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

VALENTINA GARCIA-LEE, MARTHA ELENA DÍAZ-HERNANDEZ and JESÚS CHIMAL-MONROY
Inhibition of WNT/ß catenin is necessary and sufficient to induce Scx expression in developing tendons of chicken limb

Valentina Garcia-Lee¹#, Martha Elena Díaz-Hernandez#¹,²,³ and Jesús Chimal-Monroy¹*

¹Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. México.

#Both authors contributed equally to this study.

Shorth running title: **WNT is necessary and sufficient to induce Scx**

Key Words: Scleraxis, tendon differentiation, limb development, TGFβ, Wnt signaling.

**Abbreviations**

AER apical ectodermal ridge
EDC Extensor Digitorum Communis tendon
TGFβ Transforming growth factor-beta
WNT Wingless-related integration site

*Corresponding author: 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
Present address: ²Department of Orthopaedics, Emory University, Atlanta, Georgia, and ³Atlanta VA Medical Center, Decatur, Georgia.
Abstract
The cell differentiation of the musculoskeletal system is highly coordinated during limb development. In the distal-most region of the limb, the WNT and FGF signaling released from the apical ectodermal ridge maintain the mesenchymal cells in the undifferentiated stage. Once the cells stop receiving WNT and FGF signaling, they respond to differentiation signals. Particularly during tendon development, the 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 closely involved in tendon differentiation. However, whether the 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 the Scx gene expression. According to our results, the blockade of WNT/β catenin promoted the Scx gene expression. In contrast, the inhibition of TGFβ/SMAD signaling did not maintain the Scx gene expression. Interestingly, the blockade of both WNT/β-catenin and TGFβ/SMAD signaling at the same time promoted the Scx gene expression. Thus, as our results suggest, the inhibition of WNT/β signaling is necessary and sufficient to induce Scx gene expression.
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 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 common precursor cells differentiate into Scx+ tenocytes when they initiate tendon differentiation program, whereas precursor cells near to the skeletal elements become Sox9+ beginning 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 $Tgf\beta_2^{-/-}$, $Tgf\beta_3^{-/-}$ and $Tgf\beta_2\text{-Prx1Cre}$ demonstrated that $Scx$ expression depends on TGFβ signaling. Deletion of $Tgf\beta_2R2$ in PRX1-Cre and $Tgf\beta_2^{-/-}/Tgf\beta_3^{-/-}$ 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., 1998). 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 $Sno$; 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 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. 1a, 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. 1a, 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. 1a, 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. 2b, c). Interestingly, our findings showed that the Scx gene expression was induced after treatment with DKK alone (Fig. 1c). 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. 2d, 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. 3a, 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. 3a-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. 3d-f). However, *N-Myc* induction by WNT3A was detected to be closer to the ectoderm (Fig. 3d, 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 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. 4a, b). The inhibition of WNT/β-catenin signaling by DKK induced *Scx* gene expression, mainly regionalized in mesenchymal cells beneath the ectoderm (Fig. 4a, c). As was expected, we observed that WNT3A inhibits the *Scx* gene expression (Fig. 4a, 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<sup>-/-</sup>, *Tgfβ3<sup>-/-</sup>, *Tgfβ2<sup>-/-</sup>/Tgfβ3<sup>-/-</sup>* 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 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 ld2. 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 ld2, 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 & 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 (Huckle, 2003). 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 (Ganan 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).

**Acknowledgments**

This work was supported by grants 53484 and 168642 (CONACyT), 1887 (Fronteras de la Ciencia-Conacyt), IN213314 and IN211117 (DGAPA-Universidad Nacional Autónoma de México). The authors are grateful to Dr. Jessica Cristina Marín-Llera for her critical comments on the manuscript. Valentina García-Lee was the recipient of a scholarship from CONACyT and was a student in the PhD program: Programa de Maestría y Doctorado en Ciencias Médicas, Odontológicas y de la Salud, Universidad Nacional Autónoma de México.
References

AKIYAMA, H., KIM, J. E., NAKASHIMA, K., BALMES, G., IWAI, N., DENG, J. M, ZHANG, Z., MARTIN, J.F., BEHRINGER, R.R., NAKAMURA, T., AND DE CROMBRUGGHE, B (2005). Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. Proceedings of the National Academy of Sciences of the United States of America. PNAS 102 (41) 14665-14670

BAFFI, M. O., MORAN, M. A., & SERRA, R (2006). Tgfbr2 regulates the maintenance of boundaries in the axial skeleton. Dev Biol. 15;296(2):363-74

BLITZ, E., SHARIR, A., AKIYAMA, H., & ZELZER, E (2013). Tendon–bone attachment unit is formed modularly by a distinct pool of Scx-and Sox9-positive progenitors. Development (Cambridge). 140 (13):2680-90

BRENT, A. E., BRAUN, T., & TABIN, C. J (2005). Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development. Development. 132: 515-528.

COHN, M. J., & TICKLE, C (1996). Limbs: A model for pattern formation within the vertebrate body plan. Trends Genet. 12(7):253-7.

CSERJESI, P., BROWN, D., LIGON, K. L., LYONS, G. E., COPELAND, N. G., GILBERT, D. J., JENKINS N.A., AND OLSON, E. N (1995). Scleraxis: A basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. Development. 121(4):1099-110.

EDOM-VOVARD, F., & DUPREZ, D. (2004) Signals Regulating Tendon Formation during Chick Embryonic Development. Dev. Dyn. 229:449–457.

GAÑAN, Y., MACIAS, D., BASCO, R. D., MERINO, R. AND HURLE J. M (1998). Morphological diversity of the avian foot is related with the pattern of msx gene expression in the developing autopod. Dev. Biol. 196, 33-41

GEETHA-LOGANATHAN, P., NIMMAGADDA, S., CHRIST, B., HUANG, R., & SCAAL, M (2010). Ectodermal Wnt6 is an early negative regulator of limb
chondrogenesis in the chicken embryo. *BMC Dev Biol* 10:32

GEETHA-LOGANATHAN, P., NIMMAGADDA, S., & SCAAL, M (2008). Wnt signaling in limb organogenesis. *Organogenesis*. 4(2):109-15

GREGG, B. C., ROWE, A., BRICKELL, P. M., & WOLPERT, L (1989). Ectodermal inhibition of cartilage differentiation in micromass culture of chick limb bud mesenchyme in relation to gene expression and cell shape. *Development* 105(4):769-77.

HAMBERGER, V., & HAMILTON, H. L (1951). A series of normal stages in the development of the chick embryo. Dev Dyn.195: 231-72

HEALY, C., UWANOGHO, D., & SHARPE, P. T (1999). Regulation and role of Sox9 in cartilage formation [In Process Citation]. *Dev Dyn*. 215: 69–78

HURLE, J. M., ROS, M. A., GAÑAN, Y., MACIAS, D., CRITCHLOW, M., & HINCHLIFFE, J. R (1990). Experimental analysis of the role of ECM in the patterning of the distal tendons of the developing limb bud. *Cell Diff and Dev*. 30 (2): 97-108

HURLE, J. M., ROS, M. A., HINCHLIFFE, J. R., CRITCHLOW, M. A., & GENIS-GALVEZ, J. M (1989). The extracellular matrix architecture relating to myotendinous pattern formation in the distal part of the developing chick limb: An ultrastructural, histochemical and immunocytochemical analysis. *Cell Diff and Dev*. 27(2):103-20

KARDON, G (1998). Muscle and tendon morphogenesis in the avian hind limb. *Development*. 125: 4019-4032

KENGAKU, M., CAPDEVILA, J., RODRIGUEZ-ESTEBAN, C., DE LA PEÑA, J., JOHNSON, R. L., BELMONTE, J. C. I., & TABIN, C. J (1998). Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. *Science*. 280: 1274-1277

KISHIMOTO, Y., OHKAWARA, B., SAKAI, T., ITO, M., MASUDA, A., ISHIGURO, N., SHUKUNAMI, C., DOCHEVA, D., AND OHNO, K (2017). Wnt/β-catenin signaling suppresses expressions of Scx, Mkx, and Tnmd in tendon-derived
cells. *PLoS ONE*. 12(7):e0182051.

LORDA-DIEZ, C.I., MONTERO, J.A., MARTINEZ-CUE. C., GARCIA-PORRERO, J.A., HURLE, J.M (2009). Transforming growth factors beta coordinate cartilage and tendon differentiation in the developing limb mesenchyme. *J Biol Chem*. 284(43):29988-29996.

LORDA-DIEZ, C.I., MONTERO, J.A., CHOE, S., GARCIA-PORRERO, J.A., HURLE, J.M 2014 Ligand- and stage-dependent divergent functions of BMP signaling in the differentiation of embryonic skeletogenic progenitors in vitro. *J Bone Miner Res*. 29(3):735-748.

MERINO, R., GAÑAN, Y., MACIAS, D., ECONOMIDES, A. N., SAMPATH, K. T., & HURLE, J. M. (1998). Morphogenesis of digits in the avian limb is controlled by FGFs, TGFβs, and noggin through BMP signaling. *Dev Biol*. 200 (1): 35-45

MURCHISON, N. D., PRICE, B. A., CONNER, D. A., KEENE, D. R., OLSON, E. N., TABIN, C. J., & SCHWEITZER, R. (2007). Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development*.

PRYCE, B. A., BRENT, A. E., MURCHISON, N. D., TABIN, C. J., & SCHWEITZER, R. (2007). Generation of transgenic tendon reporters, ScxGFP and ScxAP, using regulatory elements of the scleraxis gene. *Dev. Dyn*. 236:1677–1682

PRYCE, B. A., WATSON, S. S., MURCHISON, N. D., STAVEROSKY, J. A., DÜNKER, N., & SCHWEITZER, R. (2009). Recruitment and maintenance of tendon progenitors by TGFβ signaling are essential for tendon formation. *Development*. 136: 1351-1361

QI, Z., HEIDI, E., VERONIQUE, L., & DE CROMBRUGGHE, B. (1997). Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. *Developmental Dynamics*. 209:377–386

SAUNDERS JR., J. W. (2002). Is the progress zone model a victim of progress? *Cell*. 110 (5) 541-543.

SCHWEITZER, R., CHYUNG, J. H., MURTAUGH, L. C., BRENT, A. E., ROSEN, V.,
OLSON, E. N., … TABIN, C. J. (2001). Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development*.

SEO, H. S., & SERRA, R. (2007). Deletion of Tgfbr2 in Prx1-cre expressing mesenchyme results in defects in development of the long bones and joints. *Dev Biol.* 310(2): 304–316.

SOEDA, T., DENG, J. M., DE CROMBRUGGHE, B., BEHRINGER, R. R., NAKAMURA, T., & AKIYAMA, H. (2010). Sox9-expressing precursors are the cellular origin of the cruciate ligament of the knee joint and the limb tendons. *Genesis*. 248(11): 635-44.

SOLURSH, M., SINGLEY, C. T., & REITER, R. S. (1981). The influence of epithelia on cartilage and loose connective tissue formation by limb mesenchyme cultures. *Dev Biol.* 86(2): 471-82

SUGIMOTO, Y., TAKIMOTO, A., AKIYAMA, H., KIST, R., SCHERER, G., NAKAMURA, T., HIRAKI, Y., SHUKUNAMI, C. (2012). Scx+/Scx9+ progenitors contribute to the establishment of the junction between cartilage and tendon/ligament. *Development (Cambridge)*. 140(11): 2280-8.

TABIN, C. AND WOLPERT, L (2007) Rethinking the Proximodistal Axis of the Vertebrate Limb in the Molecular Era. *Genes Dev* 21(12):1433-42

TAKIMOTO, A., ORO, M., HIRAKI, Y., & SHUKUNAMI, C. (2012). Direct conversion of tenocytes into chondrocytes by Sox9. *Exp Cell Res* 318(13):1492-507.

TEN BERGE, D., BRUGMANN, S. A., HELMS, J. A., & NUSSE, R. (2008). Wnt and FGF signals interact to coordinate growth with cell fate specification during limb development. *Development*. 135(19):3247-57.

TOZER, S., & DUPREZ, D. (2005). Tendon and ligament: Development, repair and disease. *Birth Defects Research Part C - Embryo Today: Reviews*. 75:226–236

WOLPERT, L. (1990). Signals in limb development: STOP, GO, STAY and POSITION. *J Cell Sci Suppl.* 13:199-208

YAMAMOTO-SHIRAISHI, Y. I., & KUROIWA, A. (2013). Wnt and BMP signaling
cooperate with Hox in the control of Six2 expression in limb tendon precursor.

*Developmental Biology*. 77 (2): 363-374

FIGURE LEGENDS

**Figure 1. Wnt-canonical and endogenous TGFβ signaling regulate the 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 4h 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 positive round region observed in the limb bud represents the area of inhibition regulated by the in the implanted beads (WNT3A, ALK inh and SMAD inh) or the induction (DKK) of the Scx gene expression.

**Figure 2. Inhibition of 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, the 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 positive round region observed in the limb bud represents the area of inhibition regulated by the in the implanted beads (WNT3A, ALK inh and SMAD inh) or the induction (DKK) of Scx gene expression.

**Figure 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).

**Figure 4. Scx gene expression depends on the mesenchyme position in the 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.

**Figure 5. A model of the necessary and sufficient regulation of Scx gene expression in the 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).
NECESSARY and SUFFICIENT Inhibition of WNT

- WNT
- TGFB

- DKK
- Scx
- ALK Inh
- SMAD Inh

Inhibition of WNT
- is sufficient

TGFB
- is necessary