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Mesenchymal Stem Cell-Dependent Formation of Heterotopic Tendon-Bone Insertions (Osteotendinous Junctions)

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Key Words. Gene therapy • Marrow stromal stem cells • Mesenchymal stem cells • Signal transduction • Skeleton • Somatic cell therapy • tendon • bone

ABSTRACT

Ligament-to-bone and tendon-to-bone interfaces (entheses, osteotendinous junctions [OTJs]) serve to dissipate stress between soft tissue and bone. Surgical reconstruction of these interfaces is an issue of considerable importance as they are prone to injury and the integration of bone and tendon/ligament is in general not satisfactory. We report here the stem cell-dependent spontaneous formation of fibrocartilaginous and fibrous entheses in heterotopic locations of the mouse if progenitors possess a tenogenic and osteo-/chondrogenic capacity. This study followed the hypothesis that enhanced Bone Morphogenetic Protein (BMP)-signaling in adult mesenchymal stem cells that are induced for tendon formation may overcome the tendon-inherent interference with bone formation and may thus allow the stem cell-dependent formation of tendon-bone interfaces. The tenogenic and osteo-/chondrogenic competence was mediated by the adeno- and/or lentiviral expression of the biologically active Smad8 signaling mediator (Smad8ca) and of Bone Morphogenetic Protein 2 (BMP2). Modified mesenchymal progenitors were implanted in subcutaneous or intramuscular sites of the mouse. The stem cell-dependent enthesis formation was characterized histologically by immunohistological approaches and by in situ hybridization. Transplantation of modified murine stem cells resulted in the formation of tendinous and osseous structures exhibiting fibrocartilage-type OTJs, while, in contrast, the viral modification of primary human bone marrow-derived mesenchymal stromal/stem cells showed evidence of fibrous tendon-bone interface formation. Moreover, it could be demonstrated that Smad8ca expression alone was sufficient for the formation of tendon/ligament-like structures. These findings may contribute to the establishment of stem cell-dependent regenerative therapies involving tendon/ligaments and to the improvement of the insertion of tendon grafts at bony attachment sites, eventually. STEM CELLS *2010;28:1590–1601*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Ligaments link bone to bone, whereas tendons connect muscles to bone. Both tissues transmit the forces developed by muscle contractions across joints, stabilize these or produce motion. Tendons originate in muscle (musculo-tendinous junction) and insert into bone forming an osteotendinous junction (OTJ, enthesis). Early pioneering work by Biermann and coworkers distinguishes two different forms of the OTJ, according to its site of long bone attachment [1, 2], but more recent work by Benjamin and coworkers introduced broader terminologies for the entire musculo-skeletal system. These authors classify entheses as being either of the fibrous or fibrocartilaginous type, depending on the character of the tissue at the tendon/ligament-bone interface [3–5].

Author contributions: S.S.-O.: collection and/or assembly of data; F.W.: collection and/or assembly of data; A.H.: conception and design, collection and/or assembly of data, manuscript writing; A.W.: collection and/or assembly of data; S.L.: collection and/or assembly of data; B.N.: collection and/or assembly of data; V.S.: collection and/or assembly of data; W.L.: essential administrative support; A.D.G.: collection and/or assembly of data, interpretation of data; J.R.: provision of study material, collection and/or assembly of data; T.H.: essential administrative support; F.T.: provision of study material; E.W.: provision of study material; P.C.: collection and/or assembly of data; G.G.: conception and design, manuscript writing, final approval of the manuscript. S.S.-O., F.W., and A.H. contributed equally to this article.

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Entheses on the meta-/diaphysis of long bones are of the fibrous type, those on epiphyses of long bones or the short bones of the wrist and ankle are of the fibrocartilaginous type [6]. The specialized structure of the OTJ prevents collagen fiber bending, fraying, shearing, and failure [7]. At the bony insertion site, elastic and soft tendon/ligament anchors to hard bone, both of which are materials of rather diverse physical properties. Therefore, structures adjacent to the enthesis are involved in the prevention of injury or destruction due to mechanical loading forming an entire "enthesis organ." This term has been coined to point out that additional structures adjacent to an enthesis are responsible for stress dissipation at the attachment site [3]. For example, the Achilles enthesis organ consists of a tendon insertion associated with a complex of adjacent fibrocartilages, a bursa and a fat-pad. A recent study describes 14 of such complex enthesis organs in the organism [8].

The fibrocartilaginous (direct) type of enthesis is composed of four zones: a dense fibrous connective tissue tendon or ligament zone, uncalcified fibrocartilage, mineralized fibrocartilage, and bone [9]. This zonal concept has recently been modified in which one or more of these zones may be missing [10]. The outer border of calcification is indicated by a basophilic tidemark that separates fibrocartilage and mineralized fibrocartilage, similar to the tidemark found in articular cartilage. In contrast, a fibrous (indirect) enthesis lacks both fibrocartilage intermediate zones. The tendon or ligament directly attaches the bone (predominantly in adult persons) or the periosteum (predominantly in children) [4].

The integration of healing tendon or ligament into bone after surgical reconstruction is an issue of considerable importance as the integration of tendon grafts is often not satisfactory [11]. Approaches with Bone Morphogenetic Protein 2 (BMP2) to enhance tendon healing in a bone tunnel resulted in an acceleration of the healing process and a higher pullout strength [12, 13]. BMP12 seemed to be able to promote the formation of a fibrocartilage insertion in a rat model [14] and Martinek et al. [15] used adenoviral ex vivo administration of the *BMP2* gene to improve osteointegration of semitendinosus tendons and to replace the anterior cruciate ligament in a rabbit model.

In this study, we assess the competence of multipotent mesenchymal stem cells (MSCs) for the factor-dependent generation of heterotopic tendon/ligament and of tendon-bone interfaces. Several reports have already described strategies for a MSC-dependent repair of tendon tissue. MSCs were applied on a scaffold or directly injected into the tendon defect [16–19]. However, depending on the cell-to-collagen ratio, heterotopic bone formation was also observed in the repair sites. Routine applications of MSCs for the management of acute tendon injuries in horses have also been reported [20]. In addition, MSCs may serve as vehicles for gene delivery, and we recently demonstrated that Smad8/BMP2-modified MSCs may contribute to the heterotopic tendon-like tissue formation and to orthotopic healing of a rat Achilles tendon defect [21]. We now developed the hypothesis that higher expression levels of BMP2 in the presence of Smad8 might lead to bone formation and the concomitant development of tendon-bone interfaces in addition to tendon/ligament-like tissue. Such a codevelopment of bone and tendon should lead to interactions between hard and soft tissue, which we wanted to investigate. We hoped that an interaction between tendon and bone would result in the formation of tendon-to-bone junctions that might in the long-term be translated into clinically relevant settings. In addition, we hoped and expected that increased viral vector-borne Smad8ca expression would be sufficient for the development of pure tenogenic tissue, which is a clinically attractive perspective for tendon and ligament injuries.

In this study, we provide evidence, indeed, that tendon-bone insertions may form spontaneously when adult stem cells possess both tenogenic and bony capacity. The viral vector-dependent modification of murine MSC-like cells (C3H10T[1/2]) with constitutively active Smad8 (Smad8ca) and BMP2 leads to the spontaneous formation of heterotopic fibrocartilaginous tendon-bone junctions, whereas the similar viral modification of primary human MSCs (hMSCs) resulted in heterotopic tendon-bone insertions without obvious fibrocartilaginous elements. Moreover, high-level expression of Smad8ca alone leads to the formation of tendinous/ligamentous structures without bony elements. These findings may contribute to the establishment of stem cell-dependent regenerative therapies involving tendon/ligaments and the insertion of tendon grafts at bony attachment sites, eventually.

Materials and Methods

Adenoviral Constructs

The conserved MH1 domain from rat Smad8 has been deleted to obtain a biologically active Smad8 (Smad8ca) as described [21]. For generation of infectious recombinant adenoviruses for *Smad8ca* and *BMP2* a commercially available system was used (Adeno-X, Clontech-Takara, Saint-Germain, France, http://www.takarabioeurope.com/index.html). The recombinant Adeno-X vector DNAs were transfected into low-passage HEK293 cells (ICLC HTL04001). Two to three days later, a cytopathic effect was observed. The infected cells were collected, washed, and lysed in sodium desoxcholate (0.5% w/v). After centrifugation (1,250*g*, 10 minutes) the supernatant was collected and infectious recombinant adenoviral particles were purified (CsCl gradient; 1.7 g/cm^3; 120,000*g*; 24 hours). Viral stocks were stored at 4°C.

Lentivirus Production

The three HIV-1-derived vectors harboring *BMP2, Smad8wt,* or *Smad8ca* were obtained by replacing the *lacZ* gene of the pHRCMVlacZ plasmid [22] by a cassette containing *BMP2, Smad8wt,* or *Smad8ca* followed by an internal ribosome entry sequence and the enhanced green fluorescence marker gene. To produce lentiviral vectors, each transfer vector together with the HIV-1 packaging plasmid pCMVΔR8.2 and the pMD. G plasmid encoding the vesicular stomatitis virus envelope glycoprotein were cotransfected by the calcium phosphate method into HEK 293T cells. Supernatants were collected 48 hours post-transfection and concentrated 100 times by ultracentrifugation. The titer of each batch of lentiviral vectors was determined by measuring the fluorescence of transduced HEK 293T cells by flow cytometry.

Cell culture and Infection of MSCs

Murine C3H10T[1/2] cells were cultured in high-glucose Dulbecco's Modified Eahle's Medium (DMEM) containing 10% fetal bovine serum (Sigma-Aldrich, Taufkirchen, Germany; http://www.sigmaaldrich.com/germany) as described [23]. Human bone marrow stromal cells (MSCs) were isolated from bone marrow aspirates from patients with the consent of the ethics committee of the Hannover Medical School (MHH). Mononuclear cells were isolated by a Biocoll gradient (Biochrom Ltd., Berlin, Germany, http://www.biochrom.de/). The colonies of plastic-adherent cells were subcultured and replated at a density of 2,000–5,000 c/cm^2. MSC characteristics were confirmed by fluorescence-activated cell sorting (FACS) analysis of cell surface molecules as detailed in Supporting Information and by in vitro differentiation. For infection, adenoviruses were used at a

multiplicity of infection (MOI) of 100 (lac Z: MOI 10), lentivirus infections were performed at MOI 25. Five days after infection, cells (C3H10T[1/2]: 1×10^6, hMSCs: 3×10^5) were seeded onto one collagen sponge ($3 \times 3 \times 3$ mm^3). Ectopic implantations were performed subsequently.

Ectopic Implantation Model

Cell-collagen implants seeded with modified C3H10T1/2 or hMSCs were mounted on individual type I collagen sponges (#ID-2205, Duragen, Integra Life Sciences, Neu-Ulm, Germany, http://www.integra-ls.com/home/) and transplanted into the thigh muscle or subcutaneosly into female C3H (for C3H cells) or nude (for human MSCs) mice (4- to 8-week old). Before transplantation animals were anesthetized intraperitoneally with 1% (w/v) ketamine plus 0.2% (w/v) rompun mixture (ketamine: BDT, rompun: Bayer, Germany, http://www.bayer.de/de/produkte-von-a-bis-z.aspx; 280 μl/per mouse). Skin was swabbed with Braunol (Braun, Melsungen, http://www.bbraun.com/) and cut, and an intramuscular pocket was formed in the thigh muscle and filled with the collagen sponge containing cells. The sponge was inserted and positioned near the muscle belly. Skin was sutured. The mice were killed 4 weeks after transplantation. Explanted transplants were fixed in 4% formalin solution overnight and stored in 70% ethanol or immediately dehydrated and paraffin embedded. These experiments were approved by the local administration of Lower-Saxony (#33.11.42502-14-051/07).

Immunoblot Analysis

Cell pellets of transfected HEK 293T, C3H10T[1/2], or MSCs (shock-frozen in liquid nitrogen) were analyzed as described [23]. Flag antibodies were purchased from Sigma and monoclonal antibodies against human BMP2 were from R&D Systems GmbH (Wiesbaden, Germany, http://www.rndsystems.com/).

Histological Analysis

Explanted tissues with engrafted stem cells were immersion fixed in 4% neutral buffered formalin for 24 hours, embedded in paraffin, cut at 5 μm using a motorized microtome (Leica Mikrosystems, Wetzlar, Germany, http://www.leica-microsystems.com/) and stained with hematoxylin and eosin (H&E), Masson-Golder, Toluidin-blue, or von Kossa following routine procedures. Images were acquired using a bright-field microscope (Olympus, Hamburg, Germany, http://www.olympus.de/microscopy/, BX51, Model U MDOB3 with soft-imaging software Olympus Cell D) and processed with Adobe Photoshop CE. No specific feature within an image was manipulated.

In Situ Hybridization

DIG (Digoxigenin-11-UTP)-labeled cRNA probes of defined length (antisense and sense) were generated by in vitro transcription of linearized plasmids according to the manufacturer's instructions (Roche, Penzberg, Germany, http://www.roche.de). Template DNAs were as follows: rat-Smad8 [21], human collagen type Ia1, collagen type IIa1, and Collagen type IIIa1 [24]. The sections were deparaffinized and treated with proteinase K (20 μg/ml, 30 minutes at 37°C; Roche). The sections were incubated in prehybridization buffer (50% formamide, 1× SSC, 10% dextran sulfate, 1 mg/ml yeast tRNA, 1× Denhardt's for 1 hour at 42°C in a humidified chamber). Then, the denatured and DIG-labeled probe in prehybridization buffer was added. After 24 hours, the sections were washed and incubated with an anti-DIG antibody (AP Roche). Staining was with BCIP and NBT (Roche) in the dark at 37°C.

Immunohistology

Immunohistochemical analyses were performed with deparaffinized tissue sections.

Heat-induced antigen retrieval was by autoclaving 10 minutes in 10 mM trisodiumcitrate, 0.05% Tween 20, pH 6.0. Sections were incubated with the primary antibody for 30 minutes (polyclonal rabbit anti-mouse collagen type I; polyclonal rabbit anti-human collagen type III also reacting with mouse collagen III; Abcam, Cambridge, U.K., www.abcam.com/). Immunohistochemical staining was with the Vectastain Elite ABC Kit (VectorLabs, Peterborough, U.K., http://www.vectorlabs.com/). Collagen II immunohistochemical detection was with monoclonal mouse anti-collagen type II antibodies (Quartett, Berlin, Germany, http://www.quartett.com/). Staining was performed here with Vector M.O.M. (VectorLabs). The detection of human β_2-microglobulin was with rabbit anti-human β_2-microglobulin antibodies (Novocastra, Newcastle, U.K., www.novocastra.co.uk) followed by peroxidase-labeled goat-anti-rabbit IgG. For color development, diaminobenzidine was used. Counterstaining was performed with methyl green.

Results

Adenovirus-Dependent Expression of Smad8ca and BMP2 in Mesenchymal Progenitors (C3H10T[1/2]) Mediates the Formation of Tendon-Bone-Muscle Insertions After Heterotopic Transplantation

The heterotopic implantation of murine mesenchymal progenitors (C3H10T[1/2]) stably expressing the biologically active signaling mediator Smad8 (Smad8ca) and BMP2 resulted in the formation of tenogenic tissue without bony elements [21]. Subsequently, we developed the notion that a higher, viral vector-mediated expression level of BMP2 might overcome an inherent tendon-matrix-dependent negative impact on BMP-signaling [21, 25]. Moreover, bone formation in the presence of tendinous or ligamentous tissue might also have an impact on tendon-bone interface formation, which would be advantageous for regenerative modalities of tendon-bone lesions.

To investigate the stem cell competence for tendon-bone interface formation by heterotopic implantations, we used the adenovirus-mediated expression of Smad8ca and/or BMP2 in murine mesenchymal progenitors C3H10T[1/2] and the lentiviral-dependent expression of the identical factors in primary human bone marrow-derived MSCs (hMSCs). Implantations and their outcome are summarized in Table 1.

The adenovirus-mediated protein expression of Smad8ca and BMP2 in mesenchymal progenitors C3H10T[1/2] was evaluated 24 hours postinfection by western blot analysis and indicated high-level expression of both factors (Fig. 1A). Then, the *Ad-Smad8ca* and *Ad-BMP2*-modified progenitors were seeded on a collagen sponge as detailed in "Materials and Methods" section and were implanted either subcutaneously or into intramuscular pockets. In general, results obtained were comparable and independent of the location of the heterotopic transplant, although, the formation of fully developed ossicles containing bone marrow was only observed with intramuscular transplantations.

Control transplantations of murine C3H10T[1/2] progenitors modified with adenoviral vectors harboring the *E. coli lac*Z gene did not exhibit de novo tissue formation. Four weeks postimplantation, C3H10T[1/2] cells were still predominantly located in the periphery of the sponge and were not

Table 1. Summary of the heterotopic transplantations performed in this study

Cells: mice	Implantations C3H10T 1/2 C3H	Phenotype	Implantations hMSCs nude	Phenotype
Control	19	Bone: none	17	Bone: none
		Tendon: none		Tendon: none
		OTJ: none		OTJ: none
Viral vector encoding: BMP2	4	Bone: fully developed ossicle (4/4)	6	Bone: bony particles (6/6)
		Tendon: none		Tendon: none
		OTJ: none		OTJ: none
Viral vector encoding: Smad8wt	2	Bone: none	8	Bone: none
		Tendon: none		Tendon: none
		OTJ: none		OTJ: none
Viral vector encoding: Smad8ca/BMP2	27	Bone: fully developed ossicle (25/27)	8	Bone: bony particles (8/8)
		Tendon: crimp pattern (27/27)		Tendon: crimp pattern (8/8)
		OTJ: 24/27		OTJ: 5/8
Viral vector encoding: Smad8ca	24	Bone: none	7	Bone: none
		Tendon: crimp pattern (18/24)		Tendon: crimp pattern (4/7)
		OTJ: none		OTJ: none

Transplantations have been performed as described in “Materials and Methods” section. The phenotypic results are detailed in the “Results” and “Discussion” sections.
Abbreviations: BMP2, Bone Morphogenetic Protein 2; hMSCs, human mesenchymal stem cells; OTJ, osteotendinous junction.

involved in substantial tissue development (Fig. 1B). The implantation of the nonactive wild-type form of Smad8 (Smad8wt) also did not result in substantial de novo tissue formation (data not shown). In contrast, intramuscular transplantation of adenovirally modified progenitors expressing both Smad8ca and BMP2 led to the heterotopic formation of an ossicle with inserting tendinous elements (Fig. 1C). The ossicle shows a fully developed bony collar surrounding a bone marrow and hypertrophic cartilage undergoing enchondral ossification (Fig. 1C). In addition, a tenogenic element of considerable size is inserting into the mineralizing ossicle (Fig. 1C, between the dashed blue lines). Mineralization has been confirmed by von Kossa-staining (Fig. 1D). The inserting tendinous element is characterized by a tendon-typical crimp pattern (Fig. 1E, 1F) and flattened tenocyte-like cells (Fig. 1F). Interestingly, the tendinous structure is aligned parallel to the longitudinal axis of the striated host muscle. This tendon/muscle alignment is also observed in another implant (Fig. 2) indicating that mechanical force may play a role in the orientation of the new tendon-like structure. Before inserting into bone, the crimp pattern of the tendinous element converts into a population of toluidine blue-positive, supposedly cartilage-like tissue containing chondrocytes (Fig. 1E, 1F; black arrowheads). With longer implantation times (two months), tendon-bone-interface formation was also observed, albeit with the substantial development of adipogenic tissue (Supporting Information Fig. 1). This result may be explained with the reduced mechanical loading conditions at the heterotopic intramuscular sites of implantation.

The chondrogenic nature of the OTJ was substantiated with another implant (Fig. 2A). Here, a substantial tendon-like element is located between the host muscles and the bony ossicle (Fig. 2A, dashed blue line). Again, crimp pattern and flattened tenocytic cells denote the tendinous nature (Fig. 2B) and the upper boxed region in Figure 2A shows the formation of a putative fibrocartilage type of OTJ (Fig. 2C). Here, a short tendon-like element (black arrowheads) approaches the bony ossicle. In the proximity of the bony collar, the morphology of the tendinous elements converts into a fibrocartilage-like structure containing proliferating chondrocytes (PC). To investigate as to whether or not the cell type inserting into the bony elements possesses chondrocytic properties or rather consists of periosteal cells, we applied nonradioactive in situ hybridization. We used probes specific for collagen I, which is expressed in ostoblasts and tenocytes and probes specific for the chondrocyte-specific collagen II, respectively. As anticipated, collagen I expression is found in most of the cells associated with the bone and tendon-like structures (Fig. 2D). In contrast, a notable collagen II expression is predominantly monitored only in cells in the tendon-bone attachment site, close to the bony elements of the ossicle (Fig. 2E; dark blue). The proliferative nature of these cells may be deduced/inferred from areas where cells are organized in short stack-like structures as observed in PCs of the growth plate, which are arranged into columnar stacks. The collagen II expression of these cells substantiates that inserting cells between tendon and bone possess chondrocytic characteristics and that the OTJ formed in this particular location belongs to the fibrocartilage type.

The Modified Mesenchymal Progenitors Directly Contribute to Heterotopic Tendon Formation

To answer the question as to whether or not the modified mesenchymal progenitors directly contribute to tendon formation, the recombinant Smad8 expression was investigated with nonradioactive in situ hybridizations. For this analysis, we used the intramuscular heterotopic ossicle and its tendinous structures, which have already been described in Figure 2. In situ hybridization with a Smad8-specific probe was performed with two regions of the implant exhibiting distinct tenogenic structures (Fig. 3).

Interestingly, Smad8ca expressing cells are predominantly located in tendinous cells proximal to the bone, while the more distally located tenocyte-like cells do not exhibit a notable Smad8 expression (Fig. 3C, 3E). Those Smad8ca-expressing cells are located in tendon-like structures is indicative for a direct contribution of Smad8ca-modified stem cells to tendon development. However, distally located cells do not

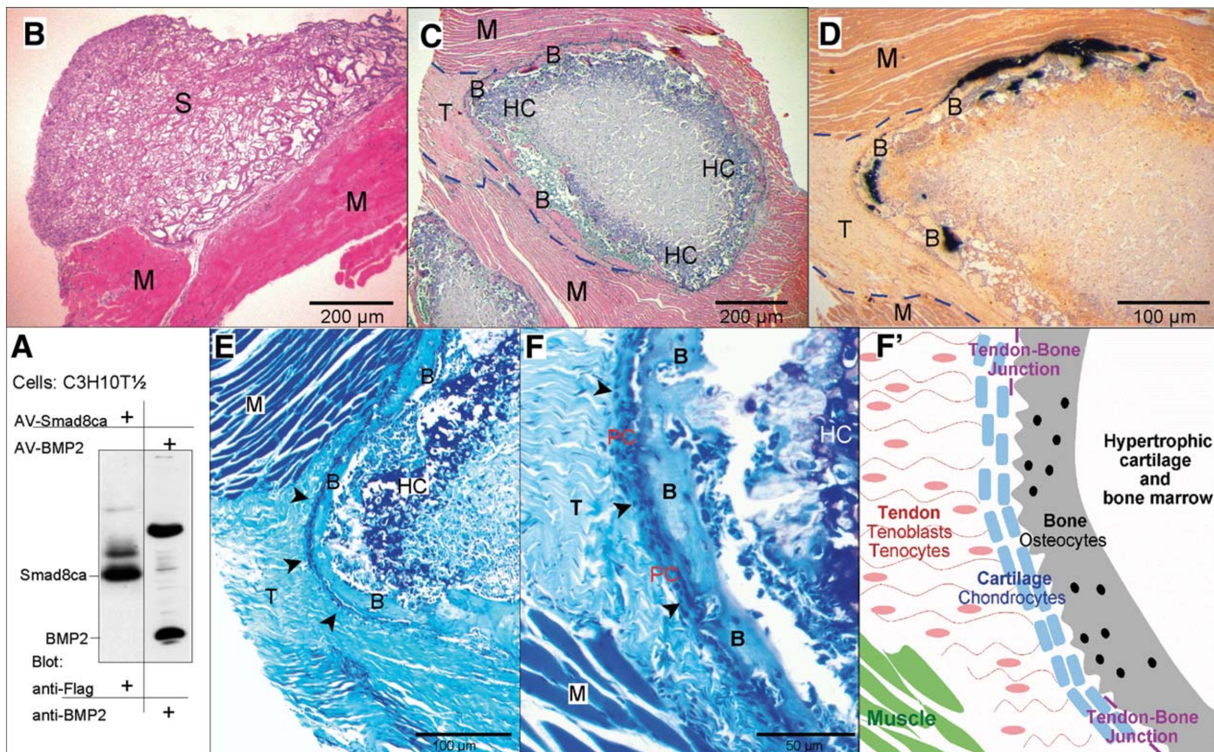

Figure 1. Heterotopic intramuscular transplantation of adenovirally modified mesenchymal progenitors (C3H10T[1/2]) expressing Smad8ca and BMP2 results in tendon-bone-muscle insertions. Murine mesenchymal progenitors C3H10T[1/2] have been infected with recombinant adenoviral particles mediating the expression of BMP2 and Smad8ca. One day before transplantation, cells were seeded on a collagen sponge (Duragen). Four weeks after implantation mice were sacrificed. The explants were embedded in paraffin, sectioned, and histologically characterized. **(A):** Western Blot analysis of adenoviral vector-mediated expression of Smad8ca and BMP2 in C3H10T[1/2]. After 24 hours of infection, BMP2 expression is assessed by anti-BMP2 antibodies, the Flag-tagged Smad8 by anti-Flag-blotting. The multiplicity of infection was 100. **(B):** Intramuscular control transplantation of a collagen sponge with control adenovirus (*Ad-lacZ*)-infected C3H10T[1/2] cells. C3H10T[1/2]. **(C):** Intramuscular transplantation of adenovirally modified C3H10T[1/2] cells expressing Smad8ca and BMP2 leads to the heterotopic formation of an ossicle with inserting tendinous elements (overview, H&E staining). The inserting tendinous element is located within the dashed blue lines. **(D):** Mineralized structures are demonstrated by von Kossa staining. **(E):** Before inserting into bone, the crimp pattern of the tendinous element embeds a population of toluidine blue-positive cells (black arrowheads; magnification of **[C]**). **(F)**: Fibrocartilage OTJ-like structure formed by implanted mesenchymal progenitors (magnification of **[E]**). The toluidine blue-positive, chondrocyte-like cells between tendinous and bony tissue are indicated (black arrowheads). **(F′)**: Schematic drawing of **(F)** indicating the position of the OTJ. Abbreviations: B, bone; BMP2, Bone Morphogenetic Protein 2; HC, hypertrophic chondrocytes; M, muscle; PC, proliferating chondrocytes; S, collagen sponge; T, tendon-like structures.

exhibit a notable Smad8ca-expression could either be explained by host stem cells immigrating from the surrounding host muscle-tissue and contributing to tendon formation or by a loss of adenoviral expression of recombinant Smad8ca in the mature and flattened tenocyte-like cells found in the more distal regions of the bony ossicle. The morphology of the cells indicates that the maturation of the tendon elements proceeds from bone to muscle: The more tenoblast-like tendon progenitor cells exhibiting a less flattened phenotype are located proximal to the bony ossicle, whereas flattened and mature tenocyte-like cells are predominantly found adjacent to host muscle tissue. In the next experiments, we assessed tendon maturation in this heterotopic system in more detail.

Implantation of Smad8ca- and BMP2-Modified Progenitors Exhibit a Bidirectional Mode of Tendon and Bone Formation

As described earlier, implantations of Smad8ca- and BMP2-modified progenitors seem to develop a mature tendon-like phenotype proximal to the surrounding host tissue, whereas less differentiated tissue is predominantly located below the mature structures and proximal to the newly formed bone. We assumed that this would be more apparent at earlier harvesting dates of the implants. Indeed, an overview of a 2 week heterotopic implant displays predominantly mature tendon-like elements proximal to host muscle-tissue and is characterized by expression of collagen type I, Supporting Information Figure 3c.

In comparison with the 4 week implants, immature tenogenic structures are more abundant. They are characterized by the epxression of collagen type III (Supporting Information Fig. 3d) and are mixed with early centers of cartilage (positive for collagen type II, Supporting Information Fig. 3e) and bone (expressing collagen type I, Supporting Information Fig. 3c) formation, located adjacent to the mature tenogenic structures, closely associated with the collagen carrier of the implant (Supporting Information Fig. 3).

Adenovirus-Mediated Expression of Smad8ca Alone Is Sufficient for the Heterotopic Formation of Tendon-Like Elements

Initially, we observed Smad8ca- and BMP2-dependent tendon formation in stable progenitor lines when coexpressed from nonviral vectors. The question was now whether adenovirally modified murine mesenchymal progenitors C3H10T[1/2] that express considerably higher protein levels of Smad8ca are able to form tendinous elements after heterotopic intramuscular implantation. Indeed, adenovirally expressed Smad8ca in

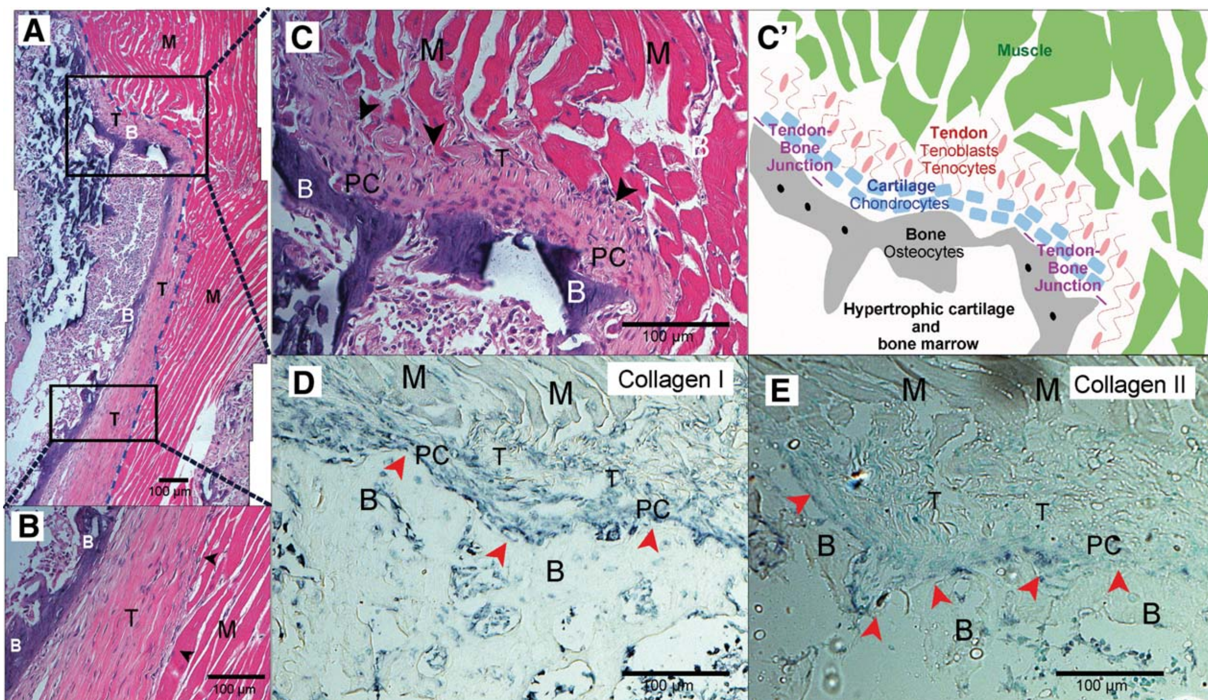

Figure 2. Heterotopic intramuscular transplantation of adenovirally modified mesenchymal progenitors (C3H10T[1/2]) expressing BMP2 and Smad8ca form a fibrocartilage type of osteotendinous junction (OTJ). Nonradioactive in situ hybridizations with collagen I and II specific probes. **(A):** Intramuscular heterotopic ossicle with tendinous elements after transplantation of adenovirally modified C3H10T[1/2] progenitors expressing Smad8ca and BMP2 as described in Figure 1 (H&E staining; overview). The tendinous element is located between the dashed blue line and the bony ossicle. **(B):** Tendon-like structures of the heterotopic implant (magnification of the lower rectangle in **[A]**) **(C):** Osteotendinous junction (OTJ) of the heterotopic implant (magnification of the upper rectangle in **[A]**). Tendon-like tissue with flattened tenocyte-like cells is indicated (black arrowheads). On approaching the bony collar of the ossicle **(B)** tenocytes/-blasts are replaced by proliferating chondrocyte-like cells. **(C′):** Schematic drawing of **(C)** indicating the position of the OTJ. **(D, E):** In situ hybridization of section **(C)** with collagen I or collagen II-specific probes. **(D):** Collagen I mRNA is expressed in tendon- and in the bone-forming cells (dark blue). **(E):** Collagen II mRNA is predominantly expressed in the chondrocyte-like cells proximal to the ossicle (dark blue). Weak counter-staining with methylgreen (light green). The bone-tendon boundary in **(D)** and **(E)** is indicated (red arrowheads). Abbreviations: B, bone; HC, hypertrophic chondrocytes; M, muscle; PC, proliferating chondrocytes; T, tendon.

mesenchymal progenitors C3H10T[1/2] seems to be sufficient for the formation of tendinous structures without bony elements (Fig. 4A–4C). These tenogenic structures are characterized by a crimp pattern and by flattened tenocyte-like cells and also display expression of Smad8 as elucidated by in situ hybridization with a Smad8-specific probe (Fig. 4D, 4E). The latter is again indicative for a direct contribution of the modified progenitors to the formation of tendon-like structures. Additional implants indicating Smad8ca-dependent formation of tendon-like elements are demonstrated in the Supporting Information Figure 2.

Lentivirally Smad8ca- and BMP2-Modified Human MSCs (Bone Marrow) Are Also Able to Form Tendon-Bone-Muscle Insertions After Heterotopic Intramuscular Transplantation

In general, murine C3H10T[1/2] stem cells have been considered as a valid model system for MSCs, however, this requires a substantiation of the findings in primary human adult stem cells. We used primary human MSCs from bone marrow (hMSCs), which have been collected from bone samples of patients undergoing orthopedic surgery. The male and female donors were healthy and aged between 18 and 80 years. Mononuclear cells from the aspirates were isolated with standard technologies (see "Materials and Methods" section). The adherent cells were subjected to FACS-analysis to confirm their MSC-surface marker repertoire. The isolated MSCs were CD14−, CD34−, CD45− CD44+, CD73+, CD90+, CD105+, and CD166+ and also underwent adipogenic, chondrogenic, and osteogenic differentiation under the standard conditions (see "Materials and Methods" section; Supporting Information Fig. 5).

For the viral modification of human MSCs, a lentiviral infection system was chosen as prospective gene or cell therapies with adenoviral infection systems may be problematic due to the inherent immunogenic properties of adenoviruses [26]. Implantations and their outcome are summarized in Table 1.

Lentiviral infections were performed at MOI of 25. Five days after infection, the lentivirally modified hMSCs were trypsinized, and 3×10^5 hMSCs were seeded onto one collagen sponge ($3 \times 3 \times 3$ mm^3). Twenty-four hours later, heterotopic implantations were performed into nude mice as described earlier. The capacity of the lentiviral infection system to yield high-level expression of Smad8ca and BMP2 was confirmed in hMSCs 48 hours after infection with western blotting (Fig. 5A).

Control infections, here with infectious lentiviral particles mediating Green Fluorescent Protein (GFP) expression, do not lead to a notable hMSC-dependent de novo tissue formation at heterotopic locations (Fig. 5B). The intramuscular transplantation of lentivirally modified hMSCs expressing Smad8ca and BMP2 leads to the formation of bony structures with inserting tendon-like elements (Fig. 5C, 5D). In general, only early stages of bone formation and inserting tendon-like structures are

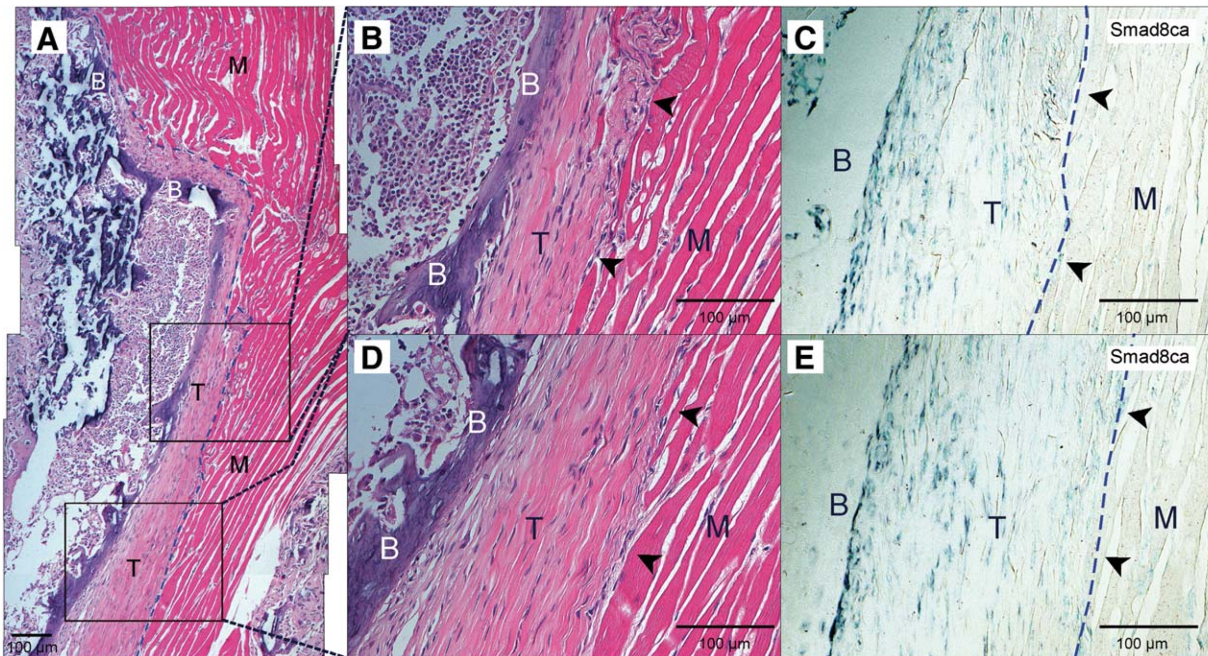

Figure 3. Implanted and modified mesenchymal progenitors C3H10T[1/2] contribute to heterotopic tendon formation. Nonradioactive in situ hybridizations with Smad8-specific probes. **(A):** Intramuscular heterotopic ossicle with tendinous structures as described in Figure 2A (overview, H&E staining). The tendinous element is located by the dashed blue line and the bony ossicle. **(B):** Tendon-like structures (magnification; upper black rectangle in **[A]**). Arrowheads indicate the muscle-tendon-like tissue border. **(C):** Recombinant Smad8-expressing cells are predominantly located proximally to the bony elements of the ossicle. In situ hybridization of the region shown in (**[A]**, upper rectangle) and in **(B)** with a Smad8-specific probe. Distally located cells do not exhibit a notable Smad8-expression. **(D):** Tendon-like structures (magnification; lower black rectangle in **[A]**). The arrowheads indicate the muscle-tendon-like tissue border. **(E):** Corresponding Smad8 in situ hybridization **(D)**. Again, only tenoblasts/-cytes located proximal to bone display a notable Smad8 expression. This is indicative for a contribution of Smad8ca-modified progenitors to tendon development. Abbreviations: B, bone; M, muscle; T, tendon.

monitored (Fig. 5C, 5D; red arrowheads) and ongoing angiogenesis as well (Fig. 5D, black arrowheads). The hMSCs allowed the formation of bone particles, but a fully developed ossicle as seen for the modified C3H10T[1/2] progenitors has never been observed. The organized structure of extracellular matrix and tendon-like structures can be identified (Fig. 5C, 5D), however, only very early stages of the crimp pattern may be monitored (Fig. 5D). The cell numbers and cell morphologies in the tendinous structures indicate early tenoblastic characteristics.

Interestingly, the phenotype of Smad8ca- and BMP2-dependent tendon-bone interface formation never indicated any fibrocartilage structure at the attachment site. The human MSC-dependent formation of tendon and bone structures in this heterotopic system implies that considerably more time for the development of mature tenogenic and bony elements is required than for the tissue formation initiated from the murine C3H10T[1/2] progenitors under comparable implantation conditions. Another reason may also be attributed to the fact that due to size limitations of the scaffold only a third of the murine cell numbers could be transplanted.

Based on immunohistology with human-specific anti-β2-microglobulin-antibodies, hMSCs do contribute to the formation of bony and tendinous elements as well (Fig. 5E–5H). Bone-lining cells and embedded osteocytes in bone are characterized by the expression of human β2-microglobulin (Fig. 5E, 5F; red arrowheads) and many of the tenoblast-like cells also stain positive for human β2-microglobulin (Fig. 5G, 5H; blue arrowheads).

As shown before (Fig. 4), virally expressed Smad8ca alone in C3H10T[1/2] was sufficient for the heterotopic generation of tendon/ligament-like tissue. Similarly, lentivirally modified human MSCs, which express Smad8ca seem to acquire the competence to form tendinous elements (Fig. 6), however, as described earlier, at the same time (4 weeks) after transplantation, hMSC-mediated tissue formation exhibits an earlier state of tendinous development in comparison with the murine progenitors as demonstrated both by H&E and Masson-Goldner staining (Fig. 6A–6E).

In conclusion, lentiviral modification of hMSC yields similar results as obtained for the murine progenitors, however, the time needed for hMSCs-dependent de novo tissue formation is considerably longer. Human MSCs seem to possess the competence for the Smad8ca-dependent tendon/ligament development and for the Smad8ca/BMP2-dependent formation of tendon-bone attachment sites as well, however, without fibrocartilage elements.

Discussion

The conversion of the tendon-like tissue into putative cartilage with chondrocyte-like cells und subsequently, the integration into bone tissue indicates that a fibrocartilage type of OTJ may be created by heterotopically implanted Smad8ca/BMP2-modified murine mesenchymal progenitors C3H10T[1/2]. In addition, it implies that the simultaneous presence of both tenogenic and osteogenic capacities may be sufficient for the stem cell-dependent, spontaneous formation of tendon-bone interfaces or attachment sites.

In heterotopic implants, differentiation of tendon and cartilage/bone seems to largely develop in opposing directions. The grafted modified stem cells are predominantly trapped on the surface of the collagen carrier. Chondrogenic and osteogenic

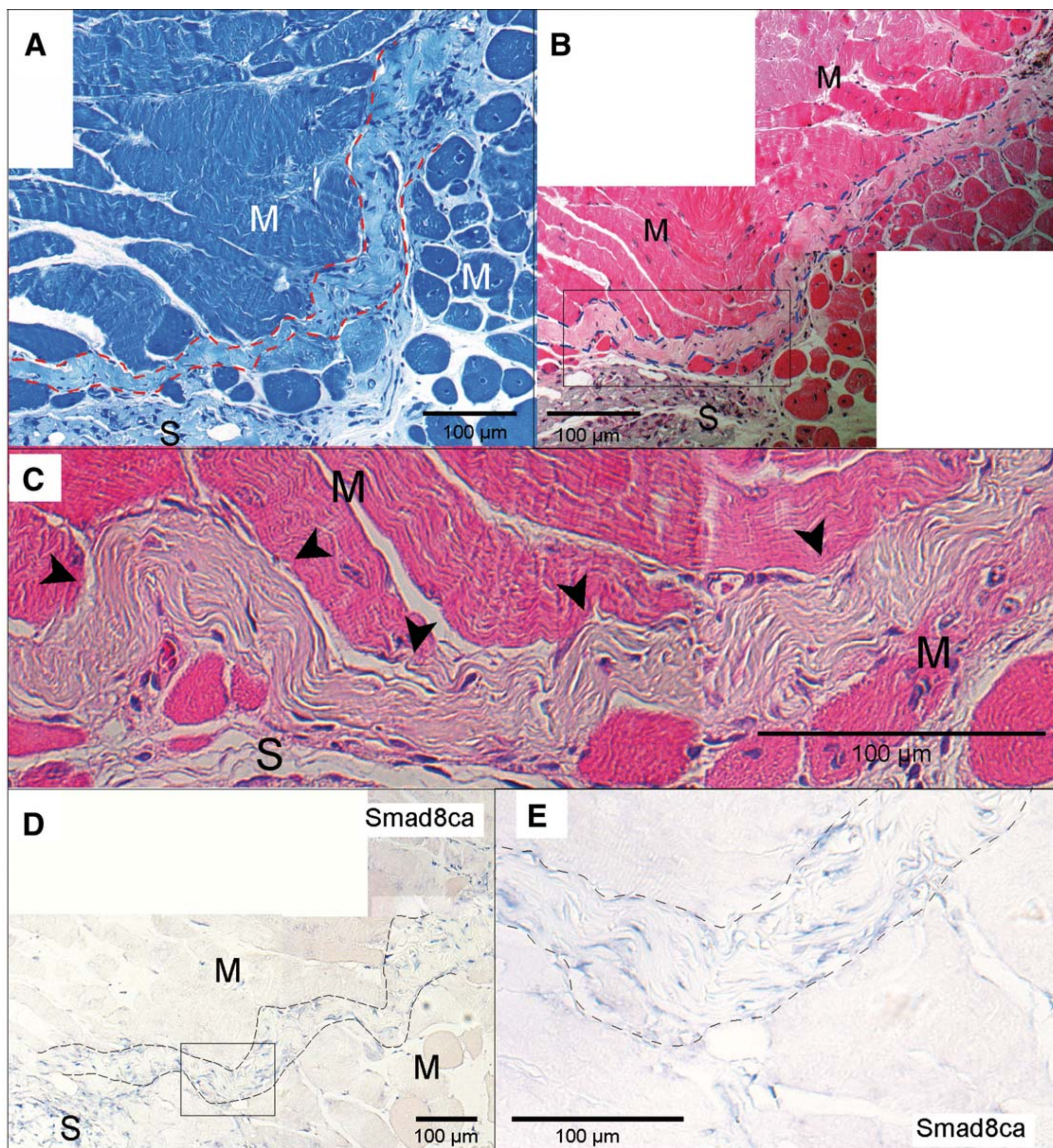

Figure 4. Adenovirus-mediated expression of Smad8ca in murine C3H10T[1/2] progenitors is sufficient for the heterotopic formation of tendon-like elements. **(A):** Adenovirally modified murine mesenchymal progenitors C3H10T[1/2], which express Smad8ca form tendinous elements after heterotopic intramuscular implantation (overview, toluidine-blue staining). **(B):** Tendinous element after heterotopic intramuscular implantation of Smad8ca expressing Smad8ca-modified progenitors (C3H10T[1/2]) (overview, H&E staining). **(C):** The tendinous element boxed in **(B)** is enlarged and displays its crimp pattern and flattened tenocyte-like cells (arrowheads). **(D):** In situ hybridization of the region displayed in **(A)** and **(B)** with a Smad8-specific probe. **(E):** Tenocyte-like and Smad8ca-expressing cells (magnification of **[D]**, rectangle). Tenocyte-like cells express recombinant Smad8ca. Abbreviations: M, muscle; S, sponge; T, tendon.

differentiation seem to be closely associated with the cells on the surface of the collagen carrier while the tenogenic progenitors develop and mature away from the carrier and towards the surrounding muscle tissue.

Different Directions for Heterotopic Tendon and Bone Maturation

In the heterotopic tendon and bone formation model presented here, tendon matures from the collagenous implant towards the surrounding muscle tissue. This was concluded from the morphological appearance of flattened tenocyte-like cells occurring proximal to the host muscle tissue and the more voluminous tenoblast-like cells located near the bony elements (Fig. 2). To substantiate this hypothesis, collagen I and III immunohistochemistry of implants after a shorter duration of implantation, that is, 2 weeks instead of 4 weeks, was applied which, indeed, demonstrated more developing and less mature tendon-like tissue (Supporting Information Fig. 3).

The differentiation and maturation of both tendon and osteogenic tissues of heterotopically implanted MSCs seems to be well separated and to be organized in different directions. At first, mesenchymal progenitors reside mainly on the surface of the collagen carrier (Figs. 1B, 5B). Cartilage and bone formation is then predominantly observed in association with the collagen carrier, whereas tendon formation and maturation develop and mature rather in connection with and adjacent to the host muscle tissue (Supporting Information Fig. 4). However, further studies are needed to understand these differences in the differentiation and development of tendinous and osteogenic tissue from the heterotopically implanted cells, and how at sites of interacting

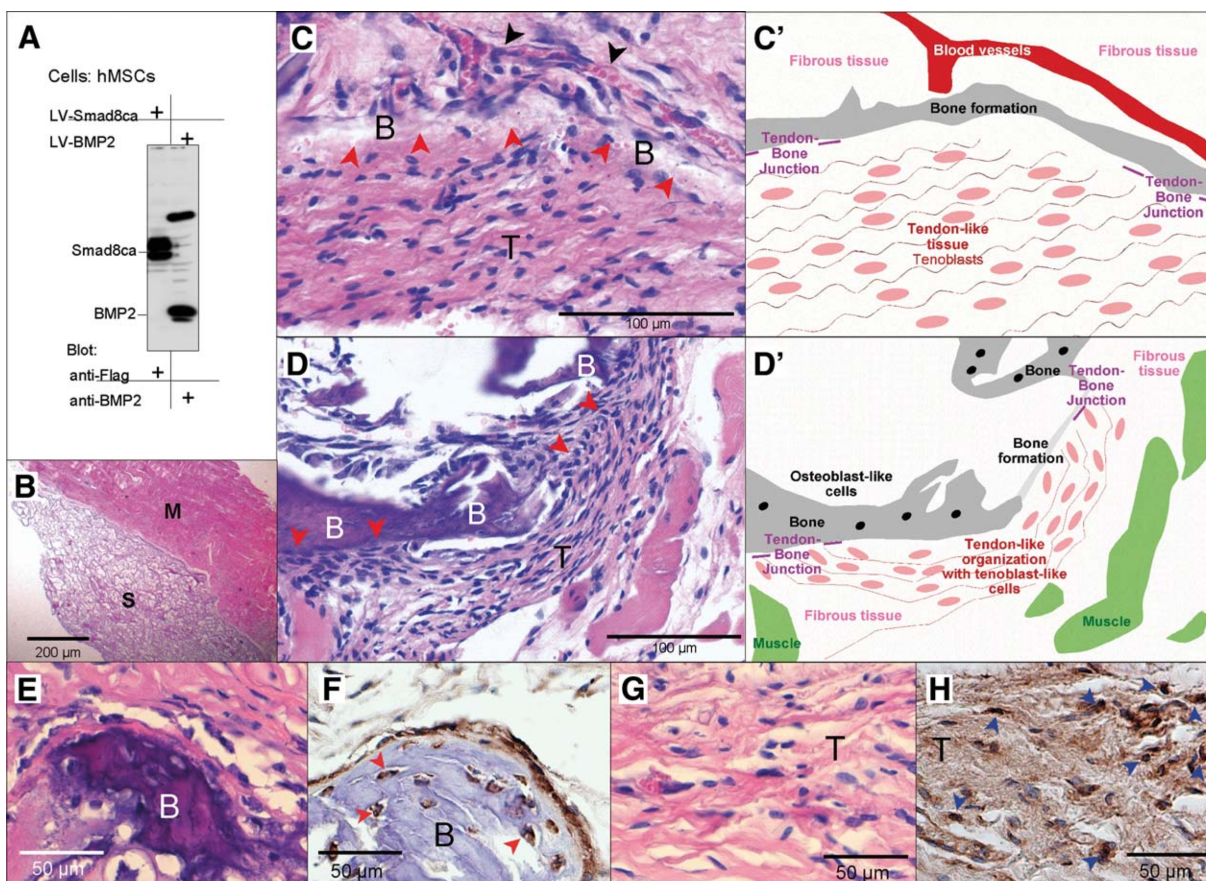

Figure 5. Heterotopic intramuscular transplantation of lentivirally modified human MSCs (bone marrow) expressing BMP2 and Smad8ca are also able to form tendon-bone-muscle insertions. Human MSCs have been isolated from bone marrow (Materials and Methods) and were lentivirally infected for BMP2 and Smad8ca expression. **(A):** Western blot analysis of lentiviral vector-mediated expression of Smad8ca and BMP2 in human MSCs. BMP2-expression is assessed by anti-BMP2 antibodies, the Flag-tagged Smad8 by anti-Flag-blotting. **(B):** Intramuscular control transplantation of a collagen sponge with control-infected human MSCs. **(C, D):** Intramuscular transplantations of lentivirally modified hMSCs expressing Smad8ca and BMP2 lead to the heterotopic formation of bony elements with inserting tendinous elements (overview, H&E staining). Red arrowheads indicate the potential formation of osteotendinous junctions (OTJ). Black arrowheads show ongoing angiogenesis. **(C′, D′):** Schematic drawings indicating the position of the OTJs. **(E):** Bony element in a heterotopic implant with modified human MSCs (H&E staining). **(F):** Immunohistochemistry with human-specific anti-β2-microglobulin of the location shown in **(E)**. Bone-lining cells and embedded osteocytes in bone are characterized by the presence of human β2-microglobulin. **(G):** Part of a tendon-like structure (H&E staining). **(H):** Immunohistochemistry with specific anti-human β2-microglobulin antibodies from the location shown in **(D)**. Several tenoblast-like cells stain positive for human β2-microglobulin (blue arrowheads). Abbreviations: B, bone; BMP2, Bone Morphogenetic Protein 2; hMSCs, human mesenchymal stem cell; M, muscle; S, collagen sponge; T, tendon-like structures.

tendinous and osteogenic tissue the formation of OTJs is brought about.

Progenitor-Dependent Formation of Tendon-Bone Interfaces

The murine progenitor-dependent formation of distinct fibrocartilage OTJs is predominantly observed if osteogenic and tenogenic tissues meet in perpendicular fashion (Figs. 1, 2C). However, tenogenic and osteogenic tissue may also meet with an acute angle (Figs. 2B, 3). Under these conditions, the formation of fibrocartilage tissue in the tendon/bone interface seems to be reduced as evidenced morphologically and by histological staining, and substantiated by collagen II expression. In contrast to murine progenitors C3H10T[1/2], we could not observe the human MSC-dependent generation of tendon/bone attachment sites with a fibrocartilage interface. This may be due to the fact that in comparison with the murine progenitors bone marrow-derived hMSCs exhibit a reduced competence for chondrocyte differentiation and cartilage formation at heterotopic sites. In fact, the efficient hMSC-dependent cartilage formation in vivo is a problem as such for regenerative strategies involving cartilage tissues. Although hMSCs spontaneously form bone on hydroxyapatite carriers, various growth factors seem to be required to promote chondrogenesis in bone marrow-derived hMSCs. Various isoforms of Transforming Growth Factor-beta (TGF-beta) or Fibroblast Growth Factor (FGF) have successfully been applied [27–29] while BMP2, in comparison with TGF-beta's, seems less favorable for cartilage formation (own investigations, data not shown).

We, therefore, hypothesize that the reduced capacity for cartilage formation in hMSCs in this investigation is the reason for the lack of fibrocartilage intermediates in the tendon-bone attachment sites and that this chondrogenic capacity would have to be additionally supplied for regenerative strategies involving the establishment of the fibrocartilage enthesis type.

Future studies have to show whether members of the TGF-β-family may provide such an additional chondrogenic capacity. Interestingly, it has recently been observed that stem cells derived from tendon may develop OTJs if they interact

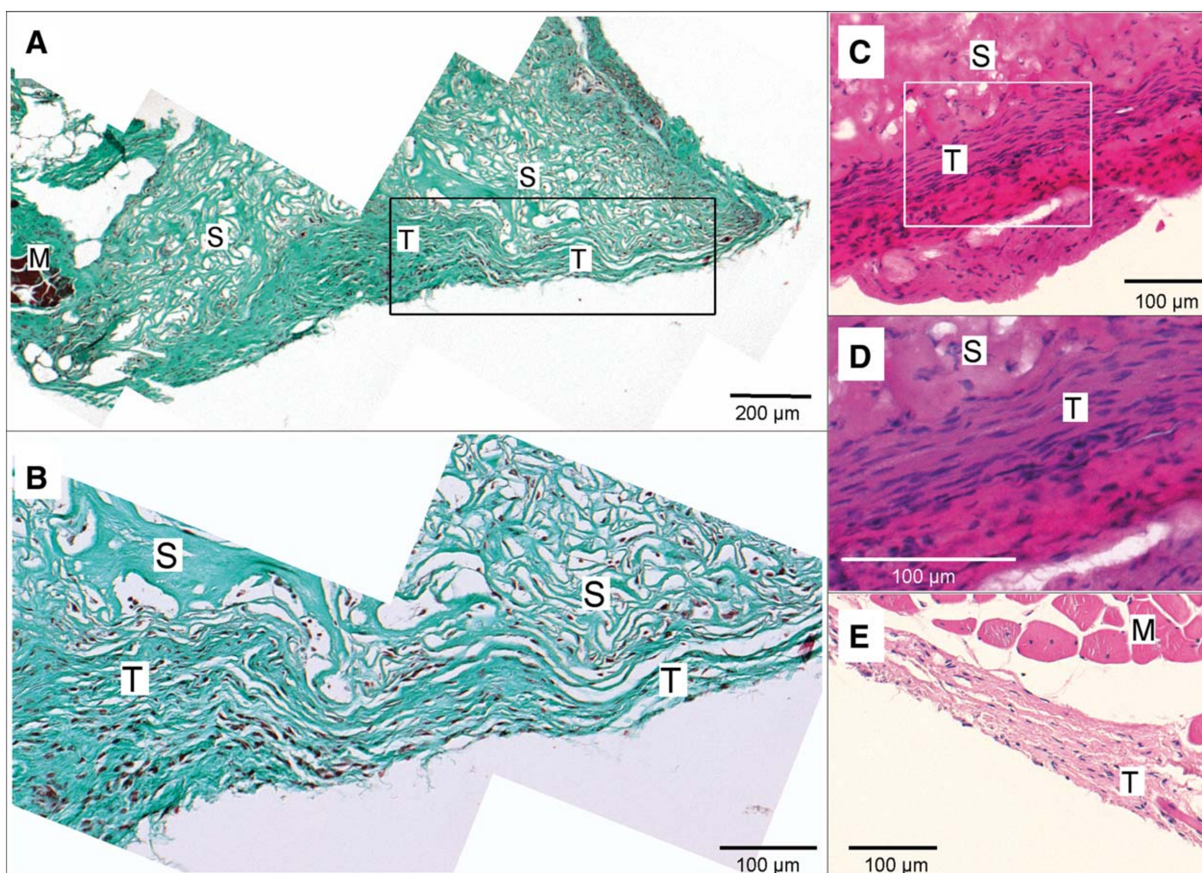

Figure 6. Lentivirus-mediated expression of Smad8ca in human mesenchymal stem cells (MSCs) is sufficient for the heterotopic formation of tendon-like elements. **(A):** Lentivirally modified human MSCs which express Smad8ca form tendinous elements after heterotopic intramuscular implantation (overview, Masson-Goldner staining). **(B):** Tendinous elements after heterotopic intramuscular implantation of Smad8ca-modified human MSCs (magnification, rectangle of **(A)**, Masson-Goldner staining). **(C):** Tendinous elements after heterotopic intramuscular implantation of Smad8ca-modified human MSCs (H&E staining). **(D):** Tenocyte-like cells (magnification of **[C]**, rectangle; H&E staining). **(E):** Tendinous elements after heterotopic intramuscular implantation of Smad8ca-modified human MSCs (H&E staining). Abbreviations: M, muscle; S, collagen sponge; T, tendon.

with bony calvariae [25], however, the type of OTJ has not been investigated closer.

In the study, we propose a TGF-beta/BMP signaling-dependent model for the formation of tendon and tendon/bone interfaces. However, another approach might involve transcription factors to drive stem cells into tendon/ligament formation. Unfortunately, only few transcription factors have been described as being expressed in tendons and Scleraxis (*Scx*) is still the only transcription factor displaying an expression pattern restricted mainly to tendons and ligaments [30, 31]. However, *Scx* is not necessary for tenocyte specification, as tendon progenitor cells are present and many tendons still form in *Scx*−/− mutant mice [32]. Therefore, *Scx* alone is not sufficient for tendon formation. In *Drosophila* sp., tendon differentiation relies upon the transcription factor "stripe," an early growth response (Egr)-like transcription factor [33–36]. Its vertebrate homologues *Egr1* and *Egr2/Krox20* are so far not openly involved in tendon formation, however, a tenogenic capacity for these factors has also not yet been excluded.

Heterotopic Formation of Tendon, Bone, and OTJs—The Question of Mechanical Loading

The heterotopic implantation of adeno- or lentivirally modified stem cells forming tendinous/ligamentous tissue by the expression of growth factors and/or particular signaling molecules as demonstrated here may imply that tendon formation predominantly is dependent on growth factor signaling rather than on mechanical loading. However, the pivotal role of mechanical stimuli for the formation and maintenance of tendons and ligaments has been well documented [37–40]. So, well-established stem cell-dependent tenogenesis models apply stem cell-seeded gels under static or dynamic tension [41]. Moreover, MSCs formatted into a type I collagen gel under constant load aligned with the load axis within tendon defects after implantation [16, 18, 42, 43]. It seems difficult to separate the influence of mechanical loading from growth factor-dependent activities as several studies have clearly shown that mechanical loading triggers the expression of a plethora of growth factors and extracellular matrix molecules defining a tenogenic fate [40, 44-46]. Indeed, the overall parallel orientation of the new tendons along with actively contracting muscles of the host in our study implies that mechanical loading exerted by the microenvironment does play a major role in the formation of tendinous structures (Figs. 1, 2). Moreover, extended implantation times (2 months) resulted in tendon-bone-interfaces formation with substantial adipogenic appendages (Supporting Information Fig. 1). This is also indicative for reduced mechanical loading conditions at the heterotopic intramuscular sites of implantations.

Conclusion

The present data indicate the remarkable competence of adult stem cells to form OTJs once they possess a tenogenic and an osteogenic capacity. Our findings support the notion of a process that is primarily driven by growth factors and signaling mediators, which, however, is further shaped and maintained by mechanical loading as described in [47]. Tendon/ligament-bone attachment sites are susceptible to injury and, unfortunately, the current regenerative technologies fail to restore the function and the anatomic structure of tendon/ligament entheses. To re-establish the biomechanical properties of tendon/ligament attachment sites, a full regeneration of this structure is required. In a recent study, characterizing the biomechanical properties of tendon/ligament-to-bone interfaces, Moffat et al. [47] suggest the construction of a multiphased scaffold to support the establishment of distinct and continuous tissue regions observed at the native interface. First results of such a biomimetic strategy have recently been reported [48, 49]. Here, we describe now the option to establish a stem cell-dependent regeneration of tendon/ligament-bone interfaces, which might be considered as another critical step in the regeneration of functional soft tissue-to-bone interfaces. The ability of adult stem cells to form tendon/ligament attachment sites if they exhibit both a distinct tendinous/ligamentous and osteogenic capacity may be used especially in combination with the biomimetic modalities suggested before to efficiently reconstruct soft tissue-to-bone interfaces.

Acknowledgments

We appreciate superior technical assistance by Inge Hollatz and Doris Stellfeld. G.G., P.C., and T.H. have been financially supported by the European Community (Key action LSH 1.2.4-3, Integrated project: GENOSTEM "Adult MSCs engineering for connective tissue disorders. From the bench to the bed side", Contract no: 503161). In addition, G.G. received funding by the German Research Foundation (DFG, Deutsche Forschungsgemeinschaft) in SFB 599.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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