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ΤΜΗΜΑ ΟΔΟΝΤΙΑΤΡΙΚΗΣ

ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ

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EFFECT OF CONTINUOUS HYDROSTATIC PRESSURE ON CHONDROCYTE DIFFERENTIATION

Κωνσταντίνος Γ. Καραμεσίνης

ΑΘΗΝΑ 2016

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Επιβλέπων Καθηγητής για την εκπόνηση της Μεταπτυχιακής Διπλωματικής Εργασίας: Επίκουρος Καθηγητής κ. Ηλίας Μπιτσάνης

Τριμελής Επιτροπή για την αξιολόγηση της Μεταπτυχιακής Διπλωματικής Εργασίας:

1. Επίκουρος Καθηγητής κ. Ηλίας Μπιτσάνης

2. Επίκουρη Καθηγήτρια κ. Ελένη Βασταρδή

3. Αναπληρώτρια Καθηγήτρια κ. Ευθυμία Μπάσδρα

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καθοδήγησή της , την υποστήριξη και συμβολή της στην επίλυση των προβλημάτων που ανέκυπταν κατά τη διάρκεια της διπλωματικής μου εργασίας.

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Ευχαριστώ επίσης το Ίδρυμα Κρατικών Υποτροφιών διότι η ολοκλήρωση της εργασίας αυτής έγινε στα πλαίσια της υλοποίησης του μεταπτυχιακού προγράμματος το οποίο συγχρηματοδοτήθηκε μέσω της Πράξης «Πρόγραμμα χορήγησης υποτροφιών ΙΚΥ με διαδικασία εξατομικευμένης αξιολόγησης ακαδ. έτους 2012-2013» από πόρους του Ε.Π. «Εκπαίδευση και Δια Βίου Μάθηση» του Ευρωπαϊκού Κοινωνικού Ταμείου (ΕΚΤ) και του ΕΣΠΑ (2007-2013).

Από τις ευχαριστίες μου δεν θα μπορούσαν να απουσιάζουν οι συμφοιτητές και αγαπημένοι μου φίλοι, *Αλίνα,* Λύδια και Αλέξης. Τους ευχαριστώ ειλικρινά για την ανιδιοτελή βοήθεια και υποστήριξη κατά τη διάρκεια αυτών των ετών, που ήταν καθοριστικές για να ξεπεραστούν ακόμα και οι πιο δύσκολες περιστάσεις. Η παρέα και φιλία μας έκαναν ιδιαίτερα όμορφη τη διαδρομή των τελευταίων τεσσάρων ετών. Κλείνοντας, θα ήθελα να ευχαριστήσω την οικογένειά μου και τους φίλους μου που είναι δίπλα μου και με έχουν στηρίξει στη μέχρι τώρα πορεία μου.

Contents

Περίληψη

Παρά το γεγονός ότι οι μηχανικές δυνάμεις επάγουν τη χονδρογένεση και επηρεάζουν τη διαφοροποίηση και την ωρίμανση των χονδροκυττάρων *in vitro*, η αλληλουχία των βιοχημικών γεγονότων που ακολουθεί την άσκηση συνεχών δυνάμεων στα χονδροκύτταρα παραμένει αδιευκρίνιστη.

Σκοπός: Να διερευνηθεί η μακροπρόθεσμη επίδραση της υδροστατικής πίεσης στη διαφοροποίηση των χονδροκυττάρων, όπως αντικατοπτρίζεται στα πρωτεϊνικά επίπεδα των μεταγραφικών παραγόντων SOX9 και RUNX2. Επίσης, να μελετηθεί η μεταγραφική δραστηριότητα του SOX9, προσδιοριζόμενη από τα επίπεδα φωσφορυλίωσής του (pSOX9).

Υλικά και Μέθοδος: 14,7kPa συνεχούς υδροστατικής πίεσης ασκήθηκαν σε ATDC5 χονδροκύτταρα για 12, 24, 48 και 96 ώρες υπό συνθήκες καλλιέργειας σε απλό θρεπτικό υλικό ή σε θρεπτικό υλικό διαφοροποίησης που περιείχε ινσουλίνη (ITS). Καλλιέργειες ATDC5 κυττάρων σε ITS θρεπτικό υλικό για τα ίδια χρονικά διαστήματα αποτέλεσαν ομάδες ελέγχου. Σε κάθε χρονικό σημείο του εκάστοτε πειράματος λήφθηκαν πρωτεϊνικά εκχυλίσματα και ακολούθησε ανοσοαποτύπωση κατά Western για την αξιολόγηση των επιπέδων των πρωτεϊνών SOX9, pSOX9 και RUNX2.

Αποτελέσματα: Παρατηρήθηκε μείωση στα επίπεδα των πρωτεϊνών SOX9 και pSOX9 μετά από άσκηση υδροστατικής πίεσης στα ATDC5 κύτταρα για χρονικό διάστημα 12 ωρών. Ακολούθως, από το χρονικό σημείο των 24 ωρών και έπειτα, τα πρωτεϊνικά τους επίπεδα σημείωσαν αύξηση. Αντίστροφο πρότυπο παρατηρήθηκε

για το μεταγραφικό παράγοντα RUNX2, η έκφραση του οποίου έφτασε στο μέγιστο επίπεδο μετά από 24 ώρες πίεσης και καλλιέργειας σε ITS θρεπτικό υλικό. Τα χρονικά σημεία των 12 και 24 ωρών θεωρούνται κρίσιμα για τη μετάβαση των χονδροκυττάρων προς τα όψιμα στάδια διαφοροποίησης κατά την άσκηση υδροστατικής πίεσης σε συνθήκες καλλιέργειας με απλό θρεπτικό υλικό και με ITS θρεπτικό υλικό, αντίστοιχα.

Συμπεράσματα: Τα αποτελέσματα της έρευνας αυτής παρέχουν ενδείξεις ότι η συνεχής υδροστατική πίεση επάγει τη διαφοροποίηση των χονδροκυττάρων μέσω μιας σειράς γεγονότων που σε μοριακό επίπεδο περιλαμβάνουν μεταβολές στους μεταγραφικούς παράγοντες SOX9 και RUNX2. Επίσης, προτείνεται ένα θεωρητικό υπόβαθρο για το μηχανισμό δράσης των λειτουργικών μηχανημάτων στην κλινική πράξη.

Λέξεις – Κλειδιά: χονδροκύτταρα, διαφοροποίηση, υδροστατική πίεση, SOX9

Summary

Background/Objective: Mechanical forces influence differentiation and maturation of chondrocytes *in vitro*. However, the series of biochemical events following continuous force application to chondrocytes remain largely unexplored. The aim of the present study was to investigate the long-term effects of hydrostatic pressure (HP) on chondrocyte differentiation, as indicated by protein levels of the chondrocyte differentiation markers SOX9 and RUNX2 transcription factors, as well as on the transcriptional activity of SOX9, as determined by pSOX9 levels.

Materials and Methods: ATDC5 cells were exposed to 14.7kPa of continuous HP for 12, 24, 48 and 96 hours at the presence of maintenance medium or insulin-supplemented differentiation medium (ITS). ATDC5 cells cultured in ITS medium for the same time-points served as controls. Cell extracts for each time-point and experimental condition were assessed for SOX9, pSOX9 and RUNX2 protein levels using Western immunoblotting analysis.

Results: Exposure of ADTC5 cells to HP resulted in an early drop in SOX9 and pSOX9 protein levels at 12 hours followed by an increase at 24 hours onwards. A reverse pattern relatively to SOX9 was observed for RUNX2 protein, which reached peak levels at 24 hours of HP treatment of chondrocytes in ITS culture. Twelve and 24 hours of HP treatment were revealed as critical time-points for the transition of chondrocytes towards late differentiation events.

Conclusions: Our data indicate that long periods of continuous hydrostatic pressure stimulate chondrocyte differentiation through a series of molecular events involving the induction of SOX9 and RUNX2 and provide a theoretical background for oro-facial functional orthopaedics.

Key-words: chondrocytes, differentiation, hydrostatic pressure, SOX9

Introduction

Excessive research has been conducted on the physiology and mechano-biology of mandibular condyle, underlining its significant role during growth and development of the mandible. Mandibular condylar cartilage is an important growth site of the mandible where endochondral ossification takes place in response to environmental and extrinsic factors (Meikle, 1973; Moss and Rankow, 1968). Animal and tissue experiments indicate that alterations in loading conditions on mandibular condyle may influence proliferation and differentiation processes in chondrocytes thus leading to modification of condylar growth and allowing treatment interventions in various dento-facial discrepancies (de Sa et al., 2013; Chen et al., 2009; Kantomaa and Pirttiniemi, 1996; Kantomaa et al., 1994).

Mechano-transduction constitutes the biological mechanism through which chondrocytes respond to mechanical stimuli. Specifically, forces exerted on chondrocytes stimulate mechano-sensitive structures, including integrins (Marques et al., 2008; Zhang et al., 2008) and G proteins (Zhang et al., 2008) and result in specific intra-cellular molecular signals (Papachristou et al., 2009; Wong and Carter, 2003). Upon activation of these signalling pathways, transcription factors and other molecules which regulate the cell cycle and determine the differentiation status of chondrocytes are either silenced or up-regulated, depending on the nature of mechanical stimuli (Papachristou et al., 2009; Ramage et al., 2009; Papachristou et al., 2006; Rabie et al., 2003).

In vitro experiments on condylar chondrocyte cultures reported that hydrostatic pressure (HP) stimulates chondrogenesis events. Specifically, there are indications that chondrocytes are rapidly directed into differentiation and subsequently maturation, which in turn possibly leads to a down-regulation of proliferation and growth (Basdra et al., 1994; Takano-Yamamoto et al., 1991).

SOX9 belongs to a large family of transcription factors (Wright et al., 1993) and it has been proved to be a key-regulator of the initial stages in the chondrogenesis pathway and chondrocyte differentiation (Akiyama et al., 2002; Lefebvre et al., 2001). Its essential role has also been demonstrated during the embryonic development and postnatal growth of mandibular condylar cartilage (Shibata et al., 2006; Rabie and Hagg, 2002). *Sox9* is expressed in the resting and proliferating cartilaginous zones by pre-chondroblasts and chondroblasts (Shibata et al., 2006; Lefebvre and Smits, 2005; Rabie and Hagg, 2002). Moreover, SOX9 prevents chondrocytes from entering the maturation state and preserves them in the proliferative stage (Amano et al., 2009; Saito et al., 2007).

In vitro experiments in chondrocytes have revealed that not only do mechanical forces affect SOX9 protein levels but also regulate its phosphorylation and subsequent activation. Upon phosphorylation, phosphorylated/activated SOX9 (pSOX9) translocates into the nucleus and activates the transcription of its target genes (Haudenschild et al., 2010; Huang et al., 2000).

Furthermore, unequivocal evidence support that RUNX2 is a downstream target of mechano-transduction pathways and its transcriptional activity has been

found to be modulated by forces exerted in bone and cartilage (Ziros et al., 2008; Franceshi and Xiao, 2003). It is considered as a bone-specific transcription factor and is required for normal skeletal development and intra-membranous bone growth (Ducy et al., 1997; Otto et al., 1997). Additionally, *Runx2* is essential for cartilage development and mediates significant events during the late stage of chondrogenesis (Inada et al., 1999; Kim et al., 1999). Particularly, *Runx2* is expressed by terminally differentiated chondrocytes in the hypertrophic cartilaginous zone and is required for chondrocyte maturation (Enomoto-Iwamoto et al., 2001; Takeda et al., 2001; Enomoto et al., 2000).

RUNX2 is detected in a similar spatial pattern and exerts the aforementioned functions in mandibular condylar cartilage as well (Rath-Deschner et al., 2010; Rabie et al., 2004). In a recent study, changes in RUNX2 protein levels have been detected upon exertion of HP for a short time-course on condylar chondrocytes, further supporting that RUNX2 is a target of mechanical signals in mandibular condyle (Huang et al., 2015).

The purpose of the present study was to investigate the long-term effects of continuous hydrostatic pressure on SOX9 expression and activation as well as on RUNX2 expression in ATDC5 chondrogenic cells.

Materials and Methods

ATDC5 cell line and culture conditions

The chondrogenic cell line ATDC5 was purchased by Sigma-Aldrich (Sigma-Aldrich, Germany). The cells were cultured in DMEM - F12 containing L-Glutamine and Hepes (#LM-D1223, Biosera, Europe) supplemented with 5% fetal bovine serum-FBS (Gibco, ThermoFisher Scientific, Germany) and 1% Penicillin-Streptomycin mixture (10000U/ml of penicillin and 10000μg/ml of streptomycin, Gibco, ThermoFisher Scientific, Germany).

For the experiments including culture of ATDC5 cells in ITS differentiation medium (ITS experiment and HP plus ITS experiment), ITS Liquid Media Supplement (#I3146, Sigma-Aldrich, Germany) was added to the standard growth medium as previously described (Tare et al., 2005). Final concentrations in the medium were: bovine insulin 10mg/L, human transferrin 5.5mg/L, and sodium selenite 5μg/L. The differentiation medium was changed with fresh every two days. ATDC5 cell cultures were maintained at 37°C in a humidified atmosphere containing 5%CO_2-95% air.

Hydrostatic pressure apparatus and experimental conditions

Continuous hydrostatic pressure (HP) was delivered to the chondrocyte monolayer through our in-house-designed hydrostatic pressure apparatus (“Continuous Flow Constant Pressure for cell culture” apparatus, manufactured by Inspiration Technology Innovation, ITI, Athens, Greece, Figure 1A). The principles of the pressure system implemented in the present study have already been described and utilized by our research group and others for the study of the effects of HP upon

chondrocytes (Basdra et al., 1994, Takano-Yamamoto et al., 1991). The apparatus consists of the hydrostatic pressure delivery system and the heat-sealed chamber (made of acrylic glass, Plexiglas) in which the petri dishes are placed. This chamber is positioned inside the incubator in order to maintain the conditions of the chamber stable (37°C in a humidified atmosphere containing 5%CO_2-95% air). The hydrostatic pressure delivery system controls the HP at the bottom of the petri dishes in the chamber through strict regulation of the infusing and outgoing premixed gas flow (Figure 1B). This system is capable of applying continuous HP to the bottom of the petri dish of magnitudes ranging from approximately 4.9kPa (50g/cm^2) to approximately 14.7kPa (150g/cm^2).

ATDC5 cells were plated in 100mm petri dishes at a density of 2.2×10^6 cells per dish. Passages p7-p10 were used for all experiments. When cells reached about 70% confluency, petri dishes were placed into the hydrostatic pressure apparatus (HP experiment and HP plus ITS experiment). Following application of continuous HP at a magnitude of 14.7kPa, cells were harvested immediately. For ITS experiment, when cells reached 70% confluency, growth medium was changed to ITS differentiation medium (changed every two days). For HP plus ITS experiment, when cells reached the desired confluency, medium was changed to ITS differentiation medium and 14.7kPa of HP was applied. For each time-point, cells from 3 petri dishes were collected. Samples collected for HP experiment were 12 hours (12h) untreated, 12h HP, 24h HP, 48h HP and 96h HP; for ITS experiment were 12 hours (12h) untreated, 12h ITS, 24h ITS, 48h ITS and 96h ITS and for HP plus ITS experiment were 12 hours (12h) control (ITS), 12h HP plus ITS, 24h HP plus ITS, 48h HP plus ITS and 96h HP plus ITS.

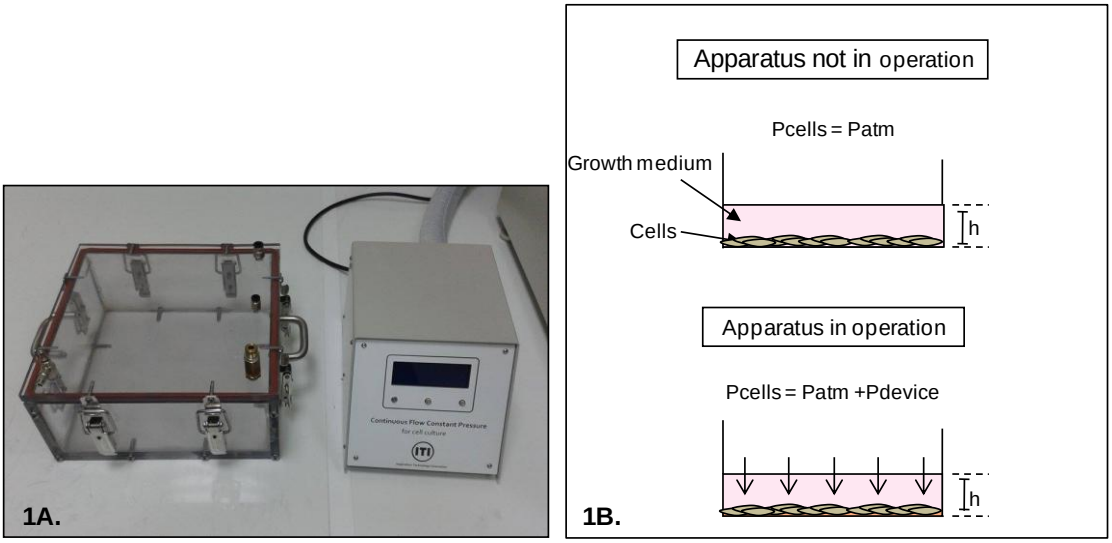

Figure 1. Illustration of the "Continuous Flow Constant Pressure" hydrostatic pressure system

A) On the left, the heat-sealed chamber in which the ATDC5 petri dishes are placed is depicted. Temperature and pH levels are maintained stable by placing the chamber inside a standard incubator. On the right, the hydrostatic pressure delivery system is shown. **B)** The hydrostatic pressure (HP) at the bottom of the petri dish, where cells are attached, equals to $P_{cells}= P_{atm}+P_{device}+P_{liquid}$; P_{atm}: Atmospheric pressure (1atm=1bar=1000mbar=1019.7g/cm2=101kPa); P_{device}: Pressure applied by the apparatus by compression of the gas phase; P_{liquid}: The pressure conferred to the cells because of the liquid (growth medium). Since the growth medium has a density of about $\rho=1000kg/m^3$ and the cells are attached in a depth of h=1 mm=10^{-3}m, then $P_{liquid}=\rho* g* h=1000kg/m^3 * 9.81m/s^2 * 10^{-3}m=0.1g/cm2=10Pa$. Thus, P_{liquid} is very close to zero and $P_{cells}= P_{atm}+P_{device}$

Western Blot analysis

Protein extraction from ATDC5 cells was performed using ice-cold Cell Lysis Buffer (#9803, Cell Signaling technology, USA). The concentration of protein extracts was assessed using the Bradford assay (#5000205, Bio-Rad, USA). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred into a nitrocellulose membrane (Porablot NCP, Macherey-Nagel, Germany). Membranes were blocked for 1 hour in room temperature in Phosphate Buffered Saline Tween-20 (PBST) containing 5% non-fat milk and incubated overnight at 4°C with the primary antibodies: anti-SOX9 (#AB5535, Merck-Millipore, Germany, 1:1000), anti-phospho-SOX9 (pSer191, #SAB4503991, Sigma-Aldrich, Germany, 1:500), anti-RUNX2 (#sc-10758, Santa Cruz Biotechnology, USA, 1:750) or anti-β-ACTIN (#MAB-1501, Merck-Millipore, Germany, 1:10000). The membranes were then incubated with the HRP- conjugated secondary antibodies for 1 hour in room temperature. Goat anti-rabbit IgG-HRP (#sc-2004, Santa Cruz Biotechnology, USA, 1:2000) was used against SOX9, phospho-SOX9 and RUNX2 whereas goat anti-mouse IgG-HRP (#sc-2005, Santa Cruz Biotechnology, USA, 1:2000) was used against β-ACTIN antibody. The detection of the immune-reactive bands was performed with the ECL Western Blotting Substrate (#32106, ThermoFisher Scientific, Germany). Relative protein amounts were evaluated using Image J software (Abramoff et al., 2004) and normalized to β-ACTIN levels. All experiments were performed 3 times and representative results of one experiment are shown.

Statistical analysis

Statistical analysis of the data was conducted with IBM SPSS Statistics for Windows, version 22.0 (IBM corp., USA). One-way analysis of variance (ANOVA), followed by Bonferroni tests were applied in order to perform multiple comparisons among the results derived from each experiment. Values of p lower than 0.05 were considered as statistical significant. The quantified data are presented at the graphs as mean value ± SD.

Results

Evaluation of SOX9 expression and activation patterns in ATDC5 cells cultured in ITS differentiation medium

Evaluation of SOX9 and phosphorylated (p)/active SOX9 protein levels in ATDC5 cells cultured in ITS medium for 12, 24, 48 and 96 hours was performed by Western immunoblotting (Figure 2A) followed by densitometric analysis (Figure 2B). A significant decrease in SOX9 protein levels upon culture of ATDC5 cells in ITS differentiation medium for 24 hours was observed in comparison to cells cultured in maintenance medium ($p<0.05$). When ATDC5 cells were cultured in ITS medium for 48 hours, SOX9 significantly increased relatively to the 24-hour time-point ($p<0.01$) and returned back to the levels of untreated cells. A significant drop in SOX9 was detected again at 96 hours of ITS culture relatively to the 48-hour time-point ($p<0.01$).

Regarding SOX9 activation, a significant increase was detected in the levels of pSOX9 upon 12 hours of culture in ITS medium, which was followed by a significant

decrease at 24 hours ($p<0.01$ and $p<0.001$, respectively). At 48 hours, pSOX9 protein levels increased significantly to a maximum peak ($p<0.001$). At 96 hours, pSOX9 decreased in relation to 48 hours of ITS culture ($p<0.001$), but remained elevated when compared to untreated cells ($p<0.001$) (Figure 2A, 2C).

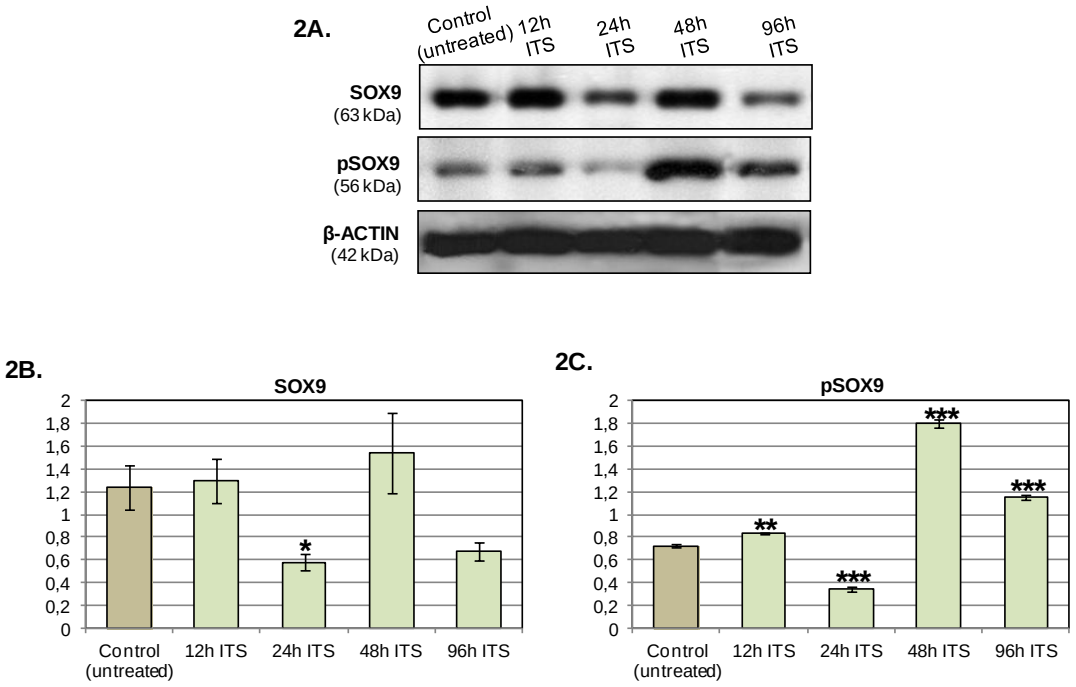

Figure 2. Effects of ITS treatment on SOX9 and pSOX9 levels in ATDC5 cells

A) SOX9 and pSOX9 protein levels following ITS treatment for the indicated time-points. **B)** SOX9 protein quantification levels following ITS treatment. Mean values of 3 different experiments are presented. Statistical significance was evaluated compared to control protein levels. **C)** pSOX9 protein quantification levels following ITS treatment. Mean values of 3 different experiments are presented. Significance levels were evaluated compared to control protein levels

*denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$

Effects of hydrostatic pressure on SOX9 expression and activation in ATDC5 cells

The effects of hydrostatic pressure on SOX9 and pSOX9 protein levels in ATCD5 cells for 12, 24, 48 and 96 hours were determined by Western immunoblotting (Figure 3A) followed by densitometric analysis (Figure 3B). A significant decrease in SOX9 protein levels was observed in ATDC5 cells upon exertion of 14.7kPa of HP for 12 hours in comparison to untreated cells ($p<0.001$). At 24 hours of HP expression levels of SOX9 significantly increased and remained elevated at 48 and 96 hours as well ($p<0.001$, respectively). At 96 hours SOX9 levels were reduced relatively to the 48-hour time-point, although they still remained increased in comparison to untreated cells ($p<0.01$ and $p<0.001$, respectively).

The exertion of HP for 12 hours caused a significant drop in pSOX9 ($p<0.001$), which was followed by a significant increase from 24 hours onwards ($p<0.001$, respectively) (Figure 3A, 3C).

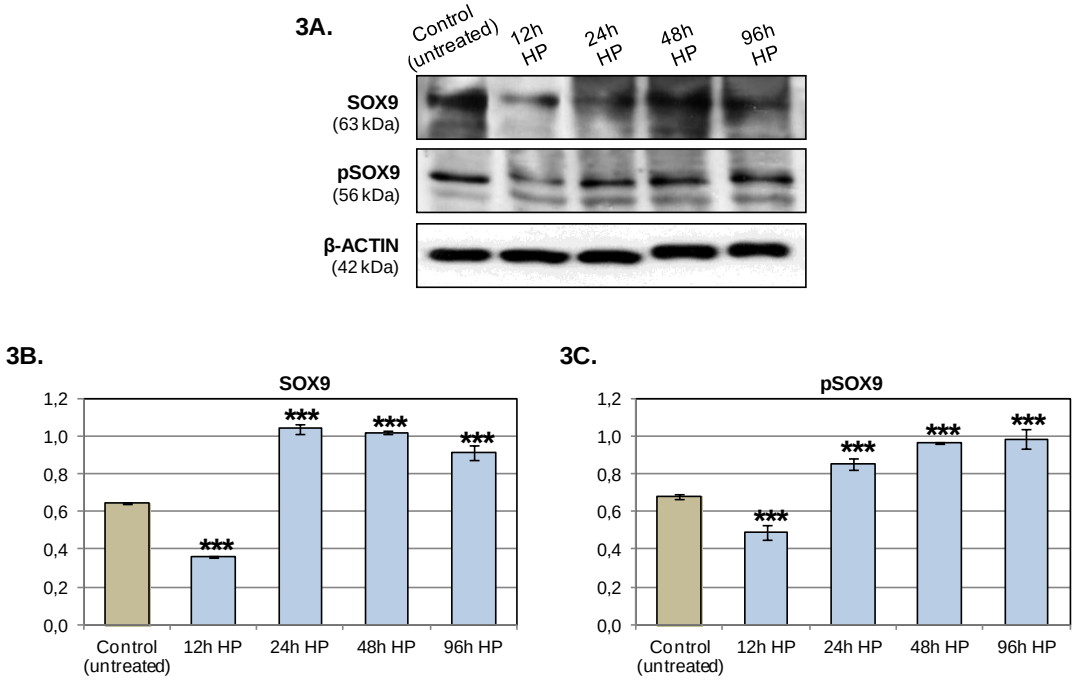

Figure 3. Effects of HP treatment on SOX9 and pSOX9 levels in ATDC5 cells

A) SOX9 and pSOX9 protein levels following HP application of approximately 14.7kPa (150g/cm^2) for the indicated time-points. **B)** SOX9 protein quantification levels following HP application. Mean values of 3 different experiments are presented. Statistical significance was evaluated compared to control protein levels. **C)** pSOX9 protein quantification levels following HP application. Mean values of 3 different experiments are presented. Statistical significance was evaluated compared to control protein levels

*denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$

Effects of hydrostatic pressure on SOX9 expression and activation in ATDC5 cells cultured in ITS medium

The effects of hydrostatic pressure on SOX9 and pSOX9 protein levels in ATCD5 cells cultured in ITS medium for 12, 24, 48 and 96 hours were determined by Western immunoblotting (Figure 4A) followed by densitometric analysis (Figure 4B). A significant increase in SOX9 was observed upon exertion of 14.7kPa of HP for 12 hours in ATDC5 cells cultured in ITS differentiation medium in comparison to control (ITS) cells ($p<0.01$). At 24 hours, the combination of HP and ITS treatments caused a significant drop in SOX9 protein ($p<0.001$). At 48 hours, it began to increase in comparison to 24 hours ($p<0.01$), but still remained at a lower level relatively to the control cells ($p<0.001$), until 96 hours, when it returned to control levels.

The protein levels of pSOX9 exhibited a similar pattern throughout the time-points (Figure 4A, 4C). An increase was initially identified, upon 12-hour combined HP and ITS treatment, compared to the control (ITS) cells ($p<0.001$). At the 24-hour time-point, pSOX9 showed a significant drop ($p<0.001$), followed by an elevation at 48 hours and a maximum peak at 96 hours ($p<0.001$, respectively).

Effects of hydrostatic pressure on RUNX2 expression in ATDC5 cells cultured in ITS medium

Since combined HP and ITS treatment exhibited significant effects on SOX9 and pSOX9 levels we proceeded to investigate any possible changes in the levels of RUNX2 protein by western immunoblotting. A decrease was noted upon exertion of HP for 12 hours in ATDC5 cells cultured in ITS medium, compared to controls (ITS) ($p<0.001$). At 24 hours, RUNX2 expression was increased to a maximum level ($p<0.001$). Thereafter, at 48 and 96 hours a decrease in RUNX2 levels was detected relatively to the 24-hour peak ($p<0.001$). However, at 48 hours RUNX2 was still elevated in comparison to control cells, whereas at 96 hours its expression reached to a level lower than the controls ($p<0.001$, respectively) (Figure 4D).

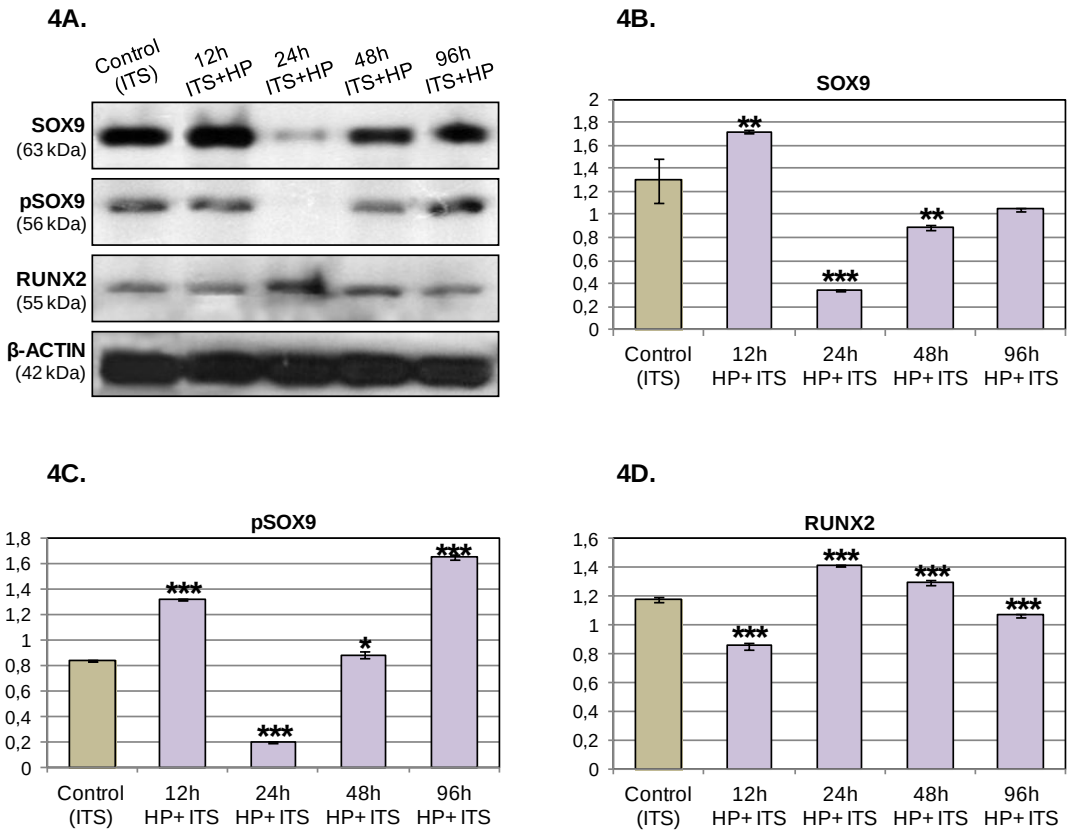

Figure 4. Effects of combined HP and ITS treatment on SOX9, pSOX9 and RUNX2 levels in ATDC5 cells

A) SOX9, pSOX9 and RUNX2 protein levels following HP (14.7kPa) plus ITS treatment for the indicated time-points. **B)** SOX9 protein quantification levels following HP plus ITS treatment. Mean values of 3 different experiments are presented. Statistical significance was evaluated compared to control protein levels. **C)** pSOX9 protein quantification levels following HP plus ITS treatment. Mean values of 3 different experiments are presented. Statistical significance was evaluated compared to control protein levels. **D)** RUNX2 protein quantification levels following HP plus ITS treatment. Mean values of 3 different experiments are presented. Statistical significance was evaluated compared to control protein levels

*denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$

Discussion

Mechanical stimuli have been well recognized as important modulators of homeostasis and development of cartilage (O'Conor et al., 2013). Furthermore, aberrations in mechano-transduction pathways have been implicated in the pathogenesis of cartilaginous diseases, malignancies (Spyropoulou et al., 2015; Papachristou et al., 2008) and cartilage degeneration as well (Papachristou et al., 2008). Previous studies have focused on short-term effects of continuous hydrostatic pressure (HP) on metabolic activity (Chen et al., 2007) and differentiation status of chondrocytes (Huang et al., 2015; Basdra et al., 1994). In the present study we investigated the long-term effects of HP on chondrocyte differentiation, as indicated by protein levels of SOX9 and RUNX2 transcription factors as well as on the transcriptional activity of SOX9, as determined by pSOX9 levels.

The pressure system used in our study resembles the *in vivo* situation of mandibular condylar chondrocytes under hydrostatic loading application (Zhang et al., 2006) and is considered an accurate and reliable method to transmit pressure upon cells (Yousefian et al., 1995; Basdra et al., 1994; Takano-Yamamoto et al., 1991). The magnitude of applied HP of 14.7kPa was selected based on previous data (Basdra et al., 1994; Takano-Yamamoto et al., 1991) so that it would be well above physiologically and well below pathologically exerted HP (Chen et al., 2007).

In the present study we utilized ATDC5 chondrogenic cell line (Atsumi et al., 1990) which has been proposed as an appropriate model for *in vitro* study of chondrogenesis and mechano-transduction signalling (Yao and Wang, 2013). Primary

chondrocyte cultures are very difficult to handle because they terminally differentiate under culture conditions (Engel et al., 1990) and it is not feasible to passage them. Especially for mandibular condylar chondrocyte cultures, the condylar cartilage tissue isolated from rats or mice is very small and a considerable number of experimental animals are required in order to establish a culture, not to mention the diverse population of cells within the culture presenting with different stages of differentiation/maturation (Weiss et al., 1986). ATDC5 cells are characterized by homogeneity, remain in a proliferative undifferentiated stage and preserve the chondroprogenitor phenotype when they are cultured in a maintenance medium (Shukunami et al., 1996). Most importantly, when ATDC5 cells are cultured in a medium supplemented with insulin, they are able to go through all the multiple stages of differentiation and maturation (Shukunami et al., 1997; Shukunami et al., 1996).

Therefore, we initially detected the protein levels of SOX9 and pSOX9 in ATDC5 cells cultured in ITS differentiation medium (ITS experiment). Contrary to previous studies (Temu et al., 2010; Altaf et al., 2006; Shukunami et al., 1997), which aimed to establish chemically-induced differentiation regimens for chondrocytes, we investigated the effects of ITS culture throughout a short time-course in order to identify the expression and activation pattern of SOX9 among time-points that corresponded to HP experiments.

The determination of SOX9 protein levels, allowed us to evaluate the differentiation status of ATDC5 cells. In ITS experiment, SOX9 expression presented with a biphasic pattern, with the significant decrease in the 24-hour time-point

indicating a transition towards the late stages of chondrogenesis. Previous studies reported a similar cyclic pattern with fluctuations in *Sox9* mRNA levels in well established chemically-induced differentiation schemes, but throughout longer time periods (Temu et al., 2010; Altaf et al., 2006).

Regarding the effects of HP, a significant decrease in SOX9 protein was detected at the 12-hour time-point followed by a significant increase at 24 hours onwards. In a recent study, where the short-term effects of HP on primary condylar chondrocytes were investigated, an elevation in SOX9 protein was detected upon 3 and 4 hours of pressurization (Huang et al., 2015). In our study, the HP applied on ATDC5 cells was almost 7 times lower, but it was implemented for a much longer time-course indicating that both magnitude and duration of the HP affect proliferation and differentiation of chondrocytes (Ragan et al., 1999). The combination of HP and ITS treatments resulted in a reverse cyclic pattern of SOX9 expression, when compared to HP experiment. The initial increase probably indicated an induction of the early events of differentiation, followed by the fall in SOX9 levels reflecting the initiation of late differentiation events.

SOX9 transcription factor is a downstream molecule of mechano-sensitive signalling cascades, including cAMP/PKA and Rhoa/ROCK pathways. During mechanical stimulation of chondrocytes, these pathways regulate its transcriptional activity via phosphorylation and subsequent nuclear accumulation of pSOX9 (Juhasz et al., 2014; Haudenschild et al., 2010). To this direction, we investigated the protein levels of pSOX9 (pSer181) in order to record SOX9 transcriptional activity. It has been reported that pSOX9 protein levels increased upon 2 hours of dynamic compression

of chondrocytes, while SOX9 protein remained unchanged. However, at 16-18 hours of dynamic compression, SOX9 increased and pSOX9 exhibited an even greater elevation (Haudenschild et al., 2010) indicating that pSOX9 is more readily responsive to mechanical signals than SOX9. Indeed, in ITS experiment pSOX9 protein levels were subjected to greater alterations than SOX9.

The exertion of HP on ATDC5 cells caused a significant decrease in pSOX9 levels at an earlier time-point (12 hours) than in ITS experiment, which coincided with the early decrease observed in SOX9 protein as well. However, the pattern that pSOX9 followed beyond the 12-hour time-point was rather different from SOX9 protein, since a gradual increase was observed. It should be noted that any discrepancies observed between pSOX9 and SOX9 patterns may be attributed to the ability of SOX9 to auto-activate its expression, through an auto-regulatory loop which includes both SOX9 and pSOX9 (Kumar and Lassar, 2009).

Collectively, the early drop detected in SOX9 and pSOX9 upon exertion of HP indicated the induction of late chondrogenic differentiation events at 12 hours. However, the combined experiment evoked changes in SOX9 expression and transcriptional activity which represent both early and late differentiation processes and rendered 24 hours as the critical time-point for the transition towards late differentiation.

We then proceeded to assess RUNX2 protein levels under the combined treatment of HP and ITS on chondrocytes. It has been reported that SOX9 down-regulates RUNX2 and suppresses its activity (Yamashita et al., 2009; Zhou et al.,

2006). Our results are in agreement with the aforementioned studies, since we observed that RUNX2 protein levels followed an inverse pattern relatively to SOX9 protein throughout the experimental time-points. Specifically, at 12 hours a significant drop in RUNX2 coincided with the increase in SOX9 levels. At the 24-hour time-point, when SOX9 fell to a minimum level, RUNX2 levels reached to a peak, confirming that 24 hours was the critical time-point for the initiation of the late events of differentiation.

In consistency with our results, *in vivo* tissue studies which examined the effects of soft and hard diet on mandibular condyle specimens have detected an increase in RUNX2 and a decrease in SOX9 in the condyles that were subjected to increased mastication forces (Papadopoulou et al., 2007; Papachristou et al., 2005). Similar experiments have revealed that soft diet results in a higher proliferative activity in the condylar cartilage, whereas hard diet induces maturation of the condylar chondrocytes (Enomoto et al., 2014).

The results of the present study support the concept behind the applied mechano-therapy in dentofacial orthopaedics, i.e., chin-cup use in the treatment of mandibular prognathism. Based on animal studies, various appliances were designed aiming to modify growth by promoting/accelerating (Tsolakis et al., 1997; McNamara and Carlson, 1979) or restricting (Asano, 1986; Janzen and Bluher, 1965) the growth potential of the mandibular cartilage. Moreover, mandibular advancement promoted SOX9 expression levels (Rabie et al., 2003), while compressive forces restricted growth in mandibular condyle (Teramoto et al., 2003).

To this end, our findings further substantiate the notion that the functional plasticity of condylar cartilage is mainly based on the mechano-responsiveness of chondrocytes, which possess the capacity to respond in terms of differentiation and/or proliferation to various mechanical signals. The exertion of continuous hydrostatic pressure seems to induce late differentiation phenomena and potentially enforces chondrocytes to enter into the maturation state more rapidly, not allowing for much proliferation and finally leading to a smaller condylar cartilage.

Conclusions

Mechanical signals regulate chondrocyte cell cycle and metabolism. In our study we investigated the prolonged effects of continuous hydrostatic pressure on the protein levels of well-established markers of chondrocyte differentiation and maturation. The patterns expressed by these molecules indicate that hydrostatic pressure, either alone or combined with insulin-supplemented medium, stimulates differentiation and induces a transition towards chondrocyte maturation. Further research is required in order to elucidate the exact molecular pathways involved in mechano-regulation of chondrocyte differentiation for future targeted pharmacological approaches.

References

1. Abramoff, M.D., Magalhaes, P.J. and Ram, S.J. (2004) Image Processing with ImageJ. *Biophotonics International*, 11, 36-42.

2. Akiyama, H., Chaboissier, M.C., Martin, J.F., Schedl, A. and de Crombrugghe, B. (2002) The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes and Development*, 16, 2813-2828.

3. Altaf, F.M., Hering, T.M., Kazmi, N.H., Yoo, J.U. and Johnstone, B. (2006) Ascorbate-enhanced chondrogenesis of ATDC5 cells. *European Cells and Materials*, 12, 64-69.

4. Amano, K., Hata, K., Sugita, A., Takiqawa, Y., Ono, K., Wakabayashi, M., Kogo, M., Nishimura, R. and Yoneda, T. (2009) Sox9 family members negatively regulate maturation and calcification of chondrocytes through up-regulation of parathyroid hormone-related protein. *Molecular Biology of the Cell*, 20, 4541-4551.

5. Asano, T. (1986) The effects of mandibular retractive force on the growing rat mandible. *American Journal of Orthodontics and Dentofacial Orthopedics*, 90, 464-474.

6. Atsumi, T., Miwa, Y., Kimata, K. and Ikawa, Y. (1990) A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells. *Cell Differentiation and Development*, 30, 109-116.

7. Basdra, E.K., Huber, L.A., Komposch, G. and Papavassiliou, A.G. (1994) Mechanical loading triggers specific biochemical responses in mandibular condylar chondrocytes. *Biochimica et Biophysica Acta*, 1222, 315-322.

8. Chen, J., Sorensen, K.P., Gupta, T., Kilts, T., Young, M. and Wadhwa, S. (2009) Altered functional loading causes differential effects in the subchondral bone and condylar cartilage in the temporomandibular joint from young mice. *Osteoarthritis and Cartilage*, 17, 354-361.

9. Chen, Y.J., Zhang, M. and Wang, J.J. (2007) Study on the effects of mechanical pressure to the ultrastructure and secretion ability of mandibular condylar chondrocytes. *Archives of Oral Biology*, 52, 173-181.

10. de Sa, M.P., Zanoni, J.N., de Salles, C.L., de Souza, F.D., Suga, U.S. and Terada, R.S. (2013) Morphometric evaluation of condylar cartilage of growing rats in response to mandibular retractive forces. *Dental Press Journal of Orthodontics*, 18, 113-119.

11. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L. and Karsenty, G. (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*, 89, 747-754.

12. Engel, F.E., Khare, A.G. and Boyan, B.D. (1990) Phenotypic changes of rabbit mandibular condylar cartilage cells in culture. *Journal of Dental Research*, 69, 1753-1758.

13. Enomoto, A., Watahiki, J., Nampo, T., Irie, T., Ichikawa, Y., Tachikawa, T. and Maki, K. (2014) Mastication markedly affects mandibular condylar cartilage growth, gene expression, and morphology. *American Journal of Orthodontics and Dentofacial Orthopedics*, 146, 355-363.

14. Enomoto, H., Enomoto-Iwamoto, M., Iwamoto, M., Nomura, S., Himeno, M., Kitamura, Y., Kishimoto, T. and Komori, T. (2000) Cbfa1 is a positive regulatory factor in chondrocyte maturation. *The Journal of Biological Chemistry*, 275, 8695-8702.

15. Enomoto-Iwamoto, M., Enomoto, H., Komori, T. and Iwamoto, M. (2001) Participation of Cbfa1 in regulation of chondrocyte maturation. *Osteoarthritis and Cartilage*, 9 Suppl A, S76-84.

16. Franceschi, R.T. and Xiao, G. (2003) Regulation of the osteoblast-specific transcription factor, Runx2: responsiveness to multiple signal transduction pathways. *Journal of Cellular Biochemistry*, 88, 446-454.

17. Haudenschild, D.R., Chen, J., Pang, N., Lotz, M.K. and D'Lima, D.D. (2010) Rho kinase-dependent activation of SOX9 in chondrocytes. *Arthritis and Rheumatism*, 62, 191-200.

18. Huang, L., Cai, X., Li, H., Xie, Q., Zhang, M. and Yang, C. (2015) The effects of static pressure on chondrogenic and osteogenic differentiation in condylar chondrocytes from temporomandibular joint. *Archives of Oral Biology*, 60, 622-630.

19. Huang, W., Zhou, X., Lefebvre, V. and de Crombrugghe, B. (2000) Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer. *Molecular and Cellular Biology*, 20, 4149-4158.

20. Inada, M., *et al*. (1999) Maturational disturbance of chondrocytes in Cbfa1-deficient mice. *Developmental Dynamics*, 214, 279-290.

21. Janzen, E.K. and Bluher, J.A. (1965) The cephalometric, anatomic, and histologic changes in Macaca mulatta after application of a continuous-acting retraction force on the mandible. *American Journal of Orthodontics*, 51, 823-855.

22. Juhasz, T., *et al*. (2014) Mechanical loading stimulates chondrogenesis via the PKA/CREB-Sox9 and PP2A pathways in chicken micromass cultures. *Cellular Signalling*, 26, 468-482.

23. Kantomaa, T. and Pirttiniemi, P. (1996) Differences in biologic response of the mandibular condyle to forward traction or opening of the mandible. An experimental study in the rat. *Acta Odontologica Scandinavica*, 54, 138-144.

24. Kantomaa, T., Tuominen, M. and Pirttiniemi, P. (1994) Effect of mechanical forces on chondrocyte maturation and differentiation in the mandibular condyle of the rat. *Journal of Dental Research*, 73, 1150-1156.

25. Kim, I.S., Otto, F., Zabel, B. and Mundlos, S. (1999) Regulation of chondrocyte differentiation by Cbfa1. *Mechanisms of Development*, 80, 159-170.

26. Kumar, D. and Lassar, A.B. (2009) The transcriptional activity of Sox9 in chondrocytes is regulated by RhoA signaling and actin polymerization. *Molecular and Cellular Biology*, 29, 4262-4273.

27. Lefebvre, V. and Smits, P. (2005) Transcriptional control of chondrocyte fate and differentiation. *Birth Defects Research. Part C, Embryo Today: reviews*, 75, 200-212.

28. Lefebvre, V., Behringer, R.R. and de Crombrugghe, B. (2001) L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. *Osteoarthritis and Cartilage*, 9 Suppl A, S69-S75.

29. Marques, M.R., Hajjar, D., Franchini, K.G., Moriscot, A.S. and Santos, M.F. (2008) Mandibular appliance modulates condylar growth through integrins. *Journal of Dental Research*, 87, 153-158.

30. McNamara, J.A. and Carlson, D.S. (1979) Quantitative analysis of temporomandibular joint adaptations to protrusive function. *American Journal of Orthodontics*, 76, 593-611.

31. Meikle, M.C. (1973) The role of the condyle in the postnatal growth of the mandible. *American Journal of Orthodontics*, 64, 50-62.

32. Moss, M.L. and Rankow, R.M. (1968) The role of the functional matrix in mandibular growth. *The Angle Orthodontist*, 38, 95-103.

33. O'Conor, C.J., Case, N. and Guilak, F. (2013) Mechanical regulation of chondrogenesis. *Stem Cell Research and Therapy*, 4, 61.

34. Otto, F., *et al*. (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*, 89, 765-771.

35. Papachristou, D., Pirttiniemi, P., Kantomaa, T., Agnantis, N. and Basdra, E.K. (2006) Fos- and Jun-related transcription factors are involved in the signal transduction pathway of mechanical loading in condylar chondrocytes. *European Journal of Orthodontics*, 28, 20-26.

36. Papachristou, D.J., Gkretsi, V., Rao, U.N., Papachristou, G.I., Papaefthymiou, O.A., Basdra, E.K., Wu, C. and Papavassiliou, A.G. (2008) Expression of integrin-linked kinase and its binding partners in chondrosarcoma: association with prognostic significance. *European Journal of Cancer*, 44, 2518-2525.

37. Papachristou, D.J., Papachroni, K.K., Basdra, E.K. and Papavassiliou, A.G. (2009) Signaling networks and transcription factors regulating mechanotransduction in bone. *BioEssays*, 31, 794-804.

38. Papachristou, D.J., Papadakou, E., Basdra, E.K., Baltopoulos, P., Panagiotopoulos, E. and Papavassiliou, A.G. (2008) Involvement of the p38 MAPK-NF-kappaB signal transduction pathway and COX-2 in the pathobiology of meniscus degeneration in humans. *Molecular Medicine*, 14, 160-166.

39. Papachristou, D.J., Pirttiniemi, P., Kantomaa, T., Papavassiliou, A.G. and Basdra, E.K. (2005) JNK/ERK-AP-1/Runx2 induction “paves the way” to cartilage load-ignited chondroblastic differentiation. *Histochemistry and Cell Biology*, 124, 215-223.

40. Papadopoulou, A.K., Papachristou, D.J., Chatzopoulos, S.A., Pirttiniemi, P., Papavassiliou, A.G. and Basdra, E.K. (2007). Load application induces changes in the expression levels of Sox-9, FGFR-3 and VEGF in condylar chondrocytes. *FEBS Letters*, 581, 2041-2046.

41. Rabie, A.B. and Hagg, U. (2002) Factors regulating mandibular condylar growth. *American Journal of Orthodontics and Dentofacial Orthopedics*, 122, 401-409.

42. Rabie, A.B., She, T.T. and Hagg, U. (2003) Functional appliance therapy accelerates and enhances condylar growth. *American Journal of Orthodontics and Dentofacial Orthopedics*, 123, 40-48.

43. Rabie, A.B., Tang, G.H. and Hagg, U. (2004) Cbfa1 couples chondrocytes maturation and endochondral ossification in rat mandibular condylar cartilage. *Archives of Oral Biology*, 49, 109-118.

44. Ragan, P.M., Badger, A.M., Cook, M., Chin, V.I., Gowen, M., Grodzinsky, A.J. and Lark, M.W. (1999) Down-regulation of chondrocyte aggrecan and type-II collagen gene expression correlates with increases in static compression magnitude and duration. *Journal of Orthopaedic Research*, 17, 836-842.

45. Ramage, L., Nuki, G. and Salter, D.M. (2009) Signalling cascades in mechanotransduction: cell-matrix interactions and mechanical loading. *Scandinavian Journal of Medicine and Science in Sports*, 19, 457-469.

46. Rath-Deschner, B., Daratsianos, N., Dühr, S., Girmann, N., Winter, J., Kroll, F., Reichert, C., Jäger, A. and Götz, W. (2010) The significance of RUNX2 in

postnatal development of the mandibular condyle. *Journal of Orofacial Orthopedics*, 71, 17-31.

47. Saito, T., Ikeda, T., Nakamura, K., Chung, U.I. and Kawaquchi, H. (2007) S100A1 and S100B, transcriptional targets of SOX trio, inhibit terminal differentiation of chondrocytes. *European Molecular Biology Organization reports*, 8, 504-509.

48. Shibata, S., Suda, N., Suzuki, S., Fukuoka, H. and Yamashita, Y. (2006) An in situ hybridization study of Runx2, Osterix, and Sox9 at the onset of condylar cartilage formation in fetal mouse mandible. *Journal of Anatomy*, 208, 169-177.

49. Shukunami, C., Ishizeki, K., Atsumi, T., Ohta, Y., Suzuki, F. and Hiraki, Y. (1997) Cellular hypertrophy and calcification of embryonal carcinoma-derived chondrogenic cell line ATDC5 in vitro. *Journal of Bone and Mineral Research*, 12, 1174-1188.

50. Shukunami, C., Shigeno, C., Atsumi, T., Ishizeki, K., Suzuki, F. and Hiraki, Y. (1996) Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor. *The Journal of Cell Biology*, 133, 457-468.

51. Spyropoulou, A., Karamesinis, K. and Basdra, E.K. (2015) Mechanotransduction pathways in bone pathobiology. *Biochimica et Biophysica Acta*, 1852, 1700-1708.

52. Takano-Yamamoto, T., Soma, S., Nakagawa, K., Kobayashi, Y., Kawakami, M. and Sakuda, M. (1991) Comparison of the effects of hydrostatic compressive

force on glycosaminoglycan synthesis and proliferation in rabbit chondrocytes from mandibular condylar cartilage, nasal septum, and spheno-occipital synchondrosis in vitro. *American Journal of Orthodontics and Dentofacial Orthopedics*, 99, 448-455.

53. Takeda, S., Bonnamy, J.P., Owen, M.J., Ducy, P. and Karsenty, G. (2001) Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. *Genes and Development*, 15, 467-481.

54. Tare, R.S., Howard, D., Pound, J.C., Roach, H.I. and Oreffo, R.O. (2005) Tissue engineering strategies for cartilage generation – micromass and three dimensional cultures using human chondrocytes and a continuous cell line. *Biochemichal and Biophysical Research Communications*, 333, 609-621.

55. Temu, T.M., Wu, K.Y., Gruppuso, P.A. and Phornphutkul, C. (2010) The mechanism of ascorbic acid-induced differentiation of ATDC5 chondrogenic cells. *American Journal of Physiology, Endocrinology and Metabolism*, 299, E325-334.

56. Teramoto, M., Kaneko, S., Shibata, S., Yanagishita, M. and Soma, K. (2003) Effect of compressive forces on extracellular matrix in rat mandibular condylar cartilage. *Journal of Bone and Mineral Metabolism*, 21, 276-286.

57. Tsolakis, A.I., Spyropoulos, M.N., Katsavrias, E. and Alexandridis, K. (1997) Effects of altered mandibular function on mandibular growth after condylectomy. *European Journal of Orthodontics*, 19, 9-19.

58. Weiss, A., von der Mark, K. and Silbermann, M. (1986) A tissue culture system supporting cartilage cell differentiation, extracellular mineralization, and subsequent bone formation, using mouse condylar progenitor cells. *Cell Differentiation*, 19, 103-113.

59. Wong, M. and Carter, D.R. (2003) Articular cartilage functional histomorphology and mechanobiology: a research perspective. *Bone*, 33, 1-13.

60. Wright, E.M., Snopek, B. and Koopman, P. (1993) Seven new members of the Sox gene family expressed during mouse development. *Nucleic Acids Research*, 21, 744.

61. Yamashita, S., Andoh, M., Ueno-Kudoh, H., Sato, T., Miyaki, S. and Asahara, H. (2009) Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. *Experimental Cell Research*, 15, 2231-2240.

62. Yao, Y. and Wang, Y. (2013) ATDC5: an excellent in vitro model cell line for skeletal development. *Journal of Cellular Biochemistry*, 114, 1223-1229.

63. Yousefian, J., Firouzian, F., Shanfeld, J., Ngan, P., Lanese, R. and Davidovitch, Z. (1995) A new experimental model for studying the response of periodontal ligament cells to hydrostatic pressure. *American Journal of Orthodontics and Dentofacial Orthopedics*, 108, 402-409.

64. Zhang, M., Chen, Y.J., Ono, T. and Wang, J.J. (2008) Crosstalk between integrin and G protein pathways involved in mechanotransduction in mandibular

condylar chondrocytes under pressure. *Archives of Biochemistry and Biophysics*, 474, 102-108.

65. Zhang, M., Wang, J.J. and Chen, Y.J. (2006) Effects of mechanical pressure on intracellular calcium release channel and cytoskeletal structure in rabbit mandibular condylar chondrocytes. *Life Sciences*, 78, 2480-2487.

66. Zhou, G., Zheng, Q., Engin, F., Munivez, E., Chen, Y., Sebald, E., Krakow, D. and Lee, B. (2006) Dominance of SOX9 function over RUNX2 during skeletogenesis. *Proceeding of the National Academy of Sciences of the United States of America*, 103, 19004-19009.

67. Ziros, P.G., Basdra, E.K. and Papavassiliou, A.G. (2008) Runx2: of bone and stretch. *The International Journal of Biochemistry and Cell Biology*, 40, 1659-1663.

Appendix 1 – Raw Data

ITS experiment - Densitometric analysis/Quantification of β-ACTIN, SOX9 and pSOX9 – Normalization of SOX9 and pSOX9 to β-ACTIN

	Actin	Sox9 #1	Sox9 #2	Sox9 #3	Sox9/Actin #1	Sox9/Actin #2	Sox9/Actin #3
Control (Untreated)	24.128	33.256	31.986	24.567	1,37831565	1,325679708	1,018194629
12h ITS	23.423	34.026	31.899	25.339	1,452674721	1,361866541	1,08179994
24h ITS	29.198	18.306	18.023	14.474	0,626960751	0,617268306	0,495718885
48h ITS	24.073	30.882	33.324	46.571	1,282848004	1,384289453	1,934574004
96h ITS	23.306	16.786	16.753	13.616	0,720243714	0,71882777	0,584227238
	pSox9 #1	**pSox9 #2**	**pSox9 #3**	**pSox9/Actin #1**	**pSox9/Actin #2**	**pSox9/Actin #3**	
Control (Untreated)	17.326	17.831	17.129	0,71808687	0,73901691	0,709922082	
12h ITS	19.677	19.444	19.289	0,840071724	0,830124237	0,82350681	
24h ITS	10.239	10.860	9.691	0,350674704	0,371943284	0,331906295	
48h ITS	43.685	42.391	44.158	1,814688655	1,760935488	1,834337224	
96h ITS	26.400	27.305	26.862	1,132755514	1,171586716	1,152578735	

HP experiment - Densitometric analysis/Quantification of β-ACTIN, SOX9 and pSOX9 – Normalization of SOX9 and pSOX9 to β-ACTIN

	Actin	Sox9 #1	Sox9 #2	Sox9 #3	Sox9/Actin #1	Sox9/Actin #2	Sox9/Actin #3
Control (Untreated)	25.381	16.411	16.217	16.417	0,64659	0,63894	0,64682
12h HP	24.094	8.893	8.718	8.551	0,36910	0,36183	0,35490
24h HP	23.793	25.408	24.663	24.225	1,06788	1,03657	1,01816
48h HP	25.908	26.633	26.225	26.191	1,02798	1,01224	1,01092
96h HP	26.205	22.655	24.176	24.616	0,86453	0,92257	0,93936
	pSox9 #1	**pSox9 #2**	**pSox9 #3**	**pSox9/Actin #1**	**pSox9/Actin #2**	**pSox9/Actin #3**	
Control (Untreated)	17.083	17.245	17.211	0,67306	0,67945	0,67811	
12h HP	11.633	12.019	11.704	0,48282	0,49884	0,48576	
24h HP	20.248	19.797	20.614	0,85101	0,83205	0,86639	
48h HP	25.158	24.809	24.993	0,97105	0,95758	0,96468	
96h HP	25.878	26.130	25.478	0,98752	0,99714	0,97226	

HP plus ITS treatment - Densitometric analysis/Quantification of β-ACTIN, SOX9, pSOX9 and RUNX2 – Normalization of SOX9, pSOX9 and RUNX2 to β-ACTIN

	Actin	Sox9 #1	Sox9 #2	Sox9 #3	Sox9/Actin #1	Sox9/Actin #2	Sox9/Actin #3
Control (ITS)	23.423	34.026	31.899	25.339	1,452674721	1,361866541	1,08179994
12h HP+ ITS	24.736	42.386	42.510	42.915	1,713534929	1,718547865	1,734920763
24h HP+ ITS	24.627	8.331	8.657	8.449	0,338287246	0,351524749	0,343078735
48h HP+ ITS	27.182	24.762	24.517	23.617	0,910970495	0,901957178	0,868847031
96h HP+ ITS	23.455	24.521	24.316	25.019	1,045448732	1,036708591	1,066680878
	pSox9 #1	**pSox9 #2**	**pSox9 #3**	**pSox9/Actin #1**	**pSox9/Actin #2**	**pSox9/Actin #3**	
Control (ITS)	19.677	19.444	19.289	0,840071724	0,830124237	0,82350681	
12h HP+ ITS	32.750	32.602	32.415	1,323981242	1,31799806	1,310438228	
24h HP+ ITS	4.749	4.752	4.875	0,19283713	0,192958947	0,197953466	
48h HP+ ITS	24.106	23.330	24.510	0,886836877	0,858288573	0,901699654	
96h HP+ ITS	38.395	39.317	38.201	1,6369644	1,676273716	1,628693242	
	Runx2 #1	**Runx2 #2**	**Runx2 #3**	**Runx2/Actin #1**	**Runx2/Actin #2**	**Runx2/Actin #3**	
Control (ITS)	27.317	28.005	27.416	1,166246851	1,19561969	1,170473466	
12h HP+ ITS	20.402	21.430	21.561	0,82478978	0,866348642	0,871644567	
24h HP+ ITS	34.659	34.764	34.927	1,407357778	1,411621391	1,418240143	
48h HP+ ITS	35.480	34.604	35.527	1,30527555	1,273048341	1,307004635	
96h HP+ ITS	25.324	24.769	25.010	1,079684502	1,05602217	1,066297165	

Appendix 2 – Statistical Analysis

ITS experiment – Normalized SOX9 levels

One-way ANOVA and Bonferroni test

Between-Subjects Factors

		Value Label	N
Groups	1,00	Control (Untreated)	3
	2,00	12h ITS	3
	3,00	24h ITS	3
	4,00	48h ITS	3
	5,00	96h ITS	3

Tests of Between-Subjects Effects

Dependent Variable: Sox9overActin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,080[a]	4	,520	12,400	,001
Intercept	17,031	1	17,031	406,221	,000
Groups	2,080	4	,520	12,400	,001
Error	,419	10	,042		
Total	19,530	15			
Corrected Total	2,499	14			

a. R Squared = ,832 (Adjusted R Squared = ,765)

Multiple Comparisons

Dependent Variable: Sox9overActin

Bonferroni

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control (Untreated)	12h ITS	-,0581	,16719	1,000	-,6568	,5407
	24h ITS	,6607*	,16719	,027	,0620	1,2595
	48h ITS	-,2932	,16719	1,000	-,8919	,3056
	96h ITS	,5663	,16719	,069	-,0325	1,1651
12h ITS	Control (Untreated)	,0581	,16719	1,000	-,5407	,6568
	24h ITS	,7188*	,16719	,016	,1200	1,3176
	48h ITS	-,2351	,16719	1,000	-,8339	,3636
	96h ITS	,6243*	,16719	,039	,0256	1,2231
24h ITS	Control (Untreated)	-,6607*	,16719	,027	-1,2595	-,0620
	12h ITS	-,7188*	,16719	,016	-1,3176	-,1200
	48h ITS	-,9539*	,16719	,002	-1,5527	-,3552
	96h ITS	-,0945	,16719	1,000	-,6932	,5043
48h ITS	Control (Untreated)	,2932	,16719	1,000	-,3056	,8919
	12h ITS	,2351	,16719	1,000	-,3636	,8339
	24h ITS	,9539*	,16719	,002	,3552	1,5527
	96h ITS	,8595*	,16719	,004	,2607	1,4582
96h ITS	Control (Untreated)	-,5663	,16719	,069	-1,1651	,0325
	12h ITS	-,6243*	,16719	,039	-1,2231	-,0256
	24h ITS	,0945	,16719	1,000	-,5043	,6932
	48h ITS	-,8595*	,16719	,004	-1,4582	-,2607

Based on observed means.
The error term is Mean Square(Error) = ,042.

*. The mean difference is significant at the 0,05 level.

ITS experiment – Normalized pSOX9 levels

One-way ANOVA and Bonferroni test

Between-Subjects Factors

		Value Label	N
Timepoints	1,00	Control (Untreated)	3
	2,00	12h ITS	3
	3,00	24h ITS	3
	4,00	48h ITS	3
	5,00	96h ITS	3

Tests of Between-Subjects Effects

Dependent Variable: pSox9overActin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3,572[a]	4	,893	1774,150	,000
Intercept	14,176	1	14,176	28161,536	,000
Timepoints	3,572	4	,893	1774,150	,000
Error	,005	10	,001		
Total	17,753	15			
Corrected Total	3,577	14			

a. R Squared = ,999 (Adjusted R Squared = ,998)

Multiple Comparisons

Dependent Variable: pSox9overActin

Bonferroni

(I) Timepoints	(J) Timepoints	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control (Untreated)	12h ITS	-,1089*	,01832	,001	-,1745	-,0433
	24h ITS	,3708*	,01832	,000	,3052	,4364
	48h ITS	-1,0810*	,01832	,000	-1,1466	-1,0154
	96h ITS	-,4300*	,01832	,000	-,4956	-,3644
12h ITS	Control (Untreated)	,1089*	,01832	,001	,0433	,1745
	24h ITS	,4797*	,01832	,000	,4141	,5453
	48h ITS	-,9721*	,01832	,000	-1,0377	-,9065
	96h ITS	-,3211*	,01832	,000	-,3867	-,2555
24h ITS	Control (Untreated)	-,3708*	,01832	,000	-,4364	-,3052
	12h ITS	-,4797*	,01832	,000	-,5453	-,4141
	48h ITS	-1,4518*	,01832	,000	-1,5174	-1,3862
	96h ITS	-,8008*	,01832	,000	-,8664	-,7352
48h ITS	Control (Untreated)	1,0810*	,01832	,000	1,0154	1,1466
	12h ITS	,9721*	,01832	,000	,9065	1,0377
	24h ITS	1,4518*	,01832	,000	1,3862	1,5174
	96h ITS	,6510*	,01832	,000	,5854	,7166
96h ITS	Control (Untreated)	,4300*	,01832	,000	,3644	,4956
	12h ITS	,3211*	,01832	,000	,2555	,3867
	24h ITS	,8008*	,01832	,000	,7352	,8664
	48h ITS	-,6510*	,01832	,000	-,7166	-,5854

Based on observed means.
The error term is Mean Square(Error) = ,001.

*. The mean difference is significant at the 0,05 level.

HP experiment – Normalized SOX9 levels

One-way ANOVA and Bonferroni test

Between-Subjects Factors

		Value Label	N
Groups	1,00	Control (Untreated)	3
	2,00	12h HP	3
	3,00	24h HP	3
	4,00	48h HP	3
	5,00	96h HP	3

Tests of Between-Subjects Effects

Dependent Variable: Sox9overActin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,999[a]	4	,250	535,007	,000
Intercept	9,470	1	9,470	20285,158	,000
Groups	,999	4	,250	535,007	,000
Error	,005	10	,000		
Total	10,474	15			
Corrected Total	1,004	14			

a. R Squared = ,995 (Adjusted R Squared = ,993)

Multiple Comparisons

Dependent Variable: Sox9overActin

Bonferroni

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control (Untreated)	12h HP	,2822*	,01764	,000	,2190	,3454
	24h HP	-,3968*	,01764	,000	-,4599	-,3336
	48h HP	-,3729*	,01764	,000	-,4361	-,3097
	96h HP	-,2647*	,01764	,000	-,3279	-,2015
12h HP	Control (Untreated)	-,2822*	,01764	,000	-,3454	-,2190
	24h HP	-,6789*	,01764	,000	-,7421	-,6157
	48h HP	-,6551*	,01764	,000	-,7183	-,5919
	96h HP	-,5469*	,01764	,000	-,6101	-,4837
24h HP	Control (Untreated)	,3968*	,01764	,000	,3336	,4599
	12h HP	,6789*	,01764	,000	,6157	,7421
	48h HP	,0238	,01764	1,000	-,0394	,0870
	96h HP	,1320*	,01764	,000	,0689	,1952
48h HP	Control (Untreated)	,3729*	,01764	,000	,3097	,4361
	12h HP	,6551*	,01764	,000	,5919	,7183
	24h HP	-,0238	,01764	1,000	-,0870	,0394
	96h HP	,1082*	,01764	,001	,0450	,1714
96h HP	Control (Untreated)	,2647*	,01764	,000	,2015	,3279
	12h HP	,5469*	,01764	,000	,4837	,6101
	24h HP	-,1320*	,01764	,000	-,1952	-,0689
	48h HP	-,1082*	,01764	,001	-,1714	-,0450

Based on observed means.
The error term is Mean Square(Error) = ,000.

*. The mean difference is significant at the 0,05 level.

HP experiment – Normalized pSOX9 levels

One-way ANOVA and Bonferroni test

Between-Subjects Factors

		Value Label	N
Groups	1,00	Control (Untreated)	3
	2,00	12h HP	3
	3,00	24h HP	3
	4,00	48h HP	3
	5,00	96h HP	3

Tests of Between-Subjects Effects

Dependent Variable: pSox9overActin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,527[a]	4	,132	1129,595	,000
Intercept	9,437	1	9,437	80967,269	,000
Groups	,527	4	,132	1129,595	,000
Error	,001	10	,000		
Total	9,965	15			
Corrected Total	,528	14			

a. R Squared = ,998 (Adjusted R Squared = ,997)

Multiple Comparisons

Dependent Variable: pSox9overActin

Bonferroni

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control (Untreated)	12h HP	,1877*	,00881	,000	,1562	,2193
	24h HP	-,1729*	,00881	,000	-,2045	-,1414
	48h HP	-,2876*	,00881	,000	-,3191	-,2560
	96h HP	-,3088*	,00881	,000	-,3403	-,2772
12h HP	Control (Untreated)	-,1877*	,00881	,000	-,2193	-,1562
	24h HP	-,3607*	,00881	,000	-,3922	-,3291
	48h HP	-,4753*	,00881	,000	-,5069	-,4437
	96h HP	-,4965*	,00881	,000	-,5281	-,4649
24h HP	Control (Untreated)	,1729*	,00881	,000	,1414	,2045
	12h HP	,3607*	,00881	,000	,3291	,3922
	48h HP	-,1146*	,00881	,000	-,1462	-,0831
	96h HP	-,1358*	,00881	,000	-,1674	-,1043
48h HP	Control (Untreated)	,2876*	,00881	,000	,2560	,3191
	12h HP	,4753*	,00881	,000	,4437	,5069
	24h HP	,1146*	,00881	,000	,0831	,1462
	96h HP	-,0212	,00881	,370	-,0528	,0104
96h HP	Control (Untreated)	,3088*	,00881	,000	,2772	,3403
	12h HP	,4965*	,00881	,000	,4649	,5281
	24h HP	,1358*	,00881	,000	,1043	,1674
	48h HP	,0212	,00881	,370	-,0104	,0528

Based on observed means.
The error term is Mean Square(Error) = ,000.

*. The mean difference is significant at the 0,05 level.

HP plus ITS experiment – Normalized SOX9 levels

One-way ANOVA and Bonferroni test

Between-Subjects Factors

		Value Label	N
Timepoints	1,00	Control (ITS)	3
	2,00	12h HP + ITS	3
	3,00	24h HP + ITS	3
	4,00	48h HP + ITS	3
	5,00	96h HP + ITS	3

Tests of Between-Subjects Effects

Dependent Variable: Sox9overActin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3,107[a]	4	,777	101,473	,000
Intercept	16,911	1	16,911	2209,342	,000
Timepoints	3,107	4	,777	101,473	,000
Error	,077	10	,008		
Total	20,094	15			
Corrected Total	3,183	14			

a. R Squared = ,976 (Adjusted R Squared = ,966)

Multiple Comparisons

Dependent Variable: Sox9overActin

Bonferroni

(I) Timepoints	(J) Timepoints	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control (ITS)	12h HP + ITS	-,4236*	,07143	,001	-,6794	-,1677
	24h HP + ITS	,9545*	,07143	,000	,6986	1,2103
	48h HP + ITS	,4049*	,07143	,002	,1490	,6607
	96h HP + ITS	,2492	,07143	,058	-,0067	,5050
12h HP + ITS	Control (ITS)	,4236*	,07143	,001	,1677	,6794
	24h HP + ITS	1,3780*	,07143	,000	1,1222	1,6339
	48h HP + ITS	,8284*	,07143	,000	,5726	1,0842
	96h HP + ITS	,6727*	,07143	,000	,4169	,9286
24h HP + ITS	Control (ITS)	-,9545*	,07143	,000	-1,2103	-,6986
	12h HP + ITS	-1,3780*	,07143	,000	-1,6339	-1,1222
	48h HP + ITS	-,5496*	,07143	,000	-,8055	-,2938
	96h HP + ITS	-,7053*	,07143	,000	-,9612	-,4495
48h HP + ITS	Control (ITS)	-,4049*	,07143	,002	-,6607	-,1490
	12h HP + ITS	-,8284*	,07143	,000	-1,0842	-,5726
	24h HP + ITS	,5496*	,07143	,000	,2938	,8055
	96h HP + ITS	-,1557	,07143	,543	-,4115	,1001
96h HP + ITS	Control (ITS)	-,2492	,07143	,058	-,5050	,0067
	12h HP + ITS	-,6727*	,07143	,000	-,9286	-,4169
	24h HP + ITS	,7053*	,07143	,000	,4495	,9612
	48h HP + ITS	,1557	,07143	,543	-,1001	,4115

Based on observed means.
The error term is Mean Square(Error) = ,008.

*. The mean difference is significant at the 0,05 level.

HP plus ITS experiment – Normalized pSOX9 levels

One-way ANOVA and Bonferroni test

Between-Subjects Factors

		Value Label	N
Timepoints	1,00	Control (ITS)	3
	2,00	12h HP + ITS	3
	3,00	24h HP + ITS	3
	4,00	48h HP + ITS	3
	5,00	96h HP + ITS	3

Tests of Between-Subjects Effects

Dependent Variable: pSox9overActin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3,623[a]	4	,906	3602,351	,000
Intercept	14,247	1	14,247	56665,899	,000
Timepoints	3,623	4	,906	3602,351	,000
Error	,003	10	,000		
Total	17,872	15			
Corrected Total	3,625	14			

a. R Squared = ,999 (Adjusted R Squared = ,999)

Multiple Comparisons

Dependent Variable: pSox9overActin

Bonferroni

(I) Timepoints	(J) Timepoints	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control (ITS)	12h HP + ITS	-,4862*	,01295	,000	-,5326	-,4399
	24h HP + ITS	,6367*	,01295	,000	,5903	,6830
	48h HP + ITS	-,0510*	,01295	,028	-,0974	-,0047
	96h HP + ITS	-,8161*	,01295	,000	-,8624	-,7697
12h HP + ITS	Control (ITS)	,4862*	,01295	,000	,4399	,5326
	24h HP + ITS	1,1229*	,01295	,000	1,0765	1,1693
	48h HP + ITS	,4352*	,01295	,000	,3888	,4816
	96h HP + ITS	-,3298*	,01295	,000	-,3762	-,2835
24h HP + ITS	Control (ITS)	-,6367*	,01295	,000	-,6830	-,5903
	12h HP + ITS	-1,1229*	,01295	,000	-1,1693	-1,0765
	48h HP + ITS	-,6877*	,01295	,000	-,7341	-,6413
	96h HP + ITS	-1,4527*	,01295	,000	-1,4991	-1,4064
48h HP + ITS	Control (ITS)	,0510*	,01295	,028	,0047	,0974
	12h HP + ITS	-,4352*	,01295	,000	-,4816	-,3888
	24h HP + ITS	,6877*	,01295	,000	,6413	,7341
	96h HP + ITS	-,7650*	,01295	,000	-,8114	-,7187
96h HP + ITS	Control (ITS)	,8161*	,01295	,000	,7697	,8624
	12h HP + ITS	,3298*	,01295	,000	,2835	,3762
	24h HP + ITS	1,4527*	,01295	,000	1,4064	1,4991
	48h HP + ITS	,7650*	,01295	,000	,7187	,8114

Based on observed means.
The error term is Mean Square(Error) = ,000.

*. The mean difference is significant at the 0,05 level.

HP plus ITS experiment – Normalized RUNX2 levels

One-way ANOVA and Bonferroni test

Between-Subjects Factors

		Value Label	N
Timepoints	1,00	Control (ITS)	3
	2,00	12h HP + ITS	3
	3,00	24h HP + ITS	3
	4,00	48h HP + ITS	3
	5,00	96h HP + ITS	3

Tests of Between-Subjects Effects

Dependent Variable: Runx2overActin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,553[a]	4	,138	477,616	,000
Intercept	20,230	1	20,230	69892,093	,000
Timepoints	,553	4	,138	477,616	,000
Error	,003	10	,000		
Total	20,786	15			
Corrected Total	,556	14			

a. R Squared = ,995 (Adjusted R Squared = ,993)

Multiple Comparisons

Dependent Variable: Runx2overActin

Bonferroni

(I) Timepoints	(J) Timepoints	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control (ITS)	12h HP + ITS	,3232*	,01389	,000	,2734	,3729
	24h HP + ITS	-,2350*	,01389	,000	-,2847	-,1852
	48h HP + ITS	-,1177*	,01389	,000	-,1674	-,0679
	96h HP + ITS	,1101*	,01389	,000	,0604	,1599
12h HP + ITS	Control (ITS)	-,3232*	,01389	,000	-,3729	-,2734
	24h HP + ITS	-,5581*	,01389	,000	-,6079	-,5084
	48h HP + ITS	-,4408*	,01389	,000	-,4906	-,3911
	96h HP + ITS	-,2131*	,01389	,000	-,2628	-,1633
24h HP + ITS	Control (ITS)	,2350*	,01389	,000	,1852	,2847
	12h HP + ITS	,5581*	,01389	,000	,5084	,6079
	48h HP + ITS	,1173*	,01389	,000	,0675	,1670
	96h HP + ITS	,3451*	,01389	,000	,2953	,3948
48h HP + ITS	Control (ITS)	,1177*	,01389	,000	,0679	,1674
	12h HP + ITS	,4408*	,01389	,000	,3911	,4906
	24h HP + ITS	-,1173*	,01389	,000	-,1670	-,0675
	96h HP + ITS	,2278*	,01389	,000	,1780	,2775
96h HP + ITS	Control (ITS)	-,1101*	,01389	,000	-,1599	-,0604
	12h HP + ITS	,2131*	,01389	,000	,1633	,2628
	24h HP + ITS	-,3451*	,01389	,000	-,3948	-,2953
	48h HP + ITS	-,2278*	,01389	,000	-,2775	-,1780

Based on observed means.
The error term is Mean Square(Error) = ,000.

*. The mean difference is significant at the 0,05 level.