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Three-Dimensional High-Density Co-Culture with Primary Tenocytes Induces Tenogenic Differentiation in Mesenchymal Stem Cells

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ABSTRACT: Mesenchymal stem cells (MSCs) have potential applications in regenerative medicine and tissue engineering and may represent an attractive option for tendon repair and regeneration. Thus far the ability of MSCs to differentiate into tenocytes in vitro has not been investigated. Experiments were performed with and without growth factors (IGF-1, TGF-β1, IGF-1/TGF-β1, PDGF-BB, and BMP-12), in co-cultures of tenocytes and MSCs mixed in different ratios and by culturing MSCs with spent media obtained from primary tenocytes. Tenogenesis was induced in MSCs through a combination of treatment with IGF-1 and TGF-β1, in high-density co-cultures and through cultivation with the spent media from primary tenocytes. Electron microscopy and immunoblotting were used to demonstrate up-regulation of collagen I/III, decorin, tenomodulin, β1-Integrin, MAPKinase pathway (Shc, Erk1/2), and scleraxis in the co-cultures and provide simultaneous evidence for the inhibition of apoptosis. In monolayer co-cultures extensive intercellular contacts between MSCs and tenocytes were observed. Cells actively exchanged vesicles, which were labeled by using immunofluorescence and immunogold techniques, suggesting the uptake and interchange of soluble factors produced by the MSCs and/or tenocytes. We conclude that MSCs possess tenogenic differentiation potential when provided with relevant stimuli and a suitable microenvironment. This approach may prove to be of practical benefit in future tissue engineering and tendon regenerative medicine research.

Keywords: mesenchymal stem cell; tenocytes; tenogenesis; 3-D co-culture; ultrastructure

More than 30 million tendon and ligament injuries are reported globally every year.[1] These range from acute injuries caused primarily by trauma or inflammatory processes, to chronic injuries caused mainly through repetitive mechanical stress and excessive strain.[2] Tendons and ligaments have a very limited intrinsic potential for repair and regeneration poses a complex challenge for clinicians.[3,4] Gold standard treatments include allografts, autografts, xenografts, and prosthetics. However, these methods have failed to provide a functionally and mechanically adequate tendon with a long-term positive outcome.[5] The main reason is the formation of a biomechanically inferior fibrous, scar-like tissue[6] leading to significant functional impairment and disability of the patients not only in sport but also in everyday life.[7] This loss of mechanical function is the consequence of the formation of a distorted ECM with misaligned collagen fibrils in the reclaimed material.[8] The highly organized tendon extracellular matrix is produced by tenocytes, the specialized and differentiated cells of tendons. Therefore, new treatment strategies for tendon repair require appropriate cell sources.

Mesenchymal stem cells (MSCs) are increasingly studied for their potential in regenerative medicine and tissue engineering due to their pluripotency. They also represent an attractive option for tendon repair and regeneration.[9] MSCs are found in almost every organ and tissue.[10,11] However, adipose-derived MSCs have become increasingly popular in basic stem cell biology and experimental regenerative medicine research.[12,13] Under appropriate cell culture conditions they can differentiate into several tissues including bone, cartilage, and adipose tissue.[14,15] However, in order to trigger differentiation, in vitro growth factors are needed for these induction protocols.[16] Although several growth factors such as IGF-1, TGF-β1, BMP-12, and PDGF-BB have been described as having positive effects on tenocyte proliferation and differentiation,[17–20] so far there is no compelling evidence that tenogenesis can be induced in MSCs.

There is evidence, however, that re-implanted MSCs in vivo undergo site-specific differentiation and, for example, in bone tissue can enhance bone formation.[21] Further, we and others have previously demonstrated that co-culture with differentiated primary tissue cells can induce differentiation of MSCs into fully differentiated cells without adding growth factors.[22–24] These results suggest that differentiation of MSCs is not solely modulated by growth factors but also by direct cell-to-cell contact, active intercellular materials exchange, and an appropriate culture microenvironment. There have been few studies on the signaling pathway activation in tenocytes that initiate proliferation and differentiation.[2] However, it is well known that the MAPKinase signaling pathway plays a pivotal role in the maintenance of cell phenotype and differentiation[25,26] and that the transcription factor scleraxis is of vital importance for maintenance of the tenocyte phenotype.[27,28] The aim of this study was to evaluate the tenogenic induction potential of primary

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derived tenocytes on MSCs in a three-dimensional co-culture system to gain a better understanding of the processes involved in tenogenic differentiation. This knowledge may improve tendon regeneration and open up new therapeutic approaches for tendinopathies.

METHODS

Animal Tissues

MSCs were isolated from canine adipose tissue biopsies and autologous primary canine tenocytes were isolated from tendon biopsies, both obtained during total hip replacement surgeries at the Clinic of Veterinary Surgery, Ludwig Maximilian University of Munich (LMU), Germany, with fully informed owner consent and project approval by LMU. Animals used were between 3 and 7 years old.

Adipose Derived Canine Mesenchymal Stem Cells

MSCs were isolated as previously described.[29] Briefly, small pieces of adipose tissue were digested with collagenase 0.2% in Ham's-F12 at 37°C for 2 h. The digested tissue was centrifuged and the pellet resuspended in cell culture medium consisting of DMEM/Ham's-F12 1:1, 10% FCS, 1% partricin solution, 1% penicillin/streptomycin solution, 75 μg/ml ascorbic acid, 1% essential amino acids, and 1% Glutamine, all obtained from Seromed (Munich, Germany). After 4 days in culture, non-adherent cells were washed off with Hank's balanced salt solution. Characterization of MCSs was performed by their ability to adhere to plastic, immunofluorescent evaluation of a set of defined markers ($CD105^+$, $CD90^+$, $CD45^-$, and $CD34^-$) and, as described previously, by their multi-lineage differentiation potential to osteoblasts, adipocytes and chondrocytes in vitro.[10,29–31]

Primary Canine Tenocytes

Tenocytes were isolated by cutting the freshly obtained tendon biopsies into 1–2 mm thick slices while carefully removing the epi- and peritendon sheath. Samples were incubated in cell culture medium and after 4–7 days, tenocytes started to migrate from the tissue explant, adhered to the Petri dish and formed colonies. Cells were split when colonies had reached 60–70% confluence. Primary tenocytes were cultured at a density of 200,000 cells in 60 mm Petri dishes in monolayer culture at 37°C with 5% CO_2. Media were changed three times per week.

High-Density Culture

Three-dimensional high-density cultures were prepared as previously described.[22] Briefly, around 1×10^6 cells were pipetted onto a nitrocellulose filter on a steel grid. This model allows the cells to aggregate, forming a distinct pellet. High-density cultures consisted of either MSCs or tenocytes alone or a combination of both (co-cultures) at different ratios (90:10, 70:30, 50:50). Cultures were either left untreated or incubated with various growth factors: IGF-1, hTGF-β1, IGF-1/TGF-β1, PDGF-BB, or BMP-12 (all obtained from Acris Antibodies GmbH, Hiddenhausen, Germany).

Histology

Osteogenesis was evaluated by von Kossa staining as previously described.[10] Adipogenesis was evaluated using Oil Red O staining of lipid vacuoles. Chondrogenesis in MSC high-density cultures was determined by staining cartilage specific proteoglycans with Alcian blue.

Immunofluorescence

Isolated canine adipose derived MSCs were evaluated by immunofluorescence in monolayer culture for MSC specific markers $CD105^+$ and $CD90^+$ and for the hematopoietic stem cell markers $CD45^-$ $CD34^-$ as previously described.[10]

Western Blotting

To examine the levels of total cell protein expression of the tenocytes, the extracts were prepared and fractionated by SDS–polyacrylamide gel electrophoresis as previously described.[26] After electrophoresis, the proteins were electro-transferred onto nitrocellulose membranes, blotted with each primary antibody, and then incubated with alkaline phosphatase conjugated secondary antibodies. After further washing, specific antigen–antibody complexes were detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate (*p*-toluidine salt; Pierce, Rockford, IL).

Electron and Immunoelectron Microscopy

Electron microscopy was performed as previously described.[29] Briefly, high-density co-cultures were fixed for 1 h in Karnovsky's Fixative and post-fixed in 1% OsO_4 solution. After dehydration, pellets were embedded in Epon, ultrathin cuts made on a Reichert-Ultracut E. and contrasted with a mixture of 2% uranyl acetate/lead citrate. For immunoelectron microscopy, cultures were fixed in 3% paraformaldehyde plus 0.25% glutaraldehyde and examined using a Zeiss electron microscope (TEM 10, Zeiss, Jena, Germany) as previously described.[32]

PKH Membrane Labeling

Ca. 1 million cells were washed three times with PBS and labeled with the fluorescent PKH membrane dye (Sigma, Munich, Germany), mixed at a ratio of 1:1 and incubated for up to 7 days in monolayer or 14 days in high-density culture, as previously described.[22] Successful staining was evaluated under an immunofluorescence microscope and images were digitally captured and stored (Leica, Bersheim, Germany).

Endocytosis/Exocytosis Assay

The capacity for materials exchange was determined using an endocytosis/exocytosis assay as previously described.[33] Briefly, 5×10^5 cells were suspended in medium and MSCs were incubated with 10 nm while tenocytes were incubated with 5 nm Goat-Anti-Rabbit gold-conjugated secondary antibodies. After 10 min, cells were centrifuged and washed three times with PBS/1% BSA. Co-cultures were created with a 1:1 ratio of MSCs and tenocytes and incubated with the controls of either MSCs or tenocytes alone for 48 h in monolayer. Samples were fixed with Karnovsky, treated as described above and evaluated under a TEM 10.

Cultivation of MSCs with Supernatant Derived from Primary Tenocytes

The effects of spent media from primary tenocyte cultures on MSCs in high-density culture were studied for a period of 14 days. As controls high-density cultures of pure tenocytes, pure MSCs or MSC/tenocyte co-cultures (as positive control) at a ratio of 50:50 were incubated with normal cell culture medium or pure MSC cultures were incubated with IGF-1

and hTGF-β1 (5 ng/ml each). Cultures were evaluated by TEM 10.

Statistical Analysis

The density (specific binding) of each band was measured by densitometry using "Quantity One" (Bio-Rad Laboratories, Inc., Hercules, CA). The results are shown as the mean ± SD of a representative experiment performed in triplicate.

RESULTS

Characterization of MSCs

Adipose derived MSCs exhibited a polymorphic, fibroblast-like morphology (Fig. 1A-a). Cells formed colonies after 3–5 days in culture and actively searched for cell-to-cell contacts. Light microscopic analysis of MSC monolayer cultures treated with adipogenic differentiation media and stained with Oil Red O staining contained numerous vacuoles filled with neutral lipids (Fig. 1A-b). Upon osteogenic induction treatment, MSCs in monolayer exhibited polygonal morphologies as compared to controls. They formed nodules and deposited minerals that stained positive with von Kossa (Fig. 1A-c). Chondrogenic induced MSC high-density cultures stained positive for alcian blue revealing high amounts of cartilage specific proteoglycans (Fig. 1A-d). Ultrastructural evaluation of untreated MSCs demonstrated apoptosis or necrosis in the high-density cultures (Fig. 1B-a). In contrast adipogenic induced MSC high-density cultures exhibited adipogenesis with metabolically active cells containing many vacuoles embedded in a highly structured extracellular matrix (Fig. 1B-b). In osteogenic induced MSC high-density cultures ultrastructural evaluation revealed newly formed osteoblasts, with many cell–cell contacts and large numbers of cell organelles. The newly produced matrix consisted of thick fibrils that were well organized and densely distributed in the extracellular space (Fig. 1B-c). In chondrogenic induced MSC high-density cultures, a typical cartilage nodule formation was observed (Fig. 1B-d). Immunofluorescence was used to confirm positive expression of the stem cell specific markers $CD105^{+}$ and $CD90^{+}$ and negative expression of the hematopoietic lineage markers $CD45^{-}$ and $CD34^{-}$ (Fig. 1C).

Differentiation of MSCs to Tenocytes Through Growth Factors

Pure tenocyte cultures were evaluated as controls: Close cell-to-cell contacts were observed. The cells also exhibited large euchromatin rich nuclei and typical tenocyte-like spindle shaped morphologies and formed thin,

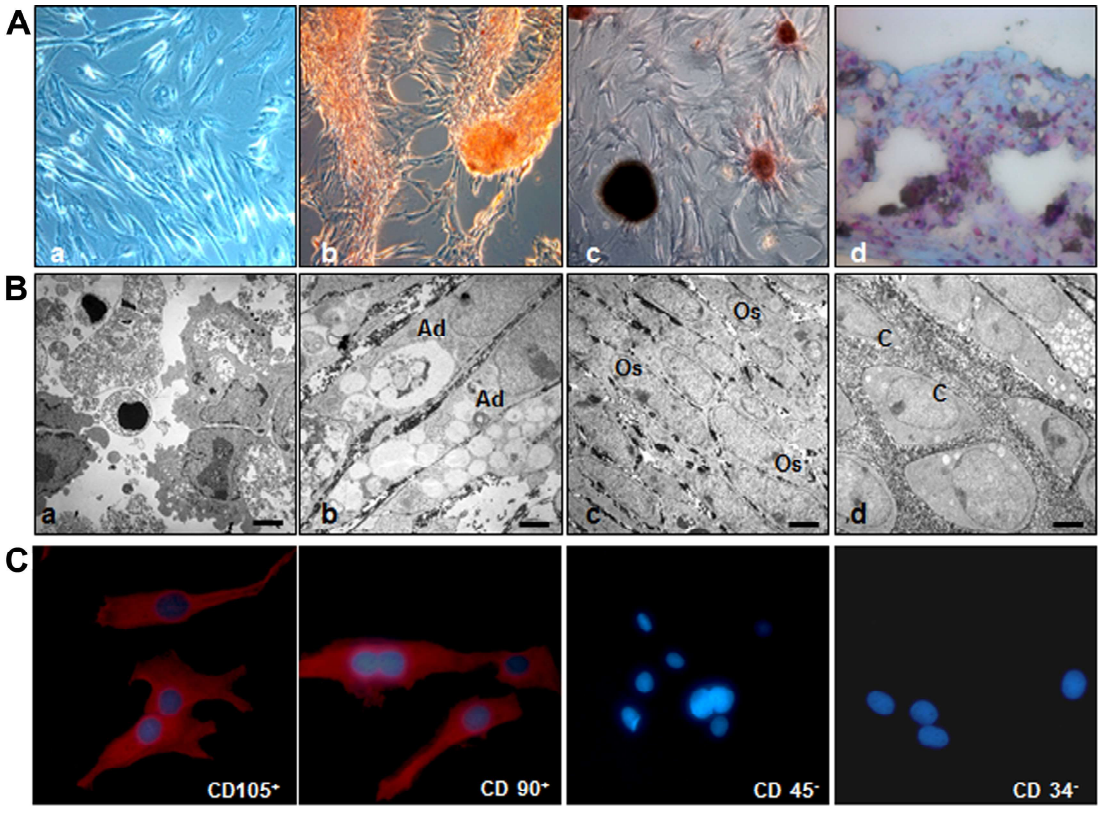

Figure 1. (A) Light micrographs of freshly isolated MSCs in monolayer (a) and lineage differentiation of MSCs to adipocytes stained with Oil Red O (b), osteocytes stained with von Kossa (c), and chondrocytes stained with alcian blue (d). Magnification (a–d): ×40. (B) Electron microscopic demonstration of untreated MSCs (a) and adipogenic (b), osteogenic (c), and chondrogenic (d) induction of MSCs in high-density culture. Magnification: ×5,000, bar = 1 μM. (C) Immunofluorescence labeled MSCs in monolayer show positive signals for stem cell specific markers $CD105^{+}$ and $CD90^{+}$ and no signal for hematopoietic stem cell markers $CD45^{-}$ and $CD34^{-}$. Magnification: ×40.

elongated processes. Bundles of highly structured, fibrillar extracellular matrix were visible in the intercellular space (Fig. 2A). In contrast untreated MSCs exhibited morphological signs of apoptosis including membrane blebbing, free cellular organelles and other cellular debris (Fig. 2A). MSC cultures treated with IGF-1 alone showed little signs of intercellular contacts, the intercellular space filled only to some extent with unstructured extracellular matrix compounds (Fig. 2A-a). In the cultures treated with TGF-β1 alone a similar picture was observed: G-cells were separated by large intercellular spaces that were filled with unorganized extracellular matrix (Fig. 2A-b). Interestingly, treatment with a combination of IGF-1/TGF-β1 produced different results. Here, cells had mainly spindle-shaped morphology, exhibited extensive intercellular contacts and produced a well-organized extracellular matrix (Fig. 2A-c). After 14 days, intercellular spaces had become tightly packed with matrix components and there were clear hints of a highly active cell metabolism, that is, a well-developed protein synthesis apparatus, very similar to the pure tenocyte culture. Cells cultured with PDGF-BB alone had some intercellular contacts, appeared partly spindle-shaped and some cells contained vacuoles (Fig. 2A-d). After 7 days culturing time with BMP-12, cells and matrix appeared unstructured and little intercellular communication could be seen (Fig. 2A-e). To evaluate these ultrastructural results in more detail, immunoblotting was performed for tenogenic specific markers, signaling pathway activation and signs of apoptosis induction. The amount of the predominant collagens in tendon tissue, collagen types I and III (Millipore, Schwalbach, Germany), showed similar results. In pure tenocyte cultures both collagens were synthesized both after 7 and 14 days (Fig. 2B). In untreated and treated MSCs with either IGF-1 or TGF-β1 alone no synthesis of either collagen type I or III was observed (Fig. 2B).

Decorin (Millipore) expression was highest in tenocyte controls and MSCs treated with IGF-1/TGF-β1, where it was markedly increased after 14 days (Fig. 2B). In contrast there was no decorin synthesis in untreated MSC cultures and in cultures treated with

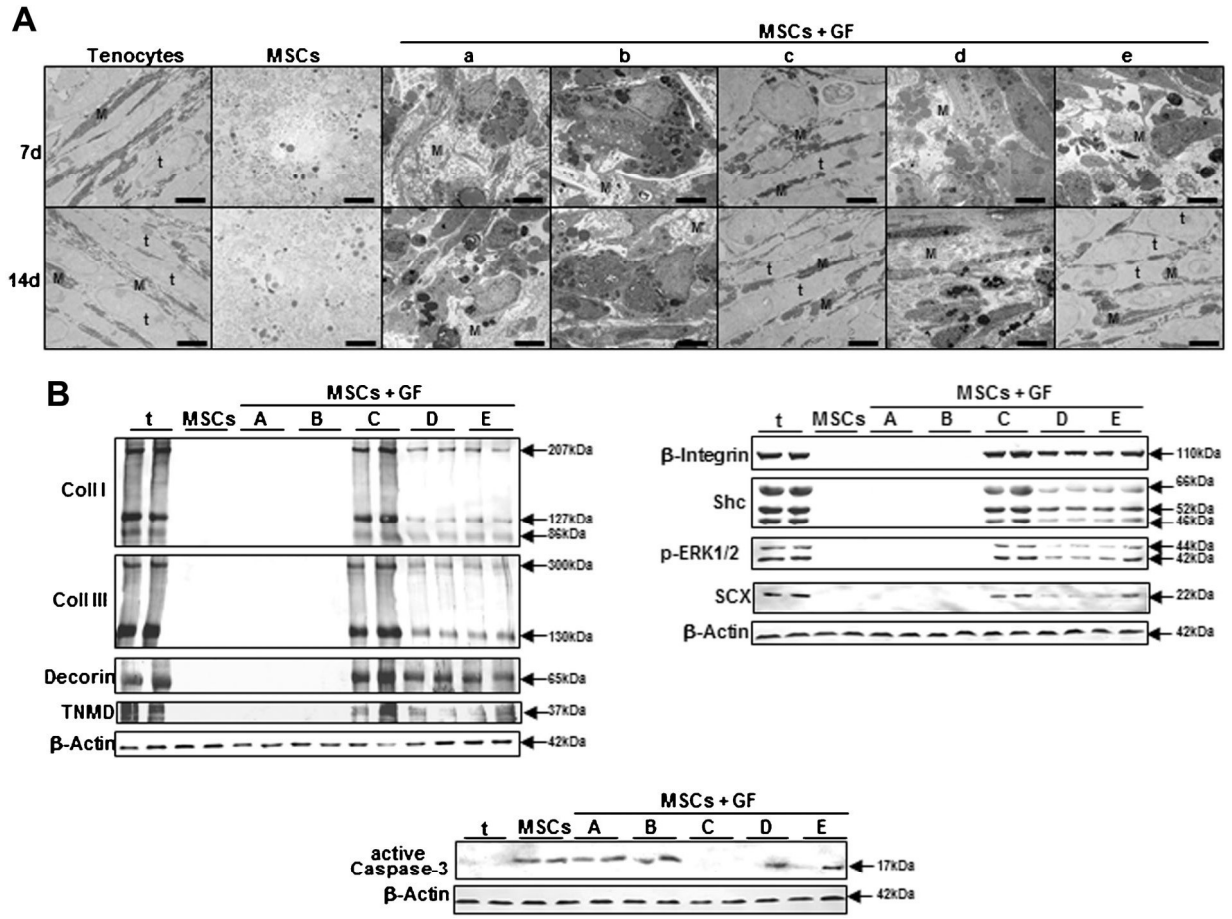

Figure 2. (A) Electron microscopic analysis of high-density cultures treated for 7 and 14 days. Pure tenocyte and MSC cultures served as untreated controls; MSCs treated with various growth factors (GF) as follows: (a) 10 ng/ml IGF-1; (b) 10 ng/ml TGF-β1; (c) IGF-1/TGF-β1 (5 ng/ml each); (d) 10 ng/ml PDGF-BB; (e) 10 ng/ml BMP-12. t, tenocytes; M, matrix. Magnification: ×5,000, bar = 1 μm. (B) Western blot analysis confirmed the results obtained using ultrastructural techniques. Immunoblots of whole cell lysates (500 ng protein per lane) were probed with antibodies against, collagen types I and III, Decorin, tenomodulin, β1-integrin, Shc, activated ERK1/2, scleraxis, and activated caspase-3. Each experiment was performed in triplicate. Expression of the housekeeping gene β-actin (Sigma) was not affected.

IGF-1 or TGF-β1 alone. In cultures treated with PDGF-BB or BMP-12 some decorin expression could be seen with synthesis levels considerably lower than in the pure tenocyte cultures. Synthesis of the tendon specific marker tenomodulin (TNMD) (Santa Cruz, CA) was highest in tenocyte control cultures; however concentration decreased slightly after 14 days (Fig. 2B). No TNMD expression could be seen in untreated MSCs and MSCs treated with IGF-1 or TGF-β1 alone. In contrast in MSC cultures treated with the combination of IGF-1/TGF-β1 some TNMD-expression was observed after 7 days, which increased to levels similar to control cultures at 14 days. Activation of MAPKinase signaling cascade members Shc and extracellular regulated kinases 1/2 (ERK1/2) (BD Biosciences, Erembodegem, Belgium) is shown in Fig. 2B. In pure tenocyte cultures and in MSCs treated with the combination of IGF-1/TGF-β1, high levels of Shc and ERK1/2 expression could be observed. In contrast in untreated MSCs and in MSCs treated with IGF-1 or TGF-β1 alone, no expression was observed and only marginal quantities of ERK1/2 and Shc could be detected in the PDGF-BB and BMP-12 cultures. The tendon-specific transcription factor scleraxis (Abcam plc., Cambridge, UK) was upregulated in pure tenocyte cultures and in MSCs treated with the combination of IGF-1/TGF-β1 (Fig. 2B). In contrast scleraxis expression was low in PDGF-BB and BMP-12 cultures and completely lacking in untreated MSCs and in MSCs treated with IGF-1 or TGF-β1 alone. To demonstrate the influence of growth factors on the induction of the apoptotic signaling cascade in MSCs, cultures were evaluated for activated caspase-3 (R&D Systems, Abingdon, UK). Cleaved caspase-3 was expressed in the untreated MSC cultures and in cultures treated with either IGF-1 or TGF-β1 alone already after 7-day culture period. After an incubation period of 14 days, activated caspase-3 was also expressed at high levels in the cultures incubated with PDGF-BB and BMP-12.

High-Density Co-Culture with Tenocytes Induces Tenogenesis in MSCs

In three-dimensional culture, primary tenocytes exhibited a typical tenocyte morphology of elongated cells with long, thin pseudopodia and synthesized a highly organized ECM consisting of thick fibrils; in contrast MSCs underwent apoptosis with degradation of cells, membrane blebbing, and formation of apoptotic bodies (Fig. 3A). In co-cultures with primary tenocytes and MSCs, spindle-shaped cells were observed with numerous cell processes and intercellular connections and large quantities of highly structured, thick ECM fibrils in the pericellular space. However, interestingly in co-cultures containing more than 50% MSCs, this is in the 90:10 and 70:30 co-cultures, formation of numerous vesicles was additionally observed (Fig. 3A). Immunoblotting of collagen types I and III, tenomodulin, the signal and adhesion molecule β1-integrin, the MAPK signaling pathway proteins Shc and ERK1/2 confirmed the ultrastructural results (Fig. 3B). Pure tenocyte cultures exhibited collagen types I and III expression both after 7 and 14 days culture. In MSC cultures only marginal expression of both collagen types I and III was observed both after 7 and 14 days culture. In contrast, in all co-cultures, high production of both collagen types I and III was observed. Expression of both collagen types I and III increased with the percentage of tenocytes in the co-culture, reaching levels comparable to pure tenocyte cultures in the co-culture containing 50% tenocytes (Fig. 3B). The tendon typical proteoglycan TNMD was highly expressed after both 7 and 14 days culture in pure tenocyte cultures and in all co-cultures (Fig. 3B). After 14 days, TNMD levels were highest in co-cultures containing 50% tenocytes. β1-integrin (Millipore) is an important mediator between matrix and cells and its expression was observed both after 7 and 14 days in pure tenocyte cultures and all co-cultures, with expression levels similar to tenocyte cultures (Fig. 3B). Low quantities of β1-integrin were found in pure MSC cultures. After 7 and 14 days increased activation of the MAPKinase signaling members, adaptor protein, Shc and ERK1/2 were observed in pure tenocyte cultures and all co-cultures, with expression levels similar to tenocyte cultures (Fig. 3B).

Tenogenesis in Co-Culture Is Enhanced by Intensive Cell–Cell Interaction

To further evaluate and possibly explain where tenogenic effects observed in co-culture between MSCs with primary tenocytes originated, further experiments were carried out. First, the two cell types were labeled with two different colored PKH fluorescent membrane dyes, and brought into 50:50 co-cultures in monolayer. Already after 1 day in culture, extensive intercellular contacts and cellular interactions could be observed. MSCs and primary tenocytes started developing long cytoplasmic processes and actively sought cell contact with the other cell type (Fig. 4A:a–c). Cell–cell interactions increased after 3 days and active vesicle exchange between the different cell types was seen. Green vesicles could be observed in red cells and vice versa (Fig. 4A:d,e). Active cell-to-cell communication resulting in enhanced proliferation of MSCs in the vicinity of primary tenocytes continued until incubation ended after 7 days (Fig. 4A-f).

Endocytosis/exocytosis assays were carried out to evaluate materials exchange between the cells. MSCs were incubated with 10 nm gold particles, primary tenocytes with 5 nm gold particles and then incubated either alone or in 1:1 co-culture over 48 h. Ultrastructural evaluation with transmission electron microscopy demonstrated active exchange of gold particles between MSCs and tenocytes (Fig. 4B). Indeed, in the co-cultures, gold particles of different sizes could be

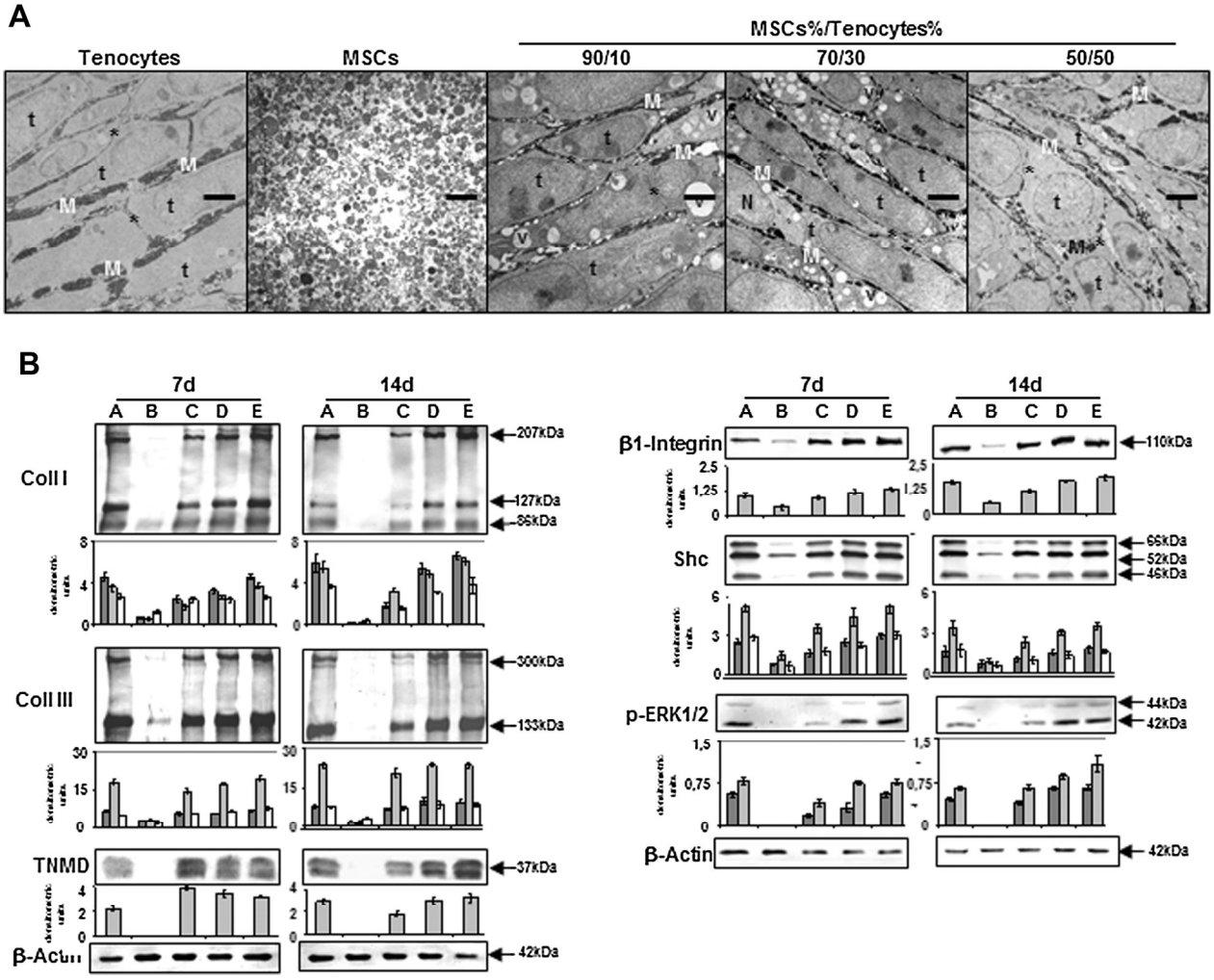

Figure 3. (A) Electron microscopic analysis of high-density MSC/tenocyte co-cultures after 14 days. V, vacuoles; M, matrix; t, tenocyte. Magnification: ×5,000, bar = 1 μM. (B) Immunoblots of whole cell lysates (500 ng protein per lane) were probed using antibodies against collagen types I and III, tenomodulin, β1-integrin, Shc, and activated ERK1/2. (a) 100% tenocytes; (b) 100% MSCs; (c) 90%MSC/10%Ten; (d) 70%MSC/30%Ten; (e) 50%MSC/50%Ten. Each experiment was performed in triplicate. Expression of the housekeeping gene β-actin was not affected.

observed in the extracellular space, within the cytoplasm and in newly formed vacuoles of the same cell.

Supernatant Derived from Tenocytes (Monolayer Culture) Has Tenogenic Induction Potential on MSCs in High-Density Culture

Recent studies have demonstrated that spent media can stimulate MSC differentiation. Thus we evaluated whether MSCs undergo tenogenesis in high-density when cultured only with supernatant obtained from primary tenocytes in monolayer culture. In control cultures of untreated tenocytes, MSCs treated with IGF-1/TGF-β1 (each 5 ng/ml) and co-cultures containing 50% primary tenocytes and 50% MSCs, ultrastructural evaluation demonstrated highly metabolically active cells, producing a well organized, tenogenic extracellular matrix, were as pure MSC cultures became apoptotic (Fig. 5A). Interestingly, in MSC cultures treated with the supernatant obtained from tenocytes, a similar pattern of tenogenic induction as in control cultures was observed. Immunogold labeling of the cultures demonstrated production of collagen type I and tenomodulin (Fig. 5A). The results obtained with electron and immunoelectron microscopy were confirmed with immunoblotting. In supernatant treated cultures, high upregulation of tenogenic specific ECM components, activation of signaling pathways and tenogenic specific transcription factor scleraxis were observed at levels comparable to control cultures of untreated tenocytes, MSCs treated with IGF-1/TGF-β1 and co-cultures containing 50% primary tenocytes and 50% MSCs (Fig. 5B).

DISCUSSION

The aim of this study was to evaluate the tenogenic induction potential of primary derived tenocytes and various growth factors on MSCs in a three-dimensional co-culture system to gain a better understanding of the processes involved in tenogenic differentiation of MSCs. As trauma of a tendon poses a

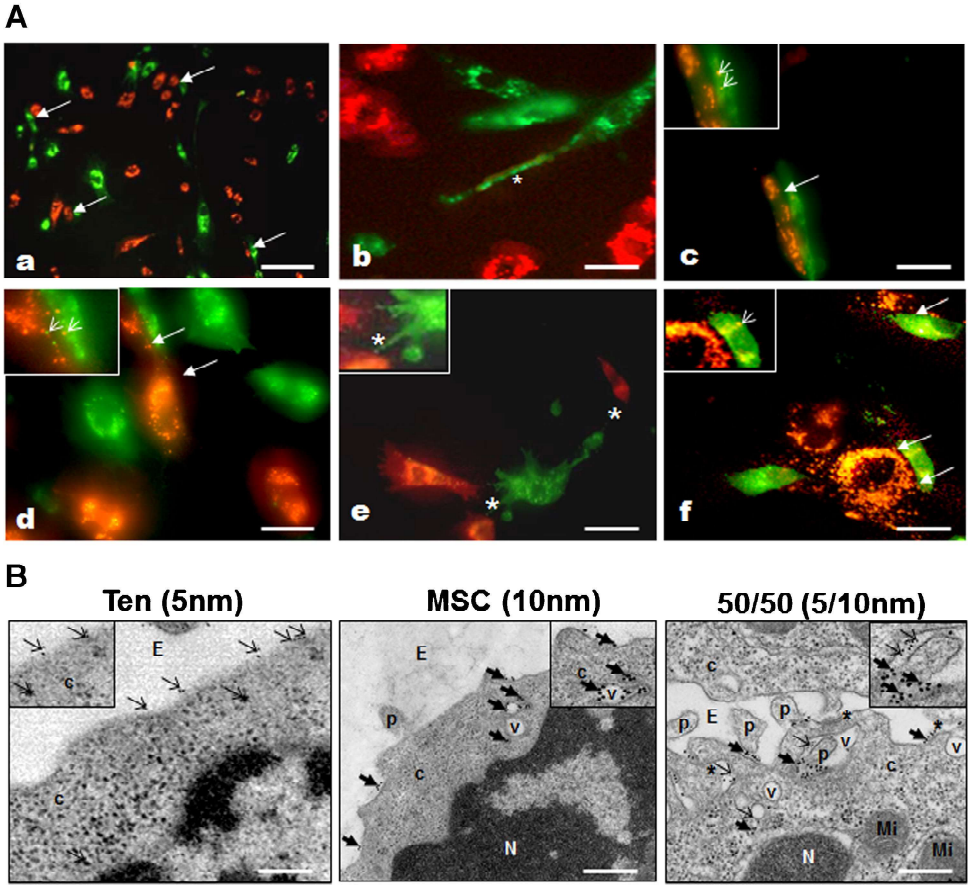

Figure 4. (A) PKH membrane labeling of MSCs (red) and tenocytes (green) in monolayer co-culture at a ratio of 1:1. Intercellular contacts (arrows) can be seen already after 1 day in culture (a–c). These increased after 3 days (d,e) and remained until incubation ended after 7 days (f). Co-cultured cells actively sought cell–cell contact (asterisks) through formation of pseudopodia and exchanged vesicles (insets, arrowheads). Magnification (a–f): ×20, bar: 30 μm. (B) After incubation of MSCs with 10 nm gold particles (thick arrows) and tenocytes with 5 nm gold particles (thin arrows), they were either incubated alone or in co-culture for 48 h at a ratio of 1:1. Ultrastructural evaluation of the co-cultures demonstrated gold particles (Amersham, Braunschweig, Germany) of different sizes within the cytoplasm (c) of the same cell, in vacuoles (v) and in the extracellular space (E). Mi, mitochondria. Magnification: ×20,000, bar = 0.2 μM.

great clinical challenge, due to the limited regeneration capacities of tendon in vivo,[3,4] new, adequate, alternatives and effective methods for tendon tissue regeneration are desperately needed. Indeed, tendon disease such as tendinitis makes up more than 30 million orthopedic cases worldwide each year.[1] Gold standard techniques for tendon repair comprise grafting of tendon tissue with allografts, autografts, xenografts and/or prosthetics.[5,34] However, full biomechanical efficacy has not been achieved.[34] The main reason for this is the low regeneration potential of tendon tissue due to its poor vascularization and weak proliferation potential of tenocytes leading to formation of scar-like tissue.[6] Therefore, recent approaches for tendon regeneration have focused on optimizing in vitro methods for tenocytes and growth factors.[34] However, as tenocyte amounts from biopsies are limited an alternative cell source with tenogenic induction potential and abundant amount of cells would be preferable and more effective for large defect repairs.[9,19]

MSCs are pluripotent and can be obtained in large quantities from adipose tissue.[12,13,29] However, the tenogenic induction potential of MSCs has not been evaluated. Therefore, in this study we evaluated a simple tenogenic induction cocktail of growth factors for MSCs. IGF-1, TGF-β1, and PDGF-BB are the main growth factors associated with tendon tissue during regeneration.[17,18,20] Treating with these alone did not induce tenogenic potential in MSCs; no tendon specific matrix was formed and cells did not resemble tenocytes. Violini et al.[19] used equine derived bone marrow stem cells to show that BMP-12 exerts tenogenic differentiation in MSCs. We also observed up-regulation of tenogenic markers such as tenomodulin and decorin with BMP-12. Several studies have shown that cell–matrix interactions are primarily mediated via multi-functional β1-integrins,[35,36] which function as signal transduction molecules[37] stimulating the MAP-Kinase pathways.[26,38] Since inhibition of cell–matrix interactions leads to tenocyte apoptosis,[39–41] we excluded all single growth factor treatments, including

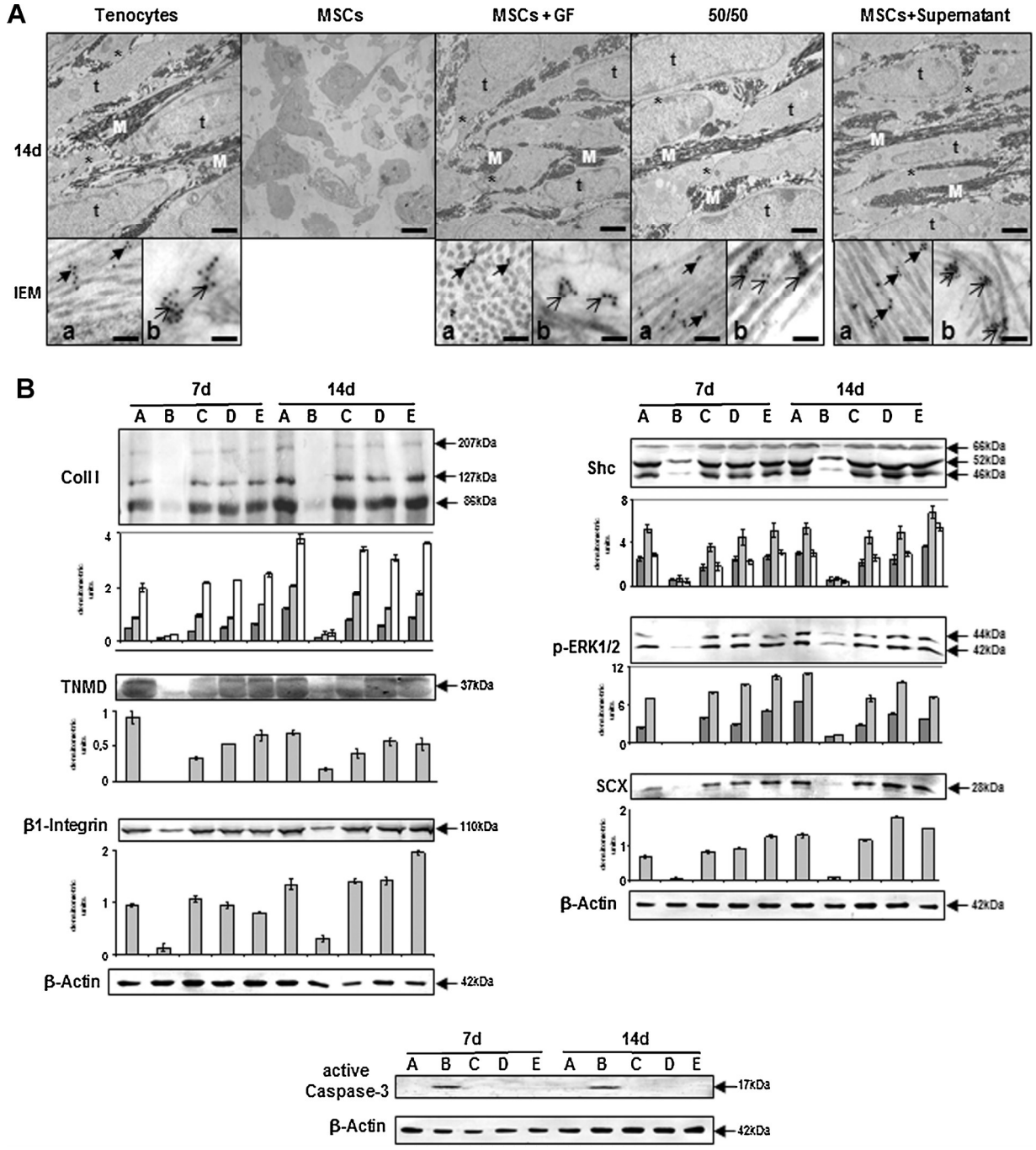

Figure 5. (A) Electron microscopic analysis of high-density cultures and co-cultures of MSCs/tenocytes after 7 and 14 days in culture. Untreated MSCs underwent apoptosis or necrosis. In contrast, pure tenocyte cultures, MSCs cultures treated with IGF-1/TGF-β1 (GF) (5 ng/ml each), MSC/tenocyte 1:1 co-cultures or MSCs cultures treated with the supernatant from monolayer tenocytes demonstrated clearly tenogenic matrix production (M) and tenocytes (t). Immunolabeling (IEM) of these cultures showed collagen type I (a) and tenomodulin (b) within the matrix. Magnification: ×5,000, bar = 1 μM; IEM: ×15,000, bar = 0.3 μM. (B) Immunoblots of whole cell lysates (500 ng protein per lane) were probed using antibodies against collagen type I, tenomodulin, β1-integrin, Shc, activated ERK1/2, scleraxis and activated caspase-3. (a) 100% tenocytes; (b) 100% MSCs; (c) MSCs with IGF-1/TGF-β1 (GF) (5 ng/ml each); (d) 50%MSC/50%Ten; (e) MSCs cultivated with tenocytes supernatant. Each experiment was performed in triplicate. Expression of the housekeeping gene β-actin was not affected.

BMP-12, as an appropriate induction cocktail for tenogenesis of MSCs.

We also observed up-regulation of the tenogenic transcription factor scleraxis in MSC cultures treated with the combination of IGF-1 and TGF-β1, comparable to pure tenocyte cultures. Scleraxis plays a pivotal role in tendon differentiation and matrix production.[27,28] Up-regulation of scleraxis by a combination of IGF-1 and TGF-β1 further underlines the positive tenogenic differentiation potential of both of these growth factors on the MSC cultures.

We next evaluated the intrinsic tenogenic potential of tenocytes on MSCs. In three-dimensional high-density co-culture, tenocytes exhibited a high tenogenic inductive potential on MSCs even at a very low tenocyte concentration in the culture of only 10%. Co-culture studies on other cells have shown that close contact in culture enhances specific cell lineage formation.[22,42] In a previous study we have demonstrated that high-density co-culture of MSCs and primary osteoblasts enhances osteogenesis.[22] Interestingly, in the same study, we demonstrated that osteogenic medium obtained from osteoblast cultures was sufficient for induction of osteogenesis in MSCs in high density culture.[22] This study has demonstrated that a similar effect can be observed in MSCs treated with the supernatant obtained from tenocyte monolayer cultures. We could also show by PKH staining and immune electron microscopy that soluble factors are segregated from the tenocytes and absorbed by the MSCs. Studies by other groups have also demonstrated this effect.[43,44] A study by Luo et al.[23] has demonstrated that indirect co-culture between bone marrow obtained MSCs and tenocytes enhances gene expression of tenogenic markers in MSCs.

In conclusion, adipose tissue derived MSCs possess the potential for tenogenic differentiation in the presence of appropriate cellular stimuli. Stable tenogenic induction can be achieved with a combination of IGF-1/TGF-β1, co-culture of MSCs and tenocytes or through culturing MSCs with spent media from pure tenocyte cultures. Therefore, our results point to new ways in which MSCs might be manipulated in culture for future therapeutic strategies for tendon tissue engineering.

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