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Adenosine A_1 receptor stimulation enhances osteogenic differentiation of human dental pulp-derived mesenchymal stem cells *via* WNT signaling

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Abstract In this study, mesenchymal stem cells deriving from dental pulp (DPSCs) of normal human impacted third molars, previously characterized for their ability to differentiate into osteoblasts, were used. We observed that: i) DPSCs, undifferentiated or submitted to osteogenic differentiation, express all four subtypes of adenosine receptors (AR) and CD73, corresponding to 5′-ecto-nucleotidase; and ii) AR stimulation with selective agonists elicited a greater osteogenic cell differentiation consequent to A_1 receptor (A_1R) activation. Therefore, we focused on the activity of this AR. The addition of

Abbreviations: ADA, adenosine deaminase; A_1R, adenosine A_1 receptors; $A_{2A}R$, adenosine A_{2A} receptors; $A_{2B}R$, adenosine A_{2B} receptors; A_3R, adenosine A_3 receptors; ALP, alkaline phosphatase; ARS, Alizarin Red S staining; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS21680, 2-*p*-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine; Cl-IBMECA, 1-[2-chloro-6-[[(3-iodophenyl) methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-β-D-ribofuranuronamide; DPCPX, 8-cyclo pentyl-1,3-dipropylxanthine; DKK-1, Dickkopf-1; DPSCs, dental pulp stem cells; Dvl, dishevelled; GSK-3β, glycogen synthase kinase-3β; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; MEM, α-Minimal Essential Medium; MAPK, mitogen-activated protein kinase; MRS1220, *N*-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-*c*]quinazolin-5-yl]benzene acetamide; MSC, mesenchymal stem cells; MSCGM, Mesenchymal Stem Cell Growth Medium; NECA, 5′-*N*-ethylcarboxamidoadenosine; PI3K, phosphatidylinositol 3-kinase; PSB603, 8-[4-[4-(4-chloro phenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine; RUNX-2, Runt-related transcription factor-2; Wnt, wingless; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol

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15–60 nM 2-chloro-N^6-cyclopentyl-adenosine (CCPA), A_1R agonist, to DPSCs at each change of the culture medium significantly increased the proliferation of cells grown in osteogenic medium after 8 days in vitro (DIV) without modifying that of undifferentiated DPSCs. Better characterizing the effect of A_1R stimulation on the osteogenic differentiation capability of these cells, we found that CCPA increased the: i) expression of two well known and early osteogenic markers, RUNX-2 and alkaline phosphatase (ALP), after 3 and 7 DIV; ii) ALP enzyme activity at 7 DIV and iii) mineralization of extracellular matrix after 21 DIV. These effects, abolished by cell pre-treatment with the A_1R antagonist 8-cyclopentyl-1,3-dipropyl-xanthine (DPCPX), involved the activation of the canonical Wnt signaling as, in differentiating DPSCs, CCPA significantly increased dishevelled protein and inhibited glycogen synthase kinase-3β, both molecules being downstream of Wnt receptor signal pathway. Either siRNA of dishevelled or cell pre-treatment with Dickkopf-1, known inhibitor of Wnt signaling substantially reduced either DPSC osteogenic differentiation or its enhancement promoted by CCPA. Summarizing, our findings indicate that the stimulation of A_1R may stimulate DPSC duplication enhancing their osteogenic differentiation efficiency. These effects may have clinical implications possibly facilitating bone tissue repair and remodeling.

Introduction

Bone tissue engineering/regeneration is one of the most investigated areas of research aimed at providing potential new clinical applications to cure bone defects and to accelerate repairing of damaged skeletal tissues. Mesenchymal stem cells (MSCs) deriving from different sources (embryonic tissues, bone marrow, umbilical cord blood, adipose and muscle tissues) are currently studied, due to their biological capability to differentiate into osteogenic lineage (Seong et al., 2010). Also dental pulp stem cells (DPSCs), obtainable from teeth usually discarded during orthodontic treatments, are receiving extensive attention, since they are able to differentiate, either *in vitro* or *in vivo* (in animal models), into bone forming cells, osteoblasts (Bakopoulou et al., 2011; Nampo et al., 2010; Huang et al., 2009).

In order to increase the available cell source for an efficient application to bone tissue engineering therapies reducing, at the same time, the likelihood of spontaneous differentiation into divergent lineages, it is important to develop well-defined and proficient protocols, identifying agents able to improve this aspect. Several growth factors and signaling molecules may influence the growth and differentiation of DPSCs, including adenine-based purines. They are ubiquitous substances produced and released from most cells and tissues. At the extracellular level, nucleotides and nucleosides such as ATP or adenosine, respectively, interacting with specific receptors, regulate a wide variety of physiological/pathological processes (Burnstock, 2011; Burnstock and Ulrich, 2001). The receptors for adenine-based purines are classified into two groups; P1 receptors, which are primarily activated by adenosine, and P2 receptors, which respond to nucleotides. The P1 receptors are further subdivided into 4 receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3). Also, the P2 receptors are subdivided into seven P2X ligand-gated ion channels ($P2X_{1-7}$) and eight P2Y G-protein-coupled receptors ($P2Y_{1,2,4,6,11-14}$) (Burnstock, 2007). Each of these receptors has been cloned, characterized and displays distinct tissue expression and pharmacology. As for bone, extracellular ATP has been indicated as able to modulate differentiation and function of osteoblasts from this source *via* different P2Y and P2X receptor subtypes (for a review, see Orriss et al., 2010). Like in other cells, ATP is released from the osteoblasts in physiological condition and even more after cell damages (Orriss et al., 2009) and is rapidly metabolized into adenosine (Evans et al., 2006). More recently, it has been reported that A_{2B} receptors and also A_1R, even though to a lesser extent, may play a role in favoring proliferation and osteogenic differentiation of human primary bone marrow stromal cells (Costa et al., 2011), a role that has been confirmed by *in vivo* studies carried out using A_{2B} receptor (A_{2B}R) knockout mice (Carroll et al., 2012). Nothing is known about the possible influence of adenine-based purines on DPSC growth and osteo/odontogenic differentiation. Thus, we started our research focusing on A_1R activity, evaluating whether the selective stimulation of these receptors may affect the proliferation and/or the mineralization potential of DPSCs *in vitro*, in order to better understand the mechanisms involved in and to contribute to a proper use of these cells for regenerative dentistry.

Materials and methods

Materials

2-Chloro-N^6-cyclopentyladenosine (CCPA), 2-*p*-(2-carboxyethyl)phenethylamino-5′-N-ethyl carboxamidoadenosine (CGS21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), Dickkopf-1 (DKK-1), apyrase from potato, adenosine deaminase (ADA) bovine recombinant as well as the other chemicals, unless differently indicated, were purchased from Sigma-Aldrich (Milan, Italy).

1-[2-Chloro-6-[[(3-iodophenyl) methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-β-D-ribo furanuronamide (Cl-IBMECA), *N*-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-*c*] quinazolin-5-yl]benzene acetamide (MRS1220), 5′-*N*-ethylcarboxamidoadenosine (NECA) and 8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine (PSB603) and, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) were obtained from Tocris Bioscience (Space Import–Export, Milan, Italy). Disposable materials for tissue cultures were from Falcon (Steroglass, Perugia, Italy). α-Minimal Essential Medium (MEM) was from Sigma-Aldrich (Milan, Italy) whereas Mesenchymal Stem Cell Growth Medium (MSCGM™) and Mesenchymal Stem Cell Osteogenic Differentiation Medium were purchased from Lonza (Basel, Switzerland).

Cell culture

Surgical pulp samples (n = 10) were obtained from 8 patients (3 females and 5 males), with a mean age of 17 years, at the Oral Science Nano and Biotechnology Department (University of Chieti), during orthodontic treatment. Informed consent was obtained from each of them. Dental pulps were isolated after tooth extraction as previously reported (Teté et al., 2008; D'Alimonte et al., 2011), digested (1 h; 37 °C) in MEM containing 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Milan, Italy), 500 μg/ml clarithromycin (Menarini, Florence, Italy), 3 mg/ml collagenase type I (Sigma) and 4 mg/ml dispase (Roche, Monza, Italy). Cells were separated by filtering through a 70-μm strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA), then resuspended in MSCGM and centrifuged (10 min; 1200 rpm). Cell pellet was resuspended in the same medium and plated in 25 cm^2 flasks. Cultures were incubated at 37 °C in a 5% CO_2, and the medium was changed twice a week. Experiments were performed only in the first six cell passages.

Experimental protocol

To measure DPSC proliferation rate, cells were seeded at 2×10^3 cell/well onto 96-well plates whereas they were plated at 4×10^4 cell/well onto 6-well plates to evaluate mineralization by Alizarin Red S (ARS) staining and non tissue specific alkaline phosphatase (ALP) activity assays or at $2.5–5 \times 10^5$ cell/well onto 100 mm plates to study the expression of immunophenotype markers by flow cytometry, osteogenic markers by real time PCR analysis or adenosine receptors (AR), to induce transient RNA silencing (siRNA) for dishevelled protein and to evaluate intracellular signaling by western blotting. The assays were performed at different times, as indicated in the Results section. AR agonists were administered at different concentrations to the cells, starting 24 h after seeding. Afterwards, they were added to the cultures at each medium change for the indicated period. When present, the AR antagonists were adedd 30 min before the respective receptor agonists.

Flow cytometry

Cell staining of surface antigen CD73

Cells (5×10^5 cells/sample) were incubated with 100 μl of 20 mM ethylenediaminetetraacetic acid (EDTA) at 37 °C for 10 min. Cells were washed with 3 ml of washing buffer and centrifuged (4 °C, 400 ×*g*, 8 min). Subsequently, samples were resuspended in 100 μl washing buffer containing the appropriate amount of surface antibody (phycoerythrin-conjugated anti-CD73, purchased from Becton Dickinson, San Jose, CA); samples were incubated for 30 min at 4 °C in the dark. Tubes were washed (3 ml of washing buffer), centrifuged (4 °C, 400 ×*g*, 8 min) and cells were resuspended with 1 ml 0.5% paraformaldehyde, incubated for 5 min at room temperature, washed, centrifuged (4 °C, 400 ×*g*, 8 min) and stored at 4 °C in the dark until the acquisition. Ten min before the analysis, a specific solution, containing 7-amino-actinomycin D (Becton Dickinson) was added to the samples to exclude dead cells. Finally, cells were analyzed on a FACSCalibur flow cytometer (BD), using CellQuest™ software (Becton Dickinson).

Flow cytometry measurement

Quality control included regular check-up with Rainbow Calibration Particles (6 peaks) and CaliBRITE beads (both from Becton Dickinson). Debris was excluded from the analysis by gating on morphological parameters; 20,000 non-debris events in the morphological gate were recorded for each sample. All antibodies were titrated under assay conditions and optimal photomultiplier (PMT) gains were established for each channel (Lanuti et al., 2012). Data were analyzed using FlowJo™ software (TreeStar, Ashland, OR). Mean Fluorescence Intensity Ratio (MFI Ratio) was calculated dividing the MFI of positive events by the MFI of negative events (Miscia et al., 2009).

Cell proliferation

Cell proliferation was assayed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Milan, Italy), according to the manufacturer's instructions. The absorbance was measured at 490 nm, using a microtiter plate reader (Spectracount™, PerkinElmer Life, Waltham, MS, USA).

RNA isolation, RT-PCR and real time-PCR analysis

Total RNA from was isolated by using the RNeasy Plus Universal Mini Kit (Qiagen Inc., Valencia, CA) according to manifacturer's instructions. The quality of total RNA was assessed by measuring the A260/280 ratio using a spectrophotometer. For the reverse transcriptase reaction, M-MLV Reverse Transcriptase reagents (Sigma-Aldrich) were used. First strand cDNA was synthesized from 1.5 μg of total RNA using the RT-PCR system RETROscript™ (Ambion, Monza, Italy) with random hexamers. The resultant cDNA (2 μl) was amplified in a 100 μl reaction volume containing PCR reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxy-dNTP, 1 μM oligonucleotide primers (MWG Biotech, Ebersberg, Germany), and 2.5 U AmpliTaq Gold™ DNA polymerase (Applied Biosystems). The final cycle was followed by a 10-min incubation at 72 °C. PCR primers, annealing temperatures, and product sizes are shown in Table 1. Reaction was also performed without the reverse transcriptase step as control for genomic contamination. PCR products were separated by 1.5% agarose gel electrophoresis in gels containing ethidium bromide and visualized with UV light. Direct sequencing of the RT-PCR bands was performed for the analysis of the RT-PCR bands. Real-Time PCR was carried out with the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Expression of Runt-related transcription factor-2 (RUNX-2) and ALP was evaluated at 3, 7 and 14 days in DPSCs cultured in undifferentiating or osteogenic medium. Commercially available TaqMan Gene Expression Assays (RUNX-2, Hs00231692_m1, ALP, Hs01029144_m1) and the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were used according to standard

Table 1 Primer sequences used for RT-PCR analysis.

Gene	Accession number	Forward primer	Reverse primer	T (°C) annealing	Product (bp)
hA1	NM_000674	TCTTCCTCTTTGCCCTCAGCT	GCTCAGAACACTGTTGCCTCT	55	525
hA2a	NM_000675.4	AGGGCTAAGGGCATCATTG	GGATACGGTAGGCGTAGATGA	58	519
hA2b	NM_000676	TGACTTCTACGGCTGCCTCTT	TGACTTGGCTGCATGGATCT	55	487
hA3	NM_000677	TTTGCTGGCTGGTGTCATT	AGGCATAGACGATAGGGTTCA	55	452
hGAPDH	NM_002046	GAGTCCACTGGCGTCTTCAC	GGTGCTAAGCAGTTGGTGGT	55	190

protocols. Beta-2 microglobulin (B2M, Hs99999907_m1, Applied Biosystems, Foster City, CA, USA) was used for template normalization and duplicates were set up for each samples.

Western blot analysis

Cells were harvested at 4 °C in a lysis buffer (in mM: Tris buffer 50, NaCl 150, PMSF 1.0; 1% Nonidet-P40, 5 μg/ml leupeptin, 5 μg/ml aprotinin), disrupted by sonication and centrifuged (14,000 rpm, 5 min, 4 °C). Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Milan, Italy). Samples (50 μg), diluted in SDS-bromophenol blue buffer, were boiled (5 min) and separated on 12.5–15% SDS-polyacrylamide gels. Proteins were transferred on a polyvinylidene fluoride membrane, blocked with PBS/0.1% Tween20/5% non-fat milk (Bio-Rad Laboratories) for 2 h at 4 °C, incubated overnight at 4 °C with specific primary antibodies [polyclonal rabbit anti-A_1 dilution 1:1000 (Abcam, Cambrige, UK), anti-A_{2A} dilution 1:200, anti-A_{2B}, dilution 1:200, anti-A_3, dilution 1:200 (Alomone Labs, Jerusalem, Israel); anti-dishevelled (Dvl), dilution 1:200 (Santa Cruz Biotechnologies, DBA Italia, Segrate (MI), Italy); anti-phospho-glicogen synthase kinase3β (p-GSK3β, ser9), dilution 1:1000; (Cell Signalling Technology, Euroclone, Milan, Italy)] and then exposed to donkey anti-rabbit HPR-conjugated secondary antibody for 1 h at room temperature (GE Healthcare Life Sciences, Milan, Italy; final dilution 1:5000). The specificity of the antibodies used to determine the presence of AR was previously established (Costa et al., 2011; Gharibi et al., 2012). To determine the equal loading of samples, the blots were stripped and re-probed with an anti-ß-actin antibody (dilution 1:100, incubation for 1 h at room temperature; Santa Cruz Biotechnologies). For protein phosphorylation (p-GSK3β), cells were serum-starved for 24 h, washed three times in PBS and incubated in serum-free media with or without drugs for the indicated periods, as described in the Results section. The stimulation was terminated by washing in ice-cold PBS containing 1 mM sodium orthovanadate, followed by lysis in ice-cold immunoprecipitation assay buffer containing protease inhibitors. Immunocomplexes were visualized using the enhancing chemiluminescence (ECL) detection system (GE Healthcare Life Sciences) and quantified by densitometric analysis (Molecular Analyst System).

Quantification of mineralization

Cells grown under undifferentiating condition were detached (0.025% trypsin/0.04% EDTA dissolved in PBS, 10 min, 37 °C) and re-seeded on culture plates. Three days after replating, the cells were switched to Mesenchymal Stem Cell Osteogenic Differentiation Medium. Visualization of calcium deposition and extracellular matrix mineralization was obtained by ARS staining assay performed after 21 days *in vitro* (DIV). This assay was carried out according to the method by Gregory et al. (2004). Unless differently indicated, the passages were performed at room temperature. Cells were washed with PBS, fixed in 10% (v/v) formaldehyde (Sigma-Aldrich) for 30 min and washed twice with abundant dH_2O prior to addition of 1 ml of 40 mM ARS (pH 4.1) per well. After cell incubation (20 min) under gentle shaking, unincorporated dye was aspirated, cells were washed with dH_2O (4 ml) four times further, while shaking for 5 min. Then water was carefully discarded and plates were stored at −20 °C prior to dye extraction. For staining quantification, 800 μl 10% (v/v) acetic acid was added to each well. Cells were incubated for 30 min with shaking, and then scraped from the plate, transferred into a 1.5-ml vial and vortexed for 30 s. The obtained suspension, overlaid with 500 μl mineral oil (Sigma-Aldrich), was heated to 85 °C for 10 min, then transferred to ice for 5 min, carefully avoiding opening of the tubes until fully cooled, and centrifuged at 20,000 ×*g* for 15 min. Five hundred microliters of the supernatant was placed into a new 1.5-ml vial and 200 μl of 10% (v/v) ammonium hydroxide was added to neutralize the acid, assuring a pH between 4.1 and 4.5. Aliquots (150 μl) of the supernatant were read in triplicate at 405 nm by a spectrophotometer (Spectramax SM190, Molecular Devices, Sunnyvale, CA, USA). For quantification of staining, samples were treated as previously described (Gregory et al., 2004).

Alkaline phosphatase activity assay

Tissue-nonspecific ALP activity was determined in DPSCs during osteogenic differentiation as previously reported (Laflamme et al., 2010). Cultured cells were washed with PBS, lysed with 1 ml of Tris buffer (10 mM, pH 7.5, 0.1% Triton X-100). Cell lysates were centrifuged (2000 rpm; 1 min); then, 20 μl of supernatant solution from each sample was combined with 20 μl of 1 mM *p*-nitrophenyl phosphate (*p*-NPP, Sigma; solution at pH 10.3 with $MgCl_2$-diethanolamine buffer) used as a substrate and dispensed into 96-well plates. The samples were incubated in the dark at room temperature for 30 min; then, the reaction was halted by adding 10 μl of 2 N NaOH. The amount of *p*-NPP released was measured as absorbance at 405 nm on a microplate spectrophotometer (Spectramax SM190). The protein content of each sample was determined by the Bradford method (Bradford, 1976). The enzyme activity was expressed as nmoles of *p*-NPP released per mg of protein per 30 min.

Dvl-2 small interfering RNA transfection

Dishevelled-2 (Dvl-2) expression was knocked down with Dvl-2 small interfering RNA (siRNA; Qiagen, Valencia, CA, USA) which was designed and synthesized by Qiagen based on human DvL-2 sequence (FlexiTube GeneSolution GS1856 for DVL2, Gene Accession Number: NM_004422). siRNA transfection experiments in DPSCs were performed with the Amaxa system (Amaxa, Cologne, Germany) using Human MSC Nucleofector Kit and program C-17 for high cell survival according to the manufacturer's protocol and 600 ng of siRNA/1 × 10^6 cells (Lanuti et al., 2009). As a positive control to monitor transfection efficiency, cells were transfected with control vector pmaxGFP (green fluorescence protein) provided in Nucleofector kit and analyzed 48 h later by fluorescence microscopy and flow cytometry (see Fig. 1 of the Supplemental material). Cells were submitted to osteogenic differentiation 24 h after siRNA induction. Knocking down of Dvl-2 was revealed by western blot analysis performed at 3 days after differentiation induction (Fig. 1 Supplemental material), whereas its influence on ALP expression and activity was assayed by real-time RT-PCR and colorimetric assay, respectively, carried out at 3 or 7 days after cell commitment toward osteogenesis (see Results).

Statistical analysis

Experimental values are expressed as mean ± SEM. Statistical analysis was performed by Student's t test. *P* values were assumed as significant at 0.05. The data were analyzed by Prism3 software (GraphPad, San Diego, CA, USA).

Results

Expression of CD73 and adenosine receptors in DPSCs

In this study, we used MSCs isolated from human dental pulp that, grown under appropriate culture conditions, undergo osteogenic and, to a lesser extent, adipogenic differentiation. As we previously demonstrated (Teté et al., 2008; D'Alimonte et al., 2011), undifferentiated DPSCs are negative for hematopoietic markers (CD14, CD34, and CD45), while they express numerous surface mesenchymal markers (CD29, CD90, CD105, CD166, CD146, and STRO-1), showing an immunophenotypic profile consistent with the literature. Undifferentiated DPSCs, like MSCs deriving from other sources (Evans, 2012; Takedachi et al., 2012), also express CD73, another mesenchymal marker, to a high extent (Figs. 1A–B). Such an expression was not modified in cells submitted to osteogenic differentiation. Of interest, CD73 corresponds to the enzyme ecto-5′-nucleotidase, the activity of which leads to the conversion of extracellular AMP, in turn deriving from ATP catabolism, into adenosine.

As well, the expression of the four receptor subtypes for adenosine, that are A_1, A_{2A}, A_{2B} and A_3 receptors, was similar in DPSCs grown in either undifferentiating or osteogenic medium for 14 DIV, as elicited by RT-PCR assay or western blot analysis (Figs. 1C–D). The period of 14 DIV for carrying out these experiments was chosen as the osteogenic differentiation has already begun unequivocally, whereas the mineralization of extracellular matrix was not so intense to impair cell detachment, necessary to perform the subsequent molecular biological assays.

The stimulation of adenosine receptors differently affects the activity of tissue nonspecific ALP assumed as an index of osteogenic DPSC differentiation

We first screened the effects of the stimulation of AR by endogenous or exogenous compounds on the DPSC capability to differentiate towards an osteogenic phenotype when grown in osteogenic medium. To this aim, we evaluated the activity of ALP, an enzyme typically expressed on the basolateral membrane of osteoblasts and related precursors (Nakano et al., 2004), that functions to promote bone mineralization. In previous papers (Teté et al., 2008; D'Alimonte et al., 2011) we demonstrated that in DPSC committed to osteogenesis the activity of this enzyme was maximal at 7 DIV (see also Fig. 4). Therefore, we chose this time to perform our investigation. We observed that addition to the culture of apyrase or ADA, able to metabolize ATP/ADP or adenosine, respectively, did not affect ALP activity (Fig. 2). On the contrary, the stimulation of A_1R and A_{2B}R by the respective agonists (CCPA and NECA) caused a dose-dependent increase of the enzyme activity and that of A_{2A}R and A3R by CGS21680 or Cl-IBMECA decreased or did not affect it, respectively (Fig. 2). These stimulatory or inhibitory effects were abolished by cell pre-treatment with the respective receptor antagonists, that were unable to modify DPSC osteogenic commitment when administered alone to the cultures. Since in our hands the effect promoted by A_1R stimulation was greater than that consequent to the activation of other AR, this finding prompted us to better investigate the activity of A_1R.

Effect of A_1R stimulation on the proliferation of DPSCs grown in undifferentiating and osteogenic conditions

We first evaluated the ability of these cells to proliferate using the MTS assay as an index of cell viability and performing measurements from 2 up to 16 DIV. The duplication rate was greater in undifferentiated cells (Fig. 3A) as compared to differentiating sister cells, even though both types of cells reached a plateau phase around the 8–10th DIV. The addition of CCPA at different doses (15–60 nM) and for different periods (2, 4 and 8 DIV) significantly increased the proliferation rate of differentiating DPSCs at the 8th DIV, without substantially modifying that of undifferentiated cells (Figs. 3B–C). The proliferating CCPA-induced effect was counteracted by cell pre-treatment with 100 nM DPCPX (data not shown).

Effect of the addition of CCPA on the differentiating potential of DPSCs towards an osteoblast lineage

In order to better characterize the activity of A_1R on the osteogenic differentiation of DPSCs, we evaluated the effect of the addition of CCPA on cell culture mineralization measured by ARS staining and colorimetric detection. The

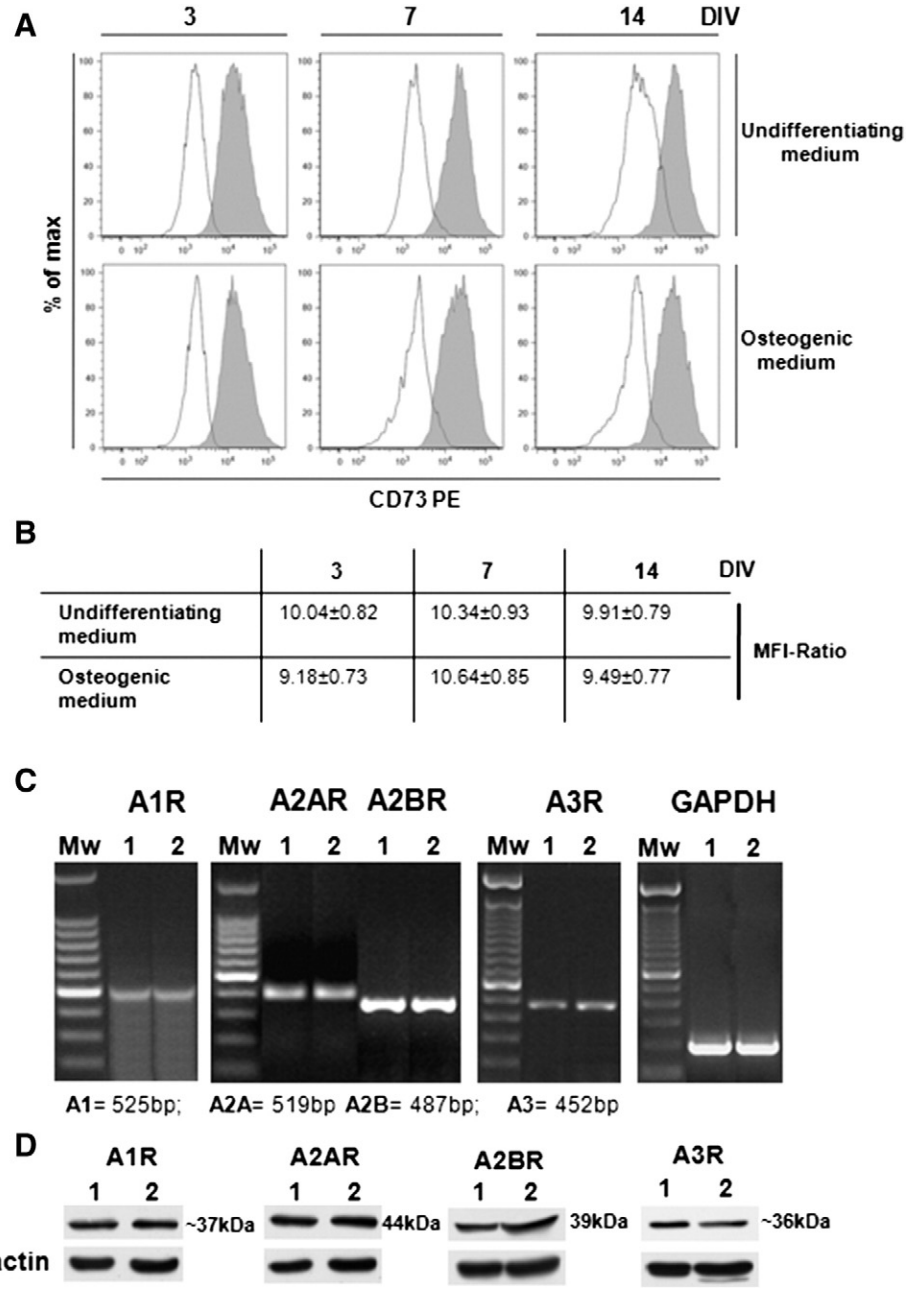


Fig. 1 Expression of CD73 and four subtypes of AR in DPSCs grown in undifferentiating or osteogenic medium. A–B) The cytofluorimetric analysis was performed in DPSCs previously incubated with phycoerythrin(PE)-conjugated antibody against CD73. The filled histogram shows the distribution of the antigen expression whereas the open histogram represents the distribution of the respective irrelevant control. The values were expressed as mean fluorescence ratio (MFI) obtained dividing the MFI of positive events by the MFI of negative events. Panel A is representative of three separate experiments performed using cells after different days *in vitro* (DIV) whereas the numeric values of MFI ratio (B) are the mean ± SEM of three independent experiments. C–D) The presence of the AR has been evaluated by RT-PCR (panel C) and western blot (panel D) analyses. Immune-detected bands have been obtained from DPSCs grown as undifferentiated cells (lane 1) or in a osteogenic conditioning medium (lane 2) for 14 DIV. Expression of GAPDH and β-actin was used as an internal control in C and D, respectively. The molecular weights (expressed as kilodaltons, kDa) reported in the figures relating to western blot analysis are those currently indicated in literature for each receptor subtype and also in the data sheets of the antibodies used for their detection. Data are representative of three independent experiments with very similar results.

exposure of cells grown under osteogenic condition to 30 nM CCPA, added to the cultures at every change of medium significantly increased extracellular calcium deposition after 14 and 21 DIV, in comparison with values determined in control (Fig. 4A). The ability of CCPA to increase extracellular matrix mineralization was confirmed by ARS staining carried out in the same cells after 21 DIV (Fig. 4E).

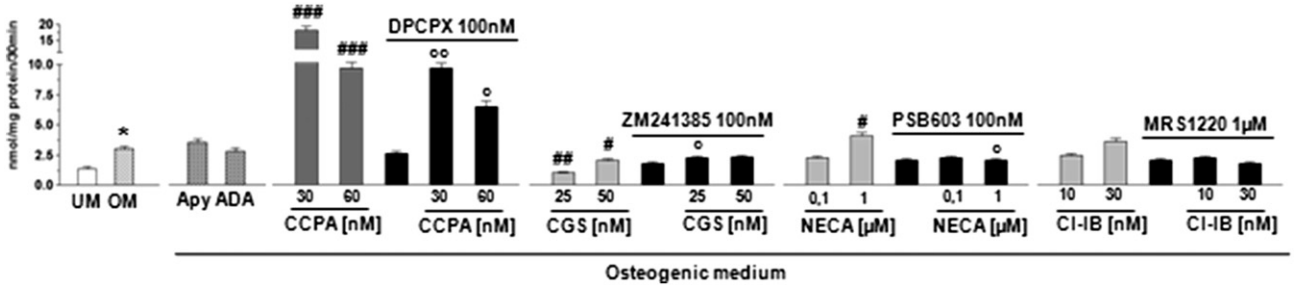


Fig. 2 Effect of apyrase, ADA and selective agonists of different subtypes of AR on ALP activity evaluated in DPSCs grown for 7 days in osteogenic medium. DPSCs were grown in undifferentiating (UM) or osteogenic (OM) medium; cells committed towards osteogenesis were also exposed to apyrase (apy, 2 U/ml), or adenosine deaminase (ADA, 0.2 U/ml) or agonists of A_1R (CCPA), $A_{2A}R$ (CGS21680), $A_{2B}R$ (NECA) and A_3R (Cl-IBMECA). The effect caused by these agonists was also evaluated in the presence of the respective AR antagonists (DPCPX, ZM241385, PSB603 and MRS1220) that were administered 30 min prior to the agonist. ALP activity was assayed as described in the Materials and Methods section and expressed as nmol of *p*-nitrophenol produced per mg of cell protein within a period of 30 min (nmol/mg protein/30 min). Values are the mean ± SEM of 3 separate experiments in which different cell samples were used. $^*p < 0.05$: statistical significance *vs.* cells grown in UM; # $p < 0.05$; ### $p < 0.001$: statistical significance *vs.* cells grown in OM; $^\circ p < 0.05$, $^{\circ\circ} p < 0.01$: statistical significance *vs.* cells grown in OM and exposed to the respective AR agonist (Student's t test).

Additionally, we assayed the expression of RUNX-2, a key transcription factor associated with osteogenesis (Komori, 2005), in parallel to that of ALP. The expression of these two early osteogenic markers was significantly increased in DPSCs grown in osteogenic medium and further enhanced by cell exposure to CCPA at 3 and 7 DIV (Figs. 4C–D). This result was confirmed by the evaluation of ALP activity, that progressively increased in DPSCs grown in osteogenic medium and was maximal at 7 DIV in comparison with that of undifferentiated cells. DPSC exposure to CCPA enhanced ALP activity over the effect caused by the addition of osteogenic medium alone both at 3 and mainly at 7 DIV (Fig. 4B), whereas the treatment of DPSCs with CCPA for a longer period (up to 14 DIV) did not further influence the enzyme activity promoted by osteogenic condition. All CCPA-induced effects were counteracted by the contemporaneous presence of the A_1R antagonist DPCPX (100 nM) (Figs. 2A–E).

Molecular pathways involved in the CCPA increased osteogenesis in DPSCs

We wondered whether the CCPA-induced increase of osteogenesis in DPSCs grown under differentiating condition was coupled to the activation of the canonical wingless (Wnt) signaling pathway, as occurs in other human MSCs, *i.e.* deriving from bone marrow and differentiating into osteoblasts (Krause et al., 2010). As shown in Figs. 5A–B, when the cells were cultured in osteogenic medium for 3 and 7 DIV, there was a significant increase in the intracellular level of Dvl, the most proximal cytosolic component known in this pathway, that is usually enhanced when a member of the Wnt family binds the cell receptors encoded by the Frizzled gene family. In the same cells and at the same times, we observed a parallel significant increase in the expression of phosphorylated glycogen synthase kinase-3beta (p-GSK-3β), another factor downstream Wnt signaling pathway, that converges on the transcriptional regulator β-catenin. Once phosphorylated, GSK-3β is no longer able to cause the phosphorylation and then the ubiquitination/degradation of β-catenin, thus favoring its translocation to the nucleus (Fig. 5C), that in turn induces the transcription of proteins involved in different body functions, including osteogenesis (Logan and Nusse, 2004; Chen et al., 2012). The exposure of our cells committed towards osteogenesis to 30 nM CCPA significantly enhanced, at 3 and 7 DIV, either the intracellular level of Dvl, or the expression of p-GSK-3β as compared to cells exposed to osteogenic medium alone (Figs. 5A–B).

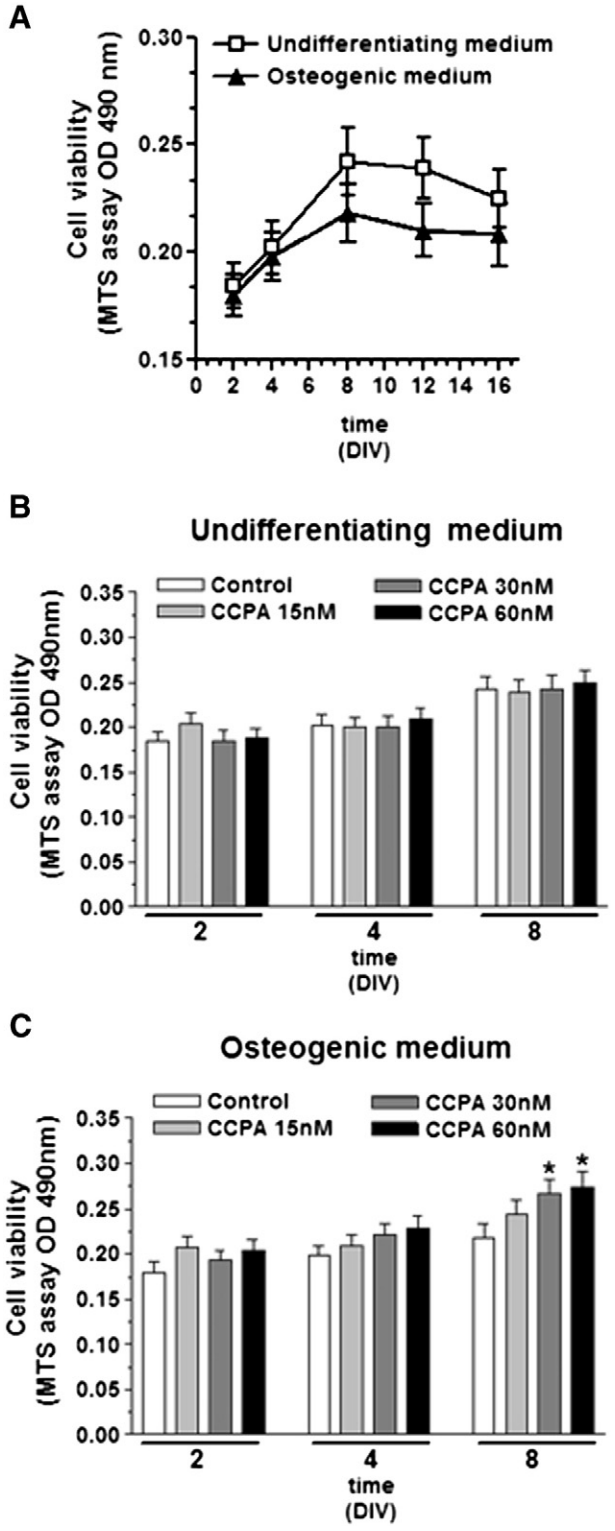
Involvement of Wnt/Dvl pathway in the CCPA induced increase in DPSC osteogenesis

To demonstrate the involvement of Wnt pathway in the CCPA-induced effects, we carried out further experiments using differentiating DPSCs: in one set of cell cultures we performed Dvl-2 siRNA transfection; in another set, normal differentiating DPSCs were pre-treated with Dickkopf-1 (DKK-1), a known antagonist of the canonical Wnt pathway, prior to the exposure to CCPA. As shown in the Supplemental material, our transfection conditions were remarkably efficient as in cells in which fluorescent GFP, assumed as a positive control, was transfected, microscopical detection and flow cytometric analysis revealed a very large amount of positively transfected cells. The success of Dvl-2 silencing was witnessed by western blot analysis showing that Dvl-2 immune-complex was significantly reduced in cells submitted to siRNA and grown for 3 DIV in osteogenic medium as compared to control cells. The exposure of these silenced cells to CCPA did not reverse Dvl-2 knocking down (Fig. 6A). Similarly, there was no increase in the expression and activity of ALP evaluated in the same silenced cells after 7 DIV of osteogenic differentiation and CCPA was unable to induce stimulatory effects on the enzyme expression/activity (Fig. 6B), as previously demonstrated in control cells (see also Fig. 5). Accordingly, the exposure of normal DPSC to the Wnt inhibitor DKK-1 reduced the differentiation of DPSCs exposed to osteogenic medium alone and abolished

CCPA-increased mineralization of differentiating DPSCs, evaluated as ARS staining and colorimetric detection (Fig. 6C).

Discussion

In this study we demonstrated that DPSCs may be induced to a greater differentiation into osteoblasts by selective stimulation of A_1R.



We started our study based on the known capability of DPSC to differentiate into osteoblasts (Huang et al., 2009; Ulmer et al., 2010; D'Alimonte et al., 2011; Estrela et al., 2011). Here, we confirmed data demonstrating that the proliferation rate of MSCs of dental origin, even when grown in osteogenic condition, is rapid reaching a plateau around 8–10 DIV. This time is approximately half of that used by stem cells deriving from other sources, in particular from bone marrow (Huang et al., 2009; Alge et al., 2010; Costa et al., 2011), that represent the benchmark for studies dealing with properties of MSCs. Thus, DPSCs are a good model to evaluate the influence of different substances on their growth and differentiation towards an osteoblastic phenotype. These aspects are currently under intense evaluation for future clinical applications in tissue engineering and bone regeneration (Ulmer et al., 2010; Estrela et al., 2011).

We directed our attention to purines, ubiquitous substances that, acting as signaling molecules, are able to affect different body functions, including osteogenesis (Gartland et al., 2012). A number of papers have recently demonstrated that different receptors to ATP are present in either osteoblasts or osteoclasts and participate in bone remodeling processes (Orriss et al., 2010; Gartland et al., 2012; Noronha-Matos et al., 2012). On the contrary, knowledge on the functions of its metabolite, adenosine, at bone level is at the very beginning.

Here, we showed that DPSCs, like MSCs deriving from human or rodent bone marrow (Evans et al., 2006; Gharibi et al., 2011; Costa et al., 2011), are provided with the four subtypes of AR. In our cells, their expression was not modified following DPSC differentiation, whereas in MSCs from human or rodent bone marrow there was a dominant expression of $A_{2B}R$ in undifferentiated cells and/or during later stages of their osteoblastic differentiation (Costa et al., 2011; Gharibi et al., 2011). The discrepancy may likely be due to differences in the source from which MSCs were derived (*i.e.* animals or aged women with osteoarthrosis *versus* young healthy persons, in our case). The prevalence of $A_{2B}R$ reasonably justifies also the greater influence exerted by these receptors compared to that of A_1R on the osteogenic differentiation of MSCs used in the cited papers. We further observed that either undifferentiated or differentiating DPSCs express CD73, corresponding to 5′-ectonucleotidase enzyme, in equivalent manner, differently from other MSCs in which the enzyme expression/activity decreases during differentiation (Costa et al., 2011; Liu et al., 2009a; Liu et al., 2009b).

Fig. 3 Effects of CCPA on DPSC cell growth. Proliferation rate was assessed by MTS assay in DPSCs grown under undifferentiating or osteogenic condition (panel A) over a period from 2 to 16 days *in vitro* (DIV). During a period ranging from 0 to 8 DIV some undifferentiated (panel B) or differentiating (panel C) cultures were exposed to CCPA administered at different doses at each medium change. The proliferation rate was measured as the absorbance detected at 490 nm in untreated cultures (control) and in cultures exposed to CCPA. Results are expressed as mean ± S.E.M. of three independent experiments, in which different cell samples were used. *$p < 0.05$: statistical significance *vs*. control (Student's t test).

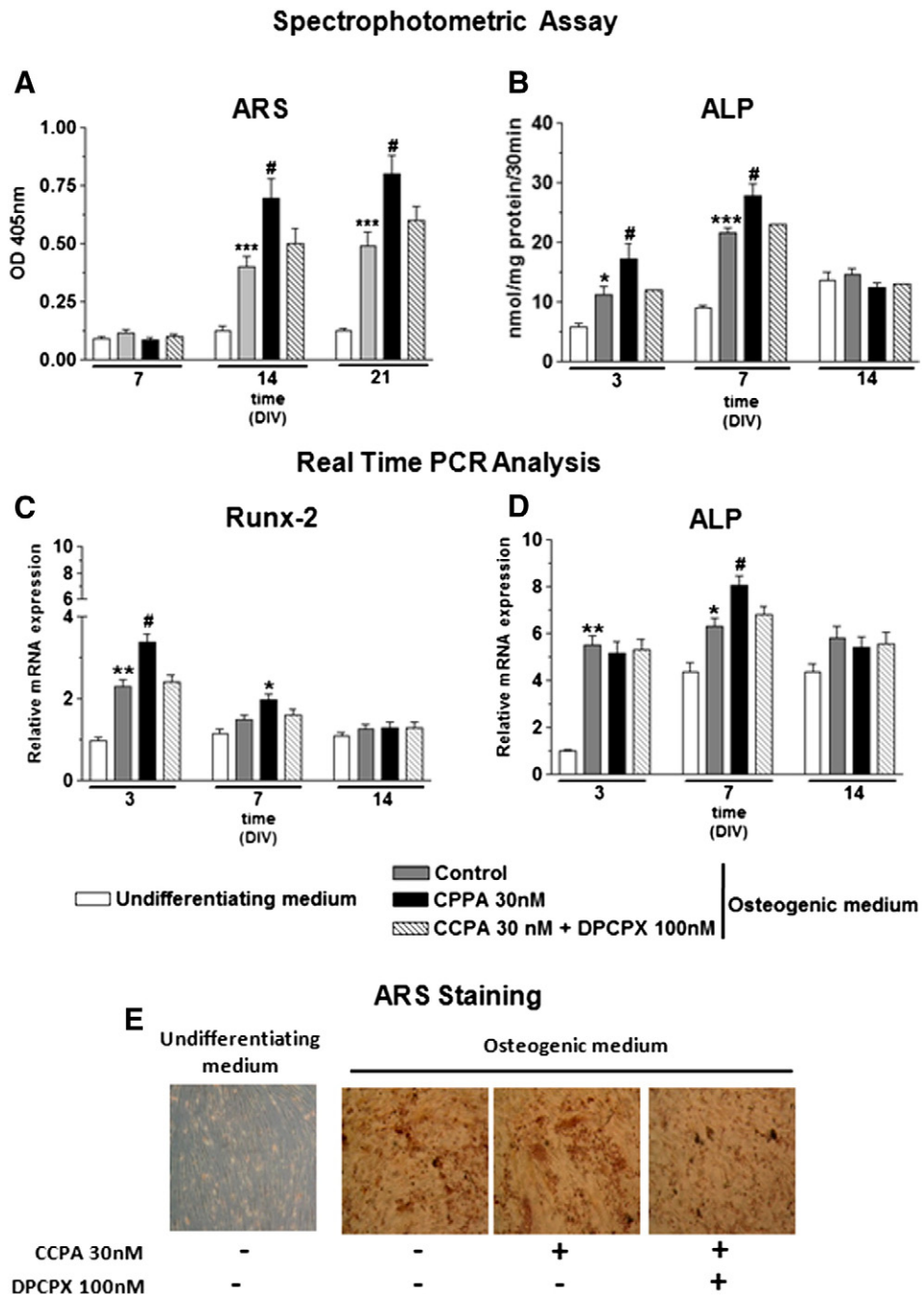
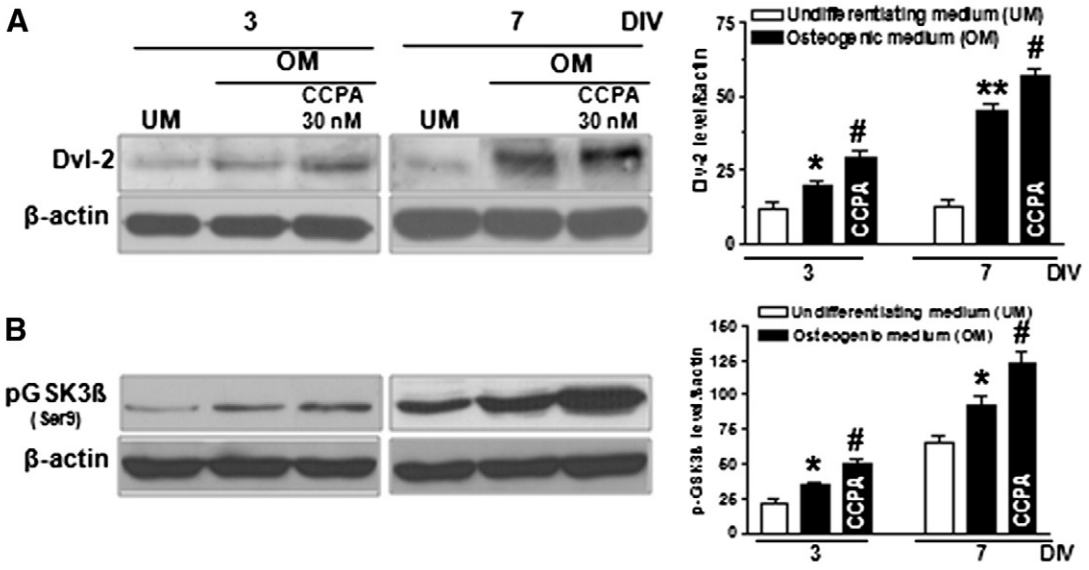
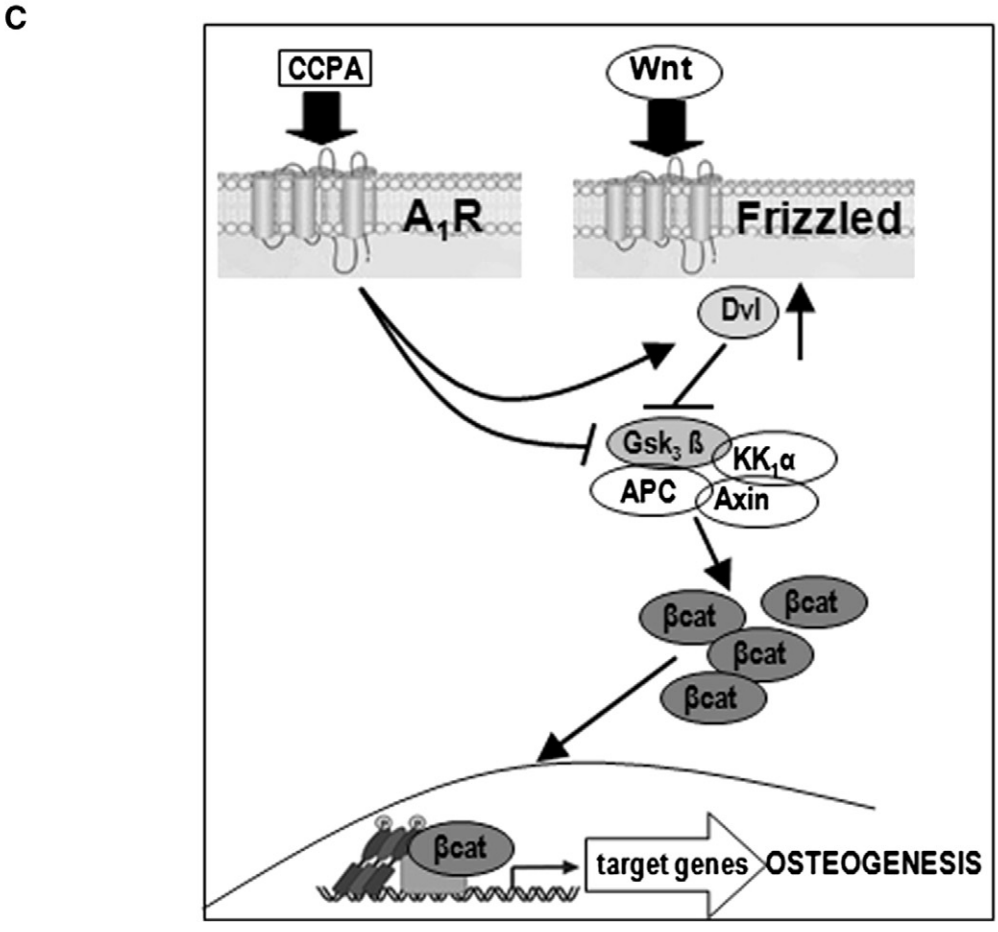


Fig. 4 Effects of the treatment with CCPA, assessed after different periods (7–14 and 21 DIV), on the osteogenic differentiation of DPSCs evaluated as extracellular matrix mineralization (panels A and E), ALP activity (panel B) and expression of osteogenic markers such as RUNX-2 and ALP (panels C–D). In some experiments, cells were pre-treated with DPCPX, A_1R antagonist, added 30 min prior to the addition of CCPA. A and E) Mineralization of DPSCs, grown for 21 DIV in osteogenic medium, evaluated by cell staining with Alizarin red S (ARS, panel E) and quantification of staining *via* extraction with ammonium hydroxide (panel A) at different periods (7–14–21 DIV). The amount of released dye was measured by a microplate reader at 405 nm. The values, expressed as units of optical density (O.D.), are the mean ± S.E.M. of three independent experiments, in which different cell samples were used. B) DPSCs were grown in the absence or in the presence of CCPA. After different periods (3, 7 and 14 DIV), ALP activity was assayed as described in the Materials and methods section and expressed as nmol of *p*-nitrophenol produced per mg of cell protein within a period of 30 min (nmol/mg protein/30 min). Values are the mean ± SEM of three separate experiments. C–D) ALP and RUNX-2 expression evaluated as real time PCR. This analysis was performed on total RNA extracted from DPSCs cultured in undifferentiating medium and osteogenic differentiating medium, as described in Materials and methods, some of differentiating cells being exposed to CCPA for different period of time (3–7–14 DIV). Data are the mean ± SEM of three separate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$: significantly different from undifferentiated DPSCs; # $p < 0.05$: significantly different from DPSCs growing in osteogenic medium (Student's t test).

Thus, DPSCs are able to respond to the stimulation by extracellular purine nucleosides which, for the most part, are formed from the corresponding nucleotides, commonly released from cells in physiological and even more in pathological conditions. However, as observed elsewhere (Costa et al., 2011; Shimegi, 1998), the activity of the enzymes metabolizing extracellular ATP does not produce amounts of endogenous adenosine able to influence the osteogenic differentiation of DPSCs grown in normal (non stressful/pathological-like) culture conditions. In fact, cell exposure to apyrase or ADA, that increased or decreased adenosine levels in the culture medium, or to AR antagonists alone did not modify the osteogenic commitment of our cells. In contrast, the pharmacological and selective





stimulation of AR subtypes, in particular of A_1R or $A_{2B}R$, increased DPSC osteogenic differentiation, with a greater effect by A_1R activation. Since in our cells the specific stimulation of A_3R caused no modification of their osteogenic commitment, this also excludes that CCPA, even at the highest dose (60 nM), reported as able to interact with A_3R in human cells (Klotz et al., 1998), may enhance DPSC differentiation by interfering with these receptors. Noteworthy, A_1R influenced also the proliferation of differentiating DPSCs, as previously observed in other cell types of mesenchymal origin from humans or rodents (Costa et al., 2011; Shimegi, 1998), without affecting cell cycle of DPSCs grown in undifferentiating medium. Since they show a very rapid duplication, we hypothesize that the proliferative effect of CCPA might be obscured.

So far, literature on A_1R has been in favor of a prevailing role played by these receptors in the activation of osteoclasts (Kara et al., 2010; He and Cronstein, 2012) and some findings showed that the blockade of these AR may induce favorable effects on osteoporosis or fracture healing. However, our results showed that the stimulation of A_1R favors the commitment of MSCs towards osteogenesis. This effect might reinforce a similar one promoted by purines acting on $A_{2B}R$, whose expression seems to increase with the age of cells/patients.

Noteworthy, the A_1R-induced enhancement of osteogenesis in DPSCs is coupled to the activation of Wnt signaling pathway and this is the first time to our best knowledge that such an involvement has been documented. So far, we and others have demonstrated that A_1R stimulation is coupled to the activation of the mitogen-activated kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3K)/Akt pathways (Schulte and Fredholm, 2003; Ciccarelli et al., 2007; D'Alimonte et al., 2007), in addition to the well known Gi protein-mediated inhibition of the adenylate cyclase/cAMP system. In this concern, previous data showed that the stimulation of this enzyme system induced osteogenesis in human but not in rodent derived MSCs (Siddappa et al., 2008, 2010). Accordingly, osteogenic effects caused by the stimulation of either A_1R or $A_{2B}R$ have been evaluated in terms of adenylate cyclase activity (Costa et al., 2011; Gharibi et al., 2011). Surprisingly, the effects evoked by the two AR stimulation on MSC osteogenesis were similar whereas the invoked mechanism of action on cAMP formation was the opposite. Tentavive explanations looked at the other known molecular pathways coupled to these receptors such as MAPK or PI3K/Akt signals. Our findings demonstrated that there may be another possibility, as the stimulation of A_1R leads to the activation of Wnt pathway. At present, there is an increasing interest in the activity of Wnt signal in different cells and in particular in the modulation of MSC differentiation into osteoblasts (Bain et al., 2003; Gregory et al., 2005), even though results on this aspect are still conflicting. In fact, a number of papers demonstrated that activation of the canonical Wnt pathway may inhibit MSC differentiation into osteoblasts (Eijken et al., 2008), promoted by bone-morphogenetic protein 2 or dexamethasone (Silverio et al., 2012; de Boer et al., 2004). This is true also for MSC from dental tissues (Liu et al., 2009a; Liu et al., 2009b; Scheller et al., 2008). In order to reconcile these opposite findings, it should be considered that Wnt signal sustains cell differentiation when activated during the first days/weeks of this process, whereas the stimulation of this cascade in more mature osteoblasts seems to cause an arrest of MSC differentiation (van der Horst et al., 2005). In our opinion, a later involvement of $A_{2B}R$ activating the adenylate cyclase/cAMP system may support a complete and successful osteogenic differentiation of MSCs. This hypothesis, however, deserves further investigation.

Conclusions

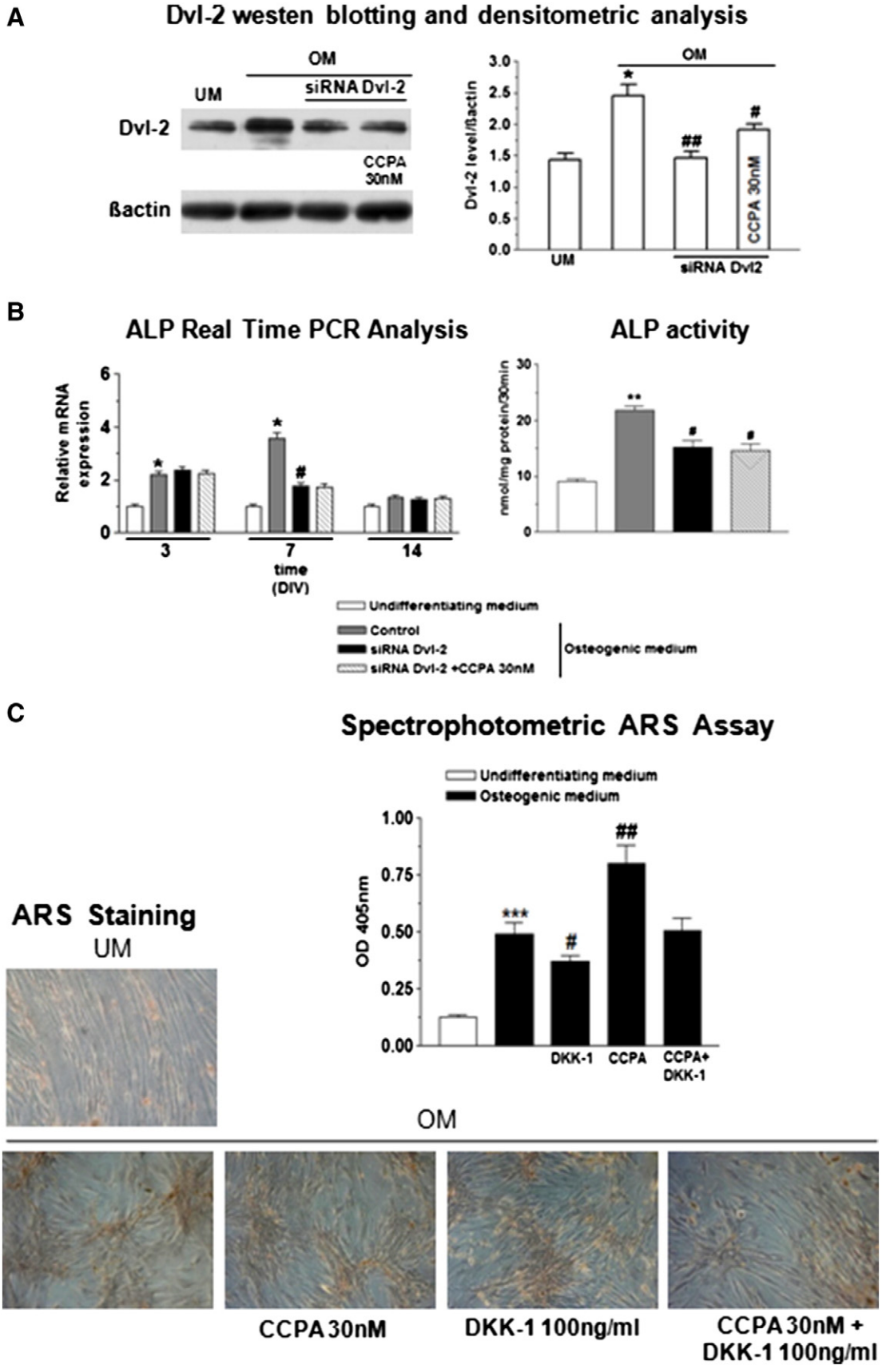
In summary, DPSCs are a good model to study the contribution of MSCs in bone remodeling and regeneration. Also, purinergic signaling, that has been shown to regulate proliferation, cell death, differentiation and successful engraftment of stem cells originating from diverse origins (Glaser et al., 2012), may contribute to modulate osteogenesis and bone damage repair. More in particular, the role played by adenosine, that has long been neglected at the bone level, seems now to attract an increasing interest. Surely, there is still much work to do in order to understand either the function of AR or the complexities of the signal pathways activated as a consequence of their stimulation or inhibition. But we are confident that this kind of research may open a new interesting scenario aimed at a better comprehension of the processes and substances involved in bone remodeling and repair.

Conflict of interest statement

The authors have no conflict of interest to disclose.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.04.002.

Fig. 5 Pharmacological activation of A_1R stimulates the accumulation of Dishevelled-2 (Dvl-2) protein and induces GSK3β phosphorylation in the Wnt pathway in differentiating DPSCs. A–B) DPSCs growing in osteogenic medium were exposed to CCPA for different times. Levels of Dvl-2 or phosphorylated GSK3β were determined by Western blot analysis (50–60 μg of proteins were loaded per lane). Immunoblots, reprobed with antibody against β actin, to assure equal sample loading, were quantified by densitometric analysis, the values of which, normalized to β actin, are reported in the histograms. Densitometric values are the mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$: significantly different from undifferentiated DPSCs; # $p < 0.05$: significantly different from DPSCs growing in osteogenic medium (Student's t test). C) Simplified scheme representing the canonical Wnt pathway as related to the A_1R stimulation. Dvl: dishevelled protein; when activated (directly by Wnt ligands, able to stimulate frizzled receptors, or indirectly *via* A_1R stimulation), increase of cytosolic Dvl levels leads to phosphorylation of GSK3β on serine 9, that results in the inhibition of the GSK3β activity. Thus, β-catenin is no longer phosphorylated and in this way avoids the subsequent ubiquitination/degradation. β-catenin from cytosol translocates into the nucleus, where it activates genes related to osteogenesis.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CCPA
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ARS Staining
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Fig. 6 Pharmacological inhibition of Wnt signal by siRNA of dishevelled-2 (Dvl-2) protein or DKK-1, inhibitor of this pathway, attenuates the osteogenic differentiation of DPSCs either grown in conditioning medium alone or exposed to CCPA. A) Significant knocking down of Dvl-2 was shown by western blot analysis. Silenced cells grown for 3 days in osteogenic medium, in the presence or not of CCPA, failed to elicit increased Dvl-2 expression as compared to normal DPSCs cultured in differentiating condition. Immunoblots, reprobed with antibody against β actin to assure equal sample loading, were quantified by densitometric analysis, the values of which, normalized to β actin, are reported in the histograms. Densitometric values are the mean ± SEM of three independent experiments. $*p < 0.05$: significantly different from DPSCs grown in undifferentiating medium (UM); $\#p < 0.05$, $\#\#p < 0.01$: significantly different from DPSCs growing in osteogenic medium (OM) (Student's t test). B–C) Expression and activity of ALP (evaluated at 7 DIV) as well as ARS staining (measured after 21 DIV) were assumed as reference tests to demonstrate the effect of Wnt signal inhibition on DPSC osteogenic differentiation. In the panel B, experiments were performed in DPSCs silenced for Dvl-2 whereas in the panel C Wnt signal was inhibited by the antagonist DKK-1. CCPA was applied to the cultures at each medium change. When present, DKK-1 was added 30 min prior to the receptor agonist. ALP expression (left) was evaluated as real time PCR performed on total RNA extracted from DPSCs cultured in undifferentiating medium and osteogenic differentiating medium. ALP activity (right) was assayed as described in the Methods section and expressed as nmoles of *p*-nitrophenol produced per mg of cell protein within a period of 30 min (nmol/mg protein/30 min). Values are the mean ± SEM of three separate experiments. ARS staining (shown by the five pictures, representative of at least three separate experiments with similar results) was also quantified by a spectrophotometric assay carried out on cells after 21 DIV. The amount of released dye was measured by a microplate reader at 405 nm. The values, expressed as units of optical density (O.D.), are the mean ± S.E.M. of three independent experiments, in which different cell samples were used. $*p < 0.05$, $**_p < 0.01$, $***p < 0.001$: significantly different from undifferentiated DPSCs; $\# p < 0.05$, $\#\#p < 0.01$: significantly different from DPSCs growing in osteogenic medium (Student's t test).

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