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Osteoarthritis and Cartilage

ShcA promotes chondrocyte hypertrophic commitment and osteoarthritis in mice through RunX2 nuclear translocation and YAP1 inactivation

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SUMMARY

Objective: Chondrocyte hypertrophic differentiation, a key process in endochondral ossification, is also a feature of osteoarthritis leading to cartilage destruction. Here we investigated the role of the adaptor protein Src homology and Collagen A (ShcA) in chondrocyte differentiation and osteoarthritis.
Methods: Mice ablated for ShcA in osteochondroprogenitor cells were generated by crossing mice carrying the Twist2-Cre transgene with $ShcA^{flox/flox}$ mice. Their phenotype ($n = 5$ to 14 mice per group) was characterized using histology, immuno-histology and western-blot. To identify the signaling mechanisms involved, *in vitro* experiments were conducted on wild type and ShcA deficient chondrocytes (isolated from $n = 4$ to 7 littermates) and the chondroprogenitor cell line ATDC5 ($n = 4$ independent experiments) using western-blot, cell fractionation and confocal microscopy.
Results: Deletion of ShcA decreases the hypertrophic zone of the growth plate (median between group difference −11.37% [95% confidence interval −17.34 to −8.654]), alters the endochondral ossification process, and leads to dwarfism (3 months old male mice nose-to-anus length −1.48 cm [-1.860 to −1.190]). ShcA promotes ERK1/2 activation, nuclear translocation of RunX2, the master transcription factor for chondrocyte hypertrophy, while maintaining the Runx2 inhibitor, YAP1, in its cytosolic inactive form. This leads to hypertrophic commitment and expression of markers of hypertrophy, such as Collagen X. In addition, loss of ShcA protects from age-related osteoarthritis development in mice (2 years old mice OARSI score −6.67 [-14.25 to −4.000]).
Conclusion: This study reveals ShcA as a new player in the control of chondrocyte hypertrophic differentiation and its deletion slows down osteoarthritis development.

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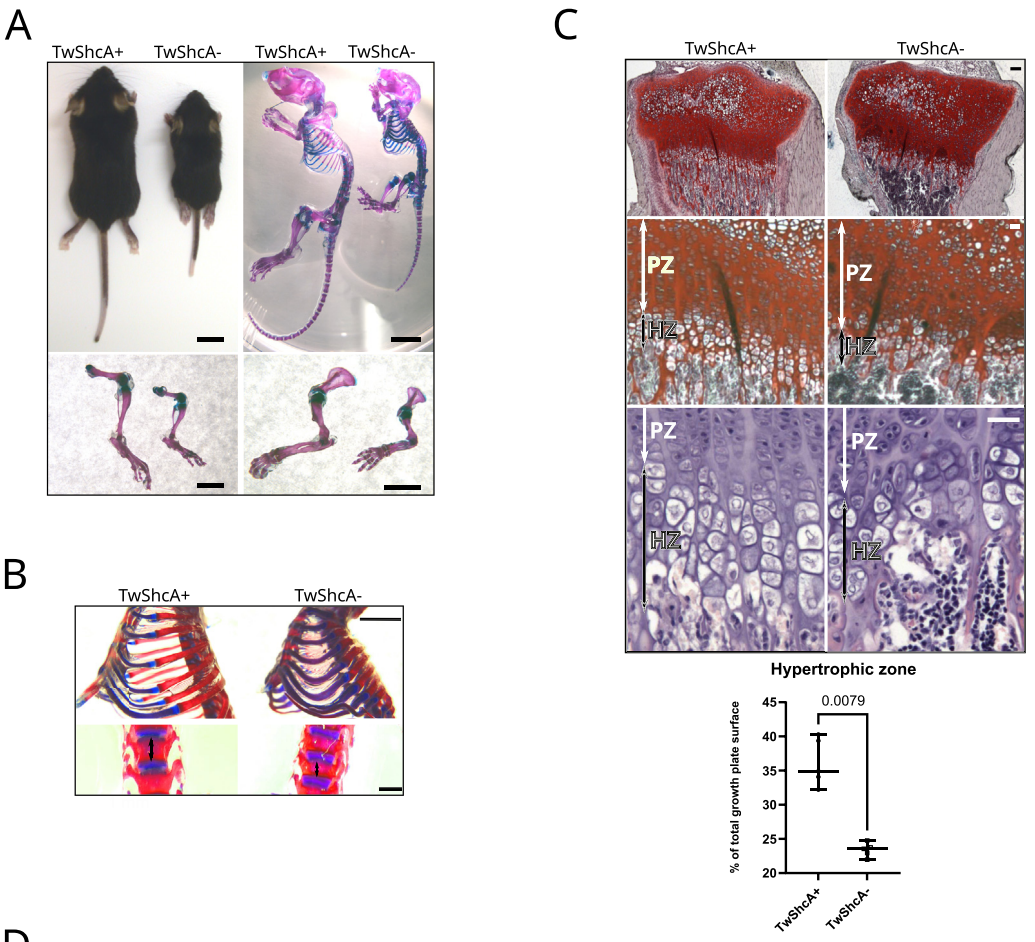

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Fig. 1

Introduction

Osteochondroprogenitor cells derive from mesenchymal stem cells and give rise to either chondrocytes or osteoblasts[1]. Chondrocyte differentiation and hypertrophy are key events in long bones and vertebral skeleton formation allowing skeletal growth from embryogenesis to skeletal maturity[2]. During endochondral ossification (EO), chondrocytes produce transient cartilage scaffolds for new bone formation in the growth plate (GP)[2]. The GP is a highly organized cartilage structure in which chondrocytes proliferate and differentiate in pre-hypertrophic and then hypertrophic chondrocytes. Hypertrophic chondrocytes orchestrate cartilage extracellular matrix (ECM) remodeling, its calcification and osteoblasts infiltration which lead to replacement of cartilage by bone and longitudinal bone growth. Defective hypertrophic differentiation and ossification can lead to impaired longitudinal growth and thus dwarfism[3].

Chondrocyte hypertrophy is also a feature of osteoarthritis (OA) as quiescent articular chondrocytes can undergo an aberrant terminal hypertrophic differentiation[4]. The switch from a quiescent to a hypertrophic phenotype is accompanied by the pathologic remodeling of the ECM leading to articular cartilage destruction.

In articular cartilage, and in the GP, resting chondrocytes, synthesize an ECM rich in collagen type II (col2a1) and proteoglycans. In addition to changes in cell morphology, collagen type II expression decreases during chondrocyte maturation, and the hypertrophic chondrocyte initiates the synthesis of collagen type X, together with proteolytic enzymes which damage ECM integrity and lead to cartilage destruction[2,4,5].

Although the mechanisms involved are not fully understood, multiple factors, including matrix proteins, growth factors like Insulin-like Growth Factor-I (IGF-I) or Fibroblast Growth Factor (FGF), transcription factors, and intracellular signaling proteins have been involved in chondrocyte hypertrophy[3,6–8]. Among intracellular signaling pathways, the MAPK/ERK1/2 pathway can be activated by various stimuli including growth factors, and is involved in chondrocyte differentiation from the pre-hypertrophic stage to the late hypertrophic stage during EO[9]. Furthermore, the MAPK/ERK1/2 pathway phosphorylates and activates RunX2 (Cbfa1), a master transcription factor for chondrocyte hypertrophy and an indispensable collagen type X transactivator[10,11].

Src homology and Collagen A (ShcA) is a cytosolic adaptor protein that binds to the cytoplasmic tail of growth factor receptors and ECM receptors once activated[12]. ShcA recruitment to the plasma membrane leads to the activation of the Ras:Raf:MEK1:ERK1/2 pathway and to the connection between ECM and the cytoskeleton, both being important determinants of chondrocyte differentiation[9,12–14]. ShcA is expressed in hypertrophic chondrocytes but its precise role during chondrogenesis remains unknown[15]. ShcA has the potential to signal downstream of several plasma membrane receptors, such as the IGF-I- receptor, the FGF receptor-1, the discoidin domain receptor 1[16–18]. These receptors are necessary for chondrocyte hypertrophic differentiation and their downregulation leads to EO alteration and dwarfism phenotypes or protects from OA[19–21]. We thus hypothesized that ShcA might integrate growth factor and ECM signaling in chondrocyte differentiation. We generated TwShcA− mice in which ShcA is selectively ablated in osteochondroprogenitor cells (condensed mesenchyme-derived chondrocytes and osteoblasts) by breeding Twist2 transgenic mice with ShcA$^{flox/flox}$ mice[13]. We used *in vitro* experiments to study intracellular signaling mechanisms involved.

Method

Study flow charts, more detailed methods and complete statistical analysis are provided in the Supplemental section.

Mice

C57BL/6 mice carrying a ShcA allele into which loxP sites are integrated have been generated by gene targeting in embryonic stem cells (ShcA$^{flox/flox}$) (authorization APAFIS#15477)[13]. Specific p66, p52 and p46 ShcA inactivation in osteochondroprogenitor cells was achieved by crossing transgenic mice carrying the Twist2-Cre transgene (The Jackson laboratory) with ShcA$^{flox/flox}$ mice[22]. Genotyping of the wild type (TwShcA+) and ShcA mutant (TwShcA−) mice by polymerase chain reaction (PCR) was performed as described using primers specific for ShcA[13].

Histology and immunostaining experiments

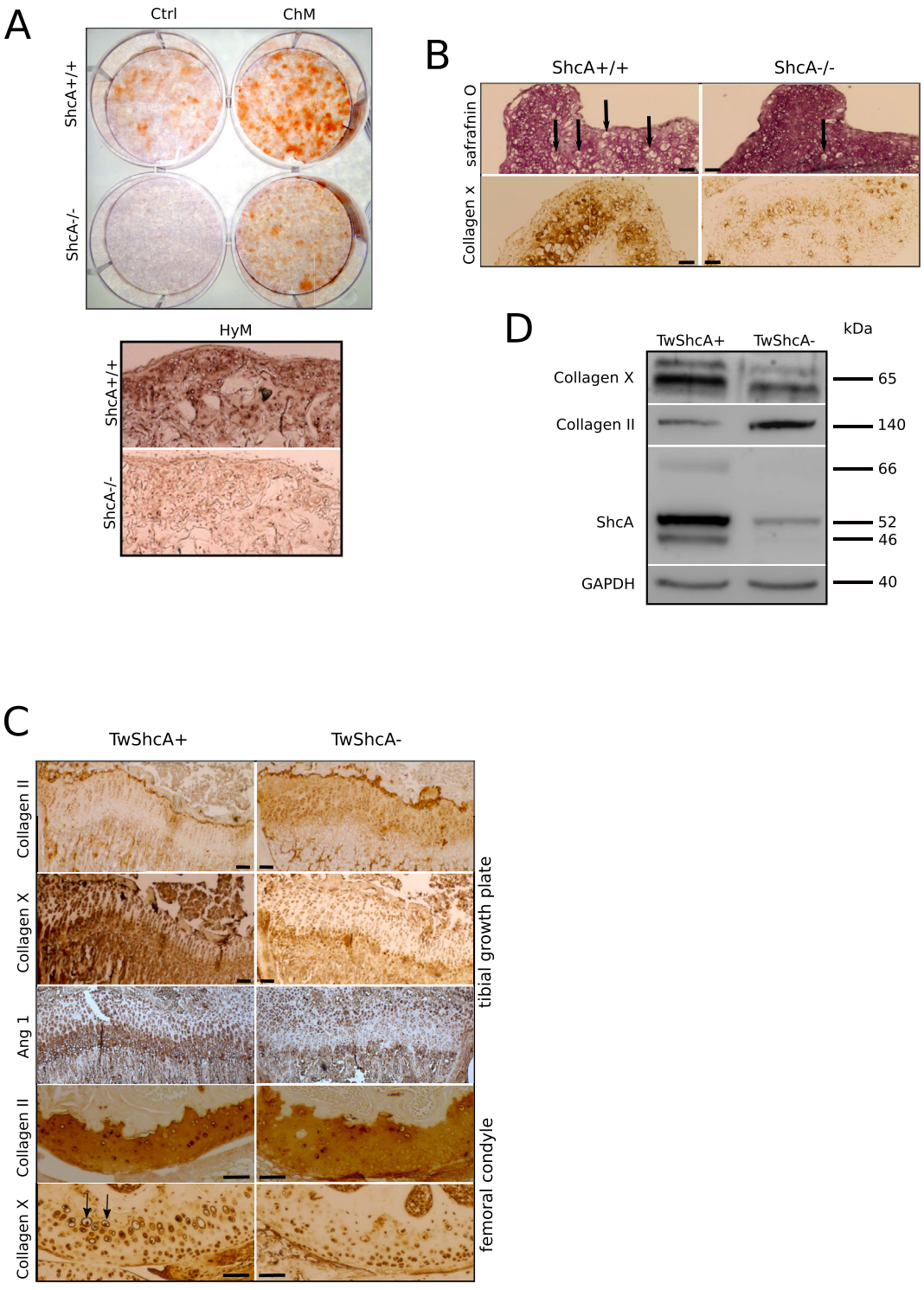
Longitudinal joint sections at 5 μm thickness were processed for Safranin-O/Fast Green, hematoxylin/eosin or immunohistochemical staining according to standard methods. The Vectastain kit and the DAB detection system (Clinisciences) were used. The stained specimens were photographed digitally under a microscope. For quantitative analysis of the hypertrophic zone of the GP, images taken through the microscope were processed using Image J®.

To evaluate OA severity, two histopathology scorings were applied after Safranin O and Fast Green staining: the OsteoArthritis Research Society International (OARSI) and the modified Mankin scoring systems[23,24]. Averaged scores were used in statistical analyses.

Chondrocyte isolation and culture

The costal cartilage as well as femoral and tibial articular cartilage were isolated from TwShcA+ and TwShcA− male and female mice 7–10 days after birth and primary murine

Dwarfism phenotype and decreased hypertrophic chondrocyte maturation in growth plate from TwShcA− mice. A) Representative body appearance and alizarin red- and alcian blue-stained bone and cartilage (upper panel) and alizarin red- and alcian blue-stained hind legs (lower left panel) and front legs (lower right panel) in 1 month old male mice that express (TwShcA+) or lack ShcA in osteochondroprogenitor cells (TwShcA−). Scale bars 10 mm. B) Alizarin red- and alcian blue-stained rib cages (upper panel) and spines (lower panel) from 1 month male mice that express (TwShcA+) or lack ShcA (TwShcA−) in osteochondroprogenitor cells. Dark double headed arrow: alizarin-stained vertebral bone. Scale bars 5 mm (upper panel) and 2 mm (lower panel). C) Safranin O-fast green (upper and middle panels) and hematoxylin eosin (lower panel) stainings and percentage of hypertrophic zone surface relative to total growth plate surface in tibial growth plate from 1 month old male mice that express (TwShcA+) or lack ShcA in osteochondroprogenitor cells (TwShcA−) ($n = 5$ mice in each group). PZ: proliferating zone, HZ: hypertrophic zone, scale bars 50 μm. Values are median [95% CI]. Mann−Whitney test. D) Quantification of body size and weight in male and female mice that express (TwShcA+) or lack ShcA in osteochondroprogenitor cells (TwShcA−) at 4, 8, 12 and 16 weeks of age ($n = 5$ to 14 mice in each group). Values are median [95% CI]. Mann−Whitney test.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Collagen II
Collagen X
tibial growth plate
femoral condyle

Fig. 2

chondrocytes from hyaline cartilage were extracted and cultured as previously described[25].

Cells culture

Primary murine chondrocytes were cultured in DMEM medium supplemented with 10% FBS and 2 mM L-glutamine. The mouse chondroprogenitor cell line ATDC5 (Sigma–Aldrich) was cultured in DMEM/F12 medium supplemented with 5% FBS and 2 mM L-Glutamine. Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. To delete ShcA, ATDC5 cells were transfected 24h after seeding with either scrambled siRNA, or siRNA against p66, p52 and p46 isoforms of ShcA (Dharmacon) at a final concentration of 20 nM using lipofectamine 3000 (Thermo Fischer Scientific) and used 48 h post-transfection.

Differentiation protocols

Dedifferentiated primary murine chondrocytes were used to perform a two-dimensional (monolayer culture which favors hypertrophy), or a three-dimensional (pellet culture) model of chondrogenic differentiation *in vitro* as previously described[26]. An *in vivo* hypertrophic differentiation model was also used as previously described[27].

Cell fractionation

Cell lysates from isolated chondrocytes at third passage and from ATDC5 cells were fractionated as previously described and analyzed by western-blot[28].

Western blot

SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to standard procedures. ImageQuant®LAS 4000 Imaging System (Amersham) was used to visualize protein expression. Optical densitometry was performed with Adobe Photoshop and Image J®.

mRNA expression analysis

RNA was isolated using TRIzol reagent (Sigma, St Louis, Mo) according to the manufacturer's instructions. 50 ng of RNA were converted to cDNA using the High-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). PCR amplification was performed using SYBRGreen PCR master mix (Kappa biosystems, Wilmington, MA) according to the manufacturer's instructions.

Confocal microscopy

Isolated chondrocytes at first passage were seeded on glass slides, and 48 h later were fixed with 3% paraformaldehyde, and incubated with anti-RunX2 (#8486, Cell signaling), anti-YAP1 (#4912, Cell signaling), anti-IgG control primary antibodies and Alexa Fluor 488 (Fischer Scientific) secondary antibodies. Immunofluorescence-labeled cells were analyzed using a Leica TSC SPE confocal microscope with the × 63 oil immersion objective.

Statistical analysis

Values resulting from at least three independent biological units are reported as between group difference of median [95% confidence interval of difference] in the text and median [95% confidence interval] in the figures. Statistical significance was determined using a Mann–Whitney test, Tukey box plot analysis showing a non-normal distribution (GraphPad Prism 9.3.1 ®, *San Diego, CA*). Description of the study experimental units and the number used in each experiment, statistical test results, and study flow charts are provided in the supplemental section.

Results

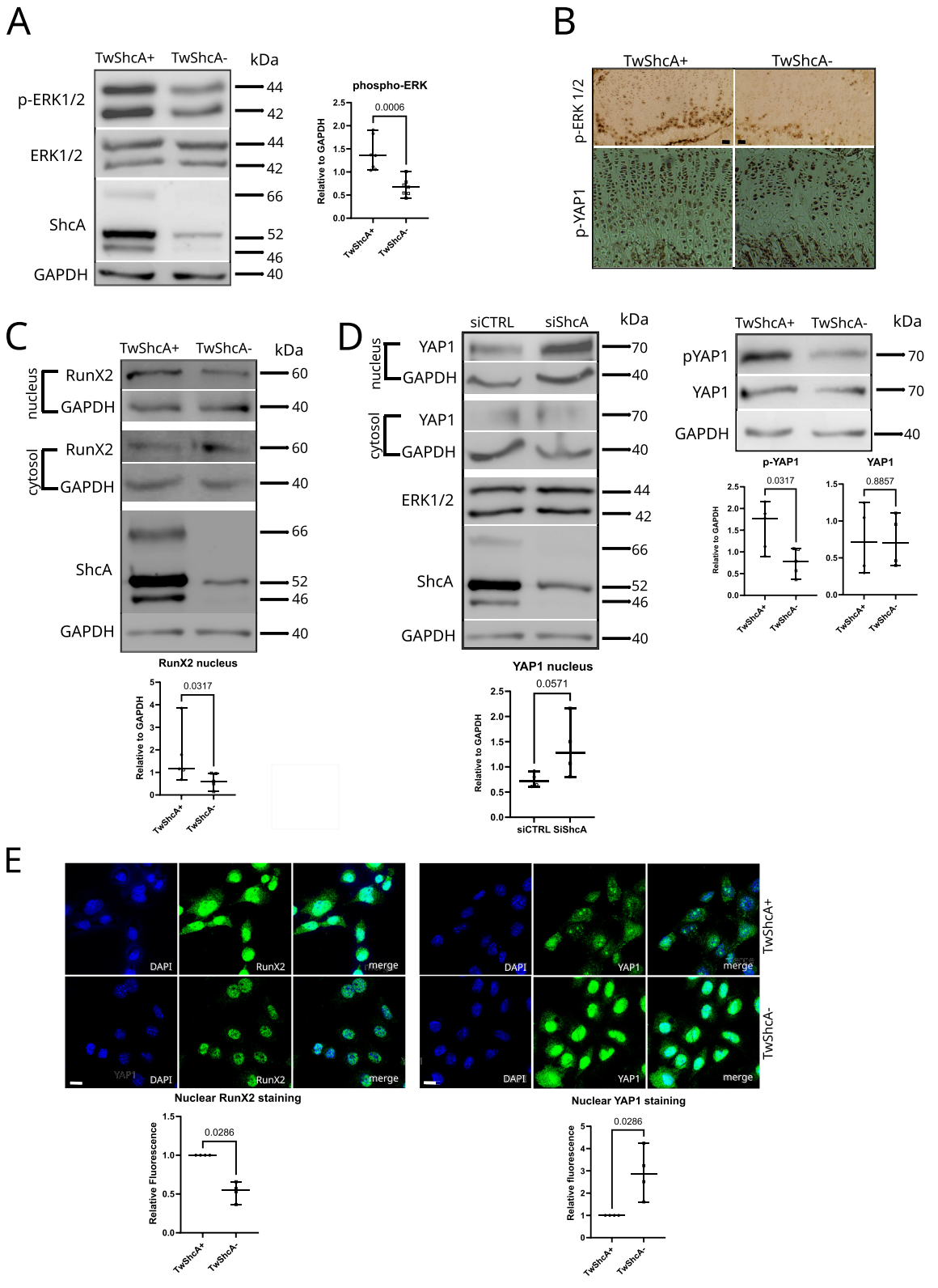
Deletion of ShcA in osteochondroprogenitor cells leads to dwarfism

In TwShcA– mice, the three isoforms of ShcA, p66, p52 and p46 were efficiently reduced in chondrocytes isolated from knee, vertebral and costal cartilage but not in non-cartilaginous tissues like lungs or the spleen (supplemental figure (A) and (B)). TwShcA– mice showed a dwarfism phenotype with a decrease in body size and weight compared to control littermates (TwShcA+) during growth till adulthood both in males and females (3 months old male mice nose-to-anus length, median between group difference −1.48 cm [95% confidence interval −1.860 to −1.190]) [Fig. 1(A) and (D)]. Vertebral bodies and hind and front legs lengths were also significantly decreased [Fig. 1(A) and (B)].

Alizarin red and alcian blue stainings for bone and cartilage tissue respectively showed an increased cartilage-to-bone ratio in the rib cage and the spine from TwShcA– mice [Fig. 1(B)]. Histological analysis of safranin O- and hematoxylin-eosin-stained tibial GP indicated an altered GP organization in the absence of ShcA [Fig. 1(C)]. In 1 month old TwShcA– mice, the proliferating zone appeared disorganized and non-columnar and the hypertrophic zone was decreased (−11.37% [−17.34 to −8.654]) [Fig. 1(C)]. The decrease of the hypertrophic zone was still present in 3 months old TwShcA– but to a lesser extent (supplemental fig. (C)).

These data are indicative of an altered EO process and suggest that ShcA is required to complete the whole chondrocyte hypertrophic differentiation process.

Decreased hypertrophic chondrocyte maturation and collagen X expression in TwShcA– mice and ShcA deficient cells. A) Alizarin red (upper panel) and Alkalin phosphatase (lower panel) stainings of dedifferentiated chondrocytes isolated from TwShcA+ (ShcA+/+) and TwShcA– (ShcA–/–) mice cultured in monolayer in chondrogenic medium (ChM) or control medium (ctrl) for 2 weeks (upper panel) or cultured on a collagen I sponge in hypertrophic medium and implanted in mice (lower panel) (pictures representative of $n = 3$ independent biological units). B) Safranin O and collagen X stainings of dedifferentiated chondrocytes isolated from TwShcA+ (ShcA+/+) and TwShcA– (ShcA–/–) mice cultured in pellet in chondrogenic medium for 2 weeks (picture representative of $n = 3$ independent biological units). Arrows show hypertrophic chondrocytes. Scale bars 100 μm. C) Collagen II, Collagen X and Angiopoietin 1 stainings of tibial growth plate (upper panels) and femoral condyle articular cartilage (lower panels) in TwShcA+ and TwShcA– mice. Arrows show hypertrophic-like chondrocytes. Scale bars 100 μm. D) Representative western-blot analysis of collagen X and collagen II protein levels in knee joint articular chondrocytes isolated from TwShcA+ and TwShcA– mice (representative of $n = 7$ independent biological units in each group for collagen X, $n = 5$ independent biological units in each group for collagen II).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TwShcA+
TwShcA-
Nuclear RunX2 staining
0.0286
Relative Fluorescence
Nuclear YAP1 staining
0.0286
Relative fluorescence

Fig. 3

ShcA drives chondrocyte maturation to hypertrophy and collagen X expression

We directly tested the role of ShcA in chondrocyte differentiation by performing *in vitro* experiments. When primary chondrocytes isolated from hyaline cartilage are expanded in monolayer, they typically dedifferentiate and acquire a fibroblast-like phenotype[25]. These fibroblast-like cells can be redifferentiated using chondrogenic differentiation protocols either in monolayer culture (which favors hypertrophic differentiation) or in pellet culture[26]. Using such protocols, we observed that, after dedifferentiation, chondrocytes isolated from TwShcA− mice are less prone to mineralize than those isolated from TwShcA+ mice as shown by the decrease in the alizarin red stained matrix (Fig. 2(A), upper panel). In addition, less hypertrophic-like chondrocytes were found in the pellet culture of chondrocytes isolated from TwShcA− mice (Fig. 2(B), Safranin O staining). Finally, the immunological staining of the pellets shows a decrease in collagen X staining, a marker of hypertrophy, in chondrocytes isolated from TwShcA− mice [Fig. 2(B)]. To test the resistance of ShcA deficient chondrocytes to further mature *in vivo*, ShcA deficient cells were submitted to hypertrophic differentiation *in vitro* and the resulting tissue was implanted subcutaneously in nude mice for 4 weeks. Immunohistochemical analysis show a decrease in the staining of alkalin phosphatase (ALP), a crucial pro-mineralizing enzyme synthetized by hypertrophic chondrocytes, in ShcA deficient chondrocytes compared to control chondrocytes (Fig. 2(A) lower panel). Not all hypertrophic markers are controlled by ShcA as the expression of MMP13 is not altered in the absence of ShcA (supplemental figure (H)). These *in vitro* data strongly suggest a role for ShcA in controlling chondrocyte hypertrophic commitment.

Collagen X is an ECM protein specifically synthetized by hypertrophic chondrocytes whereas collagen II is an ECM protein synthetized by quiescent chondrocytes[29]. Immuno-histological analysis of tibial GP sections from 1 month old mice indicated a marked decrease in collagen X as well as an increase in collagen II stainings in TwShcA− mice [Fig. 2(C)]. In femoral articular cartilage from one-year-old mice, immunostaining of collagen X and the number of hypertrophic chondrocytes were decreased whereas the area of collagen II immunostaining was increased in TwShcA− mice [Fig. 2(C)]. Quantifications of collagen X and collagen II protein expressions show that collagen X was decreased in chondrocytes isolated from TwShcA− mice knee joint cartilage (−0.4671 [−0.5198 to −0.4400]) whereas collagen II expression was increased (0.8238 [0.1581 to 1.228]) [Fig. 2(D)]. Interestingly, the mRNA expression of Sox9, a major transcription factor involved in collagen II expression, is enhanced in ShcA-deficient ATDC5 cells (Supplemental figure (F)).

Hypertrophic chondrocytes synthesized angiopoietin 1 which has been implicated in the recruitment of endothelial cells and the regulation of angiogenesis in the site of EO[30,31]. Immuno-histological analysis of tibial GP sections from 1 month old mice indicated a marked decrease in the staining of angiopoietin 1 in hypertrophic chondrocytes in TwShcA− mice [Fig. 2(C)]. mRNA expression of angiopoietin 1 is decreased to a similar extent in ATDC5 cells deficient for ShcA (−0.6802 [−0.9870 to −0.2496]) (Supplemental figure (E)).

Thus, the decrease of the hypertrophic markers collagen X and angiopoietin 1 parallels the inhibition of chondrocyte hypertrophic commitment in the absence of ShcA.

ShcA induces hypertrophic commitment by promoting ERK1/2 activation, RunX2 nuclear translocation and by retaining YAP1 in its cytosolic inactive phosphorylated form

One of the main downstream targets of ShcA is ERK1/2[12]. It has been reported that the MAPK/ERK1/2 pathway promotes chondrocytes differentiation from the pre-hypertrophic to the late hypertrophic stage during EO[9]. Once activated ERK1/2 phosphorylates and activates Runx2 in osteoblasts[10]. Runx2 and its target gene collagen X are essential for chondrocyte hypertrophy[11]. Thus, by activating ERK1/2, ShcA might induce RunX2 activation leading to Collagen X expression and chondrocyte hypertrophic commitment.

To test this, we first quantified ERK1/2 phosphorylation in primary chondrocytes isolated from knee joint cartilage. We found that deletion of ShcA leads to a marked decrease in p-ERK1/2 (−0.6833 [−1.156 to −0.3463]) [Fig. 3(A)]. An important decrease in the expression of phospho-ERK1/2 was also observed *in vivo* in tibial GP hypertrophic chondrocytes from 1 month old TwShcA− mice [Fig. 3(B)]. In columnar proliferating chondrocytes, the decrease was observed to a lesser extent [Fig. 3(B)].

We next tested whether ShcA promotes Runx2 nuclear translocation. Using cell fractionation and immuno-fluorescence experiments in primary chondrocytes isolated from knee joint cartilage of TwShcA+ and TwShcA− mice, we found a decrease in RunX2 expression in the nucleus (−0.5459 [−3,236 to −007,623]) [Fig. 3(C)] and a decreased nuclear staining of RunX2 in ShcA deficient cells (−0.45 [−0.6351 to −0.3417]) [Fig. 3(E)]. Total RunX2 mRNA expression was not altered (supplemental figure (D)). Thus, ShcA is required for RunX2 nuclear translocation in chondrocytes.

YAP1 is a transcriptional effector of the Hippo pathway. In cells, YAP1 is present in a cytosolic Ser/Thr phosphorylated inactive form (p-YAP1), whereas in the nucleus YAP1 regulate transcription[32]. Because YAP1 can bind to RunX2 and suppresses collagen X transcription[32,33], we tested whether ShcA retains p-YAP1 in the cytosol and thus prevents its nuclear translocation. Using primary chondrocytes isolated from knee joint cartilage of TwShcA+ and TwShcA− mice, we found a significant decrease in p-YAP1 expression in ShcA deficient cells (−0.9874 [−1.519 to −0.04967]) with no alteration in total YAP1 expression (Fig. 3(D), right panel). A decrease in the staining of phospho-YAP1 was also observed *in vivo*

Decreased ERK1/2 and RunX2 activation and increased YAP1 activation in TwShcA− mice and ShcA deficient cells. A) Western blot analysis and relative quantification of phospho-ERK1/2, ShcA and GAPDH proteins levels in knee joint articular chondrocytes isolated from TwShcA+ and TwShcA− mice ($n = 7$ mice in each group). Values are median [95% CI]. B) phospho-ERK1/2 and phospho-YAP1 stainings of tibial growth plate in TwShcA+ and TwShcA− mice. Scale bars 100 μm. C) Western-blot analysis and relative quantification of nuclear RunX2 and GAPDH protein levels in knee joint articular chondrocytes isolated from TwShcA+ and TwShcA− mice ($n = 5$ mice in each group). Values are median [95% CI]. Mann–Whitney test. D) Western-blot analysis and relative quantification of nuclear and cytosolic YAP1, of total ERK and GAPDH protein levels in ATDC5 cells down-regulated for ShcA (siShcA) and control cells (siCTRL) ($n = 4$ independent biological units in each group) (left panel) and Western blot analysis and relative quantification of total p-YAP1, total YAP1 and GAPDH in knee joint articular chondrocytes isolated from TwShcA+ and TwShcA− mice ($n = 4$ mice in each group) (right panel). Values are median [95% CI]. Mann–Whitney test. E) Representative confocal immunostaining of RunX2 (left panel) and YAP1 (right panel) in articular chondrocytes isolated from TwShcA+ and TwShcA− mice and relative fluorescence intensity analysis ($n = 4$ independent biological units). Scale bars 10 μm. Values are median [95% CI]. Mann–Whitney test.

in tibial GP hypertrophic chondrocytes from 1 month old TwShcA− mice [Fig. 3(B)]. We also tested YAP1 activation by its nuclear translocation. Using cell fractionation experiments on ATDC5 cells and immuno-fluorescence experiments on isolated chondrocytes, we found a marked increase in YAP1 nuclear translocation in ShcA deficient cells (0.5605 [−0.1121 to 1.550]) (Fig. 3(D), left panel) and a marked staining of YAP1 in the nucleus of ShcA deficient cells (1.869 [0.6055 to 3.243]) [Fig. 3(E)]. The level of ERK1/2 and RunX2 were not altered in the absence of ShcA (Fig. 3(D) and supplemental (G)). ShcA controls the activation levels of ERK1/2 and RunX2 but not their expression levels.

Taken together, our results show that ShcA controls hypertrophic differentiation and collagen X expression by promoting ERK1/2 activation and RunX2 nuclear translocation, and by retaining YAP1 in its cytosolic inactive phosphorylated form.

Deletion of ShcA in osteochondroprogenitor cells protects from aged-related OA development in mice

Aberrant terminal hypertrophic differentiation of articular chondrocytes has been implicated as a crucial step in OA pathogenesis[4]. During OA, articular chondrocytes change their phenotype to one resembling hypertrophic GP chondrocytes and OA can be regarded as an ectopic recapitulation of the EO process[4,34].

Because ShcA promotes chondrocyte hypertrophic commitment, we tested whether its deletion protects against OA in aged TwShcA− mice compared to young TwShcA− mice. As mice of the C57BL/6 background are characterized by a determined propensity to develop spontaneous OA with age[35], the TwShcA− mice were backcrossed on a C57BL/6 genetic background. With aging, tibio-femoral joints from TwShcA+ mice demonstrated erosion with loss of articular cartilage tissue staining, including in superficial and in at least portions of deeper cartilage layers, denudation, with matrix loss extending to calcified cartilage interface, and clefts to calcified zone (Fig. 4(B), left panel) compared to young mice (Fig. 4(A), left panel). Cartilage histopathology scorings, according to the OARSI and the modified Mankin scoring systems, showed a drastic increase in 2 years old TwShcA+ mice compared to young mice which validated the age-related development of osteoarthritic lesions [Fig. 4(C)].

We then characterized the effect of ShcA deletion on spontaneous aged-induced OA development. Safranin O fast green staining of tibio-femoral joints showed a slightly increased glycosaminoglycan staining of tibial plateau and femoral condyle in 1 month old TwshcA− mice compared to TwShcA+ mice [Fig. 4(A)]. Cartilage histopathology scorings showed a significant increase in aged TwShcA− mice compared to young TwShcA− mice, however the cartilage tissue was substantially less impaired compared to TwShcA+ mice at the same age (OARSI: −6.67 [−14.25 to −4.000], modified Mankin: −3.66 [−6.000 to −1.000]) [Fig. 4(B) and (D)].

These data indicate that ShcA promotes aged-related cartilage destruction and that deletion of ShcA in osteochondroprogenitor cells can slow down OA development in mice.

Discussion

Chondrocytes originate from mesenchymal stem cells-derived osteochondroprogenitors. Chondrogenesis and terminal hypertrophic chondrocyte differentiation involve integrins and numerous growth factors receptors[3,6–8]. ShcA is a ubiquitously expressed adaptor protein that binds to the cytoplasmic tail of integrins and growth factor receptors once activated[12]. Subsequently ShcA recruits and activates the Grb2:Sos:Ras:Raf:MEK1/2:ERK1/2 signaling cascade[12]. Although ShcA has been identified in hypertrophic

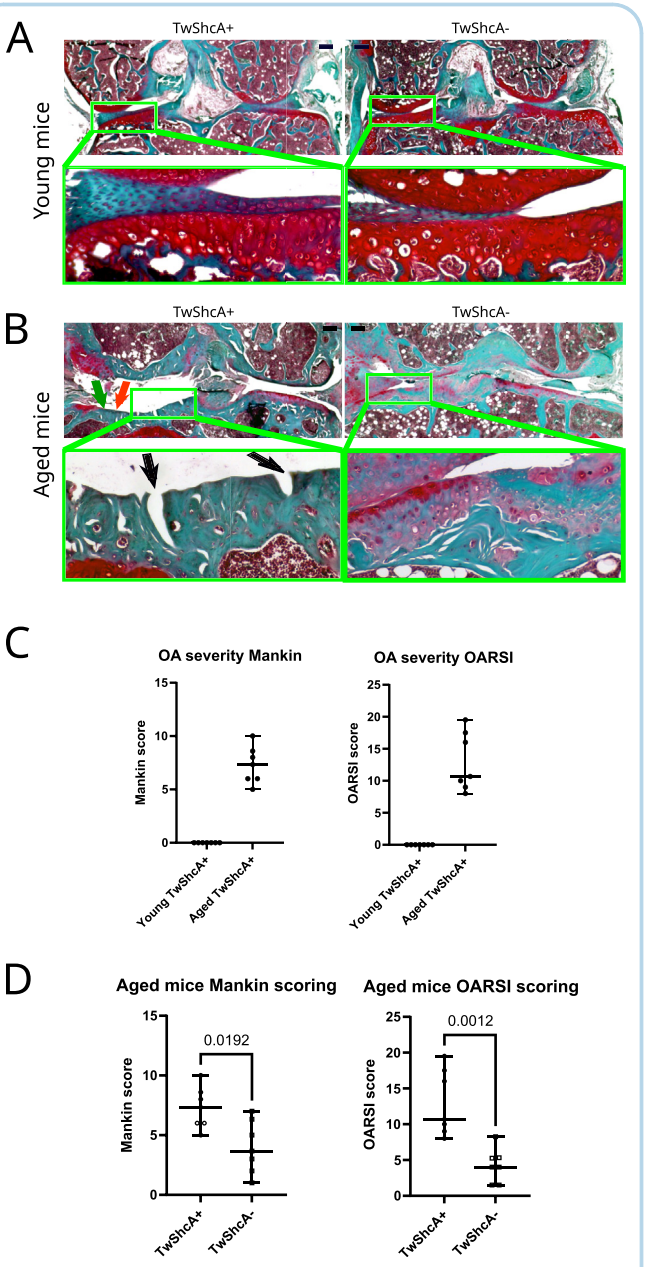

Fig. 4 Osteoarthritis and Cartilage

Inhibition of OA development in TwShcA− mice. Safranin O fast green staining of knee joint from 1 month old (young mice, A) and 2 years old (aged mice, B) TwShcA+ and TwShcA− mice. C) Osteoarthritis severity evaluation in aged TwShcA+ wild type mice compared to young TwShcA+ wild type mice using the OsteoArthritis Research Society International (OARSI) and the modified Mankin scoring systems. D) Comparison of osteoarthritis severity between aged TwShcA+ and TwShcA− mice using the OARSI and the modified Mankin scoring systems. Scale bars 250 μm (upper panels) 100 μm (lower panels). Green arrow: loss of articular cartilage, orange arrow: denudation of cartilage surface, black arrows: clefts to calcified zone. $n = 7$ male mice in each aged group, $n = 8$ male mice in each young group. Values are median [95% CI]. Mann−Whitney test.

chondrocytes, its function in chondrocyte differentiation has never been addressed[15]. By its potential to integrate multiple extracellular stimuli ShcA may behave as an important regulator of chondrocyte differentiation. Our data indicate that deletion of ShcA in osteochondroprogenitor cells leads to a reduced cartilage-to-bone ratio and a dwarfism in mice. This phenotype is characterized by an altered EO process with an important inhibition of chondrocyte hypertrophic maturation in the GP. Furthermore, in mice loss of ShcA appears protective against age-related OA, a pathology regarded as an ectopic recapitulation of the EO process. ShcA promotes chondrocyte hypertrophic commitment and OA through RunX2 activation and YAP1 inhibition. Our *in vitro* experiments using either chondrocytes isolated from TwShcA− mice or the ATDC5 chondroprogenitor cell line support the crucial role of ShcA in chondrocyte differentiation *per se*. However, osteoblasts may also have contributed to the dwarfism phenotype observed *in vivo* as our mouse model is based on ShcA ablation in osteochondroprogenitor cells[22]. The absence of ShcA in osteoblast may alter their differentiation or their recruitment to the GP and the subsequent mineralization of the ECM.

During hypertrophic maturation, composition of the ECM synthesized by chondrocytes shifts from collagen II synthesis to collagen X, creating a favorable environment for mineralization and replacement of cartilage by bone[2,36,37]. In the absence of ShcA, we observed a decrease in collagen X expression both in the GP and the articular cartilage from adult mice, but no decrease in collagen II expression, that was even increased. Thus, not only are ShcA-deficient chondrocytes refrained from undergoing hypertrophic differentiation but also they exhibit the collagen II marker of quiescence. The increase in collagen II expression in ShcA-deficient chondrocytes might be explained by an increase in Sox9 expression as we observed in ATDC5 cells. The transcription factor Sox9 activates collagen II expression and prevents the conversion of proliferating chondrocytes into hypertrophic chondrocytes by directly interacting with RunX2 and repressing its activity[38,39]. Besides activating RunX2 by the control of YAP1 nuclear access, ShcA might also activate RunX2 by inhibiting Sox9 expression. These suggest that ShcA is able to control the two antagonistic master transcription factors, Sox9 and RunX2, that are involved in cell fate determination.

The angiopoietin 1-Tie2 axis has been implicated in the recruitment of endothelial cells and the regulation of angiogenesis in the site of EO and hypertrophic chondrocytes synthesized angiopoietin 1[30,31]. In the absence of ShcA, we observed a decrease in the expression of angiopoietin 1. Besides Collagen X, ShcA is thus required to promote angiopoietin 1 expression and the whole EO process. ShcA might promote Ang-1 expression through the activation of RunX2. It has indeed been described that RunX2 controls angiopoietin 1 expression in osteoblasts[40].

ERK1/2 is one of the main downstream targets of ShcA[12]. Conditional deletion of ERK1/2 in hypertrophic chondrocyte leads to a decrease in long bones growth after birth and to an inhibition of the transition of early hypertrophic chondrocytes to terminally differentiated chondrocytes[9]. Upon ShcA knockdown, a decrease in ERK1/2 phosphorylation was observed which points out a major role of the ShcA-ERK1/2 interplay in chondrocyte signaling pathways. Interestingly, the decrease in ERK1/2 phosphorylation is mainly observed in hypertrophic chondrocytes from the GP and to a lesser extent in columnar proliferating chondrocytes. It has been reported that the main role of ERK1/2 in cartilage is to stimulate not cell proliferation but rather chondrocyte maturation and hypertrophic differentiation, and that c-Raf may be responsible for ERK1/2 activation in hypertrophic chondrocytes[9,41]. Our results demonstrate that upstream of c-Raf, ShcA is necessary to activate ERK1/2, a determinant factor for hypertrophic differentiation. Our results also showed that alternative pathways are still able to activate ERK1/2 in the absence of ShcA. This residual activation might represent a protective effect as ERK1/2 is involved in other aspects of chondrocyte homeostasis such as protection against apoptosis, biosynthesis of matrix molecules and protease inhibitors[42–44].

Runx2 has been implicated as a master transcription factor for chondrocyte hypertrophy[11]. After its nuclear translocation, RunX2 can be phosphorylated and activated by ERK1/2 leading to its binding to the promoter of Collagen X and transcriptional activation[10,11]. We found that the decreased activation of ERK1/2 in ShcA-deficient chondrocytes correlates with a decreased nuclear translocation of RunX2 whereas total RunX2 mRNA level remains unchanged. Taken together these observations suggest that ShcA activates chondrocyte hypertrophic differentiation and collagen X expression by activating ERK1/2 and by promoting RunX2 nuclear translocation.

Our study also reveals that not only ShcA drives chondrocyte maturation to hypertrophy by positively activating ERK1/2 and RunX2 but also by negatively regulating YAP1. We report that upon ShcA knockdown, the cytoplasmic inactive form of YAP1 is decreased and YAP1 nuclear translocation is increased whereas total YAP1 expression is not altered. It has been shown that YAP1 can inhibit collagen X expression by a direct interaction with RunX2[32,33].

Our data highlight the crucial role of ShcA in regulating the nuclear access of the transcription factor RunX2 and its regulator YAP1 to control protein expression. In chondrocytes, we found that ShcA retains YAP1 in its inactive form in the cytoplasm while promoting ERK1/2 activation and RunX2 nuclear translocation. RunX2 nuclear translocation activates hypertrophic commitment and collagen X transcription. The ShcA-mediated retention of YAP1 in the cytoplasm might involve the formation of a ShcA-Grb2-YAP1 complex. Indeed, it has been described that YAP1 is able to interact with SH3 domain-containing proteins through its WW domain[45]. Grb2 contains such a SH3 domain and is able to bind ShcA through its SH2 domain[46,47].

Aberrant terminal hypertrophic differentiation of articular chondrocytes has been implicated as a crucial step in OA pathogenesis[4]. During this switch, articular chondrocytes change their phenotype to one resembling hypertrophic GP chondrocytes and OA can be regarded as an ectopic recapitulation of the EO process[4,34]. However, the mechanisms involved in this phenotypic switch are far from being fully understood. Hypertrophic chondrocyte differentiation may be promoted by the inflammatory environment or altered joint biomechanics. It is also possible that chondrocytes in the articular cartilage and osteoblasts in the subchondral bone exert influence on each other through a bone-to-cartilage crosstalk leading to both cartilage destruction and fracture of the subchondral bone[48,49]. Our results show that in the absence of ShcA, articular cartilage and subchondral bone are preserved from joint degradation induced by aging and TwShcA− mice are refrained from severe OA. ShcA thus appears as an important new player in the etiology of OA. The promotion of chondrocyte hypertrophic differentiation by ShcA is certainly part of the mechanism involved in OA. But the mice model used in this study cannot rule out a role of ShcA-deficient osteoblasts, maybe through a crosstalk with articular chondrocytes.

Several initial events are involved in chondrocyte differentiation towards hypertrophy, i.e., mechanical stimuli through integrins, growth factors receptors activation[3,6–8,14]. ShcA potentially binds to the cytoplasmic tail of these receptors[12,16–18]. Our results reveal that ShcA behaves as a major regulator to integrate multiple stimuli and to complete the whole intracellular signaling process leading to hypertrophic commitment either in physiological processes like skeletal growth or in a pathological process like OA in mice. Further

studies are awaited in human cells to test the relevance of ShcA as an interesting therapeutic target.

Author contributions

Conception and design of the study: AAJ, MM, NA, BA, HX, BP, MRL. Acquisition of data: AAJ, CM, BL, EMD, AC, MM, MM, ML, LA, AS, TJ, MRL. Analysis and interpretation of data: AAJ, CM, BL, EMD, AC, MM, ML, MM, LA, AS, TJ, BP, MRL. Drafting the article: BP, MRL. Revising the article critically: AAJ, MM, NA, BA, HX, BP, MRL. Obtaining of funding: BP, MRL. Final approval of the submitted manuscript: AAJ, CM, BL, EMD, AC, MM, JH, MM, LM, LA, AS, TJ, NA, BA, HX, BP, MRL. BP (philippe.boucher@unistra.fr) and MRL (rachel.matz-westphal@unistra.fr) take responsibility for the integrity of the work as a whole, from inception to finished article.

Conflict of interest

For all authors there are no conflict of interest to declare.

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Supplementary data

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