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Control of tendon cell fate in the embryonic limb: A molecular perspective

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Abstract: The molecular cascade underlying tendon formation starts when progenitor cells begin to express the *Scleraxis* (*Scx*) gene. *Scx* knockout mice develop some but not all tendons, suggesting that additional factors are necessary for tendon commitment, maintenance, and differentiation. Other transcription factors, such as *Mohawk* (*Mkx*) or early growth response (*Egr*), maintain *Scx* expression and extracellular matrix formation during fibrillogenesis. The inhibition of wingless and int-related protein signaling is necessary and sufficient to induce the expression of *Scx*. Once the commitment of tenogenic lineage occurs, transforming growth factor-beta (TGFβ) induces the *Scx* gene expression, becoming involved in the maintenance of tendon cell fate. From this point of view, we discussed two phases of the tenogenic process during limb development: dependent and independent of mechanical forces. Finally, we highlight the importance of understanding embryonic tendon development to improve therapeutic strategies in regenerative medicines for tendons.

Introduction

The formation of the musculoskeletal system during limb development is a paradigmatic model for studying cell differentiation, morphogenesis, and patterning. At the onset of limb formation, cartilage and tendon progenitor cells arise from the lateral plate mesoderm while the limb bud forms. Concomitant with the establishment of the limb primordium, the commitment of mesodermal cells is controlled by three signaling centers that coordinate the spatial distribution and patterning of differentiating tissue. The apical ectodermal ridge (AER) regulates the proximo-distal axis and limb outgrowth, maintaining the cells underneath the AER in a multipotent, proliferative state; this region is referred to as the undifferentiated zone. The dorsal and ventral ectoderm coordinate to establish the limb's dorsal and ventral polarity. Finally, the zone of polarizing activity provides the pattern formed according to the anterior and posterior polarity of the limb (McQueen and Towers, 2020; Marin-Llera *et al.*, 2019).

The fine-tuned control of proliferation and differentiation influenced by signals from the ectoderm forms the distinct anatomical regions of the limb and its tissue components (Fig. 1A). In each anatomically-distinct area, the differentiation of mesodermal cells initiates once signals from the ectoderm cease (McQueen and Towers, 2020; Cooper *et al.*, 2011; Dudley *et al.*, 2002). Besides, mesodermal cells are kept under a proliferative, undifferentiated state by the action of fibroblast growth factor (FGF) and wingless and int-related protein (WNT) signaling (ten Berge *et al.*, 2008). Cartilage commitment occurs at the core of the limb and gives rise to skeletal elements. In contrast, tendon differentiation occurs between these skeletal elements and the ectodermal surface of the limb (Fig. 1B) (Hurle *et al.*, 1990).

Tendons are difficult to heal due to their relatively acellular and avascular nature. After an injury, tendons form scar tissue and ectopic bone without regenerating the original tendon structure with low mechanical properties. Numerous efforts to promote tendon healing techniques such as PRP (platelet-rich plasma), stem cells, scaffolds, gene therapy, gel and cell sheets, and scaffolds have been well documented (Lakhani *et al.*, 2021; He *et al.*, 2022). However, in-depth knowledge of cellular and molecular processes during tendon development is essential to improve therapeutic strategies.

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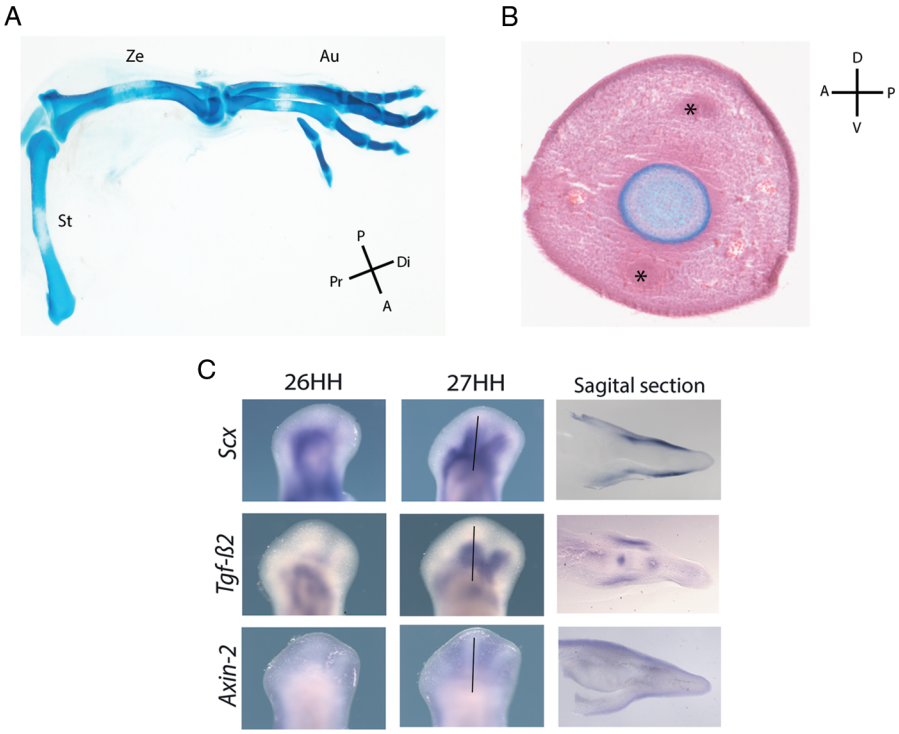

FIGURE 1. Limb anatomy and tendon cell fate. (A) Anatomical regions of a chicken hindlimb stained with Alcian blue. The most proximal element, the stylopod (St), is identified as a single skeletal element; two central skeletal elements in the zeugopod (Ze), and the autopod (Au), are characterized by the most distal and highly segmented skeletal elements. Proximal (Pr), distal (Di), posterior (P), and anterior (A). (B) Transversal section of a chicken digit stained with Alcian blue and hematoxylin and eosin. Undifferentiated mesodermal cells, blood vessels, and ectoderm surround the skeletal element. The asterisks denote the ventral and dorsal tendon blastema, positioned between the ectoderm and the skeletal element. Dorsal (D); ventral (V); anterior (A), and posterior (P). (C) *In situ* hybridization showing the expression pattern of *Scx*, *Tgfb2*, and *Axin2* in whole-mount chicken hindlimbs at 26 and 27 HH stages and sagittal section at stage 27 HH (see the black line as a reference) (*Scx*: Scleraxis; *Tgfb2*: transforming growth factor beta 2; *Axin2*: a protein involved in the negative control of WNT β-catenin signaling).

Since the early cellular processes underlying tendon formation in the limb have been characterized (Hurle *et al.*, 1990), an early molecular marker characteristic of this developmental progression has been identified (Schweitzer *et al.*, 2001). Therefore, the mechanisms that dictate the specification of mesodermal cells in tendon progenitor cells warrant further investigation.

Tendon Formation

From histological to molecular events during tendon formation

Tendons connect skeletal muscles to bones and transmit the mechanical force of muscular contraction to produce movement, whereas the ligaments align bones within joints to maintain their stability (Murchison *et al.*, 2007). Tendons are connective tissues rich in extracellular matrix (ECM) components, mainly collagen types I and III, tenascin, and fibronectin (Hurle *et al.*, 1990).

The first histological event observed during tendon differentiation is the formation of an ECM scaffold, particularly in the long autopodial tendons that correspond to extensor (dorsal) and flexor (ventral) tendons in developing autopod chick embryos (Hurle *et al.*, 1990). Together with the establishment of digital rays during tendon development, the formation of a thick ectodermal-mesodermal lamina rich in tenascin occurs. Finally, mesodermal cells condensate to originate the tendon blastema around this lamina (Hurle *et al.*, 1990).

The earliest molecular steps of tenogenesis involve the recruitment of progenitor cells and are muscle-independent. In contrast, once the tendon tissue is established, the subsequent tendon development depends on the presence of muscle; in its absence, tendon development is arrested. Thus, the first stage of tendon differentiation does not need the mechanical load; the second stage does (reviewed by Felsenthal and Zelzer, 2017).

Sox9 (SRY-Box transcription factor 9) and *Scleraxis* (*Scx*; bHLH transcription factor) are the master genes that regulate chondrogenesis (Akiyama *et al.*, 2002; Akiyama *et al.*, 2005) and tenogenesis, respectively (Schweitzer *et al.*, 2001; Liu *et al.*, 2021). Chondrocytes and tenocytes differentiate from a common precursor expressing both master genes ($Sox9^+$/Scx^+) (Sugimoto *et al.*, 2013). When $Sox9^+$/Scx^+ cells enter the tendon differentiation program, *Sox9* ceases to be expressed while *Scx* expression is maintained (Blitz *et al.*, 2013). In contrast, precursor cells near the skeletal elements become $Sox9^+$, differentiating down the cartilage lineage; the expression of *Sox9* is initially maintained while *Scx* ceases to be expressed (Takimoto *et al.*, 2012; Blitz *et al.*, 2013; Sugimoto *et al.*, 2013). Mesodermal cells commit to the tendon fate after WNT signals emanating from the dorsal

and ventral ectoderm stop receiving (ten Berge *et al.*, 2008). In this sense, the molecular processes that trigger tenogenic differentiation start with *Scx* expression, as observed in all tendon precursors of the limb (Schweitzer *et al.*, 2001).

Transforming growth factor-beta (TGFβ) family members are expressed in the tendon blastema (Merino *et al.*, 1998; Pryce *et al.*, 2009). Although the onset of the tendon differentiation program occurs in conditional deletion of *TgfbR2* in cells expressing paired-related homeobox 1 (PRRX1) and double mutants for $Tgfb2^{-/-}/Tgfb3^{-/-}$ (Pryce *et al.*, 2009), most tendons and ligaments are lost after, suggesting a role for TGFβ in maintaining *Scx* expression. Thus, its role appears as a permissive factor that induces *Scx* gene expression in committed cells to the tenogenic lineage or maintains the tendon cell fate (Garcia-Lee *et al.*, 2021; Pryce *et al.*, 2009). However, its participation in the recruitment of new progenitors cannot be ruled out because of the robust role of TGFβ in inducing *Scx* gene expression in a short time (Pryce *et al.*, 2009). Interestingly, *Scx* is needed to form long tendons and those responsible for transmitting musculoskeletal force in the limbs, trunk, and tail, but not the tendons anchoring muscle (Murchison *et al.*, 2007). Thus, other transcription factors may be required to induce tendon differentiation independently of *Scx* gene expression. Transcriptomic analysis of developing mouse limb tendon cells and gain and loss of function experiments suggest that TGFβ via the suppressor of mothers against decapentaplegic (SMAD 2/3) signaling is sufficient to induce *Scx* expression during tendon development in mouse limb explants and C3H10T1/2 cells (Havis *et al.*, 2014).

Interestingly, the overexpression of *Sox9* in tenocytes promotes its conversion to chondrocytes (Soeda *et al.*, 2010; Takimoto *et al.*, 2012). Thus, the ability of precursor cells to start the chondrogenesis or tenogenesis program depends on the inducer. However, TGFβ induces the ectopic expression of *Sox9* and *Scx* gene expression when implanted in the third interdigit in chick embryos or micromass cultures, suggesting that cell fate between chondrogenesis or tenogenesis is finely regulated via two SMAD-interacting proteins, transforming growth-interacting factor (TGIF) and ski novel gene (SnoN), that negatively regulate the TGFβ signaling pathway. TGIF directs precursor cells to enter the tendon differentiation program instead of chondrogenesis (Lorda-Diez *et al.*, 2009). Thus, cells may commit to following either the tenogenic or chondrogenic differentiation program in response to the TGFβ signaling threshold.

Role of early growth response (EGR) and Mohawk (MKX) in the tendon differentiation program

The homeodomain protein MKX and zinc-finger protein EGR are involved in tendon development (Ito *et al.*, 2010; Liu *et al.*, 2010; Lejard *et al.*, 2011; Guerquin *et al.*, 2013). *Mkx* gene is expressed after the expression of *Scx* occurs. Knocking out *Mkx* does not affect the formation of tendons but causes defects in type I collagen fibrils and other ECM components such as lumican, decorin, and fibromodulin, which affects the growth and mass of tendons (Ito *et al.*, 2010). Therefore, while SCX is necessary for the onset of tenogenesis in some tendons, MKX is not required to ensure its differentiation. Given that MKX promotes the expression of *Scx* by binding to the *Tgfb2* promoter, *Mkx* gene expression is required during tendon development (Liu *et al.*, 2015). Also, MKX regulates *Sox9* by repressing its expression (Suzuki *et al.*, 2016). Consequently, chondrocyte differentiation is inhibited, as demonstrated in $Mkx^{-/-}$ rats undergoing cell transdifferentiation from tenocytes to chondrocytes, which leads to early tendon ossification (Suzuki *et al.*, 2016).

EGR1 and EGR2 are two DNA-binding proteins involved in embryonic tendon formation. Their genes share sequence homology with the *Stripe* gene expressed in the tendons of *Drosophila* (Lejard *et al.*, 2011). *Egr1* and *Egr2* null and double *Egr1*/2 mutant mice demonstrate that both control tendon type I collagen transcription and fibrillogenesis. Furthermore, both *Egr1* and *Egr2* are sufficient to induce *Scx* gene expression. However, double *Egr1*/2 mutant mice do not exhibit a tendon phenotype; *Scx* gene expression is reduced but not inhibited, suggesting that *Egr1*/2 is not involved in the onset of tendon differentiation (Lejard *et al.*, 2011). Remarkably, the TGFβ signaling pathway is activated after the overexpression of *Egr1* in cell culture, like MKX, since Egr1 is enriched at the *Tgfb2* promoter (Guerquin *et al.*, 2013). Given that TGFβ also induces *Egr* gene expression in chicken limbs *in vivo* (Lejard *et al.*, 2011), these data support that TGFβ can induce tenogenesis. This signaling pathway seems sufficient but not necessary for initiating tendon differentiation (Garcia-Lee *et al.*, 2021).

Role of the ectoderm and wingless and int-related protein signaling in the onset of the tendon differentiation program

As mentioned above, tendons are positioned between the ectoderm and skeletal elements during limb development, and the first histological evidence of tendon formation is observed between both tissues (Hurle *et al.*, 1990). Thus, signals proceeding from the dorsal and ventral ectoderm of the embryonic limb and skeletal elements may be required to control cell differentiation and its proper location in the limb. Tendon tissue formation is disrupted after removing the ectoderm due to the reduced area of *Scx* gene expression (Schweitzer *et al.*, 2001). Besides, removing the ectoderm extends the formation of cartilage and connective tissue but not muscle (Geetha-Loganathan *et al.*, 2010). Interestingly, the ectoderm's molecular signals that inhibit *Scx* expression belong to the bone morphogenetic protein family (BMP). The inhibition of BMP signaling after applying Noggin, an antagonist of BMP, extends the expression area of the *Scx* gene and inhibits the molecular markers of muscle (Schweitzer *et al.*, 2001). Another possibility that *Scx* gene expression is lost is because cell death occurs after ectoderm removal (Fernandez-Teran *et al.*, 2013); progenitor cells are depleted. However, all this data reflects that the ectoderm regulates both the position of tendons and muscles.

Although BMP signaling plays an essential role in controlling cell differentiation of muscle, tendon, and cartilage (reviewed in Wang *et al.*, 2014), other studies indicate that WNT signaling from the ectoderm regulates the onset of tendon differentiation (ten Berge *et al.*, 2008). The WNT signaling pathway is among the signals expressed in the ectodermal tissue. WNT β-catenin signaling regulates

connective tissue formation, while the sub-ectodermal mesenchyme is maintained as a pool of progenitors. After sub-ectodermal cells are far away from the WNT-ectodermal signals, presumably *Wnt6*, the progenitors start the expression of *Scx* or *Sox9*, probably through WNT-mediated centripetal patterning of the limb by the surface ectoderm (Geetha-Loganathan *et al.*, 2010). Cells differentiate into tendons or cartilage depending on their proximity to the ectoderm as the primary source of WNT signaling with influence on the mesodermal tissue (ten Berge *et al.*, 2008). Skeletal elements are present in the most central region of the limb, and the tendon is established in the area between skeletal elements and the ectoderm (Hurle *et al.*, 1990). In this context, undifferentiated cells commit to the tendon differentiation program after the WNT signaling inhibition or after the treatment with TGFβ (Garcia-Lee *et al.*, 2021).

Interestingly, when WNT and TGFβ signaling pathways are simultaneously inhibited, the *Scx* gene expression is observed (Garcia-Lee *et al.*, 2021). This suggests that the molecular cascade of tendon differentiation begins at a certain distance from the ectoderm when the negative influence of WNT signaling in mesodermal tissue is abolished or reduced. Furthermore, TGFβ signaling is permissive as it induces the expression of *Axin2;* its genic product is involved in the negative control of WNT β-catenin signaling (Garcia-Lee *et al.*, 2021) and also involved in the maintenance of the tendon cell fate (Tan *et al.*, 2020).

Tendon maintenance depends on the mechanical load

The second stage of tendon development is mechanical load dependence. Thus, it requires the presence of muscle and its contraction. In chick embryos, zeugopod or autopod tendon development is arrested in muscle-less limbs. In contrast, in muscle-less limbs of mouse embryos, autopod tendons are normal, and zeugopod tendons are lost (Gaut and Duprez, 2016; Felsenthal and Zelzer, 2017; Bobzin *et al.*, 2021). Interestingly, the zeugopod tendons in mice with muscular dysgenesis do not degenerate but are smaller than normal, with lower *Scx* gene expression than in normal animals (Gaut and Duprez, 2016; Felsenthal and Zelzer, 2017). The mechanical stimulation in tendon stem/progenitor cells (TSPC) in culture regulates the expression of genes involved in the homeostasis of the TSPC niche, such as ECM and integrin receptors resulting in the control of the expression matrix metalloproteinases (Popov *et al.*, 2015). TGFβ is involved in the maintenance of the tendon development program and possibly in the recruitment of tendon progenitors (Pryce *et al.*, 2009). These authors propose that TGFβ from the muscles may be necessary to maintain tendon progenitors (Pryce *et al.*, 2009). The presence of muscle or muscular contractions promotes the disruption of the ECM of tendons, promoting TGFβ releasing from ECM that also regulates ECM homeostasis and *Scx* gene expression (Felsenthal and Zelzer, 2017). Mechanical load regulates *Egr1* and *Mkx* expression: the higher mechanical load increases their expression; in contrast, the lower mechanical load reduces it (Gaut *et al.*, 2016; Kayama *et al.*, 2016). Because of mechanical load, EGR1/2 and MKX maintain tendon cell fate by activating the expression of *Scx* and *Col1a1* genes during development (Guerquin *et al.*, 2013). Egr1 is enriched at the *Tgfb2* promoter (Guerquin *et al.*, 2013), and because mechanical load regulates EGR1, TGFβ signaling participates in the maintenance of tendon cell identity (Havis and Duprez, 2020; Tan *et al.*, 2020). On the other hand, *Mkx* knockout mice cannot maintain tenogenic gene expression after mechanical stimuli presenting hindlimb tendons with heterotopic ossification (Kayama *et al.*, 2016; Liu *et al.*, 2019).

Concluding Remarks

The expression patterns of *Scx*, *Tgfb2*, and *Axin2* enable determining the induction and maintenance signals underlying tendon differentiation in a temporal-spatial manner (Fig. 1C). Tendon development is a highly orchestrated process with deep-seated compensatory mechanisms. This process initially depends on the inhibition of WNT signaling as it is sufficient and necessary for *Scx* gene expression (Figs. 2A and 2B). In contrast, TGFβ signaling is permissive in inducing and maintaining the tendon differentiation process by promoting *Axin2* gene expression and *Mkx* and *Erg1*/2 loop regulation; also, the function of TGFβ in the maintenance depends on the mechanical load during tendon development (Figs. 2C and 2D).

The literature demonstrates the scientific community's interest in a safe therapeutic approach with high tendon regenerative potential. Although many aspects of tendon development have been described, more specific elements are continuously being discovered that require constant further exploration; for example, *Dact* proteins are suggested as adaptor proteins that modulate WNT and TGFβ signaling during limb development (Sensiate *et al.*, 2014). The complete signaling pathway that drives tendon formation is still unknown. Future studies in this arena should provide insight into *Scx*-mediated control of tenogenic differentiation, regulation of tendon migration to their insertion sites in muscles, and the spatial organization of tendon fibers. Translating regenerative tendon therapies from bench to bed requires a deeper understanding of the cellular processes during embryonic tendon development (Ideo *et al.*, 2020). This knowledge would provide cues to promote tendon regeneration by improving therapeutic strategies following the routes of tendon developmental programs.

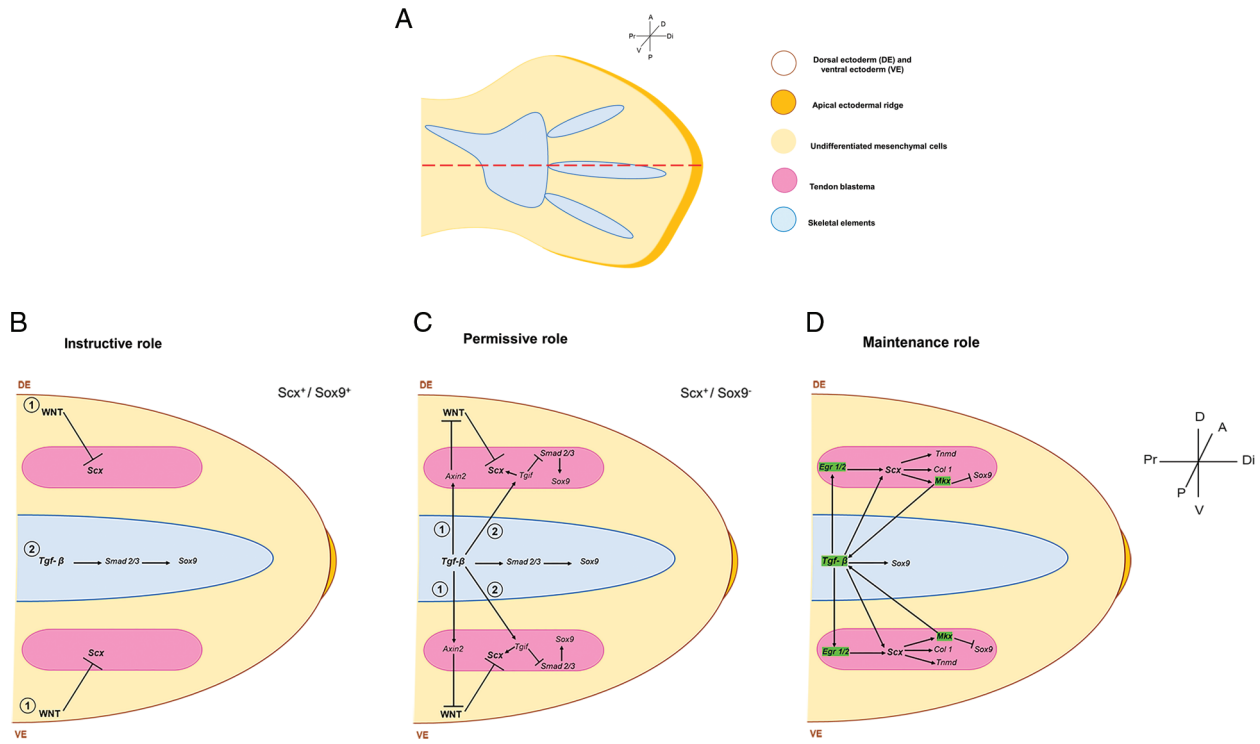

FIGURE 2. A model of induction and maintenance of the tendon program. (A) Schematic representation of the position of the sagittal section (dotted red line) shown in the models of B-D in a 28 HH chicken hindlimb. Models represent the induction and maintenance of dorsal and, presumably, ventral blastemas. (B) (1) Instructive induction of tendon fate initially depends on inhibiting WNT signaling as it is sufficient and necessary for *Scx* gene expression. (2) The TGFβ signal induces *Sox9* gene expression during the formation of the skeletal elements. (C) Following *Scx* induction in progenitor cells, the signal emanating from the skeletal elements plays a permissive role in inducing (1) *Axin* and (2) *Tgif* to promote *Scx* gene expression. (D) For maintenance, TGFβ and EGR1/2 cooperate to control tendon differentiation, while *Mkx* promotes *Scx* expression by binding to the *Tgfb2* promoter. The mechanical load induces green-highlighted genes during tendon development. Thus, TGFβ plays a role in maintaining the tendon differentiation program once established the tendon fate. (*Scx*: Scleraxis; *Mkx*: Mohawk transcription factor; *Tgfb2*: transforming growth factor beta 2; *Axin2*: a protein involved in the negative control of WNT β-catenin signaling).

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