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RESEARCH ARTICLE

Transcriptomic analysis of mouse limb tendon cells during development

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ABSTRACT

The molecular signals driving tendon development are not fully identified. We have undertaken a transcriptome analysis of mouse limb tendon cells that were isolated at different stages of development based on scleraxis (*Scx*) expression. Microarray comparisons allowed us to establish a list of genes regulated in tendon cells during mouse limb development. Bioinformatics analysis of the tendon transcriptome showed that the two most strongly modified signalling pathways were TGF-β and MAPK. TGF-β/SMAD2/3 gain- and loss-of-function experiments in mouse limb explants and mesenchymal stem cells showed that TGF-β signalling was sufficient and required via SMAD2/3 to drive mouse mesodermal stem cells towards the tendon lineage *ex vivo* and *in vitro*. TGF-β was also sufficient for tendon gene expression in late limb explants during tendon differentiation. FGF does not have a tenogenic effect and the inhibition of the ERK MAPK signalling pathway was sufficient to activate *Scx* in mouse limb mesodermal progenitors and mesenchymal stem cells.

KEY WORDS: Transcriptome, Limb, Tendon, Mouse, Scleraxis, TGF-β, SMAD2/3, ERK

INTRODUCTION

Tendons transmit forces generated from muscle to bone in order to facilitate movement. Tendons are mainly composed of type I collagen fibres organised parallel to the axis of the tendon, which provide the tensile strength of tendons. One of the difficulties in studying tendon development is that the main molecular component, type I collagen, is not specific to tendons. The discovery of the basic helix-loop-helix transcription factor scleraxis (*Scx*) as a specific tendon and ligament marker was an important step in the study of tendon development (Schweitzer et al., 2001). The loss of *Scx* activity in mice leads to a defect in the differentiation of the force-transmitting and intermuscular tendons, while not affecting the tendons anchoring muscles to the skeleton (Murchison et al., 2007). The type II transmembrane glycoprotein tenomodulin (*Tnmd*) is considered a marker of differentiated tenocytes. SCX has been shown to be required and sufficient for *Tnmd* expression (Docheva et al., 2005; Shukunami et al., 2006; Murchison et al., 2007). Two other DNA-binding proteins, namely the zinc-finger protein early growth response 1 (EGR1) and the homeodomain protein mohawk (MKX), have been shown to be involved in tendon formation (Ito et al., 2010; Liu et al., 2010; Lejard et al., 2011; Guerquin et al., 2013). However, these two transcription factors, although important for *Col1a1* transcription in tendons, are not specific to tendons.

Experiments in embryology and genetic analyses have shown that limb tendon formation relies on muscle. In the absence of muscles, stylopod (arm) and zeugopod (forearm) tendon development is initiated, but is later arrested, suggesting the requirement of signals from muscles and/or of mechanical forces to complete tendon development (Kardon, 1998; Schweitzer et al., 2001; Edom-Vovard et al., 2002; Bonnin et al., 2005). By contrast, distal (autopod) tendons form independently of muscle (Kardon, 1998; Huang et al., 2013). TGF-β and FGF are the main signalling pathways identified as being involved in stylopod and zeugopod tendon development during the muscle-dependent phase of limb tendon development (Tozer and Duprez, 2005; Schweitzer et al., 2010). However, the TGF-β and FGF signalling pathways have been shown to be involved in limb tendon development in mouse and chick embryos, respectively (Edom-Vovard et al., 2002; Pryce et al., 2009). FGF is also required and sufficient for mouse and chick axial tendon formation (Brent et al., 2003, 2005; Smith et al., 2005).

In order to identify novel tendon markers and to determine which signalling pathways are involved during tendon development, we undertook a transcriptome analysis of mouse limb tendon cells at different stages of development. We established a list of novel tendon markers. Bioinformatics analysis of the transcriptome identified the TGF-β and MAPK pathways as those most substantially modified in limb tendon cells during development. Bioinformatics data combined with TGF-β and FGF gain- and loss-of-function in mouse limb explants and mesenchymal stem cells showed that the TGF-β/SMAD2/3 and ERK MAPK signalling pathways control the commitment of progenitor cells to enter the tendon lineage.

RESULTS

Isolation of tendon cells from mouse limbs at different stages of development

In order to isolate tendon cells, we took advantage of the Scx-GFP mouse line (Pryce et al., 2007), so that *Scx*-positive cells could be isolated by flow cytometry based on GFP fluorescence. We chose the E11.5 stage to select limb tendon progenitors and the E14.5 stage to target limb tendon differentiated cells, when tendons are well individualised. We also chose an intermediate time point at E12.5, as the transitory time point between the muscle-independent and -dependent phases of tendon formation. In the absence of muscles, *Scx* is normally expressed in E11.5 limbs, defining the

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muscle-independent phase, and is then lost in E14.5 muscleless limbs at the level of the forearm and arm (Schweitzer et al., 2001; Bonnin et al., 2005). In E12.5 muscleless limbs, *Scx* expression is still present in ventral limb regions but starts to be downregulated in dorsal limb regions, at the level of the forearm (Bonnin et al., 2005; Pryce et al., 2009). We thus consider E12.5 as the transient time point between the muscle-independent and -dependent phases of forearm and arm tendon formation. Forelimbs were dissected from Scx-GFP embryos at E11.5, E12.5 and E14.5 (Fig. 1A,B). Scx-GFP^{+} cells were then separated by flow cytometry (Fig. 1C); 50, 30 and 20 embryos were needed for the E11.5, E12.5 and E14.5 stages, respectively. The dissection and cytometry steps were performed three times for each time point in order to allow triplicate Affymetrix analyses.

Microarray analyses

We performed three array comparisons: (1) E11.5 versus E12.5, corresponding to the muscle-independent phase of tendon formation; (2) E12.5 versus E14.5, corresponding to the muscle-dependent phase of arm and forearm tendon formation; and (3) E11.5 versus E14.5, corresponding to tendon progenitor cells versus tendon differentiated cells (Table 1). A total of 3282 genes (more than 10% of all transcripts in the genome array) were differentially regulated in limb tendon cells during development, between E11.5 and E14.5 (Table 1). A greater number of genes were differentially regulated during the muscle-dependent phase as compared with the muscle-independent phase (1767 versus 713; Table 1).

In total, 4888 regulated transcripts (probe sets) can be hierarchically clustered (supplementary material Fig. S1A). We then asked whether differentially regulated genes in tendon cells represented specific Gene Ontology (GO) categories, which would highlight differential biological activities during development (Table 2; supplementary material Fig. S1B). GO terms related to extracellular structure organisation, cell adhesion or response to wounding were highly represented in differentiated tendon cells at E14.5, whereas GO terms related to cell cycle were highly represented in tendon progenitor cells at E11.5 (Table 2; supplementary material Fig. S1B). In addition, very high enrichment scores and significant *P*-values were observed for the GO terms 'extracellular matrix', 'cell adhesion' and 'collagen' in tendon cells during limb development (Table 2), consistent with the massive increase of matrix synthesis during tendon development.

Genes displaying enriched expression in tendon cells during development

Few tendon-specific markers have been identified. In addition to the *Scx* and *Tnmd* tendon markers, a series of matrix proteins has been described as being expressed or/and associated with tendon development, which we previously attempted to list (Edom-Vovard and Duprez, 2004). The mRNA relative expression of the main tendon collagen, *Col1a1*, and that of the tendon-associated collagens *Col3a1*, *Col5a1*, *Col6a1*, *Col12a1* and *Col14a1* was enhanced in tendon cells between E11.5 and E14.5 (Lejard et al., 2011). This is consistent with the high enrichment score of the 'collagen' biological process during tendon development (Table 2).

One objective was to establish a list of tendon markers during development (supplementary material Table S1). We ordered the top 100 upregulated genes in E14.5 differentiated tendon cells versus E11.5 tendon progenitor cells, from high to low fold change (Table 3). We also analysed the expression of the ordered genes using Eurexpress, a transcriptome altas database for the mouse embryo. We found that the majority of the top 100 upregulated

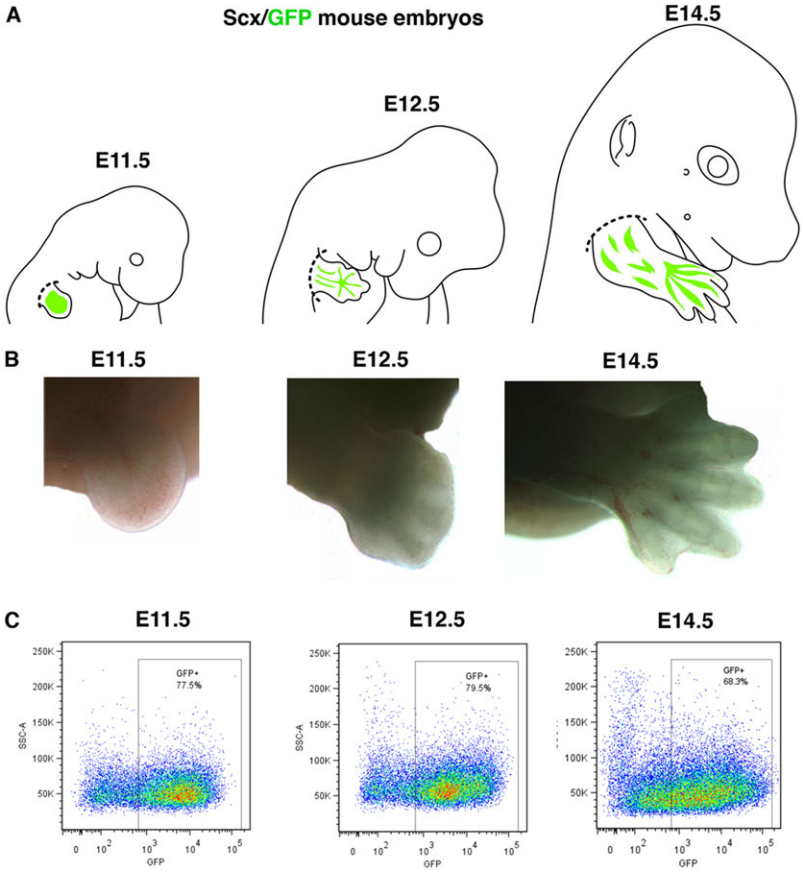

Fig. 1. Strategy of tendon cell purification from forelimbs at different stages of mouse development. (A) Forelimbs were dissected from E11.5, E12.5 and E14.5 Scx-GFP mouse embryos. Dashed lines indicate the levels of dissection for forelimbs. (B) Representative images of dissected forelimbs at the different stages. (C) GFP^{+} cells were isolated by flow cytometry. The boxed regions were used for transcriptome analysis.

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Table 1. Global reorganisation of the transcriptome of limb tendon cells during mouse development

Number of genes	E11.5 versus E14.5: tendon progenitor cells versus tendon differentiated cells	E11.5 versus E12.5: muscle-independent phase	E12.5 versus E14.5: muscle-dependent phase
Total differentially regulated	3282	713	1767
Total upregulated	2168 (66%)	582 (81.6%)	1276 (72.2%)
Total downregulated	1114 (34%)	131 (18.4%)	491 (27.8%)
Significantly differentially regulated*	282	Up	Up
	309	Up	No change
	13	Up	Down
	1050	No change	Up
	462	No change	Down
	9	Down	Up
	100	Down	No change
	27	Down	Down

*Shown are the number of genes that exhibit combinations of significant upregulation or downregulation, or no change, in E11.5 versus E14.5 compared with E11.5 versus E12.5 and with E12.5 versus E14.5.

genes were expressed in tendons (Table 3). The known tendon differentiation marker *Tnmd* was the second most differentially expressed gene on the list, displaying a 376-fold change between E14.5 and E11.5 (Table 3). In order to validate this list of tendon genes, we chose candidates not previously known to be related to tendons, starting with aquaporin 1 (*Aqp1*), a water channel protein, for which the fold change in expression levels was 57.2 between E11.5 and E14.5 in the array (Table 3). The dramatic increase of *Aqp1* mRNA levels was confirmed by RT-q-PCR (Fig. 2A). *In situ* hybridisation experiments showed that *Aqp1* is expressed in mouse forelimb tendons, similar to *Scx* expression (Fig. 2B-E). We also chose HtrA serine peptidase 3 (*Htra3*) from the array and demonstrated its expression in E14.5 mouse limb tendons by *in situ* hybridisation (Fig. 2F-H). *Aqp1* and *Htra3*, which were not previously known to be tendon related, displayed tendon-specific expression in mouse limbs (Fig. 2B-H). It should be noted that *Scx* did not appear in the array as a significantly upregulated gene, suggesting that *Scx* expression levels did not change between E11.5 and E14.5, consistent with the little variation in *Scx* mRNA expression levels in mouse limbs (supplementary material Fig. S4A). We believe that this list of genes (Table 3; supplementary material Table S1) enriched in E14.5 tendon cells constitutes an important inventory of tendon markers.

TGF-β is the main signalling pathway upregulated in limb tendon cells during development

We next aimed to identify signalling pathways modified in limb tendon cells during development. We first used Genomatix software, which established gene associations with over 400 canonical pathways. In our tendon cell array, TGF-β was the top pathway, displaying the highest number of upregulated genes in the three types of comparisons (Table 4): 59 and 102 genes of the TGF-β pathway (comprising 640 components) were significantly upregulated between E11.5 and E12.5 and between E12.5 and E14.5, respectively (Table 4). Consistent with our analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Table 2), components of the cell cycle were significantly downregulated during tendon cell differentiation between E12.5 and E14.5 (Table 4).

In order to confirm the modification of TGF-β components in tendon cells, we analysed the variation of signal transduction KEGG pathways, which defines pathways from a more conventional point of view than Genomatix. Consistent with the Genomatix analysis (Table 4), TGF-β (KEGG N°4350) was the signalling pathway (among those of the signal transduction group) that displayed the highest *P*-value in terms of being modified between the three types of comparisons (Table 5). The components of the KEGG TGF-β pathway that display significant upregulation or downregulation in expression between the three time points of comparison are listed in supplementary material Table S2 and illustrated in supplementary material Fig. S2. The mRNA expression levels of TGF-β ligands, receptors and extracellular components that were significantly differentially regulated in the arrays (supplementary material Table S2) were confirmed by RT-q-PCR analyses (Fig. 3A). Notably, all the genes encoding extracellular components [TGF-β, THBS, LTBP, decorin (DCN), TGFβi] of the 'classical' TGF-β pathway displayed significant upregulation in differentiated tendon cells versus progenitor tendon cells (supplementary material Table S2 and Fig. S2). Two of these extracellular components, namely the thrombospondin genes *Thbs2* and *Thbs4*, were expressed in mouse limb tendons (Fig. 3B-M) and are among the 100 top differentially expressed genes (Table 3).

Based on bioinformatics analyses, RT-q-PCR and *in situ* hybridisation data, we concluded that the TGF-β pathway is the most active pathway in tendon cells during mouse limb development.

Table 2. GO analysis in mouse limb tendon cells during development

	E11.5 versus E12.5		E12.5 versus E14.5	
Cluster	High at E11.5	High at E12.5	High at E12.5	High at E14.5
Transcription factor activity (GOTERM-MF-FAT)	4 (1.3×10^{-8})		15.67 (3.4×10^{-8})	
Pattern specification process (GOTERM-BP-FAT)	4 (3.8×10^{-8})			
Limb development (GOTERM-BP-FAT)	2.41 (1.1×10^{-3})		7.14 (7.3×10^{-9})	
Negative regulation of cell differentiation (GOTERM-BP-FAT)	2.66 (2.5×10^{-5})			
Cell cycle (GOTERM-BP-FAT)			17.77 (2.9×10^{-22})	
Extracellular matrix (GOTERM-CC-FAT)		27.68 (6.9×10^{-29})		38.55 (1.6×10^{-42})
Cell adhesion (GOTERM-BP-FAT)		13.5 (3.8×10^{-17})		26.58 (1.4×10^{-28})
Collagen (SP-PIR-Keywords)		9.56 (2.0×10^{-11})		12.64 (1.9×10^{-14})

Shown are enrichment scores with the *P*-value in parentheses. The DAVID bioinformatics resource 6.7 was used for this analysis.

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Table 3. Top 100 genes enriched in mouse limb tendon cells at E14.5 versus E11.5

Gene symbol	Gene name	Transcript ID	Fold enrichment for E14.5 versus E11.5	E14.5 limb expression (Eurexpress)	Human tendon expression (NextProt)
Ibsp	Integrin binding sialoprotein	NM_008318	533.56	Bone	Yes
**Tnmd*	Tenomodulin	NM_022322	376.754	Tendon	Yes
Aspn	Asporin	NM_025711	374.38	No signal	Yes
Tm4sf1	Transmembrane 4 superfamily member 1	NM_008536	210.3	Tendon	Yes
Chodl	Chondrolectin	NM_139134	151.873	No expression data	Yes
Dpt	Dermatopontin	NM_019759	116.478	Tendon	Yes
1500015O10Rik	RIKEN cDNA 1500015O10 gene	NM_024283	112.954	Tendon	–
Htra1	HtrA serine peptidase 1	NM_019564	99.275	Tendon	Yes
Slc26a7	Solute carrier family 26, member 7	NM_145947	86.5471	Tendon	–
Ifi27l1	Interferon, alpha-inducible protein 27 like 1	NM_026790	85.1394	Tendon	Yes
§*Zfp385b*	Zinc-finger protein 385B	NM_001113399	79.684	Not found in database	–
Scara5	Scavenger receptor class A, member 5 (putative)	NM_028903	68.1246	Not found in database	Yes
Adamts2	A disintegrin-like and metallopeptidase	NM_175643	67.3547	Tendon	Yes
Abi3bp	ABI gene family, member 3 (NESH) binding protein	NM_001014399	63.9134	Tendon	Yes
Gm106	Predicted gene 106	NM_001033288	63.7748	Not found in database	–
Nov	Nephroblastoma overexpressed gene	NM_010930	57.9381	Tendon/muscle	Yes
‡*Aqp1*	Aquaporin 1	NM_007472	57.276	Tendon	Yes
Clec3b	C-type lectin domain family 3, member b	NM_011606	50.2875	No signal	–
Anxa1	Annexin A1	NM_010730	49.5144	Tendon/ cartilage	Yes
Ptrf	Polymerase I and transcript release factor	NM_008986	44.047	Tendon	Yes
‡*Thbs2*	Thrombospondin 2	NM_011581	41.3212	Tendon/ connective tissue	Yes
Cd34	CD34 antigen	NM_001111059	38.7271	Vascular associated tissue	Yes
Ogn	Osteoglycin	NM_008760	38.4776	Tendon	Yes
Hapln1	Hyaluronan and proteoglycan link protein 1	NM_013500	38.014	Ubiquitous	Yes
Postn	Periostin, osteoblast specific factor	NM_015784	37.807	Tendon	Yes
**Col14a1*	Collagen, type XIV, alpha 1	NM_181277	37.7576	Tendon/ connective tissue	Yes
**Fmod*	Fibromodulin	NM_021355	37.6206	Tendon	Yes
C1qtnf3	C1q and tumor necrosis factor related protein 3	NM_030888	37.4683	Tendon	Yes
**Dcn*	Decorin	NM_007833	36.5757	Tendon	Yes
Lmna	Lamin A	NM_001002011	36.0541	Tendon, muscle	Yes
Cytl1	Cytokine-like 1	NM_001081106	35.1246	Bone	No
Rpl39l	Ribosomal protein L39-like	NM_026594	32.12	Not found in database	Yes
Cd44	CD44 antigen	NM_001039150	31.9471	Ubiquitous	Yes
Fam46a	Family with sequence similarity 46, member A	NM_001160378	30.9217	Not found in database	Yes
Comp	Cartilage oligomeric matrix protein	NM_016685	29.0474	Not found in database	Yes
Ly86	Lymphocyte antigen 86	NM_010745	28.9554	Bone	Yes
§*Klf2*	Kruppel-like factor 2 (lung)	NM_008452	28.5156	Tendon, bone, muscle	Yes
Hbb-b1/2	Hemoglobin, beta adult major and minor chains	NM_008220	28.4956	Not found in database	–
Gpr64	G protein-coupled receptor 64	NM_178712	28.2076	Tendon	Yes
Igfbp7	Insulin-like growth factor binding protein 7	NM_008048	27.4032	Not found in database	–
Mgp	Matrix Gla protein	NM_008597	26.6881	Tendon	Yes

Continued

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Table 3. Continued

Gene symbol	Gene name	Transcript ID	Fold enrichment for E14.5 versus E11.5	E14.5 limb expression (Eurexpress)	Human tendon expression (NextProt)
Fam129a	Family with sequence similarity 129, member A	NM_022018	25.6168	Tendon/ connective tissue	
Acan	Aggrecan	NM_007424	25.0067	Cartilage, enthesis	Yes
Abca8a	ATP-binding cassette, sub-family A (ABC1), member 8a	NM_153145	24.9123	Not found in database	–
Abca9	ATP-binding cassette, sub-family A (ABC1), member 9	NM_147220	24.3583	Weak signal	Yes
Kitl	Kit ligand	NM_013598	23.8154	Tendon	Yes
Ly6h	Lymphocyte antigen 6 comp	NM_001135688	23.3942	No limb expression	–
Lox	Lysyl oxidase	NM_010728	23.2075	No expression data	Yes
‡*Thbs4*	Thrombospondin 4	NM_011582	22.5476	Tendon	Yes
Kdelr3	KDEL endoplasmic reticulum protein retention receptor 3	NM_134090	21.4719	Tendon	Yes
**Bgn*	Biglycan	NM_007542	21.468	Tendon/ connective tissue	Yes
Fndc1	Fibronectin type III domain containing 1	NM_001081416	21.3487	Tendon	Yes
Fam189a1	Family with sequence similarity 189, member A1	NM_183087	20.819	Weak expression	–
‡*Htra3*	HtrA serine peptidase 3	NM_030127	20.6535	Tendon	Yes
§*Ahr*	Aryl-hydrocarbon receptor	NM_013464	20.6166	Tendon	Yes
Cbln2	Cerebellin 2 precursor protein	NM_172633	20.5779	Tendon	–
Phex	Phosphate regulating gene	NM_011077	20.2375	Skeletal muscle	–
Gfra2	Glial factor family receptor alpha 2	NM_008115	20.0138	Weak signal	Yes
Prokr1	Prokineticin receptor 1	NM_021381	19.812	Connective tissue	–
Plac9	Placenta specific 9	NM_207229	19.676	Tendon	Yes
Clu	Clusterin/similar to clusterin	NM_013492	19.542	Tendon	Yes
Egfl6	EGF-like-domain, multiple 6	NM_019397	19.2315	Tendon/ connective tissue	Yes
Mfap5	Microfibrillar associated protein 5	NM_015776	19.042	Muscles	Yes
Matn2	Matrilin 2	NM_016762	19.0283	Tendon	Yes
§*Fosl2*	Fos-like antigen 2	NM_008037	18.9957	Skeleton	Yes
Pamr1	Peptidase domain	NM_173749	18.9641	Tendon	Yes
Itih5	Inter-alpha (globulin) inhibitor H5	NM_172471	18.9351	Tendon	Yes
Kera	Keratocan	NM_008438	18.8904	Tendon	Yes
Cpxm2	Carboxypeptidase X 2 (M14 family)	NM_018867	18.3564	Tendon	Yes
Ltbp2	Latent transforming growth factor beta binding protein 2	NM_013589	18.2384	Tendon	Yes
**Col6a1*	Collagen, type VI, alpha 1	NM_009933	18.2108	Tendon/ connective tissue	Yes
Ifi27	Interferon, alpha-inducible protein 27	NM_026790	18.1595	Tendon/ connective tissue	Yes
S100a10	S100 calcium binding protein A10 (calpactin)	NM_009112	17.5541	Tendon	Yes
Adam33	A disintegrin and metallopeptidase domain 33	NM_033615	17.2731	Not found in database	Yes
Slc7a10	Solute carrier family 7 member 10	NM_017394	17.069	Connective tissue	–
Csgalnact1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	NM_172753	16.8809	No regionalised signal	Yes
Epyc/*Dspg3*	Epiphycan/dermatan sulfate proteoglycan 3	NM_007884	16.7175	Cartilage	–
Steap4	STEAP family member 4	NM_054098	16.4923	Not found in database	Yes
Angptl1	Angiopoietin-like 1	NM_028333	16.4828	Tendon	Yes
Thy1	Thymus cell antigen 1, theta	NM_009382	15.98	Connective tissue	Yes

Continued

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Table 3. Continued

Gene symbol	Gene name	Transcript ID	Fold enrichment for E14.5 versus E11.5	E14.5 limb expression (Eurexpress)	Human tendon expression (NextProt)
§*Zcchc5*	Zinc-finger, CCHC domain containing 5	NM_199468	15.8492	Muscle/tendon	Yes
Col8a2	Collagen, type VIII, alpha 2	NM_199473	15.3467	Tendon	Yes
Itih5	Inter-alpha (globulin) inhibitor H5	NM_172471	15.336	Tendon	Yes
§*Atf3*	Activating transcription factor 3	NM_007498	15.3012	Tendon	Yes
§*Nfix*	Nuclear factor I/X	NM_001081981	15.059	Muscle/ connective tissue	Yes
Trpm5	Transient receptor potential cation channel, subfamily M, member 5	NM_020277	15.0396	Tendon/ connective tissue	–
**Col6a3*	Collagen, type VI, alpha 3	XM_00147456	15.0244	Tendon	Yes
Rerg	RAS-like, estrogen-regulated, growth-inhibitor	NM_001164212	14.9248	Tendon	Yes
Chsy3	Chondroitin sulfate synthase 3	NM_001081328	14.8703	Not found in database	Yes
Ctsk	Cathepsin K	NM_007802	14.7175	Tendon/ cartilage	Yes
Cilp	Cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	NM_173385	14.6908	Tendon	Yes
4632428N05Rik	RIKEN cDNA 4632428N05 gene	NM_001159572	14.6227	Not found in database	–
Fcgrt	Fc receptor, IgG, alpha chain transporter	NM_010189	14.4576	Cartilage	Yes
**Col1a1*	Collagen, type I, alpha 1	NM_007742	14.375	Tendon	Yes
Olfm3	Olfactomedin 3	NM_153157	14.2809	Not found in database	–
Ifi35	Interferon-induced protein 35	NM_027320	14.375	Cartilage	Yes
Aoc3	Amine oxidase, copper containing 3	NM_009675	14.2681	Not found in database	Yes
Gpr133	G protein-coupled receptor 133	NM_007742	14.375	Weak, tendon	Yes
Rnase4	Ribonuclease, RNase A family 4	NM_021472	14.1177	Limb connective tissue	–
Ggta1	Glycoprotein galactosyltransferase alpha 1, 3	NM_001145821	14.0359	Ubiquitous	Yes

Genes with probe expression above 500 AU (arbitrary units) have been ordered according to fold enrichment (from high to low) of gene expression in E14.5 versus E11.5 tendon cells from the array analysis. The Eurexpress and human NextProt databases were used to define gene expression in E14.5 mouse limb tendons or in human tendons, respectively.

*Genes known to be related to tendons.

‡Genes previously not known to be tendon related and analysed by *in situ* hybridisation or/and RT-q-PCR in the present study.

§Transcription factors.

TGF-β pathway involvement in mouse limb tendon development

Bioinformatics analyses highlighted that, in addition to being upregulated during tendon cell differentiation between E14.5 and E12.5, components of the TGF-β pathway were also significantly upregulated between E12.5 and E11.5, i.e. during the muscle-independent phase of tendon formation (Tables 4 and 5). This suggested an involvement of the TGF-β pathway at an earlier stage of the tendon program than previously believed (Pryce et al., 2009). In order to validate our bioinformatics analysis of the tendon transcriptome, we employed an *ex vivo* system based on mouse forelimb explants. Forelimb bud explants were dissected either at E9.5/E10.5 to target the initial phase of tendon formation or at E12.5 to target the differentiation phase of tendon formation, and were incubated for 24 h in the presence of TGFβ2 ligand for TGF-β gain-of-function experiments or specific TGF-β inhibitors for TGF-β loss-of-function experiments. We chose TGFβ2 ligand, as opposed to TGFβ1 or TGFβ3, for the gain-of-function experiments because *Tgfb2* displayed higher levels of endogenous expression than *Tgfb1* and *Tgfb3* in mouse limbs (supplementary material Fig. S4C).

TGFβ2 was sufficient to increase *Scx* expression in E9.5/E10.5 mouse forelimb explants (Fig. 4A). Expression of the tendon-related *Col1a1*, *Col1a2*, *Thbs2* and *Thbs4* genes was also upregulated after TGFβ2 application (Fig. 4A). In E9.5/E10.5 mouse limbs, the expression levels of *Tnmd* and *Aqp1* were considered undetectable (above 31 PCR cycles; supplementary material Fig. S4B), and TGFβ2 was unable to increase their expression in early E9.5/E10.5 mouse limb explants cultured for 24 h (data not shown). In order to test the requirement of TGF-β signalling for the initiation of tendon gene expression in forelimb buds, we blocked the TGF-β signalling pathway by applying specific TGF-β inhibitors (supplementary material Fig. S5). In the presence of the SB43 inhibitor, which blocks the TGF-β pathway at the level of the ALK4, ALK5 and ALK7 (ACVR1B, TGFBR1 and ACVR1C, respectively – Mouse Genome Informatics) receptors (Inman et al., 2002), *Scx*, *Col1a1*, *Col1a2* and *Thbs2* gene expression was significantly downregulated (Fig. 4A). Blockade of the SMAD2/3 intracellular pathway using the SIS3 inhibitor (Jinnin et al., 2006) also diminished the relative expression levels of *Scx*, *Col1a1*, *Col1a2*, *Thbs2* and *Thbs4* compared with control limbs in E9.5/E10.5 mouse limb explants (Fig. 4A). Consistently, application of the SIS3 inhibitor abolished *Scx* expression in E9.5 mouse limb explants (Fig. 4B). This showed that TGF-β was sufficient and required via the SMAD2/3 intracellular pathway for *Scx*, *Col1a1*, *Col1a2*, *Thbs2* and *Thbs4* expression in E9.5/E10.5 mouse forelimbs.

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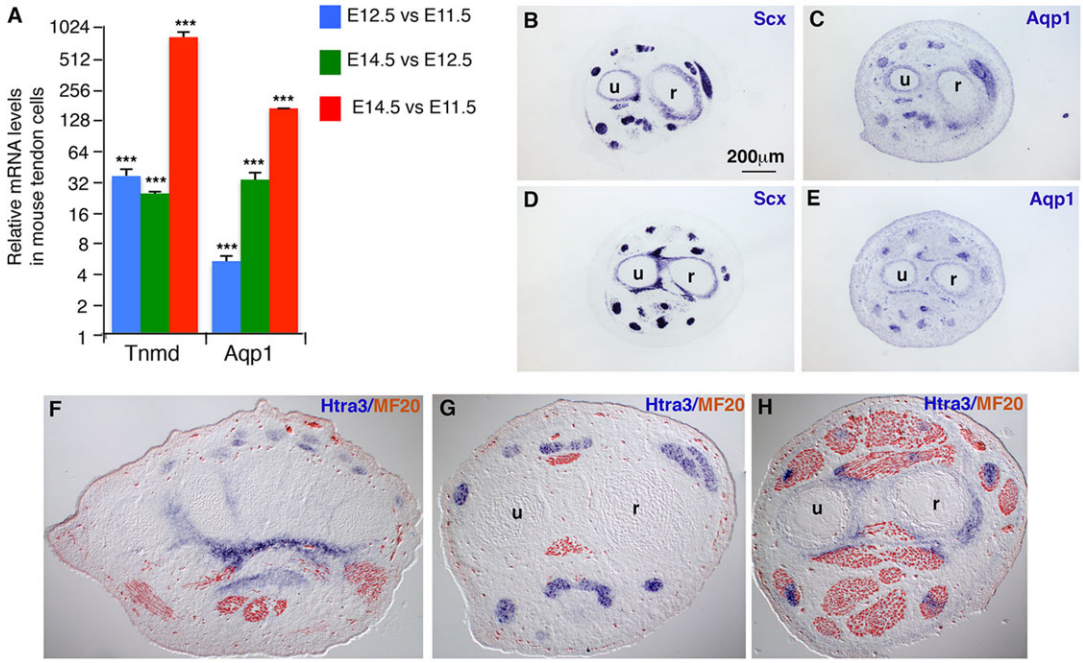

Fig. 2. Expression of *Aqp1* and *Htra3* genes in mouse limb tendons. (A) RT-q-PCR analyses of *Tnmd* and *Aqp1* expression in tendon cells at different stages of mouse limb development. The mRNA levels of tendon cells at E11.5 or E12.5 were normalised to 1 for each comparison so that the graph shows the relative increase of mRNA levels in tendon cells between E12.5 and E11.5, E14.5 and E12.5, and E14.5 and E11.5. ***P<0.001; error bars indicate s.d. (B-E) Adjacent transverse sections of forelimbs of E14.5 mouse embryos were hybridised with *Scx* (B,D) or *Aqp1* (C,E) probes. Adjacent sections are shown from distal (B,C) to proximal (D,E) zeugopod limb regions. (F-H) Transverse sections of E14.5 mouse limbs hybridised with *Htra3* probe (blue) and immunostained for the heavy chain of myosin II (MF20 antibody; brown). u, ulna; r, radius.

From E12.5, tendon differentiation is concomitant with important transcriptomic changes in mouse limb tendon cells (Table 1) and with an enriched expression of matrix and tendon genes (Tables 2 and 3). Consistently, the relative endogenous expression levels of *Col1a1*, *Col1a2*, *Thbs2*, *Thbs4*, *Tnmd* and *Aqp1* were significantly upregulated in E12.5 limbs compared with E9.5 or E10.5 limbs (supplementary material Fig. S4A,B). In E12.5 limb explants, TGFβ2 was sufficient to increase the relative mRNA levels of *Scx*, *Col1a1*, *Thbs2*, *Thbs4* and *Tnmd*, but not of *Aqp1* (Fig. 4C). However, the blockade of TGF-β receptors (SB43) or SMAD2/3 activity (SIS3) in E12.5 limb explants did not affect *Scx*, *Col1a2*, *Thbs4*, *Tnmd* or *Aqp1* gene expression, while decreasing that of *Col1a1* and *Thbs2* (Fig. 4C). This showed that in E12.5 mouse limbs TGF-β is sufficient for the expression of the tendon markers *Scx*, *Col1a1*, *Thbs2*, *Thbs4* and *Tnmd*, while being required only for *Col1a1* and *Thbs2* expression.

We conclude that TGF-β is sufficient for the expression of *Scx* and tendon-associated genes at different stages of limb development from E9.5 to E12.5, whereas the intracellular SMAD2/3 pathway is required for *Scx* and tendon-associated gene expression in early E9.5/E10.5 limbs.

Involvement of the ERK MAPK pathway in mouse limb tendon development

In addition to the TGF-β pathway, the MAPK signalling pathway (KEGG N°4010) also appeared to be significantly differentially regulated between E11.5 and E12.5 and between E11.5 and E14.5 (Table 5; supplementary material Fig. S3 and Table S3). MAPK pathways are activated by receptor tyrosine kinases, including FGF receptors (Mason et al., 2006). FGF signalling has been shown to positively regulate *Scx* expression in chick limb tendons, chick and mouse axial tendons and intermuscular tendons of chick stomach (Edom-Vovard et al., 2002; Brent and Tabin, 2004; Brent et al., 2005; Le Guen et al., 2009). No such evidence of FGF sufficiency exists during mouse limb tendon development. An observation from

Table 4. Signalling pathways (signal transduction pathways and GO tissues) modified in mouse limb tendon cells during development

Pathway (total number of genes in pathway)	E11.5 versus E12.5	E12.5 versus E14.5	E11.5 versus E14.5
Signal transduction pathways			
*TGF-β (640)	59 (3.42×10^{-10})	102 (2.86×10^{-9})	136 (6.18×10^{-9})
*Matrix metalloproteinase (236)	19 (2.38×10^{-3})	51 (1.88×10^{-9})	64 (9.60×10^{-9})
*Thrombospondin 1 (56)	9 (3.00×10^{-4})	13 (1.12×10^{-3})	17 (7.08×10^{-4})
‡Aurora kinase (130)	–	26 (7.72×10^{-14})	45 (2.28×10^{-22})
‡Cell division (201)	–	32 (7.72×10^{-14})	48 (2.76×10^{-16})
Tissues			
*Tendons (197)	39 (1.47×10^{-21})	63 (4.22×10^{-27})	85 (6.30×10^{-33})
*Ligaments (120)	35 (1.63×10^{-25})	43 (4.53×10^{-21})	58 (5.26×10^{-26})

For each comparison is shown the number of genes upregulated or downregulated with the P-value in parentheses. TGF-β is the pathway displaying the greatest number of upregulated genes in each type of comparison. Genomatix software was employed for this analysis.
*Upregulated pathways.
‡Downregulated pathways.

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Table 5. Analysis of the KEGG pathway 'signal transduction' category in limb tendon cells during development

Pathway (total number of genes in pathway)	E11.5 versus E12.5 (713 regulated genes in the transcriptome)	E12.5 versus E14.5 (1767 regulated genes in the transcriptome)	E11.5 versus E14.5 (3282 regulated genes in the transcriptome)
N°04350: TGF-β (85)	11 (2.8×10^{-3})	17 (2.2×10^{-2})	27 (5.1×10^{-3})
N°04010: MAPK (259)	17 (9.21×10^{-2})	34 (NS, 0.17)	57 (5.6×10^{-2})
N°04020: Calcium (185)	13 (6.48×10^{-2})	24 (NS, 0.2179)	36 (NS, 0.356)
N°04310: Wnt (156)	12 (5.18×10^{-2})	15 (NS, 0.7958)	34 (NS, 0.175)
N°04340: Hedgehog (49)	7 (2.77×10^{-2})	5 (NS, 0.876)	–

The DAVID bioinformatics resource 6.7 was used for this analysis. For each comparison is shown the number of differentially regulated genes in the pathway with the *P*-value in parentheses. The KEGG pathways have been ordered from high to low by *P*-value for the E11.5 versus E12.5 comparison. The two most significant *P*-values correspond to the TGF-β and MAPK pathways for the E11.5 versus E12.5 and E11.5 versus E14.5 comparisons. NS, not significant.

the bioinformatics data is that several FGF ligands appeared significantly upregulated in mouse tendon cells during limb development (supplementary material Table S3), although none of these specific FGF ligands has been reported to be linked with tendon development. Another striking observation is that most of the MAP kinases displaying significant variation in the transcriptome showed a significant decrease in expression, such as *Map3k4*, *Map2k6*, *Mapk8* (*Jnk*) and *Mapk12* (*p38*) (supplementary material

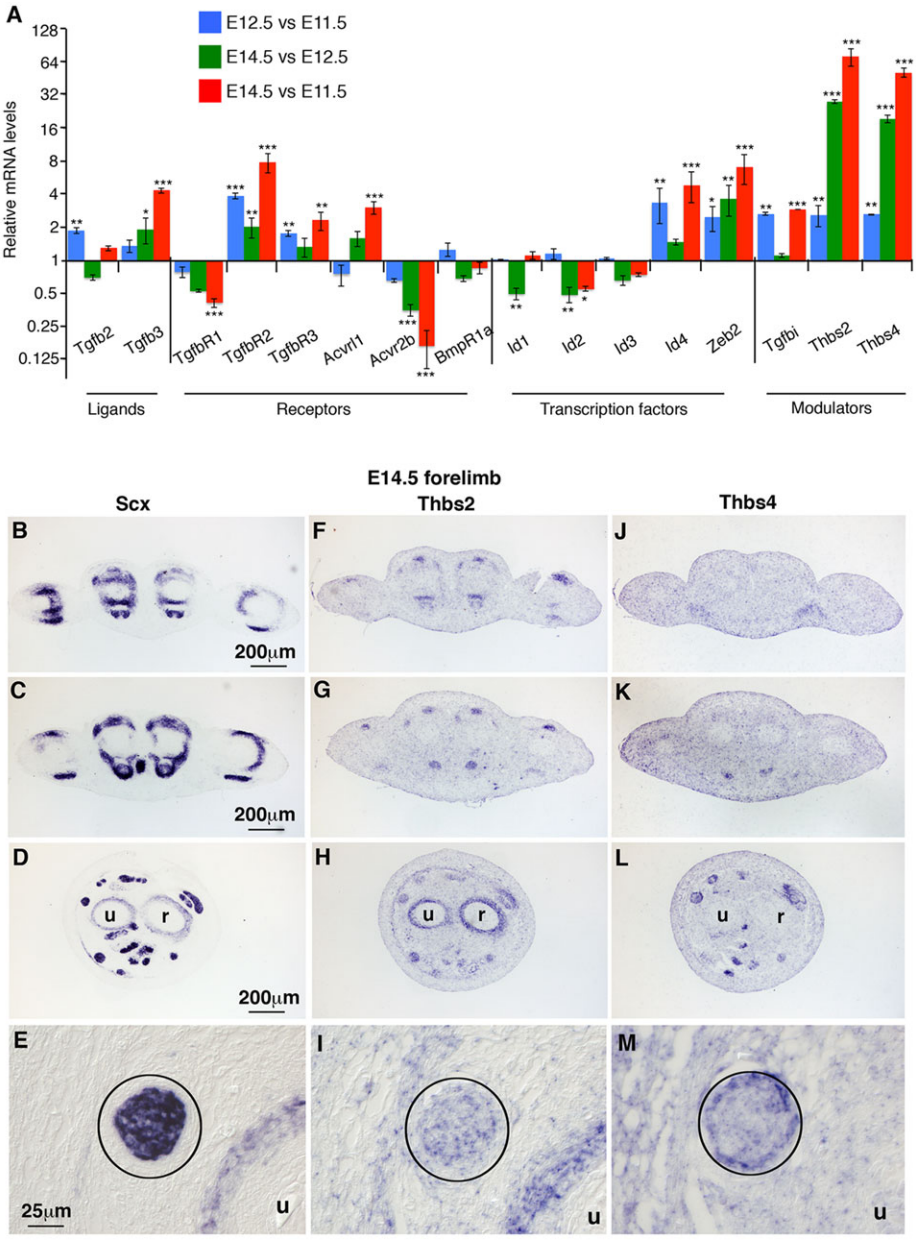

Fig. 3. Expression of TGF-β signalling pathway components in mouse FACS-sorted tendon cells and limbs. (A) RT-q-PCR analyses of TGF-β-associated gene expression levels in tendon cells at different stages of development. Shown is the relative increase in mRNA levels in Scx-GFP^{+} tendon cells at different stages of development: E12.5 versus E11.5, where mRNA levels of tendon cells at E11.5 were normalised to 1; E14.5 versus E12.5, where mRNA levels of tendon cells at E12.5 were normalised to 1; and E14.5 versus E11.5, where mRNA levels of tendon cells at E11.5 were normalised to 1. **P*<0.05; ***P*<0.01; ****P*<0.001; error bars indicate s.d. (B-M) Adjacent transverse sections of forelimbs of E14.5 mouse embryos were hybridised with *Scx* (B-E), *Thbs2* (F-I) or *Thbs4* (J-M) probes. B,F,J, C,G,K and D,H,L are groups of adjacent sections from distal to proximal limb regions. (E,I,M) Higher magnifications of tendon shown in D,H,L. The circles delineate the same areas, highlighting differences between the *Thbs2* (I) and *Thbs4* (M) expression domains in tendons. All sections are dorsal to the top and posterior to the left. u, ulna; r, radius.

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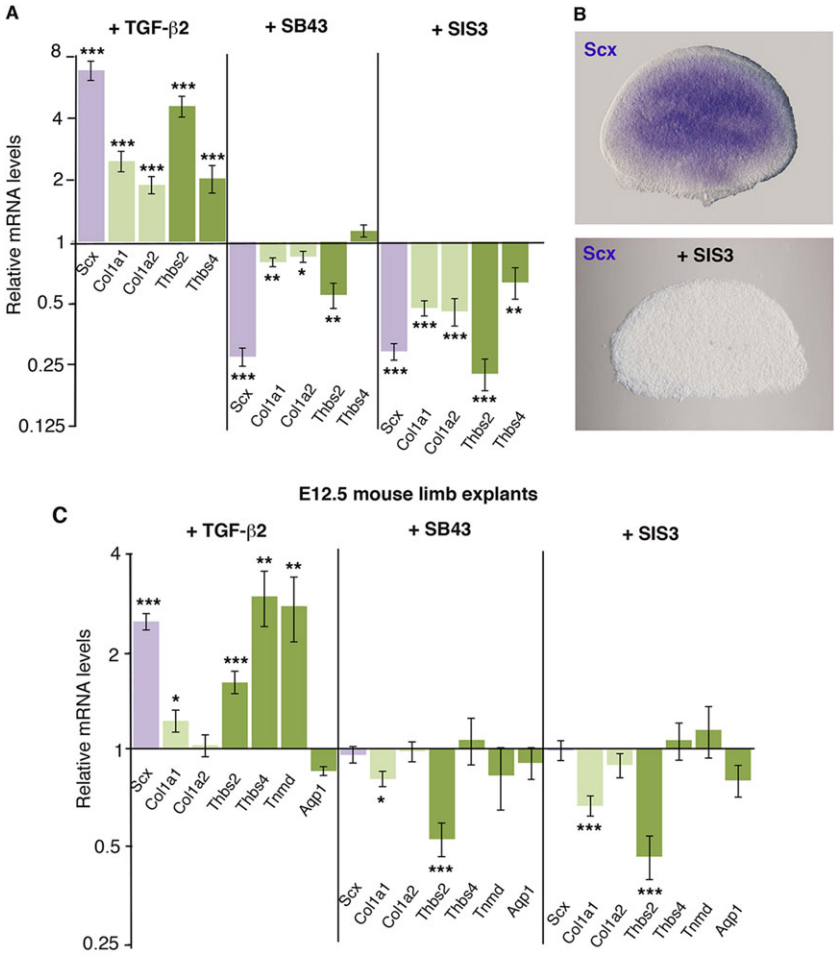

Fig. 4. Effects of TGF-β gain- and loss-of-function in mouse limb explants. (A) RT-q-PCR analyses of the expression levels of tendon markers in E9.5/E10.5 mouse limb explants cultured for 24 h with TGFβ2, SB43 or SIS3 inhibitors. (B) *In situ* hybridisation for *Scx* expression in E9.5 mouse limb explants cultured with SIS3 inhibitor for 24 h. (C) RT-q-PCR analyses of *Scx*, *Col1a1*, *Col1a2*, *Thbs2*, *Thbs4*, *Tnmd* and *Aqp1* expression levels in E12.5 mouse limb explants cultured for 24 h with TGFβ2, SB43 or SIS3 inhibitors. For each gene, the mRNA levels of control limb explants were normalised to 1. **P*<0.05; ***P*<0.01; ****P*<0.001; error bars indicate s.e.m.

Table S3). In addition, the MAP kinase phosphatase *Dusp6*, which is known to be a readout of active ERK1/2 (MAPK3/1) signalling during development (Lunn et al., 2007), was clearly downregulated in tendon cells during development (supplementary material Table S3). This decrease in the expression of *Dusp6* and of genes encoding MAP kinases in tendon cells suggested a diminution of ERK MAPK activity in tendon cells during mouse development.

In order to validate this bioinformatics data, we blocked the ERK MAPK pathway in mouse limb explants using the PD18 inhibitor, which is known to prevent ERK phosphorylation (Bain et al., 2007). *Pea3* (*Etv4*) and sprouty 2 (*Spry2*) genes are transcriptional targets of ERK MAP kinases and are considered a readout of ERK activity (O'Hagan et al., 1996; Mason et al., 2006). The dramatic loss of *Pea3* and *Spry2* expression showed that ERK signalling activity was downregulated in the presence of PD18 (Fig. 5A). Consistent with the bioinformatics data, ERK inhibition led to a significant activation of the expression of *Scx*, *Col1a1*, *Col1a2* and *Thbs2* in E9.5 mouse limb explants (Fig. 5A). FGF4 application to E9.5 mouse limb explants did not modify tendon gene expression (Fig. 5A). We conclude that inhibition of the ERK MAPK signalling pathway was sufficient to activate the expression of tendon genes, including *Scx*, in early mouse limbs.

Positive and negative cross-talk between the ERK and SMAD intracellular pathways have been highlighted in several cellular and *in vivo* contexts (Massague, 2012) (supplementary material Fig. S5). In epithelial cells, ERK1/2 activation inhibits SMAD3 transcriptional activity via phosphorylation in its linker region (Kretzschmar et al., 1999; Matsuura et al., 2005; Wrighton et al., 2009). Since mutations of these phosphorylation sites increase SMAD3 activity (Wrighton et al., 2009), we hypothesise that ERK blockade could activate SMAD3 transcriptional activity in tendon cells. To assess activity of the SMAD3 pathway we used *Smad7*, a negative-feedback regulator that is considered a general TGF-β transcriptional target gene (Massague, 2012). We did not observe any increase in *Smad7* expression following PD18 application (Fig. 5A). *Smad7* expression was also unchanged after FGF4 application (Fig. 5A), indicating an absence of cross-talk between the FGF/ERK and SMAD3 pathways in the E9.5 mouse limb context. This indicated that tendon gene activation following ERK inhibition was not a consequence of SMAD3 activation.

In order to determine whether the positive effect of TGF-β and of ERK inhibition on tendon gene expression could be additive, we systematically compared the TGF-β effect with that of simultaneous TGF-β+PD18 application on the mRNA levels of tendon genes in E9.5 mouse limb explants (Fig. 5B). We did not observe any significant increase in *Smad7* expression levels in the presence of TGF-β+PD18 versus TGF-β alone (Fig. 5B), confirming the absence of any increase in SMAD3 transcriptional activity in the context of ERK inhibition (Fig. 5A). TGF-β receptors can activate various MAP kinases, including ERK (supplementary material Fig. S5) (Massague, 2012). However, in early mouse limb explants TGF-β did not activate *Pea3* or *Spry2* expression (Fig. 5B) and did not prevent ERK inhibition based on similar *Pea3* and *Spry2* downregulation with PD18 (Fig. 5A) and TGF-β+PD18 (Fig. 5B). Regarding tendon genes, there was no difference in *Scx* mRNA

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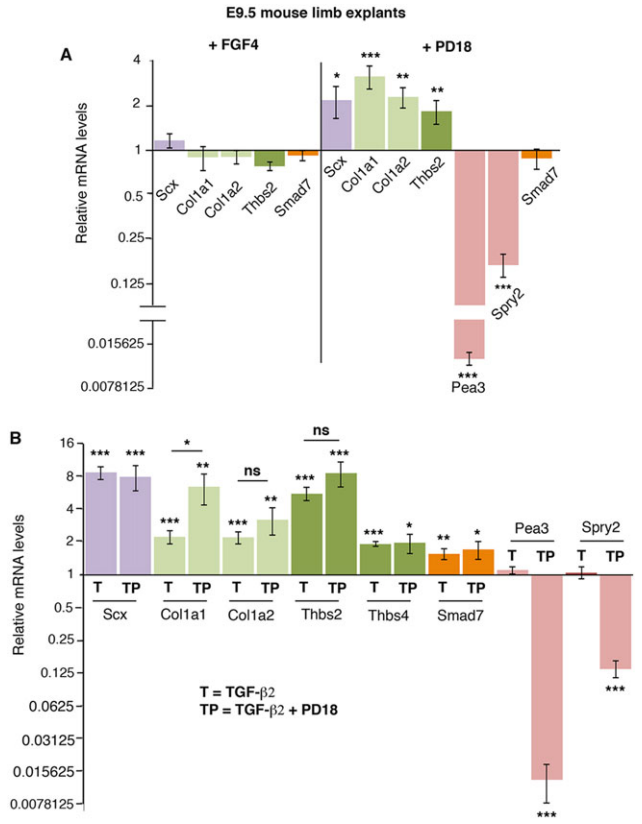

Fig. 5. Effects of FGF/ERK gain- and loss-of-function in E9.5 mouse limb explants. (A) RT-q-PCR analyses of the tendon genes *Scx*, *Col1a1*, *Col1a2* and *Thbs2* in E9.5 mouse limb explants cultured for 24 h with FGF4 or PD18 inhibitor. (B) RT-q-PCR analyses of *Scx*, *Col1a1*, *Col1a2*, *Thbs2*, *Thbs4*, *Smad7*, *Pea3* and *Spry2* mRNA levels in E9.5 mouse limb explants cultured for 24 h with either TGFβ2 (condition T) or TGFβ2+PD18 inhibitor (condition TP). For each gene, the mRNA levels of control limbs were normalised to 1. **P*<0.05; ***P*<0.01; ****P*<0.001; NS, not significant; error bars indicate s.e.m.

levels following TGF-β+PD18 application versus TGF-β (Fig. 5B). However, we did observe a significant difference in *Col1a1* expression levels, and a non-significant tendency to increase for *Col1a2* and *Thbs2* expression levels following TGF-β+PD18 co-treatment versus TGF-β alone in E9.5 mouse limb explants (Fig. 5B). This showed that there was no obvious additive effect on *Scx* expression following TGF-β application and ERK inhibition in E9.5 mouse limbs.

The positive effect of ERK inhibition on *Scx* expression observed in E9.5 limb explants was lost in E10.5 limb explants, whereas the *Col1a1* and *Thbs2* expression levels were still significantly elevated, following PD18 application at E10.5 (supplementary material Fig. S6A). FGF4 application did not have any significant effect on tendon gene expression in E10.5 mouse limbs, as in E9.5 limbs (supplementary material Fig. S6A). Lastly, in E12.5 mouse limb explants, ERK inhibition did not activate the expression of any tendon genes and even significantly inhibited *Col1a2* expression. Consistently, FGF ligand application activated *Col1a2* expression levels in E12.5 mouse limb explants (supplementary material Fig. S6B).

We conclude that FGF does not positively regulate *Scx* expression in early and late mouse limbs and that ERK inhibition is sufficient to enhance the expression of tendon genes independently of the activation of TGF-β signalling in early E9.5 mouse limbs.

TGF-β/SMAD2/3 and FGF/ERK pathway involvement in mouse mesenchymal stem cells

TGF-β and MAPK signalling pathways were modified in limb tendon cells during development (Tables 4 and 5), and TGFβ2 application or ERK MAPK inhibition was sufficient to activate tendon gene expression in mesodermal limb cells (Figs 4 and 5). In order to determine whether TGF-β ligands or ERK inhibition could also drive stem cells towards the tendon lineage, we utilised mesenchymal stem cells. These cells can differentiate into various tissues of mesodermal origin, when cultured in appropriate differentiation media (Caplan, 2007).

TGF-β has been shown to have a protenogenic or prochondrogenic effect depending on cell type or culture conditions (Lorda-Diez et al., 2009, 2013; Pryce et al., 2009). We used the multipotent murine C3H10T1/2 mesenchymal stem cells (Reznikoff et al., 1973). TGFβ2 has been shown to activate *Scx* expression in C3H10T1/2 stem cells (Pryce et al., 2009). Consistently, the relative levels of *Scx* and *Col1a1* gene expression were significantly elevated in the presence of TGFβ2 or TGFβ3 (Fig. 6A). In contrast to the tendon markers, the relative levels of the cartilage marker *Sox9* were significantly decreased in the presence of TGF-β ligands (Fig. 6A). We did not observe any increase in tendon or cartilage marker expression after simultaneous addition of TGFβ2 and TGFβ3 ligands compared with either ligand alone (Fig. 6A). The TGF-β effect on tendon and cartilage marker expression was reduced in the presence of SB43 inhibitor, which blocks the TGF-β pathway at the level of the receptors, and in the presence of SIS3 inhibitor, which blocks the SMAD2/3 intracellular pathway (Fig. 6B). We conclude that TGF-β ligand has the ability to direct mouse mesenchymal stem cells towards the tendon lineage (*Scx*) at the expense of the cartilage lineage (*Sox9*) via the SMAD2/3 pathway.

The outcome of FGF treatment on *Scx* expression in stem cells differs between studies. FGF2 treatment in mouse stem cells activated the expression of *Scx* (Ker et al., 2011), whereas FGF4 treatment in mouse tendon progenitor cells inhibits *Scx* expression (Brown et al., 2014). We assessed the efficiency of FGF4 and ERK inhibition (PD18) in C3H10T1/2 cells by *Pea3* upregulation in the presence of FGF4 and *Pea3* downregulation with PD18 (Fig. 6C). Consistent with the PD18 effect in E9.5 mouse explants (Fig. 5), ERK inhibition activated *Scx* and *Col1a1* gene expression in C3H10T1/2 cells (Fig. 6C). PD18 treatment did not affect *Smad7* expression in C3H10T1/2 cells (Fig. 6C), indicating that SMAD2/3 activity was not modified in this experimental design. ERK inhibition did not affect *Sox9* expression in C3H10T1/2 cells (Fig. 6C). FGF4 had the opposite effect and led to a significant inhibition of *Scx* and *Col1a1* gene expression in C3H10T1/2 cells (Fig. 6C).

We conclude that TGF-β ligand has the ability to direct mouse mesenchymal stem cells towards the tendon lineage at the expense of cartilage. ERK inhibition activates *Scx* expression, whereas FGF appeared to have an anti-tenogenic effect on mouse mesenchymal stem cells.

DISCUSSION

We have established the first transcriptome of mouse tendon cells during development. Bioinformatics analyses highlighted the TGF-β and MAPK signalling pathways as being the main signalling pathways regulated in limb tendon cells during development. Modification of these pathways in mouse limb explants or mesenchymal stem cells showed that TGF-β signalling was sufficient and required via SMAD2/3 to drive mouse mesodermal stem cells towards the tendon lineage *ex vivo* and *in vitro*. FGF did not have a tenogenic effect and the inhibition

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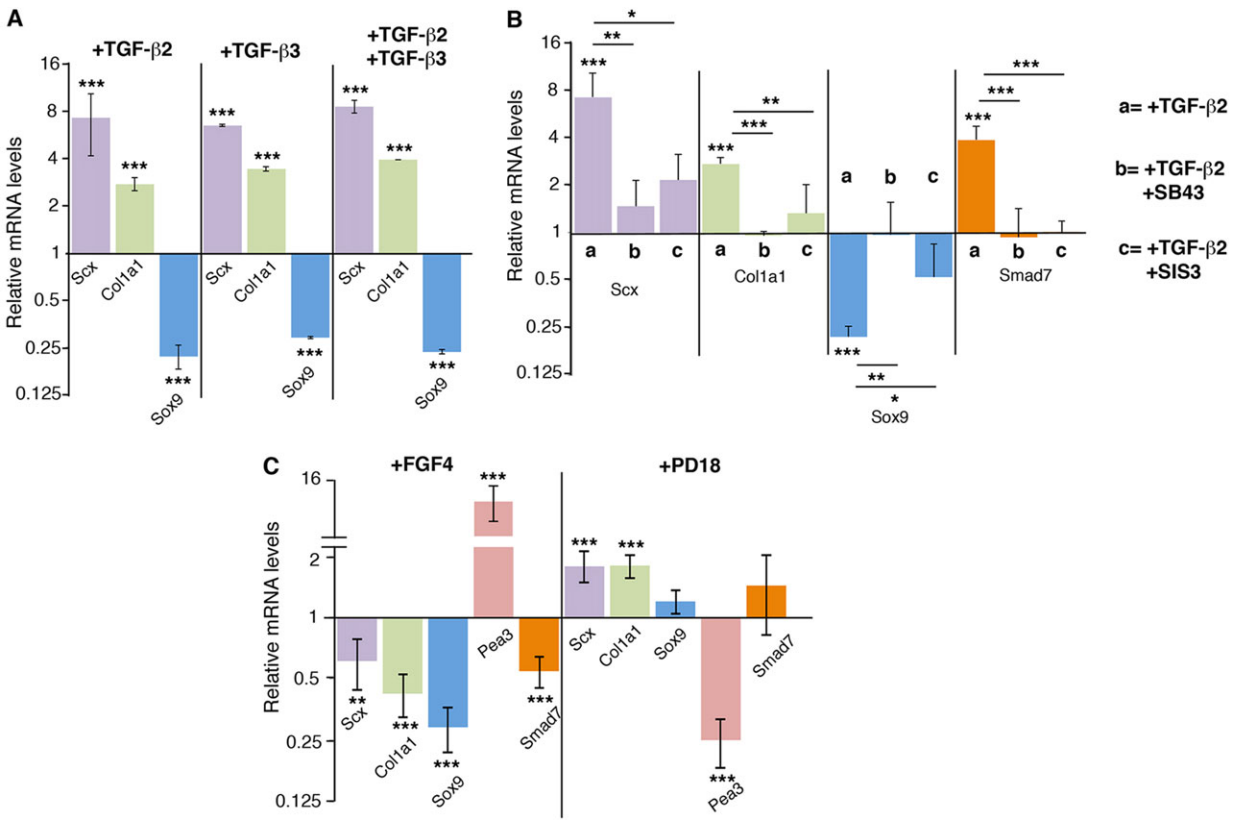

Fig. 6. Effect of TGF-β/SMAD2/3 and FGF/ERK signalling in mesenchymal stem cells. (A) C3H10T1/2 cells were cultured in the presence of TGFβ2, TGFβ3 or TGFβ2+TGFβ3 for 24 h. Relative mRNA expression was examined for the tendon markers *Scx* and *Col1a1* and for the cartilage marker *Sox9*. For each gene, the mRNA levels of non-treated C3H10T1/2 cells were normalised to 1. (B) C3H10T1/2 cells were cultured in the presence of TGFβ2 (condition a), TGFβ2+SB43 inhibitor (condition b) or TGFβ2+SIS3 inhibitor (condition c) for 24 h. The mRNA levels of non-treated C3H10T1/2 cells were normalised to 1 for condition a; the mRNA levels of TGFβ2-treated C3H10T1/2 cells were normalised to 1 for conditions b and c. (C) C3H10T1/2 cells were cultured in the presence of FGF4 or PD18 inhibitor for 24 h. The mRNA levels of non-treated C3H10T1/2 cells were normalised to 1. **P*<0.05; ***P*<0.01; ****P*<0.001; error bars indicate s.d.

of the ERK MAPK signalling pathway was sufficient to activate *Scx* in mouse limb mesodermal progenitors and mesenchymal stem cells.

Genes expressed in limb tendons cells during mouse development

We have established a list of genes that are differentially expressed in limb tendon cells during development. Differentially expressed genes displayed very high fold changes (ranging from 533 to 14 for the top 100 genes). The second most differentially expressed gene of the ordered list is *Tnmd*, which is known to be involved in tendon formation (Docheva et al., 2005; Shukunami et al., 2006). Among the top 100 enriched genes, those encoding matrix components were found to be drastically upregulated in tendon cells during development, including *Col14a1*, *Fmod*, *Dcn* and *Bgn*. Mice mutant for these matrix genes display a tendon phenotype (Ameye et al., 2002; Ansorge et al., 2009). Among this top list, *Aqp1* and *Htra3* (which were previously not known to be related to tendon) were identified as expressed in E14.5 mouse limb tendons. The AQP1 hydrophobic transmembrane water channel protein is known to be responsible for the rapid response of cell volume to changes in plasma tonicity and is involved in cell proliferation, migration and adhesion processes of many cell types (Benga, 2012). However, AQP1 function in tendon development is not yet known. The serine protease gene *Htra3*, which is known to act as a tumour suppressor (Skorko-Glonek et al., 2013), has until now never been associated with tendon formation. Lastly, among the top enriched genes, we found components of the TGF-β pathway, including the thrombospondin genes *Thbs2* and *Thbs4*, which were expressed in mouse limb tendons. Mice that lack *Thbs2* display connective tissue abnormalities, including in tendons (Kyriakides et al., 1998). The *Drosophila* equivalent, Thrombospondin (Tsp), is produced by tendon cells and is essential for the formation of the integrin-mediated myotendinous junction (Subramanian et al., 2007). We believe that these genes that are differentially expressed in limb tendon cells during mouse development (listed in Table 3; supplementary material Table S1) constitute an important list of tendon markers.

TGF-β signalling pathway involvement in mouse limb tendon development

Bioinformatics data highlighted a TGF-β activity in limb tendon cells during the muscle-independent phase (before E12.5) and the differentiation phase (after E12.5) of tendon formation. We confirmed that TGF-β was sufficient for tendon gene expression in early mouse limb explants. In addition, the SMAD2/3 intracellular pathway was required for *Scx* expression in early mouse limb explants. In early mouse limbs, *Scx* is also expressed in cartilage progenitors of the entheses (tendon attachment sites to bone) (Blitz et al., 2013; Sugimoto et al., 2013) and TGF-β signalling has been shown to be required for the specification of Scx^{+} enthesis progenitors (Blitz et al., 2013). The onset of *Scx* expression in mouse limbs is at E10 (Schweitzer et al., 2001; Sugimoto et al., 2013) and *Scx* expression was disrupted only at

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E12.5 in limb buds of *Tgfb2*$^{-/-}$;*Tgfb3*$^{-/-}$ and conditional *Tgfbr2* mutant mice (Pryce et al., 2009). However, *Scx* and *Col1a1* gene expression and tendon matrix organisation are altered in adult tendons of *Smad3*$^{-/-}$ mice (Berthet et al., 2013) and it was shown that SMAD3 is recruited to *Scx* regulatory regions in adult mouse tendons (Berthet et al., 2013), suggesting a direct role for SMAD signalling in *Scx* expression. Because we found a loss of *Scx* expression when SMAD2/3 was inhibited in mouse limb explants, we propose that in the *Tgfb2*$^{-/-}$;*Tgfb3*$^{-/-}$ and conditional *Tgfbr2* mutant mice, the endogenous SMAD2/3 intracellular pathways may be activated by alternative TGF-β superfamily ligands or receptors to initiate *Scx* expression. Moreover, it has been shown that the SMAD intracellular pathways can also be activated by alternative ligands (Guo and Wang, 2009; Massague, 2012), suggesting that non-canonical TGF-β signals or receptors could drive *Scx* expression in early mouse limbs. Based on *Scx* expression, TGF-β was also able to drive mesenchymal stem cells toward the tendon lineage, via SMAD2/3. These data converge on the idea that the TGF-β signalling pathway is sufficient and required via SMAD2/3 to initiate the commitment of undifferentiated mesodermal cells towards the tendon lineage *ex vivo* and *in vitro*, but direct targeting of SMAD genes *in vivo* will be required to determine if, during mouse development *Scx* expression is indeed dependent exclusively on SMAD2/3 signalling.

In addition to being sufficient for *Scx* expression during the early stages of mouse limb development, TGFβ2 was also sufficient for tendon gene expression in late mouse limb explants. This indicated that a TGF-β signal was involved in the positive regulation of late tendon marker expression during the tendon differentiation process. Because *Tnmd* mRNA expression levels were barely detectable before E12.5 in mouse limbs, we believe that a TGF-β signal participates in the initiation of *Tnmd* expression in E12.5 limbs. However, the intracellular SMAD2/3 pathway was only required for *Col1a1* and *Thbs2* expression and not for *Scx*, *Thbs4* or *Tnmd* expression in E12.5 mouse limbs. This showed that other signalling pathways were required for the expression of these tendon markers during the tendon differentiation process. The calcium pathway, being the third most differentially regulated (Table 5), is a good candidate pathway. The absence of *Aqp1* gene regulation by TGFβ2 also indicated the involvement of other signalling pathways during tendon cell differentiation. It is likely that mechanical forces and downstream signalling pathways are important for limb tendon differentiation after E12.5.

FGF signalling in mouse limb tendon development

Bioinformatics analyses identified the MAPK pathway as being significantly modified in tendon cells during mouse limb development. The significant decrease in expression of MAP kinases and the phosphatase *Dusp6* in the array suggested a reduction of MAP kinase activity in tendon cells during development. This tendency was surprising given the positive effect of FGF signal on *Scx* expression in chick limbs and chick and mouse somites (Edom-Vovard et al., 2002; Brent et al., 2003, 2005). In addition, the positive effect of FGF on *Scx* expression has been shown to occur via the Ets transcription factor PEA3 and the ERK MAP kinases in chick axial tendons (Brent and Tabin, 2004; Smith et al., 2005). In contrast to the chick model and mouse somites, we found that FGF does not activate *Scx* expression in mouse limb explants (Fig. 5; supplementary material Fig. S6) nor in mouse mesenchymal stem cells (Fig. 6). We even observed a significant reduction of *Scx* and *Col1a1* expression in mouse C3H10T1/2 cells in the presence of FGF4 (Fig. 6). This decrease in *Scx* expression is consistent with that observed in mouse tendon progenitor cells upon FGF4 treatment (Brown et al., 2014). Moreover, the inhibition of ERK MAPK signalling appeared to be sufficient for inducing *Scx* expression in E9.5 mouse limb mesodermal progenitors and in mesenchymal stem cells. This result is consistent with the requirement of FGF loss to promote cell differentiation in many tissues (Mathis et al., 2001; ten Berge et al., 2008; Chang et al., 2013). The ability of ERK inhibition to activate *Scx* expression was only observed in E9.5 limb explants and not at later stages. E9.5 corresponds to the developmental time when limb mesodermal progenitor cells will commit to the tendon lineage based on *Scx* expression.

To date, we conclude that ERK inhibition is sufficient to prime mouse stem cells for the tendon lineage and that the FGF signalling pathway has a different role in mouse limb tendon development than that in chick limb tendon development. Experiments are underway with the aim of furthering our understanding of the differences in the involvement of the FGF/ERK pathway in limb tendon development between the chick and mouse models.

In summary, we have established a list of genes enriched in limb tendon cells during mouse development. We have shown that TGF-β signalling is sufficient and required via SMAD2/3 to drive mouse mesodermal stem cells towards the tendon lineage *ex vivo* and *in vitro*. In contrast to chick, in the mouse FGF does not have a tenogenic effect and inhibition of the ERK MAPK signalling pathway is sufficient to activate *Scx* in mouse limb mesodermal progenitors and mesenchymal stem cells.

MATERIALS AND METHODS

Mouse lines

Scx-GFP (Pryce et al., 2007) or wild-type (Janvier, France) mouse embryos were collected after natural overnight matings. For staging, fertilisation was considered to take place at 12.00 a.m.

RNA isolation and microarray analysis

Forelimbs from E11.5, E12.5 and E14.5 Scx-GFP embryos were collected and dissociated. Cell suspensions were subjected to FACS using a MoFlo XDP flow cytometer (Beckman Coulter) with the Dako-Moflo Summit software (Dako, Agilent Technologies) or using a VantageTM SE option DiVa flow cytometer (Becton-Dickinson; laser 488 nm). The GFP^{+} fractions were collected in PBS containing 2 mM EDTA and 20% foetal calf serum. RNA quantity was monitored on Agilent RNA Pico LabCHips.

Fragmented biotin-labelled cRNA samples were hybridised on Affymetrix GeneChip Mouse Genome 430 2.0 arrays that contain 45,000 probe sets. Each probe set consists of 22 probes of 25 bp with 11 perfect matches and 11 mismatches. For each experimental group (E11.5, E12.5 and E14.5), three biological replicates were hybridised. Microarray analysis was performed using a high-density oligonucleotide array (Affymetrix) on the ProfileXpert core facility. Total RNA (100 ng) was amplified and biotin-labelled using GeneChip 3′ IVT Express target labelling, control reagents and procedures from Affymetrix. Before amplification, spikes of synthetic mRNA at different concentrations were added to all samples; these positive controls were used to ascertain the quality of the process. Biotinylated antisense cRNA for microarray hybridisation was prepared. After final purification using magnetic beads, cRNA quantification was performed with a NanoDrop (Thermo Scientific) and quality checked with an Agilent 2100 Bioanalyzer.

Biotin-labelled cRNA samples (15 µg) were fragmented, denatured and hybridised on Affymetrix arrays for 16 h at 45°C with constant mixing by rotation at 60 rpm in a GeneChip hybridisation oven 640 (Affymetrix). After hybridisation, arrays were washed and stained with streptavidin-phycoerythrin (Invitrogen) in a Fluidic Station 450 (Affymetrix) according to the manufacturer's instruction. The arrays were read with a confocal laser (GeneChip scanner 3000, Affymetrix). Then, CEL files were generated using Affymetrix GeneChip Command Console software 3.0. The

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array has been submitted to the GEO repository with accession number GSE54207.

Statistical analysis of microarray data

The microarray data were normalised with Affymetrix Expression Console software using the MAS5 statistical algorithm. Normalised data were compared and filtered using Partek Genomic Suite software 6.5. Pairwise comparisons were performed between each developmental stage (E11.5, E12.5 and E14.5). Each sample from one group was compared with each sample from the other group and only genes showing a variation of 1.5-fold were considered significantly differentially regulated.

Bioinformatics analyses of differentially expressed genes in tendon cells

DAVID was used to identify enriched GO terms. Genomatix software was used to identify signalling pathways based on literature data mining. Consequently, a Genomatix pathway includes a larger number of components than canonical pathways. The KEGG signal transduction pathways are a collection of manually drawn pathway maps representing current knowledge on the molecular interaction and reaction networks for a wide range of biological processes. DAVID was used to identify regulated KEGG pathways.

Mouse limb explant cultures

Limb buds were dissected from E9.5, E10.5 and E12.5 mouse embryos, embedded in collagen and cultured at 37°C in 5% CO_2 in Optimem medium (Diez del Corral et al., 2003). Explants were treated with recombinant human TGFβ2 (R&D Systems) at 20 ng/ml or with FGF4 (R&D Systems) at 200 ng/ml, for 24 h. The TGFβ2 signalling pathway was blocked using SB431542 (SB43, Selleck Chemicals) or SIS3 (Merck) chemical inhibitors; the ERK signalling pathway was blocked using PD184352 (PD18) chemical inhibitor (Axon Medchem). All inhibitors were diluted in DMSO (Fluka) and added to the medium for 24 h at 10 µM (SB43), 20 µM (SIS3) or 3.3 µM (PD18). Media with buffers only were used as controls. After treatments, explants were fixed and processed for RT-q-PCR or *in situ* hybridisation.

RNA isolation, reverse transcription and quantitative real-time PCR (RT-q-PCR)

Total RNAs were extracted from forelimb FACS-sorted Scx-GFP cells at different developmental stages, mouse C3H10T1/2 cells or mouse limb explants. RNA (300 ng to 1 µg) was reverse transcribed using the High Capacity Retrotranscription Kit (Applied Biosystems). RT-q-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primer used for RT-q-PCR are listed in supplementary material Table S4. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The ΔCts were obtained from Ct normalised with *Gapdh*, *Hprt* or *18S* levels in each sample. For mRNA level analyses of Scx-GFP cells at different developmental stages, three independent RNA samples originating from three FACS-sorted experiments were analysed in triplicate. For mRNA level analyses of C3H10T1/2 cell cultures, five independent RNA samples were analysed in duplicate. For mRNA level analyses in mouse limb explant cultures, 8-12 independent RNA samples were analysed in duplicate. For E9.5, E10.5 and E12.5 mouse limb explants, we pooled 14, 11 and 6 limb buds, respectively, to obtain enough material in RNA samples. Data were analysed by unpaired Student's *t*-test using Microsoft Excel.

In situ hybridisation

Forelimbs from E14.5 wild-type mouse embryos were fixed in Farnoy and processed for *in situ* hybridisation using 8 µm wax tissue sections. Mouse limb explants were fixed in 4% formaldehyde. The digoxigenin-labelled mRNA probe for mouse *Scx* was used as described (Lejard et al., 2011). cDNAs for *Aqp1*, *Thbs2* and *Thbs4* were cloned by PCR in pCRII-TOPO (Invitrogen). *Htra3* cDNA was cloned by PCR in pBluescript KS (Addgene). The probes were prepared by plasmid linearisation with *Bam*HI and probe synthesis with T7 RNA polymerase for *Aqp1* and *Thbs4*, plasmid linearisation with *Not*I and probe synthesis with Sp6 RNA polymerase for *Thbs2*, and plasmid linearisation with *Sal*I and probe synthesis with T7 RNA polymerase for *Htra3*.

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Competing interests

The authors declare no competing financial interests.

Author contributions

D.D., E.H. and I.O-M. designed experiments; E.H., M-A.B., I.O-M. and M.R. performed experiments; E.H., N.N. and C.D. performed bioinformatic analysis; J.W., M-J.G., C.B-B., F.R and R.S. contributed reagents/analytic tools; E.H., R.S. and D.D. analysed the data and D.D. and E.H. wrote the manuscript.

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Supplementary material

Supplementary material available online at
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