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Hedgehog signaling underlying tendon and enthesis development and pathology

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Abstract

Hedgehog (Hh) signaling has been widely acknowledged to play essential roles in many developmental processes, including endochondral ossification and growth plate maintenance. Furthermore, a rising number of studies have shown that Hh signaling is necessary for tendon enthesis development. Specifically, the well-tuned regulation of Hh signaling during development drives the formation of a mineral gradient across the tendon enthesis fibrocartilage. However, aberrant Hh signaling can also lead to pathologic heterotopic ossification in tendon or osteophyte formation at the enthesis. Therefore, the therapeutic potential of Hh signaling modulation for treating tendon and enthesis disease remains uncertain. For example, increased Hh signaling may enhance tendon-to-bone healing by promoting the formation of mineralized fibrocartilage at the healing interface, but pathologic heterotopic ossification may also be triggered in the adjacent tendon. Further work is needed to elucidate the distinct functions of Hh signaling in the tendon and enthesis to support the development of therapies that target the pathway.

Keywords

Tendon enthesis; Growth plate; Mineralization; Fibrocartilage; Heterotopic ossification; Hedgehog signaling; Development; Healing

Hedgehog (Hh) signaling in tendon enthesis development and mineralization

In this review, we summarize how Hh signaling regulates the development and mineralization of the tendon enthesis, how its inactivation can impair tendon enthesis healing, and how its activation can lead to pathologic heterotopic ossification. Additionally, we discuss the gaps in our mechanistic understanding of this process in the tendon and enthesis, focusing on the contrasts between its beneficial activation during development and

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its pathologic activation during disease. These insights will help move the field towards development of Hh-specific cellular and molecular strategies for improving tendon-to-bone healing and for preventing tendon heterotopic calcification.

The Hh signaling pathway

Hh signaling is initiated when a Hh ligand binds to the cell surface receptor Patched 1 (PTCH1), which reverses the repression of the cell surface protein Smoothened (SMO) in the primary cilium, a subcellular protrusion emanating from the apical surface of mammalian cells [1, 2] (Figure 1). When a Hh ligand is absent, PTCH1 represses SMO activity. After the Hh ligand binds to PTCH1, SMO is released and triggers downstream signal transduction through proteins such as GLI-Kruppel family member Gli1, Gli2, and Gli3. Gli1 activates transcriptional activity whereas Gli3 plays a transcriptional repressor role. Gli2 can either activate or repress transcriptional activity, depending on the conditions. Numerous other cellular factors are involved in Hh signaling by regulating activities downstream of Gli1/2/3. For example, suppressor of fused homolog (Sufu) functions as a negative mediator of Hh signaling and kinesin protein 7 (Kif7) exerts both positive and negative regulatory roles on Hh signaling. Furthermore, the functions of many Hh-related proteins on Hh activation or inactivation are dependent on tissue type and signaling status, an area of ongoing study [3, 4].

The Hh signaling pathway is well-recognized to govern organ patterning, early embryonic development, formation of the musculoskeletal system, and tumorigenesis [5, 6]. Early in development, a gradient in sonic hedgehog (Shh), one of the three mammalian hedgehog orthologs, regulates mesoderm patterning [7]. The influence of Shh continues at later stages, regulating the polarizing activity of cells residing in the posterior distal region of the limb bud and further controlling patterning of both the anterior-posterior and proximal-distal axes [8]. Similarly, Shh is critical for craniofacial development, mediating survival and proliferation of neural crest cells [9]. During postnatal stages of development, endochondral skeletal growth is dependent on another Hh ortholog, Indian hedgehog (Ihh), as demonstrated, e.g., by observations that Ihh mutant mice have severe dwarfism [10].

The role of Hh signaling in tendon enthesis development

Hh signaling is also critical for the formation of the enthesis, a mineralized transitional tissue which bridges soft connective tissues such as tendon, ligament, and meniscus to bone [11–13]. This specialized tissue is critical for effective force transmission between unmineralized and mineralized tissues. The enthesis has classically been categorized as either fibrous or fibrocartilaginous, depending on its structural and compositional makeup and its anatomic site [14]. The fibrous enthesis is composed of perforating mineralized collagen fibers and can often be found in soft tissue-to-bone attachments into the metaphyses and diaphyses [15]. The tendon/ligament end of the fibrous enthesis is enriched in collagen type I. As it inserts into bone periosteum or cortex, mineralization of the collagen type I matrix is observed. The fibrocartilagous enthesis is displays spatial gradients in extracellular matrix composition and mineralization (Figure 2). The tendon/ligament end of the fibrocartilaginous enthesis transitions from tendon to unmineralized fibrocartilage, then to mineralized fibrocartilage, and finally to bone, with a corresponding

shifts in constituents from collagen type I, to collagen type II and aggrecan, and finally to mineralized collagen type II and X [11]. Load transfer across dissimilar materials such as tendon and bone (which differ in modulus by two orders-of-magnitude) is an engineering challenge, as stresses are amplified at their interface [14, 16]. Fibrous entheses attach over relatively large surface areas, effectively distributing forces across the attachment footprint to reduce stress and achieve load transfer. In contrast, fibrocartilaginous entheses attach over much smaller surface areas and require a number of multiscale structural and compositional mechanisms to achieve effective load transfer (e.g., a spatial gradient in mineral content) [17–19].

The formation of the enthesis begins during embryonic development, and is orchestrated by the functions of multiple progenitor cell populations [20, 21]. Cells positive for both SRY-box transcription factor 9 (Sox9) and scleraxis (Scx) distinguish enthesis-specific progenitors from cartilage and tendon progenitors, which are positive for only Sox9 or Scx, respectively [20]. Depending on the anatomical location, Sox9-lineage enthesis progenitors ultimately either differentiate into, or are replaced by, a Hh-positive cell population marked by Gli1 [22]. In the entheses that migrate along bone shaft during development, Sox9-lineage cells are replaced by Gli1-lineage (Gli1+) cells, whereas in the entheses that remain stationary throughout development, Sox9-lineage cells differentiate into Gli1+ cells [22]. During postnatal development, Gli1+ cells initially reside in the unmineralized enthesis fibrocartilage of 4 week-old mice and then fully mineralized enthesis fibrocartilage of 8-week old mice [23, 24]. Gli1+ cells and their progenies are retained in the enthesis region throughout postnatal development, eventually populating the entire fibrocartilage region between tendon and bone (Figure 2) [24]. Lineage tracing has revealed that most of these cells lose their responsiveness to Hh signaling as the skeleton matures [21]. These findings, combined with the observation that ablation of the Gli1+ cells leads to a loss of mineralized fibrocartilage, suggest that these cells play a critical role in enthesis mineralization [24]. A number of Hh-related ligands, including Shh and Ihh regulate the expression of Gli1, depending on the development stage, but the origin of these ligands inducing Gli1 expression at the enthesis is unclear.

Cells responsive to Gli1 during enthesis development are also regulated by mechanical loading, as limb paralysis during the neonatal period leads to an increased number of Gli1+ cells in the mouse entheses [24, 25]. Since primary cilia have been reported to receive and transduce mechanical loading, activation of Gli1 during enthesis mineralization in the first few weeks after birth is also concurrent with cilium incidence, further implicating Hh signaling as responsible for enthesis mechanosensing [25]. Moreover, tendon entheses with inactivated Hh signaling cannot adapt to altered biophysical loading and also have abnormal ciliogenesis. However, the underlying mechanism of how mechanical loading, Hh signaling, and primary cilia interactively regulate each other and then contribute to tendon enthesis formation and remodeling is unclear.

The role of Hh signaling on enthesis development and mineralization has been further explored by using transgenic murine models with both loss-of-function and gain-of-function [12, 24–26]. One such model that has been widely studied utilizes conditional deletion and activation of SMO, a receptor which is necessary for the transduction of hedgehog signaling

in response to Hh ligands. SMO deletion at the tendon enthesis leads to substantial defects in tissue structure and composition, including decreased mineralization, abnormal collagen content, decreased proteoglycan content, and altered biomechanical properties [12, 24–26]. In contrast, SMO activation leads to increased protein expression of tenascin (Tnc), biglycan (Bgn), and collagen type 2 (Col2). Similarly, constitutive activation of SMO in tendon cells results in the ectopic expression of chondrogenic markers in the tendon midsubstance, while SMO deletion in tendon cells decreases fibrocartilage differentiation at the enthesis [12].

Besides Hh signaling, other signaling pathways, such as fibroblast growth factor (FGF), transforming growth factor beta (TGFβ), bone morphogenetic proteins (BMPs), and growth differentiation factor (GDF), also play important roles in tendon enthesis development. Using loss-of-function mouse models, these pathways have been shown to regulate differentiation and specification of enthesis progenitors and to coordinate enthesis tenogenesis, chondrogensis, and osteogenesis to form the fibrocartilagous transition [14, 16, 27, 28]. However, it remains unclear how these pathways interact with Hh signaling to regulate enthesis development and mineralization in particular, although crosstalk between Hh, TGFβ, and BMP signaling pathways has been proposed in other tissues and biologic processes (Figure 3) [29–35]. A better understanding of crosstalk between these factors during enthesis formation and function is necessary to help motivate Hh-related drug development for enthesis regeneration.

Similarities between tendon enthesis development and growth plate biology

Our understanding of enthesis development, particularly as it relates to mineralization, is based on the substantive literature on growth plate biology. Superficially, the enthesis and the growth plate share many similarities, e.g., there are spatially varying distributions of chondrocyte phenotypes and extracellular matrix components (Figure 4). Round “resting” chondrocytes with minimal proliferative activity reside on the unmineralized end of the growth plate [36]. A gradient of chondrocyte phenotypes is then seen, shifting from proliferating, to pre-hypertrophic, to hypertrophic (mineralizing), and finally to terminal chondrocytes (ultimately replaced by osteoblasts) [36]. At the enthesis, round quiescent chondrocytes are located on the unmineralized side and hypertrophic chondrocytes, with obvious lacuna, are seen on the mineralized end adjacent to bone [16]. Consistent with the hypertrophic phenotype, collagen X is highly expressed by the mineralizing chondrocytes in both the tendon enthesis and the growth plate [37, 38]. However, there are key differences between enthesis and growth plate biology. Most critically, in the growth plate, a combination of vascular invasion, hypertrophic chondrocyte apoptosis, and chondrocyte transdifferentiation results in the replacement of mineralized cartilage with bone [36]. These processes are not seen at the enthesis, leading to some descriptions of the enthesis as an “arrested growth plate” [27]. This difference was made clear when tracking the Gli1+ cells at the enthesis and at the adjacent secondary center of ossification: Gli1+ cells persisted through maturity at the enthesis but disappeared from the adjacent bone that was formed via endochondral ossification [24, 39].

An abundance of previous work has determined the contribution of Ihh to endochondral skeleton development and growth plate maintenance via regulating chondrocyte proliferation

and differentiation [36, 40, 41]. Furthermore, Ihh also influences osteoblast differentiation activity because of absence of osteoblasts and expression of osteoblast markers (i.e., Runx2, osteopontin) after Ihh deletion in chondrocytes [40, 41]. From the perspective of growth plate development and maintenance, Ihh and parathyroid hormone-like hormone protein (PTHrP) coordinate through a negative feedback loop to drive chondrocyte fate to leave the proliferative stage [36]. Ihh is synthesized by prehypertropic or early hypertropic chondrocytes whereas PTHrP is synthesized by prechondral cells. This creates opposing spatial gradients of the two factors. Ihh promotes chondrocyte proliferation and PTHrP production, while PTHrP prevents chondrocytes from proliferating, resulting in a negative feedback look that controls terminal chondrocyte differentiation and therefore inhibits Ihh production [36, 42]. Previous work examining enthesis mineralization has also demonstrated essential roles for Hh and PTHrP during the formation, maintenance, and healing of the enthesis [11, 24, 39, 43].

Hedgehog signaling during tendon and enthesis healing

Insufficient or aberrant Hh signaling has been associated with poor tendon-to-bone healing, enthesitis, and tendon heterotopic ossification [39, 44]. Due to the stress concentrations inherent to the attachment of mechanically dissimilar tissues, regenerating the enthesis during healing remains the goal for effective repair of connective tissues to bone. Unfortunately, fibrovascular scar tissue dominates the healing response, resulting in a persistent clinical challenge for surgically reconnecting tendon to bone, with alarmingly high failure rates [45,46]. Furthermore, conditions leading to inappropriate mineralization result in significant pain and disability at and around joints [13]. The prevalence of these conditions and the lack of effective therapeutic strategies motivate the development of novel treatment approaches, e.g., by studying molecular and cellular cues that drive enthesis development and/or developing agonists and antagonists for Hh signaling control.

As expected from the established role of Hh signaling during enthesis development, numerous studies have demonstrated the importance of Hh signaling during tendon-to-bone healing. Activation of Hh signaling has been observed at the enthesis during rotator cuff healing or after ACL reconstruction, with increased expression of Hh-related components and increased numbers of Hh-responsive cells (Table 1) [39, 47]. Specifically, expression of *Ihh*, *PTCH1*, *Smo*, and *Gli1* was significantly increased at the tendon-bone interface during the early healing phases after rotator cuff repair or ACL reconstruction [47–50]. Additionally, lineage tracing studies showed that Gli1+ cells populated the immature enthesis after needle punch injury, in contrast to the injury response at the mature enthesis, which involved only a small number of Gli1+ cells [39]. The higher numbers of Gli1+ cells at the injured enthesis of immature mice resulted in markedly better healing compared to adult mice. These cells were positive for the stem cell marker Notch1 and are thought to retain progenitor characteristics [39]. Furthermore, following injury, this population of cells was observed to form clusters adjacent to the site of injury and express Ki67, a marker of proliferation [39]. The importance of Hh signaling for enthesis healing was also demonstrated in mice with tendon- and enthesis-specific *SMO* deletion, where tendon and enthesis cells had reduced cellularity and decreased ECM and mineralization, leading to impaired healing [39]. Although no study has directly assessed the effect of Hh upregulation

on enthesis healing, a study investigating mesenchymal stem cell treatment on enthesis healing found that treatment was associated with increased Hh activity, increased Sox9-positive cells, and stronger and more wide-spreading staining for type II collagen [49].

Hh signaling also appears to be involved in the healing response of the tendon mid-substance, although the mechanisms of action remain unclear. Hh ligands, the *PTCH1* receptor, and the downstream component *Gli1* were significantly upregulated in sheath tissues of mouse tibialis anterior tendon after injury [51]. Furthermore, injured tibialis anterior tendons in mice with tendon-specific *SMO* knockout had impaired healing processes compared to wild type controls, as demonstrated by downregulated ECM-related genes, reduced cell proliferation and collagen deposition, and thinner tendon sheath tissues [51]. In contrast, Hh activation via conditional knockout of a Hh suppressor gene improved tendon healing by promoting cell proliferation and tendon sheath remodeling. Coupled with the findings that TGFβ signaling plays a critical role in tendon midsubstance healing, potential crosstalk between Hh and TGFβ signals may explain a yet-to-be determined mechanism through which tendon healing is dependent on Hh signaling [51–53].

Hh signaling in tendon/enthesis heterotopic calcification

Heterotopic ossification (HO) is the pathologic ectopic formation of bone/mineralization in a soft tissue. Records of this phenomenon date back to World War I in the context of blast and gunshot wounds [54]. Since that time, aberrant soft tissue ossification has been observed in numerous areas such as major orthopedic surgery postoperatively, traumatic brain injury, spinal cord lesions, and burns (Figure 5) [55]. Considerable research has gone into uncovering the biochemical pathways and mechanisms underlying the development of heterotopic ossification in tendon, including the contribution of hedgehog signaling. Treatment approaches to prevent HO have shown only limited efficacy.

Presumed mechanisms that drive tendon HO

BMPs have long been recognized as important signaling factors necessary for proper skeletal development and homeostasis [56]. In the canonical pathway, BMP receptor activation leads to Smad complex formation and subsequent translocation to the nucleus [57]. Type 1 BMP (BMP-1) signaling has been implicated as a mechanism for HO, particularly in studies of patients with fibrodysplasia ossificans progressiva (FOP), a rare but serious genetic disorder characterized by sporadic HO throughout the body [58]. A mutation in activin A receptor 1 (*ACVR1*), the gene encoding the BMP-1 receptor, was found to cause constitutive activation of the BMP signaling pathway and subsequent widespread HO [59]. The FOP phenotype has since been recreated in a mouse model via a *ACVR1* knockout [59]. Additionally, conditional activation of *ACVR1* driven by Scx-Cre transgene consistently caused progressive HO in ligament and tendon, further supporting this pathway's contribution to tendon-specific HO [60]. In injury-induced tendon mineralization models, BMP receptor kinase inhibitors were found to significantly reduce tendon mineralization, thus presenting a potential pharmacologic target for future HO therapy development [61].

BMP and TGFβ pathways share multiple signaling hubs and then cooperatively or counteractively regulate cell behavior and tissue homeostasis [62]. Therefore, it is not surprising that TGFβ signaling has also been reported to contribute to tendon/enthesis HO. Evaluation of muscle from HO patients and Achilles tendon from trauma-induced HO mice showed increased numbers of cells positive for pSmad2/3, indicative of activated TGFβ signaling [63]. Monocytes/macrophages associated with HO were found to produce TGFβ1 and cause aberrant chondrogenic differentiation of progenitor cells [64]. Furthermore, treatment with neutralizing TGFβ antibodies attenuated tendon and cartilage HO of BMP-induced mouse models [63, 65]. Reduction of systemic macrophage TGFβ levels successfully ameliorated HO in Achilles tendon [52]. Knockout of TGFβ receptor II in a subpopulation of mesenchymal stem cells prevented the occurrence of cartilage and tendon HO, implicating a cellular source of TGFβ leading to HO [63]. Besides mesenchymal stem cells, tendon-derived progenitor cells positive for both *Scx* and *cathepsin K* were also found to participate in pathologic tendon HO [66].

Another studied pathway for HO development involves the *Mohawk* (*Mkx*) gene, a member of a larger class of homeobox genes, which codes a tendon/ligament associated transcription factor. Although originally discovered to mediate postnatal tendon homeostasis, the *Mohawk* transcription factor has recently been implicated in HO [67]. Mice deficient in the *Mkx* gene exhibited complete penetrance of the HO phenotype by two months of age [68]. In rats, onset of the HO phenotype in *Mkx*-deficient mutants occurred even sooner than the wild type mice, suggesting a disruption during tenogenesis [69]. Increased BMP pathway genes in the *Mkx*-deficient rats suggest a common pathway between these models and the development of HO. Interestingly, hedgehog signaling was also shown to be upregulated during tendon ossification in *Mkx*-deficient mice [66].

Perturbations in extracellular matrix (ECM) maintenance have also been illustrated to lead to tendon HO. Biglycan (*Bgn*) and fibromodulin (*Fmod*) are two small leucine-rich proteoglycans (SLRPs) highly involved in tendon and bone ECM formation and maintenance [70]. Double deficient $Bgn^{-/-}/Fmod^{-/-}$ mice had abnormal collagen fibril structure and successive development of HO in quadriceps, patellar, and Achilles tendons [71]. The severity of the HO phenotype in these $Bgn^{-/-}/Fmod^{-/-}$ mice was rescued by moderate exercise, indicating an essential role of biophysical signaling in maintaining cell phenotype and ECM remodeling [72]. Besides ECM, disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) proteinases, responsible for the construction and breakdown of various ECM components, have been documented to be involved in HO. Transgenic mice of dual knockout $Adamts7^{-/-}$ $Adamts12^{-/-}$, two elements within the family of ADAMTS proteinases, were found to develop HO in adult hindlimb tendons [73]. These $Adamts7^{-/-}$ $Adamts12^{-/-}$ mice also exhibited reduced levels of *Bgn* and *Fmod*, further suggesting distorted ECM composition as a driver of tendon HO.

Traumatic insults in the form of injury or burn can recapitulate the tendon HO phenotype. The first reproducible animal model for tendon HO was described in 1953 after the observation of ossification following tenotomy of rat Achilles tendon [74]. Further work established mouse and rat models associated with other clinically relevant traumas such as burns, blast injury, and amputation [64, 75–78]. Further examination of HO formation in a

trauma model revealed significant hypoxia inducible factor-1α (HIF-1α) expression in HO tissue [79]. Since HIF-1α is a transcription factor known to be critical for the chondrocyte survival [80], the activation of HIF-1α in this trauma-induced HO model provides evidence for its role to arrest mesenchymal cells towards a chondrogenic pathway and subsequent HO formation. Besides HIF-1α, activated nerve growth factor, accompanied with axonal invasion, and TGFβ produced by monocytes or macrophages were also shown to drive osteochondral differentiation of tendon-specific stem cells after soft tissue trauma [64, 78].

Contribution of Hh signaling to HO in tendon

Aberrant Hh signaling has been found to contribute to the development of HO in tendon. Patients with progressive osseous heteroplasia (POH), a rare disease characterized by extra-skeletal bone formation similar to FOP, lack a functional α-subunit of the stimulatory G protein ($G\alpha_s$), a potent negative regulator of Hh signaling, due to a null mutation in *GNAS* [81]. Highly upregulated Hh signaling was seen at sites of ectopic bone derived from samples from POH patients [44]. In mice, loss of *GNAS* caused upregulation of Hh signaling and subsequent development of HO within the Achilles tendon akin to POH patients [44]. When analyzing a different major inhibitor of Hh signaling, *Sufu*, the development of spontaneous HO in tendon was again demonstrated [66]. In the aforementioned $Mkx^{-/-}$ mouse model of HO, tissue-specific inactivation of *SMO*, ameliorated the extent of tendon ossification [82]. Pharmacologic inhibition of Hh signaling via *SMO* antagonism or, more recently, *Gli1/Gli2* inhibition, was also successful in decreasing ectopic bone formation [66, 83].

Enthesophytes/enthesis heterotopic ossification

The formation of enthesophytes, or bone spurs at ligament or tendon insertion sites, is an understudied phenomenon, and a direct link between this pathology and Hh signaling has not yet been confirmed. The involvement of pathways closely related to Hh signaling in enthesophyte formation has not been well established. BMP signaling, for example, drives the formation of enthesophytes in spondyloarthropathies, and its inhibition reduced excessive bone formation in a model of ankylosing enthesitis [84, 85]. BMP has been shown to stimulate Ihh expression in chondrocytes, and to coordinate their proliferation and differentiation [86, 87]. Additionally, both BMP and Ihh have been heavily implicated in the formation of osteophytes, bone spurs that originate at bone edges rather than enthesis regions [88–90]. Although the pathophysiologies of enthesophytes and osteophytes are not identical, they are positively associated and have several mechanistic similarities [91, 92]. Together, these observations point to a likely role of hedgehog signaling in abnormal bone formation at entheses, an area which merits further investigation. A better understanding of the mechanisms behind enthesophyte formation is essential for the development of improved therapeutics, to improve patient outcomes for a condition that can cause chronic pain and significantly reduce mobility in affected joints [93, 94].

Targeting Hh signaling for treatment of enthesopathy/tendinopathy

The Hh signaling pathway has served as an alluring target for therapeutic modulation for a wide range of pathologies. Generally, Hh inhibitors have been used to treat pathologies

of unregulated growth, such as cancer, for which there are a number of approved Hh-related treatments. Hh-modulating approaches may also be of therapeutic benefit in the treatment of various tendon and enthesis disorders as well as to enhance tendon-to-bone healing. However, there are currently no FDA-approved Hh-modulating therapeutics for the treatment of these conditions. As such, repurposing Hh-modulating therapeutics from other areas to treat disorders of the tendon and its bony attachment presents an exciting new opportunity.

Therapeutic approaches for Hh signaling inhibition and agonism

Much of the development and application of Hh-modulating therapeutics has come from Hh inhibitor use in cancer research and treatment. Activation of the Hh signaling pathway results in upregulation of pro-angiogenic and pro-proliferative genes, and abnormal activation of Hh signaling has been linked to numerous malignancies [95, 96]. An extensive amount of preclinical and clinical research has been conducted on the use of Hh inhibitors in cancer treatment, which has led to FDA approval of several Hh inhibiting therapeutics [97]. There are numerous mechanisms by which the Hh signaling pathway can be inhibited. One method of inhibiting Hh signaling is by targeting the Hh ligand. Targeting of the Shh ligand has been achieved both through direct antibody targeting as well as through the targeting of a membrane-bound acyltransferase necessary for the palmitoylation of Shh during the final steps of protein synthesis [98–101]. The Hh signaling pathway can also be antagonized via inhibition of the cell surface receptor SMO. It has been demonstrated that cyclopamine, an alkaloid derived from *V. californicum*, can bind to SMO and inhibit Hh signaling [102, 103]. Since the discovery of the Hh-antagonizing potential of cyclopamine, numerous small molecules capable of binding to and inhibiting Smo have been developed [98]. Additionally, itraconazole, an FDA-approved antifungal drug, has been shown to target SMO and inhibit Hh signaling via the prevention of SMO accumulation in primary cilia [25, 97, 104]. Hh signaling can also be inhibited via the targeting of Gli1 transcription factors, which are downstream of the Hh ligand and its cell surface receptors. Arsenic trioxide has been shown to inhibit the activity of Gli1 and Gli2 via direct binding [105, 106]. Additionally, small molecule antagonists of GLI transcription factors have been developed [107, 108].

While numerous Hh inhibitors have been characterized, with downregulation of Hh signaling possible through several different mechanisms, comparably fewer Hh agonists have been identified. Several synthetic glucocorticoids as well as several synthetic and endogenous oxysterols are capable of upregulating Hh signaling via action on SMO [109–113]. Additionally, several small molecule SMO agonists (SAG) have been developed, including the purine purmorphamine and the benzothiophene, as well as their derivatives [114–118]. Hh signaling plays an important role in both cellular proliferation and differentiation during the development of the nervous system, as well as the maintenance of precursor cells involved in the regeneration of tissue following injury [119–121]. In the setting of damage and degeneration of peripheral nerves, subcutaneous administration of a Shh-IgG fusion protein returned nerve conduction velocities from diabetic levels to non-diabetic levels in a rat model of diabetes [122]. The benefits of the pro-angiogenic and pro-proliferative effects of Hh agonism may also extend to pathologies of ischemic origin, including those affecting the skeletal muscle, heart, and brain [96, 123]. In a mouse

model of hindlimb ischemia, intraperitoneal injection of Shh recombinant protein improved blood-flow and limb salvage [124]. Upregulation of Hh signaling in cardiomyocytes via Shh gene transfer preserved cardiac function in adult animal models of myocardial ischemia [125]. However, despite promising preclinical research supporting the therapeutic potential of Hh agonism, to date, no Hh agonists have been approved for human use.

Modulation of Hh signaling as a treatment strategy for tendon and enthesis pathologies

Given the integral roles of Hh signaling in the formation of the enthesis and in pathologies such as HO, Hh signaling is a clear therapeutic target for a wide range of tendon and enthesis conditions. For tendon-to-bone repair, Hh agonism has the potential to promote fibrocartilage formation and mineralization, as seen during enthesis development, but only if an ample supply of responding progenitor cells are available. The ability of Hh agonism to increase bone volume is also of relevance, given the decrease in bone mineral density at the enthesis following rotator cuff injury and the positive effects of bone-targeted interventions for enhancing tendon-to-bone healing [50, 126, 127]. Increased Hh signaling through systemic administration of a Hh agonist has been shown to improve fracture healing in a mouse model [126], and is therefore an attractive strategy for improving tendon-to-bone healing. However, due to the region-specific activity of Hh signaling, i.e., it is both necessary for enthesis formation and pathologic in the context of HO, treatments targeting Hh signaling must be spatially and temporally controlled. For example, Hh agonists for tendon-to-bone repair should be localized to the repair site to enhance mineralization at the healing interface, without pathologic mineralization in the adjacent tendon or the formation of enthesophytes. Therefore, more appropriate, biocompatible carriers for Hh agonists or antagonists, instead of oral intake and intramuscular/intraperitoneal injection, should be developed to spatiotemporally control their release and activities [128–131]. For the treatment of HO, our current understanding of Hh signaling in the musculoskeletal system strongly supports the use of existing Hh inhibitors to be repurposed for the treatment of HO [44]. Finally, more work is needed to understand the role of Hh signaling in tendon midsubstance healing before a Hh-targeted therapy is developed. Notably, neither Hh inhibitors nor agonists are approved by the FDA for tendon and enthesis conditions, and additional pre-clinical testing is needed to evaluate efficacy and mechanisms of actions.

Cell-based therapies dependent on Hh signaling

As described earlier, cells positive for Gli1, an essential hedgehog signaling transcription factor, build the tendon enthesis and facilitate spatially graded mineralization [24, 39]. Consistent with the premise that Gli1 is a marker of enthesis stem cells, studies in other tissues have demonstrated that Gli1-expressing cells act as stem/progenitor cells to maintain tissue homeostasis and regulate repair. A critical role for Gli1+ cells in development and response to injury is reflected in multiple tissues, including teeth, bone, bone marrow, skin, colon, kidney, and heart [132–141] (Figure 6). During tooth development, Gli1+ cells located within dental mesenchyme proliferated and differentiated into various cell types (i.e. ameloblasts, odontoblasts) required for periodontal ligament and dental pulp formation [138, 139]. Furthermore, Gli1+ cells surrounding the neurovascular bundle were activated upon injury to give rise to reparative dentin, cementum, periodontal ligament, and alveolar bone [136, 137]. Consistent with the mechanosensitivity of Gli1+ cells at the tendon enthesis,

physiological occlusal forces regulated sclerostin expression of alveolar bone osteocytes and diminished the multipotent capacity of Gli1+ cells in alveolar bone and periodontal ligament [136]. In growing bone, Gli1+ cells beneath the growth plate differentiated into bone marrow adipocytes, osteoblasts, and stromal cells during development and osteoblasts and chondrocytes during bone fracture healing [141]. In tissues such as muscle, skin, and colon, Gli1+ cells have been reported to serve as stem cells within tissue-specific niches and actively regenerate injured tissues [132, 133, 142]. In contrast, Gli1+ cells residing in bone marrow, kidney, lung, liver, and heart promoted unexpected ectopic differentiation and caused myofibroblast-driven fibrosis and osteoblast-induced calcification after organ injury [134, 135, 140]. Therefore, a role for Gli1-responsive cells as a pool of stem/progenitor cells and their multi-potency can be beneficial or detrimental, depending on the context of tissue type and injury. Tissue disease or injury can push Gli1-responsive cells towards pathology-related phenotypes and drive inappropriate tissue remodeling. Future studies should consider Gli1-responsive cells as a therapeutic target and decipher the molecular mechanisms governing their differentiation in different tissues.

Perspective

The role of Hh signaling in the development of a wide range of tissues and in the progression of malignancies has been well explored. Building on this large body of literature, recent work has explored the importance of Hh signaling in tendon and enthesis development and pathology. It is clear that Hh signaling can play either a positive or a negative role on the tendon and enthesis, depending on the context. During enthesis development, Hh signaling is necessary for the formation of a functional tendon-to-bone attachment. Presumably, Hh signaling will also be necessary for regeneration of the enthesis during tendon-to-bone healing. In contrast, aberrant Hh signaling can lead to pathologic mineralization in the tendon or the formation of enthesophytes in the joint. Tendon midsubstance healing, on the other hand, presents a paradox: Hh signaling appears to promote tenogenesis after injury, in sharp contrast to Hh signaling also promoting pathologic HO in tendon under certain circumstances. Therefore, despite significant progress in our understanding of Hh signaling during tendon/enthesis development and repair, further insights into the molecular and cellular regulation of Hh signaling is necessary before we can develop thoughtful therapeutic strategies.

Cellular therapies have tremendous potential for treatment of musculoskeletal pathologies. Transplantation of progenitor cells with the capacity to proliferate and integrate with the native tissue and deposit appropriate ECM may transform the treatment of intractable clinical problems such as rotator cuff repair. Although multiple cell sources, including stem cells derived from bone marrow, adipose tissue, and tendon, have been applied in different injured animal models, the beneficial effect of cellular therapies in tendon and enthesis disorders remains elusive. As discussed in this review, Gli1-responsive cells have been appreciated to modulate development and regeneration of a wide-range of tissues and are a promising candidate for cellular therapy. Furthermore, Gli1-reponsive cells in the tendon enthesis feature more likely as progenitors instead of stem cells and they may be more inclined to progress towards a cell phenotype with characteristics of tenogenesis, chondrogenesis, and osteogenesis (rather than myogenesis or adipogenesis)

after delivery to the healing enthesis. However, concerns remain about the use of these cells, which may localize to adjacent tissues and cause HO. Therefore, future studies should investigate factors driving their phenotype shift and manipulation of tissue niches to guide differentiation, and techniques should be developed to localize the delivery of these cells to the injury site.

Lastly, Hh-related pharmacologic approaches hold great potential for treatment of tendon and enthesis pathologies, including tendon-to-bone repair and HO. Some agonists, such as SAG, Hh-Ag1.5, and purmorphamine, have already been developed and used to activate Hh signaling for improving healing of fractured bone and injured meniscus [114, 126, 143]. Compared to the FDA-approved therapeutics of Hh antagonists, there is much more work to be done for translational application of Hh agonists. Any mechanistic differences between the different Hh agonists should be addressed, since inappropriate Hh stimulation can cause oncogenesis, unwanted osteogenesis, and fibrosis. Besides screening additional small molecules as Hh agonists and thoroughly characterizing their function, the application of these molecules in the laboratory and clinical settings needs to be optimized (e.g., delivery methods and dosing details). The pharmacological manipulation of Hh signaling is also associated with the precise delivery of drug molecules. Given the multifaceted molecular and cellular elements involved in tendon and enthesis healing, pharmacological treatment of Hh inhibition/agonism should be integrated and coordinated with other modalities (i.e., physical therapy) to achieve tendon regeneration.

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Highlights

- The formation and mineralization of enthesis fibrocartilage is regulated by hedgehog signaling.
- Hedgehog signaling can be beneficial, driving enthesis healing, or detrimental, driving tendon heterotopic ossification.
- Hedgehog signaling is a promising therapeutic target for tendon and enthesis pathologies.

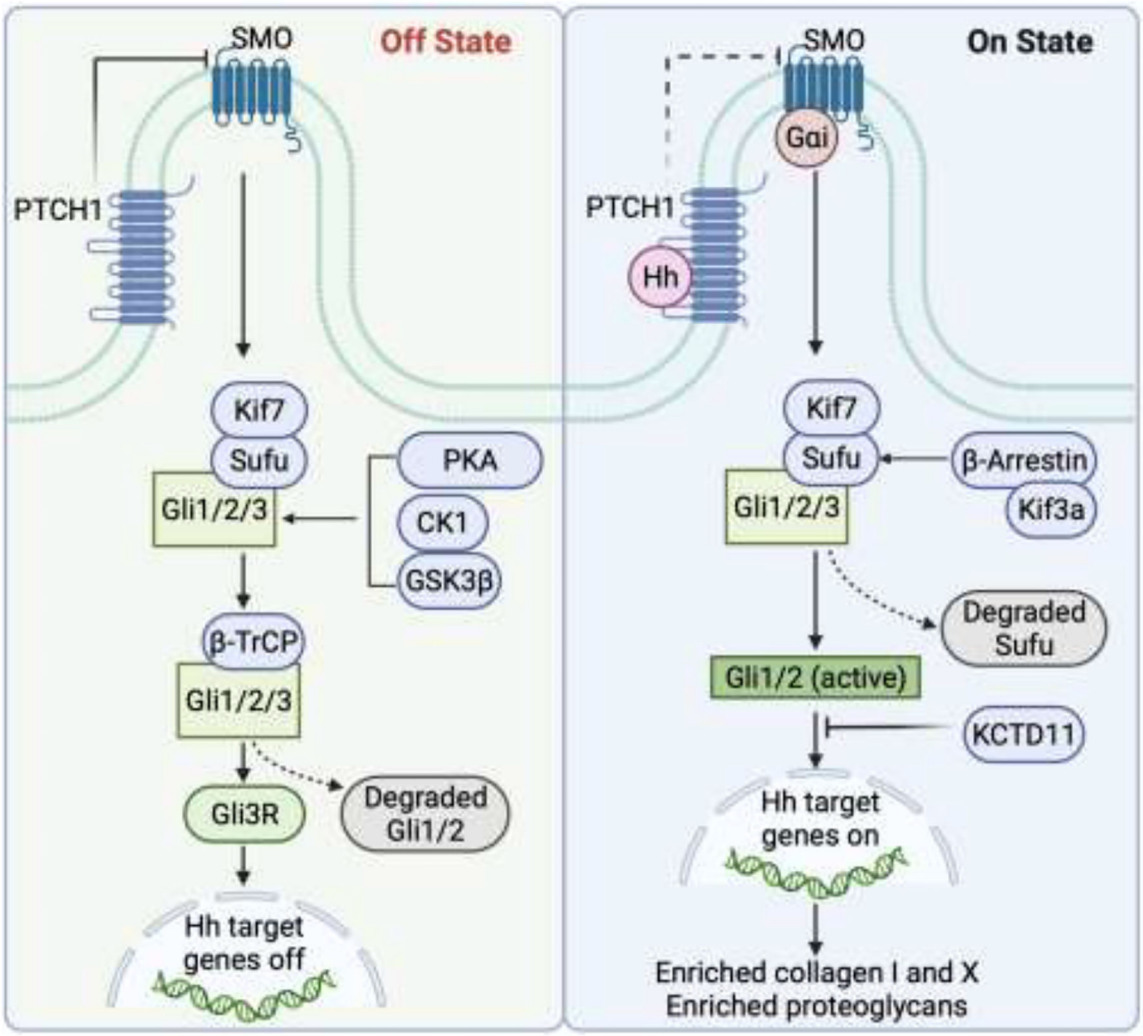

Figure 1.

Hh signaling is initiated when a Hh ligand binds to the cell surface receptor PTCH1, which results in repression and accumulation of the cell surface protein SMO in the primary cilium. SMO then triggers downstream signal transduction through proteins such as Sufu and Gli1/2/3. Other cellular factors, such as proteinase kinase A (PKA), casein kinase 1 (CK1), glycogen synthase kinase β3 (GSK3β), β-Arrestin, the kinesin family member 3a (Kif3a), beta-transducin repeats-containing proteins (β-TrCP), and the potassium channel tetramerisation doman containing 11 (KCTD11), engage in the regulation of downstream Hh transcription.

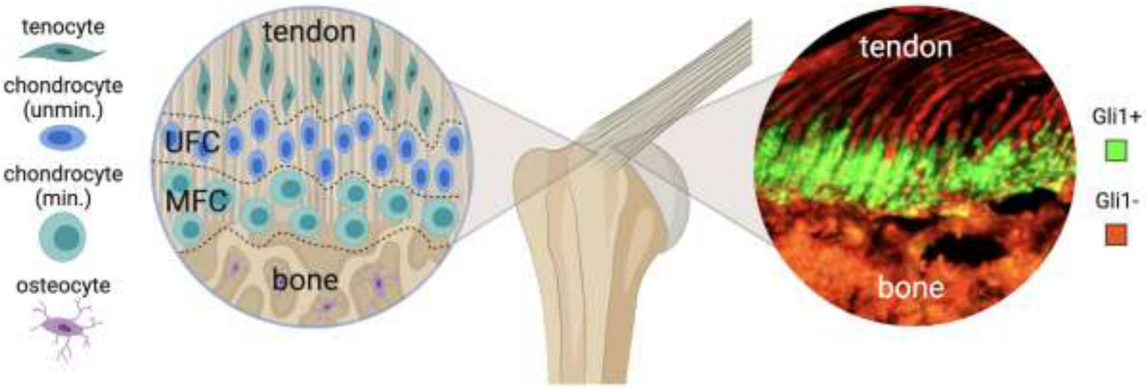

Figure 2.

Tendon attaches to bone across the enthesis, a fibrocartilaginous attachment with spatial gradients in structure, composition, and cell phenotype. Unmin.: unmineralized, Min.: mineralized chondrocytes, UFC: unmineralized fibrocartilage, MFC: mineralized fibrocartilage, Gli1+: Gli1-lineage cells, Gli-: cells not derived from Gli1 lineage.

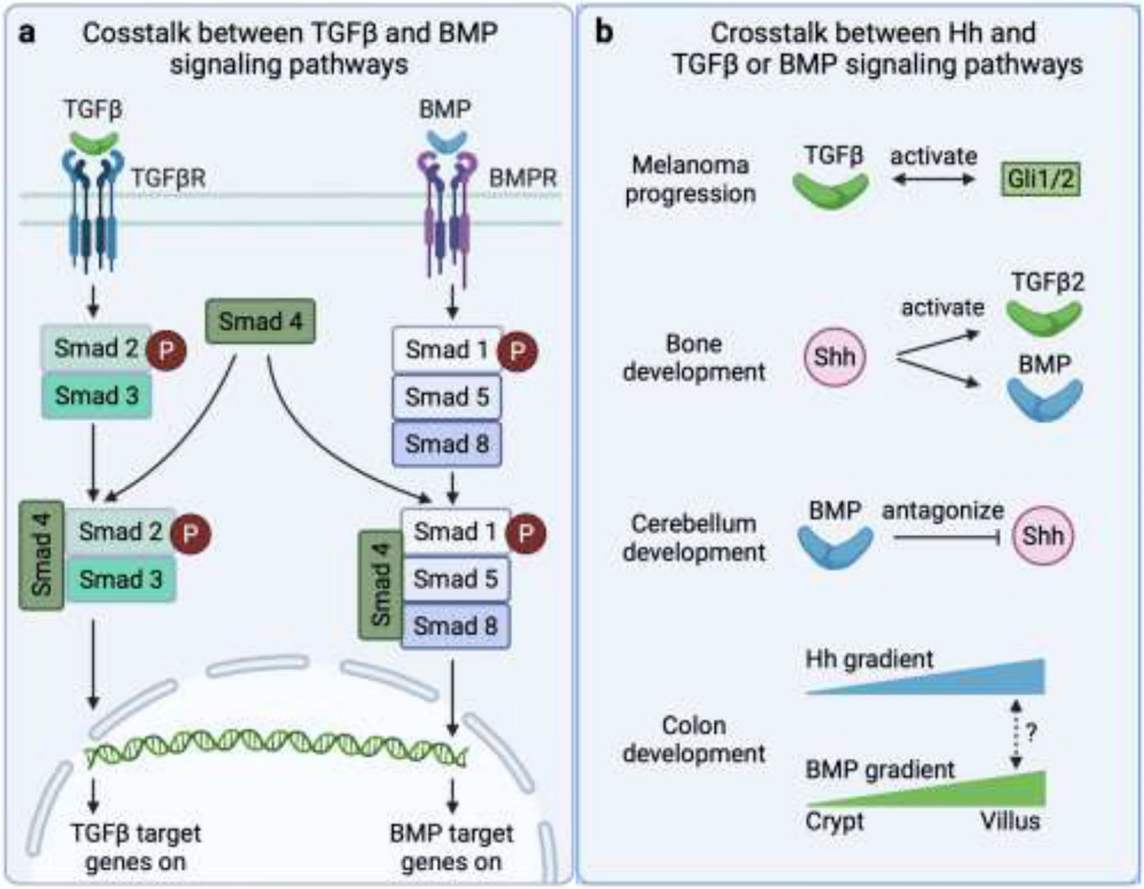

Figure 3.

Crosstalk between Hh, TGFβ, and BMP signaling pathways. **(a)** Crosstalk between TGFβ and BMP signaling pathways. **(b)** Proposed crosstalk between components of Hh and TGFβ or BMP signaling pathways.

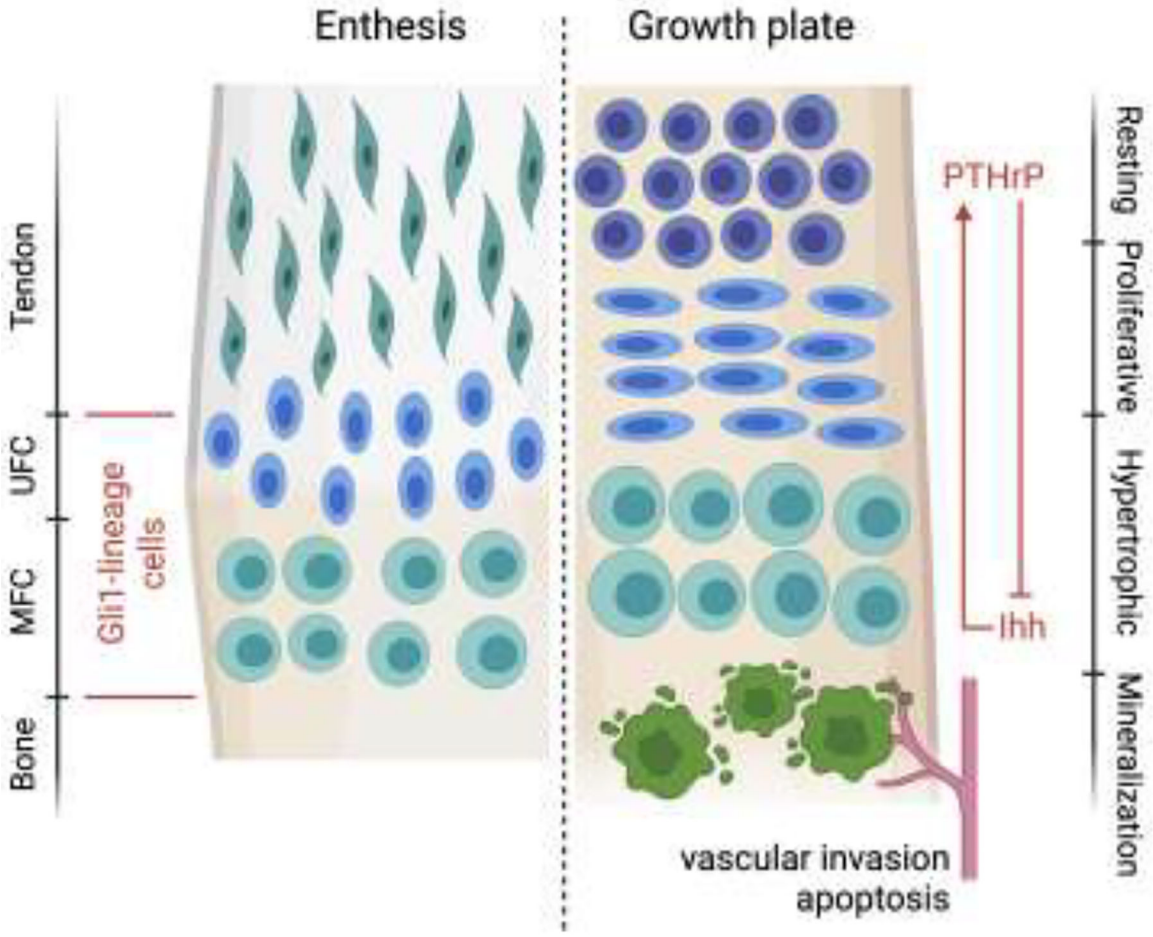

Figure 4.

The enthesis shares similarities with the growth plate, including a spatial gradient in cell phenotypes, Hh-responsive cells, and mineralizing chondrocytes. However, unlike the growth plate, vascular invasion and apoptosis does not occur in the enthesis zone of hypertrophic chondrocytes. Mineralized fibrocartilage remains at the mature enthesis, in contrast to growth plate cartilage, which is replaced by bone. UFC: unmineralized fibrocartilage, MFC: mineralized fibrocartilage.

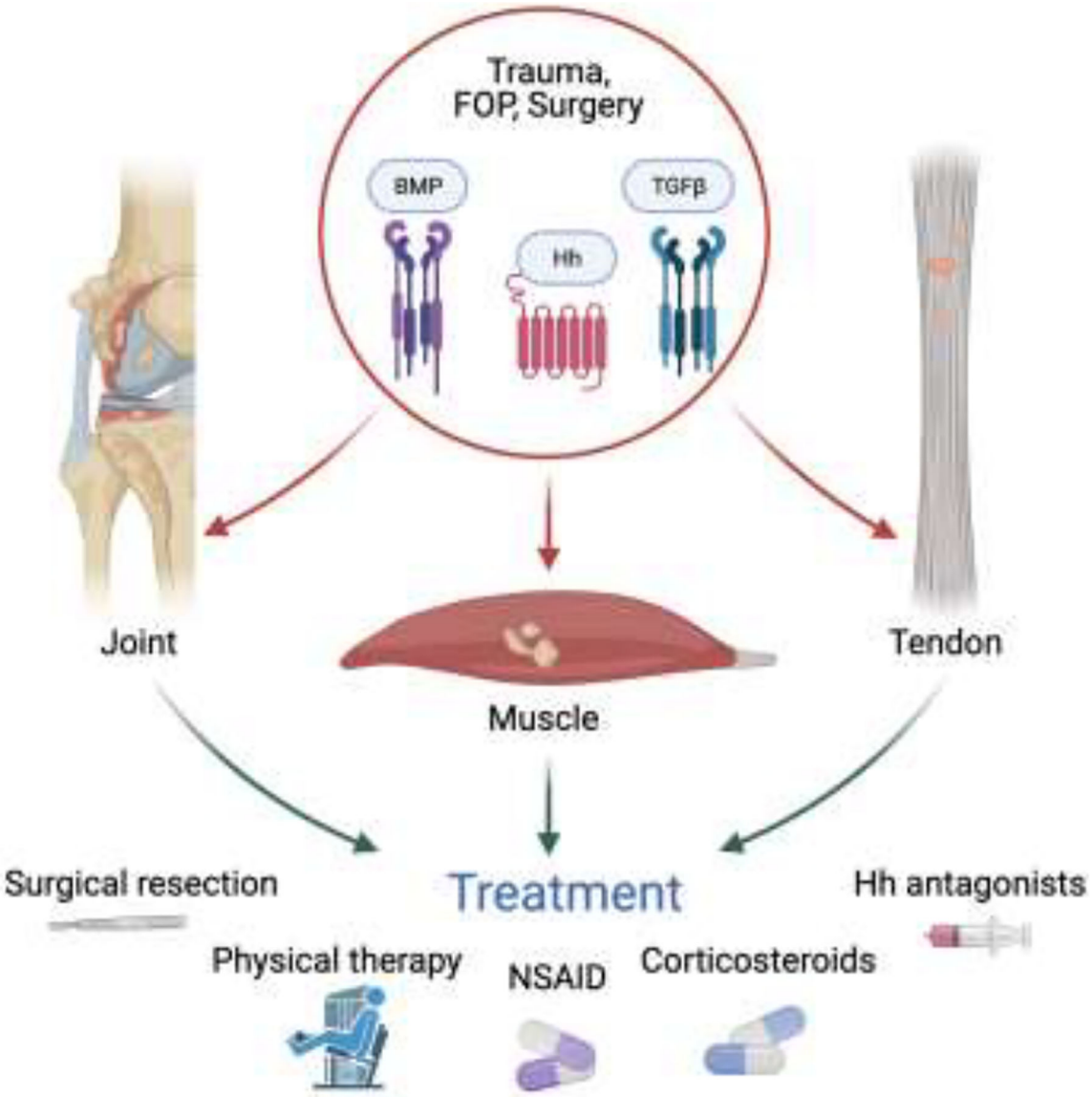

Figure 5.

Heterotopic ossification occurs after trauma, surgery, and rare genetic conditions such as fibrodysplasia ossificans progressiva (FOP) and is driven by aberrant Hh, BMP, and/or TGFβ signaling. Treatments such as surgical resection and NSAIDs are only marginally effective.

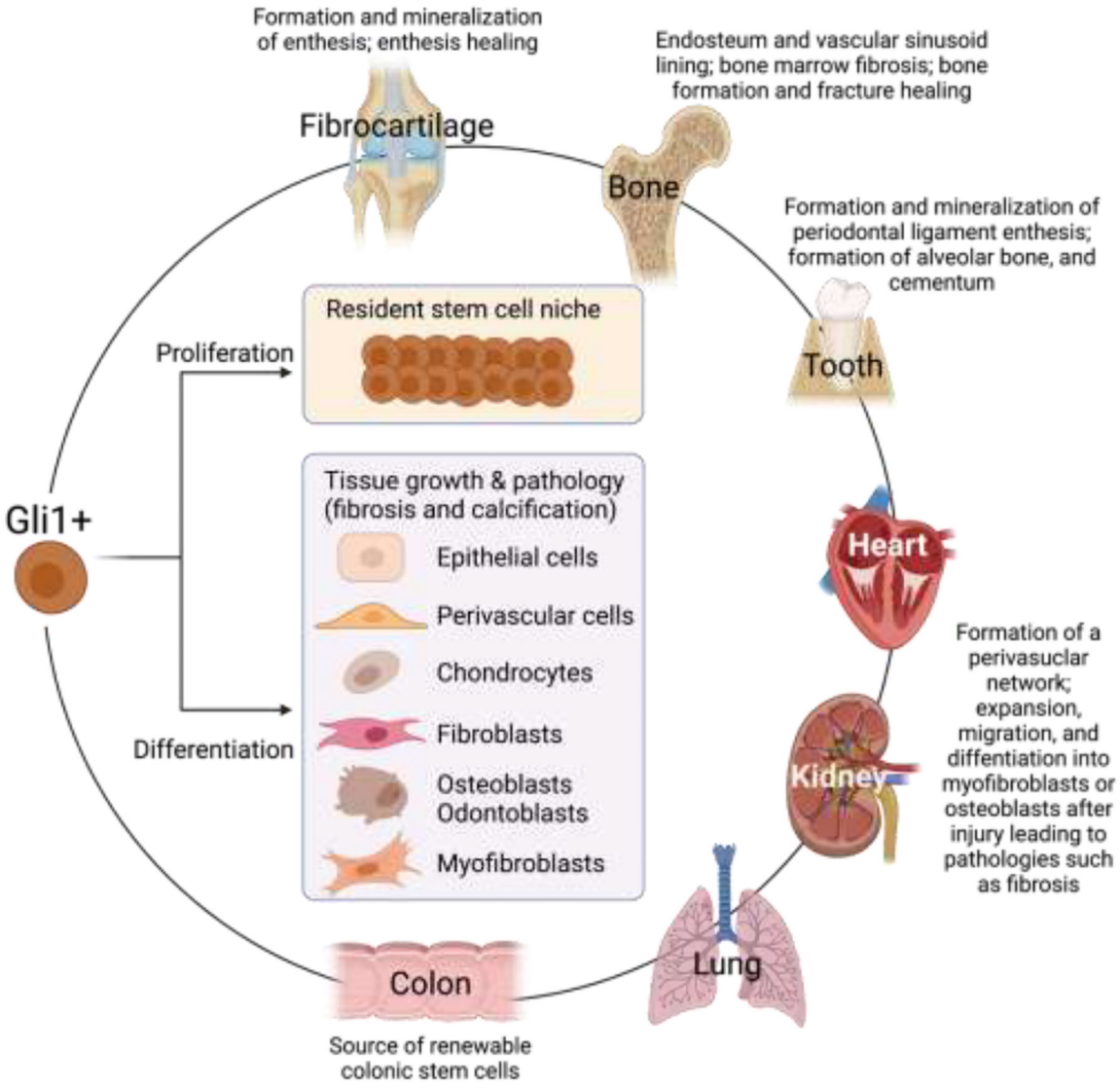

Figure 6.

A critical role for Gli1+ or Gli1-responsive cells has been described for the development and injury response of multiple tissues. Gli1-responsive cells may proliferate to maintain a stem cell niche or differentiate to populate and build a wide range of tissues.

Table 1

Hh signaling involved in tendon and enthesis healing (ACL: anterior cruciate ligament).

Tissue type	Species	Injury model	Cell population after injury	Gene expression after injury	Protein expression after injury
Supraspinatus tendon enthesis[32]	Mature mouse	Enthesis needle punch	Increasing numbers of **Hh**-lineage cells over time		Low expression of Notch 1
	Immature mouse	Enthesis needle punch	Consistently high numbers of **Hh**-lineage cells throughout healing period		High expression of Notch 1
Supraspinatus tendon enthesis[43]	Rat	Tendon transection, followed by transosseous repair	Increased Sox9-positive cells and **Ihh**-posibve cells at enthesis		Increased expression of **Gli1**, **PTCH1, Ihh**, Col2, and Sox9
Supraspinatus tendon enthesis[43]	Rat	Tendon transection, followed by transosseous repair		Similar expression of *Scx, Tnmd*; Increased expression of ***Smo***, *Col1. Acan*	
ACL enthesis[40]	Rat	Full-thick ness transection of ACL followed by reconstruction using flexor tendon graft			Increased expression of **Gli1** and **PTCH1**
ACL enthesis[41]	Mouse	Full-thickness transection of ACL followed by reconstruction with autograft		Similar expression of *Scx, Sox9*, and matrix metalloproteinases; Increased expression of *Acan*	Increased expression of **Ihh** Wnt, and PTHrP
Tibialis anterior tendon[44]	Mouse	Full-thickness transection followed by suture repair		Higher expression of *Mkx, Scx, Egr1, Col1a1, Col1a2, Tnmd, Thbs4, Tppp3, Bglap*, ***Gli1 Shh***	