Cripto favors chondrocyte hypertrophy via TGF-β SMAD1/5 signaling during development of osteoarthritis

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Abstract

Chondrocytes in mice developing osteoarthritis (OA) exhibit an aberrant response to the secreted cytokine transforming growth factor (TGF)-β, consisting in a potentiation of intracellular signaling downstream of the transmembrane type 1 receptor kinase activin receptor-like kinase (ALK)1 against canonical TGF-β receptor ALK5-mediated signaling. Unfortunately, the underlying mechanisms remain elusive. In order to identify novel druggable targets for OA, we aimed to investigate novel molecules regulating the ALK1/ALK5 balance in OA chondrocytes. We performed gene expression analysis of TGF-β signaling modulators in joints from three different mouse models of OA and found an upregulated expression of the TGF-β co-receptor Cripto (Tdgf1), which was validated in murine and human cartilage OA samples at the protein level. In vitro and ex vivo, elevated expression of Cripto favors the hypertrophic differentiation of chondrocytes, eventually contributing to tissue calcification. Furthermore, we found that Cripto participates in a TGF-β–ALK1–Cripto receptor complex in the plasma membrane, thereby inducing catalytic SMAD1/5 signaling in chondrocytes. In conclusion, we demonstrate that Cripto is expressed in OA and plays a functional role promoting chondrocyte hypertrophy, thereby becoming a novel potential therapeutic target in OA, for which there is no efficient cure or validated biomarker.

Keywords: TGF-β; BMP; ALK; Tdgf1; cartilage; bone; calcification; joint; ageing

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Introduction

Osteoarthritis (OA) is a painful and disabling condition of the joints affecting millions of people. Ageing is the primary risk factor, but how ageing results in OA remains an enigma. OA is characterized by degeneration of the articular cartilage, which has a very limited reparative capacity [1]. Therefore, detection of early tissue damage is essential to stop the progression of the disease. Unfortunately, existing diagnostic tools have low sensitivity and specificity and currently there is no validated cure for OA.

In healthy articular cartilage, chondrocytes maintain the fine balance between the production and degradation of the extracellular matrix (ECM). However, during OA, modifications in chondrocyte metabolism and environment (i.e. inflammation, hypoxia, or mechanical stress) disrupt this equilibrium. Chondrocytes then become hypertrophic and increase the secretion of matrix metalloproteinases (MMPs), leading to degeneration, mineralization, and vascularization of the matrix. The underlying mechanism driving this phenomenon remains unclear.

The transforming growth factor beta (TGF-β) family of dimeric proteins plays crucial roles during cartilage and bone development and homeostasis [2–4]. TGF-β signaling is initiated upon interaction of soluble active TGF-β ligands with membrane receptors bearing intrinsic serine/threonine kinase activity. TGF-β binds to the constitutively active TGF-β type II receptor (TβRII), which subsequently associates with and trans-phosphorylates the TGF-β type I receptor (TβRI), also named activin receptor-like kinase 5 (ALK5). Upon ALK5 activation, a specific set of intracellular mediators named SMAD2/3 become phosphorylated, associate with SMAD4, and translocate into the nucleus to modulate gene expression [5]. Importantly, in chondrocytes [6] and other cell types,
such as endothelial cells [7], TGF-β can recruit the bone morphogenetic protein (BMP) receptor ALK1, thereby inducing SMAD1/5/8 activation. Induction of either SMAD2/3 or SMAD1/5/8 leads to different transcriptional responses, including the expression of genes related to ECM components or genes mediating osteogenic differentiation, respectively. Importantly, TGF-β signal transduction is fine-tuned by the so-called co-receptors, including Cripto [8], which modify the ligand–receptor affinity but lack an intracellular enzymatic motif to originate intracellular cascades upon activation [9]. Cripto (Cripto-1, teratocarcinoma-derived growth factor-1 or TDGF-1) is a small, glycosylphosphatidylinositol (GPI)-anchored protein that plays key roles during embryonic development, in part by regulating signal transduction initiated by the TGF-β family member Nodal [10]. In adult healthy tissue, its expression is very low or absent, but high levels are found in several human tumors [11,12]. Mechanistically, Cripto has been shown to act as an obligatory co-receptor for certain TGF-β ligands such as Nodal, leading to phosphorylation of SMAD2/3, while it can also inhibit SMAD2/3 phosphorylation induced by activin-A/B or TGF-β [10,13]. TGF-β is a central regulator of chondrocyte progenitor proliferation, differentiation, and ECM production, whereas its main activity is the prevention of hypertrophic differentiation [14,15]. Disruption of TGF-β signaling has been implicated in OA [16]. In ageing and OA cartilage, the ALK1/ALK5 ratio is increased, leading to preferential activation of the SMAD1/5 signaling pathway, which mediates the expression of matrix metalloproteinase (MMP)-13, thereby contributing to the degradation of the cartilage [6,17,18]. TGF-β signaling has been linked to other changes observed in the osteoarthritic joint (osteophyte formation, subchondral bone alterations, vascularization, and fibrosis) [19]. Ablation of endogenous TGF-β activity suppresses osteophyte formation in vivo but in contrast aggravates cartilage degeneration in OA-mouse models [20]. Therefore, selective interference with TGF-β receptor pathological signaling in the joint tissues may have therapeutic promise.

In this paper, we demonstrate that expression of the TGF-β co-receptor Cripto is increased during experimentally induced OA and in human osteoarthritic cartilage. Furthermore, we describe a novel function of Cripto forming a membrane receptor complex with TGF-β and enhancing the activation of SMAD1/5/8 and subsequent mineralization in chondrocytes. Our findings hold promise for the further development of novel therapies based on Cripto targeting, to treat this so far incurable disease.

Materials and methods

Ethics

Collection and handling of human tissues (RAAK study) were performed in accordance with the guidelines of the Medical and Ethics Committee in Leiden, The Netherlands and the code of conduct of the Dutch Federation of Biomedical Scientific Societies (protocol No P08.239).

Animal procedures involving OA models were carried out after approval had been obtained from the Animal Ethics Committee of the Radboud University Nijmegen (DEC Nos RU-DEC 2010-036 and RU-DEC 2007-131). Isolation and studies involving embryonic mouse metatarsals were approved in protocol #14238, 16 December 2004 (Medical and Ethics Committee in Leiden, The Netherlands).

In vivo OA modeling

Three different in vivo experimental models were used in this study: ageing-induced OA, destabilization of the medial meniscus-induced OA, and STR/ort. In brief, C57BL/6N mice aged 6, 8, 12, 16 or 20 months (n = 8–11) and genetically predisposed STR/ort mice of ages 2, 4, 8, and 12 months (n = 7 or 8) were used to analyze the expression pattern of Tdgf1 and related family members in relation to ageing. Histological examination of the knee joints was performed after dissection, fixation, decalcification in formic acid for 1 week, and then embedding in paraffin wax. Sections were cut at 5 μm and stained with Safranin O and Fast Green, as described previously [21].

Histological evaluation was performed using a modified OARSİ score [22]. In brief, medial and lateral tibiae and femora within one joint were stained as mentioned above and examined independently (five sections each). Grade (maximum score of the pathology) and stage (extent of the total damage) were scored and averaged per joint and cartilage surface, to obtain a mean grade and mean stage per joint and cartilage surface. Finally, the total OA score was calculated as the sum of the individual OA scores of each cartilage surface.

For surgical destabilization of the medial meniscus (DMM), C57BL/6N mice at 10–12 weeks of age were used to induce OA by transecting the anterior attachment of the medial meniscus to the tibia of a right knee joint, as previously described [16]. Following the DMM operation, mice were sacrificed at different time points (2, 4 or 6 weeks) after surgery, including at least three up to 11 mice at each time point. Knee joints of sacrificed mice were isolated for histology and compared with those from control mice (n = 3). OA was confirmed by histological evaluation as described previously [23]. The corresponding left knee joint of each mouse was used as a control for comparison of DMM-operated versus non-operated knee joints.

Gene target identification

To obtain RNA samples, the cartilage dissected from the joint was instantly frozen in liquid nitrogen and crushed using a dismembrator. The crushed material was dissolved in RLT buffer supplied with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Samples were treated with proteinase K, and subsequently RNA was
isolated further using the RNeasy Mini Kit according to the manufacturer’s protocol. Since the RNA yield obtained from mouse cartilage is extremely low, we used the WT-Ovation RNA Amplification System (NuGEN, Bemmel, The Netherlands) with 50 ng of total RNA, for which RNA from different animals needed to be pooled together. The amplified RNA was subsequently loaded in a custom-made PCR Array (RT² Profiler PCR array, SAB Biosciences, Qiagen) following the instructions provided by the supplier.

Cell culture and reagents

ATDC5 cells (Riken Cell Bank, Saitama, Japan) were cultured in DMEM/F12 medium supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 95% air and 5% CO₂ at 37 °C. Immortalized C28/2 human chondrocytes [17], HEK293T cells, and C2C12 (ATCC, Rockville, MD, USA) murine myoblast cells were maintained in DMEM medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), and penicillin/streptomycin (Invitrogen). TGF-β3 was a kind gift from Dr A Hinck (University of Pittsburgh, PA, USA). Recombinant BMP9 and Cripto were purchased from R&D Systems (Minneapolis, MN, USA) and Cell Signaling Technology (Leiden, The Netherlands), respectively. Human anti-ALK1 (investigational drug anti-hALK1, PF-03446962; Pfizer, New York, NY, USA) has been described previously [24] and was used at a final concentration of 1 μg/ml.

In vitro mineralization assays

We used three different in vitro models of chondrogenic mineralization. For the first model, 5 × 10^4 ATDC5 or C28/2 cells were seeded in 48-well plates to form a monolayer and were subsequently incubated for 21 days in mineralization medium (DMEM/F12) containing insulin–transferrin–selenium (ITS; Gibco), 0.2 mM ascorbic acid (Sigma-Aldrich/Merck, Darmstadt, Germany), and 10 mM β-glycerophosphate (Sigma-Aldrich). Alternatively, ATDC5 cells were incubated in 21 days in differentiation medium containing ITS and 0.2 mM ascorbic acid, and then switched to mineralization medium including 10 mM β-glycerophosphate for 4 days. For 3D chondrogenic micromass assays, stably transduced ATDC5 cells were trypsinized and washed once with PBS. 3 × 10^5 cells were counted per micromass and resuspended in 10 μl of culture medium. Very carefully, 10 μl drops were deposited in the center of wells in a 24-well plate and placed in an incubator for 2 h. Next, 500 μl of DMEM-F12 5% FBS containing 1x ITS (Gibco) was carefully added to each well. After 24 h, the medium was replaced by DMEM-F12 5% FBS containing 1x ITS, supplemented with 0.2 mM ascorbic acid and 10 mM β-glycerophosphate and TGF-β (10 ng/ml), for the time indicated. In all differentiation experiments, the medium was refreshed every 4 days. Afterwards, cells were washed twice with PBS and fixed with 3.7% formaldehyde for 5 min. Fixed cells were washed twice with distilled water and measurement of calcium deposition was performed using Alizarin Red staining (ARS), as described previously [25]. Precipitates that originated from three independent ARS assays were dissolved using 10% cetylpyridinium chloride and absorbance was measured at 570 nm. Representative images were acquired using a Leica DM IL LED microscope (Leica Biosystems, Amsterdam, The Netherlands) loaded with a 10× magnification objective and coupled to a digital camera.

Fetal mouse bone explants (metatarsals assay)

Fetal wild-type C57BL/6N or conditional Tdgf1 knockout (Tdgf1tm2.2Mms) mouse embryos at 15.5–17.5 days post-implantation (dpi) were used for this experiment. The middle three metatarsals from the embryonic hind limbs were dissected under sterile conditions and cultured independently in 48-well plates containing 200 μl of αMEM supplemented with 10% FBS, 0.05 mg/ml ascorbic acid, l-glutamine, 0.05 mg/ml gentamycin, 0.25 mg/ml amphotericin B, 1 mM β-glycerophosphate, and 0.2% bovine serum albumin (BSA) Cohn fraction V, as described previously [26,27]. In addition, the osteogenic medium described above was supplemented with recombinant factors (TGF-β3, 10 ng/ml; Cripto, 200 ng/ml) and incubated for 6 days. For experiments analyzing the effect of genetic deletion of Cripto (Tdgf1), increasing volumes of supernatant containing control viruses or Cre-expressing lentiviruses were added to the metatarsal cultures at day 0 and cultured for 4 days, prior to stimulation as described above. During culture, lengths of hypertrophic, mineralized, and proliferative cartilage were measured under an inverted microscope. In these embryonic rudiments, the first zone of hypertrophic cartilage is visible as a clear area in the center, as demonstrated previously [28]. The mineralized cartilage is the dark area that develops in the center of the hypertrophic zone, and the proliferative zone is the cartilage at the ends of the embryonic rudiment.

Statistical analyses

Student’s t-test was used for statistical analysis, with p < 0.05 considered as statistically significant. All experiments were performed at least in triplicate, unless otherwise indicated.

Technical details for Immunohistochemical staining, immunofluorescence, and Mankin scoring; RT-qPCR; Western blotting; iodination and ligand affinity labeling of cell surface receptors; and Lentivirus production are provided in Supplementary materials and methods.

Results

The expression of the TGF-β co-receptor Cripto is increased prior to osteoarthritis development

In order to find novel druggable targets to revert pathological TGF-β signaling in OA, we analyzed secreted
TGF-β signaling modulators that are not essential for signaling but can fine-tune TGF-β signaling in a cell and/or tissue-type and dynamic manner. Since human cartilage tissues can be obtained only at the end stage of the disease, articular cartilage from C57Bl/6N mice that spontaneously develop OA during ageing was

Figure 1

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analyzed over a period of 20 months. Of note, we found that the mRNA levels of Tdgf1, encoding the TGF-β co-receptor Cripto, were strikingly increased towards the onset of OA (Figure 1A), which was confirmed at the protein level in joints of 8-month-old mice (Figure 1B). Interestingly, other soluble factors related to the activity of Cripto (i.e. Nodal, Lefty1, Lefty2, Cer1) were also induced at early stages, suggesting a common underlying mechanism. In order to correlate the expression of Cripto with OA onset and development, joints from ageing C57Bl/6N mice were scored following a modified OARSI method [22]. Importantly, Tdgf1 expression was found to be induced prior to the appearance of early histopathological signs of OA in the joints of ageing mice, which become significant at 12 months (Figure 1C and supplementary material, Figure S1A), coinciding with an induction in the expression of the chondrogenic markers aggrecan (Acan), collagen type X alpha 1 chain (Col10a1), collagen type II alpha 1 chain (Col2a1), and matrix metalloproteinase 13 (Mmp13) (Figure 1D). To further validate the expression of Tdgf1 during OA, we monitored the expression of this transcript in additional murine models of spontaneously accelerated STR/ort and induced (DMM) OA. In the well-characterized genetic STR/ort model [29], we found an initial early induction in 4-month-old STR/ort mice, followed by a progressive downregulation of Tdgf1 (Figure 1E), similar to the expression pattern of a number of chondrogenic differentiation markers (Figure 1F), thus preceding the development of severe OA histopathology (supplementary material, Figure S1B). In a mouse model of induced OA consisting in the mechanical destabilization of the medial meniscus (DMM), Tdgf1 expression was progressively reduced after 4 and 6 weeks, coinciding with a downregulation in chondrogenic genes (supplementary material, Figure S2A,B). Finally, in order to validate these observations in human OA lesions, we scored the expression of Cripto in a small subset of articular cartilage biopsies collected from OA patients following stratification by Mankin score [30–32] (supplementary material, Figure S3A). As shown in Figure 1G, Cripto was expressed in chondrocyte-like cells expressing the hypertrophic marker COL10A1 present in the superficial and deeper layers of the articular cartilage, and its expression was enhanced in severely damaged joints of OA donors (Figure 1H and supplementary material, Figure S3B). These results confirm the expression of the TGF-β co-receptor Cripto in human cartilage and relate it to the development of OA.

Cripto mediates chondrogenic hypertrophy in vitro

We aimed next to investigate the functional role of Cripto in OA. Therefore, we made use of different protocols to achieve chondrocyte hypertrophy and calcification in vitro. First, we cultured ATDC5 mouse prechondrocytes as monolayers for 21 days in the presence of insulin–transferrin–selenium (ITS), ascorbate, and β-glycerophosphate (Figure 2A). In this model, the cells were stained positively for calcium deposits using Alizarin Red (AR) from day 14 onwards (Figure 2B). This staining correlated with a significant increase in the expression of the chondrogenic markers Col10a1, Col2a1, and alkaline phosphatase (Alp). Importantly, Cripto expression was elevated towards late differentiation stages (Figure 2C and supplementary material, Figure S4). A shift in the use of the TGF-β type I receptors ALK5 and ALK1 has been shown during the progression of OA [6]. In this in vitro model, we observed a trend towards upregulation of both genes during the progression of differentiation. Since we detected Cripto expression at later stages of chondrocyte differentiation, we developed a two-step differentiation protocol to study the correlation between Cripto expression and cell calcification. ATDC5 cells were exposed to ITS for 21 days and thereafter supplemented (or not) with ascorbate and β-glycerophosphate until day 25 (Figure 2D) to induce cell calcification. Using this protocol, Tdgf1 mRNA and protein expression was highly induced from day 25 onwards in response to supplementation of the medium with ascorbate and β-glycerophosphate (Figure 2E,F and supplementary material, Figure S4), therefore suggesting a correlation of Cripto with chondrocyte hypertrophy and cartilage ossification. Finally, we validated our findings in a 3D model of chondrocyte hypertrophy, consisting in 3D micromass cultures of highly condensed ATDC5 cells, as reported elsewhere [33]. To study the role of Cripto in hypertrophy, we first stably knocked down Tdgf1 using two different short hairpin RNA (shRNA) constructs (Figure 2G). Notably, genetic
Figure 2. Cripto expression analysis during chondrogenic differentiation in vitro. (A) Schematic overview of the experimental design (one-step protocol). ATDC5 cells were cultured in the presence of chondrogenic differentiation medium containing insulin–transferrin–selenium (ITS), ascorbic acid (Asc), and β-glycerophosphate (βGP) for 21 days. At 0, 7, 14, and 21 days, the medium was refreshed. (B) Alizarin Red staining (ARS) to visualize calcium deposits was performed on ATDC5 cells cultured in monolayer under chondrogenic differentiation conditions at days 0, 7, 14, and 21 (objective magnification 10×). (C) Relative mRNA expression corresponding to Tdgf1 (encoding Cripto), Acvrl1 (encoding ALK1), Tgfbr1 (encoding ALK5), collagen type X alpha 1 chain (Col10a1), collagen type II alpha 1 chain (Col2a1), and alkaline phosphatase (Alp) in ATDC5 cells at 0, 7, 14, and 21 days of culture under chondrogenic conditions (one-step model). (D) Schematic overview of the experimental design (two-step protocol). ATDC5 cells were cultured in the presence of chondrogenic differentiation medium containing ITS for 21 days. From day 21, Asc and βGP were added to the medium. At 0, 7, 14, 21, and 25 days, the medium was refreshed. (E) ARS was performed on ATDC5 cells cultured in monolayer under chondrogenic differentiation conditions at days 0, 3, 14, and 25 (or 25 days with Asc and βGP, 25+) (objective magnification 10×). (F) Relative mRNA expression of Tdgf1, Acvrl1, Tgfbr1, Col10a1, Col2a1, and Alp in ATDC5 cells at 3, 14, and 25 days (with or without Asc and βGP, 25+) of culture under chondrogenic conditions (two-step protocol). (G) ARS performed in 3D chondrogenic pellets of ATDC5 stably knocked down for Tdgf1. Two control lines and two Tdgf1 shRNAs are shown. Relative expression of Tdgf1 and quantification of ARS upon solubilization are shown. (H) ARS performed in 3D chondrogenic pellets of ATDC5 stably overexpressing Tdgf1. Two control lines and one overexpression (OE) line are shown. Relative expression of Tdgf1 and quantification of ARS upon solubilization are shown. *p < 0.05; **p < 0.001; ***p < 0.0001.
depletion of Tdgf1 effectively inhibited the deposition of calcified matrix by condensed chondrocytes. Moreover, stable overexpression of Tdgf1 significantly augmented the calcification of 3D chondrocyte micropellets, as assessed by AR staining (Figure 2H). Our results suggest that increased expression of Cripto in hypertrophic chondrocytes favors calcification.

**Cripto modulates chondrocyte hypertrophy ex vivo**

In order to functionally verify a physiological role for Cripto in mediating chondrocyte hypertrophy, we made use of an *ex vivo* metatarsals culture system. In this model, mouse embryonic (E15.5–17.5) bone metatarsals are resected and cultured in osteogenic medium, subsequently expanding *ex vivo* in a process that resembles endochondral ossification and chondrocyte differentiation [26,27]. After culture in osteogenic medium, the length of the dark mineralized area is measured and related to the total bone length. Using this model, we first investigated the effect of recombinant Cripto in chondrocyte hypertrophy. Of note, after 7 days of culture, all metatarsals from the control group had grown in length and showed an enlarged dark area in the center, corresponding to the hypertrophic area. Ectopic addition of TGF-β effectively suppressed expansion of the hypertrophic area (Figure 3A,B). Furthermore, pre-incubation with recombinant Cripto rescued the inhibitory effect of TGF-β, suggesting that Cripto could counteract the effect of TGF-β during chondrocyte differentiation and hypertrophy, and possibly during the development of OA. To further investigate the role of Cripto during chondrocyte hypertrophy, we used middle metatarsals isolated at E17.5 from conditional Tdgf1 knockout embryos (Tdgf1^tm2.2M handic). As assessed by immunofluorescence labeling of Cripto and the hypertrophic chondrocyte marker COL10A1, double-positive cells expressing Cripto and COL10A1 were found to be accumulated at the boundaries of the hypertrophic area (Figure 3C). Next, we genetically deleted Cripto using increasing concentrations of lentivirus encoding the Cre recombinase (or a control virus) and cultured these mouse metatarsals in osteogenic medium for 10 days. Immunofluorescence analysis showed that Cripto was efficiently downregulated in a dose-dependent manner following lentiviral infection (Figure 3C). Moreover, the genetic deletion of Cripto significantly reduced the expression of COL10A1, as well as the length of the mineralized area, compared with control infected metatarsals (Figure 3D,E). We obtained similar results by interfering with Cripto expression at an earlier embryonic stage using metatarsals isolated at E15.5 (supplementary material, Figure S5). Our results demonstrate a functional role for Cripto tuning the hypertrophic response of chondrocytes to TGF-β signaling.

**Cripto fine-tunes TGF-β signaling by enhancing SMAD1/5 activation**

Finally, we investigated the molecular mechanisms by which Cripto modulates cellular responses to TGF-β in chondrocytes. A mechanism proposed to mediate OA development involves a shift in intracellular signaling induced by TGF-β, from the activation of intracellular SMAD2/3 via the ALK5 receptor, towards the phosphorylation of SMAD1/5 via ALK1 [6]. However, the mechanisms underlying this shift remain elusive. Given that Cripto can modulate TGF-β signaling in other cell types [34–36], we hypothesized that Cripto may modulate the balance between TGF-β and BMP signaling through ALK1/ALK5 in OA chondrocytes. First, we investigated the effect of Cripto on TGF-β–induced signaling in ATDC5 chondrocytic cells. Pre-incubation with increasing concentrations of recombinant Cripto led to enhanced phosphorylation of SMAD1/5 upon TGF-β stimulation in a dose-dependent manner (Figure 4A). We confirmed the effect of Cripto on phosho-SMAD1 by immunofluorescence (Figure 4B). Moreover, we analyzed the expression of phospho-SMAD1 and phospho-SMAD2 in mouse metatarsals genetically depleted for Cripto. As shown in supplementary material, Figures S6 and S7, addition of Cre lentiviruses led to a marked inhibition of Cripto expression. Interestingly, we observed an overall decrease in phospho-SMAD1-stained cells in the absence of Cripto (supplementary material, Figure S6), whereas phospho-SMAD2-stained cells were augmented (supplementary material, Figure S7), in line with previous reports describing an inhibitory effect of Cripto on TGF-β signaling [36,37].

Given that TGF-β–induced SMAD1/5 activation is mediated by ALK1, which also transduces signaling induced by BMP9 [38,39], we studied the effect of Cripto on BMP9 signaling. As shown in Figure 4C, pre-incubation with recombinant Cripto also enhanced BMP-9–induced SMAD1/5 phosphorylation, suggesting a functional interaction between ALK1 and Cripto. We then hypothesized that Cripto facilitates a physical interaction between TGF-β and ALK1. To demonstrate this, we performed ligand–receptor binding affinity assays using radiolabeled TGF-β, HEK293T cells overexpressing TβRII-myc and either ALK1-HA or ALK5-HA were incubated with radioactive TGF-β in the presence of recombinant Cripto. Of note, we detected TGF-β receptor complexes containing either ALK1 or ALK5 in the absence of recombinant Cripto, whereas pre-incubation with Cripto enhanced the association of TGF-β with a membrane complex containing TβRII, ALK1, and Cripto (Figure 4D,E). Our results indicate that Cripto preferentially interacts with ALK1 in receptor complexes at the membrane, thereby favoring SMAD1/5 phosphorylation in response to either TGF-β or BMP9. To functionally validate our hypothesis, we used a human–specific ALK1 neutralizing antibody (anti-hALK1) [24] as a drug candidate to fine-tune TGF-β signaling through ALK1 in chondrocytes. As shown in supplementary material, Figure S8, incubation of human C28/I2 chondrogenic cells with anti-hALK1 led to a significant decrease in cell mineralization in C28/I2 cells overexpressing Cripto, as measured by ARS (supplementary material, Figure S8A). These data were in line with inhibition of the expression of the
Figure 3. Cripto enhances chondrocyte hypertrophy ex vivo. (A) E17.5 metatarsals incubated for 7 days with osteogenic medium, recombinant Cripto (200 ng/ml), and/or TGF-β (10 ng/ml). The hypertrophic/calcified area is visualized as the central dark area. A representative picture is shown (objective 40x). (B) Statistical analysis of the hypertrophic area/total length calculated for all conditions shown in A (n = 7). (C) Immunofluorescence labeling for Cripto (red channel), collagen type X alpha 1 chain (COL10A1, green channel), and cell nuclei (DAPI, blue channel) in E17.5 Tdgf1tm2.2Mms metatarsals incubated for 10 days in osteogenic medium with control- or Cre-lentivirus particles. (D) Representative bright-field images of E17.5 Tdgf1tm2.2Mms metatarsals incubated for 10 days with control lentiviruses or increasing concentration of lentivirus particles encoding the Cre recombinase. Objective 40x. (E) Quantification analyses for the experimental conditions depicted in D (n = 12). *p < 0.05; **p < 0.001; ***p < 0.0001.
Figure 4  Legend on next page.
chondrogenic markers MMP13, COL10A1, and COL2A1 (supplementary material, Figure S8B). Taken together, these data show that this mechanism, which may be shared by other cell types, mediates the shift in TGF-β signaling previously described to alter chondrocyte homeostasis, eventually contributing to OA development.

Discussion

Osteoarthritis (OA) is a painful and disabling condition of the joints affecting millions of people, for which there is no validated cure or a useful biomarker, despite the extensive search for druggable targets to modulate the response of chondrocytes within the affected joints. It has been shown that TGF-β signaling plays a key role in chondrocyte differentiation and activation, and disruption of the TGF-β pathway has been associated with OA. Particularly, in osteoarthritic chondrocytes, TGF-β ligands progressively shift from canonical ALK5 signaling (thus inducing SMAD2/3 activation and subsequent anabolic signaling) towards a membrane complex containing ALK1 (leading to SMAD1 activation and catabolic responses). Unfortunately, the mechanistic details underlying this pathological shift have not yet been identified.

To the best of our knowledge, this report is the first demonstration of a functional role of the TGF-β family co-receptor Cripto in cartilage homeostasis. We first showed increased expression of Tdgf1 (encoding Cripto) in three different animal models of OA and in tissue sections from OA donors. We found a correlation between Cripto expression and disease severity, specifically in articular chondrocytes. Interestingly, Cripto (Tdgf1) was not detected in our previous microarray analysis, or in our RNA sequencing analysis of 35 paired samples of OA-affected cartilage [31]. To determine whether the different cohorts studied or discrepancies between mRNA and protein expression levels may underlie our different observations will require further studies.

Next, we used different in vitro models of chondrocyte hypertrophy and differentiation to demonstrate that Cripto is expressed in terminally differentiated (hypertrophic) chondrocytes and its inhibition partially prevents cell calcification. Mechanistically, we have shown that Cripto promotes TGF-β signaling via ALK1–phospho SMAD1 by physically taking part in a receptor complex involving TGF-β, ALK1, and TβRII, and also augments BMP9-induced SMAD1/5 activation (Figure 5). Finally, we validated our findings using gain and loss of Cripto in an ex vivo model of endochondral ossification, where Cripto modulates the differentiation of growth plate chondrocytes. Taken together, our results highlight the potential of Cripto as a therapeutic target and biomarker for OA.

Cripto has been shown previously to bind to TβRII, thereby compromising TGF-β signaling via ALK5 [36]. In chondrocytes in vitro, we observed a minor effect on SMAD2 phosphorylation, in comparison with the stimulatory effect on SMAD1/5. Moreover, Cripto also enhanced BMP9-induced activation of SMAD1/5, thus supporting the effect via ALK1. Therefore, it is possible that the function of Cripto is determined by the relative expression of certain BMP and TGF-β membrane receptors, which differ in different cell types. Our studies are consistent with a role for Cripto in stimulating chondrocyte hypertrophy via enhanced SMAD1/5 activation in response to both TGF-β and BMP9. The increased expression of Cripto combined with the previously documented shift in the ALK1/ALK5 ratio suggests that Cripto exacerbates the impact of this shift toward BMP signaling. Our studies demonstrating the existence of Cripto complexes with ALK1 suggest that this effect is direct. A shift in the TGF-β ALK1/ALK5 ratio has been previously described in endothelial cells (ECs), where activation of ALK5 inhibits EC proliferation and migration, whereas ALK1 induction results in increased migration and proliferation [40]. Whether Cripto may favor ALK1 signaling in ECs remains to be studied. Interestingly, we have recently determined that circulating ECs from patients with a very rare disease named fibrodyplasia ossicinans progressiva (FOP) express higher levels of Cripto [25]. FOP is due to enhanced SMAD1/5 signaling and subsequent heterotopic ossification linked to congenital mutations in the TGF-β family receptor ACVR1 (ALK2) [41]. Furthermore, Cripto may act in an autocrine/paracrine manner, as Cripto can be shed from the cell membrane in response to different stimuli [42,43]. Therefore, in our metatarsal assays, even though we observe a main effect in double-positive COL10A1/Cripto cells (likely chondrocytes), we cannot exclude that soluble Cripto may influence the surrounding cells too (for example, osteoblast progenitors), thereby modulating tissue calcification. Moreover, chondrocytes may not be the only source of Cripto in the OA joint. As such, macrophages [35], hematopoietic stem cells [44], and muscle satellite cells [34], at least, express Cripto in musculoskeletal tissues. Further research may be required to understand...
whether membrane-bound and soluble Cripto may have differential effects depending on the cell type and milieu of TGF-β ligands to which the cells are exposed.

In summary, our studies provide biochemical and genetic evidence that Cripto is a central mediator of OA progression and chondrocyte hypertrophy via modulation of BMP/TGF-β signaling. Further research may explore the potential of Cripto in normal cartilage development and maintenance, as a biomarker for OA in serum or synovial fluid of patients, and as a target for the development of specific antagonists to prevent chondrocyte hypertrophy.

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Author contributions statement

AGV, GSD, EBD, AVC and YR designed and performed experiments and analyzed and visualized the data. KL performed experiments and analyzed and visualized the data. GSD, MJG and PtD wrote the manuscript. YR, MG and IM provided human samples. MJG, PVK and PtD supervised the study. GSD, MJG and PtD acquired funding.

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