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TGF β 2-induced tenogenesis impacts cadherin and connexin cell-cell junction proteins in mesenchymal stem cells

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Abstract

Tenogenic differentiation of stem cells is needed for tendon tissue engineering approaches. A current challenge is the limited information on the cellular-level changes during tenogenic induction. Tendon cells in embryonic and adult tendons possess an array of cell-cell junction proteins that include cadherins and connexins, but how these proteins are impacted by tenogenic differentiation is unknown. Our objective was to explore how tenogenic induction of mesenchymal stem cells (MSCs) using the transforming growth factor (TGF) β 2 impacted protein markers of tendon differentiation and protein levels of N-cadherin, cadherin-11 and connexin-43. MSCs were treated with TGF β 2 for 21 days. At 3 days, TGF β 2-treated MSCs developed a fibroblastic morphology and significantly decreased levels of N-cadherin protein, which were maintained through 21 days. Similar decreases in protein levels were found for cadherin-11. Connexin-43 protein levels significantly increased at 3 days, but then decreased below control levels, though not significantly. Protein levels of scleraxis and tenomodulin were significantly increased at day 14 and 21, respectively. Taken together, our results indicate that TGF β 2 is an inducer of tendon marker proteins (scleraxis and tenomodulin) in MSCs and that tenogenesis alters the protein levels of N-cadherin, cadherin-11 and connexin-43. These findings suggest a role for connexin-43 early in tenogenesis, and show that early-onset and sustained decreases in N-cadherin and cadherin-11 may be novel markers of tenogenesis in MSCs.

Keywords

Tendon; Tenogenesis; Stem cells; Cadherin; Connexin; Tissue engineering

1. Introduction

Tendons are musculoskeletal tissues that transfer forces from muscle to bone, and are essential to locomotion and movement. Tendons are frequently injured and have poor

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healing ability [1,2]. Loss of function and re-rupture are common following tendon injury, and motivate regenerative tissue engineering strategies using stem cells. Mesenchymal stem cells (MSCs) have promise for tissue engineering based on their potential to differentiate into several musculoskeletal tissue lineages [3], including tendon [4,5,6,7,8,9]. However, limited understanding of tenogenic differentiation (tenogenesis) is a challenge for using stem cells in tendon tissue engineering approaches. Tenogenesis appears to be influenced by several biochemical [6] and mechanical [7] factors, and their combined effects [8,9], but their impacts on MSC behavior are not fully understood.

Mature and developing tendons contain cells that are linked via cadherins and connexins (cell-cell junction proteins). Cadherins (cad) can complex with each other, bind to the intracellular actin cytoskeleton, sense forces, and trigger downstream signaling cascades [10]. Adult and embryonic tendon cells have been found to possess N-cad and cad-11 [11,12]. Cad-11 may be necessary for regulation of cell condensation, shape, and alignment in the developing embryonic tendon [12]. Connexins (i.e., gap junctions) allow the intercellular exchange of ions and small molecules by forming direct channels between the cytoplasm of adjacent cells. In tendon, connexins (cxn) are mechanotransducers capable of altering cellular activity in response to loading [13,14]. Cxn-43 has been found in adult [15,16] and embryonic tendons [17], and limb buds [18]. Cells in mature tendon appear to communicate via cxn-43 and cxn-32, which link cells in a 3-dimensional network that can collectively respond to mechanical stimuli [15]. Taken together, developing and mature tendon cells possess N-cad, cad-11 and cxn-43 cell-cell junction proteins, but there is limited information on how these proteins are altered by tenogenic induction in MSCs.

The three isoforms of transforming growth factor (TGF) β , 1, 2, and 3, all affect musculoskeletal tissues, but have distinct effects on differentiation and cell behavior. TGF β 1 is involved in extracellular matrix remodeling and collagen secretion during tendon healing and scar formation [19]. TGF β 1 [20,21] and TGF β 3 are inducers of chondrogenesis and cartilage formation [22,23,24]. TGF β 2 appears to be tenogenic: it upregulated tenogenic gene expression in MSCs [9,25], was found in embryonic tendon [26], maintained tenogenic commitment of embryonic tendon cells [27], and induced tenogenic markers *in vivo* [28]. Thus, TGF β 2 is an especially promising tenogenic growth factor.

To evaluate tenogenic differentiation, a few markers have been identified. Scleraxis (Scx) is a transcription factor upregulated during early embryonic tendon formation and is necessary for tenogenic commitment of tendon progenitor cells [29]. Tenomodulin (Tnmd) is thought to be a late stage tendon marker, is highly expressed in adult tendons, and is regulated by Scx [30,31]. Tenascin-C (TNC) is a glycoprotein present in developing and adult tendons [32]. Alongside these protein markers, cell morphology serves as additional evidence of tenogenic differentiation. *In vivo*, tenogenically differentiating embryonic tendon progenitor cells possess a highly organized actin cytoskeleton network [33], similar to adult tendon cells [11]. At present, it is not known if biochemical factors such as TGF β 2 can initiate changes to actin organization that lead to a more tendon-like appearance in MSCs.

The objective of this study was to investigate how TGF β 2 affects cell-cell junctions and tenogenic marker protein production, as well as actin cytoskeleton organization in MSCs.

We hypothesized that tenogenesis of MSCs results in altered levels of cell-cell junction proteins. Therefore, we examined proteins levels of N-cad, cad-11, and cxn-43, as well as tendon marker proteins Scx, Tnmd, and TNC throughout 21 days of TGF β 2 treatment. We demonstrated pronounced morphological changes in TGF β 2-treated MSCs, as well as significant changes in cell-cell junction protein levels as early as day 3, and significant increases in tenogenic marker proteins at day 14 and 21. Our results indicate that TGF β 2-induced tenogenesis impacts the cell-cell junctions produced by MSCs.

2. Methods

Cell Culture and TGF β 2 Supplementation

Murine MSCs (C3H10T1/2, ATCC, Manassas, VA), a model MSC used in prior studies investigating tenogenesis [27,34], were cultured in standard growth medium (Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and 1% Penicillin/Streptomycin) until 70% confluent, and used between passage 3 and 9. MSCs were trypsinized, and 5000 cells/cm² were seeded into each well of a 12-well plate and incubated for 24 h to allow for initial cell attachment. Cells were washed with warmed phosphate buffered saline (PBS) (Gibco, Grand Island, NY), the medium was switched to low-serum medium (DMEM, 1% FBS, 1% Penicillin/Streptomycin), and allowed to equilibrate for 24 h. Cells were rinsed with warm PBS and cultured for 3, 7, 14, and 21 days (d) in low-serum medium (controls) or low-serum medium supplemented with 50 ng/mL recombinant human TGF β 2 (PeproTech, Rocky Hill, NJ). The medium was changed every third day. Experiments were repeated a minimum of 3 times.

Western Blot Analysis

Cells were collected for western blot (WB) analysis in RIPA cell lysis buffer and HALT protease inhibitor (Invitrogen, Carlsbad, CA). Sodium dodecyl sulfate (SDS) was added at a 1:1 ratio and samples were sonicated, heated to 100° C for 5 minutes, and loaded into Novex Wedgewell 4-20% Tris Glycine Mini Gels (Invitrogen). To normalize total protein content (due to increased cell proliferation with TGF β 2 treatment), 20 μ L of control and 10 μ L of treated cell lysate were loaded per lane. Cell lysate collected from each well of the 12-plate was run in its own lane (3 wells of each condition were run per individual experiment). Samples probed for TNC required 4-12% Tris Glycine gels (Invitrogen) due to the large protein size, and were run in duplicate, though all 3 wells of each condition were tested for each experiment and are included in the densitometry data. Following electrophoresis, gels were transferred to nitrocellulose membranes (Invitrogen), blocked in 5% milk in tris buffered saline (Boston Bioproducts, Ashland, MA) with 0.1% Tween20 (TBST) (Acros Organics, Morris Plains, NJ), and incubated overnight at 4° C on an orbital shaker with appropriate primary antibodies in 5% bovine serum albumin (BSA) in TBST. Primary antibodies raised in rabbit were purchased for N-cad, cxn-43, β -actin (Abcam, Cambridge MA), cad-11 (Cell Signaling Technologies, Danvers, MA), Tnmd, Scx, and TNC (Invitrogen), and used at concentrations of 1:1000 to 1:10,000. Blots were washed 3x for 5 min in TBST and incubated for 1 h at room temperature with goat anti-rabbit HRP-linked secondary antibody (Invitrogen). Blots were then washed in TBST, developed using ECL chemiluminescence reagents (Invitrogen), imaged (Syngene, Frederick, MD), and analyzed

via band densitometry in ImageJ (NIH, Bethesda, MD), with all intensities normalized to their respective β -actin bands.

Fluorescence Staining and Confocal Microscopy

To observe changes in cell morphology, cells were cultured and supplemented with TGF β 2 as described above, but on glass coverslips. At 3, 7, 14, and 21d the medium was removed, cells were rinsed with PBS, and fixed in 10% formalin overnight at 4°C. Cells were washed with PBS, permeabilized with 0.1% Triton-X (Acros Organics), and stained with FITC-phalloidin and 4,6-Diamidino-2-phenylindole (DAPI) (Life Tech., Waltham, MA) to observe the actin cytoskeleton and cell nuclei, respectively. Coverslips were mounted on slides and imaged on a spinning-disk confocal microscope (Nikon/Andor, Melville, NY).

Statistical Analysis

Proteins were initially normalized to their respective β -actin bands. Since not all timepoints and experiments could be run on the same gel, TGF β 2-treated groups were normalized to their respective controls at each day, and analyzed using 2-tailed, unpaired t-tests (Prism 8, GraphPad, LaJolla, CA). Significance was set at $p < 0.05$. Results are reported as mean $\pm$ standard deviation.

3. Results

TGF β 2 induced a fibroblastic morphology in MSCs

MSC morphology appeared fibroblastic when treated with TGF β 2, compared to controls (Figure 1). As early as 3d after TGF β 2 treatment, the actin cytoskeleton appeared more elongated and the MSCs were less circular (Fig. 1 A, B). This trend continued at 7d, accompanied by increased cell proliferation (Fig. 1 C, D). At 14 and 21d, control cells continued to proliferate and showed some level of localized actin filament alignment, but largely resembled earlier timepoint controls (Fig. 1 E, G). TGF β 2-treated cells appeared to have a high degree of local actin cytoskeleton alignment and elongation, along with a visible increase in cell number at 14 and 21d (Fig. 1 F, H), compared to controls.

TGF β 2 induced tenogenic marker proteins and altered cell-cell junction levels in MSCs

TGF β 2 treatment significantly increased levels of tenogenic marker proteins Scx and Tnmd, compared to untreated controls (Fig. 2 C, D). In TGF β 2-treated MSCs, Scx trended higher at 3 ($p=0.08$) and 7d ($p=0.1$), and was significantly increased at 14d ($p<0.0001$) and 21d ($p=0.001$), compared to controls. From 3 to 14d, Tnmd trended higher ($p=0.07-0.09$) and was significantly higher at 21d ($p=0.0004$). TNC was not detected until 14 and 21d, and showed similar levels in control and treatment groups (Fig. 2 E).

Significant changes were also observed in the levels of cell-cell junction proteins with TGF β 2 treatment (Fig. 3). In TGF β 2-treated MSCs, N-cad protein levels decreased significantly at 3 ($p=0.0006$), 7 ($p=0.009$), 14 ($p=0.01$), and 21d ($p=0.0008$), compared to controls (Fig. 3 C). Similarly, cad-11 protein levels (Fig. 3 D) were significantly decreased at 3 ($p=0.02$), 7 ($p=0.0003$), 14 ($p=0.0007$), and 21d ($p=0.03$). Cxn-43 protein levels

increased significantly at day 3 (Fig. 3 E, $p=0.0039$), and then trended lower at all other timepoints ($p=0.1-0.3$), compared to untreated controls.

4. Discussion

Cell-cell junction proteins modulate cell migration, differentiation, adhesion, and communication [10], but their role in tenogenesis has not been identified. We demonstrated that tenogenic induction in MSCs by TGF β 2 resulted in significant changes in the levels of cell-cell junction proteins (Fig. 3). Tenogenic induction was determined by elevated protein levels of Scx and Tnmd (Fig. 2) and development of fibroblastic morphology (Fig. 1). As cell-cell junction proteins are involved in differentiation and formation of other musculoskeletal tissues [35,36], the decreases in N-cad and cad-11, and initial increase in cxn-43, are noteworthy. Our results suggest that these cell-cell junctions may play a role in tenogenesis.

Defining the exact set of marker proteins to determine when MSCs are tenogenically differentiated is a challenge in itself. Of the various tendon markers, Scx and Tnmd have been repeatedly linked to tenogenic differentiation [27,29,37,38]. To our knowledge, this is the first study to demonstrate Scx and Tnmd protein production in TGF β 2-treated MSCs over 21d (Fig. 2 A-D). Significant increases in Scx and Tnmd protein levels, coupled with the development of fibroblastic morphology (Fig. 1), suggest that TGF β 2 effectively induced tenogenic differentiation in MSCs. Our findings in TGF β 2-treated MSCs support prior studies that found Scx upregulated early in tenogenesis [27]. Tnmd is a late stage marker of tenogenesis [37], and consistent with this, we found Tnmd to increase at day 21. Tnmd appeared to increase only after the initial increase in Scx at day 14. Another potential tendon marker, TNC, was undetected at 3d and 7d and produced at similar levels at 14d and 21d by control and treated MSCs (Fig. 2 E). In prior studies, TNC was found in adult tendon and increased in response to injury [39,40], but it is not known to be a specific early stage tendon marker. Based on TNC, it is possible that the MSCs are not terminally differentiated by 21d, or that TNC is not a specific tenogenic marker. The observed accompanying changes in N-cad, cad-11 and cxn-43 protein levels may provide a novel, additional set of marker proteins to indicate tenogenic induction in MSCs.

Cadherins have been found to regulate chondrogenesis and osteogenesis. N-cad has been shown to direct chondrogenesis in the developing limb bud and in MSCs [20,35,41,42,43]. N-cad is required for early mesenchymal cell condensation and N-cad levels peak within the first 24 hours of chondrogenic induction [42], but decrease significantly by day 3 of development in the chick limb [41]. TGF β 1 has been shown to drive chondrogenesis in human mesenchymal progenitor cells by modulating N-cad to influence cell condensation [20]. TGF β 1 supplementation led to an initial increase in N-cad, followed by a decrease in N-cad, as cells committed to a chondrogenic fate, while N-cad disruption led to severe disruptions in cartilage formation [20]. Our results show that TGF β 2 treatment decreased N-cad protein in MSCs, but without the initial increase seen in prior work with TGF β 1. This newly identified distinction may be unique to tenogenesis or to the impact of TGF β 2 treatment, or related to both. However, when comparing tenogenesis to chondrogenesis, our findings suggest a similarity of decreased N-cad at later stages of differentiation [20]. The

combinatorial effects of N-cad and cad-11 have been found in osteogenic differentiation and modulation of bone growth, as evidenced by the severe phenotypic deficiencies of double knockout mice lacking these cadherins [44]. Independently, N-cad appears to promote osteogenic induction of human MSCs [45], and N-cad is expressed in mature osteoblasts [46]. However, N-cad overexpression limits further osteogenesis [47]. Cad-11 also appears to play an osteogenic role during development, with reduced osteoblast differentiation and bone density observed in cad-11 knockout mice [36]. However, cad-11 may also be needed for cell condensation in tendon development [12,48]. Up until now, how cad-11 changes during tenogenesis was unknown. Our results suggest cad-11 production is lowered with tenogenesis, a reasonable observation given its osteogenic capabilities, but cad-11 does not decrease to the nearly undetectable levels of N-cad, which supports prior evidence that cad-11 is involved in tendon formation [12]. Taken together, the dramatic and rapid (by 3d) decrease in N-cad following TGF β 2 treatment is a novel finding. The persistence of almost undetectable levels of N-cad in TGF β 2-treated MSCs during 21d of culture suggests that N-cad levels decrease during tenogenesis, possibly distinguishing this process from differentiation toward alternative lineages, such as bone. Based on our findings, it is possible that N-cad and cad-11 are involved in tenogenic induction, but their role in regulating tenogenic markers will need to be identified in future studies.

Significant changes were also observed in cxn-43 protein levels. Cxn-43 was significantly higher in TGF β 2-treated MSCs at 3d, and then trended lower at later timepoints, compared to controls (Fig. 3 E). Communication via cxn-43 has been proposed as a differentiation-guiding mechanism of cartilage and tendon during embryonic limb development [18]. In adult tendons, cxn-43 was found localized to where cell processes meet as well as between cell bodies [15]. Cxn-32 was also found in adult tendon, but only between cell bodies. Both cxn-43 and cxn-32 have been implicated as mechanotransducers in tendon cells and may mediate stimulatory or inhibitory effects on collagen production in response to tensile loading [13,14]. Future studies will aim to identify the effects of tenogenic induction on additional gap junction proteins (e.g., cxn-32) and cxn-43 by combining mechanical stimulation of cells with TGF β 2 supplementation. Mechanical stimuli are also thought to regulate tenogenesis [5], but the mechanotransductive signaling pathways are still not fully understood. It is possible that the TGF β 2 pathway in coordination with cadherins and connexins may play a role in mechanotransduction, and will be investigated in future studies.

In conclusion, our results highlight the impact of TGF β 2 on tenogenesis and identify novel changes in N-cad, cad-11 and cxn-43. A limited understanding of the many factors involved in tenogenesis and tendon development has been a challenge for stem cell-based tissue engineering approaches. The levels of these cell-cell junction proteins may be used as additional markers to assess tenogenic induction of MSCs. Future work to understand how cell-cell junctions mediate the response of stem cells to biochemical and mechanical factors is needed to direct tenogenesis and inform tendon tissue engineering strategies.

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- Mesenchymal stem cells (MSCs) treated with TGF β 2 appeared fibroblastic
- TGF β 2 significantly increased scleraxis and tenomodulin protein production in MSCs
- N-cadherin and caderin-11 protein levels decreased in MSCs with TGF β 2 treatment
- TGF β 2 induces tenogenesis in MSCs and impacts cell-cell junction protein levels

Figure 1.

Representative images (20x) of MSCs. Actin cytoskeleton (green) and cell nuclei (blue) are shown in MSCs at 3, 7, 14, and 21d (A-H). TGF β 2-treated MSCs (B, D, F, H) had increased proliferation, and appeared more fibroblastic and elongated, compared to controls (A, C, E, G).

Figure 2.

Representative WB images of tendon marker proteins produced by MSCs. WB showed increases in Scx, Tnmd, and TNC following 3d, 7d (A), 14d, and 21d (B) of TGFβ2 treatment. Quantified band densitometry showed significant increases in Scx (C) and Tnmd (D), while TNC (E) levels were similar to controls. All bands were normalized to β-actin and to their respective timepoint controls. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Mean $\pm$ standard deviation.

Figure 3.

Representative WB images of cell-cell junction proteins produced by MSCs. WB showed decreases in N-cad and cad-11, and changes in cxn-43 following 3d, 7d (A), 14d, and 21d (B) of TGFβ2 treatment. Quantified band densitometry showed significant decreases in N-cad (C) and cad-11 (D), while cxn-43 increased at 3d (E). All bands were normalized to β-actin and to their respective timepoint controls. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Mean $\pm$ standard deviation.