Mechanical loading stimulates chondrogenesis *via* the PKA/CREB-Sox9 and PP2A pathways in chicken micromass cultures

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Abstract

Biomechanical stimuli play important roles in the formation of articular cartilage during early foetal life, and optimal mechanical load is a crucial regulatory factor of adult chondrocyte metabolism and function. In this study, we undertook to analyse mechanotransduction pathways during *in vitro* chondrogenesis. Chondroprogenitor cells isolated from limb buds of 4-day-old chicken embryos were cultivated as high density cell cultures for 6 days. Mechanical stimulation was carried out by a self-designed bioreactor that exerted uniaxial intermittent cyclic load transmitted by the culture medium as hydrostatic pressure and fluid shear to differentiating cells. The loading scheme (0.05 Hz, 600 Pa; for 30 min) was applied on culturing days 2 and 3, when final commitment and differentiation of chondroprogenitor cells occurred in this model. The applied mechanical load significantly augmented cartilage matrix production and elevated mRNA expression of several cartilage matrix constituents, including collagen type II and aggrecan core protein, as well as matrix-producing hyaluronan synthases through enhanced expression, phosphorylation and nuclear signals of the main chondrogenic transcription factor Sox9. Along with increased cAMP levels, a significantly enhanced protein kinase A (PKA) activity was also detected and CREB, the archetypal downstream transcription factor of PKA signalling, exhibited elevated phosphorylation levels and stronger nuclear signals in response to mechanical stimuli. All the above effects were diminished by the PKA-inhibitor H89. Inhibition of the PKA-independent cAMP-mediators Epac1 and Epac2 with HJC0197 resulted in enhanced cartilage formation, which was additive to that of the mechanical stimulation, implying that the chondrogenesis-promoting effect of mechanical load was independent of Epac. At the same time, PP2A activity was reduced following mechanical load and treatments with the PP2A-inhibitor okadaic acid were able to mimic the effects of the intervention. Our results indicate that proper mechanical stimuli augment *in vitro* cartilage formation *via* promoting both

differentiation and matrix production of chondrogenic cells, and the opposing regulation of the PKA/CREB–Sox9 and the PP2A signalling pathways is crucial in this phenomenon.

Keywords: mechanotransduction; chondrocyte differentiation; cartilage formation; extracellular matrix; H89; okadaic acid; Epac

Abbreviations: CREB, cAMP response element binding protein; DMMB, dimethyl methylene blue; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; Epac, exchange protein directly regulated by cAMP; FBS, foetal bovine serum; GAG, glycosaminoglycan; HAS, hyaluronan synthase; HDC, high density cell culture; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MSC, mesenchymal stem cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, N-methyl-*D*-aspartate; PBS, phosphate buffered saline; OA, okadaic acid; PBST, phosphate buffered saline supplemented with 1% Tween-20; PG, proteoglycan; PKA, protein kinase A; PKC, protein kinase C; PP, phosphoprotein phosphatase; PP2A, protein phosphatase 2A; TB, toluidine blue; TRPV, transient receptor potential receptor vanilloid

1. Introduction

The vertebrate skeletal system is designed to provide a solid, yet dynamic scaffold on which muscles can act, and at the same time to absorb and distribute mechanical forces generated by movements. Chondrocytes in articular cartilage are constantly exposed to compressive forces during normal activity, which is essential for maintenance of metabolic processes and healthy composition of cartilage extracellular matrix (ECM). Type II collagen and large proteoglycan (PG) aggregates with glycosaminoglycan (GAG) side chains are the main load-bearing constituents of the ECM, which account for the tensile and compressive stiffness of the tissue, respectively; furthermore, large amounts of cations and interstitial water bound to GAGs are also important factors in cartilage matrix biomechanics [1]. Compressive forces and other types of stress that affect articular cartilage during locomotion initially affect the interstitial fluid and ECM macromolecules, which results in fluid movement, followed by deformation of the ECM and the encapsulated chondrocytes [2]. Consequently, the fact that moderate cyclic or dynamic mechanical stimuli (less than 200 kPa) beneficially affect production of cartilage ECM components and cartilage thickness has been extensively investigated (reviewed by Bader and colleagues [3]).

Mechanotransduction is a complex series of events by which cells sense mechanical stimuli using mechanoresponsive elements (mechanosensation), transform them into appropriate biochemical signals (mechanochemical coupling), and initiate downstream signalling pathways that lead to cellular responses (signal transmission and response). For chondrocytes, mechanotransduction results in quantitative modulation of the rates of ECM synthesis and degradation, thus enabling them to alter the composition of the cartilage matrix [4]. While mechanotransduction pathways of mature skeletal tissues have been studied in great detail [5], our knowledge is considerably limited regarding how mechanical forces (*e.g.*

embryonic or foetal movements) affect developing cartilage and cellular differentiation in general. There is evidence that application of mechanical stimuli can determine the developmental fate of mesenchymal stem cells (MSCs) [6]. In particular, appropriate physical stimuli are reported to be essential for limb development: synovial joint and articular cartilage formation in chicken and murine embryos require proper mechanical forces by muscle contractions [7, 8]. Optimal intermittent biomechanical activation is also essential for cartilage regeneration during osteoarthritis (reviewed by Sun [9]). For a recent paper that reviews the current state-of-the-art of this field, please see [10]. However, it is still a challenge to clarify downstream targets of physical forces in mechanotransduction pathways during early cartilage development.

Chondrogenesis is a complex series of events including rapid proliferation, condensation and nodule formation of chondroprogenitor cells that requires coordinated activation of many intracellular signalling pathways [11]. The SRY-type HMG box transcription factors Sox9, L-Sox5 and Sox6 are essential for the mRNA expression of cartilage matrix-specific proteins (*e.g. COL2A1*, aggrecan core protein). Required for condensation and for maintaining the chondroprogenitor phenotype of differentiating mesenchymal cells, these transcription factors are strongly expressed throughout chondrogenesis [12]. Since the Sox transcription factors, Sox9 in particular, are pivotal elements of chondrogenesis, many signalling events regulate its expression and/or function in chondrogenic cells. Among other factors, the Sox9 promoter is known to be regulated by the cAMP response element binding protein (CREB) that binds to a CRE site upstream of Sox9 [13]. Apart from transcriptional control, direct physical association between Sox9 and CREB, the prototypical downstream transcription factor of the cAMP-dependent Ser/Thr protein kinase A (PKA), was recently demonstrated by Zhao and colleagues [14]. The PKA holoenzyme has a tetrameric structure that consists of a regulatory subunit dimer and 2

catalytic subunits [15]. The key intracellular signalling target for cAMP, PKA is known to phosphorylate the Sox9 protein (at Ser 211), which induces its translocation into the nucleus and enhances its transcriptional activity [16]. In fact, most of the effects of cAMP on many cellular processes are mediated by changes in the expression of target genes [17]. Indeed, there is a complex synergism between Sox9 function and the cAMP–PKA–CREB pathway in both mature and differentiating chondrocytes [14]. Earlier results of our laboratory and others also confirmed the involvement of PKA in the regulation of *in vitro* chondrogenesis [18, 19]. In contrast, the role of exchange proteins directly activated by cAMP (Epac1 and Epac2) has only partially been mapped in chondrocytes [20]. The chondrogenesis-promoting effect of PKA *via* Sox9 and CREB phosphorylation is counterbalanced by the activity of protein phosphatase 2A (PP2A), a negative regulator of chondrogenesis [19].

Previous attempts to decipher signalling pathways that may be involved in chondrocyte mechanotransduction were primarily focused at plasma membrane proteins with putative mechanosensor functions (*i.e.* receptors and ion channels that are primarily exposed to mechanical stimuli), including purinergic P2 receptors [21], α5β1 integrins [22], transient receptor potential channel vanilloid-4 [23], *N*-methyl-D-aspartate type glutamate receptors (NMDAR) [24] and stretch-activated Ca^{2+} channels [25]. As far as signalling events downstream of plasma membrane receptors are concerned, the involvement of the Src and focal adhesion kinases (FAK), the extracellular signal-regulated kinase (ERK) and the phosphatidylinositol 3-kinase/Akt pathways have been reported in various models [26]. Of note, the majority of the above studies were performed on mature articular chondrocytes, rather than differentiating chondrocytes; research aimed at identifying downstream mechanotransduction signalling pathways during chondrogenesis was largely lacking. In particular, the cAMP–PKA–CREB axis and its crosstalk with PP2A have not been implicated in mediating the effects of mechanical stimuli in differentiating chondrogenic cells.

Therefore, in the present work we aimed at investigating the effects of short-term uniaxial cyclic mechanical loading on chondrogenic differentiation and *in vitro* matrix production in chicken chondrifying high density cultures (HDC) by using a custom-made bioreactor. We provide evidence that cyclic mechanical load promotes chondrogenesis and stimulates cartilage ECM production, and that the observed effects can be attributed to the activation of the PKA/CREB–Sox9 signalling and concurrent inhibition of the PP2A pathway.

2. Materials and methods

2.1. Primary high density chondrifying cell cultures

The concept of a simple and well-reproducible *in vitro* experimental model of hyaline cartilage formation was originally described by Ahrens and colleagues [27]. In these micromass cell cultures (HDC), chicken embryonic limb bud-derived chondroprogenitor cells spontaneously differentiate to chondrocytes on days 2 and 3 of culturing, and a well-detectable amount of hyaline cartilage ECM is produced by culturing day 6.

To establish HDC of chondrifying mesenchymal cells, Ross hybrid chicken embryos of Hamburger–Hamilton stages 22–24 (approx. 4.5-day-old) were used. Work on early chick embryos *in vitro* does not require a license from the Ethics Committee of the University of Debrecen. Distal parts of forelimbs and hindlimbs of embryos (from ~100 eggs for each experiment) were isolated and dissociated in 0.25% trypsin–EDTA (Sigma-Aldrich, St. Louis, MO, USA; pH 7.4) at 37°C for 1 h. The enzymatic digestion was terminated by the addition of equal volumes of foetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) and digested limb buds were filtered through a 20-μm pore size plastic filter unit (Millipore,

Billerica, MA, USA) to yield a single-cell suspension of chondrogenic mesenchymal cells. After a brief centrifugation (800×*g* for 10 min), cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 10% FBS at a concentration of 1.5×10^7 cells/mL and 100 µL droplets were inoculated into plastic 6-well plates (Orange Scientifique, Braine-l'Alleud, Belgium). After allowing the cells to attach to the surface for 2 h in a CO_2 incubator (5% CO_2 and 80% humidity), 2 mL of DMEM supplemented with 10% FBS, 0.5 mM L-glutamine and antibiotics/antimycotics was added. Day of inoculation was considered as day 0 of culturing. Cultures were kept at 37°C in a CO_2 incubator for 6 days. The medium was changed on every second day.

2.2. Pharmacological modulation of PKA, PP2A and Epac activities

For some colonies, the PKA-inhibitor H89 (Cell Signaling Technology, Boston, MA, USA) was added to the medium on days 2 and 3 of culturing for 4 h at a final concentration of 20 µM (stock: 20 mM dissolved in DMSO). To block PP2A activity, some cultures were treated with okadaic acid (OA; Tocris Bioscience, Bristol, UK) for 4 h on days 2 and 3 at the concentration of 20 nM (stock: 20 µM dissolved in DMSO). Epac1 and Epac2 were inhibited by administering HJC0197 [28] (Biolog Life Science Institute, Bremen, Germany) into the medium at the final concentration of 10 µM (stock: 10 mM dissolved in DMSO) on days 2 and 3 of culturing for 4 h. Control cultures were treated with equal volumes of the vehicle (DMSO) on culturing days 2 and 3 for 4 h with no alterations compared to untreated colonies (not shown). When the loading scheme was co-applied with H89, OA or HJC0197, colonies received mechanical stimuli for the last 30 minutes of the 4-hour-long treatments with the compounds on both culturing days 2 and 3.

2.3. Mechanical stimulation

Micromass cultures grown in 6-well plates were subjected to uniaxial cyclic compressive force (approx. 600 Pa, 0.05 Hz) on culturing days 2 and 3 for 30 min using a custom-made mechanical stimulator unit (for a detailed description of the bioreactor, please see the Supporting information and Figs. S1 and S2). Moved by an electromagnet during operation, the equipment applies cyclic load transmitted by the culture medium to HDC cultured in 6-well culture plates. The loading scheme comprises two main components *via* perturbation of culture medium: (a) uniaxial compressive force; (b) a shear stress component resulting from fluid flow. Each loading cycle lasts for 20 sec: (1) pedicles dip approx. 2 mm into culture medium thus *pedicles are not in direct contact with micromass cultures*; (2) after 1 sec, pedicles are lifted up, (3) 18 sec break before the next cycle. Mechanical stimulation was carried out on both culturing days 2 and 3 for 30 min in a CO_2 incubator (37°C, 5% CO_2, 80% humidity). Control cultures were grown under identical culture conditions without mechanical stimulation.

The following measurements were performed to characterise the physical properties of the mechanical stimulator equipment. A three axis low-g micromachined accelerometer (MMA7260Q, Freescale Semiconductor, Inc., Tempe, AR, USA) was mounted to the falling part of the bioreactor, and a MPX5010D type silicon pressure sensor (Freescale Semiconductor) was assembled to the bottom of one of the wells of a culture plate. Data acquisition was performed by an NI USB-6212 bus-powered USB M Series multifunction data acquisition module (National Instruments, Austin, TX, USA) at 10^3 samples/sec rate. Data acquisition and analysis software was developed in the NI-Labview programming environment. The accelerometer and the pressure sensor were calibrated in a standard

manner. In addition, the displacement was also measured with a Casio Exilim FH25 high speed camera (at 10^3 fps).

2.4. Qualitative and semi-quantitative determination of cartilage matrix production

Micromass cultures established from 100 μL droplets of the cell suspension (containing 1.5×10^6 cells) were inoculated onto the surface of 30-mm round cover slips (Menzel-Gläser, Menzel GmbH, Braunschweig, Germany) placed into 6-well culture plates. For qualitative and semi-quantitative evaluation of cartilage matrix production, 6-day-old cell cultures were stained with dimethyl-methylene blue (DMMB; pH 1.8; Aldrich, Germany) or toluidine blue (TB; pH 2; Reanal, Budapest, Hungary) metachromatic dyes as previously described [29]. DMMB and TB metachromatic staining procedures were carried out on separate colonies from the same experiments; DMMB-stained specimens are only shown as visual representations of TB assays. Optical densities of extracted TB samples were determined from 3 cultures of each experimental group in 5 independent experiments.

2.5. Measurement of cell proliferation and mitochondrial activity

Rate of cellular proliferation in HDC was determined by measuring the radioactivity of incorporated ^{3}H-thymidine during a 16-hour-long period on day 3 (as described earlier [29]) started promptly after mechanical loading or at the end of 4-hour-long treatments with H89 or OA. For the investigation of mitochondrial activity, cells cultured in 6-well plates were used and MTT-assay was performed immediately after mechanical loading or after treatments on day 3 as it was described previously [29]. Untreated 3-day-old HDC that had

not received mechanical loading were used as controls for both assays. Measurements were carried out in 6 samples of each experimental group in 3 independent experiments.

2.6. Reverse transcription followed by polymerase chain reaction (RT-PCR)

3-day-old cell cultures were dissolved in Trizol (Applied Biosystems, Foster City, CA, USA) and total RNA was harvested in RNase-free water and stored at –70°C. Mechanically stimulated cultures as well as colonies treated with H89 or OA were collected directly after loading or the 4-hour-long treatments on day 3. Reverse transcription was performed by using High Capacity RT kit (Applied Biosystems). For the sequences of primer pairs and details of polymerase chain reactions, see Table 1. Amplifications were performed in a thermal cycler (Labnet MultiGene™ 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, USA) as follows: 95°C, 2 min, followed by 35 cycles (denaturation, 94°C, 30 sec; annealing for 45 sec at optimised temperatures as given in Table 1; extension, 72°C, 90 sec) and then 72°C, 7 min. PCR products were analysed using a 1.2% agarose gel containing ethidium bromide. Optical densities of PCR product signals were determined by using ImageJ 1.40g freeware (http://rsbweb.nih.gov/ij/).

2.7. SDS–PAGE and Western blot analysis

3-day-old cell cultures were washed in physiological saline and were harvested. Mechanically stimulated cultures as well as colonies treated with H89 or OA were collected immediately after loading or at the end of the 4-hour-long treatments on day 3. Total cell lysates for Western blot analyses were prepared as previously described [30]. 40 μg protein was separated in 7.5% SDS–polyacrylamide gels for the detection of Sox9, P-Sox9, HAS2,

HAS3, CREB, P-CREB, PKA Cα, PP2A/C, aggrecan and GAPDH. Proteins were transferred electrophoretically to nitrocellulose membranes and exposed to primary antisera. Polyclonal anti-Sox9 antibody (in 1:600) and anti-GAPDH antibody (in 1:1000) were purchased from Abcam (Cambridge, UK); anti-P-Sox9 antibody (in 1:600) and anti-HAS3 antibody (in 1:600) were obtained from Sigma-Aldrich; anti-HAS2 antibody (in 1:200) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-CREB antibody (in 1:800), anti-P-CREB antibody (in 1:800), and anti-aggrecan antibody that recognises the CAT-301 proteoglycan (in 1:500) were purchased from Millipore; anti-PKA Cα antibody (in 1:600) and anti-PP2A/C antibody (in 1:600) were obtained from Cell Signaling Technology. After washing for 30 minutes with PBST, membranes were incubated with the peroxidase-conjugated secondary antibody anti-rabbit IgG (Bio-Rad Laboratories, CA, USA) in a 1:1500 dilution. Membranes were developed using enhanced chemiluminescence (Millipore). Optical densities of signals were measured by using ImageJ 1.40g freeware.

2.8. Immunocytochemistry

Following mechanical stimulation with or without pre-treatment with the PKA-inhibitor H89 or the PP2A-blocker OA on day 3, immunocytochemistry was performed on cells cultured on the surface of coverglasses to visualise the intracellular localization of Sox9, P-Sox9, CREB and P-CREB. Unstimulated 3-day-old HDC were used as controls. Cultures were fixed in Saint-Marie's fixative (99% ethanol and 1% anhydrous acetic acid) and washed in 70% ethanol. After rinsing in PBS (pH 7.4), nonspecific binding sites were blocked with PBS supplemented with 1% bovine serum albumin (Amresco LLC, Solon, OH, USA), then cultures were incubated with polyclonal Sox9 (Abcam), P-Sox9 (Sigma), CREB or P-CREB (Millipore) antibodies at a dilution of 1:400 at 4°C overnight. For visualisation of the primary

antibodies, anti-rabbit Alexa555 secondary antibody (Life Technologies Corporation, Carlsbad, CA, USA) was used at a dilution of 1:1000. Specificity of antibodies was confirmed by applying control peptides that were identical to antigens against which the antibodies were raised; in these experiments, no aspecific signals were detected (data not shown). Cultures were mounted in Vectashield mounting medium (Vector Laboratories, Peterborough, England) containing DAPI for nuclear DNA staining. Photomicrographs of the cultures were taken using an Olympus DP72 camera on a Nikon Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan). Images were acquired using cellSense Entry 1.5 software (Olympus, Shinjuku, Tokyo, Japan) with constant camera settings to allow comparison of fluorescent signal intensities. Images of Alexa555 and DAPI were overlaid using Adobe Photoshop version 10.0 software.

2.9. In vitro PKA and PP2A enzyme activity assays

Following mechanical stimulation, 3-day-old cultures were harvested, sonicated, and after centrifugation at 10,000×*g* for 10 min at 4°C, supernatants of samples were used for *in vitro* enzyme activity measurements. Unstimulated cultures were used as controls. PKA and PP2A enzyme activity measurements were carried out on separate cell lysates from the same experiments.

PKA enzyme activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP (MP Biomedicals, Santa Ana, CA, USA) into histone H2A substrate (Sigma-Aldrich). Reaction mixtures (40 μL each) contained 50 mM 2-(N-morpholino)ethanesulphonic acid (pH 6.8), 10 mg · mL^{-1} histone IIA, 10 mM magnesium acetate, 1 mM EGTA, 5 mM cAMP, 0.12 mM ATP and [γ-^{32}P]-ATP adjusted to approximately 1 million cpm per reaction mixture, and appropriate amount of supernatant (protein content: 2.0–2.5 mg · mL^{-1}).

Reaction mixtures were prepared with or without 20 μM H89 (Sigma-Aldrich). Enzyme activity measurements were performed at 30°C. After 10 min of reaction time, determination of ^{32}P incorporation into histone IIA substrates was carried out by pipetting 30 μL aliquots of the reaction mixture on filter paper squares (Whatman P81; 2×2 cm), which were then washed three times in 0.5% phosphoric acid, air-dried and radioactivity was measured in a liquid scintillation counter. In total cell lysates of chondrogenic cell cultures, PKA activity was determined as enzyme activity that was inhibited by H89 [31].

PP2A activity was assayed with ^{32}P-labelled myosin light-chain (5 μM) as described previously [32]. Briefly, pellets were suspended in 10 mM Tris/HCl (pH 7.4), 0.1 mM EGTA, 0.25 mM dithiothreitol, 0.1 mM PMSF, 0.1 mM DFP, 0.1 mg · mL^{-1} leupeptin and 1 mM benzamidine (buffer A) and further diluted in buffer A supplemented with 1 mg · mL^{-1} BSA. One unit of the protein phosphatase activity releases 1 μmol of P_i from the phosphosubstrate per min at 30 °C.

2.10. Measurement of cAMP concentration following mechanical intervention

Changes in cAMP concentration in cultures exposed to mechanical load were determined directly after stimulation on day 3 using a cAMP Direct Immunoassay Kit (Abcam). The kit utilises recombinant Protein G-coated 96-well plate to anchor cAMP polyclonal antibody on to the plate. cAMP–HRP conjugate directly competes with cAMP from sample binding to the cAMP antibody on the plate. Untreated control colonies and cultures exposed to mechanical loading grown in 6-well culture plates were dissociated in 500 μL of 0.1 M HCl. After this step, measurements were performed according to the protocol provided by the manufacturer.

2.11. Statistical Analysis

All data are representative of at least three independent experiments. Averages are expressed as mean ± SEM (standard error of the mean) where applicable. Statistical analysis was performed by unpaired Student's *t*-test for determining the effects of mechanical stimuli *versus* unstimulated cultures; or two-way analysis of variance (ANOVA) for assessing differences among untreated cultures and colonies that received mechanical stimulus and/or H89 or OA. Threshold for statistically significant differences as compared to control cultures was set at $*P < 0.05$.

3. Results

3.1. Physical characteristics of the custom-made mechanical stimulator equipment

To comprehend the physical properties and forces that the equipment exerts on differentiating cartilage colonies, a measurement campaign was performed using a three axis accelerometer, a high-speed digital camera and a pressure sensor. More than 100 measurements were performed through the campaign. All acceleration and pressure data were in a perfect correlation with each other. We could identify the different phases of the process described in Section 2.3: in the first phase (Fig. 1A; left to the vertical line at 10 ms) the pedicle was travelling in the air; during the second phase (Fig. 1A; right to the vertical line at 10 ms) the piston reached the liquid and decelerated (see Fig. 1A). The pressure value (600 Pa) was derived from the acceleration data (both series of measurements). The measured pressure data show the increase of the pressure until the pedicle reaches its bottom position (Fig. 1B).

The acceleration as well as the pressure measurements were averaged at each sampling time point. From the averaged acceleration data we could calculate the pressure at the bottom of the dish using a simple physical model of dynamic flow (Eq. 1.).

$$\frac{1}{2}\rho v^2 + p = \frac{1}{2}\rho u^2 \qquad \text{Eq. 1.}$$

$$R^2\pi v = 2R\pi h u\,,$$

where v is the speed of the piston, ρ is the density of the fluid, p is the pressure at a given depth, u is the speed of the fluid around the perimeter of the piston, R is the radius of the piston and h is the distance between the piston's plane and the bottom of the dish. After some algebra, we can calculate the pressure as:

$$p = \frac{1}{2}\rho v^2\left(\frac{R^2}{4h^2} - 1\right). \qquad \text{Eq. 2.}$$

The speed was calculated from the acceleration data and plotted in Fig. 1C, wherein red line shows the result of the optical measurements, and blue shows the calculated speed from the accelerometer data. Both yielded very similar results.

Part of the pressure originates from the viscous flow of the culture medium between the circular pedicle and the flat bottom of the well. This lubrication problem was resolved using the Navier–Stokes equation. This is given in [33] as follows:

$$p(r) - p(0) = 3\rho v^2\,\frac{R^2 - r^2}{h^3}\,, \qquad \text{Eq. 3.}$$

where r is the distance from the centre of the piston. In Fig. 1B, the blue curve indicates the average of the measured, whereas the green one represents the calculated pressure. The green curve displays both pressure contributions; the one resulting from dynamic flow (Eq. 2) and the other arising from viscous flow (Eq. 3). Please note that the pressure fraction calculated from Eq. 3 is about one-tenth of the contribution that comes from the fluid flow (*i.e.* Eq. 2). From the Navier–Stokes equations [33] we were able to calculate the shear stress at the

bottom of the dish, which was about 5 Pa in our case. Note that this is about one-tenth of the contribution of the viscous part. For further information on the physical properties of the custom-made bioreactor, please see the Supporting Information.

3.2. Mechanical stimulation enhances cartilage formation and reduces proliferation in chicken micromass cultures

In primary chicken chondrifying HDC, differentiation and final commitment of chondroprogenitor cells take place predominantly on days 2 and 3 of culturing [27]. To monitor the effects of intermittent cyclic mechanical load exerted by our custom-made bioreactor on the differentiation of chondroprogenitor cells, loading scheme (0.05 Hz, 600 Pa) was applied on these two days of culturing for 30 min. Mechanical perturbation at higher frequencies or for longer periods completely abrogated the cultures (data not shown), while the above-described intermittent loading regime applied to differentiating chicken chondrogenic cells on culturing days 2 and 3 for 30 min significantly enhanced the amount of metachromatic cartilage matrix by day 6 (to 145±3% of control cultures; Fig. 2A) as revealed by DMMB and TB staining procedures.

When we looked at whether the loading scheme altered the expression of molecular markers of chondrogenesis, we found that it considerably increased both mRNA and protein expression of Sox9, the major cartilage-specific transcription factor (to 160% and 150% of unstimulated controls, respectively; Fig. 2B–C); moreover, it almost doubled the level of the more active Ser 211 phosphorylated form of Sox9 (P-Sox9; Fig. 2C), implicating the activation of mechanosensitive kinases and/or deactivation of phosphoprotein phosphatases (PP) upstream of Sox9.

Next, we monitored the mRNA and protein expression of an array of cartilage matrix-specific molecules following mechanical load. The mRNA expression of aggrecan *(ACAN)* and collagen type II *(COL2A1)*, the main components of cartilage ECM, were only modestly increased following mechanical stimulation (Fig. 2B), and the amount of the large aggregating proteoglycan aggrecan was elevated also on immunoblots (Fig. 2C). These results implied that mechanical load primarily enhanced the amount of sulphated glycosaminoglycans (GAG) and proteoglycans (PG) that manifested in increased metachromasia, revealed by DMMB and TB staining procedures. Therefore, mRNA expressions of hyaluronan synthase *(HAS)* enzymes 2 and 3, as well as chondroitin 4-O-sulphotransferase 1 (*CHST11*), xylosyltransferase (*XYLT1* and *XYLT2*) and 1,4-N-acetylglucosaminyltransferase *(EXTL1)*, which are involved in the synthesis of hyaluronan, catalysis of transfer of UDP-xylose to target proteins, and synthesis of sulphated GAGs, respectively, were also investigated. We detected only a moderate upregulation of these enzymes at the mRNA level, compared to an approx. 1.5- and 2-fold elevation of the protein expression of HAS2 and HAS3 in cell cultures exposed to mechanical stimulation (Fig. 2C).

Since augmented cartilage matrix formation could have resulted from altered cell numbers following mechanical load, two additional measurements were performed. Cellular viability that reflects on the actual number of cells present in HDC as a function of their mitochondrial dehydrogenase activity was determined directly after the cyclic load on day 3, and no change could be observed in this parameter. Rate of DNA synthesis was also monitored by determining the radioactivity of incorporated ^{3}H-thymidine; this assay reflects on the rate of cells entering the S phase during a 16-hour-long interval directly after mechanical load on day 3. In contrast to the mitochondrial activity assay, a 60% decrease in cell proliferation was detected followed by mechanical loading as revealed by ^{3}H-thymidine incorporation assays (Fig. 2D).

3.3. Enhanced cartilage matrix production is mediated via the PKA/CREB–Sox9 pathway during chondrogenesis in HDC

Since mechanical stimulation doubled the amount of phosphorylated Sox9 in chondrifying cultures (Fig. 2C), and as Sox9 is known to be a substrate for PKA [34], we aimed to investigate the role of this kinase in mechanotransduction pathways during *in vitro* chondrogenesis. The 30-min-long loading regime applied to differentiating cells of HDC on days 2 and 3 of culturing substantially elevated PKA catalytic subunit β (PKA Cβ) mRNA and protein levels on day 3 (Fig. 3A–B). As the archetypal downstream target for PKA is CREB that is known to regulate the Sox9 promoter [13]; furthermore, as it is reported to directly associate with Sox9 during osteochondral differentiation [14], we also looked at its expression following the loading regime. Although CREB expression (either at mRNA or at the protein level) did not respond to these treatments, its phosphorylation status (at Ser 133) was found to be augmented (approx. 2-fold) following mechanical stimulation (Fig. 3A–B), which further supported our hypothesis that mechanosensitive kinases and/or phosphatases were involved.

Therefore, we measured the PKA enzyme activity and found a steep (approx. 10-fold) increase following mechanical stimulation in a cell-free *in vitro* enzyme activity assay (Fig. 3C). Moreover, a five-fold increase in cAMP concentration of lysates was measured following mechanical loading (0.2 pmol/well) *versus* untreated control cultures (0.04 pmol/well) as revealed by the cAMP Direct Immunoassay Kit. When the PKA inhibitor H89 was applied on culturing days 2 and 3 for 4 h at 20 μM, a marked decrease (to 67±8% of control) in the amount of metachromatic cartilage matrix was observed (Fig. 3D). However, in the presence of H89, mechanical load elevated cartilage formation compared to colonies

treated with H89 only to control levels (100%±11; Fig. 3D), implying that the PKA pathway was required for mechanical load-induced matrix formation, but also suggesting the involvement of other mechanosensitive pathways.

When the proliferation rate of HDC exposed to mechanical load was investigated, partially unexpected results were gained (Fig. 3E). 4-hour-long treatment with H89 significantly reduced the rate of cell division, which is in a perfect correlation with data published in the literature [19]. However, although mechanical load alone also considerably decreased proliferation rate (see Fig. 2), co-application of the loading scheme with PKA-inhibition significantly increased the number of dividing cells compared to colonies treated with H89 only (Fig. 3E).

On the other hand, inhibition of PKA activity did not interfere with the mRNA expression of genes encoding cartilage ECM components (although *COL2A1* was found to be downregulated), nor did it alter mRNA transcript levels of the key transcription factors Sox9 and CREB (Fig. 3A). Consequently, no change in the mRNA expression patterns of the above factors could be observed when the loading scheme was applied to cultures pre-treated with H89 (Fig. 3A). In contrast, marked changes were detected at the protein level. Treatment with H89 caused a substantial decrease (to ~40%) in Sox9 protein expression, which was only partially rescued by mechanical loading (Fig. 3B). Furthermore, the level of Sox9 phosphorylated at Ser 211 was also found to be decreased, and remained at this lower level despite mechanical stimulation, implicating that PKA is one of the key kinases involved in enhanced Sox9 phosphorylation following the loading scheme (Fig. 3B). We also looked at protein expression and phosphorylation status of CREB and found partially similar changes to what was observed for Sox9: treatment with H89 considerably attenuated the protein expression level of CREB, which remained unchanged following mechanical stimulation (Fig. 3B). However, unlike Sox9, phosphorylated CREB became completely undetectable

after treatment with H89 and mechanical loading was able to partially compensate this inhibition (to ~70% of the control; Fig. 3B), implicating the involvement of other mechanosensitive signalling molecules (*e.g*. phosphoprotein phosphatases) in the regulation of CREB phosphorylation during mechanotransduction.

3.4. Decreased PP2A activity following mechanical stimulation may also contribute to enhanced cartilage matrix formation during chondrogenesis

As P-CREB is known to be a substrate also for PP2A, and since we reported earlier that inhibition of PP2A activity resulted in elevated PKA activity as well as enhanced proliferation and chondrogenesis in HDC [19, 35], we also looked at the role of this phosphatase in mediating mechanical stimuli during chondrogenesis. In contrast to PKA, PP2A expression was not altered following mechanical load either at the mRNA or at the protein level (Fig. 4A–B). However, despite unchanged protein levels, PP2A enzyme activity was found to be significantly reduced after the loading scheme as revealed by *in vitro* phosphatase assays (Fig. 4C). Since these data suggested that mechanical load exerted an inhibitory effect on PP2A activity, further experiments were performed with the Ser/Thr PP inhibitor okadaic acid (OA), which is known to be a potent inhibitor of PP2A in chondrogenic cultures when applied at 20 nM [35]. We have reported previously that 4-hour-long treatments with 20 nM OA on culturing days 2 and 3 significantly enhanced metachromatic cartilage ECM accumulation [35] (to 155±7% of the control; Fig. 4D); in this study, we aimed to identify whether treatments with OA can mimic and/or modulate the effects of mechanical stimulation.

Combined application of OA with the loading scheme further increased the amount of metachromatic ECM compared to colonies received either OA or mechanical intervention

only (to 186±9% of the control; Fig. 4D), suggesting that the effects of the two treatments were additive. Then, we looked at the mRNA and protein expression of chondrogenic markers and found that *SOX9* mRNA levels remained unchanged; *ACAN* and *COL2A1* were only slightly upregulated after mechanical load and OA-treatment; and no synergism between the two kinds of stimulation could be observed (Fig. 4A). By contrast, more pronounced changes were detected at the protein level (Fig. 4B). Although mechanical load itself resulted in increased Sox9 protein levels and a more than 2-fold elevation was detected after OA-treatments alone, combined application of the loading scheme with OA did not result in any further increase of bands as revealed by Western blot analyses. Similar results were obtained with the phosphorylated form of Sox9 (Fig. 4B).

Interestingly, cross-talk between the PP2A and PKA signalling pathways was found in chondrogenic cells as inhibition of PP2A activity by OA slightly elevated the protein level of PKA; furthermore, co-application of OA and the loading scheme resulted in an almost 5-fold increase in the amount of PKA detected on Western blots (Fig. 4B). Moreover, OA treatments mimicked the effects of mechanical stimulation on the amount of CREB protein as well as phosphorylated CREB as revealed by Western blotting. However, co-application of OA and mechanical load did not influence either the protein level or the amount of phosphorylated CREB compared to control cultures (Fig. 4B).

Since treatment with 20 nM OA is known to enhance proliferation rate of chicken micromass cultures [35], whereas mechanical load alone caused a marked inhibition of cell proliferation (see Fig. 2), we also wanted to evaluate the combined effect of OA and the loading scheme and found that the antiproliferative effect of the loading regime could be completely prevented by pre-treatment with OA as revealed by ^{3}H-thymidine incorporation assays (Fig. 4E).

3.5. Basal Epac activity could be a negative regulator of chondrogenesis, independent of mechanical intervention

Apart from the prototypical cAMP dependent protein kinase PKA, Epac proteins (also known as Rap guanine nucleotide exchange factors) have recently been implicated as novel mediators of cAMP [36]. To investigate whether Epac1 and Epac2 were expressed and functional in chondrifying HDC, further experiments were carried out. As shown in Fig. 5A, mRNA transcripts of both Epac1 and Epac2 were detected in micromass cultures throughout the culturing period, with Epac1 exhibiting a slight decline and Epac2 a mild increase as chondrogenesis proceeds. Furthermore, when the specific Epac1 and Epac2 inhibitor HJC0197 (10 μM) was administered to the medium on culturing days 2 and 3 for 4 h, metachromatic cartilage matrix production was significantly augmented (to 158±5%) as revealed by DMMB and TB staining procedures (Fig. 5B). When treatments with HJC0197 were combined with mechanical stimuli, the positive effects of the two kinds of intervention proved to be additive, yielding an almost 2-fold (189±2%) elevation of metachromatic cartilage ECM production (Fig. 5B). These results suggested that Epac proteins negatively affected chondrogenesis in the given experimental setup, and their regulation was independent of the loading regime.

3.6. Mechanical stimulation enhances nuclear signals of the transcription factors Sox9 and CREB in a PKA and PP2A-dependent manner

Mechanical load applied to chondrogenic cells did not influence the intracellular distribution of CREB, and it was found not to be altered in cultures treated with H89 (Fig. 6Aa). However, stronger nuclear signals for P-CREB were observed following the loading

scheme, which could be prevented by pre-treatment with H89 (Fig. 6Ab). These observations further support the concept of the involvement of PKA in mediating chondrogenesis-promoting effects of mechanical stimulation.

In cultures exposed to 30-min-long mechanical stimulation, accumulation of the Sox9 transcription factors in the nucleus of differentiating chondrocytes was observed compared to control colonies in parallel to enhanced cartilage matrix production, as revealed by immunocytochemical staining procedures (Fig. 6Ac). Furthermore, the amount of P-Sox9 was also elevated in the nuclei following the loading scheme (Fig. 6Ad). Treatment with H89 alone diminished signals for Sox9, and co-application of the loading regime was not able to compensate for the observed decrease. Interestingly, H89 alone did not seem to alter the amount of nuclear P-Sox9 as compared to unstimulated colonies, while in the presence of H89, mechanical load failed to elevate nuclear P-Sox9 levels (Fig. 6Ad).

However, since 4-hour-long treatments with 20 nM OA on culturing days 2 and 3 alone considerably enhanced signals for both P-Sox9 (Fig. 6Ba) and P-CREB (Fig. 6Bb) and in this respect mimicked the effects of the loading scheme, no further changes in the cellular localisation and distribution of either P-Sox9 or P-CREB could be detected in case of the combined treatment using immunocytochemistry.

4. Discussion

Biomechanical forces are key factors that keep mature cartilage in a healthy condition. During normal activity, articular cartilage *in vivo* undergoes cyclic compression (at a frequency of approx. 1 Hz during locomotion) and chondrocytes are exposed to uniform compressive force within the range of 3 to 10 MPa [37]. The requirement of a well-defined movement pattern during *in vivo* joint and articular cartilage formation in murine and avian

embryos has also been known [38, 39]. To investigate the effects of external mechanical stimuli on skeletal tissues, many different research groups have designed and developed bioreactors that can apply compressive forces (both constant and intermittent), hydrostatic pressure, tensile strain and shear stress (reviewed by Grad and colleagues [40]). The main advantage of the custom-made bioreactor applied in this study is that it exerts biomechanical stimuli that are combined in nature (hydrostatic pressure and shear stress) and thus more closely models the biomechanical environment articular chondrocytes encounter during locomotion.

The applied mechanical loading regime resulted in a significant increase of cartilage matrix *in vitro,* as well as enhanced Sox9 and CREB expression and phosphorylation, reflecting on the activation of these transcription factors with a fundamental role in the regulation of chondrogenesis. Interestingly, however, neither collagen type II nor aggrecan core protein mRNA transcript levels exhibited a similar increase, implicating that an enhanced GAG-synthesis may be accounted for the observed effect in metachromatic cartilage matrix formation. Indeed, a modest elevation in mRNA expression of key enzymes involved in GAG-synthesis (*HAS, CHST11*) was detected. Similar findings have been reported when mechanical stimulus was applied on hydrogel cultures of MSCs [41]. Moreover, the expression of 1,4-N-acetylglucosaminyltransferase *(EXTL1),* which encodes a protein that functions in the chain polymerization of heparan sulphate, as well as xylosyltransferase (*XYLT1* and *XYLT2*) that is responsible for the biosynthesis of chondroitin sulphate and heparan sulphate components of PGs, were found to be considerably upregulated in response to mechanical stimuli. In fact, several *in vivo* studies have been performed that indicate the positive effects of physiological joint loading on articular cartilage ranging from increased PG and collagen type II content to reversal of osteoarthritis (extensively reviewed by Bader and colleagues [3]).

Among posttranslational modifications, reversible phosphorylation of specific tyrosine and/or serine/threonine residues of key signalling proteins is a dynamic process which is essential for controlling cellular proliferation, differentiation and survival. Mechanical stimulation doubled the amount of the Ser 211 phosphorylated form of Sox9 transcription factor upstream of genes involved in ECM synthesis, rendering it to be more active and increasing its nuclear shuttle. For PKA is known to phosphorylate the Sox9 protein in chondrocytes [16]; furthermore, as there is a complex synergism between Sox9 function and the cAMP–PKA–CREB pathway in both mature and differentiating chondrocytes [14], we undertook to investigate the role of this signalling pathway in mechanotransduction of differentiating chondrogenic cells. PKA is a positive regulator of chondrogenesis and consequently, the negative effect of the potent PKA inhibitor H89 on metachromatic matrix production in chicken micromass cultures, along with attenuated cellular proliferation rate and phosphorylated CREB levels, has also long been known, underpinning the central role of this pathway in controlling *in vitro* chondrogenesis [19]. There are sporadic data suggesting that the PKA–CREB axis mediates activation *via* mechanical stimuli in different models. Mechanical forces were shown to trigger the cAMP–PKA-dependent signalling pathway in cultured foetal type II epithelial cells [42]. Furthermore, earlier studies also suggest the hypothesis that CREB may participate in strain-induced gene expression in endothelial cells [43]. Indeed, the PKA pathway was found to be differentially regulated following mechanical stimulation also in osteoblasts [44].

When chondrocytes isolated from rat ribs cultured *in vitro* were subjected to mechanical stress (tensile force), a significant increase in intracellular cAMP levels was reported [45]. In bovine articular cartilage explants, low-level compressive loading was found to transiently elevate mRNA levels of aggrecan *via* a cAMP-dependent mechanism; however, no direct evidence concerning the putative link between PKA and mechanotransduction was

established [46]. The role of elevated cAMP levels (and the possible involvement of PKA) in mechanotransduction pathways of bovine articular chondrocytes is also discussed in another study [2]. R_p-cAMP, the R_p-diastereomer of adenosine-3',5'-cyclic monophosphothioate, which is a membrane-permeable inhibitor that blocks cAMP-mediated effects, was able to prevent upregulation of certain clusters of genes in response to static compression [1], suggesting a determining role of signalling pathways that involve PKA. In our study, the loading regime resulted in fivefold elevation of cAMP levels compared to untreated control HD cultures. However, apart from studies using inhibitors to implicate the role of PKA in mediating mechanotransduction pathways in chondrocytes, no proof-of-concept data have been published.

We found that the competitive ATP antagonist H89 that is generally accepted to be a potent PKA inhibitor [47] decreased metachromatic matrix accumulation compared to untreated control cultures, and mechanical load was only partially able to rescue the diminished cartilage formation, suggesting that although the PKA pathway was required for mechanical load-induced matrix formation, the involvement of other mechanosensitive pathways is likely. Indeed, H89-treatment reduced the amount of P-Sox9 (at Ser 211) in differentiating chondrocytes, which could not be compensated by mechanical stimuli. In mature chondrocytes, PKA is known to phosphorylate the Sox9 protein, which enhances its transcriptional activity; furthermore, the fact that H89 (at 20 μM) reduces P-Sox9 levels has also been described [34]. However, this is the first study to report that enhanced cartilage formation in response to mechanical stimulation is related to elevated P-Sox9 levels, which can be attributed to augmented PKA enzyme activity levels assayed in cell-free samples from mechanically induced cartilage colonies.

Phosphorylation of the Sox9 protein by PKA in chondrocytes also triggers its nuclear translocation [34]; and indeed, the loading regime considerably enhanced the nuclear signal

of phosphorylated Sox9 in a PKA-dependent manner. Similar results were detected for the quintessential downstream PKA substrate CREB: its phosphorylation and the nuclear signal of P-CREB were also strongly enhanced in response to the loading regime, which could be prevented by co-application of H89 during mechanical load. Of note, P-CREB levels were only partially decreased in cultures that received a combined treatment by mechanical load and H89. Nevertheless, this contradiction can be resolved by considering the fact that CREB is also substrate for other kinases; in particular, PKCα was reported to catalyse CREB phosphorylation [48]. Moreover, PKA is known to regulate chondrogenesis in chicken limb bud-derived micromass cultures *via* a PKCα-dependent manner [49]. Cross-talk between these pathways or inhibition of protein phosphatases during mechanotransduction may explain why mechanical loading-induced CREB phosphorylation could only partially be inhibited by the application of H89.

Until the discovery of Epac proteins in 1998 the effects of the secondary messenger cAMP were considered to be mediated only by PKA and cyclic nucleotide gated ion channels [50]. Representing a novel category of cAMP mediators, Epac proteins act as specific guanine nucleotide exchange factors (GEFs) for the Ras GTPase family members Rap1 and Rap2 and consequently mediate a wide array of cellular functions including cell division, differentiation, secretion and growth [51]. However, our current understanding of the roles of Epac in chondrocytes is confined to its involvement in mediating anti-inflammatory activity exerted by adenosine 2A receptors during hyaluronan-induced inflammation [20]. We report here for the first time that mRNA transcripts for both Epac1 and Epac2 isoforms could be detected throughout the entire culturing period of chondrifying micromass cultures. Furthermore, application of the potent membrane-permeant Epac1 and Epac2 inhibitor HJC0197 [28] on days 2 and 3 for only 4 h substantially augmented *in vitro* cartilage matrix production, implicating Epac proteins and associated downstream signalling as negative

regulators of chondrogenesis in the model applied in this study. However, since we could not find interference between the effects of the loading regime and those of Epac inhibition, we can assume a lack of cross-talk between these two pathways. Although we refrained from investigating the details of Epac signalling in this work, it would be interesting to study how Epac proteins control chondrogenic differentiation of chicken micromass cultures.

The steady-state phosphorylation status of signalling proteins is governed by the opposing activities of protein kinases and phosphatases. Clearly, PP activity must be just as important as kinase activity in modulating cellular signalling. However, knowledge concerning the role of PPs during chondrogenesis has been very limited (see [19, 29, 35, 52]). Consequently, their precise involvement in mediating mechanotransduction pathways during chondrogenesis is a completely yet unexplored field. PP2A is known to be a negative regulator of chondrogenesis as its inhibition by 20 nM OA has substantially increased metachromatic cartilage matrix production, elevated cellular proliferation and induced rounding of chondrogenic cells [35]. Furthermore, we also found earlier that OA-treatments increased the phosphorylation of CREB in chicken micromass cultures, suggesting that CREB is a common target for both PKA and PP2A in chicken HDC [19]. Based on the above, a mechanical load-induced decrease in PP2A activity could also be accounted for the observed difference in CREB phosphorylation. In fact, we detected an approx. 30% decrease of PP2A activity following cyclic load in cell-free lysates, which is in a perfect correlation with the decrease observed in endothelial cells following cyclic strain by Murata and colleagues [53]. Furthermore, the effects of the combined treatment with OA and mechanical stimulation on the amount of metachromatic ECM production were additive, suggesting that enhanced PKA-activity following the loading scheme was able to further increase matrix deposition. Treatments with OA considerably enhanced not only the protein level but also the phosphorylation status of Sox9 compared to untreated control cultures; however, pre-

treatment with 20 nM OA during mechanical load was unable to further elevate signals for phosphorylated and unphosphorylated Sox9 compared to cultures that only received cyclic stimulation. Similar effects were observed for the protein expression and phosphorylation status of CREB; although OA-treatments have been shown to enhance the amount of P-CREB in HDC [19], combined treatments did not alter any of these parameters. On the other hand, the amount of PKA protein was slightly higher in OA-treated cultures *vs*. controls, and a very strong upregulation was detected in cultures that received the combined treatment with OA and mechanical load. Based on these findings, mechanical stimuli may exert their effects by activating the PKA–CREB pathway and at the same time by inhibiting PP2A signalling in chondrogenic cells.

While the effects of mechanical load on chondrogenesis and cartilage ECM production could be well explained by suggesting that they are primarily mediated by the PKA and PP2A pathways, the regulation of cellular proliferation rate in cultures exposed to mechanical stimuli was not straightforward. Although the mechanical loading scheme significantly reduced the rate of cell division and at the same time augmented metachromatic matrix production, modulation of either the PKA or the PP2A pathway yielded controversial results. While blockade of PKA activity by H89 also significantly reduced proliferation rate in HDC [19], combined treatments resulted in an increased rate of DNA-synthesis, but at the same time ECM production was suppressed. Conversely, when the PP2A-inhibitor OA was applied, cell proliferation was enhanced [19], which was slightly stimulated further by co-application of OA with the loading scheme, but at the same time, metachromatic matrix production rate was massively increased. These data suggest that there is no direct correlation between the regulation of proliferation rate and the rate of matrix synthesis in HDC following mechanical load. It seems to be the case that the response of the proliferation rate of chondrogenic cells in chicken limb bud-derived HDC to experimental modulation appears to

be context-dependent, and it may vary according to the particular constellation of other signalling pathways that are concurrently active at any time.

One possible mechanism by which mechanical stimuli may exert their positive role during *in vitro* chondrogenesis is *via* the primary cilium. Ultrastructural studies have confirmed that mature chondrocytes possess an immotile primary cilium that has a microtubule axoneme core made up of a ring of nine microtubule doublets, but devoid of the central pair of microtubules (thus designated as 9+0) that motile cilia always have (called a 9+2 axoneme) [26]. Primary cilia have been well documented to serve as a nexus that integrates chemical and mechanical signals; in particular, they are considered as mechanosensors of fluid flow in many cell types including kidney epithelial cells, cholangioctyes or endothelial cells [54]. Primary cilia are also present on human mesenchymal stem cells, on arthritic chondroprogenitor cells (CPCs) [26] and on chondrocytes where they are unique sensory organelles that project into the pericellular matrix and interact with their close environment (*i.e.* collagen types II and IV) *via* integrins, G proteins, and various Ca^{2+} channels; many of which have been implicated as mechanoreceptors [55]. Furthermore, the PKA pathway has been recently reported to be involved in the regulation of the primary cilium length in chondrocytes [56].

In light of the results discussed herein concerning mechanotransduction pathways in differentiating chondroprogenitor cells in HDC that involve the activation of the cAMP–PKA–CREB axis and concurrent inhibition of PP2A activity, we propose the following hypothesis (Fig. 6). External stimuli can have multiple targets on chondrogenic micromass cultures, but the most likely mechanism is deformation of the developing matrix and alteration of the shape of the differentiating chondrocytes within, with subsequent perturbation of the cytoskeletal organization. The chondrocyte plasma membrane has long been known to harbour mechanoreceptor complexes that include integrins mediating

attachment to ECM components (*i.e*. collagen, laminin, and fibronectin) that are associated with stretch-activated ion channels and voltage-gated calcium channels [4]. In chondrocytes, integrins play important roles also in cartilage remodelling and chondrogenesis through interactions with the focal adhesion kinase and the cytoskeleton [57]. Mechanically induced alterations in the cytoskeleton can directly initiate the formation and/or activation of specific signal transducers within the focal adhesion complex [58]. Based on our data, the next component of mechanotransduction pathways can be the activation of adenylate cyclase which, by producing cAMP triggers PKA. Such a putative link has been established in primary embryonic osteoblasts, where signal transduction pathways to mediate the upregulation of osteopontin in response to mechanical stimuli were shown to be dependent on the activation of PKA [58]. Further, our data suggest that the dramatically increased PKA activity following mechanical stimulation could be accounted for the observed loading-induced changes in HDC including enhanced cartilage ECM production, elevated Sox9 and CREB phosphorylation as well as stronger nuclear signals for these factors, since most of these effects could be prevented by the PKA-inhibitor H89. Moreover, CREB can directly activate Sox9 through physical interactions at the Ser 133 residue [14].

At the same time, mechanical intervention exerted negative effects on PP2A, the phosphatase counterpart of the PKA pathway, which could also contribute to the observed positive effects of the loading scheme. Furthermore, our results indicate cross-talk between the PKA and PP2A pathways [19]. In fact, it has long been known that by dephosphorylating PKA at Thr 197 that is required for optimal enzyme activity, PP2A can reduce the enzyme activity of PKA [59]; conversely, since mechanical load reduces PP2A activity, PKA activity can increase, enhancing chondrogenesis. These two pathways converge on the same key downstream targets Sox9 and CREB that are both positive regulators of chondrogenesis and thus may mediate the effects of mechanical stimuli. Although the presence of primary cilia in

limb bud-derived differentiating chondrocytes has not been confirmed, it is logical to hypothesise an interrelation between primary cilia and PKA-mediated mechanotransduction pathways in cells of HDC. However, further studies are needed to elucidate this putative connection.

5. Conclusions

In summary, the findings presented here unequivocally provide evidence that *in vitro* chondrogenesis in chicken HDC was enhanced and differentiation of chondrogenic cells was stimulated in response to the intermittent loading scheme applied by our self-designed mechanical stimulator equipment in a PKA/PP2A–CREB–Sox9 dependent manner. These findings may prove to be useful for future research aimed at exploiting the putative regeneration capacity of chondroprogenitor cells found in both healthy [60] and diseased articular cartilage [61]. Extrapolation of our results to cartilage regeneration or bioengineering from human mesenchymal stem cells may also contribute to recent advances in this field.

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Conflict of Interest Statement

All authors disclose that there are neither any financial nor any personal relationships with other people or organisations that could inappropriately influence (bias) their work. There are no conflicts of interests.

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Figures

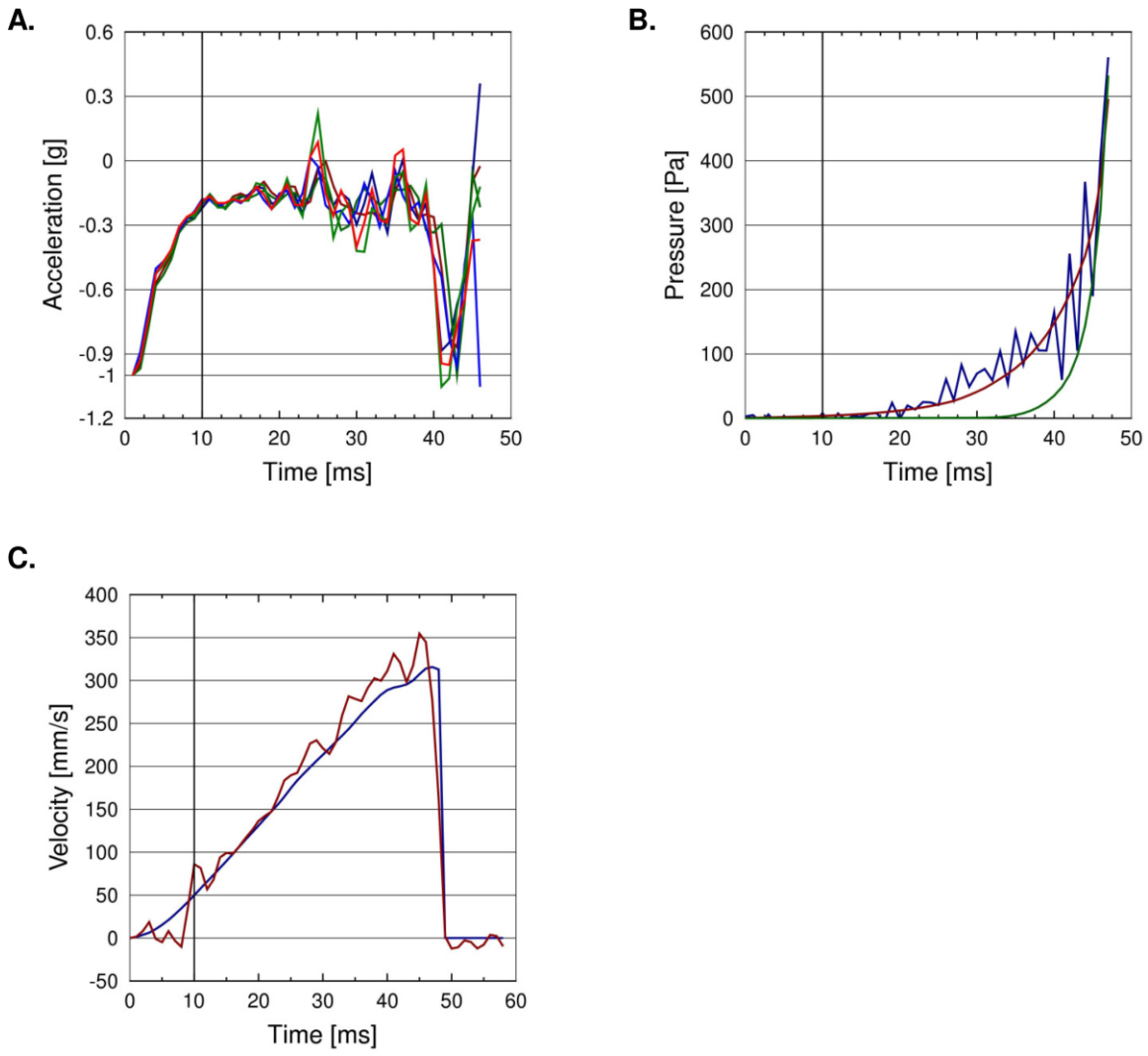

Fig. 1. Main parameters of the custom-made uniaxial mechanical stimulator equipment. (A) Acceleration of the pedicle, measured by a three axis accelerometer. (B) Calculated (green) and measured (blue) pressure at the bottom of the dish as a function of time. The red curve represents average values of the blue curve to enable easier data interpretation. (C) Calculated speed of the pedicle as a function of time. The red and blue curves were calculated from the optical and the accelerometer measurement data, respectively.

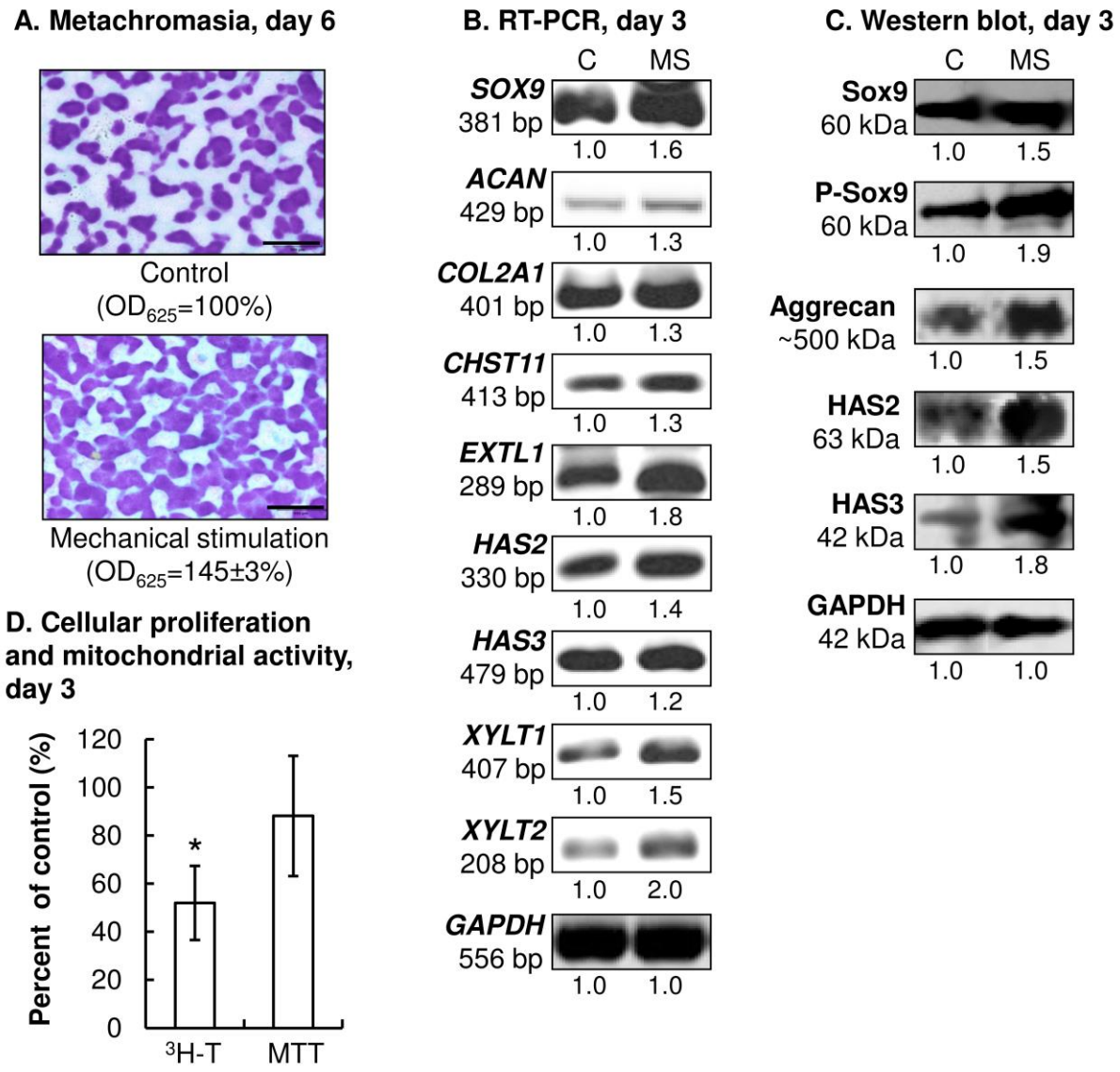

Fig. 2. Effects of mechanical stimulation on cartilage matrix production; mRNA and protein expression of Sox9 transcription factor, genes and proteins involved in matrix synthesis; as well as mitochondrial activity and proliferation rate of HDC. (A) Metachromatic cartilage areas in 6-day-old cultures were visualised with DMMB staining. Metachromatic (purple) structures in photomicrographs represent cartilaginous nodules formed by many cells and extracellular matrix rich in polyanionic GAG chains. Original magnification was 4×. Scale bar, 500 µm. Optical density (OD_{625}) was determined in samples containing TB extracted with 8% HCl dissolved in absolute ethanol. Data are expressed as mean ± SEM. (B) mRNA expression of *SOX9* and main matrix marker genes on day 3.

GAPDH was used as an internal control. (C) Protein expression of Sox9, HAS2 and 3, and aggrecan, and phosphorylation status of Sox9 in HDC on day 3 of culturing. GAPDH was used as a loading control. (D) Effects of mechanical stimulation on mitochondrial metabolic activity and cellular proliferation in HDC on culturing day 3. Statistically significant difference between proliferation rate of cells in cultures that received the loading regime *vs*. control cultures is marked by asterisk (*$P < 0.05$). Data are expressed as mean ± SEM. For panels (B) and (C), numbers below bands represent integrated densities determined by ImageJ freeware. (C, control; MS, mechanical stimulation applied on days 2 and 3 for 30 min; ^{3}H-T, ^{3}H-thymidine incorporation assay for cellular proliferation; MTT, assay for mitochondrial activity measurements.)

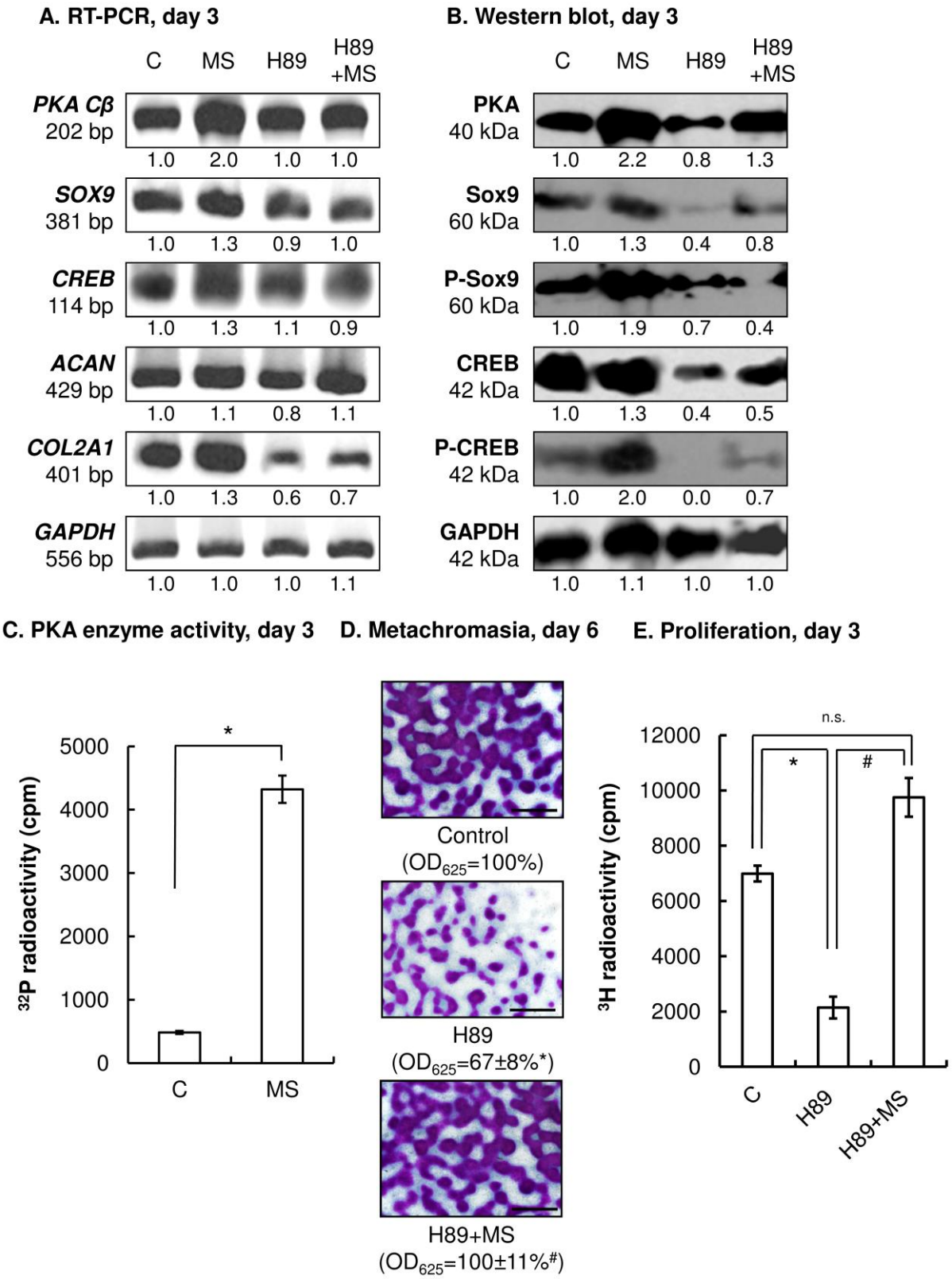

Fig. 3. Effects of the PKA-inhibitor H89 with or without mechanical load on mRNA and protein expression of key factors, PKA enzyme activity, cartilage matrix production, and proliferation rate. (A) mRNA expression of *PKA Cβ*, *SOX9*, *CREB*, *ACAN*, and

COL2A1 on day 3. *GAPDH* was used as an internal control. (B) Protein expression and phosphorylation status of PKA, Sox9 and CREB on day 3 of culturing. GAPDH was used as a control. (C) PKA enzyme activity determined in a cell-free *in vitro* assay from lysates of untreated controls and cultures following mechanical stimuli. Statistically significant difference between PKA enzyme activity in cultures that received the loading regime *vs*. control cultures is marked by asterisk (*$P < 0.05$). (D) Metachromatic cartilage areas in 6-day-old cultures were visualized with DMMB. Original magnification was 4×. Scale bar, 500 μm. Optical density (OD_{625}) was determined in samples after TB extraction. Data are expressed as mean ± SEM. Asterisk (*) represents significant difference as compared to control cultures; hashmark (#) represents significant difference compared to colonies treated with H89 only (*,$^{\#}$ $P < 0.05$). (E) Effects of H89-treatments with or without mechanical stimulation on cellular proliferation rate in HDC on culturing day 3. Statistically significant difference between proliferation rate of cells in cultures that were treated with H89 *vs*. control cultures is marked by asterisk; hashmark (#) represents significant difference compared to colonies treated with H89 only (*,$^{\#}$ $P < 0.05$). For panels (A) and (B), numbers below bands represent integrated densities determined by ImageJ freeware. (C, control; MS, mechanical stimulation applied on days 2 and 3 for 30 min; H89, treatment with 20 μM H89 on days 2 and 3 for 4 h.)

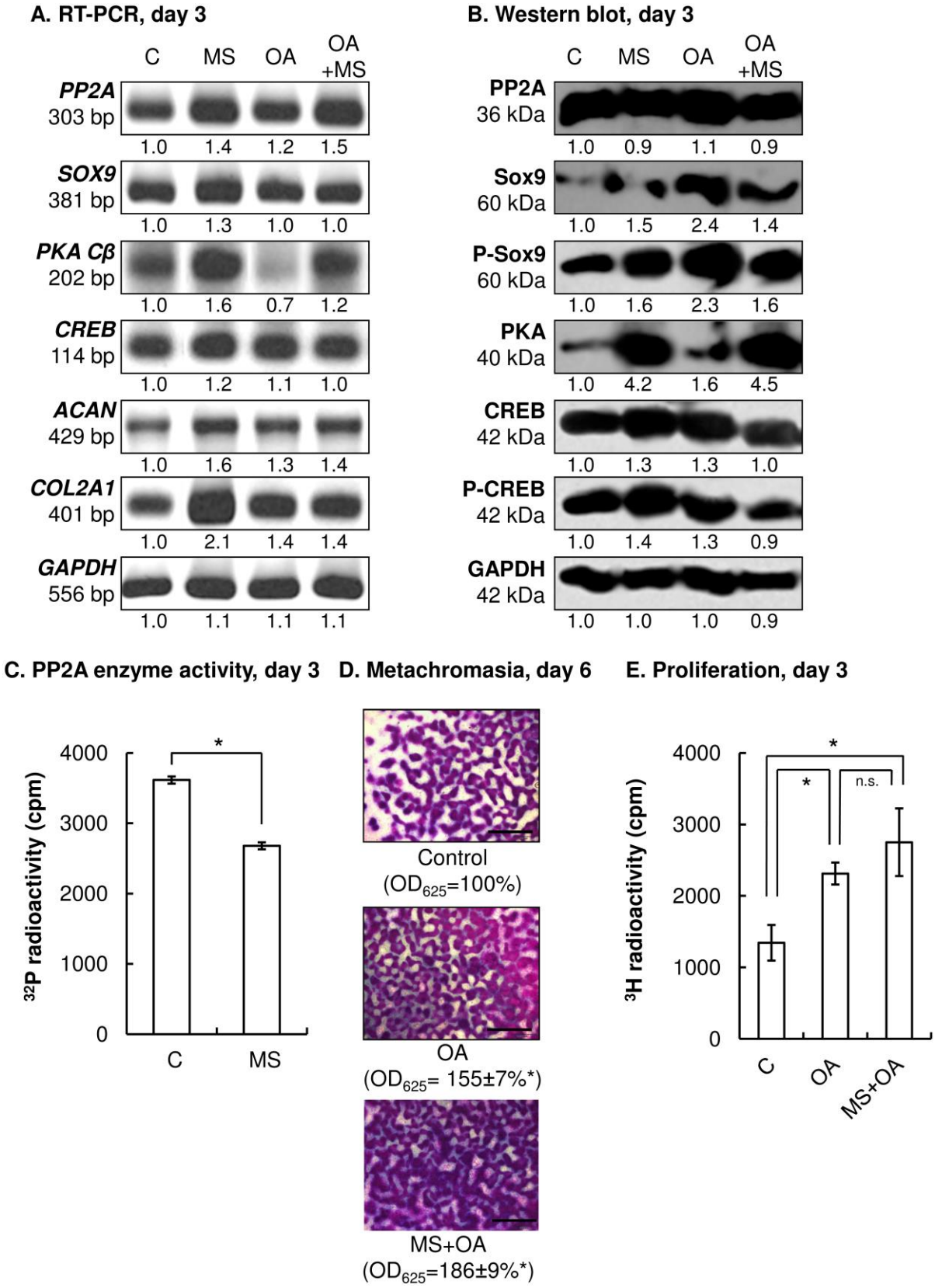

Fig. 4. Effects of the PP2A-inhibitor OA with or without mechanical load on mRNA and protein expression of key factors, PP2A enzyme activity, cartilage matrix synthesis, and

proliferation rate. (A) mRNA expression of *PP2A*, *PKA Cβ*, *SOX9*, *CREB*, *Acan*, and *COL2A1* on day 3. *GAPDH* was used as a control. (B) Protein expression and phosphorylation status of PP2A, PKA, Sox9 and CREB on day 3 of culturing. GAPDH was used as a loading control. (C) PP2A enzyme activity determined in a cell-free *in vitro* assay from lysates of untreated controls and cultures following mechanical perturbation. Statistically significant difference between PP2A enzyme activity in cultures that received the loading regime *vs*. control cultures is marked by asterisk (*$P < 0.05$). (D) Metachromatic cartilage areas in 6-day-old cultures were visualized with DMMB. Original magnification was 4×. Scale bar, 500 µm. Optical density (OD_{625}) was determined in samples after TB extraction. Data are expressed as mean ± SEM. Asterisks (*) represent significant differences as compared to control cultures (*$P < 0.05$). (E) Effects of OA-treatments with or without mechanical stimulation on cellular proliferation rate in HDC on culturing day 3. Statistically significant differences between proliferation rate of cells in cultures that were treated with OA and/or received the loading regime *vs*. control cultures are marked by asterisks (*$P < 0.05$). For panels (A) and (B), numbers below signals represent integrated densities determined by ImageJ freeware. (C, control; MS, mechanical stimulation applied on days 2 and 3 for 30 min; OA, treatment with 20 nM OA on days 2 and 3 for 4 h.)

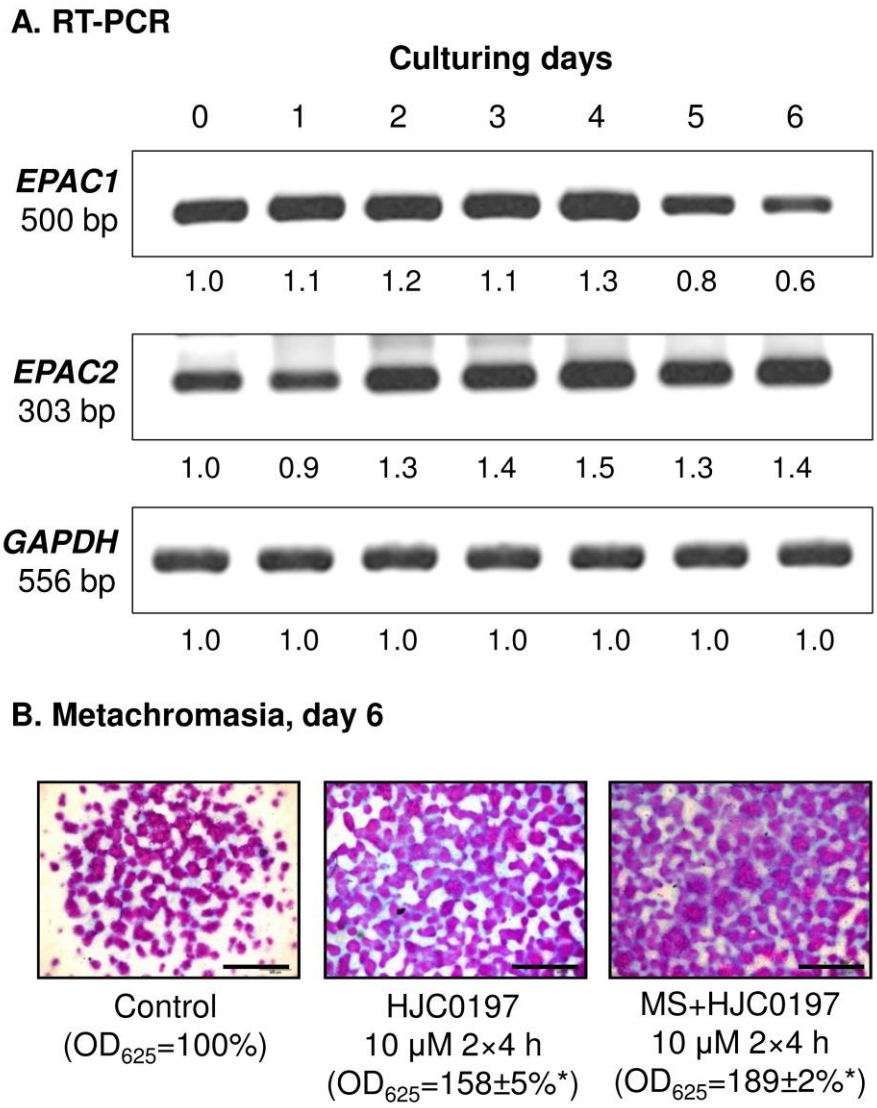

Fig. 5. EPAC1 and EPAC2 mRNA expression in chondrogenic cultures and the effects of the Epac-inhibitor HJC0197 on cartilage matrix production. (A) mRNA expression of EPAC1 and EPAC2 in HDC on various days of culturing. *GAPDH* was used as a control. (B) Metachromatic cartilage areas in 6-day-old cultures were visualized with DMMB. Original magnification was 4×. Scale bar, 500 µm. Optical density (OD_{625}) was determined in samples after TB extraction. Data are expressed as mean ± SEM. Asterisk (*) represents significant difference as compared to control cultures (*$P < 0.05$).

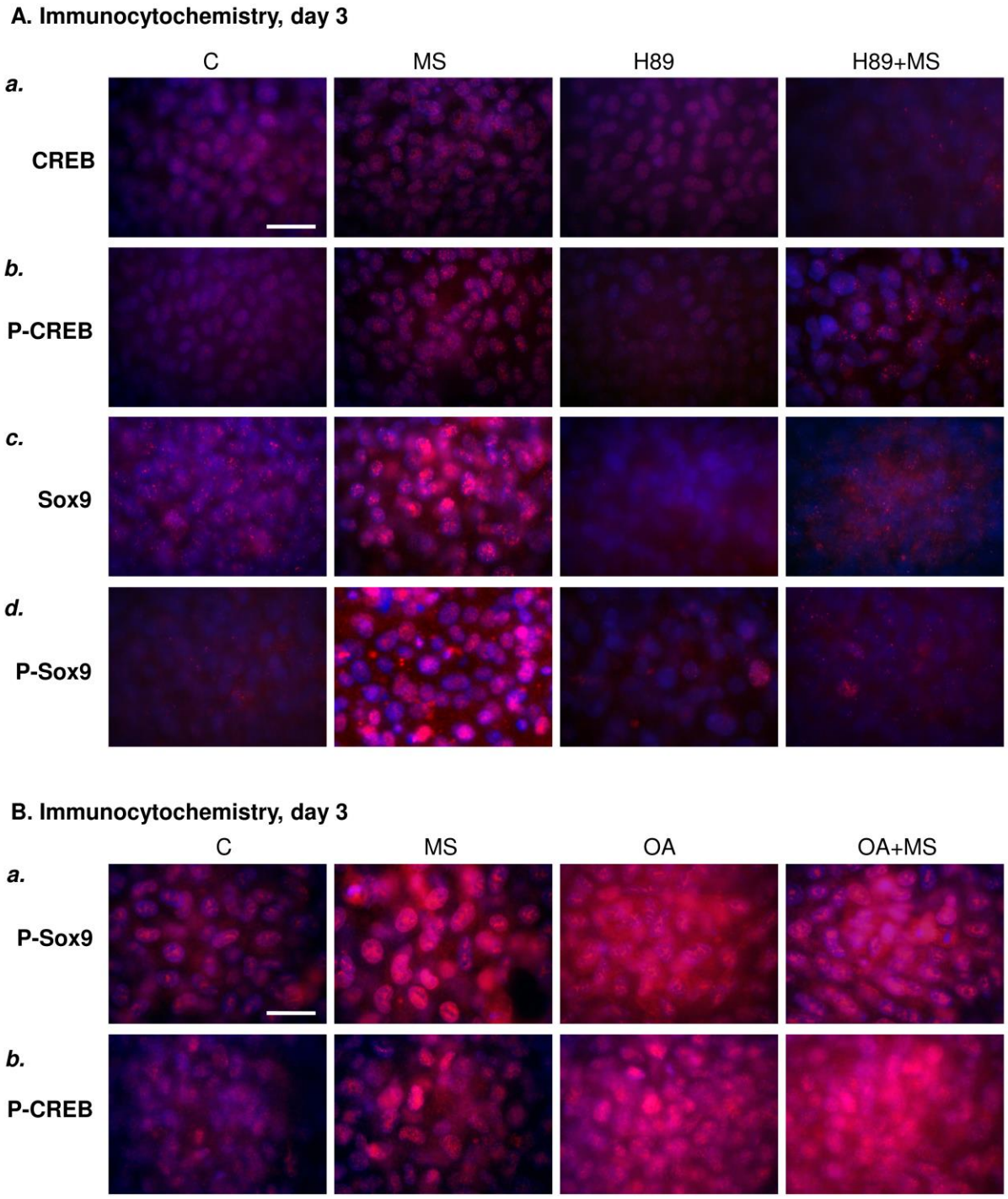

Fig. 6. Intracellular localisation of CREB, P-CREB, Sox9 and P-Sox9, in response to mechanical load, with or without pre-treatment with the PKA-inhibitor H89 or the PP2A-inhibitor OA. (A) Intracellular distribution of (a) CREB, (b) P-CREB, (c) Sox9 and (d) P-Sox9 in control conditions and following treatments (mechanical load and/or 20 μM H89) detected by immunocytochemistry in 3-day-old cultures. (B) Intracellular distribution of (a) P-CREB and (b) P-Sox9 in control conditions and following treatments (mechanical

stimulation and/or 20 nM OA) detected by immunocytochemistry in 3-day-old cultures. Primary antibodies were visualised using Alexa555-conjugated anti-rabbit secondary antibodies. Nuclear DNA was stained with DAPI. Data shown are representative out of 3 independent experiments. Scale bar, 50 μm. (C, control; MS, mechanical stimulation applied on days 2 and 3 for 30 min; H89, treatment with H89 on days 2 and 3 for 4 h; OA, treatment with OA on days 2 and 3 for 4 h.)

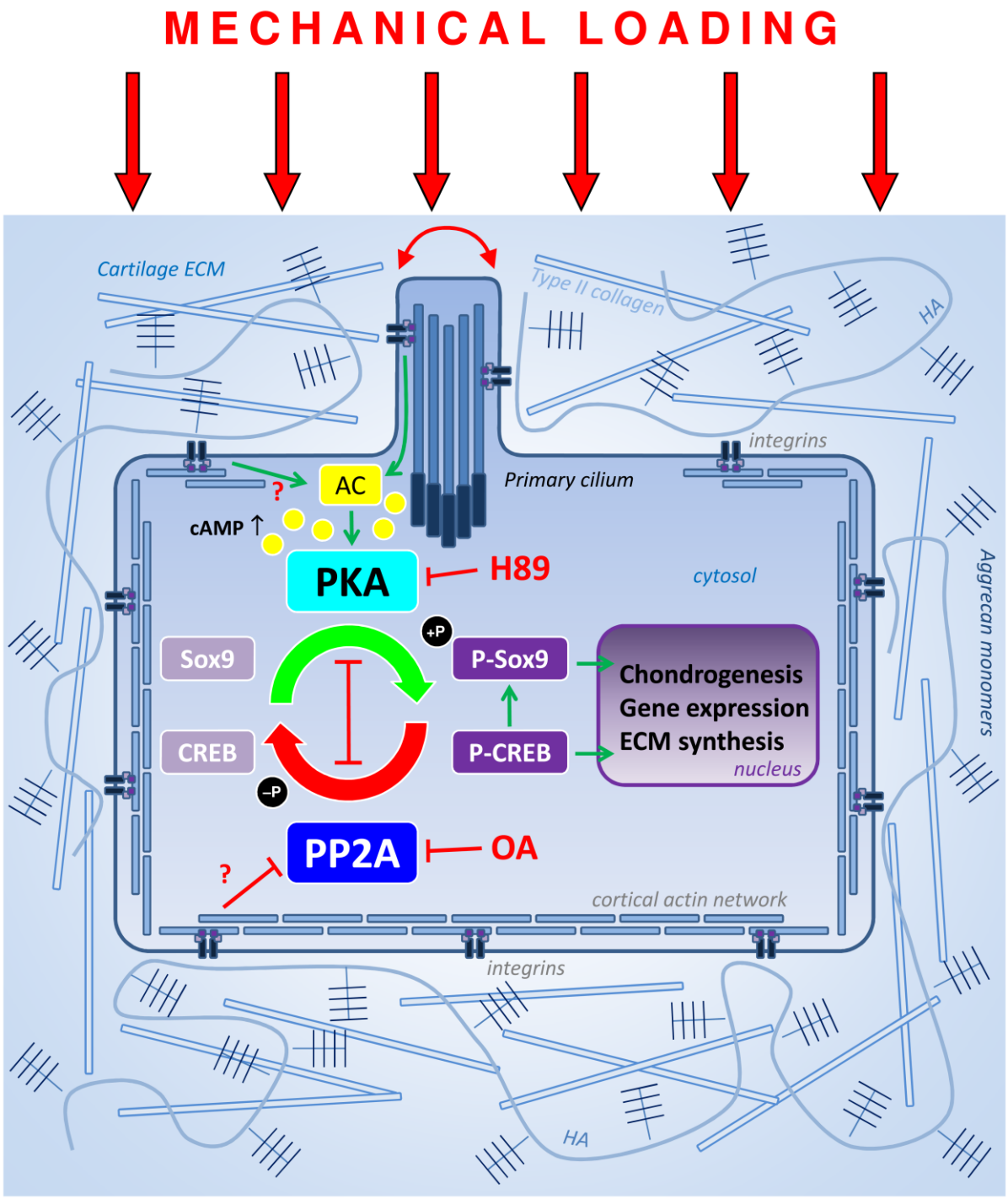

Fig. 7. Proposed hypothesis to explain mechanotransduction pathways *via* PKA/CREB–Sox9 and PP2A in differentiating chicken embryonic chondroprogenitor cells.

Mechanical loading affects the cells both directly and indirectly, by exerting pressure on the developing ECM that is comprised of type II collagen, hyaluronic acid (HA) and aggrecan. Conformational changes in ECM components and the cytoskeleton are sensed by

mechanoreceptor complexes likely containing integrins throughout the plasma membrane and also on putative primary cilia. Mechanical signals are then transduced into chemical signals by activating the adenylate cyclase (AC), which leads to the accumulation of cAMP (please see [1, 2, 45, 46]) that activates protein kinase A (PKA). Increased PKA activity results in enhanced Sox9 and CREB phosphorylation and nuclear translocation; these in turn facilitate chondrogenic differentiation and ECM matrix production. These effects could be abolished by the PKA-inhibitor H89. At the same time, PP2A activity is decreased. Since this phosphatase is a negative regulator of chondrogenesis and balances the effects of PKA on many common targets, reduced PP2A activity further enhances the chondrogenesis-promoting effects of the mechanical stimulation-induced activation of the PKA pathway. The effects of reduced PP2A activity following mechanical loading could be mimicked by the application of OA. See text for further details. Green arrows represent activation, whereas red connections mean inhibition. Hypothetic links between signalling components are labelled by question marks.