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Inhibition of WNT/β-catenin is necessary and sufficient to induce *Scx* expression in developing tendons of chicken limb

VALENTINA GARCÍA-LEE[#], MARTHA E. DÍAZ-HERNANDEZ[#,§] and JESÚS CHIMAL-MONROY*

Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México

ABSTRACT The cell differentiation of the musculoskeletal system is highly coordinated during limb development. In the distal-most region of the limb, WNT and FGF released from the apical ectodermal ridge maintain mesenchymal cells in the undifferentiated stage. Once the cells stop receiving WNT and FGF, they respond to differentiation signals. Particularly during tendon development, mesenchymal cells enter the cell differentiation program once *Scleraxis* (*Scx*) gene expression occurs. Among the signals that trigger the cell differentiation programs, TGFβ signaling has been found to be closely involved in tendon differentiation. However, whether *Scx* gene expression depends merely on TGFβ signaling or other signals is still not fully understood. In the present study, considering that WNT/β-catenin is an inhibitory signal of cell differentiation, we speculated possible antagonistic or additive effects between canonical Wnt/β-catenin and TGFβ/SMAD signaling pathways to control *Scx* gene expression. We found that the blockade of WNT/β-catenin promoted *Scx* gene expression. In contrast, the inhibition of TGFβ/SMAD signaling did not maintain *Scx* gene expression. Interestingly, the blockade of both WNT/β-catenin and TGFβ/SMAD signaling at the same time promoted *Scx* gene expression. Thus the inhibition of WNT/β-catenin signaling appears to be necessary and sufficient to induce *Scx* gene expression.

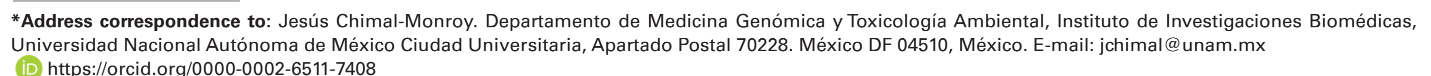
KEY WORDS: *scleraxis, tendon differentiation, limb development, TGFβ, Wnt signaling*

Introduction

The cellular events that control the onset of commitment and cell differentiation during the formation of the musculoskeletal system are highly coordinated in limb development (Cohn & Tickle, 1996; Wolpert, 1990). The mesenchymal cells underneath the apical ectodermal ridge (AER) and dorsal and ventral ectoderm (DE and VE, respectively) are maintained in undifferentiated stage by the action of the WNT and FGF signals released from AER and WNT signaling from DE and VE (ten Berge *et al.,* 2008). The first events of cell differentiation occur when undifferentiated cells abandon the region underneath AER (Tabin and Wolpert, 2007). Once the skeletal elements are established in the core of the limb bud, the first indication of tendon formation occurs between the skeletal elements and the ectoderm (Hurle *et al.*,1989). Its formation is characterized by the appearance of the mesenchymal lamina, an extracellular matrix scaffold, in which condensation of the pre-tendinous mesenchyme cells occurs, giving rise to the tendon blastema (Hurle *et al.*, 1990). In the tendon blastema, the earliest molecular marker of tendons is the bHLH transcription factor *Scleraxis* (*Scx*) (Cserjesi *et al.*, 1995; Pryce *et al.*, 2007; Pryce *et al.*, 2009; Schweitzer *et al.*, 2001). The phenotype observed in the *Scx*-null mutant mice demonstrated that *Scx* is needed for the formation of long tendons and those responsible for transmitting musculoskeletal force in limb, trunk, and tail (Murchison *et al.*, 2007).

The *Sox9* is well-established a key transcription factor for chondrogenesis (Healy, *et al.*, 1999; Qi *et al.*,1997). The existence of a common cell precursor for tenocytes and chondrocytes expressing *Scx*+/*Sox9*+ has been suggested (Sugimoto *et al.*, 2012). The com-

Abbreviations used in this paper: AER, apical ectodermal ridge; EDC, extensor digitorum communis tendon; TGFβ, transforming growth factor-beta; WNT, wingless-related integration site.



***Address correspondence to:** Jesús Chimal-Monroy. Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México Ciudad Universitaria, Apartado Postal 70228. México DF 04510, México. E-mail: jchimal@unam.mx
https://orcid.org/0000-0002-6511-7408

#Note: both authors contributed equally to this study.
§Present address: Department of Orthopaedics, Emory University, Atlanta, Georgia and Atlanta VA Medical Center, Decatur, Georgia, USA.

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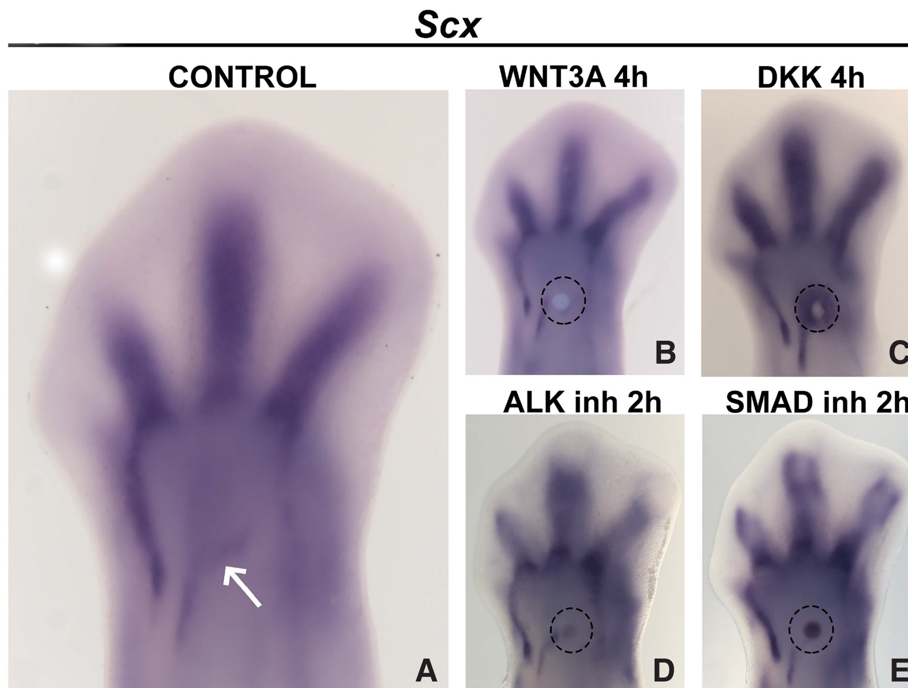

Fig. 1. Wnt-canonical and endogenous TGFβ signaling regulate *Scx* gene expression. In situ *hybridizations of* Scx *gene expression at stage 28HH after different treatments. The effect on* Scx *expression by WNT3A, DKK, ALK inhibitor, and SMAD inhibitor treatments during 4 h is shown.* **(A)** *The white arrow denotes the growing blastema of the EDC in controls.* **(B)** *Notice that WNT3A inhibits while* **(C)** *DKK induced Scx gene expression. Inhibition of the endogenous TGFβ signaling by inhibition of* **(D)** *ALK receptors or* **(E)** *SMAD resulted in the inhibition of Scx gene expression. The white arrow indicates the growing blastema of the EDC in control. The dashed black circle represents the area of inhibition regulated by the implanted beads (WNT3A, ALK inh and SMAD inh) or the induction (DKK) of* Scx *gene expression.*

mon precursor cells differentiate into *Scx*$^{+}$ tenocytes when they initiate tendon differentiation program, whereas precursor cells near to the skeletal elements become *Sox9*$^{+}$ initiating the chondrocyte differentiation program (Blitz *et al.,* 2013; Sugimoto *et al*., 2012). Interestingly, in null mice for *Sox5*/6, chondrogenic transcription factors, ectopic expression of *Scx* is observed in areas that must form cartilage tissue (Brent *et al*., 2005). On the other hand, TGFβ is expressed in mesenchymal condensations preceding the formation of both tendon and cartilage and is also expressed in developing joints (Baffi *et al*., 2006; Merino *et al*., 1998). Mutant mice embryos of *Tgfβ2*$^{-/-}$, *Tgfβ3*$^{-/-}$ and *Tgfβr2-Prx1Cre* demonstrated that *Scx* expression depends on TGFβ signaling. Deletion of *TgfβR2* in PRX1-Cre and Tgfβ2$^{-/-}$/Tgfβ3$^{-/-}$ mice embryos showed failure on joint precursor cells and the connective tissues such as long-range tendons of limb, trunk, tail, and head are mostly at a loss (Pryce *et al*., 2007; Seo & Serra, 2007). As mentioned above, the *Scx* gene expression is observed at the earliest tenogenic stage, but under those experimental conditions, it is no longer maintained. The transitory *Scx* expression suggests that the initial *Scx* induction is TGFβ-independent (Pryce *et al*., 2009).

The requirement of ectoderm for tendon development has been demonstrated (Kardon, 1998; Yamamoto-Shiraishi & Kuroiwa, 2013). The tendon formation is disrupted after ectodermal removal (Edom-Vovard & Duprez, 2004; Schweitzer *et al*., 2001; Solursh *et al*., 1981; Tozer & Duprez, 2005). This ectodermal influence on connective tissue differentiation has been attributed to WNT family members. WNT/β-catenin signaling coordinates the cell fate of connective tissue formation, maintaining sub-ectodermal mesenchymal cells as a pool of progenitors (ten Berge *et al*., 2008). The mesenchymal cells enter to tendon cell differentiation program when the levels of the WNT/β-catenin signaling are reduced (ten Berge *et al*., 2008). Interestingly, the removal of the dorsal ectoderm of the early limb results in the downregulation of *Scx* gene expression. Instead, ectopic *Sox9* gene expression extends to the normal sites of *Scx* gene expression (Schweitzer *et al*., 2001). In contrast, the overexpression of *Wnt6* inhibited the process of chondrogenesis. (Geetha-Loganathan, *et al*., 2010). Although the regulation of *Scx* by *Wnt6* was not evaluated, its expression could also be inhibited. Furthermore, the overexpression of *Sox9* in tenocytes promotes its conversion to chondrocytes (Akiyama *et al*., 2005; Soeda *et al*., 2010; Takimoto *et al*., 2012). On the other hand, TGFβ promptly induces the ectopic expression of *Scx* and *Sox9* in the interdigital tissue of chick embryo or micromass cultures. It seems that tenogenesis or chondrogenesis depends on the expression of TGFβ-signaling repressors such as *Tgif1* and *SnoN*; both are Smad-interacting proteins that negatively regulate the TGFβ signaling pathway (Lorda-Diez *et al*., 2009). In the presence of *Tgif1*, TGFβ induces common precursor cells to enter the tendon differentiation program instead of chondrogenesis (Lorda-Diez *et al*., 2009). Thus, the tenogenic differentiation program can change to another cell fate.

On this basis, this study aimed to determine whether the *Scx* gene expression depends on the activation of TGFβ signaling and inactivation of WNT/β-catenin signaling pathways or both. We found that inactivation of WNT/β-catenin signaling is necessary and sufficient to induce *Scx* gene expression.

Results and Discussion

Nusse and collaborators demonstrated that the inhibitory role of limb ectoderm on chondrocyte differentiation and maintenance of the undifferentiated stage in mesenchymal cells depends on WNT3A (ten Berge *et al*., 2008). However, it has not been demonstrated whether WNT/β-catenin signaling is also relevant in regulating *Scx* gene expression *in vivo*. On the other hand, in tendon-derived cells isolated from the Achilles tendons of 6-week old rats, WNT3A inhibits *Scx* gene expression (Kishimoto *et al*., 2017). Thus, on these bases, we decided to evaluate whether WNT3A was able to

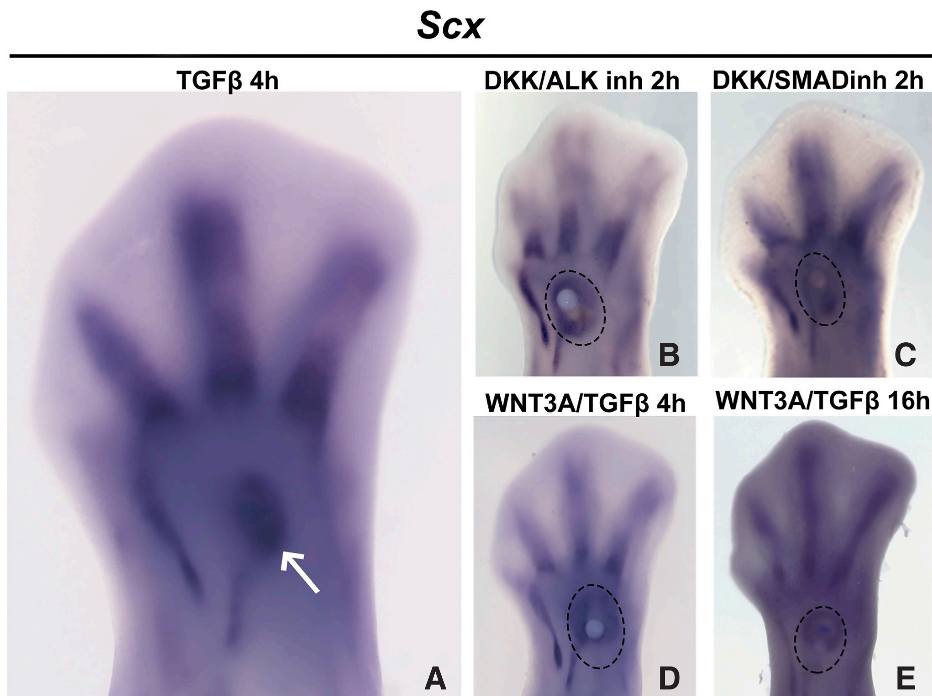

Fig. 2. Inhibition of the WNT/β-catenin is necessary and sufficient to induce *Scx* gene expression. *In situ hybridizations of* Scx *expression at stage 28HH after different treatments.* **(A)** Scx *induction by single TGFβ treatment at four h. Double treatments of DKK with* **(B)** *ALK inhibitor or* **(C)** *SMAD inhibitors at two h. Double treatment of TGFβ and WNT3a is shown at* **(D)** *4 h, and* **(E)** *16 h. Notice that with DKK treatments,* Scx *gene expression is induced even in the absence of endogenous TGFβ signaling. The white arrow indicates the induction of* Scx *gene expression in TGFβ-treatment. The dashed black circle represents the area of inhibition regulated by the implanted beads (WNT3A, ALK inh and SMAD inh) or the induction (TGFβ) of* Scx *gene expression.*

regulate *Scx* gene expression *in vivo*. For all experiments, protein-soaked beads were implanted in the presumptive tenogenic area limited by the proximal phalange three and the distal border of the EDC tendon. Therefore, our results evidenced that after four h of WNT3A treatment, *Scx* gene expression was inhibited (Fig. 1 A,B), suggesting that WNT/β-catenin signaling was able to regulate *Scx* gene expression *in vivo*. Thus, to determine whether endogenous WNT/β-catenin signaling participates in *Scx* gene expression, we used DKK-soaked beads. Results showed that single DKK treatment promoted *Scx* gene expression on the dorsal side at four h after treatment (Fig. 1 A,C). On this basis, limb mesodermal cells must abandon the influence of WNT signaling released from the ectoderm to express the *Scx* gene.

On the other hand, because it is known that TGFβ signaling induces *Scx* gene expression (Pryce *et al*., 2007; Seo & Serra, 2007), we decided to evaluate whether endogenous TGFβ signaling induces *Scx* gene during tenogenic commitment *in vivo*. For this, a specific inhibitor of activin receptor like-kinases ALK5, ALK4, and ALK7 receptors (ALK inhibitor) or the inhibitor of Smad2/3 phosphorylation small molecule (SIS3) were used to evaluate the endogenous function of TGFβ on *Scx* gene expression. Results showed that, in both treatments, *Scx* gene expression was inhibited (Fig. 1 A,D,E), suggesting that endogenous TGFβ signaling, mediated by SMAD, regulates the *Scx* gene expression. However, the effect of *Scx* inhibition was transient, since, after eight h of treatment, the Scx expression was recovered. Thus, to inhibit *Scx* gene expression, continuous inhibition is needed (data not shown).

On this basis, the inhibition of WNT/β-catenin signaling and the activation of the TGFβ signaling may be necessary to promote *Scx* gene expression (Fig. 2A). However, the sole inhibition of WNT/β-catenin signaling may be necessary and sufficient to promote *Scx* gene expression. Therefore, we hypothesized that the *Scx* gene expression would be induced even in the absence of TGFβ signaling. Accordingly, a central experiment to prove it is the simultaneous blockage of WNT/β-catenin and TGFβ signaling (Fig. 2 B,C). Interestingly, our findings showed that the *Scx* gene expression was induced after treatment with DKK alone (Fig. 1 C). Consequently, it is reasonable to propose that inhibition of WNT/β-catenin signaling is necessary and sufficient to induce the tendon differentiation program by activating *Scx* gene expression. In contrast, TGFβ signaling is necessary but not sufficient to induce *Scx* gene expression.

Moreover, we evaluated whether the activation of WNT3A in prospective tendon cells inhibits the ability of TGFβ signaling to promote *Scx* gene expression. Remarkably, TGFβ was necessary to promote *Scx* gene expression, although WNT/β-catenin signaling was present. TGFβ promoted *Scx* gene expression from four h post-treatment and even for 16 h post-treatment (Fig. 2 D,E). One possible interpretation of these results is that in these cells, TGFβ may promote the expression of genes involved in the negative control of WNT/β-catenin signaling. *Axin2* is expressed in the mesenchyme adjacent to the ectoderm (Fig. 3A). It is a central scaffold protein of the β-catenin ubiquitination complex. It results in the inactivation of canonical WNT downstream target genes. Here, we decided to evaluate whether TGFβ regulates *Axin2* expression. Results showed that TGFβ-soaked beads increase the expression of *Axin2* (Fig. 3 A,B). As a control, we evaluated whether WNT3A was able to induce the expression of *Axin2*. Under these conditions, *Axin2* was induced in an extended area of expression at four h (Fig. 3 A-C). Additionally, we evaluated the expression of *N-Myc*, a target gene of the WNT/β-signaling. *N-Myc* was broadly expressed in the mesenchyme around of chondrogenic areas (Fig. 3D). We found that TGFβ and WNT3A treatments induced *N-Myc* gene expression (Fig. 3 D-F). However, *N-Myc* induction by WNT3A was detected to be closer to the ectoderm (Fig. 3 D,F).

We found that canonical WNT and TGFβ signaling induced the expression of *Axin2* and *N-Myc* close to the ectoderm. We hypothesized the existence of a gradient of inductive signals

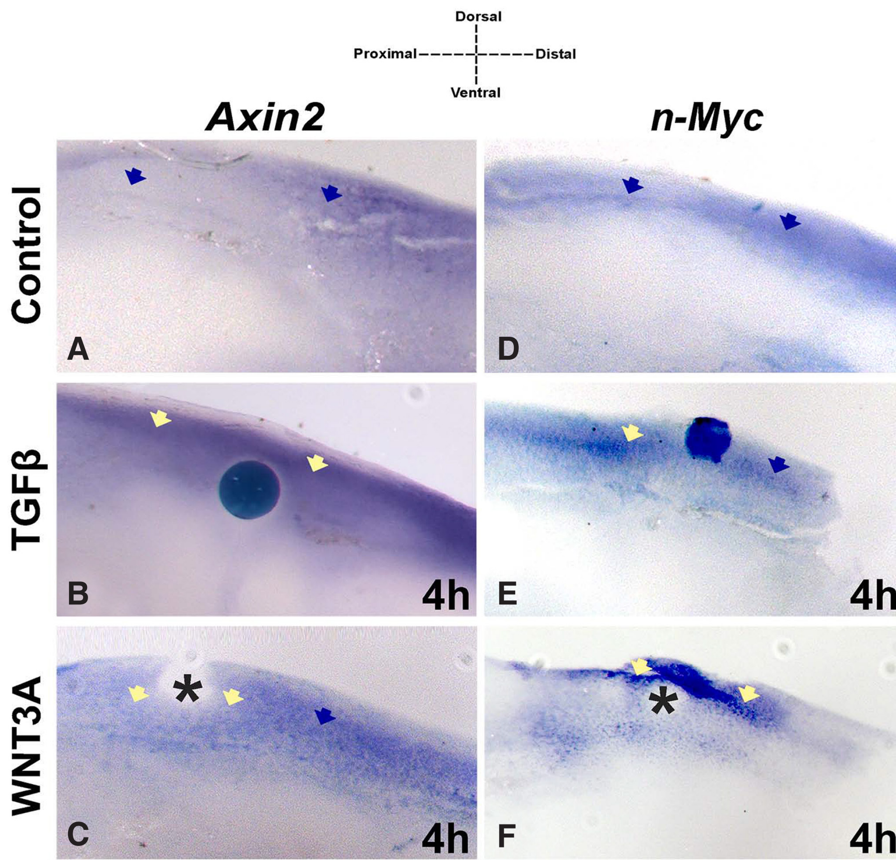

Fig. 3. Regulation of canonical WNT target genes by TGFβ and WNT3A signaling. In situ *hybridizations of* Axin2 *and* N-Myc *at stage 28HH in control limbs or after different treatments. Sagittal sections were performed after* in situ *hybridization in the EDC tendon region. Notice the changes in the area of expression of* Axin *and* N-Myc *after 4h of TGFβ or WNT3A treatments compared with controls. Blue arrows indicate areas of normal expression, while beige arrows denote induction of expression. The asterisk denotes bead position in (C) and (F).*

from the ectoderm to the skeletal elements. The response of mesenchymal cells to the tendon differentiation program depends on that canonical WNT, and TGFβ signals compete in promoting *Scx* gene expression in the cells close to the ectoderm but away from the skeletal elements. Results showed that TGFβ induced *Scx* gene expression only in mesenchymal cells beneath the ectoderm (Fig. 4 A,B). The inhibition of WNT/β-catenin signaling by DKK induced *Scx* gene expression, mainly regionalized in mesenchymal cells beneath the ectoderm (Fig. 4 A,C). As was expected, we observed that WNT3A inhibits the *Scx* gene expression (Fig. 4 A,D). Thus, these results demonstrate that mesenchymal cells underneath ectoderm begin the tendon differentiation program, probably depending on the balance of inhibitory signals. In this study, we suggest that that WNT/β-catenin signaling may be acting in a gradient from ectoderm to skeletal elements.

Although TGFβ is a potent inducer of *Scx* gene expression *in vitro*, the mutant mice embryos for *Tgfβ2*$^{-/-}$, *Tgfβ3*$^{-/-}$, Tgfβ2$^{-/-}$/Tgfβ3$^{-/-}$ and *Tgfβr2-Prx1Cre or TgfβR2* in PRX1-Cre demonstrated that even in the absence of TGFβ signaling, the expression of *Scx* occurs at the earliest tenogenic stage but, importantly, it is no longer maintained (Pryce *et al.*, 2007; Seo & Serra, 2007). This transitory expression of *Scx* indicates that its initial induction is TGFβ-independent. These data suggest an essential

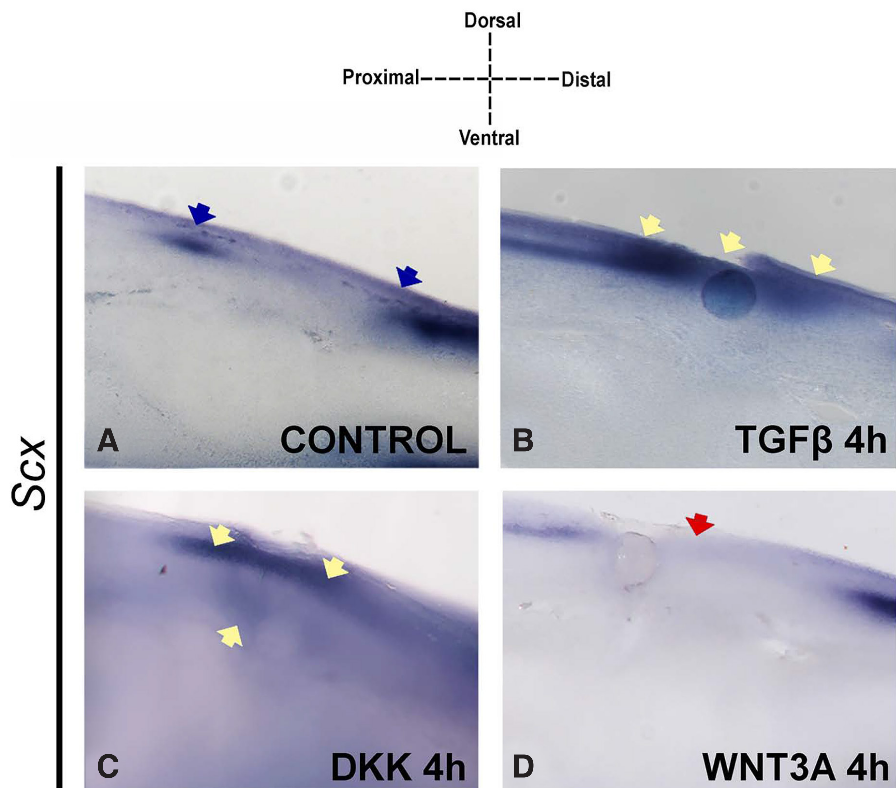

Fig. 4. *Scx* gene expression depends on mesenchyme position in tissue depth from the ectoderm to the skeletal elements. In situ *hybridization of* Scx *gene expression at stage 28HH in a control limb or after different treatments.* **(A)** *Sagittal sections were performed after* in situ *hybridization in the EDC tendon region. Notice that the induction of* Scx *gene expression by* **(B)** *TGFβ, and* **(C)** *DKK after four h is promoted close to the dorsal ectoderm.* **(D)** *In the presence of WNT3A, the Scx gene expression was inhibited in the same lapse of time. Blue arrows indicate the normal expression, beige arrows denote the induction, and the red arrow points the inhibition of Scx gene expression.*

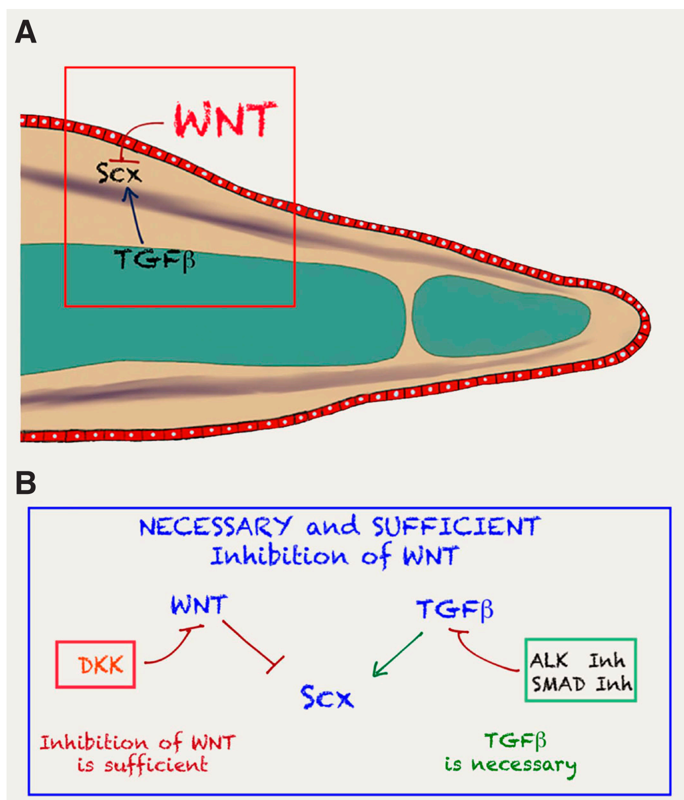

Fig. 5. A model of the necessary and sufficient regulation of *Scx* gene expression in developing tendons. (A) *Schematic representation of a sagittal section of the autopod of the chick embryo. The antagonistic interactions between WNT/β-catenin and TGFβ signaling to regulate* Scx *gene expression are shown. WNT is released from the ectoderm (red cells) and TGFβ from the skeletal elements. Both signalings regulate* Scx *gene expression.* **(B)** *Diagram representing the integration of antagonistic interaction between WNT/β-catenin and TGFβ signaling. The inhibition of WNT signaling by DKK is sufficient to regulate Scx gene expression (red letters). The inhibition of TGFβ signaling by ALK receptors or SMAD antagonists is necessary to induce Scx gene expression (green letters). The simultaneous blockage of both signalings demonstrates that inhibition of WNT/β-catenin is necessary and sufficient to induce* Scx *gene expression in the developing tendons (blue square).*

role in the maintenance of *Scx* gene expression in the early tendon progenitors (Pryce *et al.*, 2009). On this basis, TGFβ may also act as a permissive factor. However, this permissive induction is not at the earliest differentiation stages, as was demonstrated here by double treatments with DKK and ALK or SMAD inhibitors. Interestingly, in the presence of *Tgif1*, precursor cells induced by TGFβ enter to the tendon differentiation program instead of chondrogenesis (Lorda-Diez *et al.*, 2009). Thus, this factor may regulate the threshold of TGFβ necessary to induce tenogenesis or chondrogenesis.

On this basis, other signals such as BMP signaling may be able to trigger *Scx* gene expression. Accordingly, *Scx* gene expression depends on the time of BMP action and on the presence of different repressors in limb mesenchymal cells that block the chondrogenic effect by BMP signaling, such as *Id2*. Thus, the divergent response of limb mesenchymal cells to differentiate in tendon or cartilage lineage depends on the expression profile present in cells (Lorda-Diez *et al.*, 2014). In the present study, our results allowed us to identify that the inhibition of WNT/β-catenin signaling is necessary and sufficient to promote *Scx* gene expression. The experiments of inhibition of the WNT/β-catenin signaling by the action of DKK together with the inhibitors of TGFβ signaling indicate that it is not possible to repress the *Scx* gene expression by blocking TGFβ signaling. Therefore, the first step to trigger the molecular cascade leading to tendon formation, initiate when the mesenchymal cells underlying the ectoderm stop receiving the negative influence of this tissue mediated by WNT signaling. It may influence the gene expression profile of limb mesenchymal cells. Classical studies demonstrated the inhibitory effect of ectoderm on chondrogenesis and tendon development (Kardon, 1998; Wolpert, 1998; Yamamoto-Shiraishi & Kuroiwa, 2013). However, removing the ectoderm leads to the ectopic expression of *Sox9* underneath the ectoderm, but the *Scx* gene expression was not evaluated. On this basis, we speculate that many of $Sox9^+$ cells observed in that study may also be Scx^+ (Geetha-Loganathan *et al.*, 2010). Cells close to ectoderm may initiate the tendon differentiation program, and then convert in $Scx^+/Sox9^-$, but close to skeletal elements, they become $Scx^-/Sox9^+$ to start cartilage differentiation (Sugimoto *et al.*, 2012; ten Berge *et al.*, 2008). Further studies are needed to evaluate whether canonical WNT signaling may regulate the expression of *Tgif1* and *Id2,* allowing limb mesenchymal cells to divert to tenogenic lineage.

In conclusion, we propose that the initiation of the cell differentiation program towards tendon or cartilage tissues depends on their proximity to the ectoderm or the core of the limb, respectively. The inhibition of WNT signaling seems to be necessary and sufficient to induce *Scx* gene expression, whereas TGFβ is necessary to induce *Scx* gene expression (Fig. 5 A,B). It may act as a permissive factor for the tendon differentiation program. Thus, it remains to be clarified if it is required the generation of a gradient of WNT and TGFβ signaling between the ectoderm and the skeletal elements to establish tendon formation between the ectoderm and skeletal elements.

Materials and Methods

Animals

Fertilized White Leghorn chicken eggs were obtained from Alpes, Puebla, Mexico. They were incubated at 38°C, and the embryonic chick hindlimbs at stage 28HH (Hamburger & Hamilton, 1951) were used for all experiments. For treatments, heparin beads (Sigma-Aldrich, St. Louis, MO, USA) were soaked in 1 mg/ml of human recombinant WNT3A or 1 mg/ml DKK (Preprotech, Mexico City, Mexico). DKK1 is a high affinity antagonistic for the WNT co-receptor LRP6. Affi-gel agarose beads (Bio-Rad Laboratories Inc., USA) were soaked in 200 ng/ml TGFβ1 (Peprotech). AG1-X2 acetate beads (Bio-Rad Laboratories Inc., USA) were soaked in 50mM SB431542 (ALK inhibitor) or 20mM SIS3 (SMAD inhibitor). Protein- or chemical-soaked beads were implanted on the dorsal side of the hindlimb at the level of the third metatarsal. All handlings were performed in the presumptive tenogenic area limited by the proximal phalange three and the distal border of the Extensor Digitorum Communis (EDC) tendon (Pryce *et al.,* 2009). For all experiments, the right hindlimb was exposed for surgical manipulation, and left hindlimb was used as a control. After treatment, the chick embryos were returned to the incubator and collected after 2h or 4h for gene expression analysis by *in situ* hybridization.

cDNA probes and in situ *hybridization*

The following probes were used for *in situ* hybridization: *Scx*, *Axin2,* and *N-Myc. Scx* riboprobe was kindly provided by Cliff Tabin (Harvard University). Probes for *Axin2* and *N-Myc* were generated by RT-PCR.

The specific primers used to amplify *Axin2* (403bp) were (accession number NM_204491.1): Forward-TCGAGAACAACAGCATCGTC, Reverse-GACCTGTACCCGTTCTC-CAA. *N-Myc* (481bp) (accession number NM_001030952.1) Forward- AGC GAC TCG GAA GAA GAA CA, Reverse-CGT CCG ATT GGA TAG ACA GAA. Briefly, RNA was obtained from chick limbs at 28 HH stage. Single-strand cDNA was synthesized with RNase H-free reverse transcriptase kit (Invitrogen, Carlsbad, USA). The P-GEM T-easy vector (Promega, Madison, USA) was used to clone fragments, and clones were obtained using MAX efficiency DH5α™ competent cells (Invitrogen). For whole-mount *in situ* hybridization, RNA probes were labeled with UTP-digoxigenin (Roche) as described previously (Gañan *et al.,* 1998). Samples were treated with 28 μg/mL proteinase K (pK) for 28 min at 20°C for all probes. They were stained with BM Purple AP (cat. 11442074001 Roche, Switzerland) and stored at 4% paraformaldehyde.

Vibratome sectioning

After *in situ* hybridization, the samples were embedded in 4% agarose in PBS. Sections of 40μm were performed in a vibrating blade microtome Leica VT1000S (Leica Biosystems, Wetzlar, Germany). The images of the whole mount and sectioned *in situ* hybridizations were obtained in the SMZ1500 microscope (Nikon, Tokyo, Japan) using the Axiovision® software (Zeiss, Oberkochen, Germany).

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