrificed with CO_2 at 1, 4, 8, and 12 weeks after the implantation. Before sacrifice, mice were injected with pimonidazole hydrochloride (Hydroxyprobe, Inc., Burlington, MA, http://www.hypoxyprobe.com) at 4 weeks after implantation. All animals were cared for and processed according to the guidelines of the Cantonal Veterinary Office (Basel, Switzerland) for the care and use of laboratory animals. Each analysis was performed independently in four engineered tissues for each MSC primary.

Flow Cytometry

Transduced cells were analyzed for CD8a (sFlk-1-expressing or control CD8a-expressing MSC) by staining with an allophycocyanin-conjugated anti-mouse CD8a (clone 53–6.7; BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) [23].

Enzyme-Linked Immunosorbent Assay

MSCs cultured in 60-mm dishes were incubated with 1 ml of culture medium for 4 hours. The supernatants were filtered through 0.45-μm syringe filters and frozen. Soluble mouse Flk-1 and human VEGF production were quantified in cell culture supernatants using species-specific Quantikine immunoassay enzyme-linked immunosorbent assay kits (R&D Systems, Abingdon, UK, https://www.rndsystems.com/).

Biochemistry

Engineered tissues cultured in vitro for 3 weeks were analyzed for content of glycosaminoglycan (GAG) and DNA measured spectrophotometrically using dimethylmethylene blue [25, 26] and the CyQUANT Kit (Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com) [27], respectively.

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction

Total RNA of cells was extracted by using Trizol (Thermo Fisher) and the standard single-step acid-phenol guanidinium method. RNA was treated with DNAse-I using the DNA-free Kit (Thermo Fisher). cDNA was generated from 3 μg of RNA by using 500 μg/ml random

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primers (Promega, Wallisellen, Switzerland, http://www.promega.com) and 1 μl of 50 U/ml Stratascript reverse transcriptase (Stratagene, Agilent Technologies, Basel, Switzerland, http://www.agilent.com), in the presence of deoxynucleotides. Real-time reverse transcriptase- polymerase chain reactions were performed and monitored using the ABI Prism 7700 Sequence Detection System (Thermo Fisher), and the gene expression data for all tested genes (types II and X collagen [*COL2A1* and *COL10A1*], Gremlin [*GREM1*], *TGFβ1*, and Indian Hedgehog [*IHH*]) were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

HUVEC Proliferation and Human Dermal Microvascular Endothelial Cell Tube Formation In Vitro Assays

Human umbilical vein endothelial cell (HUVEC) proliferation assay was performed as previously described by Centola et al. [28]. Briefly, supernatants were collected from control and sFlk-1 MSCs after 48 hours in monolayer culture and stored at −20°C until analysis. HUVECs were cultured in 96-well plates at a density of 5×10^3 cells per well in growth medium (M199 supplemented with 20% FBS, 100 μg/ml endothelial cell growth supplement, 50 IU/ml heparin, 100 IU/ml penicillin, and 100 μg/ml streptomycin) for an initial 2 days and in low-FBS medium (assay medium, consisting of M199 with 5% FBS, 10 IU/ml heparin, and 1% penicillin/streptomycin), supplemented with recombinant human VEGF (R&D Systems) at different concentrations (0–20 ng/ml), mixed in equal volume to the supernatants for the following 2 days. HUVEC metabolic activity was measured by MTS assay (Cell Titer 96, Promega). Data are presented by using optical density arbitrary units, from $n = 3$ independent specimens.

In vitro tube formation assay was performed with human dermal microvascular endothelial cells (HDMECs) as previously described [29], either by using control or sFlk-1 MSC conditioned media (Endothelial Cell Basal Medium MV [PromoCell, Heidelberg, Germany, http://www.promocell.com/] with 5% FBS and 1% penicillin-streptomycin solution) extracted after 48 hours or by direct coculture of control or sFlk-1 MSC. Tube-like structures were visualized with CD31 staining (Dako, Glostrup, Denmark, http://www.dako.com) and a secondary goat anti-mouse antibody (Alexa Fluor 488, Thermo Fisher); nuclei were stained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com/).

Histology

Engineered tissues were fixed with 4% formaldehyde and embedded in paraffin or optimal cutting temperature medium. Paraffin sections (5 μm thick) were stained for Safranin-O and for immunohistochemistry for types II and X collagen, bone sialoprotein, and metalloproteinase-13 (MMP-13). Histological sections stained for CD31 [30] were analyzed for vessel quantification in the total implantation bed area using NIS-Elements 4.0 software (Nikon Instruments Inc., Melville, NY, https://www.nikoninstruments.com). The vessel number was then normalized to area, with values reported as vessels/mm^2.

Cryosections (10 μm thick) were incubated with the primary antibodies pan human antinuclei (BD Pharmingen, Franklin Lakes, NJ, http://www.bd.com) and with a secondary antibody labeled with Alexa Fluor 546 (Thermo Fisher). 4′,6-Diamidino-2-phenylindole was used as nuclear staining.

Histological Scoring System

The proposed histological score system for the assessment of the quality of in vivo engineered cartilage tissues derives from a modification of the categories published by Centola et al. [28]. Scoring rates are assessed blindly by three different observers.

Intensity describes the uniformity and darkness of Safranin O-fast green stain: no stain, 0; weak staining of poorly formed matrix, 1; moderately even staining, 2; even dark stain, 3.

Density represents the distance between cells/amount of matrix: high cell densities with no matrix in between, 0; high cell densities with little matrix in between, 1; moderate cell density with matrix, 2; low cell density with moderate distance between cells and an extensive matrix, 3.

Morphology represents the cell appearance: condensed/necrotic/pycnotic bodies, 0; spindle/fibrous, 1; mixed spindle/fibrous with rounded chondrogenic morphology, 2; majority rounded/chondrogenic, 3.

Calcifications observed by alizarin red staining within the cartilage newly formed: strong presence of abnormal calcifications inside the neo-formed cartilage, 0; presence of some calcification spots inside the newly formed cartilage, 1; presence of very few calcification spots inside the repaired cartilage, 2; complete absence of calcifications throughout the newly formed cartilage, 3.

Final score classifies the following:

- Not-formed cartilage (mainly fibrotic tissue): 0–6
- Inhomogeneous and fibrocartilaginous tissue, with few and nonconnected areas of newly formed cartilage: 6.1–11
- Good cartilaginous tissue, covering less than 60% of the tissue cross-sectional area: 11.1–15
- Very good quality and homogeneity of newly formed cartilage: 15.1–18

Statistical Analysis

Data were analyzed with the statistical software Prism 5.0 (GraphPad Software Inc., La Jolla, CA, http://www.graphpad.com/), using one-way analysis of variance with Bonferroni correction. (In the figures, $*$ indicates $p < .05$ and $**$ indicates $p < .01$.) Data are presented as mean $\pm$ SD.

RESULTS

Transduced MSCs Maintain Their Proliferation and Differentiation Capacity

MSCs were transduced with retroviruses expressing sFlk-1 together with a truncated version of mouse CD8a, used as a convenient marker sortable by fluorescence activated cell sorting as described elsewhere [22], or CD8a alone as a control, with a transduction efficiency of 82% $\pm$ 7% and 89% $\pm$ 3%, respectively. Genetically modified MSCs released 1.37 $\pm$ 0.14 ng/10^6 cells of mouse sFlk-1 per day. Consistent with our previous findings [23], transduction caused a slight, not statistically significant, reduction in MSC proliferation during the first 2 passages (total cell doublings in 14 days: naïve, 5.6 $\pm$ 0.5; CD8a, 4.5 $\pm$ 0.8; sFlk-1, 3.9 $\pm$ 0.2; p = N.S.). MSC chondrocytic differentiation in vitro, in the presence of TGFβ3, was not impaired by transduction (GAG/DNA ratio: naïve, 10.3 $\pm$ 6.1; CD8a, 10.6 $\pm$ 10.1; sFlk-1, 17.7 $\pm$ 14.8; p = N.S.) (supplemental online Fig. 1A). In the absence of TGFβ3 supplementation, no chondrogenesis was observed in any population (supplemental online Fig. 1A). Pellets by naïve and CD8a MSCs also released similar amounts of VEGF, at both 20% and 2% oxygen (supplemental online Fig. 1B). On the basis of these results, in subsequent experiments only naïve MSCs were used as controls.

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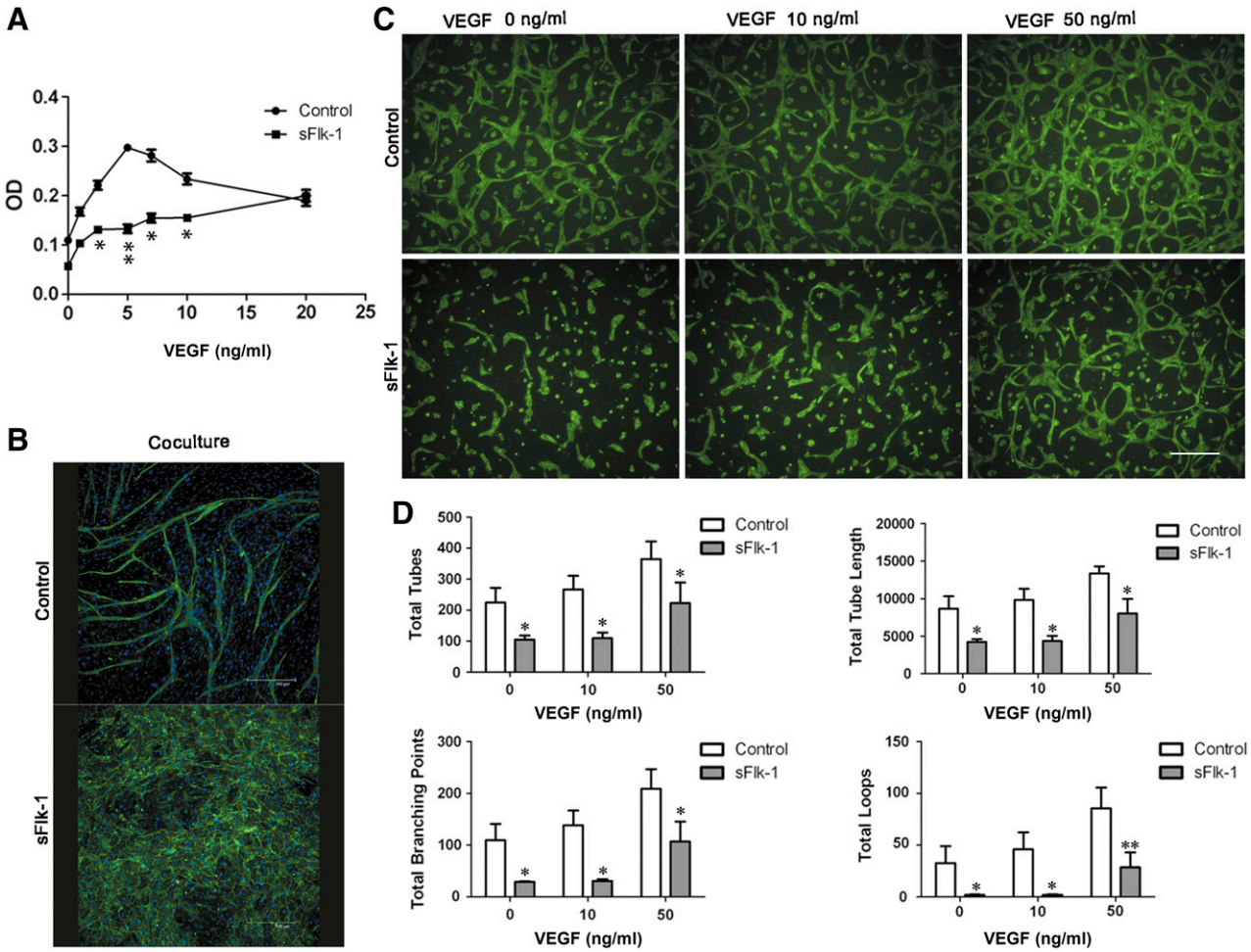

Figure 1. Inhibitory effect of sFlk-1 expressed by bone marrow-derived mesenchymal stromal/stem cells (MSCs) on in vitro endothelial organization and proliferation. Human umbilical vein endothelial cell proliferation assay performed by using supernatants collected by naïve (control) or sFlk-1-expressing MSCs. **(A):** Cell metabolic activity was measured on the basis of the colorimetric MTS assay and represented by using optical density units. **(B):** Immunofluorescence staining for CD31 in green showing the capillary-like structure formation in the direct coculture of either control or sFlk-1-expressing MSCs with human dermal microvascular endothelial cells. **(C):** Representative images of tubular structure formation of endothelial cells (stained with calcein assay medium in green after 48 hours) cultured in medium conditioned by control or sFlk-1 expressing MSCs supplemented with 0, 10, or 50 ng/ml of VEGF. Scale bars = 300 μm. **(D):** Total tube number, length, branching points, and loops were quantified by image analysis. Ten images per sample were analyzed. Data are presented as mean $\pm$ SD (n = 4 samples/group from 2 independent experiments). $*$, $p < .05$; $**$, $p < .01$. Abbreviations: OD, optical density; VEGF, vascular endothelial growth factor.

Interestingly, although VEGF protein was strongly reduced in the supernatants of sFlk-1 constructs at both 20% and 2% oxygen (supplemental online Fig. 1B), VEGF expression at the mRNA level was not downregulated in sFlk-1 constructs at either oxygen percentage (values ranging from 0.05- to 0.18-fold of GAPDH), confirming the potency of sFlk-1 in sequestering released VEGF.

sFlk-1 MSCs Inhibit Angiogenesis In Vitro

The biological activity of sFlk-1 MSCs to block VEGF-induced angiogenesis was first tested in vitro. Supernatants conditioned by sFlk-1 MSCs for 48 hours inhibited proliferation of HUVECs induced by VEGF concentrations up to 10 ng/ml (Fig. 1A), showing that sFlk-1 produced by transduced cells was biologically active. Because sFlk-1 works by competitive inhibition, the effect could be overcome by very high VEGF concentrations (20 ng/ml). Moreover, the direct coculture of HDMECs with sFlk-1 MSC blocked the in vitro formation of capillary-like structures compared with control MSCs (Fig. 1B). Effective inhibition was confirmed with conditioned media in the absence of MSCs: sFlk-1 secreted by transduced MSCs substantially reduced HDMEC organization into tubular structures in a three-dimensional (3D) collagen/fibrin gel-based culture model compared with control supernatants (Fig. 1C), as shown by the decreased tube number and length, as well as by the lower branching points and number of loops (Fig. 1D). The effects of 10 ng/ml of VEGF were completely abolished, while they were reduced with 50 ng/ml, showing a dose-dependent blockade by sFlk-1.

sFlk-1 MSCs Inhibit Angiogenesis In Vivo

Control or sFlk-1 MSCs were then loaded into collagen sponges and immediately implanted subcutaneously in nude mice for assessment of vascularization. After 12 weeks, control constructs appeared macroscopically vascularized, whereas sFlk-1 implants were predominantly avascular, with some blood vessels visible only in discrete peripheral regions (Fig. 2A). Quantification of vessels after CD31 immunohistochemical staining in the total area of

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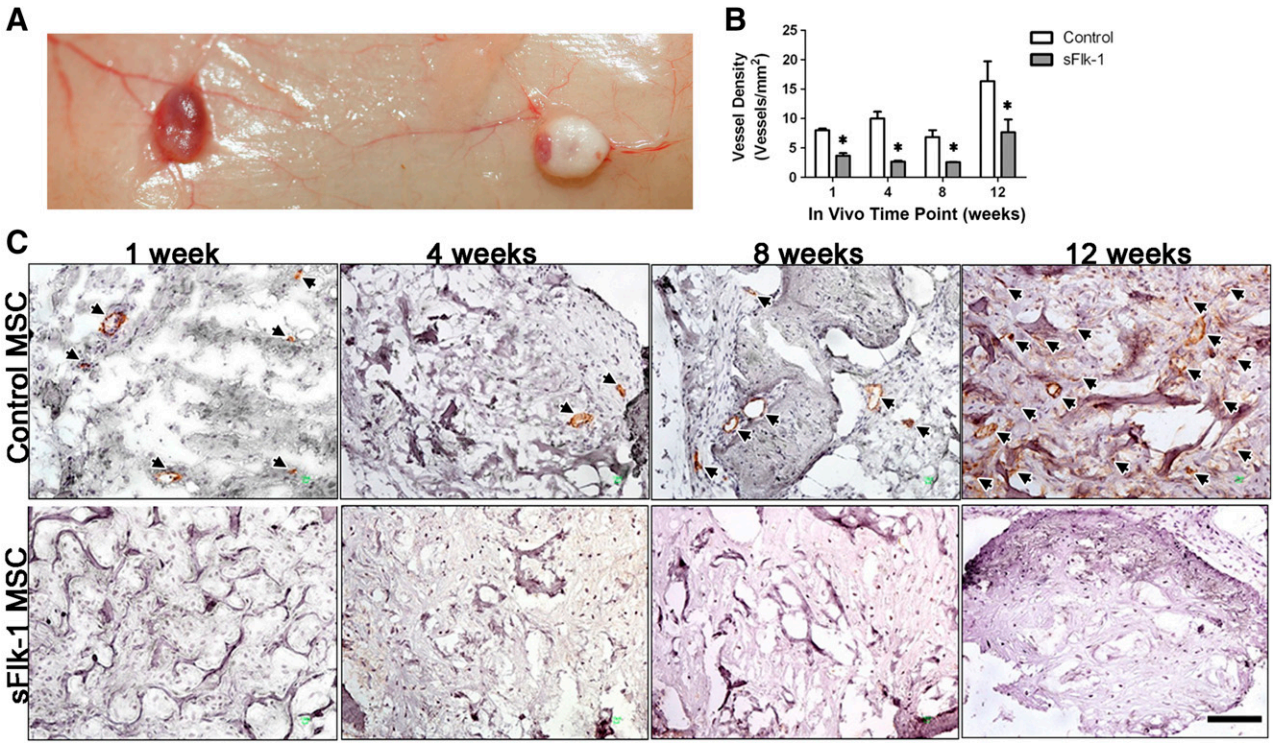

Figure 2. In vivo blocking of angiogenesis. **(A):** Representative macroscopic picture of the explants of engineered tissue generated by naïve (left) or sFlk-1-expressing (right) MSCs after 12 weeks in vivo. **(B):** Vessel density quantified within the total area of the implant generated by control (naïve) or sFlk-1 MSCs at different time points by histomorphometric analysis (n = 3–4). $*$, $p < .05$. **(C):** Immunohistochemistry for mouse CD31. Representative images at $\times$20 magnification include the central implant area generated by naive (top row) or sFlk-1 (bottom row) MSCs at 1, 4, 8, or 12 weeks. Black arrowheads indicate the blood vessels. Scale bar = 100 μm. Abbreviation: MSC, bone marrow-derived mesenchymal stromal/stem cell.

explanted tissues indicated a greatly reduced vessel density with sFlk-1 MSCs compared with control cells throughout the 12-week observation time (Fig. 2B). Histological analysis revealed no vessel infiltration within the actual sFlk-1 constructs up to 12 weeks (Fig. 2C), whereas the few vessels that could be quantified were confined in the surrounding connective tissue at the periphery of the implant. To the contrary, constructs based on control MSCs displayed vessel ingrowth up to the center of the implants already after 1 week, further progressing until 12 weeks (Fig. 2C).

sFlk-1 MSCs Undergo Spontaneous Chondrogenesis In Vivo Without Hypertrophy

Histologically, no cartilage formation was observed in collagen sponges loaded with control MSCs from any of the five donors tested, at any time point (Fig. 3A, 3B). Instead, implants based on sFlk-1 MSC from the same donors displayed foci of chondrogenesis in all cases. In these regions, cells acquired a typically chondrocytic morphology within round lacunae, surrounded by abundant extracellular matrix positively stained for both GAG and type II collagen (Fig. 3A, 3B). Together, these features indicate the hyaline, as opposed to fibrous, nature of the generated cartilage tissues [31]. Formation of cartilaginous structures was already visible by 4 weeks and further increased in quantity and staining intensity by 12 weeks (supplemental online Table 1). The newly formed cartilage only sporadically displayed foci of calcification in scattered peripheral regions but was neither resorbed nor remodelled into osteoid tissue. Immunofluorescence staining for human nuclei indicated that sFlk-1 MSCs persisted in the formed cartilaginous tissue up to 12 weeks after in vivo implantation, whereas control MSCs could be detected at a much lower frequency up to 4 weeks in vivo and a negligible fraction survived by 12 weeks (Fig. 3A, 3B). The result is consistent with the loss of implanted MSC not sufficiently preconditioned in vitro with differentiating stimuli [3]. Interestingly, MSCs from only 3 of the same 5 donors were capable of in vitro chondrogenesis in pellet cultures stimulated with the strong inducer TGFβ3. This indicates the robustness of spontaneous chondrogenesis enabled by VEGF blockade, despite the known interdonor variability in the chondrocytic differentiation potential of MSCs.

After 12 weeks in vivo, cartilaginous constructs generated by sFlk-1 MSCs remained negative for markers of hypertrophic transformation, namely type X collagen and bone sialoprotein (Fig. 4). Phenotypic stability was further confirmed by the absence of expression of MMP-13 (Fig. 4), a hallmark of cartilage remodeling during endochondral ossification [3].

Low Oxygen Tension Triggers MSC Chondrogenesis Even in the Absence of TGFβ

Staining with pimonidazole hydrochloride (Hypoxyprobe-1), a marker of hypoxia, indicated that cells in the tissues formed by sFlk-1 MSCs were exposed to oxygen levels lower than 1%, consistent with the absence of vascularization (supplemental online Fig. 2). On the basis of this finding, we tested whether hypoxic conditions could trigger chondrogenesis in vitro and possibly dispense with the requirement for TGFβ3 stimulation to differentiate MSCs into chondrocytes. In the presence of TGFβ3, naïve MSCs generated cartilaginous pellets, whose quality was independent of oxygen tension, as shown by GAG and type II collagen

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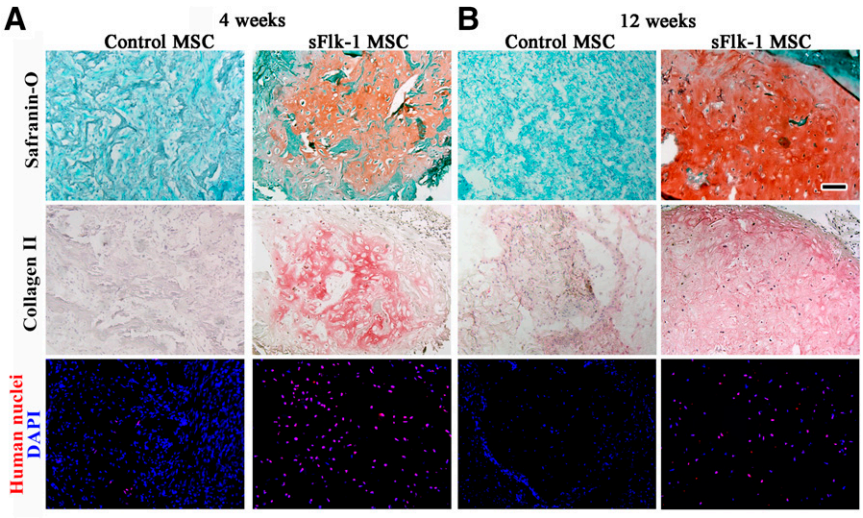

Figure 3. In vivo chondrogenesis. Histological staining with Safranin-O for glycosaminoglycans and immunohistochemistry for type II collagen of engineered tissue generated by naïve (control) or sFlk-1 MSCs after 4 **(A)** or 12 **(B)** weeks in vivo. Fluorescence staining with DAPI (in blue) and a specific anti-human nuclei antibody (in red) of constructs generated by control or sFlk-1 MSCs after 4 **(A)** or 12 **(B)** weeks in vivo. Scale bar = 100 μm. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; MSC, bone marrow-derived mesenchymal stromal/stem cell.

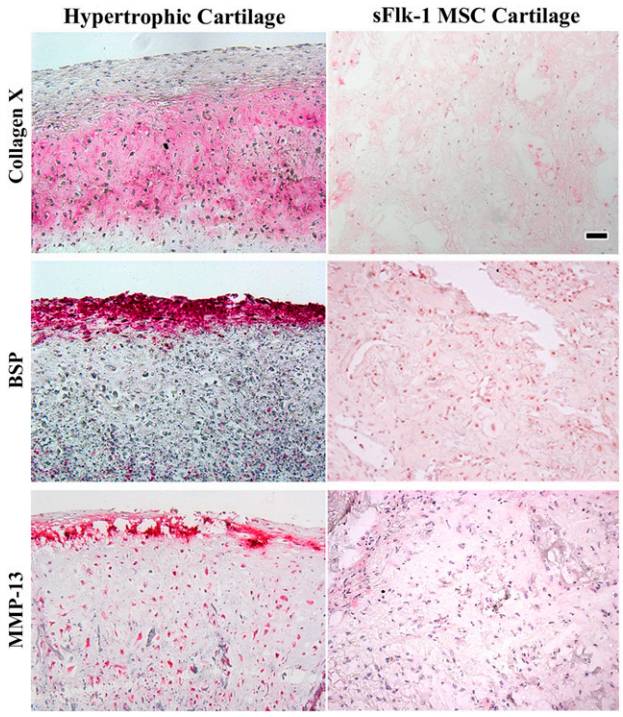

Figure 4. In vivo cartilage stability. Immunohistochemistry for type X collagen, BSP, and MMP-13 on sections of hypertrophic cartilage generated in vitro by MSCs (as a positive control) and on sections of the cartilaginous constructs generated in vivo by sFlk1 MSCs 12 weeks after implantation. Scale bar = 50 μm. Abbreviations: BSP, bone sialoprotein; MMP-13, metalloproteinase-13; MSC, bone marrow-derived mesenchymal stromal/stem cell.

staining, as well as gene expression analysis (Fig. 5A, 5C). In the absence of TGFβ3, cells cultured in 20% oxygen generated only fibrous tissue, whereas reduction of oxygen to 2% induced clear foci of chondrogenesis after 2 weeks of culture ($n = 3$ independent donors), as shown by positive staining for GAG and type II collagen (Fig. 5B) and a marked upregulation of type II collagen mRNA to levels up to threefold higher than even in the conditions treated with TGFβ3 (Fig. 5D). Hypoxia-mediated induction of chondrogenesis was also associated with the activation of the TGFβ pathway. In fact, not only did it cause a marked (350-fold) upregulation of *TGFβ1* expression compared with 20% oxygen (Fig. 5D), but type II collagen upregulation was prevented by treatment with SB-431542, an inhibitor of the TGFβ type I receptor activin receptor-like kinase-5 (15-fold reduction). Hypoxic culture in 2% oxygen in the absence of TGFβ3 stimulation also induced a gene expression pattern supportive of a stable chondrocytic phenotype because it reduced expression of hypertrophic markers, namely type X collagen and Indian Hedgehog, and it increased expression of Gremlin-1, which has a role in reducing hypertrophy by blocking the BMP pathways [32]. To verify whether sFlk-1 expression might have a direct effect on the chondrogenic commitment of MSCs, we compared their gene expression for collagen II and *TGFβ1* to that of naïve cells. In the absence of TGFβ3 supplementation and of hypoxia, sFlk-1 MSCs did not show increased expression of either gene compared with control MSCs (collagen II, $1.5 \times 10^{-5} \pm 2.510^{-6}$ vs. $3 \times 10^{-4} \pm 2 \times 10^{-4}$; *TGFβ1*, $2.6 \times 10^{-1} \pm 3 \times 10^{-2}$ vs $1.6 \times 10^{-1} \pm 1.2 \times 10^{-1}$).

Discussion

In this study, we demonstrated that blocking VEGF signaling by sFlk-1 overexpression in uncommitted human MSCs results in efficient inhibition of angiogenesis and in robust spontaneous chondrogenic differentiation in vivo. Hyaline-like cartilage was already formed after only 4 weeks and was maintained up to the last observation (12 weeks) with features of phenotypic stability, despite the otherwise intrinsic propensity of MSC to reach a hypertrophic phenotype and undergo endochondral ossification [2, 3].

It is well established that vascular regression during limb mesenchymal cell condensation is required for chondrogenesis [33] and that antiangiogenic peptides (e.g., endostatin/collagen XVIII) play an important role for the development and maintenance of cartilage [34]. In this context, our work provides the

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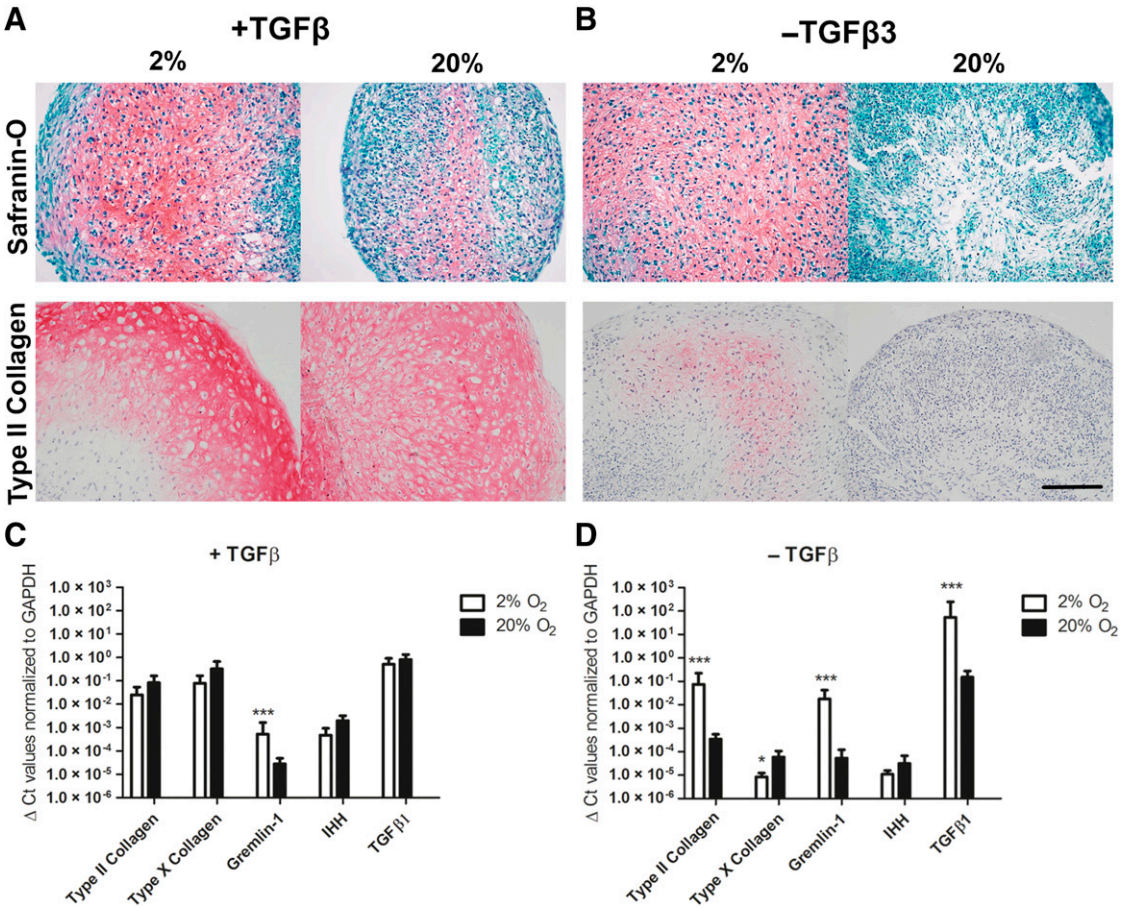

Figure 5. In vitro chondrogenesis at different oxygen tensions. Histological staining with Safranin-O and immunohistochemistry for type II collagen on constructs generated in vitro by naïve MSC cultured with **(A)** or without **(B)** TGFβ3 supplementation at 2% or 20% of oxygen tension. Scale bar = 50 μm. Expression levels of the mRNA for type II and X collagen, Gremlin-1, *IHH TGFβ1* were quantified in pellets generated by naïve bone marrow-derived mesenchymal stromal/stem cells **(C, D)** cultured in the two different oxygen tensions. ΔCt values were normalized to expression of the *GAPDH* housekeeping gene, and results are shown as mean $\pm$ SD (n = 6 samples/group from 3 independent experiments). $*$, $p < .05$, $***$, $p < .001$. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHH, Indian hedgehog; TGFβ, transforming grown factor-β.

unprecedented proof that the sole blockade of angiogenesis, without the delivery of any exogenous morphogen, is sufficient to robustly direct adult human mesenchymal progenitors into the chondrogenic lineage. Previous studies, using murine muscle-derived stem cells (MDSCs) engineered to sequester VEGF by overexpressing soluble VEGFR-1 (sFlt-1), demonstrated enhanced cartilage healing in osteochondral defect [15] or osteoarthritis [14] models, but only if a potent chondrogenic morphogen (BMP-4) was additionally delivered; in contrast, our data show spontaneous chondrogenesis in the absence of any growth factor treatment. This discrepancy may be due to (a) the as-yet uncontrolled complexity of coupling progenitor differentiation with cartilage repair in a pathologic joint environment and/or (b) a fundamental biological difference between the two species and classes of progenitors (human MSCs vs. murine MDSCs).

Mechanistically, induction of MSC chondrogenesis by sFlk-1 overexpression could be explained by an indirect effect of inhibition of angiogenesis, or by a direct autocrine regulation, because VEGF receptor is expressed by human MSCs [35] and immature [36] as well as hypertrophic [4] chondrocytes. The in vitro experiments in supplemental online Fig. 1 indicate that sequestering VEGF by sFlk-1 expression did not directly regulate the chondrogenic capacity of MSCs, consistent with a previous report using MDSC-releasing sFlt-1 [15]. Because one of the immediate consequences of inhibition of angiogenesis is the generation of a hypoxic environment, we investigated the effects of hypoxia in promoting in vitro chondrogenesis. Low oxygen tension was previously shown to promote MSC proliferation and maintenance of a progenitor state during monolayer expansion [37, 38], as well as to enhance TGFβ-induced chondrogenesis during 3D culture of MSCs [17, 37–41]. To the best of our knowledge, here we provide the first demonstration that reduction of the oxygen tension from 20% to 2% is sufficient to prime MSC chondrogenesis in vitro, in the absence of any exogenously supplemented morphogen. Combined with the proof that hypoxia is established in the implanted constructs in vivo and that sFlk1 does not directly affect chondrogenesis in vitro in 20% oxygen conditions, these findings strongly suggest that VEGF blockade primes MSC to form cartilage indirectly by inhibiting angiogenesis and generating a hypoxic environment. Analysis of TGFβ expression and inhibition of TGFβ signaling further suggest that the hypoxic conditions activated chondrogenesis by inducing TGFβ upregulation, as recently observed in a chondrocyte culture model [42], thereby dispensing with the need to provide stimulation with exogenous morphogens. Further studies are required to define the temporal kinetics

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of TGFβ production at the protein level and whether possibly increased quantities would be found embedded within the deposited extracellular matrix or released in the culture medium.

The cartilage tissue formed in vivo by sFlk-1 MSCs did not develop hypertrophy up to 12 weeks after implantation and displayed no signs of endochondral ossification, which otherwise occurs spontaneously in the same model [3]. This result was mirrored by the gene expression pattern consistent with a stable chondrocytic phenotype reached by MSCs cultured in hypoxic conditions in vitro and in the absence of TGFβ3, with basal levels of type X collagen and Indian Hedgehog. Interestingly, we also found that the chondrogenic effect of hypoxia was associated with the activation of the TGFβ pathway and with the upregulation of Gremlin-1, an inhibitor of BMP signaling. On the basis of developmental events occurring in early limb mesenchyme [43] and recent findings reported by using adult human MSCs [40], the combination of these two molecular settings provides a plausible explanation for the observed induction of chondrogenesis in the absence of hypertrophy.

A reduced delivery of specific nutrients could also have contributed to the triggering of MSC chondrocytic differentiation upon inhibition of angiogenesis. For example, limited availability of glucose could have forced its reduced consumption, which physiologically parallels chondrogenesis by mesenchymal progenitors [38]. Moreover, reduced vascularization implies that MSCs would not be exposed to possible inhibitors of chondrogenesis present in serum or released by platelets [44, 45], as well as to proinflammatory factors (e.g., interleukin-1b) produced by ingrowing granulation cells [46] and known to be detrimental for chondrocyte differentiation and cartilage formation [47]. Similarly, the absence of remodeling of the resulting cartilage into bone tissue could be mediated by the impairment of monocytes/chondroclasts, whose recruitment, differentiation, and activity are known to be dependent on VEGF [48, 49]. Although these alternative and possibly concomitant mechanisms cannot be excluded, our data point to the key role of hypoxia and TGFβ upregulation in mediating the effect of sFlk-1 overexpression.

From a translational perspective, our findings are relevant for new therapeutic approaches to articular cartilage repair, based on the generation of an avascular environment for resident or implanted MSCs. To move in this direction, further studies are required to test sFlk-1 MSCs in a cartilage repair model mimicking the biological (e.g., inflammatory component) and physical (e.g., mechanical loading) complexity of a joint environment [50] and with a longer observation period. Moreover, safety issues, related to the use of genetically modified MSCs, need to be addressed. For example, sustained stable expression of a transgene could be obtained by using self-inactivating lentiviral vectors, with the great advantage of decreasing the oncogenic potential as compared with retroviral ones [51, 52]. Alternatively, the therapeutic strategy could be implemented by a scaffold-based delivery of humanized monoclonal anti-VEGF antibodies, as recently described for the enhanced formation of cartilage by human nasal chondrocytes [28].

Conclusion

From a broader biological perspective, our study outlines the general paradigm to induce a specific differentiation of MSCs by engineering their intrinsic capacity to modify and control key features of the site of implantation. This would in turn result in MSC exposure to multiple microenvironmental regulatory signals and would thus differ from the typical direct delivery of individual morphogenic cues. The concept, underpinning the critical mutual interactions occurring between grafted cells and the host environment and possibly targeting the artificial recapitulation of developmental events in an adult organism [53], was here exemplified in the context of angiogenic signals, but it could be extended to other stem or progenitor cell systems, as well as to other features of the recipient site.

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Author Contributions

A.M.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; C.M.M.d.C.: collection and assembly of data, data analysis and interpretation, manuscript writing; S. Ghanaati, S. Gueven, M.C., R.T., M.B., C.S., A. Barbero, and U.H.: collection and assembly of data; S.S.: provision of study material or patients; J.C.K.: data analysis and interpretation; A. Banfi and I.M.: conception and design, financial support, data analysis and interpretation, manuscript writing

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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