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Pharmacological Regulation of In Situ Tissue Stem Cells Differentiation for Soft Tissue Calcification Treatment

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ABSTRACT

Calcification of soft tissues, such as heart valves and tendons, is a common clinical problem with limited therapeutics. Tissue specific stem/progenitor cells proliferate to repopulate injured tissues. But some of them become divergent to the direction of ossification in the local pathological microenvironment, thereby representing a cellular target for pharmacological approach. We observed that HIF-2alpha (encoded by EPAS1 inclined form) signaling is markedly activated within stem/progenitor cells recruited at calcified sites of diseased human tendons and heart valves. Proinflammatory microenvironment, rather than hypoxia, is correlated with HIF-2alpha activation and promoted osteochondrogenic differentiation of tendon stem/progenitor cells (TSPCs). Abnormal upregulation of HIF-2alpha served as a key switch to direct TSPCs differentiation into osteochondral-lineage rather than teno-lineage. Notably, Scleraxis (Scx), an essential tendon specific transcription factor, was suppressed on constitutive activation of HIF-2alpha and mediated the effect of HIF-2alpha on TSPCs fate decision. Moreover, pharmacological inhibition of HIF-2alpha with digoxin, which is a widely utilized drug, can efficiently inhibit calcification and enhance tenogenesis in vitro and in the Achilles's tendinopathy model. Taken together, these findings reveal the significant role of the tissue stem/progenitor cells fate decision and suggest that pharmacological regulation of HIF-2alpha function is a promising approach for soft tissue calcification treatment. STEM CELLS *2016;34:1083–1096*

SIGNIFICANCE STATEMENT

Calcification of soft tissues, such as heart valves and tendons, is a common public health concern with limited understanding and treatment options. Manipulating endogenous stem cells with small molecules has been proposed as a therapeutic strategy, but practical approaches are still unavailable. We have elucidated that HIF-2a acts as a crucial mediator of soft tissue calcification, by directly inhibiting Scx expression and regulating stem cells lineage differentiation. And we show that a pharmacological inhibitor of HIF-2a, digoxin, which is a widely utilized drug, can efficiently inhibit calcification and enhance tenogenesis in Achilles tendinopathy model. These findings are of great value on uncovering the mechanism of soft tissue calcification and developing future therapeutics based on tissue stem cells fate regulation.

INTRODUCTION

Abnormal deposition of calcium in soft tissues is known as calcification. Ectopic calcification leads to elastic mismatch and mechanical complications that can increase cardiac stress and induce rupture in arteries and tendons, resulting in substantial morbidity and mortality [1]. Despite the clinical burden of ectopic calcification-related human disease, treatment options are still limited due to lack of knowledge on its pathological mechanisms.

The causes of soft tissue calcification remain unknown, although certain genetic components and environmental factors are thought to play key roles. Inflammation is considered to be closely associated with soft tissue calcification. Lymphocytes, macrophages, and dendritic cells infiltrate into plaques and release cytokines that induce calcification [2]. A positive feedback loop may form when inflammation triggers mineralization and mineralized crystals in turn induce inflammation [3]. Meanwhile, hypoxia has also

been proposed as an inductive factor of tendon calcification, given that calcification mostly appears at the avascular area, known as the "critical zone." However, the underlying mechanism behind the association of inflammation and hypoxia with ectopic calcification has not yet been uncovered. HIF-2alpha is one of the factors regulated by both inflammation and hypoxia, which was reported to be associated with osteoarthritic calcification [4, 5]. The GEO data we analyzed also showed that HIF-2alpha was upregulated in human tendinopathy and implicated its involving in soft tissues calcification.

Conversely, the roles of tissue stem/progenitor cells in soft tissue calcification diseases have not yet been fully understood. Previously, it was thought that soft tissue calcification is a passive and degenerative process, but it is now considered to be an active tissue repair process mediated by local stem/progenitor cells [6, 7], which in fact recapitulates many features of embryonic endochondral ossification. Transcription factors such as Scleraxis (Scx), EYA1/2, EGR1, and Mohawk (Mkx) have been identified as crucial instructive factors for tendon differentiation [8–10]. During embryonic development, the differentiation of tenocytes and chondrocytes from the same progenitor pool are well coordinated through transcriptional control by Scx and SRY-box containing gene 9 (Sox9), an important regulator of cartilage formation [11, 12]. However, the underlying mechanism by which stem/progenitor cells switch their lineage commitment from tenogenesis to osteochondral ossification under pathological conditions remains an enigma. Identification of factors that regulate stem/progenitor cell differentiation pathways either directly or indirectly is crucial for developing new therapies to prevent calcification in soft tissues.

Furthermore, in situ manipulation of stem/progenitor cells may allow us to control their lineage fate, thereby providing a novel therapeutic strategy. Some studies have attempted to achieve this with growth factors, but this approach has not been successful partly because of difficulties in delivering growth factors and partly because of the nonspecific pleiotropic effects of growth factors on multiple signaling pathways. One alternative to growth factors are small molecules that can promote stem cell self-renewal and/or differentiation, which can be identified by high throughput screening and molecular analysis of relevant signaling pathways.

Here, we investigated the roles of HIF-2alpha and endogenous stem cells on soft tissues calcification. Moreover, the underlying mechanism and its related therapeutic strategy for soft tissue calcification were also systematically investigated.

Materials and Methods

Study Approval

All procedures and protocols were informed consent and approval from the by the Ethics Committee under 2nd Affiliated Hospital, School of Medicine, Zhejiang University. The human tendon tissues (5–12 mm cubic) were collected from the damaged edge from ruptured tendon during the reparative surgery: One was from the Achilles's tendon of a 54-year-old male, one was from supraspinatus tendon of 60-year-old female. An independent control group was obtained comprising four samples of Achilles's tendon collected from patients undergoing amputation. The five human mitral valves (20–30 mm cubic) were collected from patients with rheumatic heart disease undergoing valve replacement. The mean age of the rheumatic heart disease patients was 62 years (range 47–84 years).

Animal Experiments

All experiments were conducted with approval of the Zhejiang University Institutional Animal Care and Use Committee. Scleraxis-green fluorescent protein reporter (Scx-GFP) mice and Scx homozygous knockout mice were kindly provided by Dr. R. Schweitzer (Oregon Health & Science University, Oregon, USA) [13] and breed in Model Animal Research Center of Nanjing University. Type I collagenase (17100017,Gibco, Grand Island, NY, http:// www.invitrogen.com) was injected into the midpoint of the right and left Achilles tendons in 8-weeks-old rats (50 U/leg) and Scx-GFP mice (12.5 U/leg). Then, digoxin (1.5 ug/leg for rat, 0.375 ug/leg, Sigma 1200000, Sigma, St. Louis, MO, http://www.sigmaaldrich.com) was injected subcutaneously into the right legs every 3 days up to 8 weeks after the collagenase injection and the left leg was given sterile saline (self control). Skin around Achilles tendons was lifted up and drug was injected into loose connective tissue around Achilles tendons without causing injury of the tendon tissue. The animals were submitted to euthanasia at indicated time period post operatively when x-ray examinations were taken and tendon tissues were processed for histological, immunohistochemical, and gene expression studies.

Datasets Analysis

The dataset of *EPAS1* expression in nonlesional and lesional tendons from patients with tendinopathy was downloaded from GSM 26051 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc5GSM 26051) [14]. Expression data were available for 23 samples.

X-ray and μCT Analysis

Achilles tendon were dissected from mice, fixed overnight in 4% paraformaldehyde and analyzed by x-ray analysis (In Vivo F/FX, Kodak, New York, American) and high-resolution μCT (μCT 100, Scanco Medical, Basserdorf, Switzerland). Briefly, the micro-CT imaging system was operated at 70 kVp and 114 μA, which was equipped with a 5 μm focal spot x-ray tube. Two-dimensional CT images were reconstructed in 1024-1024 pixel matrices using a standard convolution-back projection procedure. Images were stored in 3D arrays with an isotropic voxel size of 12 μm. The resulting gray-scale images were segmented using a low-pass filter to remove noise, and a fixed threshold was used to extract the mineralized bone phase. An equivalent hydroxyapatite density was calculated for the mineralized lesion based on the use of a manufacturer-specific calibration curve. Micro-CT parameters, whose definition and nomenclature were established by the American Society of Bone and Mineral Research (ASBMR), were measured using the image processing software version V5.07a by Scanco Medical (Basserdorf, Switzerland) [15].

Histological and Histochemical Examination of Calcific Samples

For histological analysis of calcific samples, tendons, or valves fixed in 4% (paraformaldehyde 16005 sigma) paraformaldehyde for 1 day and then decalcified in 10% (EDTA E6758, sigma) EDTA-decalcification solution for 14 days. After

embedding in paraffin, sections were deparaffinized in xylene, washed, and hydrated with washing ethanol. Finally, the paraffin-embedded sections were stained with standard hematoxylin and eosinor safranin O staining. For fluorescence observation, the histological sections were first stained with hematoxylin h9627,sigma and the positive cells were observed under fluorescence microscopy. General histological scoring was performed using hematoxylin and eosin staining. Six parameters (fiber structure, fiber arrangement, rounding of nuclei, inflammation, vascularity, cell population) were semiquantitatively assessed. This scoring system is a modification of a previous study. Safranin O staining and Bonar score scoring was performed to examine tendon destruction. For immunohistochemical staining of HIF-2alpha in cartilage tissue, paraffin sections were deparaffinized in xylene, washed, and hydrated with washing ethanol. Sections on slides were incubated for 2 hours at room temperature with primary antibodies (Supporting Information Table 1). Next, samples were incubated with HRP horse radish peroxidase linked secondary antibodies for 30 minutes. Then, we use DAB 3, 3′-diaminobenzidine solution to visualize Immunoreactive proteins.

Immunofluorescence Microscopy

We observed paraffin-embedded sections with HIF-2α ab199, Abcam, Cambridge, UK and other antigens using standard immunofluorescence microscopy. Specifically, Paraffin-embedded sections were deparaffainized, hydrated, permeabilized, and blocked for 30 minutes with 0.1% (v v-1) Triton X-100 and 1% (w/v) bovine serum albumin. After Sections were washed followed by for 1 hour incubation with a primary antibody or control IgG. Sections were incubated with fluorescein-conjugated secondary antibody (invitrogen CA11008s, invitrogen, Invitrogen Inc., Carlsbad, CA) for 30 minutes and observed under a fluorescence microscope. Cell nuclei were stained with DAPI (blue).

Pixel intensity was calculated with Image-Pro Plus Software (Media Cybernetics). For the correlation between the expression of HIF-2alpha and ScxGFP, 130 areas (40–50 for each mouse; three mice) were randomly selected in at least five different sections, and the corresponding average pixel intensities were quantified. The background pixel intensity from sections stained with only the secondary antibodies was subtracted. Linear regression and correlation (Pearson *r*) were determined with GraphPad Prism 6.04 software.

Hypoxia Probe

To detect hypoxic cells in tendons, we used the reductive 2-nitroimidazole compound pimonidazole PIM from hypoxyprobetm-1 plus kit (HP1-100,Chemicon International, Temecula, CA, which is a chemical marker for hypoxia, when administered in vivo, forms stable adducts in hypoxic regions that then can be identified with an anti-PIM antibody HP1-100, Chemicon International [16]. After 1 week and 8 week of collagenase injection, the mice were injected i.p. with PIM hydrochloride (Chemicon International, Temecula, CA) at 120 mg/kg dose, and the tendons and thymus harvested after 3 hours postinjection. The Achilles tendons and thymocytes were embedded in paraffin. Sections were deparaffinized in xylene, washed, and hydrated with washing ethanol. We stained paraffin-embedded sections with a mouse monoclonal anti-PIM antibody (Hypoxyprobe-1 Kit; Chemicon International, Temecula, CA) or a mouse IgG1 isotype control antibody for 1 hour at 37 C in the presence of a protein blocker (DakoCytomation, Carpinteria, CA). Then, sections were incubated with HRP linked secondary antibodies for 30 minutes. Finally use DAB solution visualize Immunoreactive proteins.

Cell Culture

We obtained human fetal Achilles tendon samples from an aborted embryo (age 5 months) following the approved guidelines set by the Women's Hospital School of Medicine Zhejiang University Institutional Review Board and Institutional Animal Care and Use Committe e [17]. Tenocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, low glucose; Gibco, Grand Island, NY, http://www.invitrogen.com) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, http://www.invitrogen.com-Gibco) and 1% penicillin-streptomycin (Gibco). For TSPCs selection: the cells were cultured in DMEM supplemented with 20% FBS and penicillin-streptomycin to form colonies. Primary TSPCs were washed three times with serum-starved medium and incubated for 3 hours with serum-starved medium (3% FBS). Serum-starved TSPCs were either untreated or treated with IL-1β (5 ng/ml) and digoxin (50 nM, 100 nM) for indicated time periods. Newly confluent cells were maintained under normoxic conditions or were exposed to hypoxia for 24 hours to 2 weeks in a GasPak anaerobic chamber (BBL GasPak Pouch; Becton Dickinson, Sparks, MD) at 37 C with oxygen equal to 2%.

Infection and Transfection

Expression vectors encoding Epas1-CA and Epas1-DN were purchased from addgene [18]. Sequence of Anti-EPAS1 short hairpin RNA-1 (sh1): Forward oligo: 5′ CCGGCAGCATCTTTGATAGCAGTCTCGAGACTG CTATCAAAGATGCTGTTTTTG 3′; Reverse oligo: 5′ AATTCAAAAACAGCATCTTTGATAGCAGTCTCGAGACTGCTATCAAAGATGCTG 3′. Sequence of Anti-EPAS1 short hairpin RNA-2(sh2): Forward oligo: 5′ CCGGCGGGCCAGGTGAAAGTCTACTCGAGTAGACTTTCACC TGGCCCGTTTTTG 3′; Reverse oligo: 5′ AATTCAAAAACGGGCCAGGTGAAAGTCTACTCGAGTAGACTTTCACCTGGCCCG 3′. For retroviral transductions, cells were passaged and seeded in six-well plates in medium containing 75% retroviruscontaining supernatant (DMEM) and 25% L-DMEM medium. After 12 hours, remove the virus-containing medium. Four days after the transduction, cells were replated in selection medium containing 2 g/ml puromycin, then used without further experiment.

Differentiation and Staining

We tested the multidifferentiation potential of the TSPCs of osteogenesis and tenogenesis as described previously [19]. Primary TSPCs were either untreated or treated with IL-1β (5 ng/ml) and digoxin (50 nM, 100 nM) during induction. Osteogenic differentiation was induced by ascorbic acid, dexamethasone, and bglycerol phosphate. After 1 week, ALP activity was assayed using a BCIP/NBT alkaline phosphatase color development kit (Beyotime Institute of Biotechnology). DAPI (Beyotime Institute of Biotechnology) was used to stain nuclei. Calcium deposits were detected by staining with 2% alizarin red S (pH 4.2; Sigma) after 2 weeks. To quantify the stained nodules, the stain was solubilized with 0.5 ml 5% SDS in 0.5 N HCl for 30 minutes at room temperature. Absorbance was measured at 405 nm. Tenogenesis differentiation was induced in confluent TSPCs cultures by treated with ascorbic acid for 14 days.

RT-PCR

Total cellular RNA was isolated by lysis in TRIZOL (Invitrogen) and 1 μg of total RNA was used for cDNA synthesis (BioRad Laboratories). cDNA samples were diluted 1:10 and real-time PCR was performed using primers (Supporting Information Table 2), SYBR Green Supermix (Takara) and the iCycler Real-time PCR Detection System (Bio-Rad).

Western Blotting

All proteins were detected in whole cell lysates using following antibodies: anti-HIF-2alpha (Abcam Inc.), anti-gapdh (Cell Signaling, Danvers, MA), anti-actin (Cell Signaling, Danvers, MA).

Statistical Analysis

All quantitative data are presented as means $\pm$ s.e.m. One-way analysis of variance (ANOVA) and Student's t test was performed to assess whether there were statistically significant differences in the results between groups. Values of $p < .05$ were considered to be statistically significant. The significance level is presented as either *, $p < .05$ or **, $p < .01$.

Results

HIF-2alpha is Highly Expressed in Stem Cells Localized Within Calcified Tendons and Mitral Valves

From a global gene expression profiling of human tendinopathy (GSE26051), which consists of 23 patients undergoing surgical procedures for the treatment of chronic tendinopathy, we selectively examined several important processes, such as bone formation and vascularization, based on gene ontology (GO) function enrichment analysis. Based on previous analyses of other tissues [5], we further screened transcription factors among the genes upregulated under pathological conditions and selected HIF-2alpha as our primary target for detailed investigations. EPAS1 was significantly upregulated in human lesional tendons compared to nonlesional controls (Fig. 1A). To explore the role of HIF-2alpha in soft tissue calcification, we compared the expression levels of HIF-2alpha from uncalcified and calcified human tendons and mitral valves. We confirmed the calcific lesions in tendon and mitral valve biopsies according to histological staining (Fig. 1B, 1C). Increased EPAS1 protein levels were evident in calcific tissues as determined with immunostaining for HIF-2alpha (Fig. 1B, 1C), while HIF-1alpha stabilized mostly in cells which located at uncalcific sites rather than calcific sites (Supporting Information Fig S2A-2C). To further confirm the dysregulation of HIF-2alpha in tendinopathy, we induced tendinopathy in rats by collagenase injection into Achilles's tendons. By 8 weeks after collagenase injection, rats developed calcified tendinopathy [20], as evident from safranin O staining and Bonar Score [21] (Fig. 1D). Immunostaining and Western blot analyses revealed a similar upregulation of HIF-2alpha expression in calcific tendons, as early as 2 weeks after collagenase injection (Fig. 1D-1F and Supporting Information Fig. S1A). Notably, HIF-2alpha was primarily localized in intermediate cells between healthy tenocytes (which were stained light green in SO staining and negative for OCN staining) and osteoblasts (which were stained purple in SO staining and immunoactive for OCN staining) (Fig. 1D).

To further characterize the cellular identity of these intermediate cells, we utilized a transgenic reporter mouse strain that expresses the GFP marker driven by Scleraxis promoter (ScxGFP) [22], which is a marker for tenocytes and their progenitors. Immunostaining analyses for the chondrogenic master gene Sox9 in calcific tendons from ScxGFP mouse revealed that loss of ScxGFP and gain of Sox9 expression gradually spread from normal tendon tissues to the calcific regions, with a transient Scx+/Sox9 + stage (Fig. 1G). Previous studies revealed a critical role of HIF-2alpha in chondrocytes hypertrophy and cartilage degradation in endochondral ossification [4, 5], a process which has been partly recapitulated during ectopic calcification [6, 7]. We examined HIF-2alpha activation in chondrocytes which resides in calcific tendons by immunostaining for both HIF-2alpha and COL2A1, a marker of chondrocytes. Surprisingly, ovoid-like cells which express high level of HIF-2alpha and chondrocytes which synthesis COL2A1 appeared to be two distinct populations that closely associated with each other (Fig. 1H and Supporting Information Fig. S1B). To examine whether cells located at these transitional regions contain tendon progenitor/stem cells (TSPCs), we performed immunostaining for stro-1, CD90, and CD44 (markers related to TSPCs [23]) to visualize their distribution throughout the tendon. We observed a robust activation of HIF-2alpha in TSPCs predominantly located in the vicinity of calcified sites (Fig. 1I, 1J). Collectively, these findings demonstrate that HIF-2alpha expression level is increased in stem/progenitor cells localized within calcified tendons and heart valves.

HIF-2alpha is Activated by Pro-Inflammatory Cytokines, not Hypoxia, in Tendinopathy

To mechanistically analyze how HIF-2alpha is induced in calcified tissues, we examined *EPAS1* mRNA and protein expression levels in primary human TSPCs cultured under pathological conditions, such as hypoxia and interleukin (IL)-1β treatment. As described previously, both the proinflammatory cytokine IL-1β and hypoxia (2% O_2) elevated EPAS1 protein expression levels in vitro, but only IL-1β enhanced *EPAS1* mRNA levels (Fig. 2A, 2B, Supporting Information Fig. S3A and 3B). In contrast, HIF-1alpha only stabilized under hypoxia treatment (Supporting Information Fig. S2D). To detect proinflammatory stress in vivo, we performed immunostaining for v-rel avian reticuloendotheliosis viral oncogene homolog A (p65 or RELA), since NF-κB is known to be potent regulators of *Epas1* expression induced by IL-1β [4, 5]. And to detect hypoxic cells in calcified tendons, we utilized pimonidazol (PIM) as a chemical marker of hypoxia, which forms stable adducts in hypoxic regions after administration in vivo, and which can be subsequently detected by immunostaining [24]. Interestingly, we find that high level of HIF-2alpha and accumulation of p65 in transitional regions between healthy tendon tissues and calcified sites, whilst lacking staining of the hypoxia marker PIM (Fig. 2C and Supporting Information Fig. S4A-4F). This thus indicated that proinflammatory condition, rather than hypoxia, is associated with HIF-2alpha activation in vivo. Accordingly, we examined the association of tendinopathy phenotype with IL-1β expression and hypoxia. Various metalloproteinases such as MMP3, MMP9 are upregulated in diseased tendons, which contributes to degeneration of

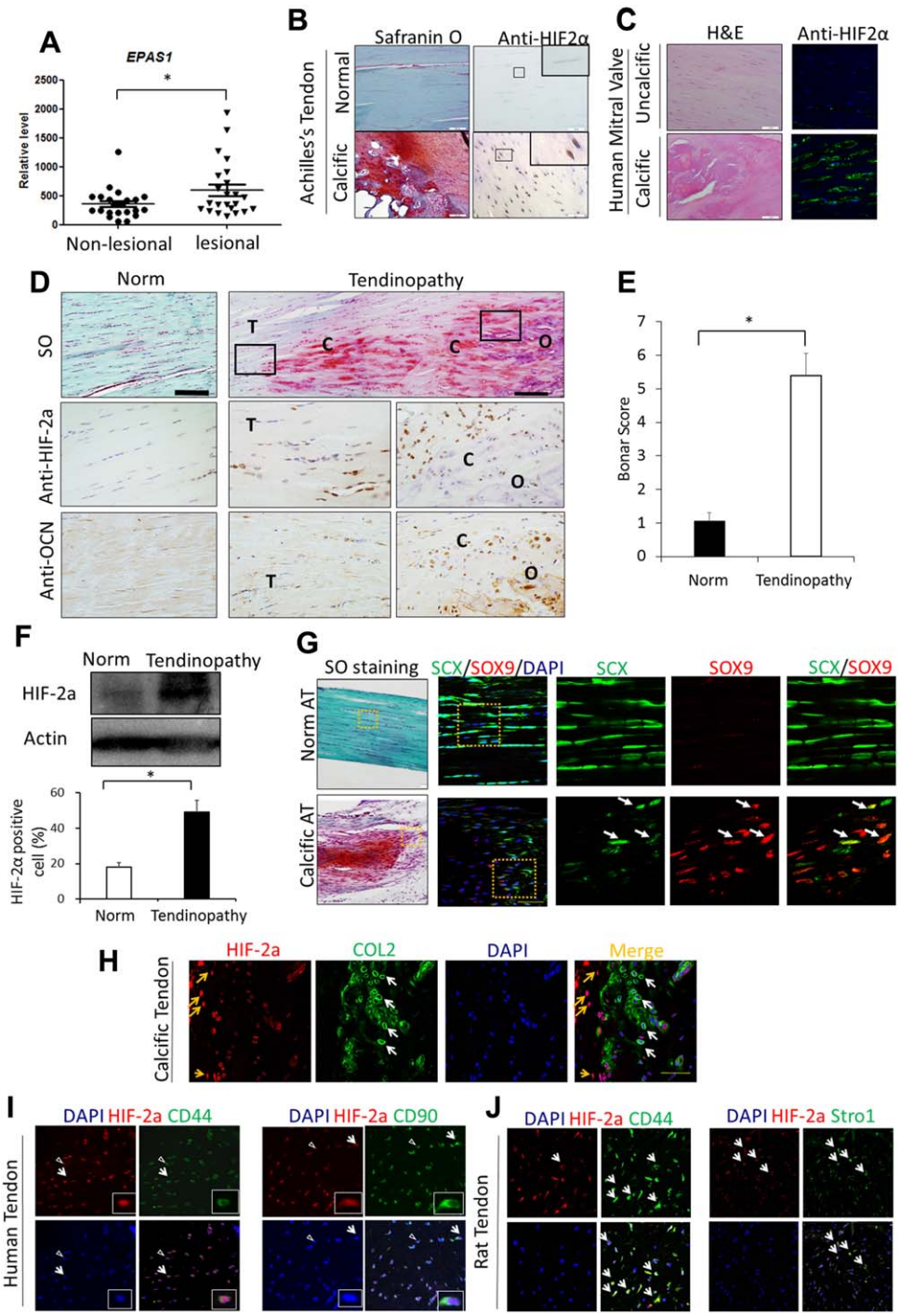

Figure 1. Upregulation of HIF-2alpha in human calcific mitral valves and tendons. **(A):** *EPAS1* mRNA expression detected on microarray analysis of human tendinopathy and normal tendon samples (*, $p < .05$, two-tailed Student's test, $n = 23$ per group). **(B):** Representative images of Safranin O staining (left) and immunohistochemical staining of HIF-2alpha (right) in normal and calcific human tendons. Scale bar, 200 μm. **(C):** H&E staining (left) and immunohistochemical staining of HIF-2alpha (right) in uncalcific and calcific human mitral valves. Scale bar, 50 μm. **(D):** Safranin O staining (up), immunohistochemical staining of HIF-2alpha (middle) and OCN (down) of rat normal and tendinopathy samples. Areas of tenocytes (T), chondrocytes (C), and osteoblasts (O) are indicated in tendinopathy samples. Scale bar, 200 μm. **(E):** Bonar score from both normal and tendinopathy rat Achilles's tendons. Values are means $\pm$ s.e.m. ($n \geq 6$) **(F)** Up: Western blot analyses of *EPAS1* from both normal and calcified rat Achilles's tendons. Down: The histogram provides a quantitative summary of the data in (D), presented as means $\pm$ s.e.m. ($n \geq 3$) **(G):** Safranin O staining and immunofluorescence staining showing SOX9 activation in calcific tendons of Scx-GFP mice. $SCX^{+}/SOX9^{+}$ cells are indicated by white arrowheads. Scale bar, 50 μm. **(H):** Immunofluorescence staining showing close association of HIF-2alpha (yellow arrowheads) and COL2A1 (white arrowheads) in calcific rat tendons. Scale bar, 50 μm. **(I, J):** Immunofluorescence staining showing HIF-2alpha activation in stem/progenitor cells ($CD44^{+}/CD90^{+}$/Stro-1^{+}) of calcific human (I) and rat (J) tendons. Scale bar, 50 μm. The box is present to indicate an area which is zoomed in. Abbreviation: H&E, hematoxylin and eosin.

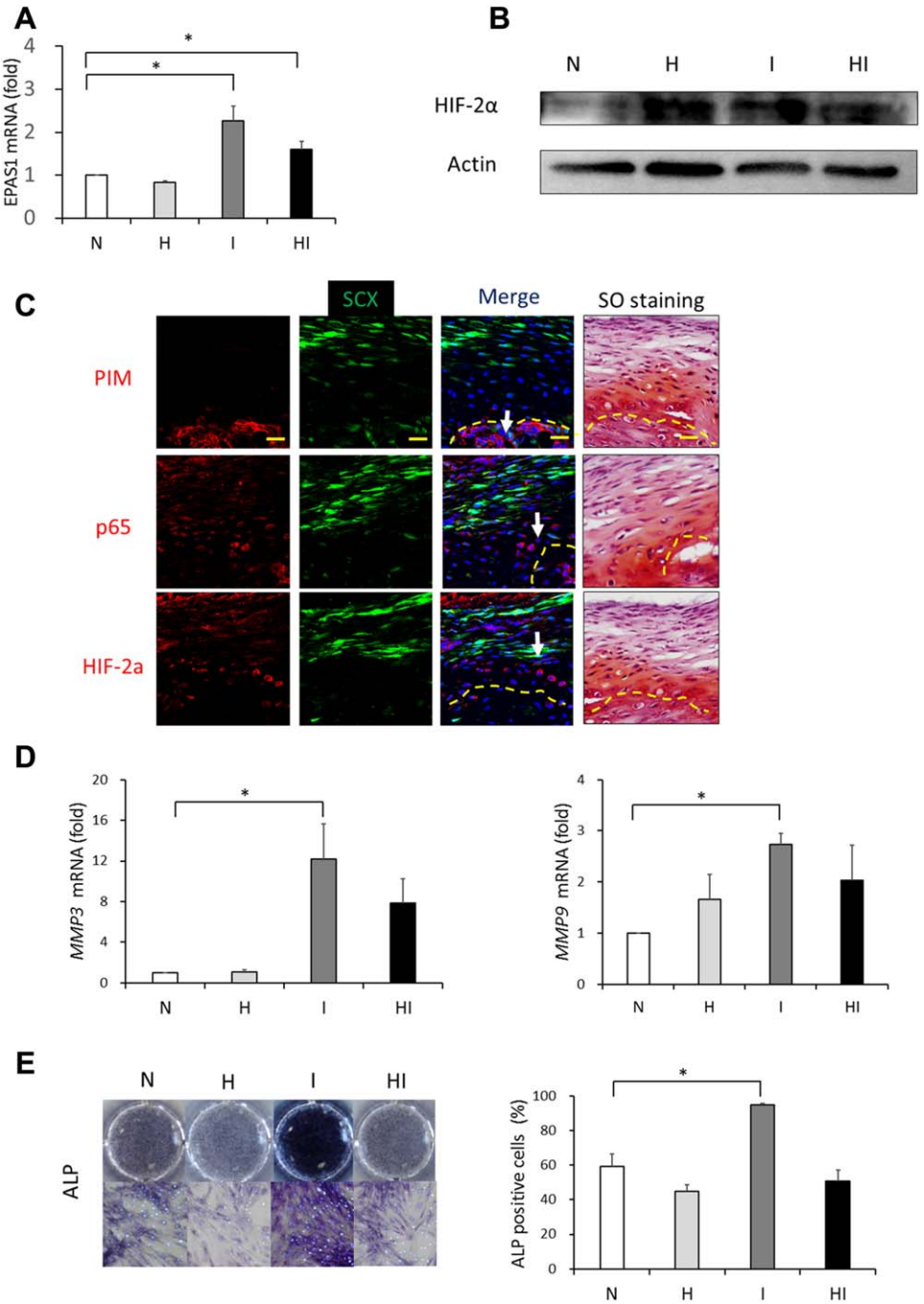

Figure 2. Regulatory mechanisms of *EPAS1* activation in tenocytes. (A, B): qPCR (A) and Western blot (B) analyses of *EPAS1* in human TSPCs treated with 2% O_2 (H), 5 ng/ml IL-1β (I) or combined (HI) for 48 hours with actin as loading control. Values in (A) are means ± s.e.m. ($n \geq 3$). *, $p < .05$ as compared to untreated control. **(C):** Safranin O staining and immunofluorescence staining of hypoxia probe (PIM), p65 and HIF-2alpha in Scx-GFP mice calcific tendons. Examples of cells that were either PIM^+, $p65^+$, HIF-2alpha$^+$ are indicated with white arrowheads. Scale bars, 50 μm. The dash lines are present to indicate transitional area between uncalcified and calcified tissues. **(D):** qPCR analyses of MMPs in human TSPCs treated 2% O_2 (H), 5 ng/ml IL-1β (I) or combined (HI) for 48 hours. Values are means ± s.e.m. ($n \geq 3$). *, $p < .05$. **(E):** ALP staining in human TSPCs cultured in osteogenesis induction medium in the presence of 2% O_2 (H), 5 ng/ml IL-1β (I) or combined (HI) for 1 weeks. Values are means ± s.e.m. ($n \geq 3$). *, $p < .05$. One-way analysis of variance (ANOVA). Abbreviations: ALP, Alkaline phosphatase; MMPs, matrix metalloproteinases; PIM, pimonidazole; TSPCs, tendon stem/progenitor cells.

tendon extracellular matrix [25]. We found that IL-1β is associated with significantly increased expression of *MMP3* and *MMP9* in primary cultured human TSPCs, while hypoxia alone is not (Fig. 2D). It indicated that IL-1β, rather than hypoxia, contributes to degeneration of tendon extracellular matrix through triggering expression of various catabolic factors. In addition, IL-1β treatment increased calcification parameters in primary cultured human TSPCs, which were decreased on hypoxia treatment (Fig. 2E). These results are similar with the findings that hypoxia maintain the stemness and decreases

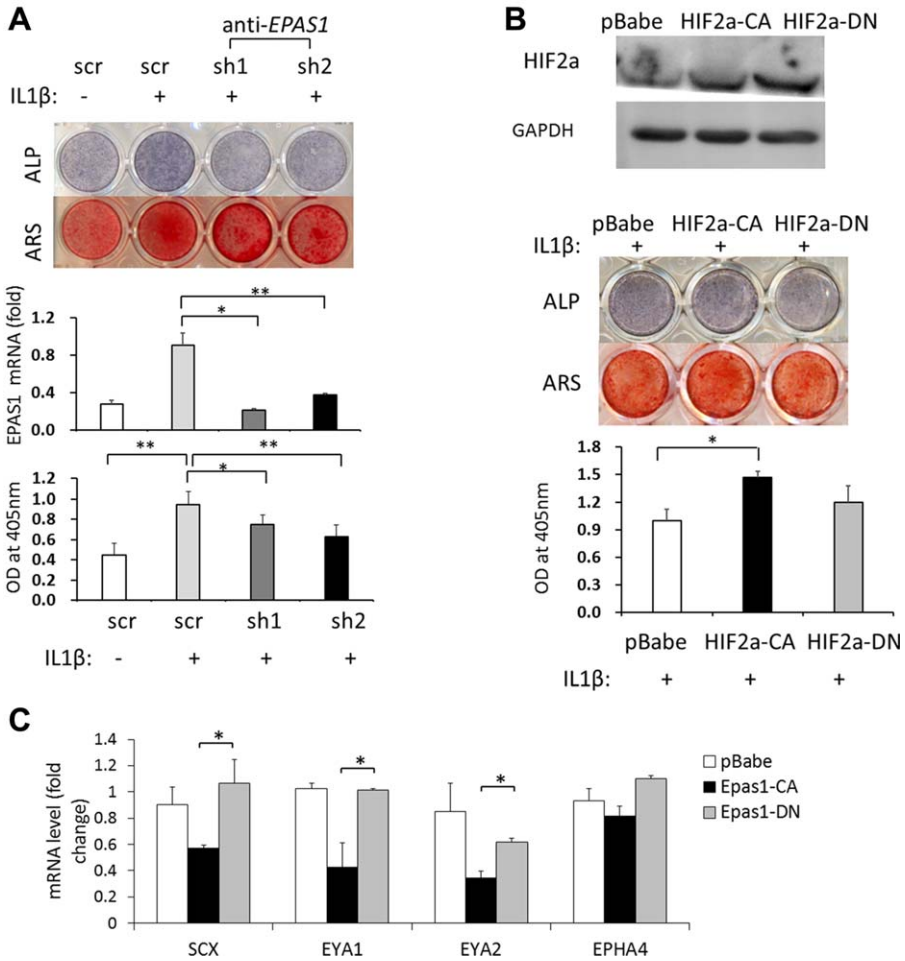

Figure 3. HIF-2alpha promotes calcification and suppresses tenogenesis in human TSPCs. **(A):** ALP and ARS in human TSPCs retrovirally transfected with control or shRNA specific for *Epas1* after culture for 2 weeks with osteogenesis induction and IL-1β (5 ng/ml) treatment. mRNA levels of HIF-2alpha were confirmed by qPCR. Values are means $\pm$ s.e.m. ($n \geq 3$). **(B):** Analyses of ALP and ARS in human TSPCs retrovirally transfected with empty vector or HIF-2alpha mutants at oxygen-dependent hydroxylation residues causing enhancement (P405A and P531A, HIF2alpha-CA) and abrogation (Δ820-870, HIF2alpha-DN) under the culture conditions used in (A). Protein levels of HIF-2alpha were confirmed by Western blot, with the GAPDH level as loading control. Values are means $\pm$ s.e.m. ($n \geq 3$). **(C):** qPCR analyses of *SCX, MKX, EYA1, EYA2, SIX2, EPAH4*, and *COL14* in human TSPCs retrovirally transfected with empty vector, HIF2alpha-CA, or HIF2alpha-DN after treated with tenogenesis induction and IL-1β (5 ng/ml) for 7 days. Values are means $\pm$ s.e.m. ($n \geq 3$). *, $p < .05$, **, $p < .01$. One-way analysis of variance (ANOVA). Abbreviations: ALP, Alkaline phosphatase; ARS, Alizarin red staining; TSPCs, tendon stem/progenitor cells.

differentiation of mesenchymal stem cells [26, 27], while proinflammatory cytokines promotes osteogenic differentiation [28]. Taken together, these data suggests that HIF-2alpha activation in calcified tissues is due to proinflammatory cytokines, rather than hypoxia.

HIF-2alpha Suppresses Tenogenesis and Promotes Calcification of TSPCs In Vitro

TSPCs are identified as a cell population that possess self-renewal capacity, as well as multipotent differentiation potential toward tenocytes, adipocytes, chondrocytes, and osteocytes in vitro [23]. During development, $Scx^{+}/Sox9^{+}$ progenitors are distributed across the enthesis (tendon to bone junction) and give rise to tenocytes and entheseal chondrocytes in vivo [11, 12]. Hence, we investigated whether HIF-2alpha can alter cell fate specification of TSPCs. We constructed retroviral vectors carrying a small hairpin RNA (shRNA) specific against HIF-2alpha. A significant reduction in HIF-2alpha expression at the mRNA levels was observed for cells transfected with both shRNAs relative to controls, with a decrease of 78% and 65%, respectively (Fig. 3A). HIF-2alpha knockdown had no effect on apoptosis and transfected cells continued to proliferate (Data not shown). Knockdown of HIF-2alpha significantly decreased alkaline phosphatase activity and alizarin red staining of TSPCs cultured under osteoinductive conditions with IL-1β treatment (Fig. 3A). Under IL-1β treatment, ectopic expression of HIF-2alpha by transfection with HIF-2alpha mutants bearing mutation causing enhancement of HIF-2alpha transactivation activity (P405A and P531A, HIF2alpha-CA) resulted in increase of ossification parameters, while all ossification parameters were decreased by overexpression of a mutant of HIF-2alpha causing abrogation of HIF-2alpha transactivation activity (P405A, P531A and Δ820-870, HIF2alpha-DN) (Fig. 3B). We then examined the effect of HIF-2alpha on tenocyte lineage differentiation of human TSPCs. Overexpression of constitutive-active forms of HIF-2alpha

suppressed expression of tenogenic markers, such as *SCX, EYA1*, and *EYA2* during induction of tenogenesis (Fig. 3C). In contrast, inhibition of HIF-2alpha dominant-negative mutant rescued expression of tenogenic markers, such as *SCX, EYA1*, and *EYA2*, during tenogenesis (Fig. 3C). These data suggest that HIF-2alpha signaling plays a significant role in lineage fate specification of tenocytes and osteoblasts derived from TSPCs.

Scleraxis is Suppressed by HIF-2alpha During Calcification

Although many studies have established the critical role of Scx (Scleraxis) during tendon and heart valve development [8, 29], there is little evidence of Scx function in the pathogenesis of tendon degeneration [30]. We, therefore, investigated whether loss of Scx function contribute to ectopic calcification pathogenesis. ScxGFP was highly expressed in a majority of tenocytes in normal *ScxGFP* mouse [31], but in calcific lesions it was robustly downregulated (Fig. 4A and Supporting Information Fig. S2A). Several potential hypoxia-responsive elements were noted in the regulatory regions upstream and downstream of the transcription start site of the human *SCX* gene (Supporting Information Fig. S2B). In addition, we analyzed HIF-2alpha expression by immunofluorescent staining and found that the ScxGFP correlated with upregulation of HIF-2alpha in calcific lesions (Fig. 4A), which indicates that the absence of Scx is associated with activation of HIF-2alpha in vivo. We also found that *SCX* transcription was downregulated in calcific tendons compared with normal tendons (Fig. 4B) and in IL1β-treated human TSPCs (Fig. 4C). *SCX* expression was significantly derepressed in human TSPCs on HIF-2alpha inhibition with either infection of HIF2alpha-DN (Fig. 4D) or anti-*Epas1* siRNA ($\sim$70%, Fig. 4E). These results suggest that Scx could be a direct target of HIF-2alpha signaling.

To test the importance of Scx function directly in calcification, we induced tendinopathy in Scx-knockout mice. As homozygotes ($Scx^{-/-}$) mouse line presented severe defects in tendon formation, we utilized heterozygotes ($Scx^{+/-}$) instead. Achilles's tendon in $Scx^{+/-}$ mouse underwent more severe calcification at 8 weeks after collagenase injection compared to WT mouse (Fig. 4F). To understand the importance of Scx as a HIF-2alpha target in calcification, we overexpressed Scx in *Epas1*-CA transfected TSPCs and grew these cells in osteogenic medium. On Scx overexpression, the increase in ossification parameters resulted from HIF-2alpha activation was rescued (Fig. 4G). Together, these results provide evidence that Scx is a key downstream effector of HIF-2alpha during calcification. In response to proinflammatory stress, HIF-2alpha-mediated downregulation of Scx lead to alteration of TSPCs fate specification and ectopic calcification in tendons.

Digoxin Impedes Calcification and Promotes Tenogenesis of TSPCs In Vitro Through Inhibition of HIF-2alpha

Because digoxin inhibits the HIF-2alpha pathway in cancer cells [32], we investigated whether it could also do the same with TSPCs. A dose-response study revealed that exposure of human TSPCs to digoxin at concentrations $\geq$50 nM for 24 hours inhibited IL1β-induced expression of HIF-2alpha protein without inhibition of mRNA expression (Fig. 5A, 5B), which indicated that digoxin inhibits HIF-2alpha at the translational rather than the transcriptional level. We then cultured TSPCs in 50 and 100 nM digoxin to investigate its effect on their fate decision. Digoxin significantly decreased the activity of alkaline phosphatase and Alizarin red staining of TSPCs undergoing osteogenic induction and IL1β treatment (Fig. 5C, 5D) without significant effect on cell apoptosis and proliferation (Data not shown). Digoxin treatment also downregulated expression level of osteogenic gene, such as *OCN, OPN*, and *VEGF* (Fig. 5E). In contrast, the expression levels of transcriptional factors for tendon differentiation, such as *SCX, MKX, EYA1*, and *EYA2*, were increased under digoxin treatment (Fig. 5F). Digoxin treatment also increased the synthesis of tendon extracellular matrix, such as *TNC* and *COL4*, during tenogenic induction (Fig. 5F). Taken together, these results indicate that digoxin effectively inhibits HIF-2alpha upregulation induced by IL1β and promotes tenogenesis and inhibits calcification of TSPCs in vitro.

Digoxin Inhibits Calcification and Enhances Tenogenesis In Vivo

Commitment of TSPCs toward tenogenic differentiation or osteogenic and chondrogenic differentiation, plays a key role in tendon regeneration and pathogenesis [23]. To evaluate whether digoxin could provide any therapeutic benefit, digoxin was subcutaneously injected into the loose connective tissues around Achilles's tendon and sterile saline was injected into the other leg as control every 3 days for 8 weeks after collagenase injection (Fig. 6A). To evaluate the delivery efficiency, we analyzed protein expression levels of HIF-2alpha after 8 weeks treatment and observed successful inhibition of HIF-2alpha in digoxin treated animals (Fig. 6H). X-ray quantification showed that compared with the self-controlled group, digoxin administration led to less ectopic calcification in Achilles's tendons (Fig. 6B, 6C). We observed a general reduction of Safranin O-stained GAG deposition and Bonar score in tendons of digoxin treated animals (Fig. 6D, 6E). Consistent with the histological examination, the upregulation of COL2A1 (Fig. 6F) and α-SMA expression (Fig. 6H) was also attenuated by digoxin treatment. When we focused on the tendon regeneration, we also observed that digoxin treatment was able to rescue the downregulation of tendon-specific transcription factors *Scx* under pathological conditions (Fig. 6G). Furthermore, histological examination and immunostaining of tendon extracellular matrix proteins, such as COL3, demonstrated that digoxin treatment was beneficial for tendon matrix synthesis (Fig. 6D, 6H). Hence, digoxin can promote teno-lineage fate specification during tendon repair and inhibit calcification progression.

Discussion

The data presented in this study clearly demonstrated that the endogenous stem cells and HIF-2alpha signaling play significant roles in soft tissues calcification, and that ectopic calcification can be inhibited by digoxin via modulation of the HIF-2alpha signaling that control stem cell fate (Fig. 7). In particular, the major findings are as follows. First, data from both our in vitro and in vivo studies indicate that HIF-2alpha activation in stem/progenitor cells of calcified tissues is due to proinflammatory conditions. The gain- and loss- of function

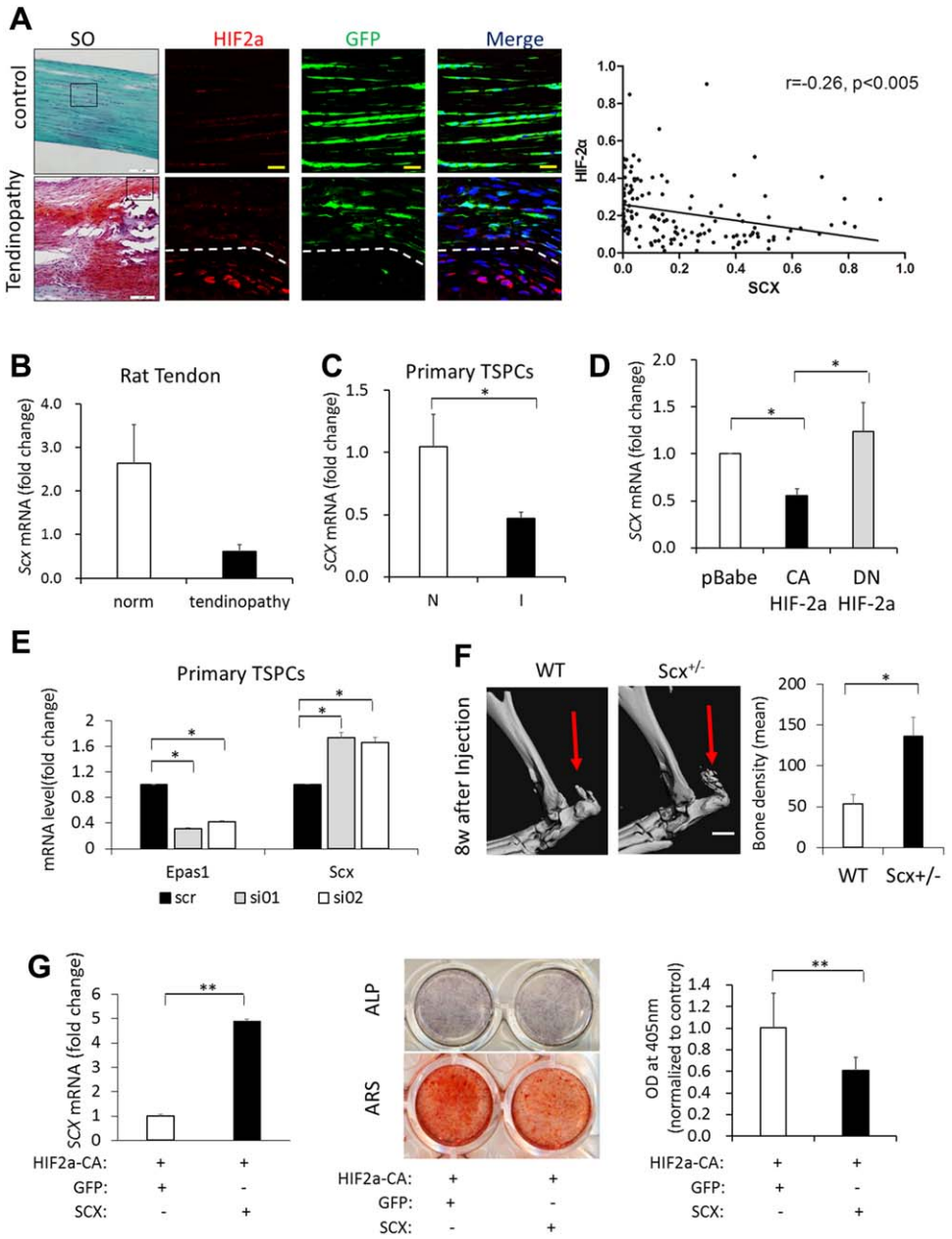

Figure 4. Scleraxis is suppressed by HIF-2alpha during calcification. **(A):** Safranin O staining (left) and HIF-2alpha immunofluorescence staining (right) in calcific tendons from Scx-GFP mice. Scale bar, 100 μm. The dash lines are present to indicate transitional area between uncalcified and calcified tissues. The average intensity of each signal was evaluated in 130 randomly selected regions of calcific tendons. The correlation between the values in each region is shown. Linear regression is indicated. Pearson r, -0.26 ($p < .005$; two-tailed t test). (B, C): qPCR analyses of *Scx* expression in rat normal and tendinopathy samples (B) and primary human TSPCs cultured under IL-1β (5 ng/ml) for 48 hours (C). Values are means $\pm$ s.e.m. ($n \geq 3$). **(D):** qPCR analyses of *Scx* expression in human TSPCs retrovirally transfected with empty vector, HIF2alpha-CA, or HIF2alpha-DN. Values are means $\pm$ s.e.m. ($n \geq 3$). **(E):** qPCR analyses of *Epas1, Scx* expression in human TSPCs whether transfected with control siRNA or siRNA specific for *EPAS1* and exposed to IL-1β (5 ng/ml) for 48 hours. Values are means $\pm$ s.e.m. ($n \geq 3$). **(F):** MicroCT examination and bone density quantification of Achilles's tendons sections from WT ($+/+$) and Scx$^{+/-}$ mice after collagenase injection for 8 weeks. Scale bar, 1 mm. Values are means $\pm$ s.e.m. ($n \geq 4$). **(G):** ALP and ARS in human TSPCs transfected with HIF2alpha-CA and empty vector or Scx after culture for 2 weeks with osteogenesis induction and IL-1β (5 ng/ml). mRNA levels of Scx were confirmed by qPCR analyses. Values are means $\pm$ s.e.m. ($n \geq 3$). *, $p < .05$. Abbreviations: ALP: alkaline phosphatase; ARS, alizarin red staining; Scx-GFP, scleraxis-green fluorescent protein reporter; TSPCs, tendon stem/progenitor cells.

studies demonstrate that HIF-2alpha plays a crucial role in cell fate decisions of TSPCs. Second, we showed that digoxin inhibits the HIF-2alpha signaling pathway in human TSPCs and that this inhibition effectively promotes tenogenesis, as well as impedes calcification both in vitro and in vivo. Third, we identified tendon-specific transcription factor Scleraxis (Scx) as a critical target of HIF-2alpha. Hence, digoxin treatment act as a modulator of the HIF-2alpha signaling during soft tissue calcification.

Transitions between cellular states are known to play critical roles in tissue development, homeostasis, and regeneration [33–37]. The conception that aberrant behavior of resident

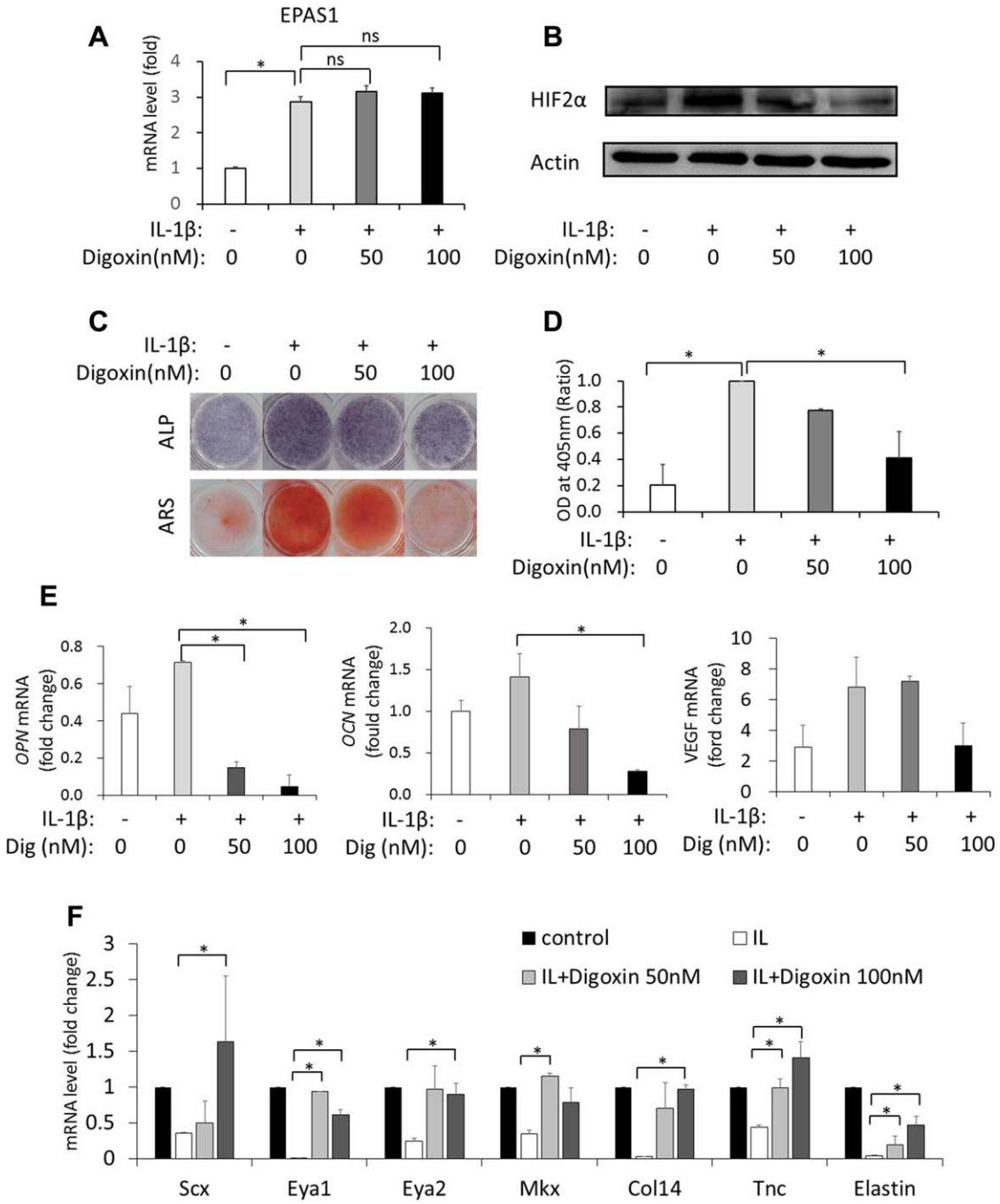

Figure 5. Digoxin inhibits HIF-2alpha to reduce calcification and promote tenogenesis of TSPCs. **(A, B):** qPCR (A) and Western blot analyses (B) of HIF-2alpha in human TSPCs treated with IL-1β (5 ng/ml) and saline or digoxin (50 nM, 100 nM) for 24 hours with actin as a loading control. Values are means ± s.e.m. ($n \geq 3$). **(C, D):** ALP and ARS in human TSPCs after culture for 2 weeks with osteogenesis induction and IL-1β (5 ng/ml) in the presence of digoxin. Values are means ± s.e.m. ($n \geq 3$). **(E):** qPCR analyses of *OPN, OCN, and VEGF* in human TSPCs after culture for 7 days with osteogenesis induction and IL-1β (5 ng/ml) in the presence of digoxin. **(F):** qPCR analyses of *SCX, EYA1, EYA2, MKX, COL14, TNC, and ELASTIN* in human TSPCs after tenogenesis induction and IL-1β (5 ng/ml) in the presence of digoxin for 7 days. Values are means ± s.e.m. ($n \geq 3$). *, $p < .05$. One-way analysis of variance (ANOVA). Abbreviations: ALP: alkaline phosphatase; ARS, alizarin red staining; TSPCs, tendon stem/progenitor cells.

mesenchymal stem/progenitor cells is actively contributing to the ectopic calcification is supported by the observation that calcified valves and vessels are characterized by ectopic osteoblast-specific gene expression [38, 39]. Mesenchymal stem cells isolated from calcific vessels and tendons have been reported to exhibit enhanced osteochondrogenic ability [40–42]. Moreover, lineage tracing study shows that vascular endothelial cells acquire multipotency and contribute to the osteoprogenitor cells when genes important for inhibition of calcification have been genetically ablated in mice [41]. Similarly, our results showed the presence of a $Sox9^{+}/Scx^{+}$ subpopulation that is localized within calcified lesions of diseased tendons.

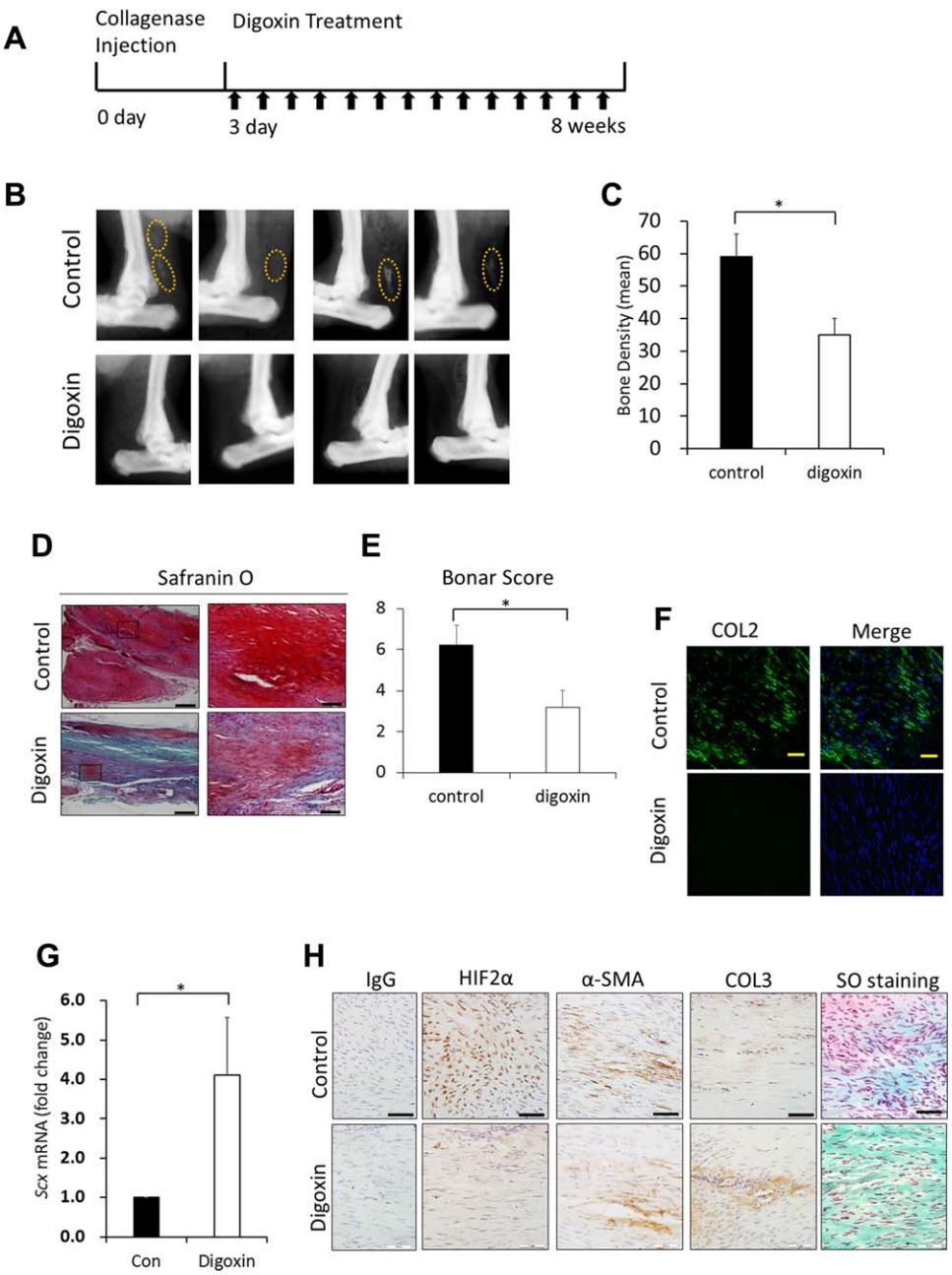

Figure 6. Digoxin treatment inhibits calcification and promotes tenogenesis in vivo. **(A):** Protocol for administration of digoxin in rats with tendinopathy induced by collagenase ($n = 4$). Digoxin or saline was subcutaneously injected into connective tissues around Achilles's tendon at 3 days after collagenase injection. Fifty microliters of a solution (65 μg/ml) of digoxin or saline was applied every 3 days for 8 weeks. **(B, C):** X-ray examination (B) and quantification (C) of calcium deposition from rats after treatment with digoxin or saline for 8 weeks. Values are means ± s.e.m. ($n = 4$). **(D, E):** Safranin O staining (D) of Achilles's tendons sections and Bonar score (E) in rats treated with digoxin or saline. Scale bar, 200 μm (left), 100 μm (right). Values are means ± s.e.m. ($n = 4$). **(F):** Immunohistochemical staining of COL2A1 from rats after treatment with digoxin or saline. Scale bar, 50 μm. **(G):** qPCR analyses of *Scx* from tendon samples of rats after treatment with digoxin or saline. Values are means ± s.e.m. ($n = 4$). **(H):** SO staining and immunohistochemical staining of HIF-2alpha, α-SMA, and COL3 from rats after treatment with digoxin or saline. Scale bar, 50 μm.

This is similar as the unique $Sox9^+/Scx^+$ progenitor pool that gives rise to both tenocytes/ligamentocytes, as well as chondrocytes at tendon-to-bone junctions during the early stages of musculoskeletal development [11]. It is possible that after tendon injury differentiated tenocytes could dedifferentiate back to the multipotential $Scx^+/Sox9^+$ embryonic stage, allowing these cells to switch lineages. Collectively, these data supports the notion that mesenchymal stem/progenitor cells play an important role in ectopic calcification, both indirectly by giving rise to a population that is less capable of regeneration and directly by producing osteochondral lineage progeny.

Microenvironmental cues, such as extracellular matrix, growth factors and cytokines, regulate the fate of tendon stem/progenitor cells [12, 23, 43–45]. In this study, we identified that active forms of IκB is accumulated within the transitional regions from tendon to calcific site, whereas hypoxia probe was

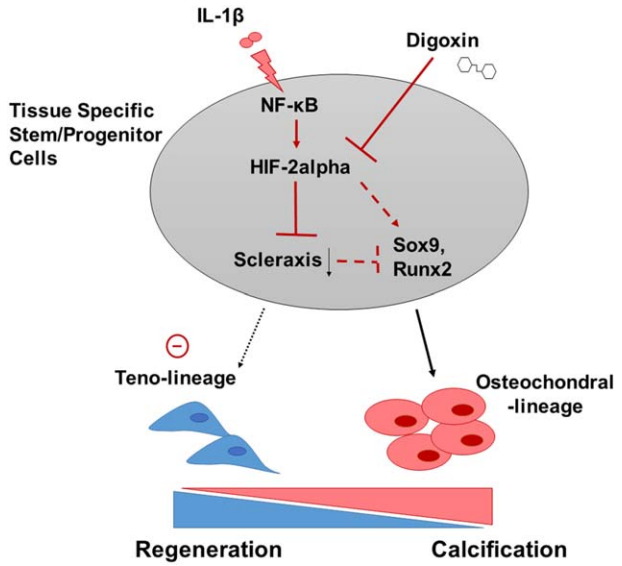

Figure 7. Schematic representation of the mechanisms through which digoxin may be affecting soft tissue stem/progenitor cells fate decision. Inflammation or injuries in tendons or heart valves result in the release of IL-1β and the downstream phosphorylation of NF-κB, causing HIF-2alpha activation. Increased level of HIF-2alpha downregulates Scleraxis, leading to decreased teno-lineage differentiation which in turn increases osteochondral lineage differentiation of stem/progenitor cells. Digoxin regulates the HIF2alpha and Scx function in stem/progenitor cells and decreases ectopic calcification in Achilles tendinopathy model.

only detected in the central of calcific lesions in vivo. Consistent with previous study [46], we also identified that TSPCs cultured under hypoxia exhibit reduced osteochondrogenic differentiation ability. Notably, we observed that overexpression of HIF-2alpha alone does not promote osteochondrogenic differentiation in TSPCs (Data not shown) unless combined with IL-1β treatment. Collectively, these data suggests that proinflammatory cytokines, rather than hypoxia, constitute one of the key factors in early stage of ectopic calcification in tendons.

HIF-2alpha has been reported to participate in endochondral ossification through transactivation of hypertrophic markers in chondrocytes [4, 47]. Nevertheless, during calcification in vivo, we have not observed a co-localization of HIF-2alpha and COL2A1, the marker of chondrocytes, thus indicating that HIF-2alpha does not directly promote chondrocyte hypertrophy in the context of tendon calcification. In contrast, we found that HIF-2alpha is widely activated in stem/progenitor cells recruited around calcific sites which suppresses Scx expression and tenogenic program of TSPCs. Downregulation of HIF-2alpha by siRNA or digoxin resulted in increased Scx expression and enhanced tenogenic differentiation of TSPCs. Previous studies also reported that HIF-2alpha promotes cell survival and proliferation in various progenitor/stem cells [16, 48, 49]. Thus, we conclude that the shift to an osteochondral-lineage of TSPCs could be partly attributed to continued proliferation of cells that did not undergo tenocyte lineage differentiation due to high levels of HIF-2alpha signaling that suppressed Scx expression. HIF-2alpha may also directly promote osteochondral differentiation of TSPCs through transactivation of master osteochondrogenic transcription factors, such as Runx2 (Supporting Information Fig. S5C) [50–52]. Unfortunately, HIF-2alpha knockout mice die prematurely at the embryonic stage [53], the function of HIF-2alpha in TSPCs need to be further validated in genetic knockout models in future researches.

Our data reveals a previously undiscovered role of Scx in the context of ectopic calcification, in addition to its well-established role in teno-lineage specification during tendon and heart valve development [29, 54]. In our previous work [55, 56], we showed that Scleraxis could inhibit osteogenesis through inhibition of BMP2 pathway. We find that pathological HIF2alpha activation downregulates the expression of Scx in tendinopathy, which could in turn enhance BMP2 signaling and osteogenesis. Cross-antagonism, which has been discovered in blood system, suggests that lineage-instructive regulators not only induce gene expression within their own lineages, but also inactivate key transcription factors of alternative cell types [57]. According to this, loss of transcription factor function should be able to trigger differentiation and promote lineage conversion [58]. This prediction is supported by evidence gathered in the blood system, such as knockdown of myeloid specifier PU.1 leading to ectopic formation of hemoglobin-producing cells [58] and knockdown of B cell lineage specifiers promoting B cell to macrophage conversion [59]. Furthermore, enhanced osteogenic differentiation and ectopic calcification in heart valves have been observed in mice deficient for key regulator of chondrogenesis [60], indicating that committed chondrogenic progenitors can be re-specified into osteogenic progenitors when key regulator of former lineage is ablated. Thus, ablation of the original lineage transcription factors which results in increased conversion toward osteogenic lineage might be a common initiating phase during ectopic calcification in soft tissues.

There is considerable interest in the concept that de-differentiation and re-differentiation of stem/progenitor cells could be potential therapeutic targets in regenerative medicine [61, 62]. The search for pharmacological agents that can be used to manipulate the differentiation pathway of endogenous adult stem cells has led to a number of recently published chemical screens [62, 63]. In this study, we took a different approach; by characterizing the role of the HIF-2alpha signaling pathway in stem/progenitor cells differentiation and then focusing on a widely-utilized drug, digoxin, which inhibits this particular signaling pathway in cancer cells [32]. Our data showed conclusively that digoxin does indeed inhibit the HIF-2alpha signaling pathway, which promotes tenogenesis and inhibits calcification in both human TSPCs and an animal tendinopathy model. Cardiac glycosides can increase calcium and phosphate excretion [64], thus raise the possibility that the reductions in tendon calcification following digoxin treatment could have been secondary to reduced renal tubular reabsorption of calcium and phosphate ions. We cannot rule out that local digoxin administration may also affect reabsorption of calcium and phosphate ions in kidney. Nevertheless, digoxin treated leg shared a same circulation system with its matched control leg within the same individual, thus the reduction in tendon calcification on digoxin treatment is probably not due to altered ions balance in circulation system. Furthermore, we did not apply digoxin in heart valve calcification treatment, in view of the fact that therapeutic plasma concentrations of digoxin in cardiac patients (≈10–30 nM) are typically lower than the concentration

(≈50–100 nM) required for maximal inhibition of HIF-2alpha expression in stem/progenitor cells after 24 hours of drug treatment. However, combination therapy with an antioxidant may result in synergistic effects and lead to increased efficacy and decreased toxicity.

Our results collectively indicate that HIF-2alpha acts as a crucial mediator of soft tissue calcification, by directly inhibiting Scx function and influencing stem cell lineage fate. This study therefore provides a clinically applicable therapeutic approach to prevent soft tissue calcification, through manipulation of the lineage specification in stem/progenitor cells with digoxin. These findings are of great value in unraveling the mechanism of soft tissue calcification, as well as in the development of future therapeutics based on manipulating stem cell lineage fate.

Conclusion

We have elucidated that lineage differentiation of TSPCs acts W.-L.S. Provision of study material from patients, Collection of data; as a crucial mediator of soft tissue calcification, which can be modulated with a widely utilized drug digoxin by inhibiting the function of HIF-2alpha.

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Author Contributions

J.-J.H.: Conception and design, Collection and assembly of data, Manuscript writing; Z.Y.: Conception and design, Collection and assembly of data; W.-L.S.: Provision of study material or patients, Collection of data; Y.-B.X., T.Z., and P.L. Collection and assembly of data; Y.-Z.C. and M.-J.K.: Provision of study. M.-J.K.: Provision of study material from patients; B.C.H.: Manuscript writing; Y.-T.Z. and W.-S.C.: Data analysis and interpretation; X.C. and H.-W.O.: Conception and design, Data analysis and interpretation. J.-J.H. and Z.Y.: These authors contributed equally to this work.

Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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