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ORIGINAL ARTICLE

Role of polyamines in hypertrophy and terminal differentiation of osteoarthritic chondrocytes

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Abstract Polyamines are naturally occurring, positively charged polycations which are able to control several cellular processes in different cell types, by interacting with negatively charged compounds and structures within the living cell. Functional genomics in rodents targeting key biosynthetic or catabolic enzymes have revealed a series of phenotypic changes, many of them related to human diseases. Several pieces of evidence from the literature point at a role of polyamines in promoting chondrocyte differentiation, a process which is physiological in growth plate maturation or fracture healing, but has pathological consequences in articular chondrocytes, programmed to keep a maturational arrested state. Inappropriate differentiation of articular chondrocytes results in osteoarthritis. Thus, we have studied the effects of exogenously added spermine or spermidine in chondrocyte maturation recapitulated in 3D cultures, to tease out the effects on gene and protein expression of key chondrogenesis regulatory transcription factors, markers and effectors, as well as their posttranscriptional regulation. The results indicate that both polyamines are able to increase the rate and the extent of chondrogenesis, with enhanced collagen 2 deposition and remodeling with downstream generation of collagen 2 bioactive peptides. These were able to promote nuclear localization of RUNX-2, the pivotal transcription factor in chondrocyte hypertrophy and osteoblast generation. Indeed, samples stimulated with polyamines showed an enhanced mineralization, along with increased caspase activity, indicating increased chondrocyte terminal differentiation. In conclusion these results indicate that the polyamine pathway can represent a potential target to control and correct chondrocyte inappropriate maturation in osteoarthritis.

Keywords Polyamines · Chondrocytes · Hypertrophy · Terminal differentiation · Osteoarthritis

Abbreviations

AR-S	Alizarin red-S
DDR	Discoidin domain receptors
DFMO	α-Difluoromethylornithine
ECM	Extra-cellular matrix
FBS	Fetal bovine serum
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
KD	Knockdown
MMP	Matrix metalloproteinase
OA	Osteoarthritis
ODC	Ornithine decarboxylase
PCR	Polymerase chain reaction
PTH	Parathormone

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Introduction

During endochondral ossification, growth plate chondrocytes proceed through tightly regulated stages, involving

proliferation, hypertrophy, apoptosis and tissue mineralization (Goldring et al. 2006; Drissi et al. 2005). The maturation of chondrocytes is arrested in articular cartilage, where their differentiation toward a more terminal hypertrophic phenotype is prevented. Chondrocytes normally keep homeostasis of articular cartilage through a limited turn-over of extra-cellular matrix (ECM) components. However, in pathological conditions, such as osteoarthritis (OA), the degenerative rheumatic disease with the highest prevalence and economic impact, articular chondrocytes display a disorganized recapitulation of endochondral ossification (Drissi et al. 2005; Kawaguchi 2008). OA chondrocytes are diverted from their homeostasis and undergo cellular reaction patterns that include proliferation, hypertrophy and apoptosis, leading to ECM degradation and calcification, and ultimately loss of tissue integrity and function. Stimuli acting on chondrocytes, such as mechanical and oxidative stress, pro-inflammatory factors and ECM degradation products, may contribute to arthritis progression by influencing a network of signaling pathways, transcription factors, matrix-degrading enzymes and paracrine/autocrine mediators, comprising cytokines and chemokines (Marcu et al. 2010; Borzi et al. 2004).

The natural polyamines, spermidine and spermine, and their precursor putrescine are small, flexible polycations that can specifically bind to nucleic acids, proteins, phospholipids and glycosaminoglycans (GAG) in vitro and influence gene expression, signaling pathways and ionic fluxes in intact cells (Gerner and Meyskens 2004; Homma et al. 2005; Bachrach 2005). Their cellular content is highly regulated by enzymatic and transport systems (Pegg 2009). In particular, ornithine decarboxylase (ODC), the first enzyme in polyamine biosynthesis, is critical for maintaining polyamine levels. Polyamine acetylation, catabolism and interconversion not only contribute to the fine regulation of polyamine levels, but also can result in the production of potentially cytotoxic H_2O_2 and aldehydes (Agostinelli et al. 2007; Casero and Pegg 2009). Polyamines can also be bound to proteins covalently, e.g. by the action of transglutaminases that can incorporate them into proteins and even form cross-links (Bachrach 2005).

Polyamines are best known as essential factors for cell proliferation, but they can be involved in other cellular responses, such as differentiation, hypertrophy and apoptosis (Cetrullo et al. 2010; Seiler and Raul 2005; Lentini et al. 2004). Actually evidence indicates that polyamines play multiple physiological roles and that alteration of their metabolism and levels may be involved in many disease states (Pegg 2009). Polyamines are ubiquitously present in cells and tissues, but their role in normal and pathological cartilage is not clear. Pioneer studies showed that increases in ODC and polyamine levels occurred in matrix-induced sequential differentiation of cartilage, bone and bone marrow in vivo (Rath and Reddi 1981) and that addition of exogenous polyamines to cultures of growing rabbit costal chondrocytes promoted GAG production, a marker of the differentiated chondrocyte phenotype (Takano et al. 1981). Moreover, parathormone (PTH) was able to induce ODC activity in quiescent chondrocytes prior to GAG synthesis, which in turn was prevented by an ODC inhibitor (Takigawa et al. 1981; Takano et al. 1983). The authors concluded that the rise in polyamine levels induced by PTH was essential for the expression of a differentiated phenotype of chondrocytes. More recently, we have found that ODC is induced in a human chondrocyte cell line by other extra-cellular messengers, such as lysophosphatidic acid and insulin-like growth factor-1 (Facchini et al. 2005a), which can regulate chondrocyte proliferation and differentiation in growth plate (Hurst-Kennedy et al. 2009; Phornphutkul et al. 2004).

It has been reported that polyamines are more concentrated in the ossifying area of epiphyseal cartilage and accumulate outside the cells where they have been proposed to be directly involved in the mechanism of calcification (Vittur et al. 1986). In addition tissue transglutaminase, which can use polyamines as a substrate, is present in growth plate cartilage, but not in articular cartilage (Aeschlimann et al. 1993); its expression correlates with events of terminal differentiation of chondrocytes and its externalization occurs before matrix mineralization.

Although the roles of polyamines in joint diseases are poorly investigated, previous researches have documented enhanced levels of polyamines in rheumatoid arthritis (Furumitsu et al. 1993) and the efficacy of an ODC inhibitor in preventing experimentally-induced arthritis in mice (Wolos et al. 1990). Recent results from our laboratory suggest that polyamine depletion induced by a specific ODC inhibitor reduces not only cell proliferation, but also p65 subunit NF-κB binding to DNA, as well as the expression of chondrocyte hypertrophy-related genes MMP-13 and IL-8 in immortalized human chondrocytes stimulated by tumour necrosis factor-α (Facchini et al. 2005b). Furthermore, polyamine depletion inhibits apoptosis-associated molecular events in monolayer cultures of human chondrocytes (Flamigni et al. 2007; Stanic et al. 2006), while intracellular accumulation of N^1,N^{11}-diethylnorspermine, a synthetic polyamine analogue, is able to facilitate chondrocyte death (Stanic et al. 2008; 2009). Altogether these results suggest that polyamines might favor some features of OA, such as transition of chondrocytes to hypertrophy and apoptosis and their response to inflammatory cytokines. In the present study, we have employed micromasses prepared from OA articular chondrocytes to investigate the effect of polyamines on molecular markers and events leading to terminal

differentiation of chondrocytes. Micromass cultures represent a convenient 3-D in vitro model recapitulating various aspects of chondrocyte maturation and OA disease (Olivotto et al. 2007; 2008; Borzi et al. 2010).

Materials and methods

Materials

α-Difluoromethylornithine (DFMO) was a generous gift of Patrick M. Woster, Wayne State University, Detroit, MI. Spermine, spermidine and all other biochemical reagents were obtained from Sigma Chemical Company (St. Louis, MO).

Cell cultures and treatments

With local Ethics Committee approval, primary chondrocytes were obtained by sequential enzymatic digestion (Olivotto et al. 2008) of tibial plateau cartilage removed from 12 OA patients undergoing knee arthroplasty, expanded in culture up to passage 1 (p1) and then used according to the particular experimental procedure.

For experiments aimed at evaluating the effect of polyamine addition/inhibition on chondrocyte differentiation to hypertrophy and terminal differentiation in 3D culture, p1 chondrocytes were seeded at 6,000 cells/cm2 and kept under regular medium (D-MEM 10% FCS) or treated with 1 mM DFMO for 72 h during the exponential phase of growth. Then, micromass seeding was performed as detailed in Olivotto et al. (2007) and micromasses were left to mature across 3 weeks with medium change every second day. Three different culture conditions were compared: control micromasses corresponding to micromasses kept in D-MEM, 10% FCS and 50 μg/ml ascorbic acid; DFMO micromasses, i.e. micromasses established with cells pre-treated with DFMO as detailed above and kept in the above detailed medium with the addition of 1 mM DFMO and polyamine-treated micromasses: i.e. micromasses kept under the addition of either spermine or spermidine at 5 μM concentration or in a range of increasing concentration (0, 0.01, 0.1, 1, 10 μM). Addition of polyamines was carried out in the presence of 1 mM aminoguanidine to control for a possible toxicity of the polyamines due to their oxidation by the amino oxidase present in the bovine serum. Micromasses were used for immunohistochemistry or western blot or real time PCR essentially as described in (Borzi et al. 2010).

In addition to the experiments performed on control/polyamine/DFMO treated micromasses, some selected experiments were performed on micromasses established from chondrocytes treated as described in (Olivotto et al. 2008) to obtain a stable and penetrant knockdown (KD) of IKKα. Briefly, IKKα KDs were achieved by spinoculation mediated transduction of early passage primary articular chondrocytes with IKKα3 retroviral vectors containing IKKα specific short-hairpin RNAs (shRNAs). The amphotyped retroviruses were prepared from Phoenix A packaging cells as described in (Olivotto et al. 2008) and in particular, IKKα3 vector was obtained with shOligos containing 19 nt complementary to a sequence starting at Nt 1288 in the coding region of IKKα (Accession# AF012890) and subcloned into the pSuper.retro(Puro) moloney retroviral vector according to the manufacturer (OligoEngine Inc.). Seventy-two hours after retroviral transduction shRNA expressing cells were selected for puromycin resistance (1.5 μg/ml) with 3 changes of media over 6 days. Results obtained with IKKα KD chondrocytes were compared to cells from the same patient infected by a retroviral vector harboring a firefly luciferase specific shRNA (GL2) as a negative control (Elbashir et al. 2001).

On the other hand, to carry out a quantitative determination of the mineralizing ability of cultures upon treatment with polyamines, p1 chondrocytes from four different patients were seeded in 10% FCS D-MEM in 4–5 replicates in 96 well culture plate and left for 96 h. Then addition of 1, 5 or 10 μM spermidine was done and left for additional 72 h before proceeding to alizarin red staining and quantitative destaining as detailed below.

Polyamine analysis

The effectiveness of DFMO in depleting the intracellular pool of polyamines was assessed by measuring the polyamine level after DFMO treatment. Polyamines were separated and quantified in acidic cellular extracts by HPLC after derivatization with dansyl chloride (Stefanelli et al. 2001). Polyamine content was expressed as nmol/mg protein.

Enzymatic assays

ODC activity was measured as detailed in (Flamigni et al. 1997). The activity of caspase enzymes was measured as described in (Facchini et al. 2005b) and expressed as mU/μg DNA, quantitated by Picogreen as detailed below.

Immunoblotting

The determination of the levels of selected chondrogenesis transcription factors, markers and regulators was carried out by western blotting, essentially as described (Flamigni et al. 1999). Briefly, total cellular lysates were obtained by extracting micromasses with lysis buffer and vigorously homogenized with disposable pestles (Sigma) and

sonication. Aliquots of 20 μg total protein were loaded in the wells of 10% polyacrylamide gels, which were subsequently transferred onto PVDF membranes and subjected to immunodetection. Signals were detected with appropriate secondary antibodies and revealed with an ECL Advance kit (Amersham, Little Chalfont, UK). SOX9 was stained with goat polyclonal antiserum (R&D Systems). Monoclonal anti-GAPDH (clone 6C5, Chemicon-Millipore) served as loading controls. Three different experiments were carried out.

Real Time PCR

Real Time PCRs were done as previously described (Olivotto et al. 2008; Borzi et al. 2010) with forward (F) and reverse (R) PCR primers including: GAPDH (NM_002046) 579–598F and 701–683R); ODC1 (NM_002539.1) 1146–1167F and 1365–1348R; Col2a1 (NM_001844 transcript variant 1): 4247–4264F and 4499–4485R; (NM_033150, transcript variant 2): 4040–4057F and 4292–4278R; SOX9 (NM_000346) 952–968F and 1069–1054R; MMP-13 (NM_002427) 496–511F and 772–756R; β-catenin (NM_001904) 1031–1052F and 1313–1293R; RUNX-2 variant transcript 3 (NM_004348) 864–883F and 968–949R; RUNX-2 variant transcripts 2 (NM_001015051) and 1 (NM_001024630) 716–735F and 820–801R; Col10a1 (NM_000493) 71–86F and 209–194R. Primers were annealed at 56°C, except Col2a1, at 58°C and SOX9, at 60°C. Five different experiments were analyzed.

Immunohistochemistry

Micromasses were prepared and analyzed by IHC along with hematoxylin counterstaining of nuclei essentially as described (Olivotto et al. 2008) and signals developed with a biotin/streptavidin amplified, alkaline phosphatase based detection system (Biogenex, San Ramon, CA) with fuchsin as substrate. Two different experiments were analyzed. Antigen unmasking was carried out with 0.02 U/ml chondroitinase ABC 20' at RT. Collagen 2 was stained with anti-Col2 mouse monoclonal antibody (5 μg/ml) (clone 2B1.5 Zymed Invitrogen). NITEGE neo-epitopes (produced after activity of aggrecanases on aggrecan) were detected with rabbit anti-sera, kindly provided by Dr John Mort (Shriners Hospital for Children, Montreal, Canada), and diluted 1:3,000. C1,2C neoepitopes (produced after collagenolytic cleavage, which in cartilage is exerted by MMP-13, the collagenase with the highest activity on collagen 2 and also by MMP-1), were detected with a C1,2C polyclonal rabbit antibody (IBEX Pharmaceuticals, Montreal, Canada) diluted 1:100. C1,2C neo-epitopes in maturing micromasses were analyzed and quantified with 7 non-overlapping images from multiple slides, which were obtained with a Nikon Eclipse 90i microscope equipped with NIS (Nikon Imaging Software) elements (Nikon Inc) as previously described (Olivotto et al. 2008). The area fraction values (i.e. the percentage area of positive signal with respect to the total area of the tissue examined) were used for statistical comparison between the different conditions.

Confocal microscopy

Subcellular RUNX-2 distribution was evaluated in two different experiments by confocal microscopy using double immunofluorescence in 4% paraformaldehyde-fixed 5 μm sections of chondrocyte micromasses. RUNX-2 staining was performed with 5 μg/ml rat anti-hRUNX-2 antibody (R&D) with signals revealed by an anti-rat TRITC antibody (1:100) (SIGMA). Nuclear staining was observed with Sybr green (1:10,000 fold dilution) (Molecular Probes, Eugene OR). The acquisition of TRITC labeled anti-hRUNX-2 antibody and Sybr green labeled DNA signals were performed using a Radiance 2000 confocal laser scanning microscope (BioRad), equipped with argon–krypton laser (excitation at 488- and 568-nm for Sybr Green and TRITC fluorescence, respectively). The Sybr green emission signal (rendered in green) was detected by a photomultiplier tube preceded by an emission filter (HQ 515/30 nm), and the TRITC emission signal (rendered in red) was detected by a photomultiplier tube preceded by an emission filter (E580LP). Optical sections were spaced approximately 1 μm along the z-axis, and were digitized with a scanning mode format of 512 × 512 pixels and 256 gray levels. Four fields (acquired with a Nikon 40 × 1.3.A objective) of two sections derived from differently treated micromasses (control, spermidine, spermine) were analyzed. Only sections passing through nuclei were submitted to confocal imaging. This was done to avoid confocal sections acquired either above or beneath the nuclear planes, which could give somewhat misleading information on fluorophore co-localization. Image processing was performed with ImageJ 1.42q software (NIH, USA) and NIS (Nikon Imaging Software) elements (Nikon Inc., USA); image rendering was performed with Adobe Photoshop. Co-localized pixels appeared as yellow–orange with varying degrees of luminosity depending on the intensity of the fluorophore signal, and the extent of co-localization. RUNX-2 nuclear localization was reported as mean intensity (i.e. usual statistical mean of intensity values of pixels, NIS Elements AR–Image Analysis) of objects identified by a binary mask created on Sybr green stained nuclei areas.

Mineralization assays

Micromasses were scored for mineralized areas by alizarin red staining as previously described (Olivotto et al. 2007). Calcium deposition in high density monolayer was quantitatively evaluated as described in (Wang et al. 2000). Prior to quantitative destaining, pictures of the cells cultured under the various conditions were taken. To compensate for the different cell number due to effects of the treatment on cell viability, alizarin red-S counts were normalized for the amount of DNA in the wells, calculated with Picogreen staining of the cells left in the wells and fluorimeter analysis, as described in (Facchini et al. 2005b).

Statistics

All data are expressed as mean ± standard error of the mean (SEM). Non-parametric statistics were employed due to the small size of data sets as is typical for primary patient samples. Means of groups were compared by Mann–Whitney *U* test with STATISTICA 7 statistical software (StatSoft, Tulsa, OK) or GraphPad Prism (GraphPad software, San Diego, CA). Data are considered as significant when $p < 0.05$.

Results and discussion

ODC activity is related to chondrocyte hypertrophy and terminal differentiation

We have previously documented the pleiotropic activity of IKKα in driving chondrocyte differentiation towards hypertrophy and terminal differentiation (Olivotto et al. 2008). IKKα ablation had a broad range of effects on OA chondrocyte physiology affecting both ECM metabolism (enhanced ECM formation as evidenced by the accumulation of highly organized Col2 fibers formation mainly due to impaired ECM remodeling) and chondrocyte differentiation towards hypertrophy and terminal differentiation (reduced cell size of chondrocytes grown in monolayer and enhanced survival of chondrocytes in differentiated micromass cultures). A role for IKKα in terminal differentiation has also been reported in the differentiation of keratinocytes (Hu et al. 2001). Unpublished data obtained in the context of differentiating chondrocytes in in vitro micromass cultures indicate that IKKα was linked to a gene expression profile exhibiting a higher than random predominance of several effectors of cell communication, ECM-receptor interaction and cellular adhesion. It could be conceivable that IKKα works as an upstream master regulator of a series of genes involved in chondrocyte differentiation. Among others, we have here investigated whether enhanced expression and activity of ODC, the key enzyme in polyamine biosynthesis, can contribute to IKKα effects. Our results reported in Fig. 1 show: (1) a statistical significant decrease in ODC mRNA expression in IKKα KD micromasses at 1 week of maturation compared to control micromasses; (2) a statistical significant decrease in ODC activity in IKKα KD high density monolayer. The correlation between IKKα KD and the reduction in both ODC mRNA expression and ODC activity suggests that ODC can represent an IKKα downstream target gene contributing to its effects on chondrocyte hypertrophy. On the other hand, to support the correlation between ODC and hypertrophy, we found a marked inhibition of RUNX-2, one of the essential transcription factors orchestrating terminal chondrogenic hypertrophy, as a consequence of the pre-treatment with DFMO, a specific inhibitor of ornithine decarboxylase. On this purpose, micromasses were prepared from growing chondrocytes kept in the presence of DFMO to reduce the intracellular polyamine pool. In fact, a pilot experiment showed that DFMO treatment of chondrocytes in their exponential phase of growth was effective in reducing the intracellular concentration (nmol/mg) of putrescine (from 0.98 to undetectable) and spermidine (from 3.2 to 0.48), while spermine content was hardly affected (from 3.46 to 3.33). Collectively these data indicate that polyamine metabolism is relevant for the induction of chondrocyte hypertrophy.

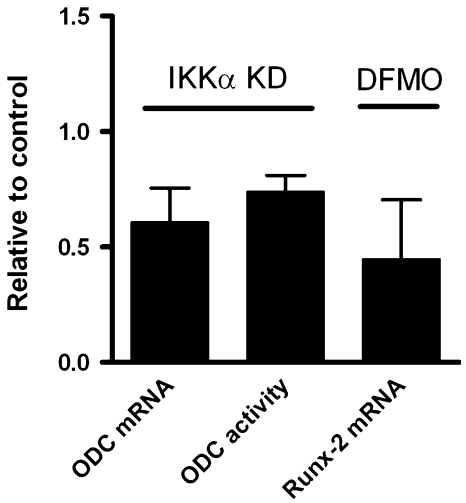
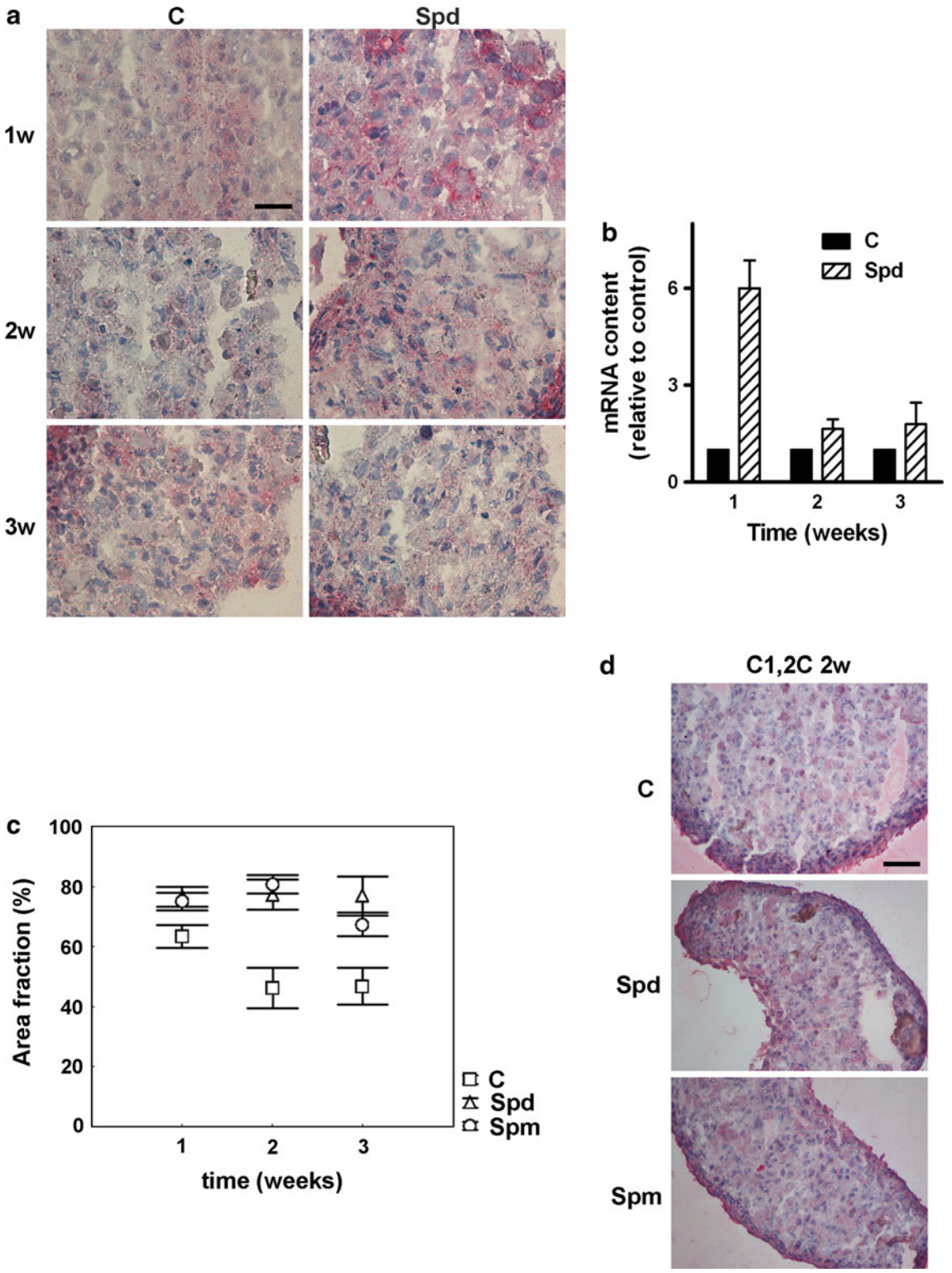


Fig. 1 ODC activity is related to chondrocyte hypertrophy and terminal differentiation. Hypertrophy occurring in micromass maturation depends on IKKα, and is prevented by its ablation. IKKα KD is associated with significant inhibition of both ODC mRNA expression (*first column* representing 12 different experiments evaluated at 1-week micromass maturation; $p = 0.0341$) and ODC activity (*second column* representing 5 different experiments; $p = 0.0225$). Conversely, the expression of RUNX-2, the pivotal transcription factor in chondrocyte hypertrophy is decreased upon DFMO mediated inhibition of ODC activity (*third column* representing 3 different experiments)



Exogenously added polyamines increase the extent and the rate of chondrogenesis and ECM remodeling

3D micromass cultures represent a convenient model to evaluate the effects of added molecules or targeted inhibition of key molecules on the progression of chondrogenesis, a tightly regulated process controlled by cellular interactions with the surrounding matrix, growth and differentiation factors, and other environmental factors that modulate cellular signaling pathways and

◀ **Fig. 2** Exogenously added polyamines increase the extent and the rate of chondrogenesis and ECM remodeling. P1 chondrocytes were seeded into micromasses in control (10% FCS D-MEM, 50 μg/ml ascorbic acid) medium (*C*) or polyamine (5 μM spermidine *Spd*, or spermine *Spm*) added medium. Chondrocyte micromasses were left to mature for 3 weeks and at 1, 2, 3 weeks time point samples were used for IHC of collagen (**a** *scale bar* 50 μm) and collagenase induced-C1,2C neoepitopes accumulation (**d** *scale bar* 100 μm, showing actual images at 2 week time point, and **c** showing cumulative results of C1,2C image analysis), as well as for real time PCR evaluation of collagen 2 expression (**b** showing cumulative data of collagen 2 expression relative to control in three different experiments). In particular, the bioactive C1,2C peptide evaluated with image analysis as described in "Materials and methods" showed significantly higher accumulation in polyamine treated samples at all time points: 1-week micromasses Spd 5 μM versus C: $p = 0.0181$ and Spm 5 μM versus C: $p = 0.0254$; 2-week micromasses Spd 5 μM versus C: $p = 0.0060$ and Spm 5 μM versus C: $p = 0.0017$; 3-week micromasses Spd 5 μM versus C: $p = 0.0152$ and Spm 5 μM versus C: $p = 0.0127$

transcription of specific genes in a temporal-spatial manner. Micromasses recapitulate the whole process of chondrocyte maturation, whose default route is terminal differentiation and endochondral ossification. An exception to this rule is represented by healthy articular cartilage, where chondrocytes are actively prevented to complete this process and are kept in a "maturational arrested state" by molecular constraints. Pivotal transcription factors, such as SOX9 and RUNX-2, and interactions among them determine whether the chondrocytes remain resting within a pre-hypertrophic extracellular matrix in healthy articular cartilage or undergo hypertrophic maturation prior to ossification as sometimes occurs in OA. An overview of many reports in literature (Goldring et al. 2006) indicates that in healthy articular cartilage chondrocytes are in a state with RUNX-2 at its least level of expression across chondrogenesis, and SOX9 in a level just below its maximum which is reached when resting chondrocytes become activated in early OA to repair early tissue damage.

Progression of chondrogenesis requires a timely controlled sequence of events: extracellular matrix deposition, extracellular matrix remodeling and production of bioactive peptides, such as collagen 2 fragments, which push the progression towards hypertrophy and terminal differentiation (Tchetina et al. 2005; Gauci et al. 2008).

A positive effect of polyamine addition on the synthesis of GAG in growing rabbit costal chondrocytes has been already reported (Takano et al. 1983). Here we show (Fig. 2a) that spermidine addition to micromasses enhances protein accumulation of collagen 2, the other key component of articular cartilage ECM and the most abundant collagen type in cartilage. Spermine yielded similar results (not shown). Noteworthy, upon spermidine or spermine addition, micromass maturation progressed at a faster rate compared to control conditions, as assessed on the basis of collagen 2 expression change in kinetics. At the mRNA level (Fig. 2b), the magnitude of the increase of collagen 2 expression yielded by 5 μM spermidine was even more marked, suggesting a higher rate of ECM remodeling in micromasses treated with exogenously added polyamines. The sequence of the events which control chondrocyte maturation has been detailed in recent papers which point at the key role of bioactive peptides arising from collagenolytic degradation of collagen 2 in boosting the activation of hypertrophy (Gauci et al. 2008; Tchetina et al. 2005). At present, the state of the art in OA pathomechanisms indicates that the very first event in cartilage ECM degradation is represented by aggrecan cleavage (van den Berg 2011), which then uncovers the discoidin domain receptors (DDR) (Vogel et al. 2006) on the chondrocytes. We investigated whether polyamine can mediate GAG degradation via induction of aggrecanase activity as assessed by evaluation of the NITEGE neoepitopes. Results indicate no polyamine induction of aggrecanase activity (not shown). However, a previous report indicated the ability of polyamines and particularly of spermidine of displacing proteoglycan

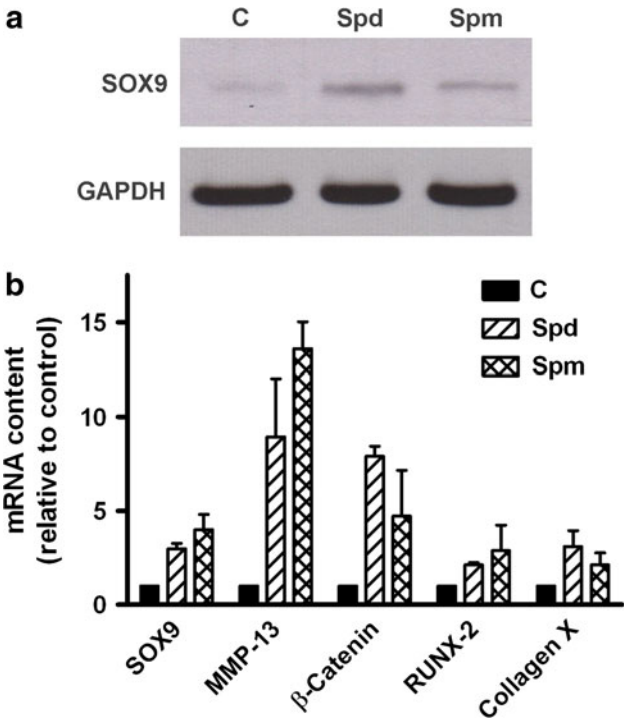


Fig. 3 Exogenously added polyamines increase the expression of key chondrogenesis regulatory transcription factors, markers and effectors. 5 μM spermidine or spermine increased the protein amount of the key chondrogenesis-driving transcription factor SOX9 in micromasses. Upper image **a** shows western blot results of one representative experiment at 2 weeks maturation. 5 μM spermidine or spermine increased SOX9 mRNA expression relative to control, along with similar effects on gene expression of the major effectors and markers of chondrocyte hypertrophy (**b** *bar graph*, showing cumulative data of 3–5 experiments). *C* control, *Spd* spermidine-treated micromasses, and *Spm* spermine-treated micromasses

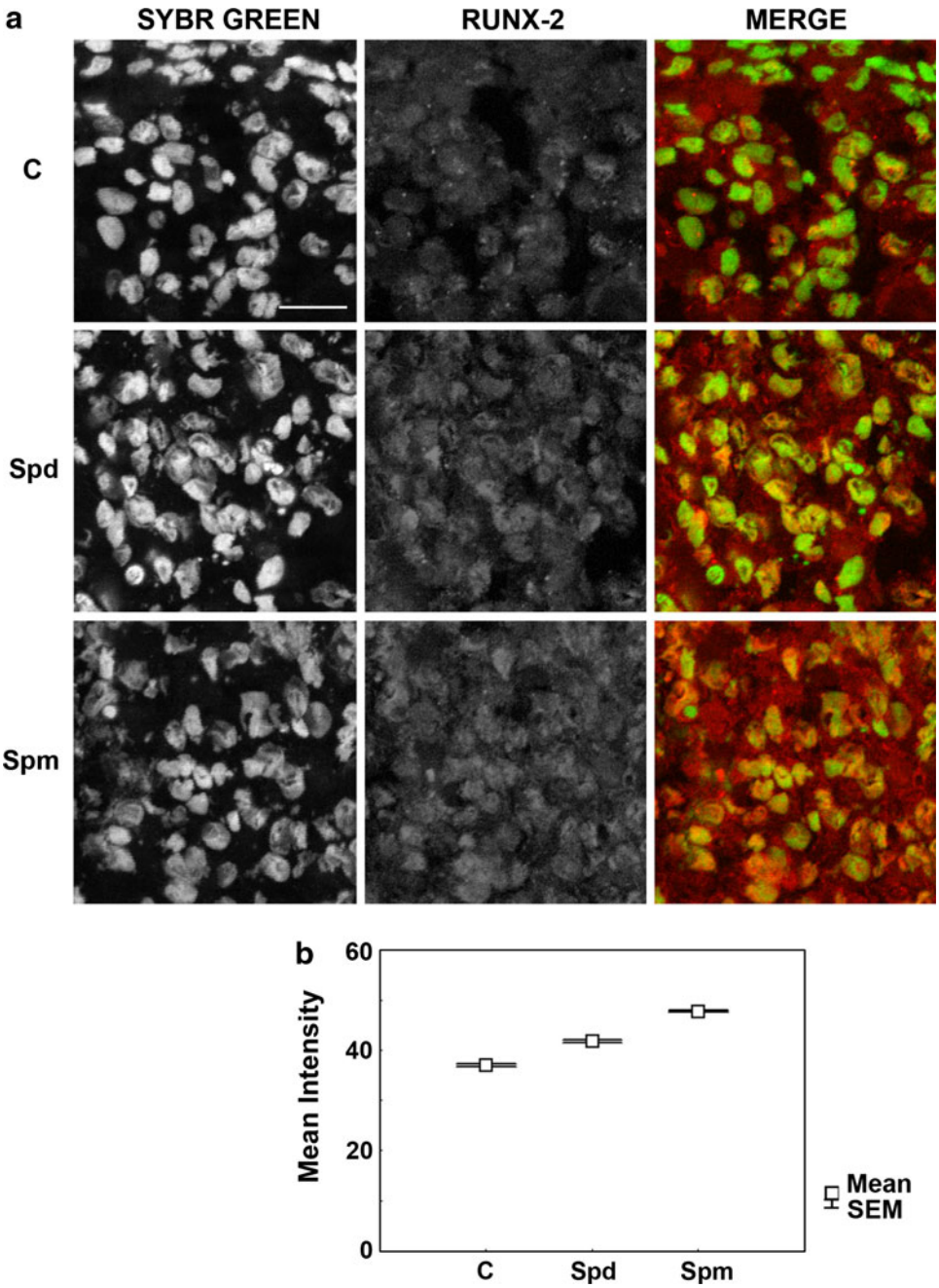


Fig. 4 Exogenously added polyamines increase the nuclear translocation of RUNX-2. Micromasses at 1, 2, 3 week of maturation were analyzed for subcellular distribution of RUNX-2, as detailed in "Materials and methods". 5 μM spermidine or spermine increased RUNX-2 nuclear translocation relative to control at 3 week time point. **a** Shows representative images acquired with confocal microscopy of subcellular distribution of RUNX-2 in 3-week micromasses: Sybr green nuclear staining, RUNX-2 staining, and merge. Original images were acquired with a 40× objective, *scale bar* 20 μm. **b** Shows Mean Intensity graph of TRITC labeled RUNX-2 in cell nuclei from control (*C*), spermidine- (*Spd*), and spermine-treated (*Spm*) micromasses. About 1 thousand cells were analyzed for mean intensity of RUNX-2 in the nuclear region, yielding a $p < 10^{-17}$ in the comparison of polyamine-treated micromasses versus control micromasses

subunits from their interaction with collagen in a cell free system (Vittur et al. 1986). It is likely and intriguing that a similar effect is also present in our experimental condition and in OA cartilage. As a consequence of GAG removal, DDR receptors become triggered by interaction with the "naked" collagen 2 molecules and mediate a series of downstream activities including the effects on cell differentiation, ECM remodeling and cell cycle control. DDR-mediated p38 MAPK activation, in particular, can be responsible for induction of MMP-13 activity (Olivotto et al. 2007), which in turn promotes collagen 2 remodeling and production of C1,2C neoepitopes. Compared to control conditions, polyamines proved indeed able to promote an enhanced C1,2C deposition, which became more evident across micromass maturation (Fig. 2c, d). Figure 2c reporting quantitative image analysis of C1, 2C analysis across micromass maturation indicates that neoepitope accumulation is significantly increased in polyamine stimulated samples, particularly at 2 and 3 weeks maturation.

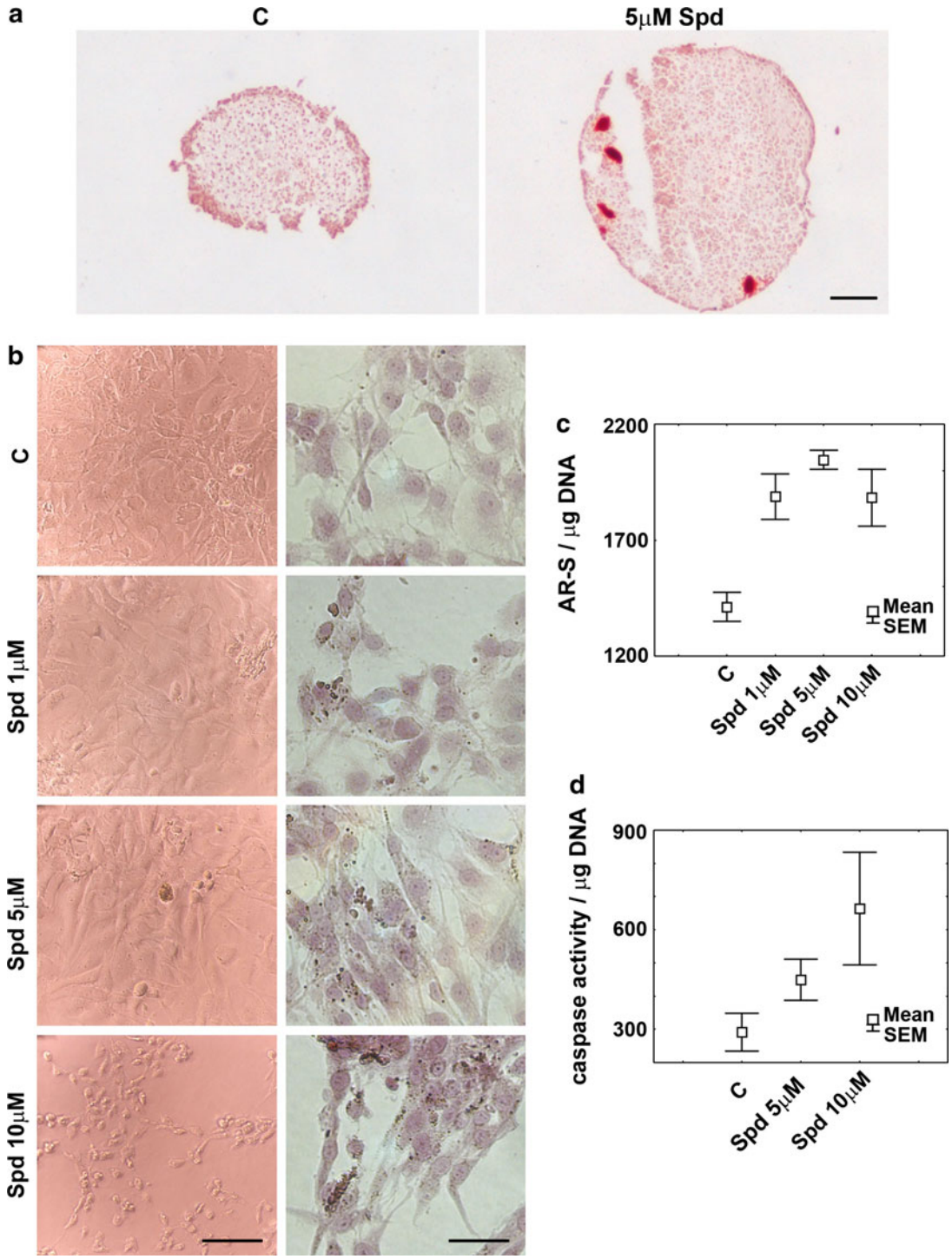


Fig. 5 Exogenously added polyamines increase terminal differentiation and mineralization. Micromasses at 3 weeks maturation upon polyamine addition showed increased mineralization as assessed with alizarin red staining (**a** shows a representative example upon 5 μM spermidine, Spd, *scale bar* 100 μm). Increased mineralization was also found in high density monolayer (4 replicate for each condition) kept under increasing concentrations of spermidine (1, 5 and 10 μM), as detailed in "Materials and methods". **b** Reports the images of chondrocyte cell cultures in the left column (*scale bar* 100 μm) and after alizarin red staining in the right column (*scale bar* 50 μm). The images show results from one representative experiment out of four experiments performed. **c** Reports cumulative data of the alizarin red bound to the cultures and normalized to the amount of DNA. Spermidine yielded a significantly increased mineralization as detected by means of alizarin red quantitative destaining (see "Materials and methods") at all concentrations tested, with $p = 0.0209$. **d** Reports evaluation of the caspase activity in high density chondrocyte monolayer culture showing increased values as a function of spermidine concentration

Exogenously added polyamines increase the expression of key chondrogenesis regulatory transcription factors, markers and effectors

Spermine or spermidine, when added at 5 μM, increased the content of SOX9 protein, a master transcription factor in chondrogenesis, as judged by Western blotting analysis (Fig. 3a). Both polyamines also increased mRNA content of SOX9, as analyzed by real time PCR (Fig. 3b). It should be noted that enhanced SOX9 expression characterizes not only growth plate chondrocytes during first stages, but also OA chondrocytes (Yang et al. 2006), which loose the maturational arrested state to re-enter their default differentiation route towards terminal differentiation (Drissi et al. 2005; van der Kraan and van den Berg 2008). Besides, both polyamines were able to enhance expression of genes related to chondrocyte hypertrophic maturation, such as RUNX-2, β-catenin, MMP-13 and collagen X.

Exogenously added polyamines increase the nuclear translocation of RUNX-2, the master transcription factors inducing chondrocyte hypertrophy

RUNX-2 is the pivotal transcription factor for chondrocyte hypertrophy and type X collagen expression as well as for osteoblast differentiation (Higashikawa et al. 2009). RUNX-2 activity is regulated at multiple levels, including transcription, translation and post-translational modification. To exert its function as a transcription factor, it needs to be directed to the nucleus via nuclear localization signal (Jonason et al. 2009). Polyamine addition to chondrocyte micromasses proved to be effective not only in inducing RUNX-2 mRNA (Fig. 3b) and protein (not shown), but also in increasing its nuclear localization significantly (Fig. 4). The enhanced nuclear localization of RUNX-2 in samples stimulated with polyamines can result from a positive feedback loop involving the sustained increased production of collagen 2 bioactive peptides (Fig. 2c, d).

Exogenously added polyamines increase terminal differentiation and mineralization

To see whether exogenously added polyamines could favor terminal differentiation and mineralization, sections of micromasses were treated with alizarin red staining as described (Borzi et al. 2010). Addition of spermidine (5 μM) proved to be effective in increasing the amount of calcium deposits (see Fig. 5a, showing a comparison between 3-week micromasses in control conditions or treated with spermidine). To provide statistical confidence to our findings, experiments were also carried out in high density monolayer culture conditions to exploit the method described in Wang et al. (2000). This method allows the accurate quantitation of the calcium deposits in the cultures, since alizarin red-S (AR-S) binds calcium stoichiometrically (2 mol of calcium per dye mole). It should be noted that the AR-S values were normalized to the number of cells remained in the wells. Indeed, the cells appear to dose-dependently suffer because of spermidine addition, with occurrence of features suggestive of apoptosis, such as chromatin condensation and detachment (Fig. 5b). Spermidine yielded a significant increased mineralization as detected by means of alizarin-red quantitative destaining (Fig. 5c). On the other hand, this increase in mineralization was accompanied by an increase in terminal differentiations as shown by a parallel rise in effector caspase activity (Fig. 5d). Collectively these data provide additional evidence that delivery of polyamines accelerates differentiation of chondrocytes down to terminal differentiation, since during hypertrophy caspase-3 activation is a prerequisite for eliciting alkaline phosphatase activity and mineral formation (Olivotto et al. 2007; Pucci et al. 2007).

Conclusions

Indication of a differentiation promoting activity of polyamines was firstly reported in growth plate cartilage and costal chondrocytes, but the present study clearly shows that this activity is also exerted with articular chondrocytes. The ODC-polyamine pathway could therefore represent a useful target to control the differentiation of chondrocytes. The findings of this paper and other previous results from our group indicate that this pathway could be usefully targeted to control inappropriate differentiation of osteoarthritic chondrocytes, to restore the functionality of the tissue or to finely tune the outcome of regenerative medicine approaches based on the transplantation of chondrocytes or on the use of mesenchymal stem cells. On the other hand, targeted delivery of these molecules could be exploited to enhance endochondral ossification in regenerative medicine approaches of bone reconstruction exploiting mesenchymal stem cells firstly induced to a chondrogenic phenotype.

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