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Embryonic mechanical and soluble cues regulate tendon progenitor cell gene expression as a function of developmental stage and anatomical origin

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ABSTRACT

Stem cell-based engineering strategies for tendons have yet to yield a normal functional tissue, due in part to a need for tenogenic factors. Additionally, the ability to evaluate differentiation has been challenged by a lack of markers for differentiation. We propose to inform tendon regeneration with developmental cues involved in normal tissue formation and with phenotypic markers that are characteristic of differentiating tendon progenitor cells (TPCs). Mechanical forces, fibroblast growth factor (FGF)-4 and transforming growth factor (TGF)-β2 are implicated in embryonic tendon development, yet the isolated effects of these factors on differentiating TPCs are unknown. Additionally, developmental mechanisms vary between limb and axial tendons, suggesting the respective cell types are programmed to respond uniquely to exogenous factors. To characterize developmental cues and benchmarks for differentiation toward limb *vs.* axial phenotypes, we dynamically loaded and treated TPCs with growth factors and assessed gene expression profiles as a function of developmental stage and anatomical origin. Based on scleraxis expression, TGFβ2 was tenogenic for TPCs at all stages, while loading was for late-stage cells only, and FGF4 had no effect despite regulation of other genes. When factors were combined, TGFβ2 continued to be tenogenic, while FGF4 appeared anti-tenogenic. Various treatments elicited distinct responses by axial *vs.* limb TPCs of specific stages. These results identified tenogenic factors, suggest tendon engineering strategies should be customized for tissues by anatomical origin, and provide stage-specific gene expression profiles of limb and axial TPCs as benchmarks with which to monitor tenogenic differentiation of stem cells.

1. Introduction

Tendons are critical to musculoskeletal function, but their poor healing ability and the limitations of surgical repair have motivated stem cell-based strategies to engineer living tissue replacements. Efforts to differentiate stem cells toward a tendon lineage have been challenged by a paucity of tenogenic cues as well as a lack of phenotypic benchmarks to assess lineage commitment. Previous strategies to tenogenically differentiate stem cells have integrated soluble factors involved in adult tendon wound healing (Moreau et al., 2005; Raghavan et al., 2012), despite their association with abnormal mechanical properties, matrix content and organization (Carpenter et al., 1998). Mechanical loading has also been used (Altman et al., 2002; Kuo and Tuan, 2008), but has yet to generate functional tendons. Our objectives are to inform tendon tissue engineering with embryonic factors involved in normal tendon formation as such factors may enable stem cells to recapitulate a developmental response, and to identify phenotypic benchmarks with which to assess tenogenic differentiation.

In vivo characterizations report differences between limb and axial tendon developmental programs. Tendon progenitor cells (TPCs) express scleraxis (Scx), a bHLH transcription factor (Schweitzer et al., 2001), from embryonic day (E) 9.5 and into adulthood in mice (Maeda et al., 2011; Pryce et al., 2009). Scx-expressing cells in the axis arise from the dorsolateral sclerotome, proximal to the myotome (Brent et al., 2003). In contrast, Scx-expressing cells in the limb arise from dorsal and ventral regions of the superficial limb mesenchyme and lack compartmentalization (Schweitzer et al., 2001; Tozer and Duprez, 2005). Interestingly, limb tendons begin developing normally in the absence of muscle, but degenerate at later embryonic stages (Kardon, 1998; Kieny and Chevallier, 1979), while no muscle-independent phase is reported for axial tendons. Based on these developmental differences, we asked whether a tendon engineering strategy should be customized for anatomically specific tissues.

The mechanical strain environment for developing tendons is largely uncharacterized, but muscle-driven movements beginning at E14 (Kodama and Sekiguchi, 1984) suggest the tissues experience mechanical stimulation during differentiation. This has not been

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examined with TPCs, but mechanical loading has been shown to influence tendon marker gene expression in MSCs (Altman et al., 2002; Doroski et al., 2010; Kuo and Tuan, 2008; Subramony et al., 2013). A role for mechanical forces in regulating tendon development is also suggested by the degeneration of tendon when muscle is paralyzed during embryonic chick development (Mikic et al., 2000). However, paralysis also results in adjacent muscle and cartilage degeneration (Hall and Herring, 1990), which likely alters tissue-specific paracrine signaling during tendon development. For example, precluding limb muscle formation in embryonic chicks abolished expression of the muscle-derived factor FGF4 and resulted in loss of Scx expression, while grafting mFGF4/RCAS-expressing cells into the muscleless limbs rescued Scx expression (Edom-Vovard et al., 2002). Taken together, it appears muscle influences on tendon development are both physical and soluble, suggesting a successful strategy to direct stem cell tenogenesis will require both.

Both FGF4 and TGFβ2 have been implicated in tendon development. Disrupted FGF signaling with SU5402 inhibited Scx expression in progenitor cells of chick and murine embryonic axial and limb tendons (Brent et al., 2005; Brent and Tabin, 2004), while exogenous FGF4 treatment induced Scx expression *in vivo* at early (murine E10 and chick HH18) and late developmental stages (chick HH36-39) (Brent et al., 2005; Edom-Vovard et al., 2002). TGFβ2 is expressed in embryonic chick and murine limb tendons (Kuo et al., 2008; Pryce et al., 2009), and disruption of its expression in $TGF\beta2^{-/-}$ mutant mice leads to tendon deficiencies (Pryce et al., 2009). Additionally, TGFβ2 treatment upregulated Scx expression in C3H10T1/2 mesenchymal progenitor cells. Therefore, FGF4 and TGFβ2 appear to be important tenogenic factors, though their effects on TPCs in isolation have not been investigated.

We aimed to characterize the tenogenicity of developmental factors and define benchmarks for axial and limb TPC differentiation *in vitro* by profiling phenotypic responses of TPCs at each developmental stage in response to TGFβ2, FGF4, loading and their combinations. We hypothesized that tendon developmental factors would be tenogenic *in vitro* and that TPCs would respond to treatments differentially as a function of developmental stage and anatomical origin. We harvested limb and axial TPCs from Scx-green fluorescent protein (GFP) transgenic mice from different stages of embryonic and neonatal development, and mechanically loaded or treated cells with TGFβ2 or FGF4, and analyzed the expression of tendon marker genes. Distinct gene response profiles of TPCs were dependent on treatment, developmental stage and anatomical origin. These results characterized the tenogenicity of developmental factors, provided phenotypic signatures as benchmarks for differentiation, and demonstrated a need to customize tendon engineering strategies for tissues by anatomical origin.

2. Methods

2.1. Materials were from Invitrogen (Carlsbad, CA) unless otherwise specified.

2.1.1. TPC harvest

Postnatal day (P) 7 and pregnant Scx-GFP mice (Pryce et al., 2007) were sacrificed by CO_2 asphyxiation and decapitation with Tufts University IACUC approval. Embryos were harvested on embryonic days (E) 13–17 (Theiler, 1989). Limbs and torsos were skinned (E15 and older), cleaned of organs and cervical tissues, and washed in phosphate buffered saline (PBS) without $MgCl_2/CaCl_2$. Limb and axial tissue (with tail) were digested in 1% type II collagenase in PBS at 37 °C for 45 min while shaking at 200 RPM, and neutralized with growth medium (GM; high glucose Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S)). Digests were strained through a 40 μm cell strainer, pelleted, washed in PBS and resuspended in GM. Cells were grown to 80% confluency, trypsinized and sorted by GFP signal using a MoFlo Legacy cell sorter (Beckman Coulter, Brea, CA) to isolate TPCs. TPCs were expanded to passage 1–2 for experimentation. At least 3 independent cell pools from different embryos were harvested for each stage and anatomical origin.

2.1.2. Mechanical stimulation and growth factor treatment

TPCs were seeded at 2×10^4 cells/cm^2 on Col I-coated Uniflex® culture plates (Flexcell International, Hillsborough, NC) for loading, or on tissue culture plastic (TCP) plates for growth factor treatment. To examine the effects of combined treatments, E16.5 limb TPCs were seeded on Uniflex plates. On day (D) 0 (after 48 h in GM), medium was replaced with basal (control) medium (BM; DMEM, 1% FBS, 1% P/S). Cells were uniaxially loaded with 1% sinusoidal tensile strain at 0.5 Hz for 1 h/day (2 h/day for E16.5 limb TPCs), and/or supplemented with 100 ng/mL rhFGF4 and/or 1 ng/mL rhTGFβ2 (PeproTech, Rocky Hill, NJ). Because mechanical strains experienced by embryonic tendon during development are unknown, strains used this study were based on those shown previously to elicit a gene expression response in progenitor cells (Kuo and Tuan, 2008; Subramony et al., 2013). The loading frequency of 0.5 Hz was based on the frequency of limb muscle motor unit bursts detected in day 7 embryonic chicks by electromyographic recordings (Bekoff et al., 1975). Medium was changed every two days. Cells were harvested on D0, 1, 3 and 5 without loading or treatment on their harvest day.

2.1.3. Quantitative polymerase chain reaction (qPCR)

Samples were homogenized in TRIzol reagent and total RNA-isolated and reverse-transcribed using the Superscript III First Strand Synthesis kit. QPCR was performed with Brilliant II SYBR Green qPCR master mix on a Stratagene Mx3000P multiplex qPCR system (Agilent, Wilmington, DE). Mouse-specific primer pairs were designed, optimized and efficiency-corrected (Yuan et al., 2008) for tendon marker and housekeeping genes (Table 1). Fold-change was calculated by $2^{-\Delta\Delta CT}$ and baseline gene expression by $2^{-\Delta CT}$ (on D0 prior to treatments). Means and standard errors were calculated for the three independent cell pools for each anatomical origin, developmental stage and condition.

2.1.4. Statistical analysis

To evaluate baseline gene expression across developmental stage, gene expression was normalized to 18S, and compared using ANOVA with Tukey's post hoc test, $p < 0.05$. The effect of a treatment (loading or growth factor) on gene expression was normalized to matching (stage and anatomical location) non-treated controls, expressed as a fold-change, and evaluated using a ratio t-test, $p < 0.05$. To evaluate the effect of a single treatment (loading or growth factor) over time (D0, 1, 3, or 5) for a given developmental stage, gene expression was normalized to D0, and compared using ANOVA with Tukey's post hoc test, $p < 0.05$. To compare the effects of combined treatments (loading, growth factor, and growth factor+loading) on E16.5 limb TPCs, gene expression was normalized to non-treated controls, and compared using ANOVA with Tukey's post hoc test, $p < 0.05$. For comparisons of gene expression by anatomical origin, the fold-change values for a given stage were analyzed by unpaired t-test, $p < 0.05$.

3. Results

3.1. Baseline gene expression comparison

Baseline expression of Scx did not significantly vary between different stage limb TPCs ($p \geq 0.53$; Fig. 1A). Axial TPC Scx expression was highest at E15, which was significantly higher than all other stages of axial TPCs except E17 ($p < 0.05$; Fig. 1A). Baseline Scx expression was higher in axial than limb TPCs at E15 ($p < 0.01$)

Table 1

Gene	Forward	Reverse	Accession no.
Scleraxis	*CCTCAGCAACCAGAGAAAGTTGAGCA*	*GCCATCACCCGCCTGTCCATC*	*NM_198885.3*
Tenomodulin	*ATGAGCAATGGGTGGTCCCGC*	*ACAGACACGGCGGCAGTAACG*	*NM_022322.2*
Collagen 1α2	*ACCTCCTGGCAACCCTGGAACAA*	*TCTGGCACCTGTAGCACCAGCA*	*NM_007743.2*
Elastin	*CTTTGGACTTTCTCCCATTTATCC*	GGTCCCCAGAAGATCACTTTCTC	*NM_007925.3*
TGFβ2	*GAGTGGCCGAGCAGCGGATT*	CCCAGGTTCCTGTCTTTGTGGTGAA	*NM_009367.3*
18S	*TCAACTTTCGATGGTAGTCGCCGT*	TCCTTGGATGTGGTAGCCGTTTCT	*X00686.1*