β ig-h3 Potentiates the Profibrogenic Effect of TGF β Signaling on Connective Tissue Progenitor Cells Through the Negative Regulation of Master Chondrogenic Genes

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Tendons and cartilage are specialized forms of connective tissues originated from common progenitor cells. Initial stages of differentiation of these tissues are characterized by the formation of cell aggregates, which share many molecular markers. Once differentiated, these cells retain considerable plasticity, and chondral metaplasia of tendon and fibrous connective tissues and eventual ossification often accompany degenerative diseases in the adult musculoskeletal system. While this fact is of great relevance for regenerative medicine and aging biology, its molecular basis remains to be elucidated. Gene expression analysis in several physiological and experimental paradigms suggests that differentiation of tendon and cartilage is regulated by a balance in the expression of chondrogenic versus tenogenic genes in the connective tissue cell precursors. Transforming growth factor β (TGF β) may function both as a profibrogenic or as a prochondrogenic factor for embryonic limb mesoderm and mesenchymal stem cell cultures, but mice that are null for TGF β 2 and 3 lack tendons. Here, we identify β ig-h3 as a factor downstream TGF β signaling regulated by Smad 2 and 3, which is highly expressed in the differentiating tendons and joint capsules. Furthermore, gain- and loss-of-function experiments using limb mesoderm micro-mass cultures show that β ig-h3 downregulates the expression of cartilage master genes, including *Sox9*, *type II collagen*, and *Hif-1 α* . Positive regulation of *Sox9* and *type II Collagen* observed in micromass cultures grown under hypoxic conditions is prevented by exogenous administration of β IG-H3, and the antichondrogenic influence of β IG-H3 is lost after *Hif-1 α* silencing with shRNA. Collectively, our findings indicate that β ig-h3 promotes the fibrogenic influence of TGF β signaling, neutralizing the prochondrogenic influence of the hypoxic-inducible factor 1 activated by the hypoxic microenvironment characteristic of limb mesenchymal aggregates.

Introduction

THE VERTEBRATE LIMB DEVELOPS as a bud growing on the flank of the embryonic body, consisting of a core of mesodermal cells that are covered by an ectodermal jacket. The initial population of mesodermal cells consists of skeletogenic cell precursors derived from the lateral plate mesoderm destined to form cartilage, joints, tendons, and ligaments. Muscles, vessels, and nerves derivate from different cell populations that originate in the embryonic trunk and invade the bud at various stages of development. The potential of the skeletogenic mesoderm to differentiate into the different connective tissue cell lineages is of high relevance in regenerative medicine. However, we are only beginning to unravel the molecular control responsible for the

differentiation of limb mesoderm skeletal progenitors into fibrocytes, tenocytes, and chondrocytes (see¹).

Transforming growth factor β s (TGF β s) have been proposed as key signals modulating connective tissue differentiation in embryonic and adult systems.^{2–4} Mice that are deficient in TGF β receptor or ligands have multiple defects in connective tissue differentiation, including joint alterations and the absence of tendons and ligaments.^{4–7} We have recently shown that TGF β modulates the cartilage and tendon differentiation of limb mesodermal progenitors, regulating the expression of *Sox9* and *Scleraxis*, which are master transcription factors for chondrogenesis and tenogenesis.⁸ However, the role of this signaling pathway bears considerable complexity (see review⁹). Indeed, *in vivo* exogenous application of TGF β s to specific regions of the embryonic

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limb induces ectopic cartilages,¹⁰ but chondrogenic differentiation of high-density mesenchymal cell cultures is reverted to fibrous differentiation if TGF β s are added to the medium.⁸ It seems that TGF β s stimulate cell aggregation and the expression of *Sox9* and *Scleraxis* at initial stages of differentiation proceeds, TGF β signaling is silenced in the core of the prechondrogenic condensations and maintained in their surface, which become fibrous. The basis for the fibrous-versus-chondrogenic differentiation remains to be clarified, but some transcription factors downstream TGF β signaling, such as *Tgif1* or *Snai1*,⁸ may serve a relevant function in this process. *Tgif1* is quickly upregulated by TGF β s and induces the downregulation of *Sox9* and upregulation of *Scleraxis*, making the later the predominant signal, which in turn promotes further tendinous differentiation.⁸ This model, based on the balance between chondrogenic and fibrogenic promoters, is supported by findings in adult and developing systems.^{11,12}

Taken together, our understanding of connective tissue differentiation and morphogenesis will be strengthened by the identification of new factors that favor either the fibrous or cartilaginous differentiation of mesodermal progenitors induced by TGF β signaling. In this work, we have explored the expression and function of TGF β -induced gene (*TGFBI*), also known as *keratoepithelin* or β ig-h3 (*big-h3*) in limb mesenchyme. The expression of *big-h3* has been associated with differentiation of some connective tissues during development.^{13–16} It is a matricellular protein inducible by TGF β in different tissues.^{17,18} It is known to modulate cell adhesion, cell migration, and cell differentiation, acting as a linker between cell surfaces and specific components of the extracellular matrices.^{18–22} Humans deficient in *big-h3* suffer severe corneal dysplasia.²³ In addition, an intense downregulation of *big-h3* was identified in melorheostosis, which is a rare disease characterized by hyperostosis and other alterations of the connective tissues.¹⁹ Our results show that *big-h3* is highly expressed under the control of TGF β signaling in the developing connective tissues of the limb, including tendons, entheses, and joint capsules. Furthermore, we provide functional experiments supporting a role of this secreted protein in the differentiation of connective tissue by downregulating chondrogenic master genes.

Material and Methods

In this work, we employed Rhode Island chicken embryos from day 4 to day 10 of incubation (id) equivalent to stages 24 to 36 HH.

In situ hybridization

The limb specimens were hybridized *in situ* in whole mount or in 100- μ m vibratome-sectioned specimens. Samples were treated with 10 μ g/mL of proteinase K for 20–30 min at 20°C. Hybridization with digoxigenin-labeled antisense RNA probes was performed at 68°C. Alkaline phosphatase-conjugated anti-digoxigenin antibody (dilution 1:2000) was used (Roche). Reactions were developed with a BCIP/NBT substrate (Roche).

Micromass cultures were hybridized *in situ* in whole-mount samples following the procedure described above; however, proteinase K digestion was reduced to 3 to 5 min.

The probe for *big-h3* was obtained by PCR: *big-h3* 5'-acaaggacatcctggctacc-3' and 5'-agccaccaacgtactgaagc-3'.

Micromass cultures

Undifferentiated mesodermal cells obtained from the progress zone region located under the apical ectodermal ridge of chick leg buds at 4.5 id (25 HH) were suspended at a density of 2.0×10^7 cells/mL in the Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Micromass cultures were obtained by pipetting 10- μ L drops of cell suspension into each well of a 24-well plate. The cells were left to attach for 2 h, and then 200 μ L serum-free medium was added. The chondrogenic outcome of the cultures and gene expression were studied at different periods in control and experimental cultures. Experiments included overexpression of human *big-h3* gene; treatments with a variety of gene-silencing constructs; or treatments with different compounds, including human recombinant TGF β 1 or TGF β 2 (10 ngr/mL; both from R&D Systems); the TGF β inhibitor, SB431542 (0.01 mM; Sigma); the specific inhibitor of Smad3, SIS3 (10 μ M, Calbiochem); and human recombinant protein β IG-H3 (R&D Systems). For *big-h3* gene overexpression experiments (see below), cells were cultured with the DMEM containing 10% fetal bovine serum and 50 μ g/mL of ascorbic acid.

Results obtained with TGF β 1 or TGF β 2 were identical, and in the article, we will refer to TGF β treatments.

Treatments with β IG-H3 were performed by adding the protein to the culture medium at 10 μ g/mL. In these experiments, the persistence of the human recombinant protein in the culture was verified using the DuoSET ELISA Development kit (DY2935, R&D Systems) following the instructions of the manufacturer. Experiments adding the recombinant protein in combination with fibronectin to the surface of the culture wells were also performed, obtaining similar results. The effects of β IG-H3 in relation with the activity of the hypoxic-induced factor were analyzed in cultures of mesenchymal cells subjected to *Hif-1 α* silencing experiments (see below) or under hypoxic conditions. For hypoxia, the cultures were grown in an atmosphere of 1% oxygen, or by adding 200 μ M cobalt chloride (Sigma-Aldrich) in the culture medium.²⁴

The chondrogenic outcome of control and treated cultures was studied by Alcian blue staining (0.5% Alcian blue at pH 1.0). Gene expression analysis was performed both by quantitative polymerase chain reaction (Q-PCR) and by *in situ* hybridization.

Regulation of genes associated with chondrogenesis (*Sox9*, *Hif-1 α* , and *type 2 Collagen*) and with tenogenesis (*Scleraxis*; *type 1 Collagen*; *Tgif1*; *Mohawk*) was analyzed by Q-PCR.

Cell nucleofection

For gain-of-function experiments, we used a construct based on the coding sequence of the human *big-h3* gene cloned into the pCMV6-XL5 vector (Origene). For this purpose, limb mesodermal cells were electroporated by using the Eppendorf Multiporator system (Eppendorf) following the manufacturer's instructions. Control nucleofections using the empty vector were performed in all experiments. The overexpression efficiency was confirmed at RNA levels

by Q-PCR analysis using specific primers for the human *βig-h3* gene and at protein levels by enzyme-linked immunosorbent assay (ELISA) (see above).

For *βig-h3* loss-of-function experiments, limb mesodermal cells were transfected with a short-hairpin RNAi for *βig-h3* (sh β ig-h3) cloned into the pcU6-1-shRNA (a generous gift of Dr. Tim J. Doran) as described by Wise *et al.*²⁵ The limb mesodermal cells were electroporated as mentioned above. The level of gene silencing was evaluated by Q-PCR and by ELISA. Control transfections using a chU6-1v-irrelevant control plasmid were performed in all experiments. Silencing of *Hif-1 α* was performed following the same protocol. The gene silencing level was evaluated by Q-PCR and by Western Blot using a monoclonal antibody against HIF-1A (Abcam).

The association of *βig-h3* to fibrogenesis induced by overexpression of *Tgif1* was studied in micromass cultures of limb mesodermal cells electroporated with the coding sequence of the human *Tgif1* gene cloned into the pCMV5

vector.²⁶ Control nucleofections using the pCMV5 vector were performed in all experiments.

In all cases, control and experimental cells were cultured following the micromass culture protocol described above and processed for Q-PCR analysis. Experiments were repeated at least five times.

Real-time Q-PCR for gene expression analysis

In each experiment, total RNA was extracted and cleaned from specimens using the RNeasy Mini Kit (Qiagen). RNA samples were quantified using a spectrophotometer (Nanodrop Technologies ND-1000). First-strand cDNA was synthesized by reverse transcriptase-PCR using random hexamers and the M-MuLV reverse transcriptase (Fermentas). The cDNA concentration was measured in a spectrophotometer (Nanodrop Technologies ND-1000) and adjusted to 0.5 μ g/ μ L. Q-PCR was performed using the Mx3005P system (Stratagene)

FIG. 1. Expression of *βig-h3* in the developing limb between 4.5 (A) and 9.5 (D) id. (A) Embryonic leg bud at 4.5 id showing intense expression of *βig-h3* in the mesenchyme surrounding the central chondrogenic core and in the distal undifferentiated mesoderm (arrow). (B–D) Whole-mount *in situ* hybridizations of the leg bud autopode at 6 (B), 7.5 (C), and 9.5 id (D). Note the intense labeling of the developing tendons and the presence of domains in the distal contour of the digits (arrow heads) and in the developing joints (arrows). (E) Longitudinal section of a 6.5-id autopode showing intense expression around the tip of the digits and in the zones of joint formation. Note also a tenuous labeling of the digit cartilages marking the future joint interspaces (arrows). (F) Detailed view of a developing interphalangeal joint at id 8. Note the tenuous labeling of the joint interspace and the intense labeling in the developing fibrous capsule. (G) Longitudinal section of a digit at id 9 showing intense labeling in the insertion regions of ventral tendons and in the joint fibrous capsules. (H–J) Transverse sections of the developing digit at 7.5 (H) and 8 id (I–J), showing a positive signal at the perichondrium at zones of tendon insertion (I–J), in contrast to the absence of transcripts in zones lacking tendon insertion (H).

with automation attachment. In this work, we have used SYBRGreen- (Takara) based Q-PCR. *Gapdh* had no significant variation in expression across the sample set, and therefore was chosen as the normalizer in our experiments. Mean values for fold changes were calculated for each gene. Expression level was evaluated relative to a calibrator according to the $2^{-(\Delta\Delta Ct)}$ equation.²⁷ Each value in this work represents the mean $\pm$ SEM of at least three independent samples obtained under the same conditions. Samples consisted of 4 micromass cultures. Data were analyzed using one-way analysis of variance followed by Bonferroni tests for *post hoc* comparisons for gene expression levels in treated micromass cultures, and Student's *t*-test for gene expression levels in overexpression and silencing experiments. Statistical significance was set at $p < 0.05$. All the analyses were done using SPSS for Windows version 18.0. Q-PCR-specific primers for chick genes were as follows: for *Sox9*, 5'-gaggaagtcggtgaagaacg-3' and 5'-gatgctgaggatgactgc-3'; for *Scleraxis*, 5'-cacaacagcgtaacacc-3' and 5'-cgctcgatcttgagacg-3'; for *Tgfr1*, 5'-ctctctaccacgaggatgc-3' and 5'-gtgcaacatccaccagtagc-3'; for *β ig-h3*, 5'-accaactggctcagtgtgg-3' and 5'-agccaccaagctactgaagc-3'; for *Mohawk*, 5'-cgctctaag

aacacagtcagg-3' and 5'-acgagtcactcgtctcagc-3'; for *Col2a1*, 5'-cagcatccagatgacctcc-3' and 5'-gtctctcgtccatgtaggc-3'; and for *Col1a1* 5'-ttgtggattctcggttactgc-3' and 5'-ggctcgatgctctcttcc-3'. For human *β ig-h3*, 5'-caccatcaccaacaacatcc-3' and 5'-cttcaagcatcgtgttgagc-3'.

Results

β ig-h3 expression in the developing digits

In situ hybridization analysis of *β ig-h3* revealed a predominant expression in zones of fibrous connective tissues and tendon formation. In limb buds at 4.5 id, *β ig-h3* transcripts were observed in domains surrounding the central chondrogenic core of the limb bud and in the undifferentiated distal mesoderm of the autopodial region (Fig. 1A). In subsequent stages corresponding with the period of digit formation, specific domains of expression were found first in the pretendinous aggregates (Fig. 1B), and next in the developing tendons (Fig. 1B–D, G–J), with a similar labeling intensity in both ventral and dorsal tendons. Intense expression domains were also observed around the growing

FIG. 2. (A–B) Alcian blue-stained control micromass cultures at day 2 (A) and 4 (B) of incubation illustrating the progressive increase in Alcian blue-positive cartilage nodules throughout the course of culture. (C) Absence of cartilage nodules in a 4-day micromass culture treated for 2 days with TGFβ. The pale, uniform blue staining corresponds to noncartilaginous connective tissue. (D–F) are *in situ* hybridizations of *β ig-h3* in control cultures of 2 days (D), 4 days (E), and TGFβ-treated 4-day culture. (G) Chart showing the regulation *Sox9*, type 2 Collagen (chondrogenic markers), and *Tgfr1* (fibrogenic marker) in 4-day micromass cultures treated for 2 days with TGFβ. *** $p < 0.001$; ** $p < 0.01$: TGFβ-treated (right bar) versus control cultures (left bar). TGFβ, transforming growth factor β. Color images available online at www.liebertpub.com/tea

tips of the digit blastemas (Fig. C, E) and in the joint-forming regions (Fig. 1C–G). Interestingly, labeling in the developing joint domains reached the highest intensity in the differentiating fibrous capsules (Fig. 1F). A further remarkable expression domain was observed in the perichondrial surface of the phalanxes, at the zones corresponding with the entheses (attachment of tendons to the skeleton; Fig. 1I–J). All those domains are regions of intense TGF β signaling.²⁸

β ig-h3 in the tenogenic/fibrogenic differentiation route of limb mesoderm

To determine whether the expression of *β ig-h3* is under the control of TGF β signaling, we used micromass cultures from undifferentiated limb mesenchymal cells. This system has been widely employed to study the molecular control of mesodermal limb differentiation.²⁹ Under control conditions, mesodermal cells exhibited a default chondrogenic fate (Fig. 2A), characterized by the progressive formation of rounded Alcian blue-positive cartilage nodules (Fig. 2A, B) surrounded by internodular spaces containing fibrogenic-like connective tissue. The examination of *β ig-h3* gene expression by *in situ* hybridization revealed a moderate increase in transcripts during the course of culture (Fig. 2D, E). Q-PCR analysis showed a twofold increase between day 2 and 4 of culture (not shown).

Previously, we have shown that addition of TGF β s in the second day of these cultures inhibits chondrogenesis and promotes fibrogenesis (Fig. 2C, G⁸). Analysis of gene expression in these experimental conditions revealed that fibrous transformation of the micromass is accompanied by intense upregulation of *β ig-h3* (Fig. 2F). Expression increased by about 7.5-fold after 2 h of treatment, reaching a 10-fold increase after 6 h of treatment, which was maintained in successive periods (Fig. 3A). Upregulation was inhibited when SB431542 or SIS3 was coadministered with TGF β (Fig. 3A). The basal expression of *β ig-h3* in the culture was also intensely downregulated by the addition of SB431542 or SIS3 (Fig. 3A). In both cases, the inhibition obtained with SIS3, which inhibits Smad3 but not Smad2, was less intense than that of SB431542, which inhibits TGF β signaling at the receptor level, suggesting that both Smad2 and Smad3 are implicated in the regulation of *β ig-h3*. Together, these findings indicate that expression of *β ig-h3* is regulated by TGF β s through the canonical Smad2/3 signaling pathway and suggest that *β ig-h3* promotes fibrogenesis in a TGF β -dependent manner.

It is known that TGF β is able to simultaneously induce the chondrogenic factor *Sox9* and the tenogenic factor *Scleraxis*. We have previously shown that the transcriptional modulator *Tgfr1*, which is a co-repressor downstream of TGF β signaling, promotes the profibrogenic effect of TGF β s and represses their prochondrogenic effect.⁸ To check whether *β ig-h3* is associated with the profibrogenic effect of *Tgfr1*, we analyzed its expression in micromass cultures overexpressing *Tgfr1*. Undifferentiated mesodermal cells obtained from the distal tip of limbs at 4.5 id were transfected with an expression vector containing the wild-type human *Tgfr1* gene and grown for 3 days in high-density micromass cultures. Under these conditions, chondrogenesis is inhibited, and fibrogenesis is stimulated.⁸ As shown in Figure 3B, Q-PCR analysis at day 3 of culture revealed threefold upregulation

FIG. 3. (A) Chart showing the regulation of *β ig-h3* in micromass cultures treated with TGF β (second column from the left), TGF β in combination with the TGF β -inhibitor SB431542 (third column), the TGF β -inhibitor SB431542 (fourth column), TGF β in combination with the Smad3 inhibitor SIS3 (fifth column), and the Smad3 inhibitor SIS3 (sixth column). Note the intense upregulation of *β ig-h3* after TGF β treatments and its abrogation by both, SB431542 and SIS3. *** p < 0.001 TGF β -treated versus control cultures (first column); ** p < 0.01 SB431542-treated versus control cultures (first column); * p < 0.05 SIS3-treated versus control cultures. ### p < 0.001, TGF β plus the inhibitors SB431542 or SIS3 versus cultures treated with TGF β . (B), quantitative polymerase chain reaction (Q-PCR) chart showing the upregulation of *β ig-h3* in micromass cultures of mesodermal cells overexpressing *Tgfr1* compared to mesodermal cells transfected with the empty vector. ** p < 0.01.

of *β ig-h3* in comparison with control cultures transfected with the empty vector.

β ig-h3 downregulates the expression of chondrogenic factors

The influence of *β ig-h3* in connective tissue differentiation was further examined through gain- and loss-of-function experiments. Gain-of-function experiments were performed by overexpressing the *β ig-h3* gene (Fig. 4A) or by adding recombinant β IG-H3 to micromass cultures. In both types of experiments, chondrogenesis was reduced. As shown in Figure 4B and C, after 4 days of culture, micromass cultures overexpressing *β ig-h3* exhibited a remarkable diminution in the number and size of cartilage nodules accompanied by the formation of wider internodular spaces. Consistent with these findings, by 3 days of culture, expression of the cartilage markers *Sox9* and *type II Collagen* was downregulated,

FIG. 4. (A) β IG-H3 protein levels evaluated by ELISA in 3-day culture concentrates. Bars illustrate absorbance levels in experimental cultures subjected to β ig-h3 overexpression (right bar) in comparison with untreated control cultures (left bar). *** $p < 0.0001$. (B–C) Detailed views of 4-day control (B) and β ig-h3-overexpressing (C) micromass cultures. Note the reduced number of cartilage nodules in the experimental culture. (D) Chart showing changes in the expression of *Sox9*, *Scleraxis*, *type 2 Collagen* (*Col2 α 1*), and *type I Collagen* (*Col1 α 1*) genes in cultures overexpressing β ig-h3. For each gene, the left bar shows the expression level in control untreated culture and the right bar the expression level in the experimental culture. *** $p < 0.001$. (E) Chart showing changes in the expression of *Hif1 α* , *Mohawk*, and *Tgif1* genes in β ig-h3-overexpressing cultures. For each gene, the left bar shows the expression level in control untreated culture and the right bar the expression level in the experimental culture; ** $p < 0.01$. Color images available online at www.liebertpub.com/tea

whereas the expression of the tendon markers *Scleraxis* and *type I Collagen* remained unchanged (Fig. 4D). To better characterize the molecular modifications induced by β ig-h3, we performed a systematic Q-PCR analysis of the expression of a panel of regulatory genes encoding for profibrogenic and prochondrogenic factors. The most relevant modification observed was the downregulation of *Hif-1 α* (hypoxia-inducible factor; see Fig. 4E). Results were similar when gain-of-function experiments were induced by addition of recombinant protein to the culture medium (not shown).

Loss-of-function experiments were performed in micro-mass cultures of limb mesoderm transfected with a short-hairpin RNA for β ig-h3 (sh β ig-h3). After 48 h of culture, we obtained a 50% reduction in gene expression and protein levels of β ig-h3 (Fig. 5A). At 3 days of culture, Alcian blue staining appeared increased only at moderate levels in these experimental micromasses (Fig. 5B, C). Hence, the effect of β ig-h3 knockdown was analyzed in more detail by monitoring the expression of profibrogenic and prochondrogenic genes, as described for gain-of-function experiments. The expression of *Sox9*, *Hif1 α* , and *type 2 Collagen* was significantly upregulated by 3 days of culture, but of profibrogenic markers, including *Mohawk*, *Tgif1*, *Scleraxis*, and *type I Collagen*, was not changed (Fig. 5D, E). These findings are consistent with those obtained in the gain-of-function experiments, revealing a negative influence of β ig-h3 in

the chondrogenic differentiation of mesoderm skeletal progenitors.

The above-described findings, together with the observed functional interaction between hypoxia/*Hif-1* signaling and β ig-h3 in cancer cell lines,²⁴ prompted us to explore if the antichondrogenic influence of β ig-h3 was associated with its influence on the expression of *Hif-1 α* . As shown in Figure 6A and B, the upregulation of chondrogenic markers, including *Hif-1 α* , observed in micromasses cultured under hypoxic conditions (1% oxygen) was prevented by addition of β IG-H3 protein in the culture medium (Fig. 6A). Conversely, the antichondrogenic influence of β IG-H3 protein was abolished when mesodermal cells were previously subjected to *Hif-1 α* silencing (Fig. 6B).

Discussion

β IG-H3 is a secreted protein located in the periphery of the cell surfaces, which is associated with the control of adhesion, migration, and differentiation in different tissues.^{14,18,30} Deficiency of β ig-h3 causes severe corneal dystrophy.^{23,31} β ig-h3 has been proposed to be involved in different embryonic systems, including chondrocyte differentiation, osteogenesis, and the formation of myotendinous junction,^{13,16,32–34} but its function still remains to be clarified.¹⁸ In this work, we show that β ig-h3 acts downstream TGF β s to regulate connective

FIG. 5. Silencing *βig-h3* upregulates the expression of chondrogenic markers. (A) βIG-H3 protein levels evaluated by ELISA in 3-day culture concentrates. Bars illustrate absorbance levels in experimental cultures subjected to gene silencing with shRNA (right bar) in comparison with untreated control cultures (left bar). ** $p < 0.01$. (B–C) Alcian blue-stained control (B) and *βig-h3*-silenced micromass cultures at day 3 of incubation. Note the mild increase in Alcian blue staining after *βig-h3* gene silencing. (D–E) QPCR analysis of the expression of chondrogenic (D) and fibrogenic (E) markers in 3-day-old micromass cultures of limb mesodermal cells transfected with shRNA for *βig-h3* gene silencing. For each gene, the left bar shows the expression level in control untreated cultures, and the right bar the expression level in experimental cultures; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Color images available online at www.liebertpub.com/tea

FIG. 6. (A) Chart showing the expression level of the *Sox9*, *type 2 Collagen (Col2α1)*, and *Hif-1α* genes in normoxic cultures (left bar), hypoxic (1% oxygen) cultures (middle bar), and hypoxic cultures with added βIG-H3 protein in the medium (right bar). Note that gene regulation observed in hypoxic cultures is abolished by adding βIG-H3 protein to the medium. ** $p < 0.01$ hypoxic cultures versus control cultures; * $p < 0.05$ hypoxic cultures versus control cultures. ## $p < 0.01$ hypoxic cultures with added βIG-H3 protein versus hypoxic cultures. # $p < 0.05$ hypoxic cultures with added βIG-H3 protein versus hypoxic cultures. (B) Expression level of *Sox9* and *type 2 Collagen (Col2α1)* in control cultures (left bar), in cultures subjected to *Hif-1α* silencing (middle bar), and in cultures subjected to *Hif-1α* silencing with added βIG-H3 protein in the medium (right bar). Note that regulation of *Sox9* and *type 2 Collagen* induced by silencing *Hif-1α* is not modified when βIG-H3 protein is added to the medium. The western blot in the upper part of the panel shows the expression of HIF-1A protein in 3-day micromass cultures of control (left lane) and sh-Hif-1α-silenced mesodermal cells. Tubulin was used as a loading control. Protein reduction was estimated in $46\% \pm 7.5\%$ in three different blots by using ImageJ software. *** $p < 0.001$; ** $p < 0.01$; *Hif-1α*-silenced or *Hif-1α*-silenced plus βIG-H3 versus control cultures.

tissue differentiation in the developing limb, likely playing a function in the establishment of the borderline between fibrous and cartilage tissue.

Transcripts of *βig-h3* are very abundant around the tip of the growing digits, in tendon primordia, and in the developing joints. The digit tip in the embryo is a zone of intense recruitment of skeletogenic progenitors.³⁵ Hence, these expression domains are consistent with studies proposing a transitory function of *βig-h3* in promoting cell adhesion to establish the prechondrogenic aggregates of cartilage mesodermal progenitors.¹⁴ In the same way, the expression domains in the tendons blastemas before differentiation may also reflect a similar role of this factor in the formation of pretendinous condensations.³⁶ However, expression of *βig-h3* is maintained in the developing tendons and joint fibrous capsules for longer periods of development, and our functional study provides evidence for a role of *βig-h3* in delaying and/or inhibiting the differentiation of mesodermal progenitors toward chondrogenesis. Thus, the expression domain of *βig-h3* in the entheses is particularly remarkable, as it fits with a role in establishing the structural transit between cartilage and tendon connective tissues.³⁷ In support of this interpretation, we show that in the presence of β IG-H3, limb mesodermal micromass cultures exhibit a significant downregulation of the *Sox9* and *Hif-1 α* genes. This finding was confirmed by loss-of-function experiments with shRNAs. Downregulation of *Sox9* and *Hif-1 α* mediated by β IG-H3 occurred without precocious changes in the expression of *Scleraxis* and other tendinous markers. *Sox9* is an essential regulator of chondrogenesis,¹ and its inactivation in limb mesenchyme results in the absence of cartilage formation.³⁸ *Hif-1 α* is a key prochondrogenic factor^{39,40} that directs the differentiation of mesenchymal stem cells and fibroblasts to chondrocytes in hypoxic microenvironmental conditions.^{41,42} Furthermore, it has been shown that *Hif-1 α* positively regulates *Sox9* and *type 2 Collagen* in connective tissue progenitor cells.^{39,42,43}

Chondrogenesis and fibrogenesis are divergent, but reversible, differentiation pathways of connective tissue progenitors¹ that are regulated by gradations in the relative expression rate of the transcription factors *Sox9* and *Scleraxis*.^{8,11,12,37} From our results, β IG-H3 appears as an indirect positive modulator of fibrous differentiation, acting as a repressor of *Sox9* expression rather than promoting profibrogenic regulators such as *Scleraxis*, *Tgif1*, or *Mohawk*.^{44,45} The negative regulation of *Hif-1 α* by *βig-h3* provides a molecular explanation for its antichondrogenic influence. *Hif-1 α* , as *βig-h3*, is expressed in the limb mesenchymal aggregates.^{39,40} The absence of blood vessels in these aggregates generates a hypoxic microenvironment that induces *Hif-1 α* gene expression and stabilizes HIF-1 α protein. HIF-1 α dimerizes with its partner HIF-1 β , forming a transcription factor, which promotes cartilage differentiation.³⁹ In our study, we have observed that the antichondrogenic influence of exogenous β IG-H3 is lost in cultures of mesodermal cells subjected to *Hif-1 α* silencing. Furthermore, the positive influence of hypoxia on the chondrogenic outcome of mesodermal cultures is abolished by adding β IG-H3 to the medium. Hence, β IG-H3 may counteract the prochondrogenic influence of *Hif-1* in hypoxic zones of the mesenchymal aggregates destined to form tendons, entheses, and joint capsules.

As expected from the functional position of *βig-h3* in the TGF β signaling cascade,¹⁸ their expression pattern in the developing limb bud is coincident with that of the TGF β 2 and 3 genes and with the domains of active (phosphorylated) Smad 2 and Smad 3.^{28,35,46} TGF β s are important regulatory signals during limb skeletogenesis.³⁵ Phenotypes detected in mice with alterations in this pathway include severe joint and tendon alterations.⁴⁻⁷ At the molecular level, TGF β signaling is highly dependent on the activity of a number of different factors that are able to modulate the response of target cells to these cytokines.⁹ There is increasing evidence showing that TGF β s may act either as prochondrogenic or profibrogenic factors in a cell context-dependent fashion.^{4,8,47,48} The prochondrogenic influence of TGF β s requires a functional association with other factors such as BMPs.^{35,49} In the absence of appropriate regulators, TGF β s inhibit chondrogenesis and promote fibrogenesis. In previous studies, we identified *Tgif1* as a profibrogenic modulator of TGF β signaling.⁸ *Tgif1* is a transcriptional co-repressor downstream TGF β /Smad2-3 signaling, which blocks the positive influence of TGF β s on *Sox9* gene expression and promotes the expression of tendon matrix markers.⁸ Here, we show that profibrogenic TGF β treatment of limb mesodermal cultures induces an intense and precocious upregulation of *βig-h3* through the canonical intracellular Smad-signaling pathway. Furthermore, *βig-h3* is upregulated in micromass cultures that overexpress *Tgif1*, thus supporting the implication of *βig-h3* in fibrogenesis.

Collectively, our findings identify *βig-h3* as a TGF β -dependent antichondrogenic signal that is active in the profibrogenic pathway and is expressed in joint capsules, tendons, and in zones of transition between cartilage and these structures. This observation may be of interest in regenerative medicine to drive the differentiation of mesenchymal stem cells toward tendon/fibrous tissue.

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Disclosure Statement

No competing financial interests exist.

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