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Adenosine Receptor Subtype Expression and Activation Influence the Differentiation of Mesenchymal Stem Cells to Osteoblasts and Adipocytes

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ABSTRACT

Osteoblasts and adipocytes differentiate from a common precursor cell, the mesenchymal stem cell (MSC). Adenosine is known to signal via four adenosine receptor subtypes, and significantly, recent findings indicate that these may play a role in MSC differentiation. We therefore investigated adenosine receptor expression and activation during the differentiation of MSCs to osteoblasts and adipocytes. The $A_{2B}R$ was dominant in MSCs, and its expression and activity were transiently upregulated at early stages of osteoblastic differentiation. Both activation and overexpression of $A_{2B}R$ induced the expression of osteoblast-related genes [*Runx2* and *alkaline phosphatase* (*ALP*)], as well as ALP activity, and stimulation increased osteoblast mineralization. The expression of $A_{2A}R$ was upregulated during later stages of osteoblastic differentiation, when its activation stimulated ALP activity. Differentiation of MSCs to adipocytes was accompanied by significant increases in A_1R and $A_{2A}R$ expression, and their activation was associated with increased adipogenesis. Enhanced $A_{2A}R$ expression was sufficient to promote expression of adipocyte-related genes (*PPAR*γ and *C/EBP*α), and its activation resulted in increased adipocytic differentiation and lipid accumulation. In contrast, the A_1R was involved mainly in lipogenic activity of adipocytes rather than in their differentiation. These results show that adenosine receptors are differentially expressed and involved in lineage-specific differentiation of MSCs. We conclude, therefore, that fruitful strategies for treating diseases associated with an imbalance in the differentiation and function of these lineages should include targeting adenosine receptor signal pathways. Specifically, these research avenues will be useful in preventing or treating conditions with insufficient bone or excessive adipocyte formation.

KEY WORDS: ADENOSINE RECEPTOR; OSTEOBLAST; ADIPOCYTE; MESENCHYMAL STEM CELL; DIFFERENTIATION

Introduction

Osteoporosis and obesity are currently major health problems worldwide and, furthermore, are growing in prevalence. They are both disorders of body composition, and while they were once thought to be mutually exclusive, they are now thought to be closely related and to share several features.[1,2] One of these shared features is that osteoblasts and adipocytes differentiate from a common precursor cell in the bone marrow, the mesenchymal stem cell (MSC). The pluripotency of MSCs is well known, and their ability to differentiate into osteoblasts, adipocytes, chondrocytes, and myoblasts has been described extensively.[3] Although numerous studies[4–7] have shown that many substances, as well as mechanical agents, contribute to these differentiation processes, the mechanisms involved are yet to be completely defined. There is, however, a large body of evidence to support the notion that there is an inverse relationship between the differentiation of MSCs to osteoblasts and adipocytes. This seems to be true during attainment of peak bone mass[8,9] and in an aging population.[10] Furthermore, such a relationship has been shown in individuals with high bone mass[11] and following mechanical loading,[12] whereas secondary causes of osteoporosis (eg, diabetes, glucocorticoids, and immobility) are associated with bone marrow adiposity.[1]

Adenosine receptors exist as four subtypes, A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R, that signal through primarily the stimulation ($A_{2A}R$ and $A_{2B}R$) and inhibition (A_1R and A_3R) of cyclic adenosine monophosphate (cAMP) and are possible drug targets for many diseases, including osteoporosis.[13] We recently described the expression and function of adenosine receptors in a human osteoprogenitor cell line and in primary bone marrow MSCs and

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showed that $A_{2B}R$ is functionally dominant in these cells.[14] We also showed that adenosine has a potent stimulatory action on interleukin 6 (IL-6) release and an inhibitory action on osteoprotegerin expression, suggesting that adenosine receptors are regulators of MSC differentiation, as well as bone formation and resorption.[14]

Adenosine receptors, in particular, $A_{2A}R$, have been shown to mediate anti-inflammatory actions and have been implicated in tissue repair and damage.[15–18] $A_{2A}R$ has been shown recently to be involved in the proliferation and differentiation of murine bone marrow–derived MSCs.[19] A_2Rs are also expressed on preadipocytes, whereas A_1R is expressed predominantly on mature adipocytes.[20,21] Furthermore, it is known that A_1R activation mediates leptin secretion[22] and protects against obesity-related insulin resistance.[23]

In this study we investigated the expression of the adenosine receptor subtypes, as well as adenosine metabolic and catabolic enzymes, in MSCs as they differentiated to osteoblasts and adipocytes. Furthermore, we used receptor agonists and antagonists, as well as receptor overexpression, to dissect out the role of the receptor subtypes during these differentiation processes.

Materials and Methods

Materials

General and cell culture materials were from Sigma-Aldrich (Poole, Dorset, UK), Fisher Scientific (Loughborough, UK), Lonza (Slough, UK), Biosera (Ringmer, UK), and Invitrogen (Paisley, UK). Molecular biology reagents were from Promega UK Ltd. (Southampton, UK) or as indicated. Antisera were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Abcam (A1R, A2aR, and A3R; Cambridge UK), and Alpha Diagnostic International (A2bR; San Antonio, TX, USA).

Adenosine receptor agonists and antagonists (Tocris Bioscience, Avonmouth, Bristol, UK) were universal agonists, adenosine, and 5′-*N*-ethylcarboxamidoadenosine (NECA); A_1R agonist and antagonist, 2-chloro-N^6-cyclpentyladenosine (CCPA) and 1-butyl-8-(hexahydro-2,5-methanopentalen-3a(1H)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1H-purine-2,6-dione (PSB36); $A_{2A}R$ agonist and antagonist, 3-[4-[2-[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl] phenyl]propanoic acid (CGS21680) and 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH442416); and $A_{2B}R$ antagonist, *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)penoxy]-acetamide (MRS1706).

The natural adenosine receptor agonist adenosine is rapidly metabolized in cell culture environments; thus this work relies on the use of the more stable adenosine receptor agonist NECA, although results with adenosine are also discussed where possible. Furthermore, since a subtype-specific A_{2B} agonist is not currently available commercially for use, we have used throughout this study a universal adenosine receptor agonist (NECA), a specific A_{2A} receptor agonist (CG62180), and specific A_{2A} and A_{2B} receptor antagonists (SCH442416 and MRS1706, respectively) to dissect out the roles of the A_{2A} and A_{2B} receptors in MSC differentiation. Thus we have characterized A_{2B} receptors by relying on the lack of effectiveness of compounds that are potent and selective agonists of other receptor types.

Cell culture

Rat bone marrow MSCs resuspended in normal growth medium—α-minimal essential medium (α-MEM) L-glutamine (2 mM), penicillin (50 U/mL), streptomycin (50 μg/mL), and 10% fetal bovine serum (FBS)—were plated (2×10^6 cells/cm^2) in 75-cm^2 flasks and incubated at 37°C in 5% CO_2 and 95% air for 3 days. The medium then was changed to remove nonadherent cells and again every 3 to 4 days thereafter, and the cells were used at passages 0 to 3.

For osteoblast differentiation, cells were seeded (5×10^3 cells/cm^2) in normal growth medium, and 24 hours later, differentiation was induced with L-ascorbic acid 2-phosphate (50 μg/mL) and dexamethasone (10^{-8} M). For mineralization experiments, β-glycerol phosphate (2 mM) also was added. For adipocyte differentiation, MSCs were cultured in normal growth medium until 50% confluent, and adipogenic differentiation was induced with dexamethasone (10^{-8} M), indomethacin (60 μM), and insulin (10 μg/mL). For most experiments, the medium was replenished every 2 to 3 days. For mineralization experiments involving adenosine, the medium was changed every weekday.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was prepared using TRIzol (Invitrogen), and contaminating DNA was removed using the TURBO DNA Free Kit (Applied Biosystems, Warrington, UK). cDNA was prepared from 1 μg of RNA by incubation for 1 hour at 37°C with Moloney Murine Leukemia Virus reverse transcriptase, RNasin, deoxyribonucleotide triphosphate mix, and oligodeoxythymidilic acid [oligo(dT)$_{15}$]. qPCR was performed on an MX3000P thermal cycler (Stratagene, La Jolla, CA, USA). Each reaction contained SYBR Green Master Mix (12.5 μL; Stratagene), primer mix (1 μL; 10 pmol of each primer, Table 1), cDNA template (1 μL), and nuclease-free water (10.5 μL). The program (40 cycles) consisted of 90°C (30 seconds), 60°C (1 minute), and 72°C (30 seconds). *β-Actin* was used as an invariant housekeeping gene. The relative quantitative expression of the gene of interest (GOI) relative to *β-actin* was calculated, and this ratio for basal or untreated cells was assigned a value of 1.

Western blotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate) containing protease inhibitors cocktail solution (chymostatin, leupeptin, antipain, and pepstatin all at 10 μg/mL), 1 mM sodium orthovanadate, and 0.1 mg/mL of phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were quantified with the Bio-Rad protein assay (Bio-Rad Laboratories, Ltd., Hertfordshire, UK). Lysates (15 to 30 μg of protein) and prestained protein markers (Cell Signaling Technology, Beverly, MA, USA) were mixed with an equal volume of loading buffer (4% sodium dodecyl sulfate, 2% β-mercaptoethanol, 20% glycerol, 100 mM Tris-HCl, pH 6.8, and 0.004% pyronin Y) and subjected to SDS-PAGE. Proteins

were transferred onto polyvinylidene fluoride (PVDF) membranes and incubated overnight at 4°C with specific antisera (used at the recommended concentrations, usually 1:1000 or 1:2000). Secondary antibodies (1:5000) conjugated with horseradish peroxidase were applied for 1 hour at room temperature, and proteins were visualized with ECL Plus reagent. Blots were stripped and reprobed with anti-β-actin (1:1000) for 1 hour at room temperature. Band densities were calculated using an AlphaImager documentation and analysis system (Alpha Innotech Corporation, Santa Clara, CA, USA).

Alkaline phosphatase (ALP) activity assay

Alkaline phosphatase (ALP) activity was determined by an assay based on the hydrolysis of *p*-nitrophenylphosphate to *p*-nitrophenol (Sigma-Aldrich). Cells were washed with PBS, and 50 μL of phosphatase substrate/alkaline buffer (pH 10.3) was added to each well. After 20 minutes in the dark, absorbance (405 nm) was read on a SpectraCount microplate photometer (Canberra Packard, Ltd., Oxfordshire, UK). ALP activity was normalized to protein concentration in parallel experimental plates.

Mineralization

Mineralized matrix was stained with alizarin red S (1:100 in distilled water, adjusted to pH 4.2, and filtered). Cells were washed with PBS, fixed (4% formaldehyde in PBS) for 15 minutes, washed with distilled water, stained for 10 minutes, washed (five times) in 50% ethanol, and air dried. For quantification, cells were destained overnight in 10% (w/v) cetylpyridinium chloride at room temperature with continuous agitation. The absorbance (562 nm) was read using a SpectraCount microplate photometer.

Oil red O and Nile red staining of adipocytes

Accumulation of intracellular lipid was assessed by staining with oil red O — a 5% stock solution in isopropanol was used to prepare the working solution of 3% in water.[24] Cells were fixed (15 minutes with 4% formaldehyde in PBS), stained for 15 minutes, and then washed with 60% isopropanol and PBS. Lipid accumulation was viewed microscopically, and images were captured with a Penguin 150 CL camera (Pixera Corporation, Santa Clara, CA, USA) through Viewfinder 3.0.1 (Pixera Corporation). For quantification, the dye was extracted with 0.2 mL of isopropanol (1 minute), and the absorbance (490 nm) was read using a SpectraCount microplate photometer.

Nile red (1 mg/mL in dimethyl sulfoxide stock[24]) was diluted 1:100 in PBS immediately before use. Cells were trypsinized, fixed for 30 minutes (4% formaldehyde in PBS) at 4°C, centrifuged, resuspended in Nile red (1 mL) for 30 minutes at 4°C, and analyzed with a FACSCalibur flow cytometer (Becton Dickinson Biosciences, Oxford, UK). For each sample, 15,000 events were collected, and data were analyzed using CellQuest 3.1 software (Becton Dickinson Biosciences).

Transient transfection of A_1R, $A_{2A}R$, and $A_{2B}R$ into MSCs

Gene constructs for human A_1R, $A_{2A}R$, and $A_{2B}R$ (kind gift from KN Klotz, Institute for Pharmacology und Toxicology, University of Würzburg, Würzburg, Germany) were cloned or subcloned into the plasmid pcDNA3.1. MSCs were transfected using the Human MSC Nucleofector Kit with the Nucleofector machine (Lonza). Cells were trypsinized, and 5×10^5 cells were resuspended in 100 μL of nucleofector solution and treated with 2 μg of pcDNA3.1/A_1, pcDNA3.1/A_{2A}, pcDNA3.1/A_{2B}, pcDNA3.1 empty vector (vector control), or positive control vector pmaxGFP to monitor transfection efficiency. The cells were plated in 6-well plates, and after overnight incubation, the medium was changed and efficiency of transfection was determined by light and fluorescence microscopy. RNA was prepared 48 hours after nucleofection and subjected to qPCR analysis.

Statistical analysis

Statistical comparisons were determined using one-way ANOVA (SPSS 16, SPSS, Inc., Chicago, IL, USA) and the Tukey multiple-comparison test. Results were considered significant if $p < .05$.

Results

Undifferentiated MSCs

Initial experiments demonstrated that the *A_1R*, *$A_{2A}R$*, *$A_{2B}R$*, and possibly *A_3R* (observed only at a *Ct* value of 35 or above) mRNA, as well as adenosine metabolic and catabolic enzyme (ie, *adenosine deaminase*, *adenosine kinase*, and *CD73*) mRNA, were expressed in undifferentiated MSCs. Furthermore, Western blotting showed that three receptor protein subtypes (A_1R, $A_{2A}R$, and $A_{2B}R$) were present, with the A_3R not being apparent (Fig. 1). In addition, a cAMP assay indicated that the A_{2B} subtype was predominant, although these assays did not preclude the presence of other functional adenosine receptor subtypes (results not shown). Furthermore, the A_3R protein was not detected, whereas MSCs underwent osteoblastic or adipogenic differentiation, and at the same time there was no convincing evidence of the presence of *A_3R* mRNA during these differentiation processes (Figs. 2*D* and 4*D*), Thus we did not continue further with studies of the A_3R in MSCs.

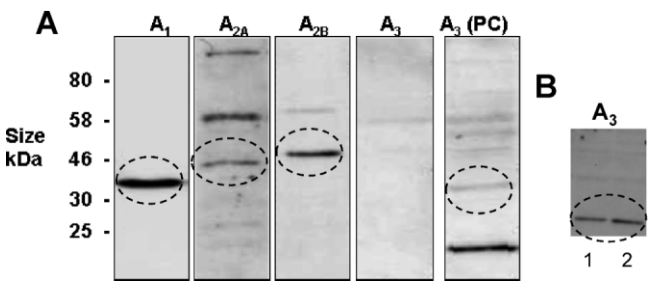

Fig. 1. Western blot analysis of adenosine receptor protein expression. (*A*) Expression of A_1R, $A_{2A}R$, and $A_{2B}R$ proteins is indicated by dashed oval shapes. Positive control (PC; BON-1) for the A_3R protein is also shown. Left margin indicates the sizes (kDa) of protein markers. (*B*) Western blot analysis of BON-1 (*lane 1*) and KRJ-1 (*lane 2*) cell extracts (positive controls) with A3R antibody. BON-1 and KRJ-1 are human neuroendocrine tumors derived from pancreas and intestine, respectively.

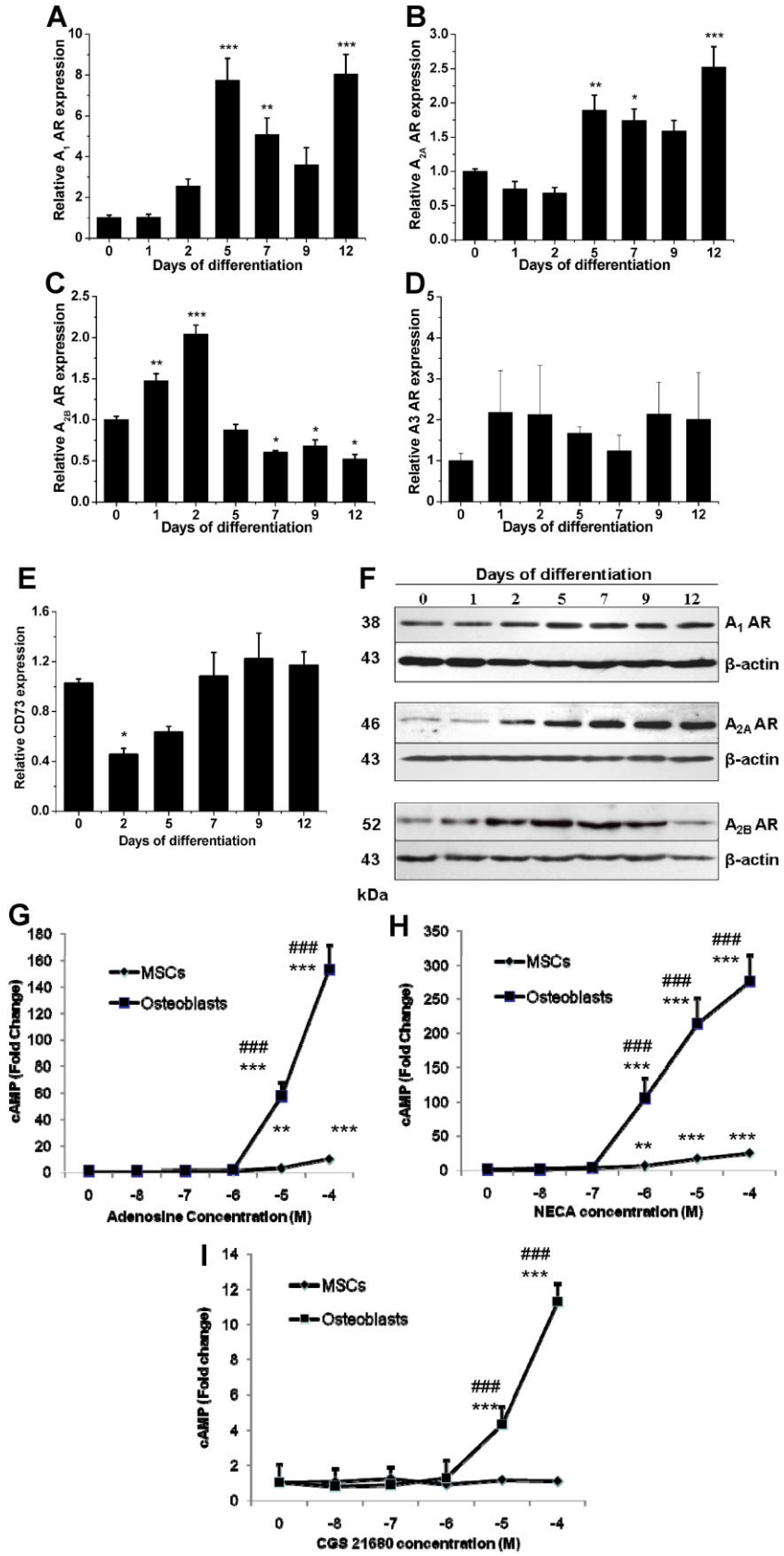

Fig. 2. Expression and function of adenosine receptors during the differentiation of MSCs into osteoblasts. mRNA expression of (*A*) A_1R, (*B*) $A_{2A}R$, (*C*) $A_{2B}R$, (*D*) A_3R, and (*E*) *CD73* (mean ± SEM of three experiments in duplicate). (*F*) Representative Western blots probed for A_1R, $A_{2A}R$, and $A_{2B}R$. (*G–I*) cAMP concentrations in MSCs differentiated to osteoblasts for 9 days and following incubation with (*G*) adenosine, (*H*) NECA, and (*I*) CGS21680 for 15 minutes in the presence of RO20-1724 (10^{-4} M) (mean ± SEM of two experiments in quadruplicate). $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ when compared with untreated cells at day 0; $^{\#\#}p < .001$ and $^{\#\#\#}p < .001$ when compared with untreated cells at the same time point.

Adenosine receptor mRNA and protein expression during osteoblastogenesis

During osteoblastogenesis, the mRNA expression of *A_1R* (Fig. 2*A*) was upregulated by up to eightfold between days 5 and 12, whereas the *$A_{2A}R$* (Fig. 2*B*) and *$A_{2B}R$* (Fig. 2*C*) mRNA showed small increases (twofold). Interestingly, *$A_{2B}R$* mRNA expression also appeared to decrease beyond day 7. No changes were observed for *A_3R* mRNA expression (Fig. 2*D*). *CD73* mRNA decreased by 50% ($p < .05$) by day 2 of osteoblastic differentiation and then progressively returned to levels seen in undifferentiated cells (Fig. 2*E*). There were no changes in adenosine deaminase expression during osteoblast differentiation.

In contrast to changes in *A_1R* mRNA expression during osteoblastogenesis, the A_1R protein remained relatively unchanged. A representative blot is shown in Fig. 2*F*, and combining densitometric values from three separate experiments confirmed that A_1R protein expression did not change significantly during differentiation to osteoblasts. This difference may be related to the already high expression of the protein in undifferentiated MSCs, which could limit further translation of mRNA. Changes in $A_{2A}R$ protein expression were similar to those seen with mRNA expression during differentiation. A relatively faint protein band was detected in undifferentiated MSCs that increased gradually in intensity during osteoblast differentiation and was maximal at days 7 to 12. The $A_{2B}R$ protein expression remained unchanged on day 1 but was strongly upregulated on days 2 and 7, but by day 12 its expression had fallen back to basal levels. A similar pattern of expression was seen for *$A_{2B}R$* mRNA during osteoblastogenesis, but the increase in protein level persisted beyond that of the mRNA, probably because the protein signal is sustained after the mRNA had been degraded.

Effects of adenosine receptor agonists on cAMP accumulation

Treatment with adenosine (natural ligand) and NECA (universal agonist) increased cAMP accumulation in a dose-related manner at all time points, indicating the predominance of A_2Rs at all stages of osteoblastogenesis (see Fig. 2*G*, *H* for day 9 time point). When compared with undifferentiated MSCs (day 0), the efficiency of adenosine and NECA in stimulating cAMP production increased during the time of osteoblastic induction. On day 0, adenosine (10^{-4} M) stimulated cAMP levels ninefold, and this was increased to 153-fold ($p < .001$) after 9 days of differentiation. The efficacy of NECA to increase cAMP also was increased significantly from 25-fold in MSCs to 275-fold at 9 days ($p < .001$) of osteoblastogenesis (Fig. 2*H*). Furthermore, MRS1706 (10^{-6} M) significantly reduced NECA (10^{-5} M) stimulated cAMP accumulation (19.4-fold reduced to 11.6-fold, respectively, $p < .001$) on day 9 of osteoblastogenesis. In addition, SCH442416 (10^{-6} M) significantly ($p < .001$) reduced this effect of NECA (21.2-fold reduced to 13.1-fold with SCH442416).

CGS21680 ($A_{2A}R$ agonist) had little effect on cAMP expression in undifferentiated MSCs (Fig. 2*I*), suggesting an absence of $A_{2A}R$. On osteoblastic induction, however, CGS21680 significantly stimulated cAMP production, with maximum efficacy on day 9 ($p < .001$; Fig. 2*I*). During osteoblastogenesis, although we have shown an increase in $A_{2A}R$ expression, the observed maximal stimulation of cAMP accumulation by NECA was approximately 20-fold more than that seen with CGS21680 at all time points. Together, these data indicate that $A_{2A}R$ and $A_{2B}R$ expression increased during osteoblastogenesis of MSCs and concur with previous data showing increased mRNA and protein expression. Nevertheless the $A_{2B}R$ remained as the dominant receptor in MSC-derived osteoblasts. Furthermore, CCPA did not affect forskolin-stimulated cAMP production (results not shown).

Effects of adenosine receptor agonists and antagonists on osteoblast markers during the differentiation of MSCs to osteoblasts

All the osteoblast markers examined (ie, *Runx2*, *ALP*, *collagen type 1*, and *osteocalcin*) increased during induced osteoblastogenesis of MSCs, and Runx2 and ALP expression increased even further in the presence of NECA (10^{-6} M). *Runx2* mRNA was increased fourfold on day 7 and was further enhanced by NECA to sevenfold (Fig. 3*A*; $p < .05$). Adenosine, however, had no effect. Both NECA (Fig. 3*B*) and adenosine (results not shown) stimulated *ALP* mRNA expression, but neither had a significant effect on the expression of *collagen type 1* or *osteocalcin* mRNA. The late osteoblast marker, *osteocalcin*, however, showed a particularly large change during osteoblastogenesis, with a greater than 10,000-fold increase in mRNA after 7 days.

Initial cell number experiments with receptor antagonists (ie, $A_{2A}R$, SCH442416, and $A_{2B}R$, MRS1706) demonstrated that concentrations up to 10^{-6} M were without effect. These concentrations were used subsequently, and exposure to antagonists alone for 2 or 5 days did not induce significant changes in *ALP* expression (Fig. 3*C*). In the presence of either SCH442416 or MRS1706, however, the NECA-induced increase in *ALP* expression was significantly reduced ($p < .01$).

Effects of adenosine receptor agonists and antagonists on ALP enzyme activity

In addition to effects on *ALP* mRNA expression, NECA (Fig. 3*D*) and adenosine (results not shown) both induced a dose-dependent increase in ALP enzyme activity over 5 to 7 days of osteoblast differentiation. The effects of NECA occurred earlier (day 2) and appeared more potent than those of adenosine, probably a reflection of the stability of NECA in comparison with adenosine. Beyond day 7 of agonist exposure, NECA and adenosine had little effect on ALP enzyme activity. Similar stimulatory effects on ALP enzyme activity were seen with the CGS21680 (Fig. 3*E*); interestingly, this increase in enzyme activity seemed to persist for up to at least 12 days. CCPA did not modulate ALP enzyme activity.

When receptor antagonists (ie, $A_{2A}R$, SCH442416, and $A_{2B}R$, MRS1706) were used, exposure to antagonists alone for 2 or 5 days did not induce significant changes in ALP enzyme activity (Figs. 2*G* and 3*F*). In the presence of SCH442416, however, NECA-induced ALP enzyme activity was partially (~75%) but significantly reversed ($p < .001$; Fig. 3*F*). In addition, the $A_{2B}R$ antagonist MRS1706 abolished the increase in *ALP* mRNA expression and ALP enzyme activity induced by NECA on days 2 and 5 of differentiation (Fig. 3*G*).

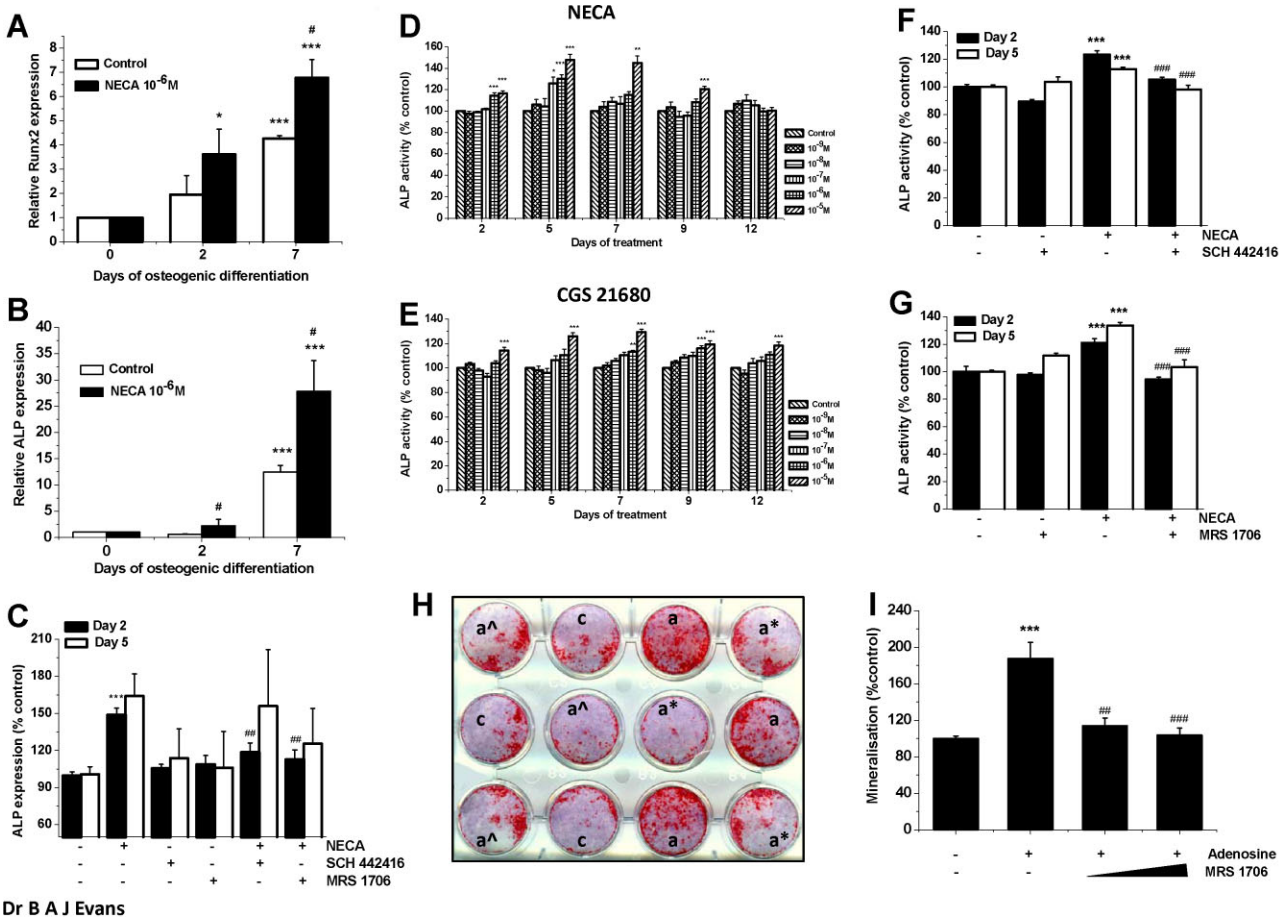

Fig. 3. Adenosine receptor signal pathway stimulation of osteogenesis in MSCs. mRNA expression of (*A*) *Runx2* and (*B*) *ALP* following treatment with NECA (10^{-6} M) for 2 and 7 days. (mean ± SEM of three experiments in duplicate). $^{*}p < .05$ and $^{***}p < .001$ when compared with day 0. $^{\#}p < .05$ when compared with vehicle at the same time point. (*C*) *ALP* mRNA expression following treatment with 10^{-6} M SCH442416 and MRS1706 alone or in the presence of NECA (10^{-6} M) for 2 and 5 days (mean ± SEM of three experiments in duplicate). $^{***}p < .001$ when compared with untreated cells. $^{\#\#}p < .01$ when compared with NECA-treated cells at the same time point. (*D*, *E*) ALP activity following treatment with NECA and CGS21680 for 2, 5, 7, 9, and 12 days (mean ± SEM of three experiments in quadruplicate). $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ when compared with vehicle at the same time point. (*F*, *G*) ALP activity following treatment with 10^{-6} M SCH442416 or MRS1706 alone or in combination with NECA (10^{-6} M) (mean ± SEM of three experiments in quadruplicate). $^{***}p < .001$ when compared with untreated cells. $^{\#\#\#}p < .001$ when compared with NECA alone–stimulated cells at the same time point. (*H*, *I*) Mineralization of MSCs following treatment with adenosine (10^{-4} M) alone or in combination with 10^{-6} M to 10^{-7} M MRS1706. (*H*) Representative photograph [control (c), adenosine (10^{-4} M, a), adenosine (10^{-4} M) + MRS1706 (10^{-7} M) (a*), and adenosine (10^{-4} M) + MRS 1706 (10^{-6} M) (a^)]. (*I*) Mineralization results following elution of alizarin red S and spectrophotometric measurement. (Mean ± SEM from three experiments in triplicate). $^{***}p < .001$ when compared with vehicle control. $^{\#\#}p < .001$ and $^{\#\#\#}p < .001$ when compared with adenosine-stimulated.

Effects of adenosine receptor agonists and antagonists on mineralization of MSC-derived osteoblasts

Since $A_{2A}R$ and $A_{2B}R$ seemed to play an important role in osteoblastogenesis, we also investigated whether activation of these receptors stimulated matrix mineralization. Moderate alizarin red S staining was observed in untreated MSCs after 10 to 12 days, and this was further enhanced ($p < .001$), respectively, by 109%, 50%, and 37% with adenosine (10^{-4} M), adenosine (10^{-5} M), or NECA (10^{-5} M) (Figs. 2*I* and 3*H* and results not shown). Mineralization, however, was unaffected by lower concentrations of NECA or adenosine. Surprisingly, CGS21680 (previously shown to induce ALP enzyme activity) had no effect on mineralization (Supplemental Fig. S1), and mineralization also was unaffected by the A_1R agonist CCPA (Supplemental Fig. S1). The $A_{2B}R$ antagonist MRS1706 inhibited the increase in mineralization induced by adenosine by up to 95% ($p < .001$; Fig. 3*I*), whereas the $A_{2A}R$ antagonist SCH442416 had no effect (Supplemental Fig. S2). The lack of effect by CGS21680 and the lack of inhibition of adenosine-induced mineralization by SCH442416 suggest that the $A_{2B}R$ was responsible for the induction of mineralization.

Adenosine receptor mRNA and protein expression during adipogenesis

During adipogenesis, *A_1R* mRNA expression was strongly upregulated with an 800-fold increase on day 12 ($p < .001$). Changes in *$A_{2A}R$* mRNA paralleled those of *A_1R* but only showed a 15- to 20-fold increase at 12 days ($p < .001$). There were no changes in the expression of *$A_{2B}R$*, *A_3R* (Fig. 4*C*, *D*), *CD73*, or *adenosine deaminase* (results not shown) during adipogenesis.

Table 1. Primer Sequences

Primers (all 5′→3′)	Forward	Reverse	Product size (bp)
A_1R	ATTGCTGTGGATCGATACC	GAATCCAGCAGCCAGCTAT	100
$A_{2A}R$	CTTCGCTATCACCATCAGCA	AAATTGCAATGATGCCCTTC	200
$A_{2B}R$	TCCATCTTTAGCCTCTTG	TCCTCTTGCTCGTGTTC	100
A_3R	TTCTTGTTTGCCTTGTGCTG	AGGGTTCATCATGGAGTTCG	129
CD73	GGACTGATTGATCCCCTCCT	TTGTCCCTGGATTTGAGAGG	192
Adenosine deaminase	AAGGAGACCTCACCCCTGAT	GTCTCATCCCCAGCCAAGT	198
Runx2	GGCCCTGGTGTTTAAATGGT	AGGCTGTTTGACGCCATAGT	178
ALP	CTGCAAGGACATCGCCTATC	CATCAGTTCTGTTCTTGGGGTA	101
Osteocalcin	CCTTCATGTCCAAGCAGGAG	GTCCGCTAGCTCGTCACAAT	152
PPARγ	TTCAGAAGTGCCTTGCTGTG	CCAACAGCTTCTCCTTCTCG	84
C/EBPα	GCCAAGAAGTCGGTGGATAA	AACACCTTCTGCTGCGTCTC	125
LPL	CTTCAACCACAGCAGCAAAA	GGCCCGATACAACCAGTCTA	148
β-Actin	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA	165

Representative Western blots for A_1R, $A_{2A}R$, and $A_{2B}R$ protein expression during adipogenesis (Fig. 4*E*) showed that in parallel with changes in mRNA expression, A_1R and $A_{2A}R$ protein expression also increased. There were no changes in $A_{2B}R$ protein expression (confirmed by combining densiometry values from three experiments) during adipogenesis, consistent with a lack of change in its mRNA.

Adenosine-mediated cAMP responses during adipogenesis

Figure 4*F–I* shows the changes in cAMP when MSCs were grown with or without differentiation medium for 9 days. At 10^{-4} M, the fold increases in cAMP for MSCs and adipocytes were, respectively, 10- and 37-fold (adenosine; $p < .001$), 25- and 55-fold (NECA; $p < .001$), and 0- and 8-fold (CGS21680; $p < .001$). Although these data indicate an overall increase in A_2R function in adipocytes compared with undifferentiated MSCs, the relative change in response to CGS21680 was particularly noticeable and is consistent with an increase in *$A_{2A}R$* mRNA and $A_{2A}R$ protein on adipocyte differentiation. Furthermore, MRS1706 and SCH442416 significantly inhibited cAMP responses to NECA (19.4-fold with 10^{-5} M NECA reduced to 11.6-fold with 10^{-6} M MRS1706 and 18.1-fold with 10^{-5} M NECA reduced to 7.4-fold with 10^{-6} M SCH442416 (both $p < .001$).

Although there was a large increase in *A_1R* mRNA during adipogenesis, this did not appear to be translated into protein, where only a small increase was observed. The A_1R agonist CCPA failed to have an inhibitory action on forskolin-stimulated cAMP levels in either MSCs or adipocyte-differentiated MSCs; this suggests low expression of the A_1R, particularly in the context of increased $A_{2A}R$ expression.

Effects of adenosine and NECA on lipid accumulation and adipocyte numbers

Treatment with NECA (10^{-4} M) increased, in a time-dependent manner, the size and number of cells that contained lipid (Fig. 5*A*). Following elution of the oil red O (a measure of total lipid content of the culture; Fig. 5*B*), the observed effects of NECA (10^{-4} M to 10^{-6} M) were dose-dependent, 10^{-6} M being the lowest concentration that caused a significant ($p < .01$) response. Adenosine (Supplemental Fig. S3) had less of an effect in comparison with NECA, and this probably reflects the short half-life, whereas NECA is regarded as being relatively stable. Treatment with adenosine (10^{-4} M) significantly ($p < .01$) increased adipogenesis from day 7, with the maximum effect on day 12. Nile red staining, however, showed that the number of adipocytes increased by up to 100% ($p < .001$) in the presence of NECA (10^{-4} M; results not shown).

Effect of NECA on the expression of adipocyte markers as MSCs differentiated to adipocytes

Adipogenic differentiation resulted in a gradual increase in the mRNA expression of *PPARγ*, *C/EBPα*, and *LPL* mRNA, which was further enhanced by the addition of NECA (Fig. 5*C–E*). *PPARγ* mRNA expression was induced significantly from 3-, 7-, and 10-fold in untreated MSCs to 6- ($p < .05$), 21- ($p < .001$), and 31-fold ($p < .01$) in the presence of 10^{-4} M NECA for 2, 7, and 12 days, respectively. Treatment with 10^{-5} M NECA also tended to have a similar stimulatory effect, although statistical significance was not reached. NECA also induced similar but more pronounced changes in *C/EBPα* and *LPL* mRNA expression.

Effect of selective adenosine receptor agonists and antagonists on lipid accumulation

Since our experiments suggested that the differentiation of MSCs to adipocytes was associated with changes in expression and activation of $A_{2A}R$ and A_1R, the effects of CCPA and CGS21680 on adipogenesis also were investigated. Both compounds stimulated adipogenesis in a time- and dose-related fashion (Fig. 6*A*, *B*). There were small but significant increases in adipogenesis in the presence of CCPA at 5, 7, and 9 days but only at the higher concentrations (10^{-5} and 10^{-6} M). Similar concentrations of CGS21680 had a much more pronounced effect on adipogenesis. The effects of CGS21680 were comparable to those observed when NECA was used (Fig. 5*A*, *B*). These results indicate an involvement of A_1R and $A_{2A}R$ in the adipogenic differentiation of

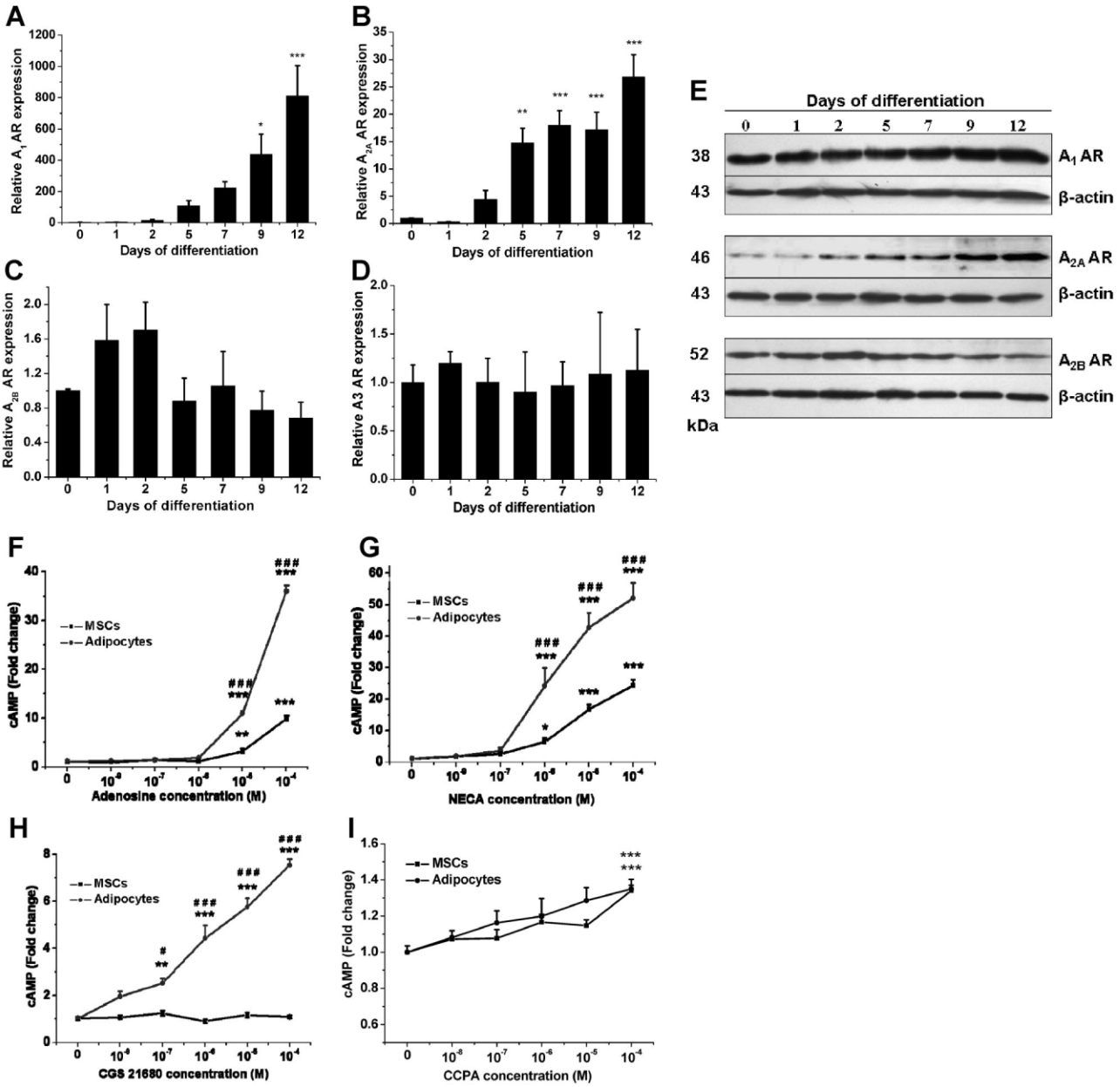

Fig. 4. Expression and function of adenosine receptors during the differentiation of MSCs into adipocytes. mRNA expression of (*A*) *A_1R*, (*B*) *$A_{2A}R$*, (*C*) *$A_{2B}R$*, and (*D*) *A_3R* (mean ± SEM of three experiments in duplicate). (*E*) Representative Western blots probed for A_1R, $A_{2A}R$, and $A_{2B}R$. (*F–H*) cAMP concentrations following incubation with (*F*) adenosine, (*G*) NECA, (*H*) CGS21680, and (*I*) CCPA for 15 minutes in the presence of RO20-1724 (10^{-4} M) (mean ± SEM of three experiments in quadruplicate). $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ when compared with untreated cells; $^{\#\#\#}p < .001$ when compared with MSCs stimulated with the same concentration of agonist.

MSCs. To confirm the involvement of these two receptors, the selective antagonists PSB36 (A_1R) and SCH442416 ($A_{2A}R$) were incubated together with NECA (Fig. 5*C*). The concentrations of antagonist used were 10^{-6} M or less because higher concentrations caused a reduction in cell number. Addition of PSB36 or SCH442416 alone (10^{-6} and 10^{-7} M) had no effect on lipid accumulation. PSB36, however, had no effect on the stimulation of adipogenesis by NECA, suggesting that A_1R may not have an important role in adenosine-mediated adipogenesis. On the other hand, SCH442416 (10^{-6} and 10^{-7} M) inhibited NECA stimulation of lipid accumulation by 80% ($p < .001$) and 75% ($p < .01$), respectively, after 12 days of treatment (Fig. 6*C*). This inhibitory action of SCH442416, however, was not seen at 7 days.

Effects of adenosine receptor overexpression on osteogenic- and adipogenic-associated genes in MSCs

Transient overexpression of A_1R, $A_{2A}R$, and $A_{2B}R$ in MSCs revealed significant changes in expression of osteoblasts and adipocyte markers (Fig. 7*A–D*). Specifically, in relation to osteoblast markers, $A_{2B}R$ overexpression increased the expression of the early marker *Runx2* ($p < .001$; Fig. 7*A*), whereas A_1R

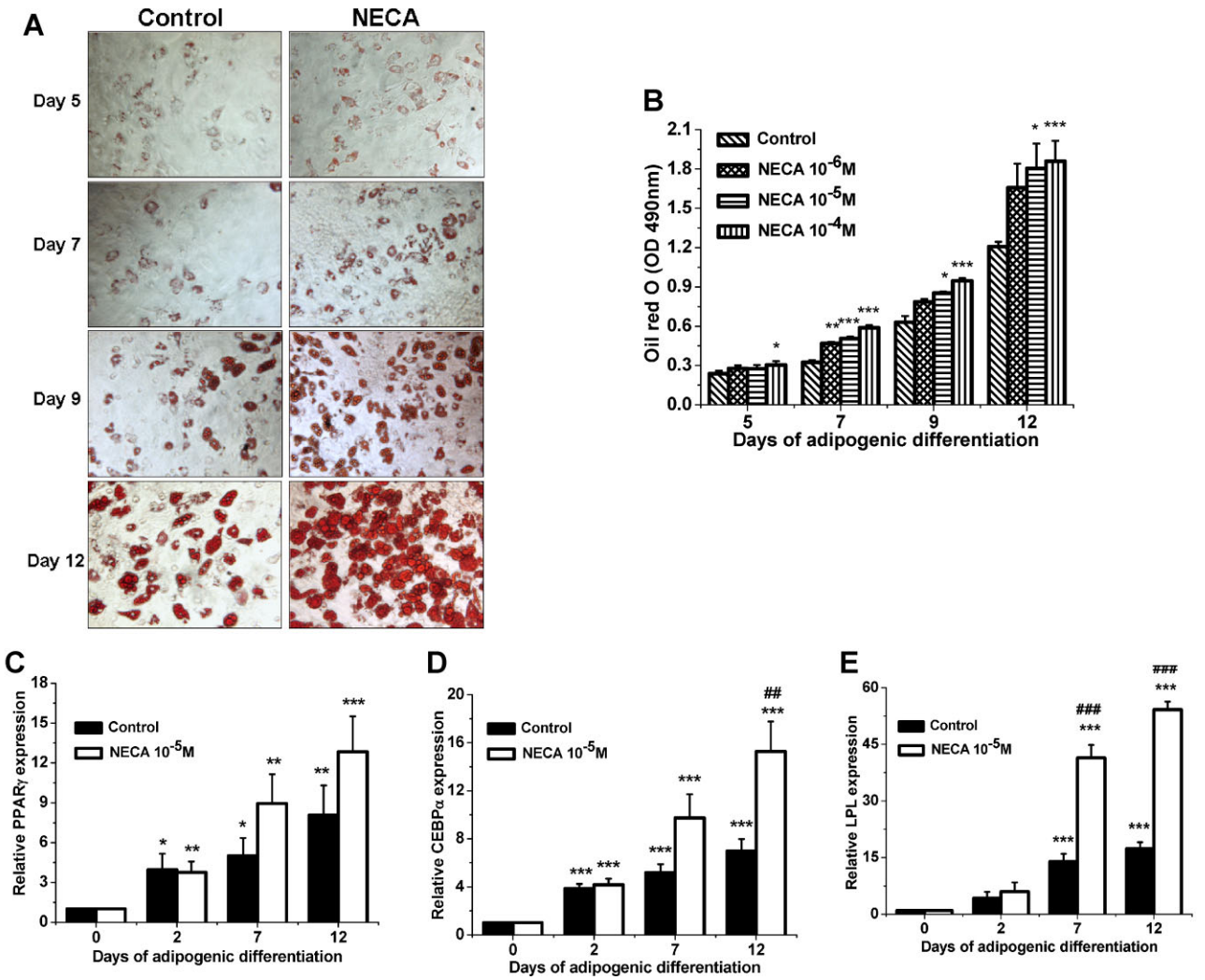

Fig. 5. NECA stimulation of adipogenesis of MSCs. Lipid accumulation (*A*) visualized by staining with oil red O (×200) (NECA 10^{-4} M compared with control) and (*B*) quantified by measuring the absorbance of the dye retained by the cells at 490 nm (mean ± SEM of three experiments in triplicate). $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ when compared with control at the same time point. (*C*) *PPARγ*, (*D*) *C/EBPα*, and (*E*) *LPL* mRNA expression following NECA treatment in adipogenic medium (mean ± SEM of three experiments in duplicate). $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ when compared with day 0 (assigned a value of 1). $^{\#}p < .05$, $^{\#\#}p < .01$, and $^{\#\#\#}p < .001$ when compared with vehicle at the same time point.

overexpression decreased the expression of the intermediate marker *ALP* (Fig. 7*B*). This latter observation could indicate that the A_1R influences differentiation of MSCs to adipocytes and thus away from the osteoblast lineage and therefore ties in with our other data. Overexpression of A_1R or $A_{2B}R$ did not change the expression of adipocyte markers (ie, *PPARγ* and *C/EBPα*; Fig. 7*C*, *D*). However, overexpression of the $A_{2A}R$ significantly increased the expression of *PPARγ* ($p < .01$; Fig. 7*C*) and *C/EBPα* ($p < .001$; Fig. 7*D*).

Discussion

There has been increasing interest recently in determining the factors that regulate the differentiation of MSCs into osteoblasts and adipocytes. Such information would enable the development of strategies for treating diseases associated with an imbalance in the differentiation and function of these lineages. Adenosine is known to regulate the physiologic function of many cells by binding to cell membrane receptors. Under basal conditions, adenosine concentrations in cells and tissue fluids are low (30 to 300 nM). When cells become stressed (eg, during hypoxic events, inflammation, and tissue injury), however, there is an accumulation of adenosine in the extracellular space[25,26] resulting in concentrations of 30 μM.[25,27] Adenosine receptors are known to be expressed by MSCs and are important for the function of these cells.[14,19] In addition, MSCs are capable of producing adenosine because they all express CD73.[28] Recently, adenosine also has been shown to help the localization of human MSCs and promote their differentiation into hepatocyte-like cells,[29] suggesting that adenosine also has a role in the differentiation of MSCs into several possible lineages. Thus we have explored how adenosine receptors are modulated as MSCs are induced to differentiate into osteoblasts and adipocytes and how these processes may be influenced by adenosine.

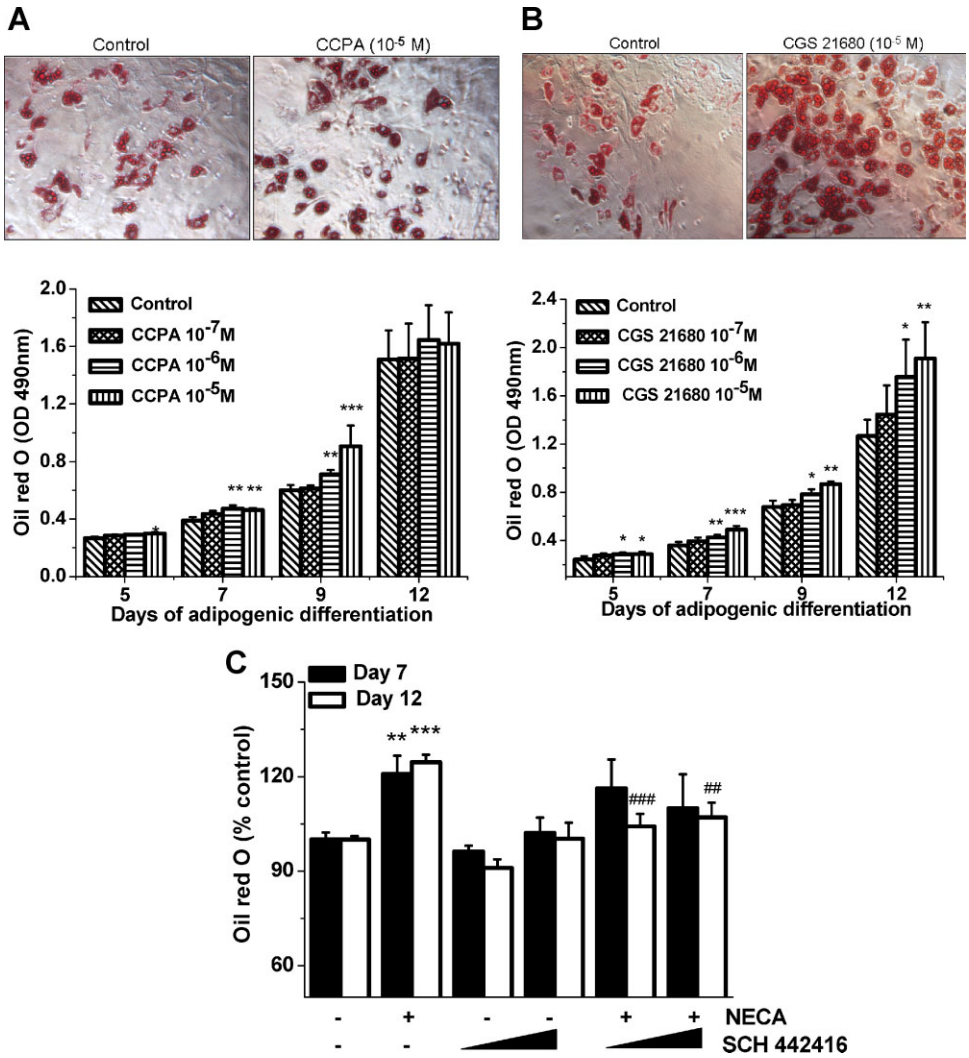

Fig. 6. A_1R and $A_{2A}R$ receptor activation promotes the adipogenesis of MSCs. MSCs incubated with adipogenic medium in the presence of (*A*) CCPA and (*B*) CGS21680 and lipid accumulation visualized by light microscopy of oil red O staining (×200) and quantified by measuring the absorbance at 490 nm (mean ± SEM of three experiments in triplicate). (*C*) Lipid accumulation in MSCs treated with SCH442416 at 10^{-6} M and 10^{-7} M in the presence or absence of NECA (10^{-5} M) in adipogenic medium (mean ± SEM of four experiments in triplicate). $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ when compared with vehicle at the same time point; $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$ when compared with NECA-stimulated cultures at the same time point.

In undifferentiated MSCs, the $A_{2B}R$ was dominantly expressed. Furthermore, its activation (unpublished observations) or overexpression in undifferentiated cells was sufficient to promote the expression of osteoblast lineage markers. Interestingly, *$A_{2B}R$* gene and protein expression appeared to be transiently upregulated as cells differentiated into osteoblasts, suggesting that this receptor may have a role in early osteoblastic differentiation. On the other hand, *$A_{2A}R$* gene and protein expression appeared to increase during later stages of osteoblastic differentiation. From these data, one can speculate that $A_{2B}R$s are more important for commitment and differentiation to osteoblasts and that $A_{2A}R$s are important for osteoblast maturation and maintenance of the osteoblast phenotype. There also was a progressive increase in $A_{2A}R$-mediated cAMP expression as MSCs were induced to differentiate to osteoblasts, which also may support a role for this receptor in later differentiation or function of osteoblasts. Activation (unpublished observations) or overexpression of $A_{2A}R$ in fact did not alter the expression of *Runx2* or *ALP* in undifferentiated MSCs, whereas it stimulated osteogenesis during the differentiation. In addition, $A_{2A}R$ has been implicated in many activities by mature osteoblasts, which might explain the upregulation of the receptors following osteogenesis.[30,31] Nevertheless, in our study, $A_{2B}R$ was the dominant receptor throughout the differentiation process, and its activation led to a stimulation of osteoblastogenesis and subsequent mineralization by osteoblasts. Our observations are supported by a recent publication by Larsen and colleagues,[32] who show that the $A_{2B}R$ is upregulated by 4.2-fold in a human bone marrow stromal cell population capable of in vivo heterotropic bone formation when compared with a second population that does not have the capacity to form such bone.

The A_1R, in contrast to the $A_{2B}R$, was found to be involved in adipogenesis and adipocyte function. A_1R expression is reported to be low in preadipocytes but increases on adipogenesis and remains at high levels in mature adipocytes.[20,21,33] Our data

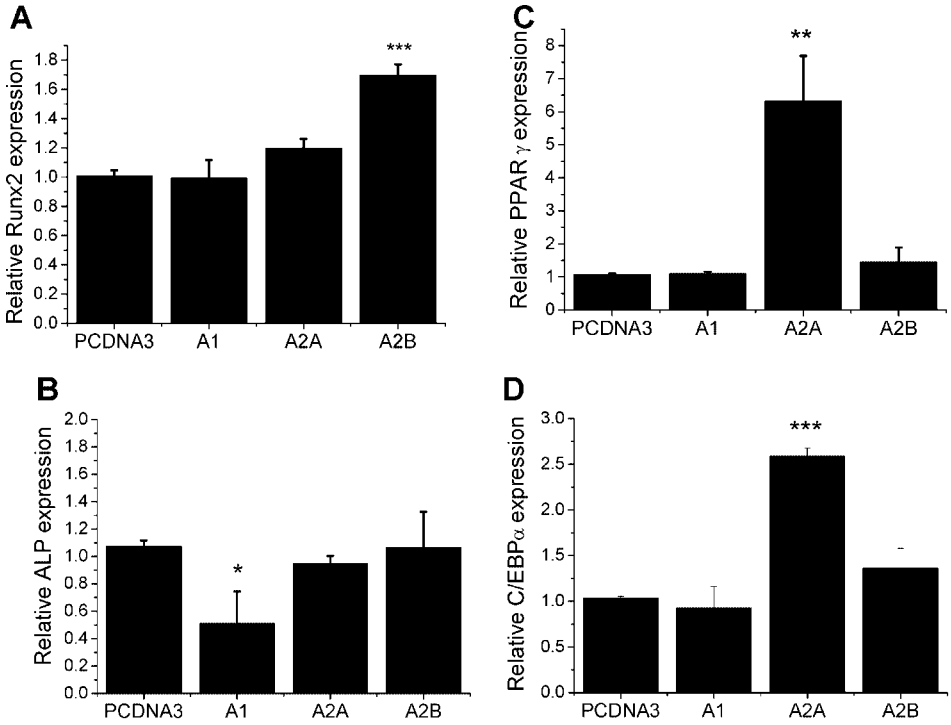

Fig. 7. Effects of adenosine receptor overexpression on osteogenic- and adipogenic-associated genes in MSCs. MSCs were transfected with positive control (pmaxGFP) and PCDNA3 plasmids containing adenosine receptor genes (A_1R, $A_{2A}R$, and $A_{2B}R$). Relative levels of (*A*) *Runx2*, (*B*) *ALP*, (*C*) *PPARγ*, and (*D*) *C/EBPα* mRNA compared with plasmid only–containing cells (mean ± SEM of three experiments in duplicate). $^{*}p < .05$, $^{**}p < .01$,and $^{***}p < .001$ when compared with values for control at the same time point.

concur with these findings. We showed that expression of the A_1R in rat MSCs was low and that it increased markedly on differentiation to adipocytes. A_1R mRNA expression increased by more than 800-fold as MSCs were induced to differentiate to adipocytes. This was accompanied by increases (but to a much lesser extent) in protein expression. On the other hand, when MSCs were differentiating to osteoblasts, there was a less than fivefold increase in A_1R mRNA and no change in protein expression. Despite the remarkable increase in expression during adipocyte differentiation, A_1R activation by the selective agonist CCPA had no effect on expression of markers of adipogenesis. A similar result was observed when MSCs were transfected with the A_1R gene. However, CCPA was found to elevate lipid accumulation by MSCs owing to either stimulation of lipogenesis or inhibition of lipolysis. Adenosine, acting through the A_1R, has been implicated in many of the physiologic functions of adipocytes and has been associated in particular with an inhibition of lipolysis.[34] Many A_1R agonists have been shown to induce antilipolytic effects.[33,35,36] Furthermore, overexpression of A_1Rs and subsequent inhibition of lipolysis in adipocytes have been suggested as a potential cause of a lower and slower rate of weight loss in obese African-American women.[37] We also have evidence that the A_1R stimulates lipogenesis during the differentiation of a preosteoblast cell line into adipocytes (unpublished observations).

Adipocyte differentiation of MSCs also was unexpectedly accompanied by increases in $A_{2A}R$ expression at both the mRNA and protein levels. This was surprising because others have reported a loss of A_2R expression and function during adipogenesis of a preadipocyte cell line (Ob1771)[21] or failed to detect A_2R expression in primary mature adipocytes.[20] Increases in $A_{2A}R$ mRNA and protein expression on induced adipogenesis also was supported by increases in cAMP stimulation in response to adenosine, NECA, and the $A_{2A}R$ agonist CGS21680. The importance of the cAMP/PKA/CREB pathway in the induction of adipocyte differentiation and maturation has been known for many years. Indeed, the cAMP pathway has been reported previously, in murine preadipocyte 3T3-L1 cells and in human MSCs, to stimulate adipogenesis and expression of adipocytic genes.[38,39] Other relevant observations are that adipocyte differentiation in mouse embryonic fibroblasts from $CREB^{-/-}$ mice is impaired,[40] and siRNA-mediated depletion of CREB and the closely related activating transcription factor 1 (ATF1) in 3T3-L1 cells results in loss of adipogenic differentiation.[41]

Activation of $A_{2A}R$ by CGS21680 stimulated adipogenesis of MSCs with similar potencies to NECA. The stimulatory affect of NECA could be blocked by SCH442416 ($A_{2A}R$-selective antagonist), suggesting an $A_{2A}R$-dependent action. We also investigated whether enhanced expression of $A_{2A}R$ is able to stimulate adipogenesis or osteoblastogenesis in the absence of differentiation medium. Our data show that enhanced expression of $A_{2A}R$ is sufficient to induce the expression of key adipogenic master switches such as *PPARγ* and *C/EBPα* that are essential to adipogenic differentiation.[42] Upregulation of these genes subsequently leads to an increase in differentiation, lipid accumulation, and activities of adipocytes. Enhanced A_1R and $A_{2B}R$ expression, on the other hand, did not effect the expression

of *PPARγ* and *C/EBPα*. These findings suggest that the $A_{2A}R$ is mainly associated with the differentiation of MSCs into adipocytes, whereas the A_1R could be involved in lipogenic activity of adipocytes rather than their differentiation.

In summary, data presented here show that adenosine can be important for both osteoblast and adipocyte differentiation. The $A_{2B}R$ is the dominant subtype in undifferentiated MSCs and during osteoblastogenesis. Osteoblast differentiation is associated with increases in the $A_{2A}R$ and $A_{2B}R$ expression, and these receptors stimulate osteoblast differentiation. The $A_{2B}R$ in particular is the major subtype in the differentiation process because it enhances the three stages of initiation, maturation, and mineralization in osteoblastogenesis of MSCs. On the other hand, the adenosine receptors involved in adipogenesis of MSCs are $A_{2A}R$ and A_1R. Despite most of the receptor subtypes being expressed in most, but not all, tissues, targeting adenosine receptors in pathophysiology with truly specific compounds is still a distinct possibility. Many serious attempts are currently being undertaken to develop new therapies in this field—for example, several companies are working in late clinical trial with $A_{2A}R$ antagonists for neurodegeneration.[13] Our work presents a better understanding of adenosine receptor expression and function in MSCs, and the changes in expression and effects of activation of A_1R, $A_{2A}R$, and $A_{2B}R$ observed make them promising targets for developing drugs to prevent or treat conditions with insufficient bone or excessive adipocyte formation. Even if these drugs could not be used systemically, their use locally at fracture sites, in osteoarthritic joints, or to prime MSCs at the time of replacement in relation to tissue engineering, for example, is a distinct possibility. Furthermore, it may be possible to target adenosine receptors indirectly by modulating downstream effectors such as ion channels[43] or putative interacting proteins such as glutamate receptors.

Disclosures

All the authors state that they have no conflicts of interest.

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Author's roles: BG was involved with conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of the manuscript. AAA was involved with collection of data and data analysis for the revised manuscript. JH was involved with conception and design, financial support, provision of study material or patients, data analysis and interpretation, manuscript writing, and final approval of the manuscript. BAJE was involved with conception and design, financial support, provision of study material or patients, data analysis and interpretation, manuscript writing, and final approval of the manuscript.

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