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Chondrogenic Differentiation of Human Mesenchymal Stem Cells Results in Substantial Changes of Ecto-Nucleotides Metabolism

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ABSTRACT

Mesenchymal stem cells (MSCs) are population of adult stem cells and attractive candidates for cartilage repair due to their chondrogenic potential. Purinergic compounds (purinergic receptors and ecto-enzymes metabolizing nucleotides), together with nucleotides/nucleosides present in the extracellular environment, are known to play a key role in controlling the stem cells biological potential to proliferate and differentiate. Despite the available literature pointing to the importance of purinergic signaling in controlling the fate of MSCs, the research results linking nucleotides and ecto-nucleotidases with MSCs chondrogenic differentiation are indigent. Therefore, the aim of presented study was the characterization of the ecto-nucleotides hydrolysis profile and ecto-enzymes expression in human umbilical cord-derived MSCs and chondrogenically induced MSCs. We described substantial changes of ecto-nucleotides metabolism and ecto-enzymes expression profiles resulting from chondrogenic differentiation of human umbilical cord-derived MSCs. The increased rate of ADP hydrolysis, measured by ecto-nucleotidases activity, plays a pivotal role in the regulation of cartilage formation and resorption. Despite the increased level of NTPDase1 and NTPDase3 mRNA expression in chondrogenically induced MSCs, their activity toward ATP remains quite low. Supported by the literature data, we hypothesize that structure–function relationships in chondrogenic lineage dictate the direction of nucleotides metabolism. In early neocartilage tissue, the beneficial role of ATP in improving biomechanical properties of cartilage does not necessitate the high rate of enzymatic ATP degradation. J. Cell. Biochem. 116: 2915–2923, 2015.

KEY WORDS: MESENCHYMAL STEM CELLS; CHONDROGENESIS; CARTILAGE; ECTO-NUCLEOTIDES; NTPDases

Mesenchymal stem cells (MSCs) are population of adult stem cells that are easy to isolate, expand in in vitro culture and they are attractive candidates for cartilage repair due to their chondrogenic potential. Currently, the research interests are directed to the complete characterization of MSCs and the understanding of the mechanisms involved in their differentiation, including chondrogenesis. Despite some advances in the knowledge of MSCs biology, their biochemical and molecular properties are still poorly defined, particularly in the aspect of purinergic signaling and its regulatory role in differentiation processes.

It is widely known that purinergic compounds (purinergic receptors and ecto-enzymes metabolizing nucleotides) are present on the MSCs surface and together with nucleotides/nucleosides (primarily ATP and adenosine) in the extracellular environment play a key role in controlling the MSCs biological potential to proliferate and differentiate (Coppi et al., 2007; Glaser et al., 2012; Ciciarello et al., 2013). ATP and adenosine outside the cell signal through the activation of purinergic receptors: P1—for adenosine, and P2—for ATP/ADP or other tri- and diphosphonucleotides. The concentration of ecto-nucleotides and ecto-nucleosides is precisely controlled by ecto-nucleotidases and other enzymes metabolizing purines. These enzymes include the following: ecto-NTPDases (nucleoside triphosphate diphosphohydrolases), ecto-NPPs (nucleotide pyrophosphohydrolases/phosphodiesterases), ecto-alkaline phosphatases, and ecto-5′nucleotidase (Zimmermann et al.,

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2012). The nucleotide kinase group of ecto-enzymes is also involved in the regulation of ecto-nucleotide concentration by transferring the phosphate moiety between nucleotides. The main function of ecto-enzymes metabolizing nucleotides outside the cell is termination of nucleotide signal due to disengagement of ligands from their receptors (Zimmermann et al., 2000).

Many results show that the purinergic receptors are diversely expressed in various types of stem cells and differentiated mature cells, whereas less is known about expression of members of ecto-nucleotidases families (Mishra et al., 2006; Roszek et al., 2013a,b; Iser et al., 2014; Kaebisch et al., 2015). Nevertheless, the existing results rise the possibility to use these ecto-enzymes as predictive markers of MSCs biological potential and/or differentiation into mature cells.

The existing literature describes the role of purinergic signaling in controlling the fate of MSCs; however, the research results linking nucleotides and ecto-nucleotidases with MSCs chondrogenic differentiation are limited. The only reference is the recent results of Ode et al. 2013 pointing at 5′-nucleotidase as key regulatory factor in osteo-/chondrogenic differentiation of MSCs and underestimated therapeutic target to guide bone and cartilage regeneration.

Chondrocytes utilize the purinergic signaling pathway as a part of the mechanotransduction cascade in which ATP and its metabolites in the extracellular space can bind and signal through a variety of purinergic P2 receptors. Different subtypes of P2X (including P2X1, P2X2, P2X3, P2X4, P2X7) and P2Y (P2Y1, P2Y2, P2Y4 and P2Y6) nucleotide receptors have been identified on articular chondrocytes (Graff et al. 2003; Burnstock et al., 2013). Early studies showed that ATP and ADP induced in chondrocytes an increase in intracellular Ca^{2+} concentration, and that calcium signaling is an important regulator of chondrogenesis (Bulman et al., 1995). Recent results have provided a detailed link between purinergic receptors and Ca^{2+} homeostasis of chondrogenic progenitor cells (CPC). Various P2 receptor subtypes were identified on CPC surface: P2Y1 can bind ADP, P2Y4 is targeted by UTP, and ATP may evoke Ca^{2+} transients via detected P2X subtypes, as well as P2Y1 and P2Y2 (Matta et al., 2015).

The aim of presented study was the ecto-enzymes expression and determination of their hydrolytic activity toward ecto-nucleotides in human umbilical cord-derived MSCs and chondrogenically induced MSCs. Since the ecto-enzymes as elements of the purinergic system are diversely expressed in different cell types, they are suggested to play different roles in stem and differentiated cells. The impact of this article is on ecto-enzymes metabolizing nucleotides because the ecto-nucleotide metabolism can affect the microenvironment, modulating important pathophysiological processes, as for example, local inflammation and immune response. Moreover, since MSCs and chondrocytes are attractive cell populations for cell-based therapies, their complete purinergic characterization is important for the better understanding of their physiology, biological potential, and for effective therapeutic applications.

MATERIALS AND METHODS

CELL CULTURE

Umbilical cord-derived human MSCs (hUC-MSCs) were purchased from PromoCell and cultured, according to the manufacturer's protocol, in Mesenchymal Stem Cell Growth Medium® with 10% Supplement Mix® (both from PromoCell GmbH, Heidelberg, Germany), 100 U/ml of penicillin, and 100 μg/ml streptomycin, at 37 °C in humidified atmosphere containing 5% CO_2.

Cultured hUC-MSCs at passage 4 were induced to chondrogenic differentiation using MSC Chondrogenic Differentiation Medium® (PromoCell GmbH) according to the protocol supplied by the manufacturer. Aliquots of 10 μl (1×10^5 hUC-MSCs) were plated per well (of 24-well tissue culture plates) and incubated for 2 h in MSC Growth Medium®. Then the medium was changed to MSC Chondrogenic Differentiation Medium® (PromoCell GmbH) to induce chondrogenesis. The induced cells formed aggregates (micromasses) after 24–48–h. The medium was changed every third day for 21 days.

ENZYMATIC ACTIVITY DETERMINATION

Ecto-nucleotidases activity assays were carried out in incubation mixtures composed of isotonic Tris–HCl buffer of pH 7.4 with 4 mmol/L Mg^{2+} and 2 mmol/L Ca^{2+}, 2.5 mmol/L levamisole (alkaline phosphatase inhibitor), 0.1 mmol/L dipyridamole (adenosine deaminase inhibitor), and 1.2 mmol/L nucleotide (ATP, ADP, or AMP) as a substrate.

The MSCs for the determination of enzymatic activity were cultured in 12-well culture plates under standard conditions. Before experiment, the culture medium was discarded and cells washed with 1 ml PBS. Enzymatic reactions were initiated with the addition of 1 ml pre-warmed incubation mixture to the growing cells. In the case of chondrogenically induced MSCs, the micromasses were collected from the wells, washed with 1 ml PBS, and then 1 ml pre-warmed incubation mixture was added. The samples were incubated for 3 h at 37 °C. Every hour, 100 μl of reaction mixture was aspirated and added to 20 μl of 5 mol/L cold $HClO_4$ to terminate the enzymatic reaction. All test samples were immediately neutralized with 5 mol/L KOH and centrifuged at 5,000*g* for 10 min. The obtained supernatants were delipidated by shaking with n-heptane (1:5, v/v), centrifuged, and analyzed for purines concentration and composition using high-performance liquid chromatography (HPLC) method.

QUALITATIVE AND QUANTITATIVE ANALYSIS OF PURINES

The qualitative and quantitative analyses of purines in the reaction mixtures were performed by the HPLC method (Czarnecka et al., 2005). The samples were separated on the Chromolith Performance RP-18e column, 4.6 mm × 100 mm (Merck KGaA, Darmstadt, Germany, Europe) under isocratic conditions. The samples were eluted using 0.1 mol/L phosphate buffer of pH 7.0 along with 25 mmol/L TBA, 5 mmol/L EDTA, and 3% methanol. The presence of purines was detected at $\lambda = 260$ nm.

RT-PCR ANALYSIS

Total RNA from undifferentiated MSCs cultures and chondrogenically induced MSCs after 3 weeks of differentiation was isolated with TRI-Reagent (Sigma–Aldrich) in accordance to the manufacturer's instructions. Micromasses were digested with 0.2% collagenase type I for 30 min at 37 °C and thoroughly pestled prior to RNA isolation.

Isolated RNA was purified from genomic DNA using RapidOut DNA Removal kit (Thermo Scientific) and the reverse transcription

was carried out using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) according to manufacturer's protocol. One microgram of RNA was incubated with reaction mixture and reverse transcriptase for 10 min at 25 °C followed by 15 min at 50 °C. To stop the reaction, samples were incubated for 5 min at 85 °C. RT-PCR of relative expression of selected genes was performed with LightCycler 480 Instrument (Roche) using LightCycler 480 SYBR Green I Master reagent (Roche) according to manufacturer's protocol, with preincubation step at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s (denaturation), 60 °C or 63 °C for 30 s (hybridization), and 72 °C for 1 s (elongation). The specificity of each reaction was confirmed on the basis of the melting curve. The relative expression of analyzed genes was calculated with LightCycler 480 Software by the $2^{-\Delta\Delta Ct}$ method and normalized using the mean result of β-actin and GAPDH as reference genes. For amplification process, the primers and annealing temperatures shown in Table I were used.

STATISTICAL ANALYSIS

Data were expressed as mean ± standard error of mean (SEM) of at least three independent experiments performed in triplicate. The statistical analysis was done using the Student's *t*- test, with $P < 0.05$ considered statistically significant.

RESULTS

Human umbilical cord-derived MSCs (hUC-MSCs) are the population of adherent cells with a fibroblast-like morphology characteristic for MSCs (Fig. 1a). Functional assays confirmed their capacity to differentiate into osteogenic, adipogenic (data not shown), and chondrogenic lineage (Fig. 1b). Chondrogenic differentiation results in condensation of cells (micromasses formation) and production of extracellular matrix, composed of proteoglycans and glycosaminoglycans among others, as was evidenced by Safranin-O staining (Fig. 1c).

Additionally, the expression of lineage-specific marker proteins was verified by RT-PCR (Fig. 2). The expression of MSCs surface markers CD73 and CD105 decreased, whereas the CD90 expression stayed at almost the same level during chondrogenic induction. Collagen 2A1 expression increased and syndecan expression was downregulated during chondrogenesis. Both undifferentiated hUC-MSCs and chondrogenically induced MSCs were subjected to our experiments.

Despite the considerable literature pointing to the importance of purinergic signaling in stem cells physiology, the data reporting the activity of ecto-enzymes metabolizing nucleotides in human MSCs are limited (see Scarfì, 2014). Therefore, we analyzed the activity of ecto-nucleotidases in MSCs from the human umbilical cord tissue, as well as in the chondrogenically induced MSCs cultured in vitro (Table II).

We detected some substantial differences in the activity for ATP, ADP, and AMP degradation between undifferentiated MSCs and chondrogenically induced MSCs. The hUC-MSC efficiently hydrolyzed AMP, whereas their hydrolytic activity toward ATP and ADP was negligible. The differentiated cells hydrolyzed mainly ADP. We carried out the qualitative and quantitative analyses of produced nucleotides and nucleosides for the determination of hydrolytic activity profile on the surface of undifferentiated MSCs and chondrogenically induced MSCs (Figs. 3,4, and 5).

Incubation of growing MSCs with ATP or ADP confirmed negligible hydrolysis of these substrates (Fig. 3a, 4a). When ATP was added to the hUC-MSCs as substrate, the nucleotide was hydrolyzed primarily to ADP during the first hour of incubation. The small amounts of produced AMP exclude the participation of NPPs in ATP hydrolysis. After 2- and 3-hour incubation, the increase in ATP concentration was observed. Chondrogenically induced MSCs presented primarily the hydrolytic activity toward ADP (Fig. 3b, 4b). ADP to AMP hydrolysis mediated by ecto-nucleotidase(s) activity seems to be specific for chondrogenically induced MSCs. On the other hand, only undifferentiated MSCs had the ability to produce adenosine from AMP due to the activity of ecto-5′-nucleotidase (Fig. 5a).

Our further studies concerning the expression analysis of enzymes metabolizing nucleotides on the surface of undifferentiated MSCs and chondrogenically induced MSCs confirmed that ecto-nucleotidases were expressed distinctly, depending on the cell differentiation into chondrogenic lineage. We demonstrated that MSCs

TABLE I. Primer Sequences and Annealing Temperatures

Marker protein/ ecto-enzyme	Forward (5′→3′)	Reverse (5′→3′)	Product (bp)	Annealing temp. (°C)
CD90	CCCAGTGAAGATGCAGGTTT	GACAGCCTGAGAGGGTCTTG	185	60
CD105	CACTAGCCAGGTCTCGAAGG	CTGAGGACCAGAAGCACCTC	165	60
Collagen 2A1	GGGAGTAATGCAAGGACCAA	ATCATCACCAGGCTTTCCAG	175	63
Syndecan	GACTGCTTTGGACCTAAATG	ACTGGGCTATGAACAAAGAA	217	63
NTPDase1	GGGAAAGACGAGGAAAGAGG	TCTGGCAATGCTTTGTTCTG	176	63
NTPDase2	CTGACCCAGTACCCCTTTGA	CAGCTGGACTGGTTGTCTCA	214	63
NTPDase3	TTTCCCTGGACACCTTCAAC	TGTATTTGGGGCCAAGTCTC	184	60
NTPDase5	GCATTTGCCAACACCTTTTT	ACAGGGCTCTCTGTGATGCT	179	60
NTPDase6	GCACTGAAGCCAGGTCTTTC	GGGCCTTTTCTCCAGGTAAC	172	60
NTPDase8	GGCCTGGAATCTCCTCCTAC	TGACTGCTGCAAAGATGTCC	195	63
5′-nucleotidase (CD73)	CGCAACAATGGCACAATTAC	CAGGTTTTCGGGAAAGATCA	196	60
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG	185	60
β-actin	ATCCACGAAACTACCTTCAA	TCTTGATCTTCATTGTGCTG	166	60

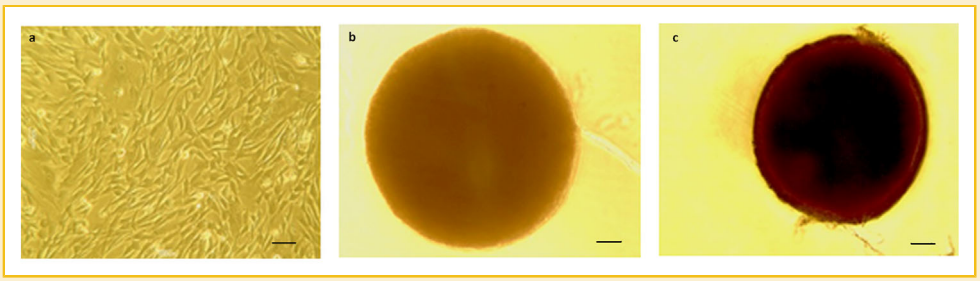

Fig. 1. Chondrogenic differentiation of hUC-MSCs. MSC cultures at passage 4 were induced with chondrogenic differentiation medium and cultured for 21 days as described in Materials and Methods section. (a) MSCs growing in standard culture medium; (b) chondrogenically induced MSCs—micromass after 21 days of culture in differentiation medium; and (c) Safranin-O stained micromass. Scale bar indicates 50 μm in picture (a) and 100 μm in (b and c).

expressed mainly 5′-nucleotidase and converted ecto-AMP to adenosine. The chondrogenic lineage-specific high expression of NTPDase1 and NTPDase3 (Fig. 6) may be responsible for ATP and ADP hydrolysis. Moreover, the soluble NTPDases 5 and 6 were expressed and it is not possible to exclude their participation in the nucleotides degradation during incubation.

DISCUSSION

Numerous studies have demonstrated the multifaceted aspects of extracellular nucleotides signaling in different biological processes in most tissues and cells, including stem cells. However, only recently, the extracellular nucleotides and their metabolites have

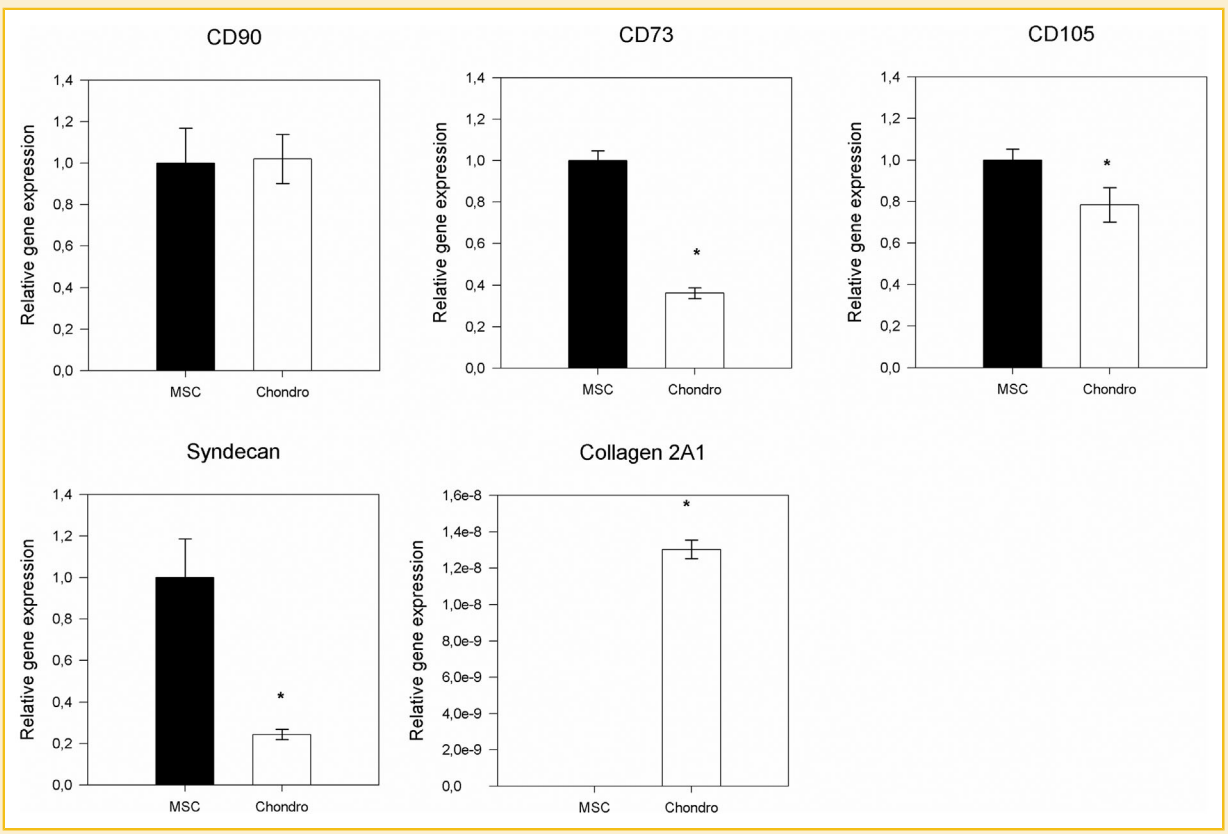

Fig. 2. Relative expression of specific markers mRNA in hUC-MSCs and chondrogenically induced MSCs. The relative expression of analyzed genes was normalized using mean result of both β-actin and GAPDH as reference genes. Data represent the fold expression of the markers in chondrogenically induced MSCs compared to MSCs, which have been normalized to 1. Bars represent mean ± SEM of at least three independent experiments performed in triplicate. Asterisk denotes significant difference from hUC-MSCs determined by the Student's *t*-test ($P < 0.05$).

TABLE II. Activities for the ATP, ADP, and AMP Degradation by Ecto-Enzymes of hUC-MSCs and Chondrogenically Induced MSCs. The Ecto-Enzymes Activity Was Determined Toward 1.2 mmol/L Nucleotide (ATP, ADP, or AMP) as a Substrate and Measured as nmole of Substrate Metabolized $\times$ Min^{-1} by 1×10^5 Cells.

Metabolized substrate and potential enzymes engaged	hUC-MSCs [nmole of substrate metabolized $\times$ min^{-1}]	Chondrogenically induced MSCs [nmole of substrate metabolized $\times$ min^{-1}]
ATP (ecto-NTPDases or ecto-NPPs)	0.316 ± 0.014	$0.644 \pm 0.030^*$
ADP (ecto-adenylate kinase or ecto-NTPDases)	0.003 ± 0.0001	$3.71 \pm 0.162^*$
AMP (ecto-5′NT)	5.51 ± 0.186	$0.597 \pm 0.113^*$

The concentrations of purines were determined using HPLC method as described in Materials and Methods section. Results are expressed as mean $\pm$ SEM.
*Significant difference from hUC-MSCs determined by the Student's *t*-test ($P < 0.05$).

been included among the molecular signals produced by MSCs, thus revealing their important roles in the maintenance, proliferation, and/or differentiation of stem cells (Glaser et al., 2012; Scarfì, 2014).

MSCs possess a significant display of purinergic receptors and ecto-enzymes on their surface enabling the concerted action of ecto-nucleotides and the precise control of their concentration, respectively (Zippel et al., 2012; Iser et al., 2014; Kaebisch et al., 2015). Enzymatic hydrolysis of extracellular nucleotides allows for signal adjustment and for modulating MSCs physiology or specifying the differentiation lineages.

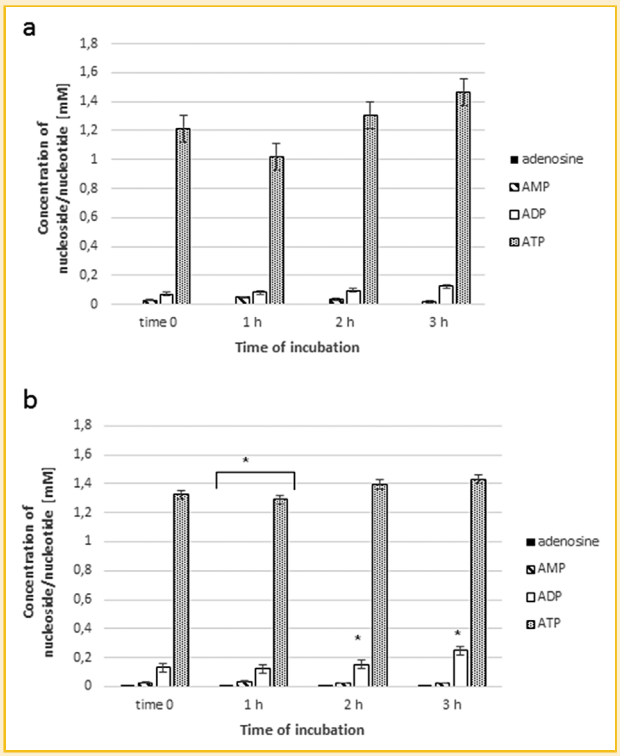

Fig. 3. The profile of ecto-enzymes activity toward ATP [(a) MSCs and (b) chondrogenically induced MSCs]. The cells were incubated with 1.2 mmol/L ATP in isotonic buffer as described in Materials and Methods section. The concentration of nucleotides/nucleosides after reaction are expressed in mmol/L. Bars represent mean $\pm$ SEM of at least three independent experiments performed in triplicate. Asterisk denotes significant difference from hUC-MSCs determined by the Student's *t*-test ($P < 0.05$).

Mature chondrocytes utilize mostly the ATP and ADP-mediated purinergic signaling pathway. Both P2X (including P2X1, P2X2, P2X3, P2X4, P2X7) and P2Y (P2Y1, P2Y2, P2Y4 and P2Y6) nucleotide receptors have been identified on articular chondrocytes (Graff et al., 2003; Burnstock et al., 2013). Early studies showed that ATP and ADP induced in chondrocytes an increase in intracellular Ca^{2+} concentration, and that calcium signaling is an important regulator of chondrogenesis (Bulman et al., 1995). Ecto-ATP and its extracellular metabolites, through purinergic receptors activation, may directly participate in the mineral matrix formation. Rosenthal et al. 2010 reported that P2 receptor agonists, such as ATP and ADP, increased PPi production and accumulation in vitro; whereas suramin, a P2 receptor antagonist, suppressed this increase. Moreover, the cultures of bovine articular chondrocytes supplemented with 62.5–250 μM ATP increased biosynthesis and accumulation of matrix components and exhibited the improved tissue growth and properties (Waldman et al., 2010; Usprech et al., 2012). Chondrocyte pellet cultures treated with ATP produced more proteoglycans and collagen that enhanced their functional properties (Croucher et al., 2000).

Recently, the involvement of the purinergic signaling pathway in isolated chondrocytes was confirmed by experiments showing that stimulation with clodronate resulted in a threefold increase in Ca^{2+} signaling, which could be efficiently inhibited through pharmacological inhibition of purinergic receptors (Rosa et al., 2014).

The above-mentioned results indicate that ATP and ADP, acting through different receptors subclasses, are able to precisely, though sometimes contrarily, regulate physiology and biological potential of stem, progenitor, and mature cells, including chondrocytes. Activity of ecto-nucleotidases is essential for controlling the level of nucleotides and its metabolites. The results presented in this article indicate that the rate of ADP hydrolysis increases considerably on the surface of chondrogenically induced MSCs when compared with undifferentiated MSCs. The chondrogenic lineage cells express the NTPDase1 and NTPDase3 mRNA at the highest level among all NTPDases analyzed. However, it is not possible to affirm that these two enzymes are exclusively responsible for the nucleotides (primarily ADP) degradation measured on chondrogenically induced MSCs surface, considering that these cells express all NTPDases at different mRNA levels. We postulate that chondrogenic lineage cells focus their nucleotides metabolism onto ecto-ADP disposal. It is also consistent with our previous results on MSC-derived osteoblasts that expressed ecto-adenylate kinase to decrease ADP level in the extracellular matrix (Roszek et al., 2013a). In the case of

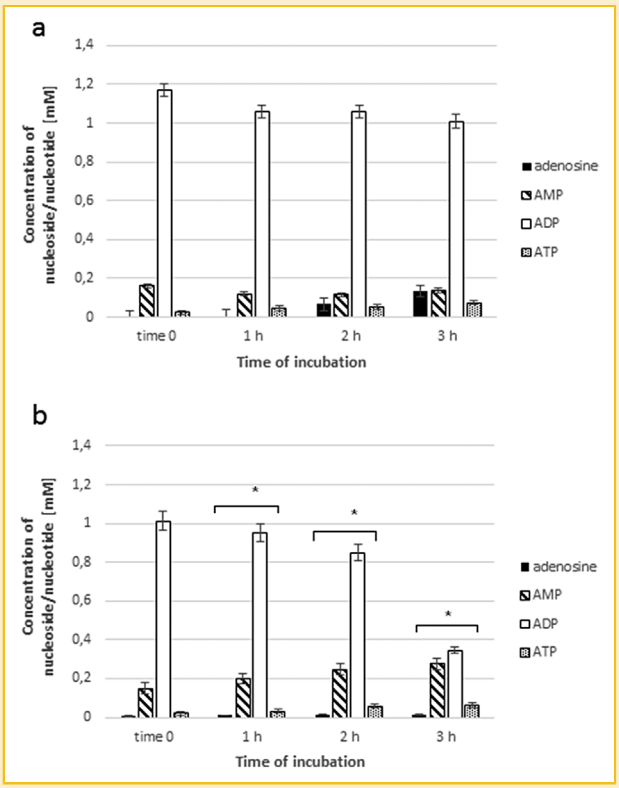

Fig. 4. The profile of ecto-enzymes activity toward ADP [(a) MSCs and (b) chondrogenically induced MSCs]. The cells were incubated with 1.2 mmol/L ADP in isotonic buffer as described in Materials and Methods section. The concentration of nucleotides/nucleosides after reaction is expressed in mmol/L. Bars represent mean ± SEM of at least three independent experiments performed in triplicate. Asterisk denotes significant difference from hUC-MSCs determined by the Student's *t*-test ($P < 0.05$).

chondrogenically induced MSCs, the decrease in ADP concentration cannot be linked with adenylate kinase activity because AMP was the main product of the enzymatic reaction and only low ATP production was observed. Ecto-ADP disposal seems to be important in the extracellular microenvironment of bone and cartilage as this nucleotide is known for its osteolytic and chondrolytic activity when acting through P2Y1 receptor activation (Hoebertz et al., 2001; Burnstock et al., 2013). Ecto-nucleotidases are also responsible for ATP removal by chondrogenically induced MSCs. However, despite the elevated level of NTPDase1 and NTPDase3 expression in chondrogenically induced MSCs, their hydrolytic activity toward ATP remains quite low. In the majority of cells, ATP acts as an anti-proliferative and pro-inflammatory agent, so it should be efficiently hydrolyzed (Bours et al., 2006; Coppi et al., 2007). Our results indicate that chondrogenically induced MSCs preferentially hydrolyze ADP, that may be explained by the diverse specificity of the chondrogenic lineage enzymes toward nucleotides. The support of this hypothesis is the fact that high 250 μM concentrations of exogenous ATP (i.e., capable of P2X7 receptor activation) did not stimulate the inflammatory reactions in the engineered cartilage. Moreover, the high ATP concentrations enhanced the biomechanical properties and functionality of cartilage (Waldman et al., 2010; Makris et al., 2014).

Our results concerning undifferentiated MSCs indicate that hUC-MSCs reveal negligible ATPase activity that is connected with the low, if any, ecto-NTPDases expression. Moreover, the addition of ATP to the MSCs culture resulted in further increase in ATP concentration in the extracellular space. We supposed that this effect might have been due to the release of intracellular ATP, similar to the results obtained in other laboratories (Coppi et al., 2007; Riddle et al., 2007). The emerging question is why MSCs do not present high ATPase activity? In our opinion, ATP signaling in MSCs is important for their mobilization, anti-inflammatory action, differentiation, and regeneration of somatic cells. Ferrari et al. 2011 showed that human MSCs were resistant to the cytotoxic effects of ATP. Gene expression profiling revealed that ATP-stimulated MSCs underwent an increasing homing capacity and upregulation of genes involved in cell migration.

The high ecto-5′-nucleotidase (CD73) activity is present on the MSCs irrespective of their source tissue. The positive expression of CD73 antigen (beside CD90 and CD105) is a minimal criterion for defining MSCs (Dominici et al., 2006). CD105 gene expression significantly decreased during chondrogenic differentiation,

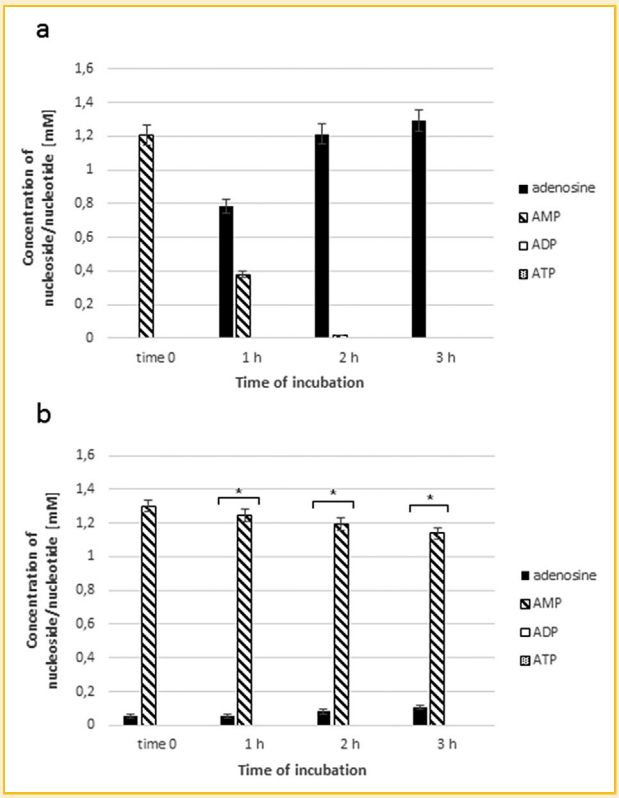

Fig. 5. The profile of ecto-enzymes activity toward AMP [(a), MSCs; and (b), chondrogenically induced MSCs]. The cells were incubated with 1.2 mmol/L AMP in isotonic buffer as described in Materials and Methods section. The concentration of nucleotides/nucleosides after reaction are expressed in mmol/L. Bars represent mean ± SEM of at least three independent experiments performed in triplicate. Asterisk denotes significant difference from hUC-MSCs determined by the Student's *t*- test ($P < 0.05$).

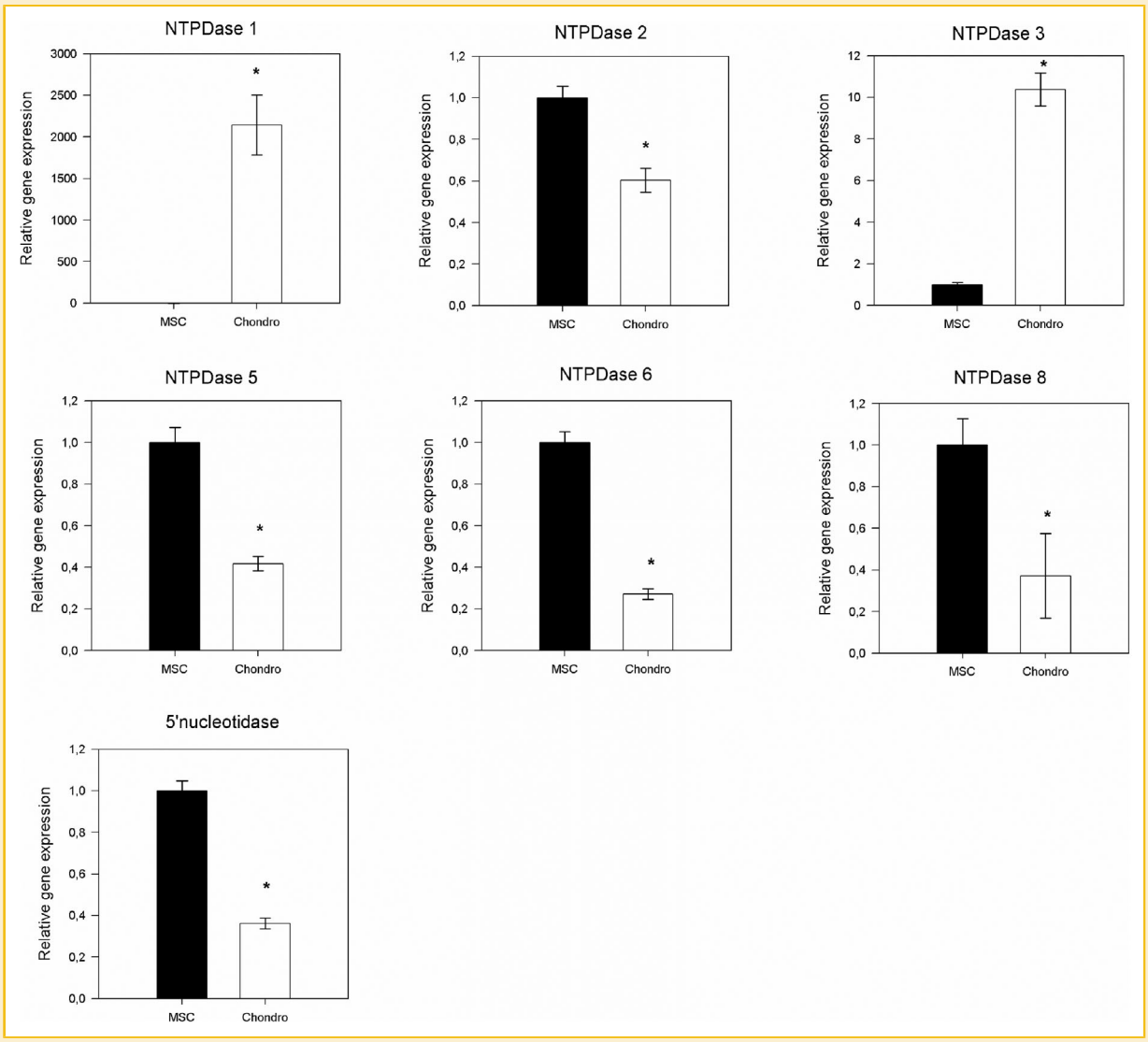

Fig. 6. Relative expression of ecto-nucleotidases mRNA in hUC-MSCs and chondrogenically induced MSCs. The relative expression of analyzed genes was normalized using mean results of both β-actin and GAPDH as reference genes. Data represent the fold expression of the markers as compared to MSCs, which have been normalized to 1. Bars represent mean ± SEM of at least three independent experiments performed in triplicate. Asterisk denotes significant difference from hUC-MSCs determined by the Student's *t*-test ($P < 0.05$).

whereas CD90 expression stayed at almost the same level. We assume that chondrogenically induced MSCs are rather pre-mature chondroblasts and the profile of markers expression confirms the assumption. Collagen 2A1 expression increased after chondrogenic differentiation but its mRNA level is still low, whereas syndecan expression was downregulated during chondrogenesis that is characteristic after the prechondrogenic condensation phase as reported in the literature (Hall and Miyake, 1995).

In the case of CD73, the substantial change in marker expression profile effects in metabolic activity changes. We present that in hUC-MSCs, the activity of 5′-nucleotidase and its expression decreases during chondrogenesis and it is in accordance with the results of Ode et al. 2013. The decreased activity of ecto-5′-nucleotidase results in the reduction of ecto-adenosine production. Adenosine enhances the proliferation rate of MSCs due to the activation of P1 receptors (Riddle et al., 2007; Katebi et al., 2009; Roszek et al., 2013b). Additionally, adenosine promotes MSCs differentiation to osteoblasts via A2B receptor activation while inhibits differentiation to chondrogenic lineages and diminishes chondrocyte ePPi formation (Rosenthal et al., 2010; Carroll et al., 2012; Scarfì, 2014). It may also switch the stem cell properties toward an immunomodulatory/regenerative phenotype (Scarfì, 2014). Thus, from their niche, MSCs give a significant contribution to the regulation of complex purinergic signaling on one hand, and to the regenerative processes within the tissue, on the other.

In conclusion, we described substantial changes of ecto-nucleotides metabolism and ecto-enzymes expression profiles resulting from chondrogenic differentiation of hUC-MSCs. The higher rate of ADP

hydrolysis, measured by ecto-nucleotidases activity, plays a pivotal role in the regulation of cartilage formation and resorption. Despite the increased level of NTPDases expression in chondrogenically induced MSCs, their activity toward ATP remains quite low. Supported by the literature data, we hypothesize that structure–function relationships in chondrogenic lineage dictate the direction of nucleotides metabolism. In early neocartilage tissue, the beneficial role of ATP in improving biomechanical properties of cartilage does not necessitate high rate of enzymatic ATP degradation.

Our results contribute to the better understanding of the biology of MSCs and their biochemical changes induced with chondrogenic differentiation. Moreover, they shed more light on involvement of purinergic signaling in the regulation of biological potential of stem and differentiated cells useful for therapeutic applications.

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