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RESEARCH ARTICLE

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ABSTRACT

The molecular programme underlying tendon development has not been fully identified. Interactions with components of the musculoskeletal system are important for limb tendon formation. Limb tendons initiate their development independently of muscles; however, muscles are required for further tendon differentiation. We show that both FGF/ERK MAPK and TGFβ/SMAD2/3 signalling pathways are required and sufficient for *SCX* expression in chick undifferentiated limb cells, whereas the FGF/ERK MAPK pathway inhibits *Scx* expression in mouse undifferentiated limb mesodermal cells. During differentiation, muscle contraction is required to maintain *SCX*, *TNMD* and *THBS2* expression in chick limbs. The activities of FGF/ERK MAPK and TGFβ/SMAD2/3 signalling pathways are decreased in tendons under immobilisation conditions. Application of FGF4 or TGFβ2 ligands prevents *SCX* downregulation in immobilised limbs. TGFβ2 but not FGF4 prevent *TNMD* and *THBS2* downregulation under immobilisation conditions. We did not identify any intracellular crosstalk between both signalling pathways in their positive effect on *SCX* expression. Independently of each other, both FGF and TGFβ promote tendon commitment of limb mesodermal cells and act downstream of mechanical forces to regulate tendon differentiation during chick limb development.

KEY WORDS: Chick, Limb, Tendon, Mechanobiology, Scleraxis

INTRODUCTION

Tendon is a connective tissue that transmits the forces generated by muscle to bone and allows body motion. Type I collagen is the main structural and functional component of tendons. The signals regulating the production and the spatial organisation of type I collagen in developing tendons have not been fully identified. Moreover, because type I collagen is not specific to tendons, it is not possible to follow tendon development by mapping collagen expression (reviewed by Gaut and Duprez, 2016). The basic helix-loop-helix (bHLH) transcription factor Scleraxis (Scx) has been identified as an early tendon marker during development. *Scx* is expressed in tendon progenitors, developing tendons and adult tendons (Schweitzer et al., 2001; Pryce et al., 2007; Mendias et al., 2012). *Scx* is not the unique master gene driving tendon development, as tendons are formed in *Scx*$^{-/-}$ mice, albeit

displaying differentiation defects (Murchison et al., 2007). Moreover, *Col1a1* expression is downregulated in tendons of *Scx*$^{-/-}$ mutant mice (Murchison et al., 2007), consistent with transcriptional regulation of the mouse *Col1a1* gene by Scx, via direct binding on the *Col1a1* promoter (Lejard et al., 2007). Tenomodulin (*Tnmd*) and *Col14a1* expression is lost in developing limb tendons of *Scx*$^{-/-}$ mice (Murchison et al., 2007). The transmembrane glycoprotein Tnmd is considered as a late tendon marker (Jelinsky et al., 2010; Sugimoto et al., 2013) and is highly expressed in embryonic day (E) 14.5 mouse limb tendon cells (Havis et al., 2014). Thrombospondin 2 and 4 (THBS2 and THBS4) were also identified in the transcriptome of mouse limb tendon cells (Havis et al., 2014) and have been shown to be involved in tendon development in mouse, *Drosophila* and zebrafish (Kyriakides et al., 1998; Subramanian et al., 2007; Subramanian and Schilling, 2014). Two other transcription factors are involved in tendon development: the homeobox protein Mkx (mohawk) (Ito et al., 2010; Liu et al., 2010; Kimura et al., 2011) and the zinc finger transcription factor EGR1 (early growth response factor 1) (Lejard et al., 2011). In contrast to *Scx*, *Mkx* and *Egr1* are not expressed during early tendon limb development and are not specific to tendons (Anderson et al., 2006; Liu et al., 2006; Ito et al., 2010; Lejard et al., 2011), but they activate *Scx* and *Tnmd* expression in various stem cell types and positively regulate type I collagen production *in vivo* (Ito et al., 2010; Lejard et al., 2011; Guerquin et al., 2013; Liu et al., 2015). In addition to the tendon-related transcription factors, two signalling pathways have been identified as being involved in tendon development: the transforming growth factor-beta (TGFβ) and fibroblast growth factor (FGF) signalling pathways (reviewed by Huang et al., 2015a; Gaut and Duprez, 2016). The TGFβ signalling pathway positively regulates *Scx* expression in early E9/E10 mouse limb explants (Pryce et al., 2009; Havis et al., 2014). TGFβ function in chick tendon development is less understood. Although TGFβ1 and 2 have been shown to increase *SCX* and *TNMD* expression in high-density cultures of chick limb cells (Lorda-Diez et al., 2009), TGFβ1 failed to activate *SCX* expression in Hamburger Hamilton stage (HH) 20/21 chick limb explants (Lorda-Diez et al., 2010). FGF positively regulates *SCX* expression in axial and foetal limb tendons during chick development (Edom-Vovard et al., 2002; Brent et al., 2003; Brent and Tabin, 2004). In contrast to the chick model, FGF has an anti-tenogenic effect in mouse embryonic tendon cells (Brown et al., 2014) and inhibition of the ERK MAPK pathway is sufficient to increase *Scx* expression in early mouse limb explants (Havis et al., 2014). Although the experimental situations in the reports described above differ between the chick and mouse models, they nevertheless suggest a differential regulation of *Scx* by FGF in the chick and mouse models.

DEVELOPMENT

Another important aspect of tendon development is its dependency on muscles. Axial, limb and head tendons require the presence of muscles for full development in chick, mouse and zebrafish embryos (reviewed by Gaut and Duprez, 2016). However, in the absence of muscle, *Scx* expression is normally initiated (and then lost) in limb and head regions of mouse, chick and zebrafish embryos (Schweitzer et al., 2001; Edom-Vovard et al., 2002; Bonnin et al., 2005; Grenier et al., 2009; Chen and Galloway, 2014; Huang et al., 2015b). The muscle dependency of *Scx* expression defines two phases for limb tendon formation: a progenitor, muscle-independent phase and a differentiation, muscle-dependent phase. This muscle dependency applies only to stylopod (arm) and zeugopod (forearm) limb tendons, as autopod (digit) tendons are dependent on cartilage in mouse embryos (Huang et al., 2015b). The molecular mechanisms underlying the muscle dependency of chick tendon development remain elusive. Although one can assume that the muscle dependency of *Scx* expression is related to muscle activity, the requirement of mechanical forces for chick limb tendon development has not been addressed and the molecular signals downstream of muscle contraction involved in tendon differentiation have not been identified.

RESULTS

The FGF/ERK MAPK pathway activates *SCX* expression in early chick limb buds

FGF positively regulates *SCX* expression via the ERK MAPK signalling pathway in chick somites (Brent and Tabin, 2004; Smith et al., 2005) and in foetal chick limbs (Edom-Vovard et al., 2002; Eloy-Trinquet et al., 2009). However, the role of FGF on *SCX* expression was not determined in chick limb undifferentiated cells during the muscle-independent phase of limb tendon development. *SCX* expression is initiated in E3 (HH20) chick limb buds (Brent and Tabin, 2004). At these stages, a source of FGF is observed in the apical ectodermal ridge (Niswander et al., 1994). We implanted FGF4 beads in early chick limb buds (E3 to E4) and analysed *SCX* expression by RT-q-PCR and *in situ* hybridisation experiments (Fig. 1A-C). *SCX* and *COL1A2* expression was upregulated as soon as 4 h after FGF4 bead implantation; this upregulation was maintained 24 h after FGF4 bead implantation (Fig. 1A). We used *ETV4* (also known as *PEA3*) and *SPRY2* as transcriptional readouts of ERK MAPK activity (O'Hagan et al., 1996; Mason et al., 2006; Havis et al., 2014). The mRNA levels of *ETV4* and *SPRY2* were increased in FGF4-implanted limbs 4 h and 24 h after grafting (Fig. 1A) and *SPRY2* expression was activated around FGF4 beads 24 h after grafting (Fig. 1C). *TNMD* is not expressed before E5 in chick limbs (Shukunami et al., 2006) and FGF4 was not able to activate *TNMD* prematurely (data not shown). This FGF4 tenogenic effect in chick limb buds contrasted with the previously demonstrated anti-tenogenic effect of FGF4 in mouse limb explants (Havis et al., 2014). We next performed chick limb bud explants in order to exclude differences due to different experimental designs and allow comparison between the chick and mouse models. Consistent with the *in vivo* FGF4 bead experiments (Fig. 1A-C), we observed that FGF4 increased the mRNA levels of *SCX* and *SPRY2* (Fig. 1D), whereas blockade of ERK MAPK with the inhibitor PD18 decreased *SCX*, *ETV4* and *SPRY2* expression in chick limb bud explants 6 h after treatment (Fig. 1D). In order to allow comparison between species, we performed equivalent mouse limb bud explant experiments and found that FGF4 significantly decreased *Scx* expression, whereas PD18 increased *Scx* expression in mouse limbs, 6 h after treatment (Fig. 1E), consistent with previously published effects of 24 h FGF4

and PD18 treatments in mouse limbs (Havis et al., 2014). We conclude that the FGF tenogenic effect observed in chick limb cells is opposite to the anti-tenogenic FGF effect observed in mouse limb cells.

We next tested whether the FGF4 effect on *SCX* in chick cells involved the SMAD2/3 pathway. We applied the SMAD2/3 inhibitor SIS3 in FGF4 gain-of-function experiments in chick limb buds (Fig. S1). Blockade of SMAD2/3 did not block the positive effect of FGF4 on *SCX* expression (Fig. S1). This result is consistent with the absence of modification of *SMAD7/Smad7* expression upon FGF/ERK MAPK manipulations in both chick and mouse limb explants (Fig. 1D,E). Smad7 is a negative-feedback regulator that is considered to be a general TGFβ/SMAD2/3 transcriptional target gene (Massague, 2012). We conclude that FGF4 positively regulates *SCX* independently of the SMAD2/3 intracellular pathway in chick limb cells.

The TGFβ/SMAD2/3 pathway activates *SCX* expression in early chick limb buds

TGFβ2 induces *Scx* expression in E10.5 mouse limb explants (Pryce et al., 2009), but TGFβ1 does not modify *SCX* expression in E3.5 (HH20/21) chick limb explants (Lorda-Diez et al., 2010). We found *TGFB2* to be expressed in ventral parts of E3 chick limb buds (Fig. 2A), as previously described (Lorda-Diez et al., 2010). Application of TGFβ2 beads in E3/E4 (HH19/21) chick limb buds increased *SCX* expression 6 h after grafting (Fig. 2B) and the mRNA levels of *SCX* and *COL1A2* were increased in grafted limbs compared with control limbs (Fig. 2C). TGFβ2 application on chick limb bud explants also increased *SCX* expression in addition to increasing *THBS2* (Fig. 2D). TGFβ2 was not able to activate *TNMD* prematurely in chick limb undifferentiated cells (data not shown), as in mouse limb undifferentiated cells (Havis et al., 2014). Blockade of TGFβ receptors (SB43) and of the SMAD2/3 signalling pathway (SIS3) decreased *SCX* expression, in addition to that of *CO1A2*, *THBS2* and *THBS4* (Fig. 2D). Consistently, *SMAD7* mRNA levels, the transcriptional readout of the SMAD2/3 intracellular pathway, were increased following TGFβ2 application and decreased with the inhibitors SB43 and SIS3 (Fig. 2D). These results show that TGFβ2 positively regulates *SCX* expression, and that the SMAD2/3 intracellular pathway is required for *SCX* expression in early chick limb undifferentiated cells.

TGFβ is known to activate the ERK MAPK pathway as a non-canonical signalling pathway (reviewed by Guo and Wang, 2009; Massague, 2012). The expression of *ETV4* and *SPRY2* was not modified upon TGFβ2 bead application (Fig. 2C). In order to confirm experimentally that the positive effect of TGFβ2 on *SCX* expression did not involve the ERK MAPK signalling pathway, we applied the inhibitor PD18 in TGFβ2 gain-of-function experiments in chick limb buds and limb explants. The blockade of the ERK MAPK pathway did not modify the positive effect of TGFβ2 on *SCX* expression in chick limbs (Fig. 2C) and in chick limb explants (Fig. 2D). We conclude that TGFβ2 activates *SCX* expression independently of the ERK MAPK signalling pathway in chick limb cells.

FGF4 positively regulates *TNMD* and *THBS2* expression during tendon differentiation

TNMD is considered as a late tendon marker in chick and mouse embryos, and is expressed during the differentiation and muscle-dependent phase of limb tendon development (reviewed by Dex et al., 2016). *Tnmd* mutant mice display an altered structure of collagen fibrils, and reduced self-renewal and increased senescence of tendon progenitors, in post-natal tendons (Docheva et al., 2005;
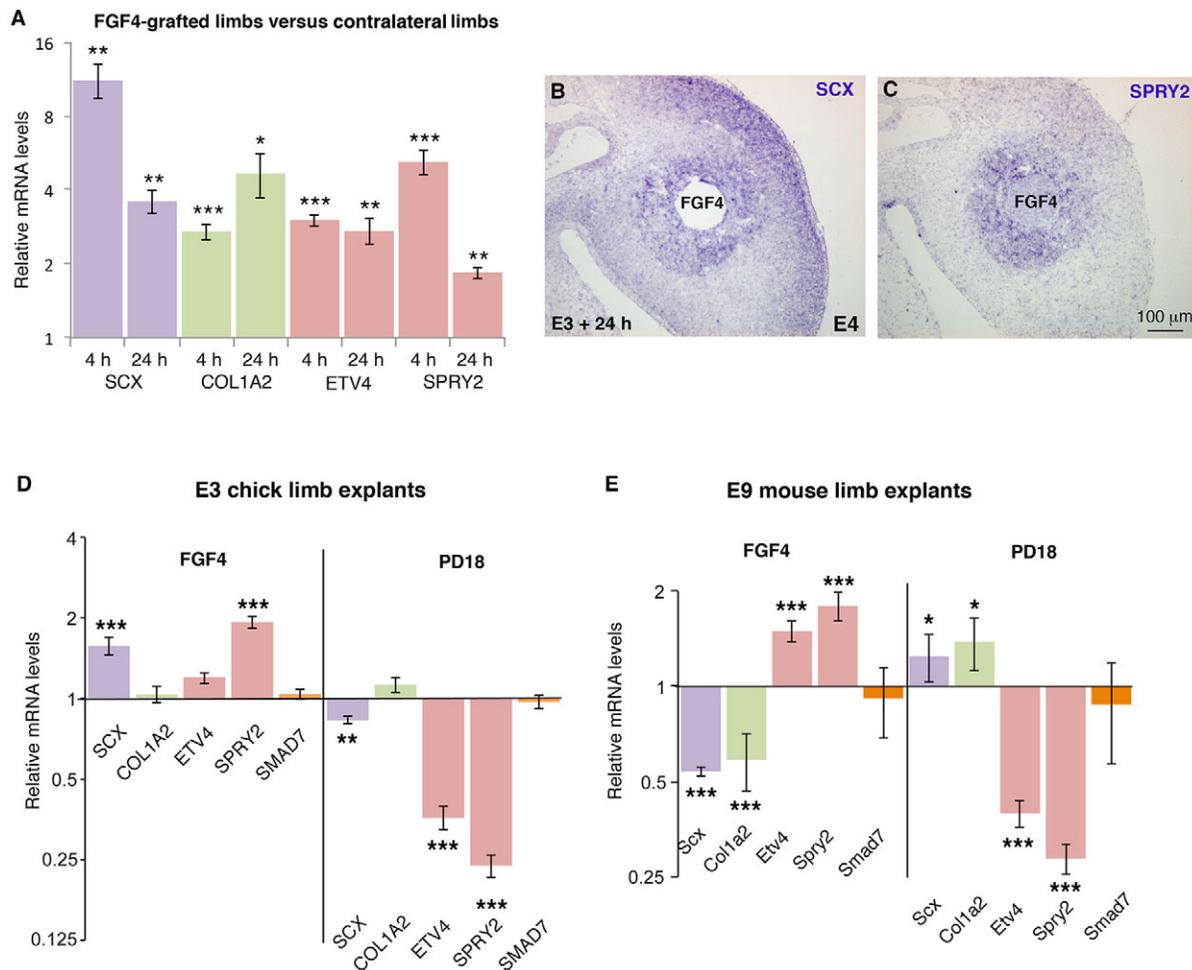
Fig. 1. Tenogenic effect of FGF on chick limb cells. (A-C) FGF4 bead application to forelimbs of E3/E4 (HH18/HH22) chick embryos. FGF4-grafted right limbs and control left limbs were processed for RT-q-PCR (*n*=6 for each time point) or for *in situ* hybridisation (*n*=3) analyses 4 h or 24 h after bead application. (A) RT-q-PCR analyses of the expression levels of *SCX*, *COL1A2* and readouts of FGF/ERK MAPK activity (*ETV4* and *SPRY2* expression) in FGF4-grafted right limbs, 4 h or 24 h after FGF4 application. For each gene, the mRNA levels in FGF4-grafted limbs are expressed relative to those of control left limbs (normalised to 1). (B,C) *In situ* hybridisation for *SCX* and *SPRY2* in E4 (E3+24 h) FGF4-grafted limbs. (D) E3 chick limb explant cultures. RT-q-PCR analyses of the relative expression levels of *SCX*, *COL1A2*, *ETV4*, *SPRY2* and *SMAD7* in E3 chick limb explants cultured for 6 h with FGF4 (*n*=10) or the inhibitor PD18 (*n*=10). (E) E9 mouse limb explant cultures. RT-q-PCR analyses of the expression levels of *Scx*, *Col1a2*, *Etv4*, *Spry2* and *Smad7* in E9 mouse limb explants cultured for 6 h with FGF4 (*n*=5) or the inhibitor PD18 (*n*=5). For each gene, the mRNA levels of treated limb explants are expressed relative to those of control limb explants (normalised to 1). *P*-values were determined by unpaired Student's *t*-test using Microsoft Excel. *$P<0.05$; **$P<0.01$; ***$P<0.001$. Error bars represent s.e.m.

Alberton et al., 2015). *TNMD* was expressed in *SCX*-positive tendons in E9 chick limbs (Fig. 3A,B, arrows), but also in dermal regions (Fig. 3B, arrowhead). Retroviral mouse Fgf4 (mFgf4/RCAS) induced ectopic *TNMD* expression in chick limbs (Fig. 3C-F), in addition to activating *SCX* expression (Edom-Vovard et al., 2002). Consistently, the relative mRNA levels of *TNMD* and *SCX* tendon genes and *ETV4* were increased in mFgf4/RCAS-limbs compared with control limbs (Fig. 3D). *THBS2*, another late tendon marker (Havis et al., 2014) was also upregulated in chick limbs upon retroviral Fgf4 (Fig. 3D,G,H). We conclude that FGF4 positively regulates *TNMD* and *THBS2* expression in chick limbs during the differentiation and muscle-dependent phase of limb tendon development.

Muscle contraction is required to maintain tendon marker expression in chick limb stylopod/zeugopod tendons

Scx/SCX expression is lost in stylopod/zeugopod muscleless limbs of mutant mice or experimental chick embryos (Schweitzer et al., 2001; Edom-Vovard and Duprez, 2004), defining the muscle-

dependent phase of limb tendon development. In the absence of muscle activity, Scx/GFP expression is diminished but not lost in zeugopod/stylopod regions of forelimbs of E18.5 paralysed *mdg* mice (Huang et al., 2015b). In order to determine the importance of mechanical signals for chick limb tendon development, we blocked muscle contraction in chick embryos using the drug decamethonium bromide (DMB). DMB acts as an acetylcholine agonist, induces depolarisation in skeletal muscles and ultimately leads to rigid muscle paralysis and to immobilised embryos (Nowlan et al., 2010). We applied DMB or control buffer in E4.5 chick embryos and analysed gene expression either by *in situ* hybridisation on sections and wholemounts or by RT-q-PCR (Fig. 4). In the absence of muscle contraction, muscles, visualised with *MYOD* expression, were present 2 days after DMB application, but displayed splitting delay 3 days after DMB application (Fig. S2). As previously described, limbs of immobilised embryos were smaller than control limbs (Nowlan et al., 2010). During the muscle-independent phase, *SCX* expression was not affected in chick limbs of immobilised embryos, 24 h after DMB application (Fig. 4A,B), consistent with
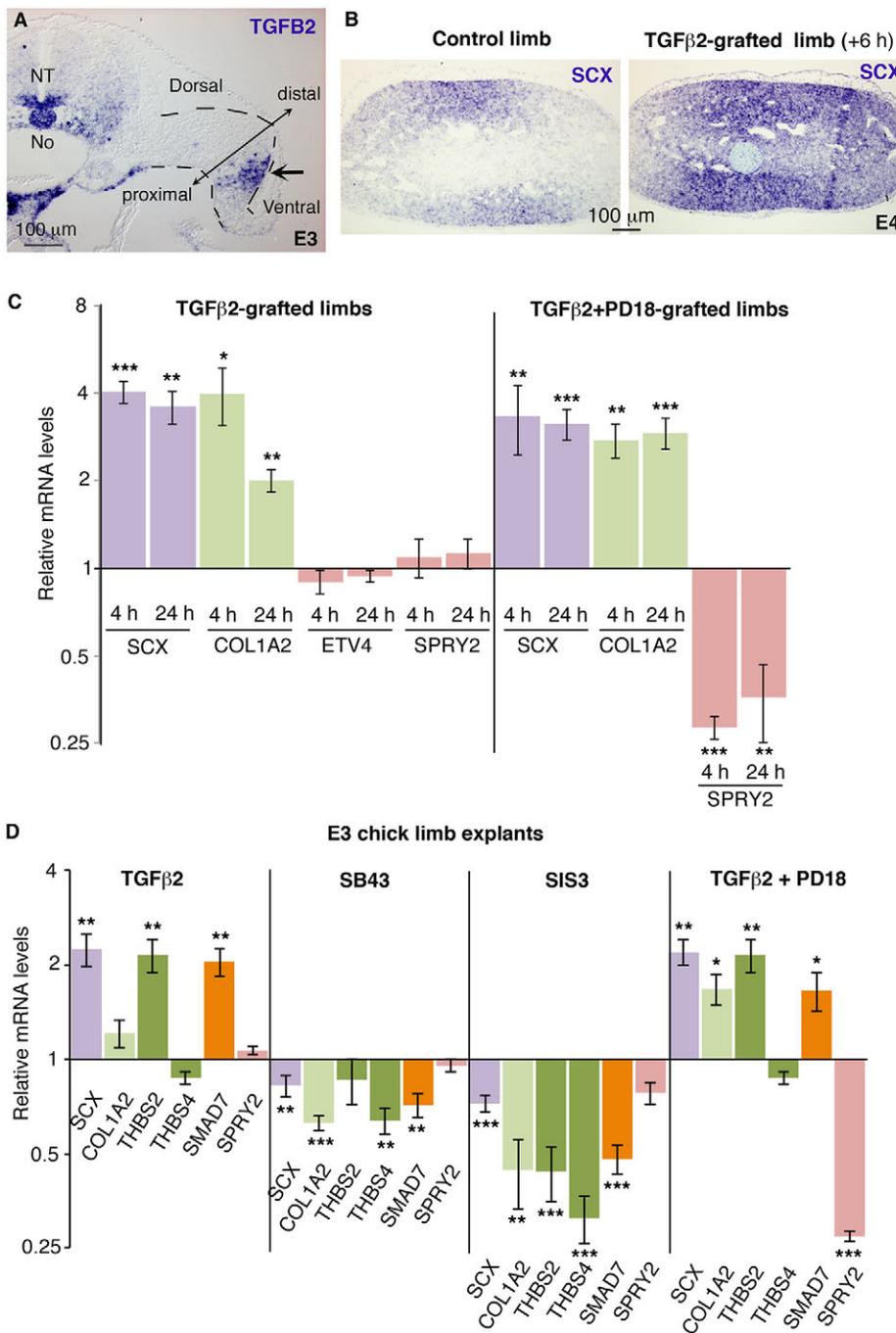
Fig. 2. Involvement of the TGFβ/SMAD2/3 pathway in chick limb tendon progenitors. (A) *In situ* hybridisation for *TGFB2* in E3 (HH19) chick embryos at the level of the forelimbs. *TGFB2* was expressed in ventral limb regions (arrow), in addition to being expressed in ventral neural tube (NT), notochord (No) and ventral aorta. Dashed lines delineate the limb bud. (B) TGFβ2 beads were grafted to forelimbs of E3.5 (HH21) chick embryos. Six hours later, transverse limb sections of TGFβ2-grafted right limbs and control left limbs were processed for *in situ* hybridisation for *SCX* (*n*=6). (C) TGFβ2 or TGFβ2+PD18 beads were grafted to forelimbs of E3/E4 (HH19/HH22) chick embryos. RT-q-PCR analyses of the expression levels of *SCX*, *COL1A2*, *ETV4* and *SPRY2* in TGFβ2- and TGFβ2+PD18-grafted right limbs, 4 h or 24 h after bead application. For each gene, the mRNA levels of bead-grafted limbs are expressed relative to those of control left limbs (normalised to 1). (D) RT-q-PCR analyses of mRNA levels for tendon genes (*SCX*, *COL1A2*, *THBS2*, *THBS4*) and readout of signalling pathways (SMAD7, SPRY2) in E3 chick limb explants cultured for 24 h with TGFβ2 (*n*=6), SB43 (*n*=7), SIS3 (*n*=9) or TGFβ2+PD18 (*n*=6). For each gene, the mRNA levels of treated explants are expressed relative to those of control limb explants (normalised to 1). *P*-values were determined by paired Student's *t*-test using Microsoft Excel. *$P<0.05$; **$P<0.01$; ***$P<0.001$. Error bars represent s.e.m.

normal *SCX* expression in muscleless limbs of E6 experimental chick embryos (Edom-Vovard et al., 2002) and E12.5 mouse *Pax3* mutants (Schweitzer et al., 2001). *SCX* expression was decreased in limbs of immobilised embryos from E6.5 (Fig. 4C-F). In order to confirm the decrease of *SCX* expression observed by *in situ* hybridization, we compared *SCX* mRNA levels in paralysed limbs versus control limbs by RT-q-PCR (Fig. 4G). RT-q-PCR analyses of whole forelimbs, forelimbs without digits, or digits only indicated a decrease of *SCX* expression in the absence of muscle contraction (Fig. 4G). The expression of *COL1A2* was slightly decreased in limbs of immobilised embryos (Fig. 4G), consistent with the general and non-tendon-specific expression of type I collagen. The decrease of *SCX* expression was more obvious in stylopod/zeugopod regions compared with digits (Fig. 4C-F),

consistent with *SCX* expression pattern in muscleless limbs of chick and mouse embryos (Schweitzer et al., 2001; Edom-Vovard et al., 2002; Bonnin et al., 2005) and with the modular development of mouse limb tendons (Huang et al., 2013, 2015b). Similar *SCX* downregulation was observed in stylopod/zeugopod tendons of hindlimbs in immobilised chick embryos (Fig. S3). *In situ* hybridisation to forelimb sections at the levels of the zeugopod (Fig. 4H,I) and digits (Fig. 4J,K) confirmed the more pronounced decrease of *SCX* expression in zeugopod compared with digits. *SCX* was also decreased in stylopod/zeugopod tendons of forelimbs, 3 days after injection of pancuronium bromide (PB), an acetylcholine antagonist, which induced flaccid muscle paralysis (Nowlan et al., 2010) (Fig. S4). The expression of the late tendon markers *TNMD* and *THBS2* was also lost in limb tendons of
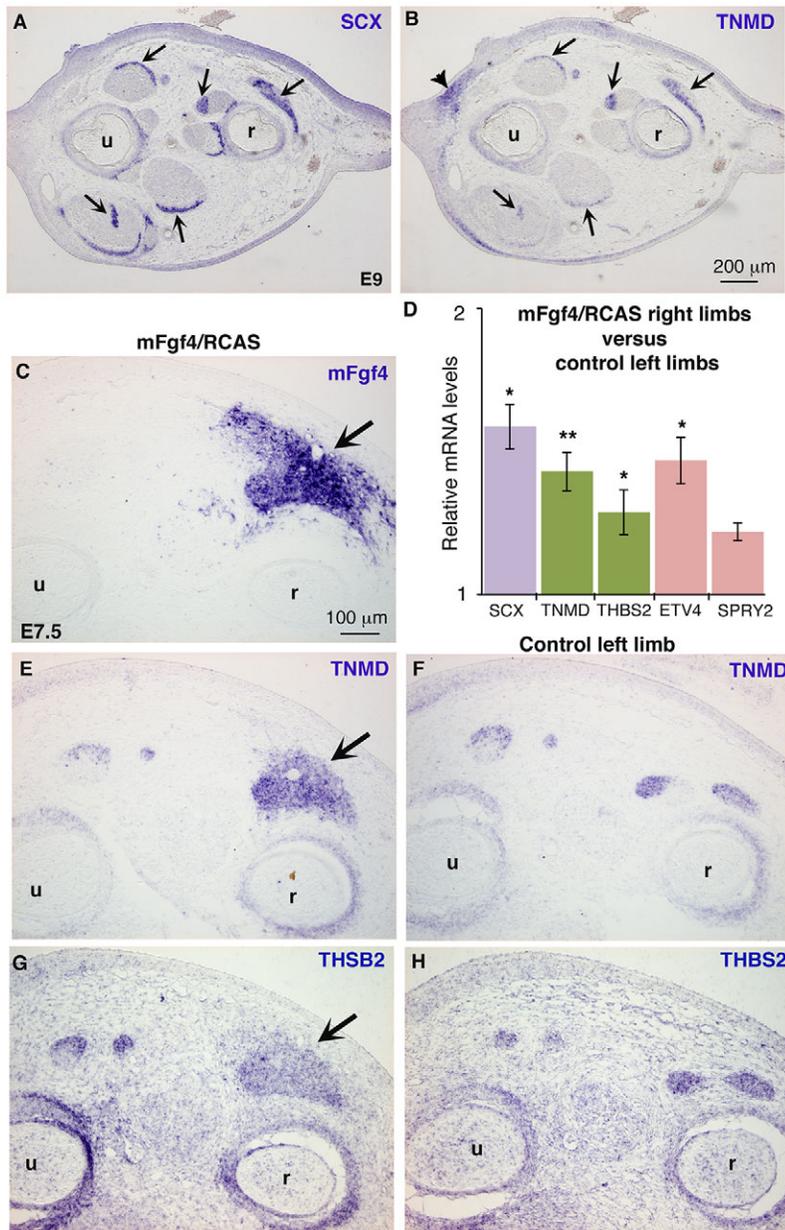
Fig. 3. FGF4 positively regulates the expression of the tendon differentiation markers *TNMD* and *THBS2*.
(A,B) Adjacent transverse sections of forelimbs of E9 chick embryos were hybridised with *SCX* and *TNMD* probes. *TNMD* was expressed in *SCX*-positive tendons (arrows). *TNMD* was also expressed in the dermis (B, arrowhead). (C-H) mFgf4/RCAS-producing cells were grafted into forelimb buds of E3.5 (HH21) chick embryos. Embryos (*n*=3) were fixed at E7.5, and grafted (C,E,G) and control (F,H) forelimbs were transversely sectioned at the level of the zeugopod. (C,E,G) Adjacent sections were hybridised with *mFgf4* probe to show the extent of virus spread and with *TNMD* and *THBS2* probes to show ectopic expression (E,G, arrows) in mFgf4-positive regions (C, arrow) compared with normal *TNMD* and *THBS2* expression in control left limbs (F,H). (D) RT-q-PCR analyses of mRNA levels in mFgf4/RCAS-infected limbs (4 days after grafting) (*n*=5). For each gene, the mRNA levels of infected (right) limbs are expressed relative to those of control (left) limbs (normalised to 1). *P*-values were determined by paired Student's *t*-test using Microsoft Excel. *$P<0.05$; **$P<0.01$. Error bars represent s.e.m. r, radius; u, ulna.

immobilised E7.5 embryos (Fig. 4L-O). We conclude that *SCX*, *TNMD* and *THBS2* expression is sensitive to mechanical signals provided by muscle contraction in stylopod/zeugopod tendons, during chick limb development.

The expression of tendon-related FGF signalling components is downregulated in paralysed limbs

In order to determine whether the FGF signalling pathway is involved in the downregulation of tendon gene expression in the absence of muscle contraction, we analysed the expression of components of the FGF/ERK MAPK signalling pathway related to tendon development, in immobilised chick embryos. During the muscle-dependent phase of limb tendon development, *ETV4*, *SPRY1* and *SPRY2* are expressed ubiquitously in chick limbs but with a high expression at muscle and tendon interface (Eloy-Trinquet et al., 2009). *FGF4* is expressed at muscle tips close to tendons (Edom-Vovard et al., 2002), whereas *FGF8* is expressed in tendons close to muscles (Edom-Vovard et al., 2001). The expression of *ETV4* and

SPRY2 was dramatically decreased in limbs of immobilised chick embryos assessed by RT-q-PCR and *in situ* hybridisation analyses (Fig. 5A-E). The *ETV4* and *SPRY2* downregulation was more pronounced in forelimbs (digit excluded) compared with digits alone (Fig. 5A). In the absence of muscle contraction, the expression of FGF ligands related to tendon development, *FGF4* and *FGF8*, was lost at muscle tips (Fig. 5F,G, arrows) and in tendons (Fig. 5H,I, arrows), respectively. These results showed that the expression of FGF ligands and transcriptional readouts of ERK MAPK activity was downregulated at the muscle/tendon interface in chick limbs, in the absence of muscle contraction.

FGF4 activates *SCX* expression in limbs of immobilised chick embryos

In order to determine whether FGF would rescue tendon gene expression in the absence of mechanical signals, we applied mFgf4-expressing retroviruses in chick limbs (mFgf4/RCAS) and injected DMB in order to prevent muscle contraction (Fig. 6A). In the
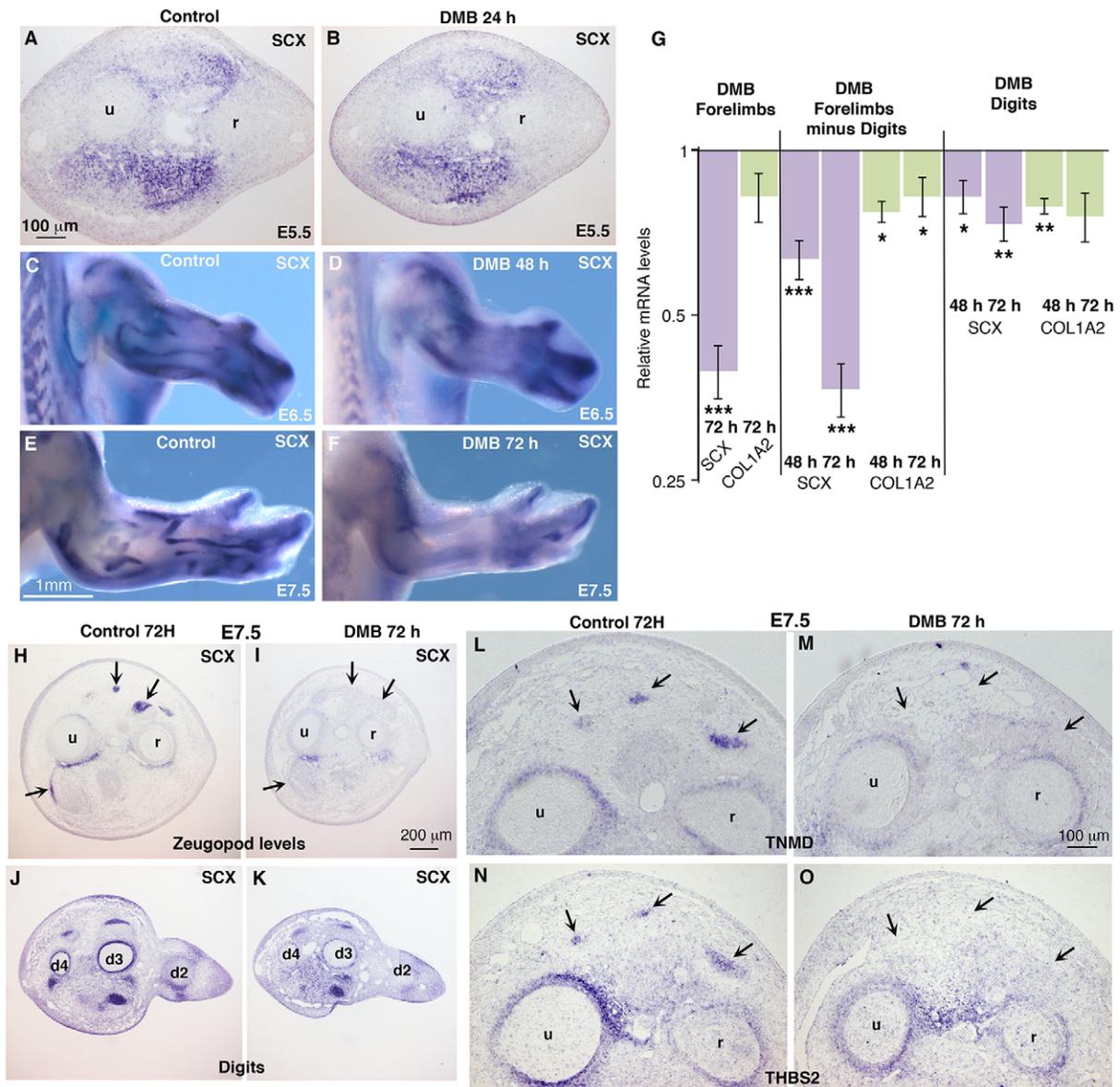
Fig. 4. Muscle contraction is required to maintain *SCX* and *TNMD* expression in chick forelimbs. DMB reagent was injected into E4.5 (HH24) chick embryos to induce immobilisation. Immobilised embryos were processed for *in situ* hybridisation (A-F,H-O) or RT-q-PCR (G) analyses. (A,B) Forelimb transverse sections of control (*n*=2) and DMB-treated embryos (*n*=4) were hybridised with *SCX* probe 24 h after treatment. (C-F) Forelimbs of control (*n*=10) and DMB-treated (*n*=10) embryos fixed 48 h (*n*=5) or 72 h (*n*=5) after application were hybridised with *SCX* probe. (G) RT-q-PCR analyses of mRNA levels for tendon genes in forelimbs (*n*=10), forelimbs with digits removed (*n*=20) and digits only (*n*=20) of DMB-treated embryos, 48 h (*n*=10) and 72 h (*n*=10) after DMB application. For each gene, the mRNA levels of treated limbs are expressed relative to those of control limbs (normalised to 1). *P*-values were determined by unpaired Student's *t*-test using Microsoft Excel. *$P<0.05$; **$P<0.01$; ***$P<0.001$. Error bars represent s.e.m. (H-O) Forelimb (H,I,L-O) and digit (J,K) transverse sections of control (H,J,L,N) and DMB-treated (I,K,M,O) embryos (*n*=4) were hybridised with *SCX* (H-K), *TNMD* (L,M) or *THBS2* (N,O) probes 72 h after treatment. r, radius; u, ulna.

absence of muscle contraction, *SCX* expression was downregulated (Fig. 6C,D). mFgf4 was able to activate ectopic *SCX* expression in limbs of immobilised embryos (Fig. 6D-F). Consistent with this, the relative mRNA levels of *SCX*, *ETV4* and *SPRY2* were significantly upregulated in mFgf4-paralysed-limbs compared with paralysed limbs (Fig. 6B). Under these experimental conditions, *TNMD* and *THBS2* expression was not changed (Fig. 6B). The relative mRNA levels of *TGFB2*, *TGFB3* and *SMAD7* were not changed in the presence of mFgf4 in immobilised embryos (Fig. 6B), indicating that TGFβ signalling was not modified under these experimental conditions. We performed a similar FGF rescue experiment in chick limb explants, in which we considered that the E5 limb explant

culture system was devoid of mechanical movements. Analysis of the relative mRNA levels in chick limb explants compared with stage-matched limbs originating from *in ovo* embryos showed a significant diminution of *SCX*, *TNMD*, *THBS2*, *ETV4*, *SPRY2* and *FGF4* gene expression (Fig. 6G), similar to that observed in immobilised chick embryos (Figs 4 and 5). Consistent with the *in ovo* FGF rescue experiments (Fig. 6A-F), the application of recombinant FGF4 in limb explant cultures induced a significant increase of the mRNA levels of *SCX*, *ETV4* and *SPRY2*, but did not affect *COL1A2* and *SMAD7* expression (Fig. 6H). The expression levels of *TNMD* and *THBS2* genes were not increased upon FGF4 treatment and even displayed a decrease of expression (Fig. 6H).
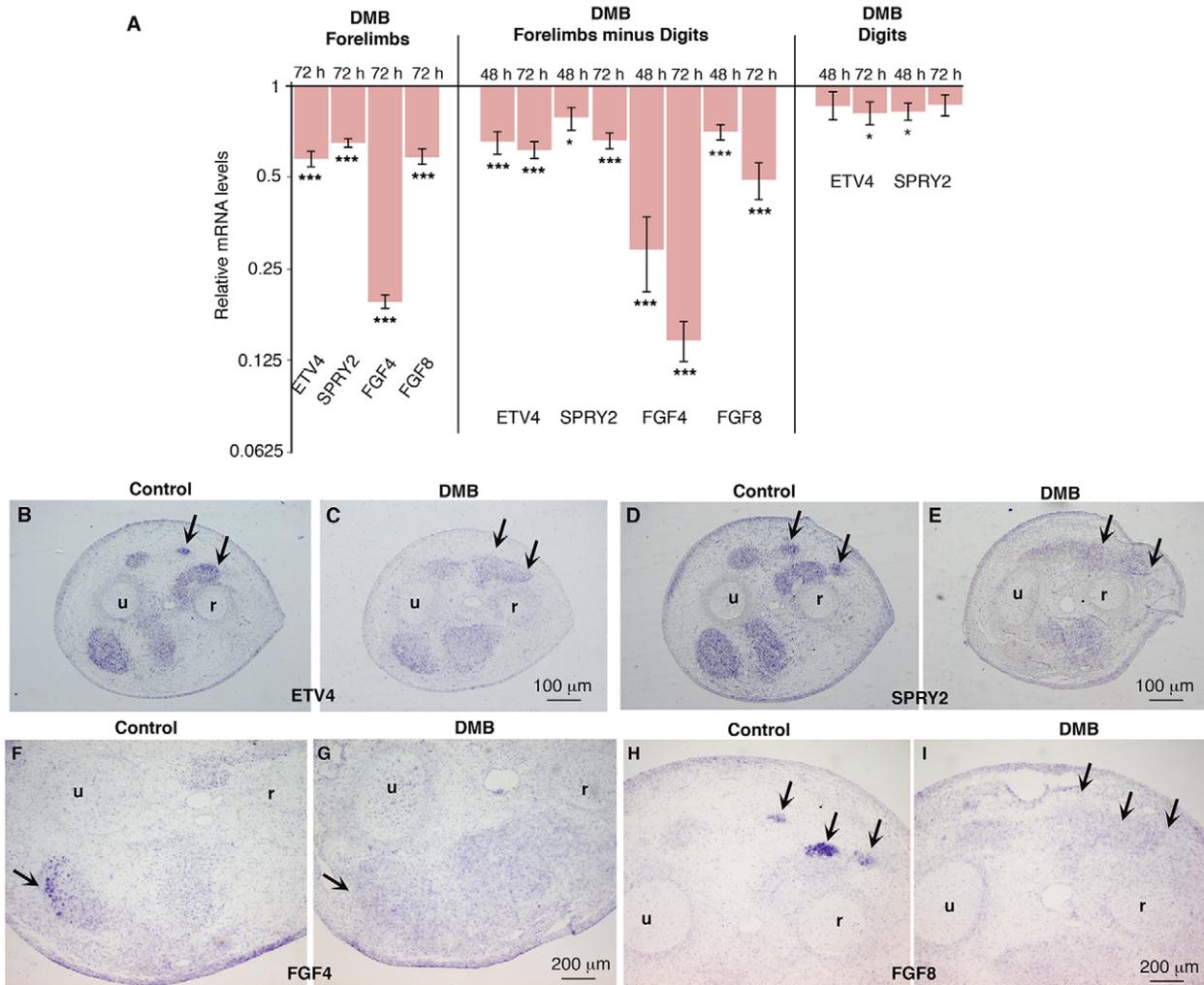
DEVELOPMENT

Fig. 5. Muscle contraction is required to maintain active FGF/ERK MAPK signalling in chick limb tendons. (A) RT-q-PCR analyses of mRNA levels for *ETV4*, *SPRY2*, *FGF4* and *FGF8* in forelimbs (*n*=9), forelimbs (digits excluded) (*n*=19) and digits (*n*=20) of DMB-treated embryos, 48 h (*n*=20) and 72 h (*n*=19) after DMB application. For each gene, the mRNA levels of treated limbs are expressed relative to those of control limbs (normalised to 1). *P*-values were determined by unpaired Student's *t*-test using Microsoft Excel. *P<0.05; ***P<0.001. Error bars represent s.e.m. (B-I) Forelimb transverse sections at the level of the zeugopod of control (B,D,F,H) and DMB-treated (C,E,G,I) embryos were hybridised with *ETV4* (B,C), *SPRY2* (D,E), *FGF4* (F,G) and *FGF8* (H,I) probes (*n*=3) 72 h after treatment. Arrows point to gene expression in control limbs (B,D,F,H) and loss of gene expression in DMB-treated limbs (C,E,G,I). r, radius; u, ulna.

We conclude that FGF4 activates *SCX* but not *TNMD* or *THBS2* expression in chick limbs in immobilisation conditions.

TGFβ2 maintains *SCX*, *TNMD* and *THBS2* expression in immobilised chick limbs

Next, we wanted to determine whether TGFβ was also sensitive to immobilisation. Both *Tgfb2* and *Tgfb3* have been shown to be involved in mouse limb tendon development (Pryce et al., 2009). In E7.5 limbs, *TGFB2* was observed in tendons, in addition to displaying expression in muscles (Fig. S5). *TGFB3* was mainly expressed in chick limb muscles, with faint expression in tendons (Fig. S5). In DMB-treated embryos, the mRNA levels of *SMAD7* and *TGFB2* were decreased in paralysed limbs compared with control limbs (Fig. 7A). *TGFB2* expression was lost in limb tendons of the zeugopod regions (Fig. 7B-E, arrows), but not in digits (Fig. 7F-I) of immobilised embryos. The diminution of the relative mRNA levels of *SMAD7* and *TGFB2* was also observed in limb explants compared with stage-matched limbs originating from *in ovo* embryos (Fig. 7J). These results show that the TGFβ/

SMAD2/3 signalling pathway was decreased in chick limb tendons under immobilisation conditions. Application of TGFβ2 to limb explants increased *SCX*, *TNMD*, *THBS2* and *SMAD7* expression compared with control limb explants (Fig. 7K). The expression levels of the transcriptional readouts of ERK MAPK activity were not modified upon exposure to TGFβ2. We conclude that TGFβ2 is sufficient to maintain the expression of *SCX* and the tendon differentiation markers *TNMD* and *THBS2* in chick limbs under immobilisation conditions.

DISCUSSION
TGFβ function in tendon development is similar in chick and mouse limbs
Our TGFβ2 gain- and loss-of-function experiments in early chick limbs and explants (Fig. 2) show that TGFβ2 is sufficient and the SMAD2/3 intracellular pathway is required for *SCX* expression in undifferentiated limb cells. These results are fully consistent with those obtained in early mouse limb explants (Pryce et al., 2009; Havis et al., 2014). These results highlight a universal role for TGFβ
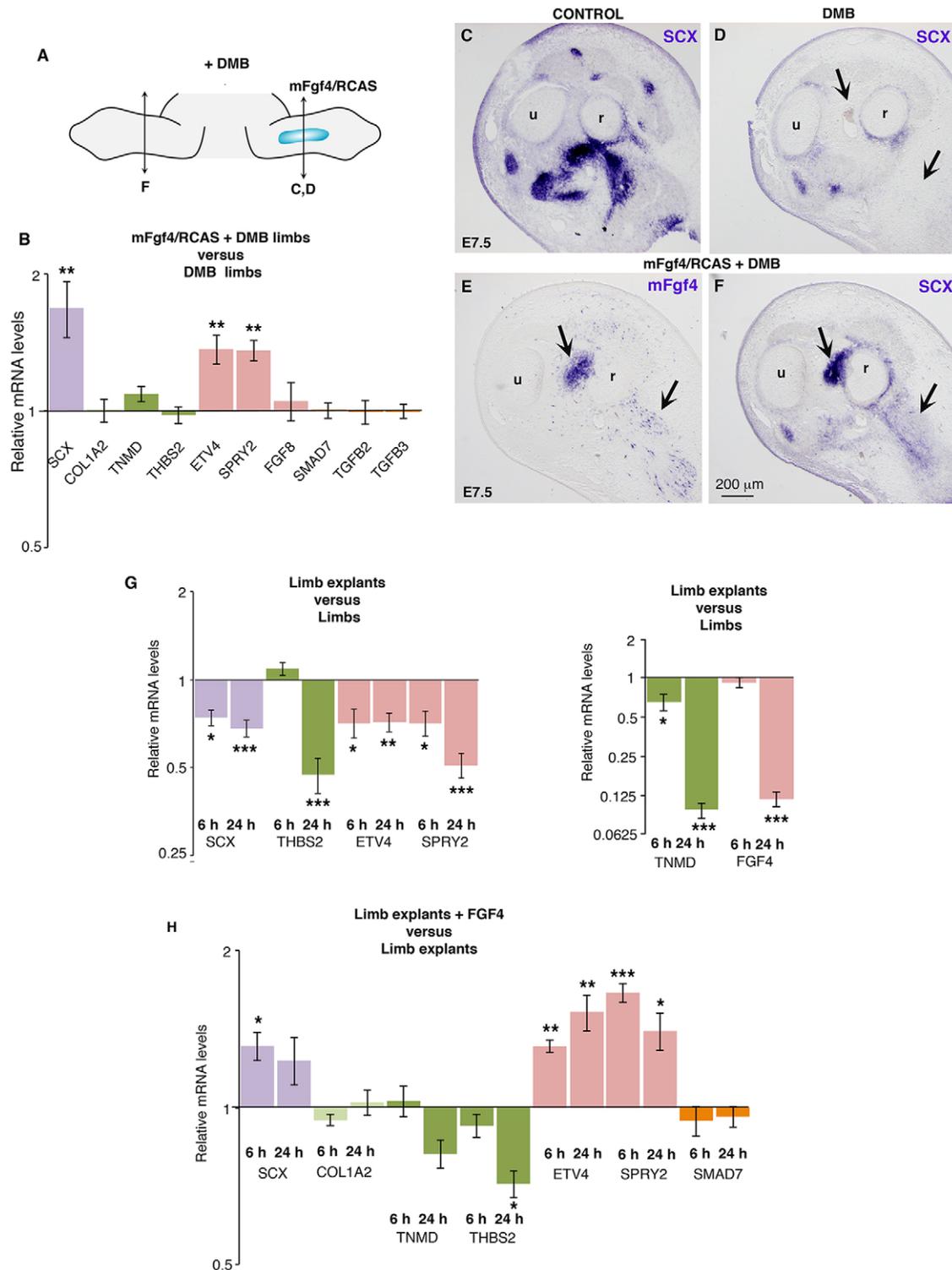
Fig. 6. FGF4 induces *SCX* expression in limbs of immobilised embryos. (A) mFgf4/RCAS-producing cells were grafted into right forelimbs of E3.5 chick embryos. These embryos were then treated with DMB at E4.5 and fixed 3 days after DMB application at E7.5. (B,D-F) The manipulated embryos were either processed for RT-q-PCR analysis (B) (*n*=7) or for *in situ* hybridisation to limb sections (D-F) (*n*=3). (B) RT-q-PCR analyses of mRNA levels for components of tendons (*SCX*, *COL1A2*, *TNMD*, *THBS2*) and of the FGF/ERK (ETV4, SPRY, FGF8) and of the TGF-β/SMAD2/3 (SMAD7, TGFB2, TGFB3) signalling pathways in mFgf4/RCAS forelimbs of DMB-treated embryos. For each gene, the mRNA levels of mFgf4/RCAS limbs are expressed relative to those of contralateral (DMB-treated only) limbs (normalised to 1). (D-F) Transverse sections of right mFgf4/RCAS forelimbs (E,F) and left forelimbs (D) of DMB-treated embryos were hybridised with probes for *mFgf4* (E) and *SCX* (D,F). (C) *In situ* hybridisation to E7.5 control limbs with *SCX* probe. r, radius; u, ulna. (G) The mRNA levels for *SCX*, *ETV4*, *SPRY2*, *TNMD*, *THBS2* and *FGF4* were compared by RT-q-PCR analysis in E5 (HH25/26) limb explants cultured for 6 h (*n*=5) and 24 h (*n*=5) versus limbs (*n*=10) of stage-matched embryos. (H) RT-q-PCR analyses of mRNA levels in E5 limb explants cultured for 6 h (*n*=7) and 24 h (*n*=5) in the presence or absence of FGF4. For each gene, the mRNA levels of E5 limb explants cultured for 6 and 24 h with no FGF4 were normalised to 1. *P*-values were determined by unpaired Student's *t*-test using Microsoft Excel. *P<0.05; **P<0.01; ***P<0.001. Error bars represent s.e.m.
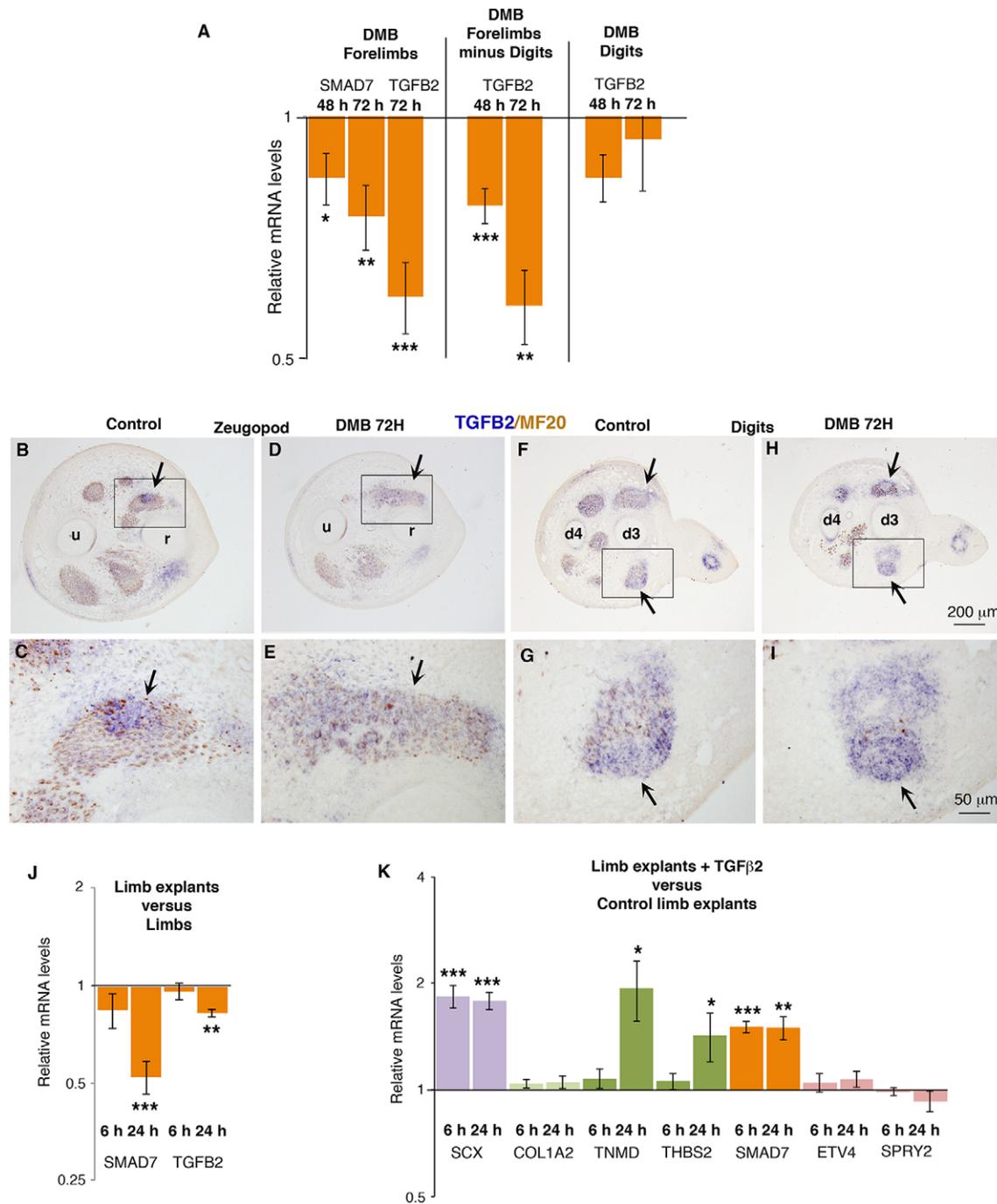
Fig. 7. TGFβ2 prevents *SCX* downregulation in limb explants. (A-I) DMB application into E4.5 chick embryos. E7.5 immobilised embryos were processed for either RT-q-PCR analysis (*n*=10) or *in situ* hybridisation to limb sections (*n*=3). (A) RT-q-PCR analyses for components of the TGFβ pathway, in paralysed limbs. For each gene, the mRNA levels of limbs of DMB-treated embryos are expressed relative to those of control embryos (normalised to 1). (B-I) Transverse limb sections at the zeugopod (B-E) and digit (F-I) levels of immobilised embryos (D,E,H,I) or control embryos (B,C,F,G) were hybridised with the *TGFB2* probe and then immunostained with MF20 antibody. C,E,G,I are high magnifications of the boxed regions of B,D,F,H, respectively. Arrows point to zeugopod (B-E) or digit (F-I) tendons in control or DMB embryos. d3, digit 3; d4, digit 4; r, radius; u, ulna. (J) The mRNA levels for *SMAD7* and *TGFB2* were compared by RT-q-PCR analysis in E5 (HH25/26) limb explants (*n*=10) cultured for 6 h (*n*=5) and 24 h (*n*=5) versus limbs of stage-matched embryos. (K) RT-q-PCR analyses of mRNA levels in E5 limb explants (*n*=15) cultured for 6 h (*n*=8) and 24 h (*n*=7) in the presence or absence of TGFβ2. For each gene, the mRNA levels of E5 limb explants cultured for 6 h and 24 h with no TGF-β2 were normalised to 1. *P*-values were determined by unpaired Student's *t*-test using Microsoft Excel. *P<0.05; **P<0.01; ***P<0.001. Error bars indicate s.e.m.

in initiating the commitment of undifferentiated limb mesodermal cells towards the tendon lineage during chick and mouse development (Fig. 8). In zebrafish embryos, blocking the TGFβ pathway (using the chemical drug SB431542) inhibits *scx*

expression (Chen and Galloway, 2014), suggesting that TGFβ is also important for the initiation of *scxa* expression in fish. The developmental TGFβ tenogenic effect is likely to be related to the recognized effect of TGFβ in positively regulating *Scx* expression in
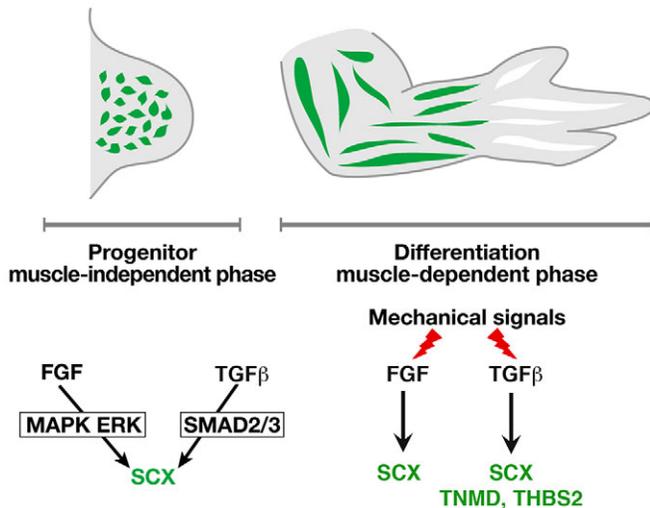
Fig. 8. FGF4 and TGFβ2 involvement in stylopod/zeugopod limb tendons during chick development. During the progenitor stage, which is independent of muscle, FGF or TGFβ ligand, independently of each other, each have a tenogenic effect on limb mesodermal undifferentiated cells. Moreover, the MAPK ERK pathway and SMAD2/3 intracellular pathways are both required for *SCX* expression in early chick limbs. During the differentiation step, which is dependent on muscle, muscle contraction is required for FGF/MAK ERK and TGFβ/SMAD2/3 activity at the muscle/tendon interface. Downstream of mechanical forces, FGF4 and TGFβ2 positively regulate *SCX* expression, and TGFβ2 (but not FGF4) regulates *TNMD* and *THBS2* expression in limb tendons.

embryonic tendon progenitors (Brown et al., 2014) and stem cell culture systems (Pryce et al., 2009; Barsby and Guest, 2013; Goncalves et al., 2013; Guerquin et al., 2013; Havis et al., 2014). We find that the positive effect of TGFβ2 on chick limb *SCX* expression is independent of ERK MAPK signalling (Fig. 2C,D), as also observed in mouse limb explants (Pryce et al., 2009; Havis et al., 2014).

Tnmd/TNMD is one of the tendon markers displaying the highest expression levels in E14.5 mouse Scx$^+$ cells but is not expressed in E11.5 mouse limb bud explants (Havis et al., 2014) or E4 chick limbs (Shukunami et al., 2006) or activated by TGFβ2 at these early stages (Havis et al., 2014). However, *TNMD* expression is activated upon TGFβ exposure in late chick (Fig. 8) and mouse (Havis et al., 2014) limb explants. This is consistent with previous reports showing *TNMD* upregulation by TGFβ ligands in 3D-culture systems of human tendon cells (Bayer et al., 2014) and of equine embryo-derived stem cells (Barsby et al., 2014), and in high-density cultures of chick limb cells (Lorda-Diez et al., 2009). It is worth mentioning that TGFβ dramatically decreases *Tnmd* expression (and activates *Scx*) in 2D-culture systems of embryonic or adult mouse tendon progenitors and in mouse mesenchymal stem cells (Guerquin et al., 2013; Brown et al., 2014; Liu et al., 2015). We believe that the opposite effects of TGFβ on *Tnmd* expression are due to the different cell contact environments in 2D-culture versus 3D-culture systems.

FGF has a tenogenic effect in chick limb undifferentiated cells, but has an anti-tenogenic effect in mouse limb undifferentiated cells

In vivo and *ex vivo* experiments demonstrated that FGF activates *SCX* expression in early chick limb buds. This is consistent with FGF function in somites of chick embryos (Brent and Tabin, 2004; Smith et al., 2005). This result observed in chick embryos is opposite to those obtained in mouse limb explants, in which FGF inhibits *Scx* expression and ERK MAPK inhibition activates *Scx* expression (Havis et al., 2014; Fig. 1E). Blockade of SMAD2/3 did not prevent the *SCX* activation by FGF4 in chick limbs (Fig. S1). Moreover, *Smad7/SMAD7* expression was not modified in any of the FGF misexpression experiments (Fig. 1D,E) (Havis et al., 2014), indicating that the TGFβ pathway is not involved in the positive or negative effect of FGF on *SCX/Scx* expression in chick and mouse, respectively. We believe that FGF has a tenogenic effect in chick undifferentiated limb mesodermal cells, but has an anti-tenogenic effect on mouse undifferentiated limb mesodermal cells. The reasons for the opposite effect of FGF signalling on limb mesodermal cells between the chick and mouse models remain unclear. However, these results are consistent with an absence or deleterious effect of FGF on tendon marker expression in 2D-culture systems of various stem cells, including mouse embryonic tendon progenitors (Brown et al., 2014), mouse mesenchymal stem cells (Havis et al., 2014), canine tendon fibroblasts (Thomopoulos et al., 2010), human amniotic fluid stem cells or adipose-derived stem cells (Goncalves et al., 2013). Consistent with the FGF tenogenic function during chick tendon development, a clear beneficial effect of FGF has been described during tendon repair in a chick digital tendon injury model. The expression of the ligand FGFb is decreased in chick tendons during the process of tendon repair (Chen et al., 2008) and ectopic application of FGF has a beneficial effect on chick tendon repair (Tang et al., 2008, 2014). We conclude that FGF positively regulates *SCX* in chick limb undifferentiated cells (Fig. 8).

FGF4 and TGFβ2 have a tenogenic effect, independently of each other, during chick limb development

FGF4 and TGFβ2 activate *SCX* expression independently of each other in early chick limb buds (Fig. 2D; Fig. S1). Although intracellular crosstalk has been identified between the ERK MAPK and SMAD2/3 signalling pathways in many biological systems (reviewed by Massague, 2012), our results indicate that these signalling pathways do not interact in the activation of *SCX* in chick limb buds. The fact that two signalling pathways activate *SCX* independently of each other indicates the presence of a safety system for tendon specification in chick limbs. This safety system is classically observed during developmental processes. Another possible hypothesis could be that two pools of *SCX*-positive cells co-exist within the chick limb buds, one pool being sensitive to the TGFβ2/SMAD2/3 signalling pathway and another one being sensitive to the FGF ERK/MAPK signalling pathway.

Limb tendon development relies on mechanical forces generated by muscle contraction

Immobilisation following muscle paralysis induces a drastic diminution of *SCX*, *TNMD* and *THBS2* gene expression in stylopod/zeugopod tendons of chick limbs (Fig. 4). This shows that tendon gene expression is sensitive to mechanical signals in chick limbs. However, in the *mdg* mouse, which is deprived of muscle activity, Scx/GFP-positive tendons are observed in stylopod/zeugopod limb regions, although they are reduced in size (Huang et al., 2015b). This difference could be due to the possibility that the GFP fluorescence can be still detected even if the *Scx* promoter is no longer active or the fact that the mouse embryos are still submitted to mechanical movements from maternal activity, whereas the pharmacologically induced immobilisation used in our experiments is more drastic. However, an alternative and plausible explanation is that these results indicate that tendon development in

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chick and mouse has differential requirements for mechanical movements. It has been demonstrated in mice that muscles are required for zeugopod tendon elongation, but only tendon size and individuation depend on mechanical forces in mouse limbs (Huang et al., 2015b). The complete loss of zeugopod tendons in chick immobilised embryos shows that mechanical signals are crucial for all the steps of chick zeugopod tendon differentiation. This can be correlated with the fact that tendon cells experience higher levels of mechanical signals in actively moving chick embryos in the egg compared with mouse embryos embedded in uterine membranes and with the faster development of the musculoskeletal system in chick versus mouse embryos.

Although differences exist between the mechanical signal requirement between chick and mouse tendon development, mechanical forces generated by muscle contraction are recognised as being required for skeletal system formation during chick and mouse development (reviewed by Shwartz et al., 2013). Immobilisation affects bone, cartilage and synovial joint morphogenesis, targeting general processes such as proliferation and differentiation leading to shape and size defects (Blitz et al., 2009; Kahn et al., 2009; Roddy et al., 2011a). A correlation has been established between biophysical stimuli patterns and skeletal regions affected upon immobilisation (Roddy et al., 2011b). Tendons that link muscles to bones are expected to experience high mechanical strains and are largely affected in immobilisation conditions. The mechanosensitivity of tendon development is maintained in adult life, as tendon cells are sensitive to mechanical signals generated by tendon loading (reviewed by Nourissat et al., 2015). *Scx* expression is downregulated in adult tendons in unloading conditions (Maeda et al., 2011), whereas *Scx* and *Tnmd* are activated under overloading conditions in mice (Mendias et al., 2012; Zhang and Wang, 2013). We conclude that tendon cells require appropriate mechanical signals during development and adult life.

FGF4 and TGFβ2 act downstream of mechanical signals to regulate tendon differentiation

In immobilised chick embryos, transcriptional readouts of both FGF/ERK MAPK and TGFβ/SMAD2/3 signalling pathways are downregulated in limb tendons. This shows that both pathways are sensitive to mechanical signals in chick limbs. These pathways are known to be sensitive to mechanical forces in other biological systems (Humphrey et al., 2014). Moreover, transcriptome profiling analyses have identified FGF and TGFβ signalling as being downregulated in developing humerus (limb bone) of immobilised mouse foetuses (Rolfe et al., 2014). In addition to being downregulated in tendons of immobilised embryos, both FGF4 and TGFβ2 prevent the decrease of *SCX* expression in chick limbs in immobilisation conditions. Rescue experiments with FGF4 or TGFβ2 in immobilised limbs do not activate TGFβ or FGF transcriptional readout, respectively, indicating that FGF4 and TGFβ2 act independently of each other to activate *SCX* expression during limb tendon differentiation. The ability of TGFβ2 to rescue *SCX* expression in chick limbs in immobilised embryos is reminiscent of the requirement of the SMAD2/3 pathway for *Scx* induction downstream of mechanical forces in tendon cells (Maeda et al., 2011). However, *TNMD* and *THBS2* downregulation was only prevented by TGFβ2 and not by FGF4. We hypothesise that TGFβ2 activates another signal required for *TNMD* and *THBS2* expression, which is not regulated by FGF4, in immobilisation conditions. In the presence of muscle contraction, FGF4 activates *TNMD* and *THBS2*, whereas in the absence of

muscle contraction FGF4 is not able to activate *TNMD* or *THBS2* expression, highlighting the independent effects of both pathways in tendon differentiation.

In summary, both FGF4 and TGFβ2 signalling molecules are involved in the commitment of undifferentiated chick limb mesodermal cells towards the tendon lineage and act downstream of mechanical forces to regulate tendon differentiation during chick limb development (Fig. 8). Both FGF4 and TGFβ2 have a tenogenic effect, independently of each other, during both muscle-independent and -dependent phases of chick limb tendon development.

MATERIALS AND METHODS
Chick and mouse embryos
Fertilised chick eggs (JA 57 strain) (EARL Morizeau, Dangers, France) were incubated at 38°C. Embryos were aged according to the number of days of incubation (embryonic day) or staged according to Hamburger and Hamilton (HH) stages (Hamburger and Hamilton, 1992). Swiss mouse embryos (Janvier Labs) were collected after natural overnight matings. For staging, fertilisation was considered to take place at midnight. The manipulation of chick and non-transgenic mouse embryos was performed in accordance with the guidelines of the French National Ethics Committee.

Bead implantation and grafting mFgf4/RCAS-expressing cells to chick limb buds
FGF4, TGFβ2, TGFβ2+PD18, FGF4+SIS3 beads or mFgf4/RCAS-expressing cells were grafted in limbs of E3/E4 chick embryos as described (Edom-Vovard et al., 2002). Embryos were harvested 4, 6 or 24 h after grafting. Further details are provided in supplementary Materials and Methods.

Chick and mouse limb explant cultures
Chick limb explants were prepared as described by Placzek and Dale (1999) and treated with TGFβ2, FGF4, PD18, SB43, SIS3 or TGFβ2+PD18 as described by Havis et al. (2014). Further details are provided in supplementary Materials and Methods.

DMB or PB application in chick embryos
Decamethonium bromide (DMB) and pancuronium bromide (PB) were prepared in Hank's solution, at final concentrations of 12 mM. DMB or PB solution (100 μl) was injected daily using a Pipetman pipette (Gilson) into the amniotic fluid next to the embryos after vitelline membrane removal in E4.5, E5.5 and E6.5 chick embryos. Control embryos were injected with Hank's solution using the same daily protocol. Immobilised or control embryos were analysed at E5.5 (24 h), E6.5 (48 h) or E7.5 (72 h). Forelimbs or hindlimbs were isolated and analysed by *in situ* hybridisation on sections or wholemounts or by RT-q-PCR analysis. For RT-q-PCR analysis, RNAs were prepared from the whole limbs, the limbs without digits or the digits alone.

RNA isolation, reverse transcription and quantitative real-time PCR
RT-q-PCR of experimental or control chick limbs, chick limb explants or mouse limb explants were performed as previously described (Havis et al., 2014). A detailed protocol is provided in supplementary Materials and Methods.

In situ hybridisation and immunohistochemistry
Control or manipulated chick limbs were fixed and processed for *in situ* hybridisation as previously described by Havis et al. (2014). The probes that were used are described in supplementary Materials and Methods. Differentiated muscle cells were detected after *in situ* hybridisation with the monoclonal antibody MF20 (non-diluted supernatant) developed by D. A. Fischman and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa.

Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.136242.supplemental

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