

Hyaluronan based gel promotes human Dental Pulp Stem Cells bone differentiation by activating YAP/TAZ pathway

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Abstract

Background. Hyaluronic acid (HA) is the major component of the extracellular matrix of human tissue, where it regulates processes such as osmotic pressure, water retention, cell migration, and differentiation. For these reasons, hyaluronans are currently used in regenerative medicine in different areas. Nevertheless, hyaluronans exist in different forms accordingly with molecular weight and degree of crosslinking, which can have a different and context-dependent effects on cellular processes. Thus, picking the most appropriate form of hyaluronan turn out to be fundamental as it can make a huge difference in tissue regeneration.

MSCs have attracted attention in tissue regeneration for their proliferation potential and ability to differentiate in several cytotypes. Among MSCs, human Dental Pulp Stem Cells (hDPSCs) were shown to be remarkably suitable for bone differentiation.

In this study, we tested the capability to induce osteogenic differentiation in hDPSCs of three hyaluronans forms: linear pharmaceutical-grade hyaluronans at high (HHA), low molecular weight (LHA), and the recently stabilized hybrid cooperative complexes (HCC), containing both sizes.

Methods. hDPSCs were treated with HHA, LHA, HCC for 7, 14 and 21 days. The effects of hyaluronans on osteogenic differentiation were evaluated by qRT-PCR and WB of osteogenic markers and by Alizarin Red S staining. CD44, the main receptor of the HA on cell surface and an upstream regulator of YAP/TAZ signaling, was analyzed by immunofluorescence. YAP/TAZ expression was measured by qRT-PCR. To confirm the involvement of YAP/TAZ pathway, YAP/TAZ inhibitor-1 was used and the loss of function of YAP/TAZ was evaluated by qRT-PCR, WB and immunofluorescence.

Results. HCC was found to be the most impacting in inducing osteogenesis, with significant effects already at 7-14 days of treatment. HCC induced strong overexpression of osteocalcin, osteopontin, and bone sialoprotein, calcification nodule formation, and CD44 up-regulation.

In addition, we showed that this biological process is associated to the activation of YAP/TAZ pathway and its target genes CTGF, ANKDR-1, RUNX-1, and RUNX-2.

Conclusions. In conclusion, in this study we show that HA's molecular weight can have a tremendous impact on HA performance for bone regeneration, and we unveil a new molecular mechanism by which HA acts on stem cells.

Background

Hyaluronic acid (HA) is a key component of extracellular matrix (ECM) in the majority of human tissues.

It interacts with other macromolecules and plays a predominant role in tissue morphogenesis, cell migration, differentiation, and adhesion. However, what we define as hyaluronic acid is not a single and unique molecule with distinct characteristic, but rather a family of macromolecules with different

molecular weight and biological activity. Indeed, HA's molecular weight may affect substantially its biochemical features and its effects on biological processes and tissue regeneration. HA-based products have already reached clinical-grade approval and are used in many fields such as orthopedics, ophthalmology, dermatology, and plastic surgery. However, most of these products do not report HA's molecular weight or report a generic reference to it, such as high, medium and low without a general consensus on what this term means. Moreover, different products with different HA's molecular weights are approved for the same indication, which, in the light of recent knowledge, makes little sense. For these reasons, a clarification on the biological properties of different molecular weights in HA is of paramount importance to improve existing products and formulate new and more effective ones. To give an example, different formulations of HA, with diverse molecule weights, stimulate Adipose Stem Cells (ASCs) to differentiate in adipose tissue [1], or preserve the stemness of human Mesenchymal Stem Cells (hMSCs) [2].

hMSCs have attracted attention in tissue regeneration for their proliferation potential and the ability to differentiate in several cytotypes such as osteoblasts, adipocytes, chondrocytes, and endotheliocytes [3]. Among hMSCs, human dental pulp stem cells (hDPSCs) were shown to be remarkably suitable for bone-endothelium co-differentiation [4]. hDPSCs can be easily extracted from dental pulp, and have several advantages compared to other types of hMSCs: better proliferative potential [5, 6], a simpler primary isolation method [7], and a higher success rate in long-term *in vitro* culture. Moreover, hDPSCs can be stored for a long time without losing their stemness [8].

YAP/TAZ (yes-associated protein/transcriptional Coactivator with PDZ binding motif) pathway is involved in transmitting extracellular mechanical signals to the nucleus to regulate different biochemical signals, among which osteogenic differentiation [9]. They interact with transcription factors, including transcription enhancer factors/transcriptional enhanced associate domain, runt domain transcription factors (RUNX), and peroxisome proliferator-activated receptors γ regulating downstream gene expression [10]. Different studies suggested that YAP/TAZ activation could be related to cell shape, stiffness of matrix, and mechanical stimulation, regulating cell proliferation, differentiation, and apoptosis [11, 12].

Here, we evaluated the biological activity of hybrid cooperative complexes (HCC) gels, in comparison to high (HHA) and low (LHA) HA molecular weight on hDPSCs. We found that HCC improves bone differentiation of hDPSCs via activation of the YAP/TAZ pathway. Our findings highlight the importance of HA's molecular weight and unveil a new molecular mechanism underlying the biological effect of HA on hDPSCs osteogenic differentiation. This opens new scenario for clinical applications of HA based-gels in regenerative medicine.

Methods

Reagents

High and low molecular weight hyaluronic acid (HA) were provided by Altergon (Altergon s.r.l., Italy). These are fermentative HA of high purity derived from *Streptococcus equi* ssp. *equi*, extensively purified at pharmaceutical grade (e.g., purity >95%, water content <10%, EU/mg <0.05, and very low metal contents). The raw materials were fully characterized through hydrodynamic analyses using Size-exclusion chromatography coupled to a triple detector (SEC-TDA, Viscotek Malvern) [13]. Hybrid cooperative complexes of hyaluronic acid (HCC), obtained through a thermal procedure for the formation of hybrid cooperative complexes of hyaluronic acid, starting from an initial mixture of an equal amount (ratio 1:1) of HHA (Mw = 1400±200 kDa; Mw/Mn = 1.4) and LHA (Mw = 100±20kDa; Mw/Mn = 1.4). The starting concentration used was 32g/L: 16 mg HHA + 16 mg LHA in 1 mL volume. The final concentration used in these experiments was 1.6 mg/mL obtained by opportunely diluting all solutions with the culture medium (DMEM Dulbecco's modified Eagle's medium, Gibco, Invitrogen, and/or OM osteogenic medium).

Dental pulp extraction and culture

All experimental procedures involved were approved by the Ethics Committee of University of Campania approved on June 12th, 2005, Internal Registry: Experimentation #914 and were performed in line with the principles of the Declaration of Helsinki.

Human dental pulps were extracted from teeth of healthy adults (aged 21–38 years) as described previously [7]. All participants signed the Ethical Committee (University of Campania Internal Ethical Committee) consent form. Every participant was pretreated for a week with professional dental hygiene. The dental crown was covered with 0.3% chlorhexidine gel (Forhans) for 2 min before the extraction. Dental pulp was obtained with a dentinal excavator or a Gracey curette. The pulp was delicately removed and immersed for 1 h at 37 °C in a digestive solution composed of 3 mg/ml of type I collagenase and 4 mg/ml of dispase in PBS containing 40 mg/ml of gentamicin. Once digested, the solution was filtered through 70 µm Falcon strainers (Becton & Dickinson). Cells were cultured in basal growth medium (standard medium) consisting of Dulbecco's modified Eagle's medium (DMEM) with 100 units/ml of penicillin, 100 mg/ml of streptomycin, and 200 mM l-glutamine (all from Gibco), supplemented with 10% heat-inactivated AB-HS (Invitrogen). Cells were maintained in a humidified atmosphere under 5% CO₂ at 37°C and the media were changed twice a week.

hDPSCs isolation and osteogenic differentiation

At the first passage of culture, cells were detached using trypsin–EDTA (GIBCO). At least 200,000 cells were incubated with fluorescent-conjugated antibodies for 30 min at 4°C, washed, and resuspended in PBS. The antibodies used in this study were: anti-CD34 PE (BD Pharmingen, Buccinasco, Milano, Italy), anti-CD90 FITC (BD Pharmingen, Buccinasco, Milano, Italy) and anti-CD45 APC-Cy7 (BD Pharmingen, Buccinasco, Milano, Italy). Isotypes were used as controls. Cells were analyzed with FACS ARIA III (BD Biosciences, San Jose, CA, USA) and data collected with Diva Software. Cells were sorted using simultaneous positivity for CD90 and CD34 using a FACS ARIA III (BD, Franklin Lakes, NJ, USA) as previously reported [7]. The purity of sorted populations was routinely 90%.

hDPSCs were treated with HHA, LHA, HCC diluted in DMEM and osteogenic media (OM). All experiments were performed at 7, 14 and 21 days. Media were changed twice a week. Cells grown in DMEM or OM without hyaluronans were used as controls.

For osteogenic differentiation, hDPSCs were cultured in osteogenic medium containing 100 nmol/L dexamethasone, 10 mmol/L beta-glycerophosphate, and 0.05 mmol/L L-ascorbic acid-2-phosphate. Osteogenic differentiation was evaluated by Alizarin Red S (ARS) and the expression of bone-related markers such as osteocalcin (OC), osteopontin (OPN), and bone sialoprotein (BSP) at 7, 14 and 21 days. For Alizarin Red S staining, samples were washed twice in PBS, fixed with 4% paraformaldehyde (PFA) for 30 min at 4°C, and stained with 2% Alizarin Red solution, pH 4.2 (Sigma Aldrich, Milan, Italy) for 20 min at room temperature. Stained cells were extensively washed with deionized water to remove any non-specific precipitation. Micrographs were taken using a microscope Eclipse TE2000-S (Nikon) and a Nikon camera. The number of bone nodules and area calculation of positive staining were measured and compared amongst groups. Quantification of the staining intensity was measured using ImageJ software (National Institutes of Health (NIH), Bethesda, MD, United States).

YAP/TAZ inhibitor-1 treatment and cell viability assay

To confirm involvement of YAP/TAZ pathway in promoting osteogenic differentiation, hDPSCs were treated with hyaluronans and YAP/TAZ inhibitor-1 (MedChemExpress.). To evaluate the cytotoxicity of YAP/TAZ inhibitor-1, hDPSCs viability was measured by the colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates at a density of 10^4 cells per well, treated with different concentrations (50, 25, 12.5, 7.25, 5, 2.5, 1.25, 0.75 nM) for 24 and 48 hours. After incubation, they were treated with 100 μ L of 1 mg/mL MTT (Sigma) in DMEM medium containing 10% FBS for 4 h at 37 °C. The medium was then replaced with 200 μ L of DMSO and shaken for 15 min. Absorbance at 540 nm was measured using a microplate ELISA reader with DMSO used as the blank.

YAP/TAZ inhibitor-1 treated hDPSCs were cultured in DMEM at 10% FBS for 24, 72 hours and 7 days. YAP, TAZ, RUNX-2, OC, OPN, and BSP gene expression were evaluated by real Time PCR. OC, OPN, and BSP protein levels were checked also by western blotting.

RNA extraction and quantitative real-time PCR analyses

hDPSCs treated with HHA, LHA, and HCC (0.16% w/v) for 7, 14, and 21 days, and with YAP/TAZ inhibitor-1 for 24, 72 hours and 7 days, were directly lysed with TRIzol® (Invitrogen, Milan, Italy). Following precipitation with isopropyl alcohol and washing with 75% ethanol, the RNA pellets were re-suspended in nuclease-free water. The concentration of the extracted RNA was determined through a Nanodrop spectrophotometer (Celbio, Milan, Italy) and 1 μ g of DNase-digested total RNA was retro-transcribed in the cDNA using Reverse Transcription System Kit (Promega, Milan, Italy). Quantitative real-time PCR was obtained by iQTM SYBR® Green Supermix (BioRad Laboratories Srl, Milan, Italy) to analyze the gene expression of some osteogenic biomarkers such as Osteocalcin (OC), Osteopontin (OPN) and Bone

sialoprotein (BSP). Also, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), were analyzed together with the two YAP/TAZ regulated genes, namely the connective tissue growth factor (CTGF) and ankyrin repeat domain-containing protein 1 (ANKRD-1). Finally, runt domain transcription factors (RUNX-1 and 2) were evaluated.

Oligonucleotide sequences used for quantitative PCR are reported in Table 1 and were obtained by Beacon Designer™ software (BioRad Laboratories Srl, Milan, Italy). Samples were analyzed in triplicate and the expression of specific mRNA relative to the control was determined after normalization with HPRT housekeeping gene (internal control). The fold-change of mRNA expression of the genes under evaluation was calculated by using the $2^{-\Delta\Delta Ct}$ comparative threshold method (ΔCt = difference of ΔCt between treated cells and untreated cells used as controls). The results were expressed as normalized fold expression, calculated by the ratio of crossing points of amplification curves of several genes and internal standard, by using the Bio-Rad iQTM5 software (Bio-Rad Laboratories Srl) as previously reported [1].

Protein levels evaluation of OC, OPN, and BSP by western blotting analyses

Following HA-based gels and YAP/TAZ inhibitor-1 treatments, hDPSCs were lysed by a Radio-Immunoprecipitation Assay (RIPA buffer) (1x) (Cell Signaling Technology), protein concentrations were evaluated using the Bradford method, and the western blotting analyses were performed according to previously described protocols [1]. In particular, 30 μ g of intracellular proteins were electrophoretically resolved on 15% SDS-PAGE and transferred to a nitrocellulose membrane (GE, Amersham, UK). This latter was blocked with 5% nonfat milk in Tris-buffered saline and 0.05% Tween-20 (TBST) and primary antibodies to detect OC (Abcam, Cambridge, UK), OPN (Abcam, Cambridge, UK) and BSP (Abcam, Cambridge, UK) were used at 1:500 dilutions and incubated for 2 h at room temperature (RT). The nitrocellulose membrane was extensively washed with TBST and immunoreactive bands were detected by chemiluminescence using corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, CAUSA), diluted 1:10000 for 1 h, at RT and reacted with an ECL system (Millipore). Protein levels were normalized versus the signal obtained with an anti-Actin antibody 1:500 dilutions (Santa Cruz Biotechnology, CA, USA) and anti-GAPDH antibody diluted 1:500 (Sigma). The semi-quantitative analysis of protein levels was carried out using the Gel Doc 2000 UV System according to the manufacturer's protocol.

CD44 expression in hDPSCs by immunofluorescence staining

Monolayers of hDPSCs were cultured and treated with HA-based gels in standard and osteogenic media in four-well covered glass chamber slides. After 48 h of treatment, the cells were fixed with 4% w/v paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and permeabilized in 0.2% Triton X-100 v/v in PBS for 1 h. Non-specific sites were blocked using blocking buffer solution (PBS containing 10% v/v bovine serum and 1% w/v BSA). The cells were then incubated with anti-CD44 antibody (Cell signaling, Leiden, The Netherlands) overnight at 4°C followed by incubation with

corresponding secondary antibody for 2 h at room temperature. Nuclei were stained with 20-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2.50-bi-1H-benzimidazole trihydrochloride hydrate, bisBenzimide (Hoechst) and actin filaments were stained using phalloidin tetramethylrhodamine B isothiocyanate. (Sigma-Aldrich, Milan, Italy). Fluorescence images were captured using a fluorescence microscopy system (Nikon, Tokyo Japan).

YAP-TAZ expression in hDPSCs by immunofluorescence staining

hDPSCs were cultured with HA-based gels in standard medium and treated with YAP/TAZ inhibitor-1 in 24-well covered glass chamber slides. After 72h of treatment, the cells were fixed with 4% w/v paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and permeabilized in 0.2% Triton X-100 v/v in PBS for 1 h. Non-specific sites were blocked using blocking buffer solution (PBS containing 10% v/v bovine serum and 1% w/v BSA). The cells were then incubated with anti-YAP and anti-TAZ antibodies (Invitrogen) overnight at 4°C followed by incubation with corresponding secondary antibody for 2 h at room temperature. Nuclei were counterstained with 20-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2.50-bi-1H-benzimidazole trihydrochloride hydrate, bisBenzimide (Hoechst). Cells were imaged with a fluorescence microscope EVOS FL Cell Imaging System (Thermo Scientific, Rockford, USA).

Cell Seeding and Differentiation in 3D

To achieve 3D tissue formation, hDPSCs were seeded on a Gingostat (GABA VEBAS, Roma, Italy, <http://www.gaba-info.it/>) scaffold. This scaffold is a lyophilized collagen type I sponge. Collagen sponges were cut under sterile conditions into 5×5×5 mm³ cubes. Scaffold cubes were placed in six-well plates and a cell suspension of 1 × 10⁶ cells contained in 100 µl medium was pipetted onto the top of each cube. Cells were allowed to adhere under a humidified atmosphere at 37°C and 5% CO₂ for 4 hours. The seeded scaffolds were then placed in the osteogenic medium with HA-based gels and cultured for 21 days in an incubator at 37°C and 5% CO₂. Media were changed twice a week.

After 3D culture, samples were fixed in 4% paraformaldehyde (PFA) and cryoprotected overnight at 4°C by immersion in a 30% (wt/vol) sucrose solution before being embedded in Tissue-Tek® O.C.T. Compound (Tissue-Tek; Sakura Finetek, Torrance, CA, <http://www.sakuraus.com.>) and frozen.

Osteogenic differentiation of frozen sections was evaluated by Alizarin Red S staining as described previously. Briefly, the samples were washed in highly purified H₂O to remove O.C.T. for 5 min and then stained with Alizarin Red solution (2%, pH 4.2; Sigma Aldrich, Milan, Italy) for 20 min at room temperature. Stained samples were extensively washed with deionized water to remove any nonspecific precipitation. Micrographs were taken with a microscope Eclipse TE2000-S (Nikon, Firenze, Italy) and a Nikon camera (Nikon, Firenze, Italy).

For immunofluorescence staining, frozen sections were permeabilized with 0.1% Triton X-100 for 15 min. Each sample was incubated in PBS containing 5% bovine serum albumin (BSA) for 30 min. at room

temperature as a blocking step. Then, after washing twice with PBS, samples were incubated with primary antibodies: mouse monoclonal to OC (1:100, Santa Cruz, Heidelberg, Germany), rabbit polyclonal to OPN (1:1000, Abcam, Cambridge, UK), overnight at 4 °C in the dark. This step was followed by incubation with the secondary antibody Tetramethylrhodamine (TRITC)-conjugates (1:1000, Abcam). Nuclear counterstaining was performed with 4,6-diamidino-2-phenylindole (DAPI). After extensive washing with PBS, coverslips were mounted onto slides. Images were collected under a fluorescence microscope (EVOS FL Cell Imaging System, Thermo Scientific, Rockford, USA).

Statistical analysis

Values are shown as the mean \pm SD of measurements of at least three independently performed experiments to avoid possible variation of cell cultures. Student's t-test was employed, and $p < 0.05$ was considered to be statistically significant.

Results

hDPSCs isolation and cell culture

hDPSCs were analyzed by flow cytometry at the first passage of culture, they were CD90⁺, CD45⁻ and CD34 expression was about 18%. hDPSCs were isolated by CD34 and CD90 co-expression, as previously reported by us [5,7]. All experiments were performed using CD34⁺CD90⁺ hDPSCs at 1^o passage of culture.

Hyaluronan based gels positively affected the expression of the osteogenic markers

To evaluate the ability of the different HA-based gels to affect osteogenic differentiation, we analyzed OC, OPN, and BSP gene and protein expression at 7, 14, and 21 days.

We found that, in standard medium, all hyaluronans prompted an up-regulation of OC gene expression at 14 days. In particular, HCC induced a very significant increase of OC gene expression than untreated, HHA and LHA treated cells ($p < 0.01$) at 14 and 21 days (Figure 1a). We next evaluated the protein expression and confirmed qRT-PCR data. In fact, after 7 and 14 days of treatment, OC levels were slightly increased with HHA and LHA respect to control, but HCC induced a stronger upregulation respect to control at 7 and 14 days (Figure 1(a)).

We next examined OPN expression, another important protein of extracellular matrix of bone. We found that, in standard medium, all hyaluronans promoted OPN mRNA up-regulation at 14 days with a stronger expression induced by HHA ($p < 0.01$). At 21 days only slight differences were evidenced (Figure 1(b)). On the other hand, OPN protein expression resulted to be already strongly upregulated with HHA and HCC at 7 days. Then, at 14 days, LHA and HCC promoted its increase whereas at 21 days, only HCC significantly upregulated OPN expression, compared to control and the other hyaluronans treatments (Figure 1(b)).

BSP is the main component of bone mineralized matrix. In our setting, we found that also BSP gene expression was strongly upregulated by HCC after 7 and 14 days of treatment (Figure 1(c)). On the other

hand, at 7 and 14 days, BSP protein levels were slightly increased by all hyaluronan based treatments and after 21 days, only HCC was effective in increasing BSP protein expression ($p < 0.05$) respect to HHA, LHA, and control (Figure 1(c)).

In osteogenic medium, there were no strong differences among treatments and controls (Figure S1).

Regarding gene expression evaluation, we found that OC gene expression was reduced for all treatments at 7 and 14 days and presented a very slight increase only in presence of HHA at 21 days respect to the control (Figure S1(a)). HHA induced an increase of OPN expression of about 5 and 35-fold respect to control at 14 and 21 days, respectively (Figure S1(b)). A slight up-regulation of OPN was evidenced with LHA at the same time. No significant differences were evidenced for HCC up to 14 days. At 21 days, HCC induced OPN up-regulation (Figure S1(b)). In addition, there was no any modulation of BSP gene expression with all treatments (Figure S1(c)). Western blotting analyses revealed that the effects of hyaluronan based treatments were not significant in hDPSCs cultured in osteogenic medium (Figure S1).

Taken together, our results show that all hyaluronan based gels positively modulated the bone-related genes and protein expression in standard medium with a paramount activity of HCC.

Hyaluronan based gels induced an increase of calcification nodules in hDPSCs

To better characterize the osteogenic differentiation, we evaluated the ability of hDPSCs to form calcification nodules after hyaluronan based gels treatment.

We found that, in the standard medium, all hyaluronans promoted the formation of calcification nodules already at 7 days. On the contrary, at 14 and 21 days, we observed that HCC and HHA led to a greater number of calcification nuclei compared to LHA and control (Figure 2).

This was confirmed also by calculating the area percentage of positive staining (Figure 2). HHA and HCC induced a significant increase in the percentage of the positively stained surface compared to those of LHA and control ($p < 0.01$ HHA vs control, $p < 0.001$ HCC vs control). In the osteogenic medium, the behavior was similar. Also here, at 7 days, calcification nodules were already detectable (Figure S2). At 14 and 21 days, HCC and HHA promoted a significant formation of calcification nodules ($p < 0.01$ HHA and HCC vs control) associated with an increase of the percentage of positively stained surface respect to those of LHA and control ($p < 0.01$ HHA and HCC vs control).

These data further confirmed that HCC, and then HHA, promoted the bone differentiation of hDPSCs in standard medium.

Hyaluronan based gels promoted expression of CD44 in hDPSCs

Because one of the main receptors of the HA is the transmembrane glycoprotein CD44, we evaluated its expression in the different treatments and conditions by immunofluorescence. As reported in Figure 3, CD44 marker was mainly distributed on the hDPSCs cell surface. In particular, in standard medium

(Figure 3), we found that CD44 was highly expressed in all hyaluronans treatments. In particular, HCC showed specific areas of positivity on hDPSCs surface. While, in osteogenic medium, the CD44 was distributed as puncta (Figure S3).

Hyaluronan based gels regulated positively gene expression of YAP/TAZ, CTGF, ANKDR-1 and RUNX-1 and RUNX-2 in hDPSCs

YAP/TAZ are known to have a key function in stem cell differentiation [14,15] but no data are available on how YAP and TAZ are regulated by Hyaluronans. In our setting, using qRT-PCR analyses, we found that YAP and TAZ are differently expressed.

Specifically, in standard medium, YAP is slightly up-regulated from 14 to 21 days by HHA, LHA, and strongly activated by HCC ($p < 0.01$) (Figure 4). On the contrary, TAZ followed a different kinetic of expression (Figure 4). Indeed, TAZ expression increased earlier, at 7 and 14 days, in particular with HHA. In the presence of LHA or HCC, TAZ mRNA levels were more down-regulated ($p < 0.01$). As expected, TAZ is inversely modulated than YAP. Additionally, we found that all hyaluronans increased CTGF and ANKDR-1 gene levels (Figure 4). CTGF mRNA levels were up-regulated by all HA-based gels up to 14 days, with a better up-regulation exerted by HCC. For ANKDR-1 gene expression, the up-regulation was found for HHA at 7 and 14 days (Figure 4). HCC already at 7 days strongly increased ANKDR-1 expression ($p < 0.01$) (Figure 4).

Then, we examined mRNA levels of RUNX-1 and 2, target genes of the YAP/TAZ pathway.

In the standard medium, all hyaluronans up-regulated RUNX-1 expression at 7 days, with a better effect exerted by HCC. RUNX-2 was positively modulated by HCC up to 14 days (Figure 4).

Taken together, these results show that hyaluronans promoted the bone differentiation via YAP/TAZ pathway.

Inhibition of YAP/TAZ pathway is associated with a reduction of osteogenic differentiation exercised by hyaluronans

To confirm that hyaluronans promote osteogenesis by activating the YAP/TAZ pathway, we treated hDPSCs with the YAP/TAZ inhibitor-1. Since this inhibitor is able to stop cell growth, we started by determining the noncytotoxic dose for hDPSCs. For this purpose, hDPSCs were exposed to different concentrations of YAP/TAZ inhibitor-1 (50, 25, 12.5, 7.25, 5, 2.5, 1.25 and 0.75 nM) for 24 and 48 hours. We did not detect appreciable differences in cell growth up to 48 hours of treatment and up to a concentration of 2.5 nM (Figure S4), which we consequently chose as work concentration.

Next, we treated the hDPSCs with hyaluronans and YAP/TAZ inhibitor-1 in standard medium and evaluated both the expression of bone markers at 24, 72 hours, 7 days and nuclear translocation of YAP/TAZ at 72 hours.

After YAP/TAZ inhibitor-1 treatment, we observed a slightly increase of YAP mRNA levels and, a drastic reduction of TAZ mRNA levels independently by times and hyaluronans treatment respect to untreated cells. Then, we assessed how the TAZ depletion affected on osteogenic differentiation. We found that all osteogenic related genes, including RUNX2, OC, OPN and BSP were strongly down-regulated. In particular, we found that inhibitor effect was stronger in cells treated with HCC than those treated with HHA and LHA for OC, and BSP at 72 hours and 7 days (Figure 5).

Then, we also examined the protein expression of bone related markers, by western blotting. We observed that at 24 and 72 hours, TAZ depletion withdrawn the expression of OC, OPN and BSP. These were detectable only at 7 days after YAP/TAZ inhibitor-1 treatment and their expression was reduced compared to untreated hDPSCs (Figure 6).

The activation of YAP/TAZ pathway led to the translocation of YAP/TAZ from cytoplasm in the nucleus. TAZ bind to YAP in cytoplasm and then the complex translocates in the nucleus. In our setting, we observed by immunofluorescence that in untreated hDPSCs, the expression of YAP and TAZ was clearly evident both at cytoplasmic and nuclear level, indicating that YAP/TAZ pathway is activated. On the contrary, after YAP/TAZ inhibitor-1 treatment, YAP and TAZ expression was strongly reduced and the localization was predominantly in the cytoplasm (Figure 7).

Effect of HA-based compounds on Osteogenic Differentiation of hDPSCs in 3D Culture

The response of hDPSCs to HA-based gels was then investigated in a microenvironment that more closely mimicked 3D structure of bone tissue. To obtain this, we seeded hDPSCs onto Gingistat collagen sponges and cultured in OM, with and without hyaluronans, for 21 days.

The Alizarin Red staining showed that all hyaluronans induced an accumulation of dye (Figure S5). In particular, HHA showed calcification nodules clearly defined indicating the presence of specific areas of matrix mineralization. Whereas, HCC treatment showed homogeneous staining (Figure S5).

The immunofluorescence staining showed that the cells in the treated samples were organized more homogeneously on the surface and the expression of OC and OPN was significantly higher in the cells treated with HHA and HCC (Figure S5).

Discussion

HA is a biopolymer largely used in clinical applications and regenerative medicine for its peculiar characteristics such as biocompatibility, biodegradability, and nonimmunogenicity [16]. Positive effects of HA are reported in several conditions, yet there is a widespread fog regarding the HA's molecule weights effects on different biological processes. This applies especially on osteogenic differentiation and osteoblast activity where the effects of HA are neglected. In this study, we show that HA formulations based on different molecular weights have different impact on hDPSCs osteogenic differentiation. We also show HA induces YAP/TAZ signaling to promote osteogenic genes and matrix formation.

hDPSCs have been extensively used as a model for osteogenesis as they are naturally committed to osteogenic phenotype [4, 5]. Indeed, they have been used to test the osteoconductivity of different biomaterials such as titanium implants, as well as the capacity of drugs to induce osteogenesis both *in vitro* and *in vivo* [17]. Moreover, they were successfully grafted in humans to repair bone defects [18]. The osteogenic differentiation is a multistep process characterized by three main stages: proliferation, matrix maturation, and mineralization. It is regulated by different genes including OC, BSP, and OPN that are temporally and spatially controlled. Osteocalcin is the most abundant protein of bone where it plays a key role in the differentiation of osteoblast progenitors, with significant up-regulation during both matrix synthesis and mineralization. BSP promotes osteoblast differentiation and functions as a calcification nucleator in the early phases of mineralization [19–23]. Osteopontin is considered as a late effector of mineralization [24]. The three hyaluronans formulations (HCC, LHA, HHA) increased OC, BSP, and OPN expression promoting osteogenic differentiation, with HCC being the most effective. HCC stimulates an upregulation of bone related markers already after 7 of treatment, and a matrix formation at 7–14 as demonstrated by Alizarin Red S staining. This is of paramount interest for clinical use of HA as these results were obtained in standard medium, without any osteogenic inducer. HCC, but also HHA, may be used to stimulate and promote the bone differentiation of endogenous stem cells improving the regeneration of damaged tissue.

HA is the main ligand of CD44 [25], thus we have then evaluated CD44 expression by hDPSCs after hyaluronans treatment. We found that HCC promoted CD44 expression in specific areas of hDPSCs membrane, suggesting that it binds to CD44 with greater efficiency than other formulations for which the CD44 distribution was not homogeneous. Previous studies have showed that CD44 is an upstream regulator of YAP/TAZ signaling [26]. We found that HCC induced a strong up-regulation of YAP, RUNX1 and RUNX2 and down-regulated TAZ already at 7 days of treatment. Moreover, HCC also promoted the gene expression of CTGF and ANKDR1, YAP/TAZ downstream target genes. This indicated that HCC increases YAP/TAZ transcriptional activity, which promotes bone differentiation. The other hyaluronans are also capable of enhancing the activity of YAP/TAZ, but to a lesser extent than HCC.

To confirm the role of hyaluronans in the activation of YAP/TAZ pathway, we inhibited this pathway using YAP/TAZ inhibitor-1. After treatment, TAZ expression was completely depleted and this was correlated with a strong downregulation of all osteogenic markers, both at gene and protein levels. In fact, we found that TAZ depletion inhibited the formation of YAP/TAZ complex in the cytoplasm and its translocation into the nucleus. Following this, downstream genes, including bone related genes were not activated. Taking into consideration all the above, we suggest that HCC binds CD44 on hDPSCs surface and induces the activation of YAP/TAZ pathway, with YAP and TAZ translocation in the nucleus and expression of bone related markers promoting bone differentiation (Fig. 8).

The model presented here resembled only in part the hDPSCs osteogenic differentiation. Bone tissue is a complex 3D structure where mineralization represents a fundamental process both in bone remodeling and calcium metabolism [27]. Therefore, we investigated the potentiality of hyaluronans to promote mineralization using an 3D culture model and loading collagen sponges with hDPSCs and hyaluronans.

Having found that, again, HCC and HHA stimulate the mineralization with an increased expression of OC and OPN, this furtherly highlights the ability of HCC, and then HHA, to stimulate osteogenesis and mineralization.

Conclusions

Taken together, our data demonstrated that HCC induces and accelerates osteogenic differentiation and improves the mineralization via YAP/TAZ pathway. HCC shows greater stability than other linear HA formulations, and a wide array of bioactive effects. HCC has the potential to stimulate the stem cells to differentiate in bone tissue without any bone inducing factors. This gel is easy to inject, due to low viscosity notwithstanding a high HA concentration respect to other commercialized formulations. It may act *in vivo* as viscous and adhesive gel to precisely stimulate the endogenous stem cells to differentiate in the injured site, supporting the translational value of the presented results towards new clinical approaches aiming at bone regeneration especially in small bone defects.

Abbreviations

HA: Hyaluronic Acid; ECM: Extracellular Matrix; ASCs: Adipose Stem Cells; hMSCs: human Mesenchymal Stem Cells; hDPSCs: human Dental Pulp Stem Cells; HCC: Hybrid Cooperative Complexes; HHA: High molecular weight Hyaluronic Acid; LHA: Low molecular weight Hyaluronic Acid; DMEM: Dulbecco's modified Eagle's medium; OM: Osteogenic medium; ARS: Alizarin Red S; OC: Osteocalcin; OPN: Osteopontin; BSP: Bone sialoprotein.

Declarations

Ethics approval and consent to participate All experimental procedures involved were approved by the Ethics Committee of University of Campania approved on June 12th, 2005, Internal Registry: Experimentation #914 and were performed in line with the principles of the Declaration of Helsinki. Human dental pulps were extracted from teeth of healthy adults (aged 21–38 years) as described previously⁷. All participants signed the Ethical Committee (University of Campania Internal Ethical Committee) consent form. Consent for publication Not applicable. Availability of data and materials All data generated or analysed during this study are included in this published article (and its supplementary information files). Competing interests The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. Funding This study was supported by Contratto di sviluppo CDS 000463 - Altergon Italia Srl and short-term grants by Bioteknet scpa. Authors' contributions M.L.N., A.S., V.V., M.C.: performing experiments, acquisition of data, analysis and interpretation of data. GFN: interpretation of data; V.D.: editing of manuscript. L.L.: provision of study materials. G.P., C.S., and V.T.: conception and design of study, interpretation of data, writing manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Primer sequences.

Gene	Name	Sequence	T _a	
OC	Osteocalcin	Forward	5-CTCCACACTCCTCGCCCTATTG-3	58°C
		Reverse	5-CTTGGACACAAAGGCTGCAC-3	
OPN	Osteopontin	Forward	5-GCCGAGGTGATAAGTGTGGTT-3	58°C
		Reverse	5-TGAGGTGATGTCCTCGTTCTG-3	
BSP	Bone sialoprotein	Forward	5-CTGGCACAGGGTATACAGGGTAG-3	60°C
		Reverse	5-ACTGGTGCCGTTTATGCCTTG-3	
YAP	Yes-associated protein 1	Forward	5-CAACTCCAACCAGCAGCAAC-3	55°C
		Reverse	5-TTGGTAACTGGCTACGCAGG-3	
TAZ	Transcriptional co-activator with PDZ-binding motif	Forward	5-TGGACCAAGTACATGAACCACC-3	55°C
		Reverse	5-TGCCTTCTATGCTCCCTCCT-3	
hCTGF	human-Connective tissue growth factor	Forward	5-AGGAGTGGGTGTGTGACGA-3	57°C
		Reverse	5-CCAGGCAGTTGGCTCTAATC-3	
hANKDR1	human-Ankyrin repeat domain-containing protein 1	Forward	5-AGTAGAGGAACTGGTCACTGG-3	57°C
		Reverse	5-TGGGCTAGAAGTGTCTTCAGAT-3	
RUNX-1	Runt domain transcription factors 1	Forward	5-AACCCAGCATAGTGGTCAGC-3	57°C
		Reverse	5-CATGGCTGCGGTAGCATTTC-3	
RUNX-2	Runt domain transcription factors 2	Forward	5-ACCAGCAGCACTCCATATCTCTAC-3	57°C
		Reverse	5-CTTCCATCAGCGTCAACACCATC-3	
HRPT	Hypoxanthine-guanine phosphoribosyltransferase	Forward	5-TGACCTTGATTTATTTTGCATACC-3	60°C
		Reverse	5-CGAGCAAGACGTTTCAGTCCT-3	

Figures

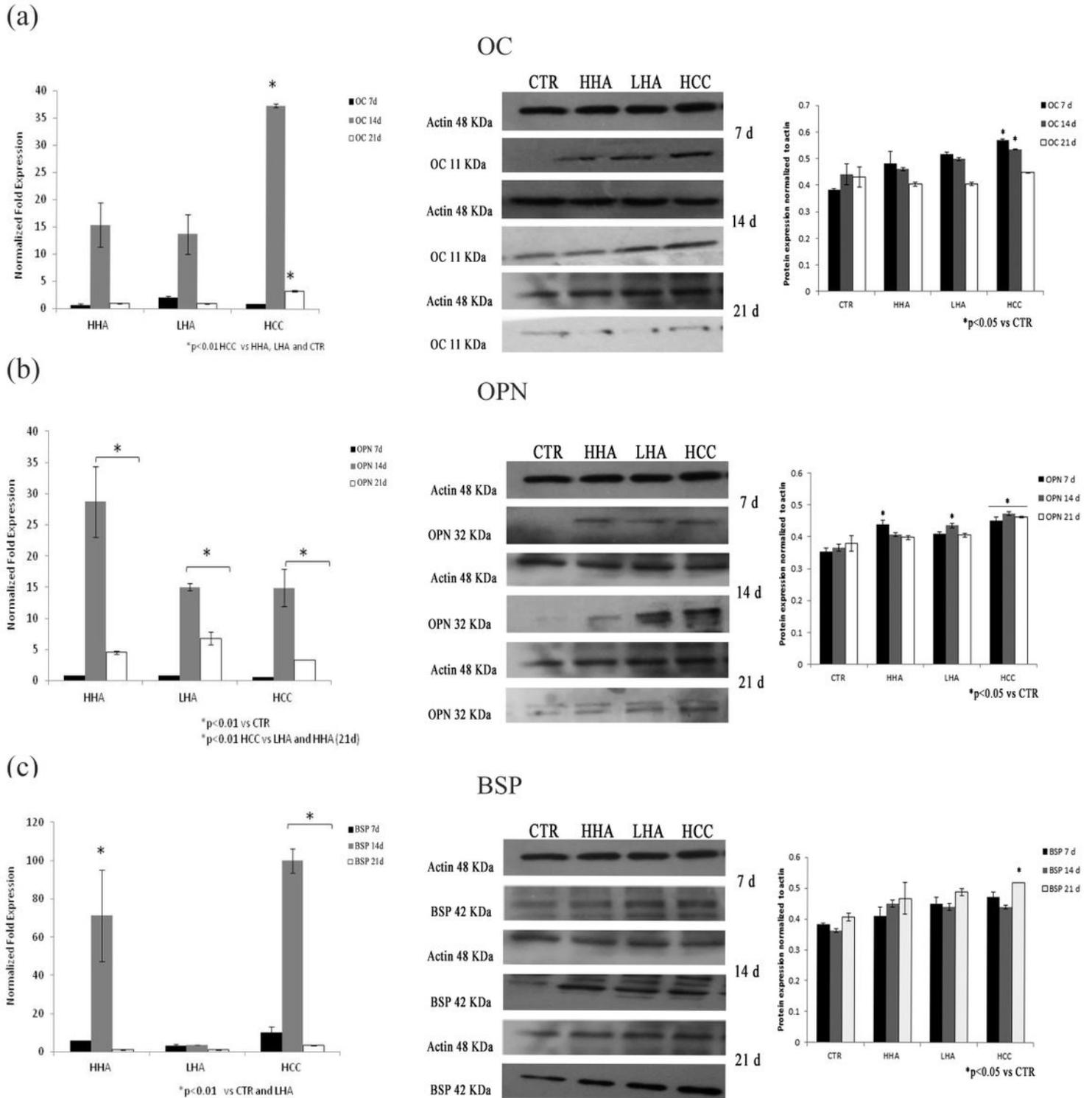


Figure 1

Analysis of OC, OPN and BSP gene expression at 7, 14 and 21 days by qRT-PCR and western blotting in standard medium. (a) OC gene expression was up-regulated with a paramount activity of HCC at 7 and 14 days of treatment both at gene and protein level; (b) OPN gene and protein expression increased after

HA treatment in 14 days; (c) BSP gene expression increased strongly at 14 days after HHA and HCC treatment, while protein expression is slightly higher in all HA treatments. The results are expressed as the mean \pm SD of three independent experiments. * p <0.01 versus CTR and other hyaluronans.

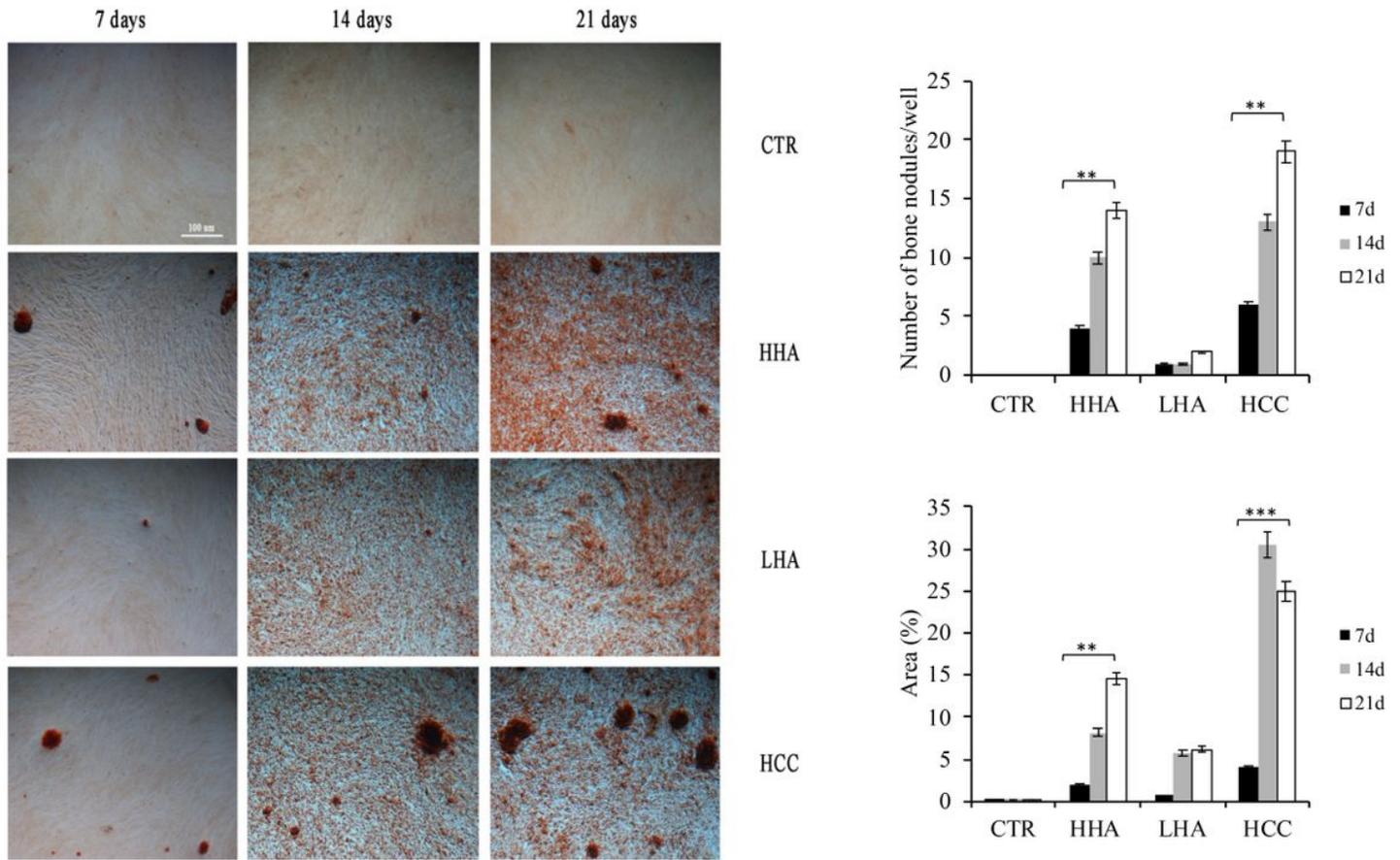


Figure 2

Calcification nodules evaluation at 7,14 and 21 days by Alizarin Red S in standard medium. Already at 7 days, Alizarin Red staining showed the formation of calcification nodules in all treated samples. At 14 and 21 days, HHA and HCC led to a greater number of calcification nuclei compared to LHA and control. This was confirmed also by calculating the area percentage of positive staining. The results are expressed as the mean \pm SD of three independent experiments. ** p <0.01 HHA vs CTR, *** p <0.001 HCC vs CTR.

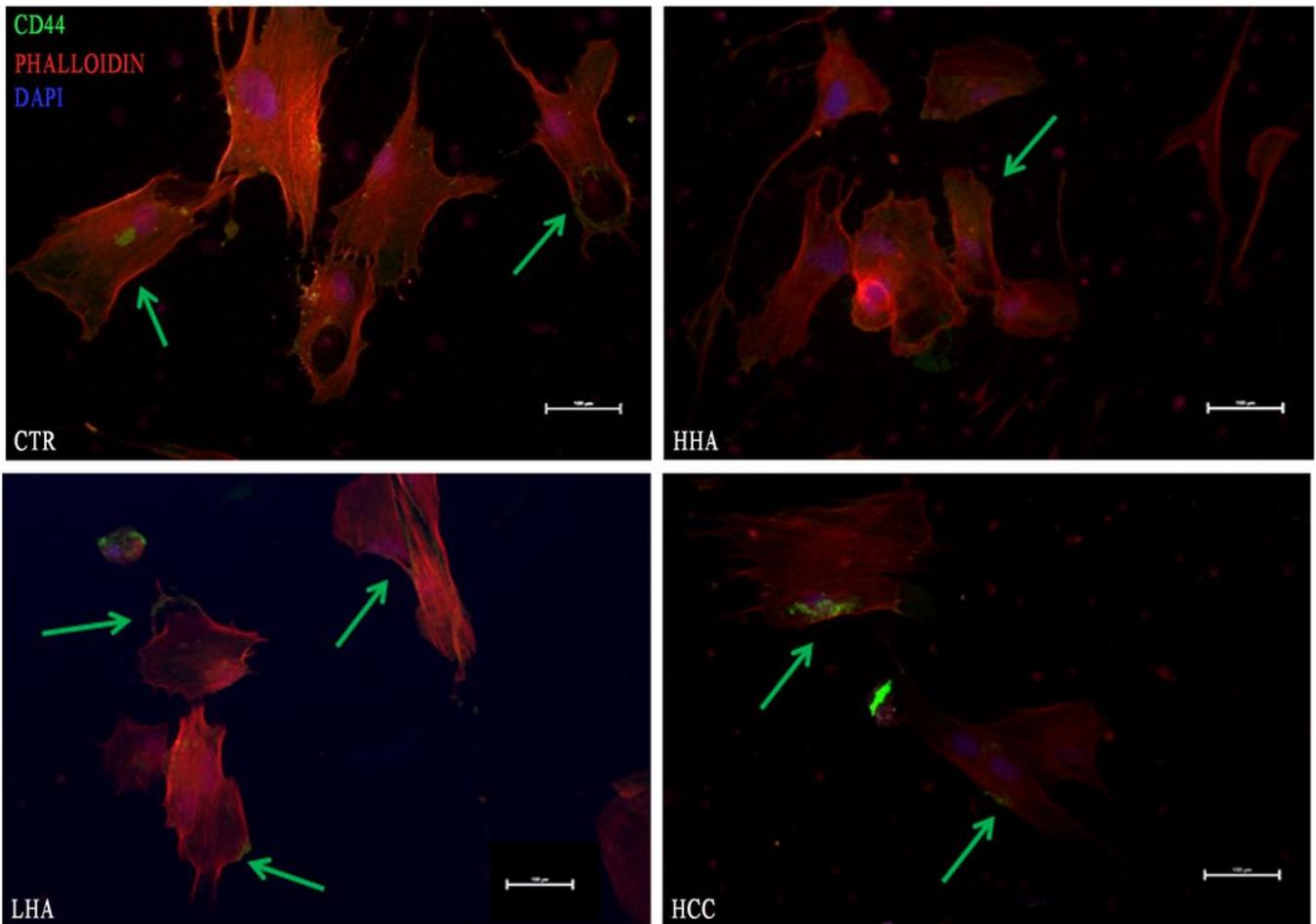


Figure 3

Evaluation of CD44 expression in hDPSCs after hyaluronan treatment in standard medium. CD44 marker was mainly localized on the hDPSCs cell surface with more areas of positivity after HCC treatment. CD44 in green, phalloidin in red; nuclei in blue.

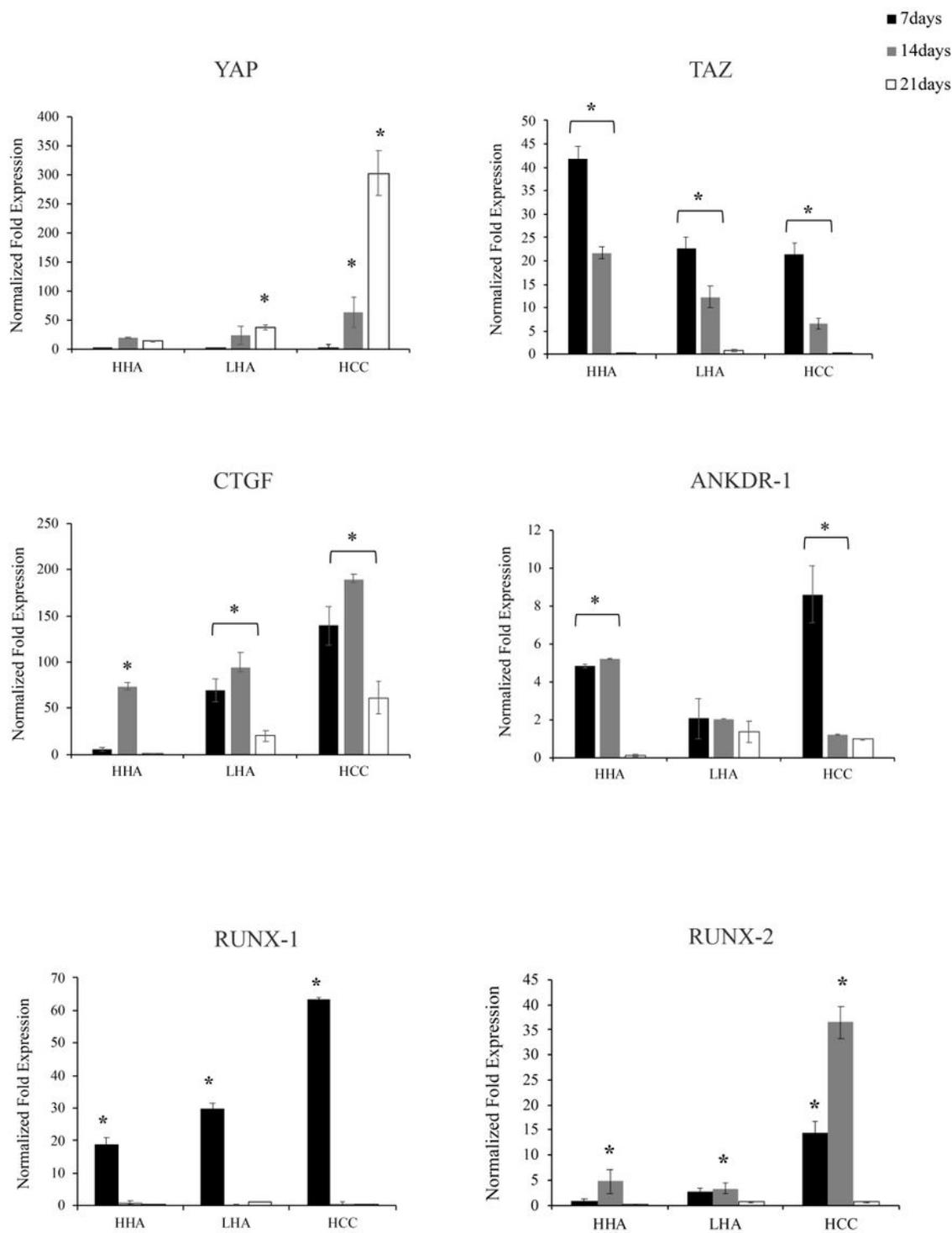


Figure 4

Analysis of YAP, TAZ, CTGF, ANKDR-1, RUNX-1 and RUNX-2 gene expression at 7, 14 and 21 days by qRT-PCR in standard medium. YAP is slightly up-regulated from 14 to 21 days by HHA, LHA, and strongly activated by HCC; TAZ expression increased earlier, at 7 and 14 days, in particular with HHA; CTGF mRNA levels were up-regulated by all HA-based gels up to 14 days, with a better up-regulation exerted by HCC; ANKDR-1 gene expression was strongly up-regulated by HCC already at 7 days; RUNX-1 gene expression

was increased by all hyaluronans at 7 days, with a better effect of HCC; RUNX-2 increased with HCC up to 14 days. The results are expressed as the mean \pm SD of three independent experiments. * $p < 0.01$ vs CTR.

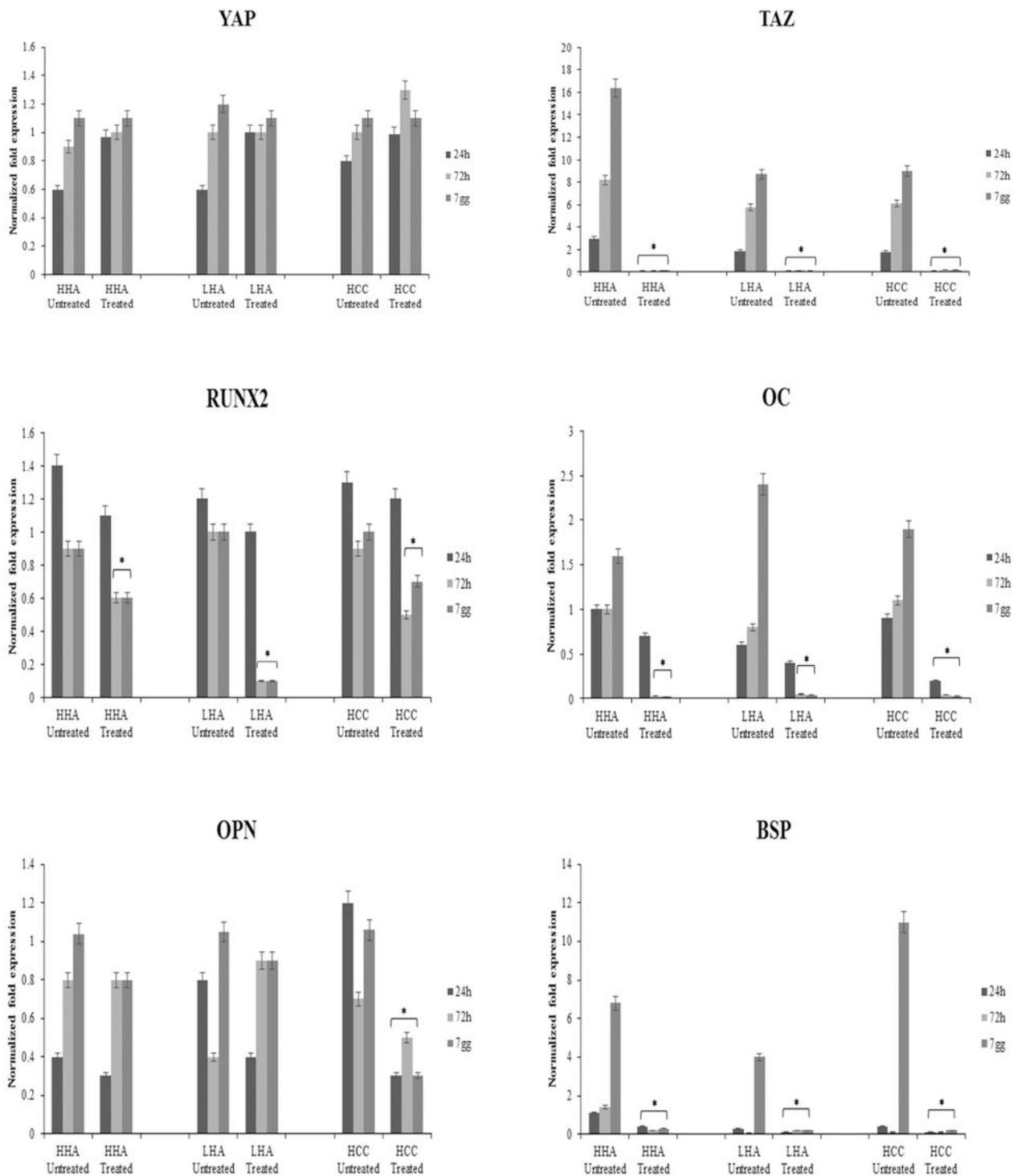


Figure 5

Analysis of YAP, TAZ, RUNX-2, OC, OPN and BSP gene expression at 24, 72 hours and 7 days by qRT-PCR in standard medium after YAP/TAZ inhibitor-1 treatment. The expression of YAP slightly increases after YAP/TAZ inhibitor-1 treatment; TAZ expression is drastically reduced in treated cells; RUNX-2 mRNA levels

are strongly downregulated in treated cells, in particular at 72h and 7d; all osteogenic related markers, OC, OPN and BSP, are strongly reduced in treated cells. The results are expressed as the mean \pm SD of three independent experiments. * $p < 0.01$ vs Untreated.

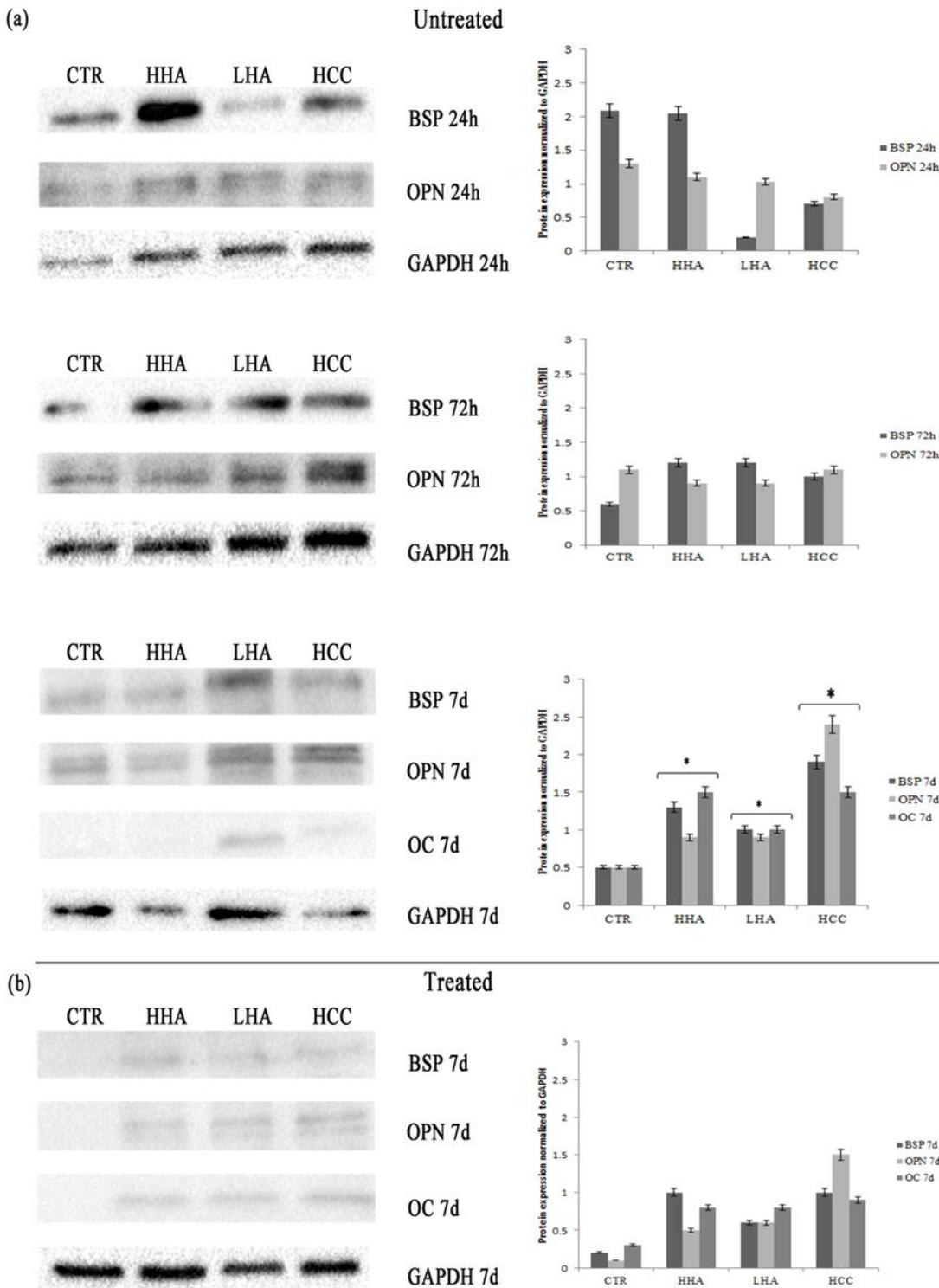


Figure 6

Analysis of OC, OPN and BSP gene expression at 24, 72 hours and 7 days by western blotting in standard medium after YAP/TAZ inhibitor-1 treatment. The expression of OC, OPN and BSP is completely depleted

in cells treated with YAP/TAZ inhibitor-1 at 24 and 72h. At 7 days only weak signal of protein expression is appreciable.

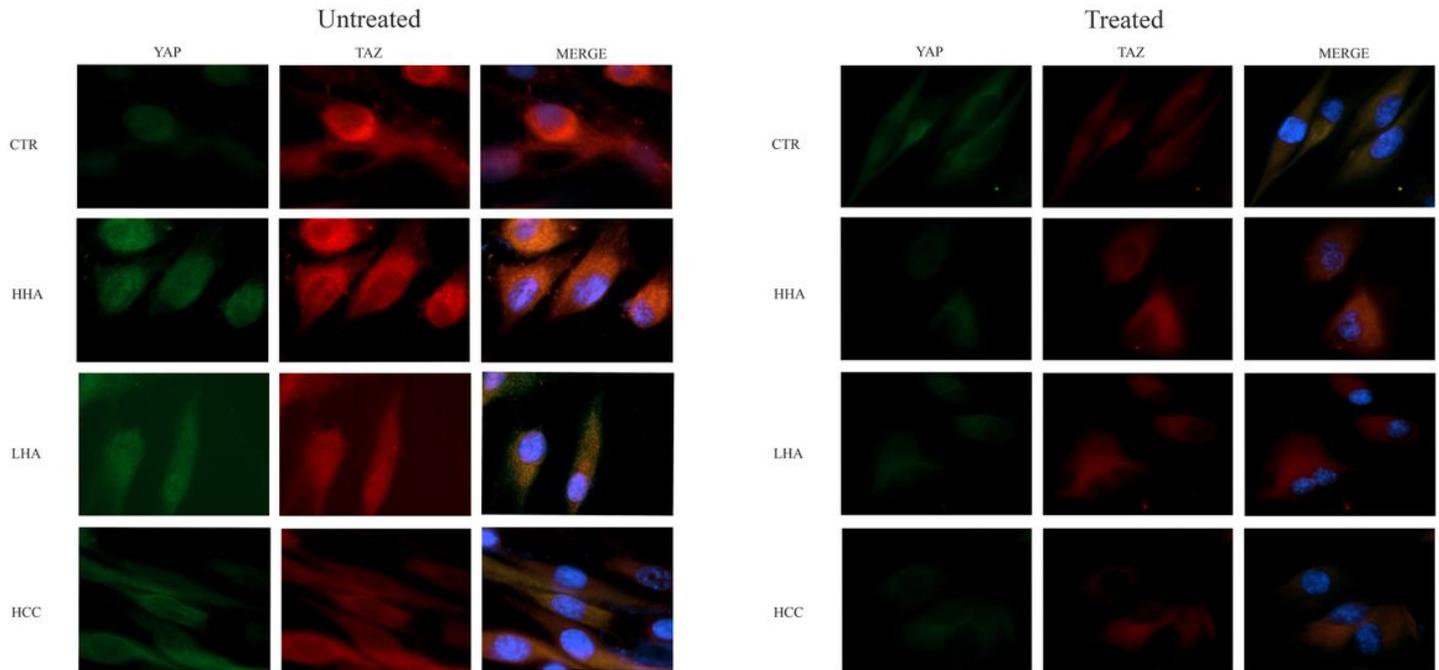


Figure 7

Analysis of YAP and TAZ, expression at 72 hours by immunofluorescence in standard medium after YAP/TAZ inhibitor-1 treatment. The expression of YAP and TAZ was clearly evident both at cytoplasmic and nuclear level in untreated cells; after YAP/TAZ inhibitor-1 treatment, YAP and TAZ expression was strongly reduced and the localization was predominantly in cytoplasm. Original magnification: 100X.

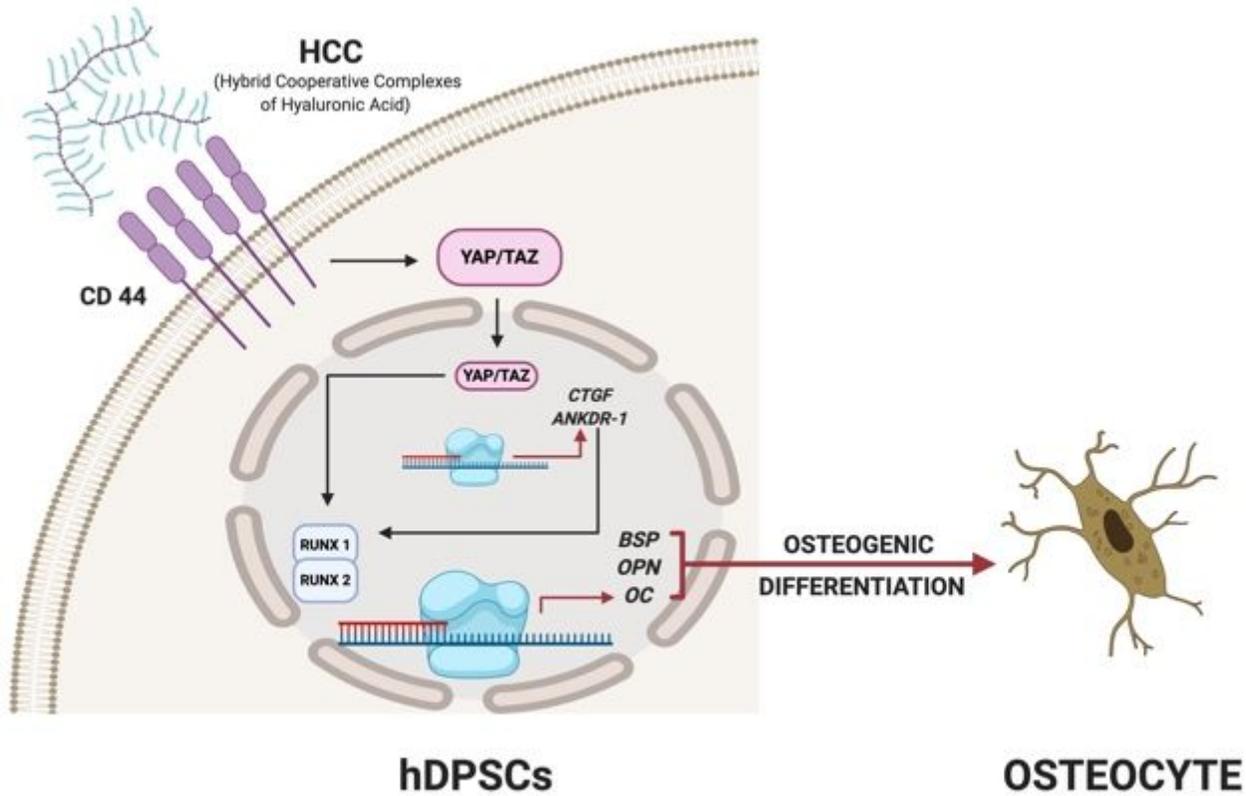


Figure 8

Proposed mechanism of HCC effect on hDPSCs. Hybrid Cooperative Complexes (HCC)-hyaluronan promotes CD44 expression on hDPSCs surface and induces activation of YAP/TAZ pathway. YAP/TAZ complex translocates in the nucleus and induces activation of bone related markers such as osteocalcin, osteopontin and bone sialoprotein, promoting bone differentiation.

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