Diverse effects of cyclic AMP variants on osteogenic and adipogenic differentiation of human mesenchymal stromal cells

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Abstract

Osteogenic differentiation of human mesenchymal stromal cells (hMSCs) may potentially be used in cell based bone tissue engineering applications to enhance the bone forming potential of these cells. Osteogenic and adipogenic differentiation are thought to be mutually exclusive and, although several signaling pathways and cues that induce osteogenic or adipogenic differentiation respectively have been identified, there is no general consensus on how to optimally differentiate hMSCs into the osteogenic lineage. Some pathways have also been reported to be involved in both adipogenic and osteogenic differentiation, as for example the protein kinase A (PKA) pathway, and the aim of this study was to investigate the role of cAMP / PKA signaling in differentiation of hMSCs in more detail. We show that activation of this pathway with dibutyryl-cAMP results in enhanced alkaline phosphatase expression whereas another cAMP analogue induces adipogenesis in long-term mineralization cultures. Adipogenic differentiation, induced by 8-bromo-cAMP, was accompanied by stronger PKA activity and higher expression of cAMP-responsive genes, suggesting that stronger activation correlates with adipogenic differentiation. In addition, whole genome expression analysis showed an increase in expression of adipogenic genes in 8-br-cAMP-treated cells. Furthermore, by means of qPCR, we show differences in peroxisome proliferator activated receptor-γ (PPARγ) activation, either alone, or in combination with dexamethasone, thus demonstrating differential effects of the PKA pathway, most likely depending on its mode of activation.

Introduction

Human mesenchymal stromal cells (hMSCs) are a good source for bone tissue engineering applications. Their isolation is relatively easy, they can be expanded *in vitro* and differentiated into osteoblasts, chondrocytes, adipocytes [1], but also into neuron-like cells [2, 3], endothelial-like cells [4, 5] and myocytes [6, 7]. Differentiation of hMSCs *in vitro* towards the osteogenic lineage can be induced with various stimuli and may also promote *in vivo* bone formation [8, 9]. Osteogenic and adipogenic differentiation are thought to be mutually exclusive, as stimuli that induce osteogenic differentiation inhibit adipogenic differentiation [10, 11] and vice versa [12]. This observation is not only important for bone tissue engineering applications, but also for the maintenance of normal bone homeostasis, where the decline in osteoblasts and the associated increase in adipocytes with aging is thought to underlie the fattening of the bone marrow and is seen as the main cause of osteoporosis [13]. Similarly, it was found that chemotherapy, resulting in bone defects as a side effect, induces a shift in the bone marrow stem cell population towards a more adipogenic genotype with higher capacity to differentiate into adipocytes [14].

Both osteogenesis and adipogenesis are induced and regulated by a number of pathways and transcription factors. Whereas osteogenesis is regulated by runt-related transcription factor-2 (runx-2) and osterix (osx) [15], adipogenesis is controlled by peroxisome proliferator activated receptor-γ (PPARγ) [16]. Although an inverse correlation between adipogenic and osteogenic differentiation of hMSCs was suggested, various pathways, activated by external stimuli that induce differentiation seem to play dual roles. For example, there are indications that BMP signaling, one of the main inducers of osteogenic differentiation in hMSCs, is also involved in adipogenic differentiation, depending on the dose and type of receptor activated [17].

Several studies have demonstrated the involvement of the cAMP/protein kinase A (PKA) pathway in osteogenesis of different cell types. In MG-63 cells, it was demonstrated that high glucose levels inhibit osteogenic differentiation, via increased levels of intracellular cAMP and phosphorylated ERK 1/2 [18]. G-protein coupled receptor (GPCR) ligands such as melatonin, prostaglandin E2 (PGE2) and parathyroid hormone (PTH) were demonstrated to increase osteogenic differentiation of MC3T3 cells, rat osteoblast-like osteosarcoma cells [19], calvaria osteoblast-like cells [20] and human osteoblasts [21] *in vitro* and PKA was shown to activate Runx2 in the rat osteosarcoma cell line UMR 106-01 [22] as well as the osteocalcin promoter in human osteoblast-like cell lines

via cAMP and forskolin [23]. In contrast, numerous studies have implicated this pathway in adipogenic differentiation. For example, Zhao *et al.* performed an siRNA screen on hMSCs and found that knockdown of *GNAS*, encoding the G protein α-subunit (Gsα), enhanced osteogenic differentiation. They demonstrated that treatment with forskolin inhibits osteogenic differentiation and treatment with dibutyryl-cAMP (db-cAMP), in combination with high concentrations of dexamethasone (10^{-7} M), enhanced adipogenic differentiation [24]. Similarly, Yang *et al.* demonstrated enhanced adipogenesis and adipogenic gene expression upon addition of PKA stimulators to MSCs in adipogenic induction medium [25]. In contrast, in our lab we have demonstrated that the same compound in the absence of dexamethasone can enhance osteogenic differentiation *in vitro* as well as improve the *in vivo* bone formation capacity of these cells [8]. In addition, we showed that intermittent exposure of hMSCs to db-cAMP decreased alkaline phosphatase (ALP) expression, which points to a more complicated role for the cAMP/PKA pathway in the balance between osteogenic and adipogenic differentiation [26].

Here, we used two different cAMP analogues (db-cAMP and 8-bromo-cAMP (8-br-cAMP)), and investigated their effects on short- and long-term cultures of hMSCs as well as their downstream activation patterns, to examine the role of PKA in osteogenic versus adipogenic differentiation in more detail.

Materials and methods

Isolation and culture of hMSCs

Bone marrow aspirates were obtained from donors after obtaining written informed consent. Human mesenchymal stromal cells (hMSCs) were isolated [27] and proliferated as described previously [28]. Briefly, aspirates were resuspended using 20G needles, plated at a density of 5.10^5 cells/cm^2 and cultured in hMSC proliferation medium, consisting of α-minimal essential medium (α-MEM, Life Technologies), 10% fetal bovine serum (FBS, Cambrex Bio sciences, Verviers), 0.2 mM ascorbic acid 2-phosphate (ASAP, Sigma Aldrich), 2 mM L-glutamine (Invitrogen, Life Technologies), 100 U/ml penicillin (Life Technologies), 10 µg/mL streptomycin (Life Technologies) and 1 ng/mL basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37 °C, in a humid atmosphere with 5% CO_2. Medium was refreshed twice a week and cells were used for further subculturing or cryopreservation upon reaching near confluence. hMSC basic medium was composed of proliferation medium without bFGF, osteogenic medium was composed of basic medium supplemented with 10^{-8} M dexamethasone (Sigma), and mineralization medium was composed of basic medium supplemented with 10^{-8} M dexamethasone and 0.01 M β-glycerophosphate (BGP, Sigma Aldrich). Adipogenic medium was composed of DMEM (Life Technologies), 10% FBS, 0.5 mM isobutyl-methylxanthine (IBMX, Sigma), 1 µM dexamethasone, 10 µM insulin (both from Sigma) and 200 µM indomethacin (Sigma).

Alkaline phosphatase (ALP) expression

To assess ALP expression, hMSCs were seeded in triplicate at 5000 cells/cm^2 in 6-well plates. Cells were kept overnight in proliferation medium to attach, after which the medium was replaced with basic medium or basic medium supplemented with 1 mM db-cAMP or 8-br-cAMP (both from Sigma, stock solutions 20 mM in water). As a negative control cells were cultured in basic medium. After 5 days, cells were trypsinized (0.25% Trypsin, Invitrogen life technologies) and 1 mL of blocking buffer (PBS with 5% bovine serum albumin (BSA, Sigma) and 0.1% sodium azide (Sigma)) was added and incubated for 30 minutes to prevent a-specific binding. After centrifugation and removal of the supernatant, cells were incubated with 50 µl of primary antibody (anti-ALP, B4-78 (Developmental Studies Hybridoma Bank, University of Iowa, USA)), diluted 1:50 in wash buffer (PBS with 1% BSA and 0.05% sodium azide) for 1 hour. For each condition, part of the cells was used for the isotype control to

determine a-specific binding. These cells were incubated with IGg-1 (BD bioscience, diluted 1:50 in wash buffer), a randomly binding antibody. Subsequently, cells were washed twice with 500 µl of wash buffer. Next, 100 µl of secondary antibody (Rat-anti-mouse IgG1-PE (BD bioscience), diluted 1:100 in wash buffer) was added. After incubation for 30 minutes, cells were washed again 3 times. 10 µl of Viaprobe (Pharmingen) was added for live/dead staining, and ALP expression was determined using a FACS Calibur measuring 10,000 events (Becton Dickinson, Immunocytometer systems). FACS data was analysed using CellQuest software. Cells with the right size (forward scatter) and granularity (side scatter) were gated and debris and dead cells were excluded from analysis. The percentage of ALP positive cells was determined, with a minimum of 7500 gated events. Experiments were repeated with cells from five different donors.

Gene expression analysis

hMSCs were seeded in triplicate at 5000 cells/cm^2 in 6-well plates and kept overnight in proliferation medium to attach. Then the medium was replaced with basic medium containing 1 mM db-cAMP or 8-br-cAMP. Cells were lysed for RNA isolation after 3, 5 or 10 days. For dexamethasone experiments, either db-cAMP or 8-br-cAMP, in combination with various concentrations of dexamethasone (10^{-8} M, 10^{-7} M or 10^{-6} M), was added. As a control, cells were cultured in basic medium. After 5 days, RNA was isolated using a Bioke RNA II nucleospin RNA isolation kit (Machery Nagel) and RNA concentrations were measured using an ND100 spectrophotometer (Nanodrop technologies, USA). cDNA was synthesized from 250 ng of RNA, using iScript (BioRad) according to the manufacturer's protocol. For quantitative PCR, a master mix containing distilled water, forward primer, reverse primer (Sigma Genosys), BSA, and SYBR green I mix (all from Invitrogen) was prepared and real-time qPCR was performed using a Light-Cycler (Roche). Light-Cycler data was analyzed using the fit points method of Light-Cycler software. The baseline was set at the lower log-linear part above baseline noise and the crossing temperature (C_t value) was determined. C_t values were normalized to the 18S housekeeping gene and ΔC_t ($C_{t,\,control} - C_{t,\,sample}$) was used to calculate the upregulation in gene expression [29]. Primer sequences are listed in table 1.

Mineralization / adipogenesis

hMSCs were seeded at 5000 cells/cm^2 in 12-well plates in triplicate and kept in proliferation medium overnight to attach to the surface. Then the medium was replaced with mineralization medium (low dexamethasone; 10^{-8} M dexamethasone) or mineralization medium with high concentration of dexamethasone (10^{-6} M), with or

without 1 mM db-cAMP or 8-br-cAMP (only during the first 5 days). As controls, cells were cultured in basic medium supplemented with BGP or in adipogenic medium. After 5 days the medium was replaced with fresh medium, but without cAMP analogues. After 28 days, the medium was removed, and cells were washed with PBS twice. 0.5 mL of 0.5N hydrochloric acid was added and incubated for at least 4 hours at room temperature on an orbital shaker. The supernatant was collected, and the amount of calcium was quantitatively determined, using a calcium assay kit (Sigma diagnostics, 587A) according to the manufacturer's protocol. The absorbance was measured using an ELx808 microplate reader (Biotek instruments). As a positive control for adipogenic cultures, 80% confluent cells were cultured in adipogenic medium. For adipogenic stainings, cells were fixed in formol (3.7% formalin plus 1g/100mL $CaCl_2.2H_2O$ (Sigma)), rinsed with water and incubated with 60% isopropanol (Sigma) for 5 minutes. Then cultures were incubated with freshly filtered Oil-Red-O solution (stock solution: 500 mg Oil Red (Sigma), 99 mL isopropanol, 1 mL water; working solution: 42 mL stock + 28 mL water). For quantification, the stain was extracted by incubating with 4% Igepal (Sigma) in isopropanol for 15 minutes on an orbital shaker at room temperature. 100 µL of the solution was used to measure the absorbance at 520 nm using an ELx808 microplate reader (Biotek instruments).

PKA activity

hMSCs were seeded at 5000 cells/cm^2 in 6-well plates in triplicate and kept in proliferation medium overnight to attach to the surface. The next day, either basic medium, or basic medium supplemented with 1 mM db-cAMP or 8-br-cAMP was added and incubated for denoted time periods. Then, cells were lysed and PKA activity was assayed using the nonradioactive PepTag assay (Promega, Madison, WI), according to the manufacturer's protocol. Briefly, cells were pelleted and lysed in extraction buffer (20 mM Tris (Sigma), pH 7.5, 5.5 mM EDTA (Sigma), 1 mM PMSF (Roche) and 10 mg/mL aprotinin (Roche)). A reaction mixture containing lysate, the PKA specific peptide substrate Peptag A1 (Kemptide), water and peptide protection solution was prepared, which was incubated for 2 minutes at 30 ^{0}C and subsequently for 30 minutes at RT. The reaction was stopped by heat-treatment at 95 ^{0}C for 10 minutes, after which phosphorylated and unphosphorylated Peptag peptides were separated by gel electrophoresis. Bands were excised, solubilized in gel solubilization solution and the amount of peptide was quantified by measuring the fluorescence intensity of the solution with the excitation wavelength set at 540 nm and

the emission wavelength set at 592 nm (Tecan). Liquefied agarose was used as a blank and data is expressed relative to the PKA activity in basic medium.

Whole genome expression analysis

hMSCs were seeded in 12-well plates at 5000 cells/cm^2 and allowed to attach for 10-15 hours in proliferation medium. The next day, either basic medium or basic medium supplemented with 1 mM db-cAMP or 8-br-cAMP was added. After 6 or 72 hours of treatment, cells were lysed directly from the plate and RNA was isolated as described above. Then, from 500 ng of RNA, cRNA was synthesized using the Illumina TotalPrep RNA amplification Kit (Ambion/Life Technologies), according to the manufacturer's protocol and RNA and cRNA quality were verified on a Bioanalyzer 2100 (Agilent). Microarrays were performed using Illumina HT-12 v3 expression Beadchips (Illumina), according to the manufacturer's protocol. Briefly, 750 ng of cRNA was hybridized on the array overnight after which the array was washed and blocked. Then, by addition of streptavidin Cy-3 (Illumina) a fluorescent signal was developed. Arrays were scanned on an Illumina iScan and raw intensity values were background corrected in Genomestudio (Illumina). Further data processing and statistical testing were performed using R and Bioconductor statistical software (http://www.bioconductor.org/). The probe-level raw intensity values were quantile normalized and transformed using variance stabilization (VSN). A linear modelling approach with empirical Bayesian methods, as implemented in Limma package [30], was applied for differential expression analysis of the resulting probe-level expression values. P-values were corrected for multiple testing using the Benjamini and Hochberg method [31] and differentially expressed genes were ranked on $\log_2$ fold change values.

Statistics

All experiments were performed in triplicates. Data were analyzed in SPSS (PASW statistics) using one-way Anova followed by Tukey's multiple comparison test ($P<0.05$ or $P<0.01$). Donor number and passage for each experiment is listed in table 2.

Results

db-cAMP induces ALP expression to a higher extent than 8-br-cAMP

To investigate the effect of both cAMP-analogues on osteogenic differentiation, we examined expression levels of the early osteogenic marker ALP, in hMSCs from 5 different donors after treatment with these compounds. As demonstrated before, db-cAMP increases the expression of this marker by approximately 2- to 3-fold, whereas 8-br-cAMP does induce ALP expression in some donors, but always to a lower extent than db-cAMP, as depicted in figure 1a.

8-br-cAMP induces adipogenic differentiation

To investigate the effects of 8-br-cAMP on differentiation of hMSCs in more detail, a long-term culture assay was performed. hMSCs from 1 donor were cultured in mineralization medium supplemented with either of the two cAMP-analogues for 5 days, after which the cells were kept in mineralization medium for 3 more weeks. In contrast with previous findings, we did not find increased calcium deposition after treatment of hMSCs with db-cAMP in the donor used, even though ALP expression levels increased upon treatment with db-cAMP. As demonstrated before using dexamethasone, hMSCs from different donors vary in their response to osteogenic stimuli [32], which is in line with our findings here. Surprisingly however, addition of 8-br-cAMP to mineralization medium resulted in the formation of lipid droplets (figure 1b), an observation that was confirmed using hMSCs from 4 other donors (data not shown). Staining of lipids with Oil-red-O and subsequent quantification showed that treatment with 8-br-cAMP resulted in almost 60% of the lipid amount in adipogenic control cultures (figure 1c), whereas db-cAMP did not induce any lipid formation. Although lipids and calcium nodules were observed in the same cultures, compared to mineralization or db-cAMP cultures, calcium deposition was severely inhibited by 8-br-cAMP-treatment.

8-br-cAMP induces expression of PPARγ

We then examined the gene expression profile of the two transcription factors controlling osteogenic and adipogenic differentiation, runx2 and PPARγ respectively, after treatment with both cAMP analogues. Figure 2 shows expression levels of these genes in hMSCs of several donors after 3, 5 and 10 days of treatment. Although for some donors runx2 expression was slightly higher after treatment with 8-br-cAMP, in general there was no

difference between the two analogues. For PPARγ however, there was a clear trend towards higher expression in 8-br-cAMP-treated cells, especially during early timepoints, where PPARγ expression was induced by 8-br-cAMP treatment in 5 out of 7 donors. Since dexamethasone is known to have distinct effects on differentiation of hMSCs, depending on its concentration (high or low concentrations induce adipogenic or osteogenic differentiation respectively), we combined 8-br-cAMP treatment with various concentrations of dexamethasone in mineralization medium. As shown in figure 3a, the amounts of lipid droplets gradually increased with increasing concentrations of dexamethasone, with 10^{-6} M resulting in the highest amounts of lipid droplets. In contrast, no lipids were present in the absence of cAMP and hardly any lipids were present in cultures with both db-cAMP and 10^{-6} M dexamethasone. In addition, gene expression profiling of runx2 and PPARγ also demonstrated that, whereas in the absence of dexamethasone neither cAMP analogue affected expression of these genes (see figure 3b), in the presence of dexamethasone, 8-br-cAMP significantly increased expression of PPARγ compared to db-cAMP. Expression of runx2 was decreased with higher concentrations of dexamethasone, and even further by 8-br-cAMP, but not by db-cAMP.

8-br-cAMP is more potent in activating PKA and cAMP-responsive genes

To elucidate if both analogues differentially activate downstream targets, we examined PKA activity after 2, 8, 24 and 48 hours of stimulation. Figure 4a demonstrates that, although there was no difference in activity after 2 hours, after 8, 24 and 48 hours PKA activity was higher after stimulation with 8-br-cAMP, which was significant after 8 and 48 hours. To explore this further, hMSCs were cultured in basic medium or basic medium supplemented with either 8-br-cAMP or db-cAMP for 6 hours, after which we performed whole genome expression profiling. Using the lists of cAMP-responsive genes as described by Zhang *et al.* [33], we examined the expression of cAMP-responsive genes in both db-cAMP- and 8-br-cAMP-treated hMSCs. Figure 4b demonstrates the fold change in expression in either 8-br-cAMP- or db-cAMP-treated cells, relative to cells in basic medium. Of 154 analyzed genes, the expression of 21 genes was increased by treatment with 8-br-cAMP, whereas the expression of 2 genes was decreased. On the other hand, treatment with db-cAMP resulted in increased expression of 13 genes and decreased expression of 9 genes. Interestingly, of the 37 genes with altered expression, 26 showed higher expression in 8-br-cAMP-treated hMSCs, whereas only 11 genes were higher expressed after treatment with db-cAMP. This suggests that 8-br-cAMP more strongly induces transcription of downstream PKA / CREB target genes.

8-br-cAMP induces adipogenic gene expression

To provide more insight in the different mechanisms of db-cAMP and 8-br-cAMP, we analyzed the global gene expression profile of hMSCs treated with both compounds for either 6 or 72 hours. Figure 5 shows the relative gene expression in 8-br-cAMP cultures, compared to db-cAMP cultures. In line with our long-term culture experiments, the expression of several adipogenic genes was higher in cells treated with 8-br-cAMP compared to db-cAMP after 6 hours and expression of these genes increased even further after 72 hours. After 6 hours of treatment, expression of several osteogenic genes was also increased in 8-br-cAMP cultures, but after 72 hours, expression of most of these genes had declined and was instead increased in db-cAMP cultures. Functions of these genes are listed in table 3. Also of interest was the large number of Wnt-related genes with differential expression. After 6 hours, positive regulators of Wnt were mainly decreased in 8-br-cAMP cultures, whereas after 72 hours the expression of both stimulators and inhibitors of the Wnt-pathway was higher in 8-br-cAMP cultures, as well as two Wnt target genes, WISP1 and WISP2. We and others have demonstrated that activation of Wnt signaling in hMSCs inhibits osteogenic differentiation [34-37], and the high number of Wnt-regulated genes in 8-br-cAMP possibly negatively correlates with osteogenic differentiation. Insulin-like growth factor (IGF) signaling and in particular the IGF binding proteins (IGFBPs), were also differentially expressed between the two types of cultures. As depicted in figure 5, after 6 hours, expression of IGF2BP3 and IGFBP5 was higher in 8-br-cAMP cultures, whereas expression of IGFBP2, -3 and-7 was lower. After 72 hours, expression of all IGFBPs was higher in 8-br-cAMP cultures. Generally, disruptions in IGF signaling result in reduced adipogenic differentiation, and most IGFBPs were shown to be present at moderate to high levels in fat tissue and to inhibit IGF or insulin induced adipogenesis [38].

Discussion

We have previously demonstrated enhanced osteogenic differentiation and increased *in vivo* bone formation of hMSCs, after treatment with db-cAMP and forskolin, both activators of the cAMP/PKA pathway [8, 39]. In contrast, here we show that another cAMP-analogue, 8-br-cAMP, induces adipogenesis in osteogenic medium. Upon addition of dexamethasone, 8-br-cAMP also induced gene expression of PPARγ and lipid droplet formation was even further increased, whereas db-cAMP did not affect expression of PPARγ or lipid formation.

As described in the introduction, cAMP/PKA signaling has been involved in both osteogenic and adipogenic differentiation, but no general consensus exists on the specific role of this pathway in hMSC cell fate. The studies by Zhao *et al.* [24] and Yang *et al.* [25] have in common that both used a relatively high concentration of dexamethasone (10^{-7} M), which may explain the contradictory results obtained in our study, where a lower concentration of dexamethasone was used (no or 10^{-8} M dex). Here, we show that treatment with 8-br-cAMP in combination with higher concentrations of dexamethasone results in higher expression of PPARγ and several other studies already described that dexamethasone may sensitize receptors or promote responsiveness of cells to other stimuli. In 1986, Rodan and Rodan suggested that dexamethasone increases the abundance of receptors and Gs in ROS 12/2.8 cells [40] and Ma *et al.* demonstrated that dexamethasone increases expression of β2-adrenergic receptors in calvarial osteoblasts, thereby increasing their responsiveness to adrenergic stimulation [41]. Watanabe *et al.* showed that both PGE_2 and forskolin induce expression of aromatase synergistically with dexamethasone, but not alone [42, 43]. In line with these data, we have also demonstrated synergistic effects between the cAMP-inducers forskolin and cholera toxin and dexamethasone in our own lab. Dexamethasone also induced intracellular cAMP levels when combined with PGE2, whereas PGE2 alone did not affect cAMP levels [26].

Besides the interplay with dexamethasone, the various downstream signaling pathways that are activated by cAMP may have distinct effect. Although it was traditionally believed that cAMP exerts its effects through PKA, around a decade ago it was recognized that cAMP also activates exchange protein directly activated by cAMP (Epac). Two Epac variants (1 and 2), also called guanine-nucleotide exchange factor (GEF) 1 and 2, have been identified, and they activate the small GTPases Rap1 and Rap2 (reviewed in [44]). It has been suggested that at low concentrations, cAMP activates PKA, whereas at higher concentrations, additional effects are exerted via Epac [44]. Specific PKA- and Epac-activating cAMP analogues have been designed, but the cAMP analogues used here

activate both PKA and Epac. Our microarray data shows that treatment with 8-br-cAMP results in higher expression of cAMP-responsive genes and in addition, 8-br-cAMP activates PKA more strongly than db-cAMP. This suggests a mechanism where 8-br-cAMP is either more stable or more potent than db-cAMP and exerts additional effects via Epac, thus resulting in adipogenic differentiation, or a mechanism where low PKA activity (db-cAMP) results in osteogenic differentiation and strong PKA activity (8-br-cAMP) leads to adipogenic differentiation.

The precise functions of PKA and Epac in adipogenic and/or osteogenic differentiation are now topic of investigation and it was demonstrated that, whereas knockdown of Epac resulted in a significant inhibition, blockage of PKA did not affect adipogenesis of 3T3-L1 preadipocytes [45, 46], thus suggesting a model in which cAMP activates Epac to stimulate adipogenesis. On the other hand, it was nicely demonstrated by Petersen *et al*. that adipogenic differentiation of 3T3-L1 cells requires activation of both PKA and Epac [47]. Using Epac or PKA-specific activators, they demonstrated that Epac nor PKA-selective stimulation is sufficient to induce adipogenic differentiation. Furthermore, they showed that PKA inhibits Rho kinase signaling and that knockdown of Rho kinase signaling results in enhanced adipogenic differentiation. Thus, activation of PKA is not required for adipogenic differentiation, as long as Rho kinase is inhibited. The involvement of RhoA and Rho-associated protein kinase (ROCK) was also demonstrated before by McBeath *et al.*, who showed that ROCK activity was associated with cell shape and accordingly, with lineage commitment [48]. Overexpression of RhoA resulted in osteogenic differentiation, whereas expression of dominant negative RhoA lead to the formation of adipocytes in the absence of any inducing factors. In addition to these findings, Li *et al*. demonstrated that, although both forskolin and 8-br-cAMP enhance dexamethasone-, insulin-stimulated adipogenesis, but in the presence of the phosphodiesterase MIX, these compounds inhibited adipogenic differentiation [49]. Most likely, the increased cAMP levels are rapidly decreased in the absence of MIX, whereas in the absence, a prolonged continuous rise in cAMP levels occurs. Furthermore, it was shown that the inhibiting effect on adipogenesis was mediated via PKA and possibly via increased phosphorylation of insulin-receptor substrate-1 (IRS-1) at ser789, thus inhibiting its activity, which is thought to be a prerequisite for adipogenic differentiation. Thus, it was suggested that a moderate increase of cAMP levels correlates with adipogenic differentiation of 3T3L1 preadipocytes, whereas high levels have a strong inhibitory effect. Our data shows a more robust effect of 8-br-cAMP on PKA and cAMP-responsive genes, correlating with adipogenic differentiation. A possible explanation for this apparent contradiction could be Epac, as

a prerequisite for adipogenic differentiation, which might be additionally activated by 8-br-cAMP, but not by db-cAMP.

The aim of this study was to evaluate the role of cAMP, / PKA signaling in osteogenic and adipogenic differentiation of hMSCs. We demonstrate here that two analogues, activating the same pathway can have differential effects on long-term differentiation of hMSCs and gene expression levels, thus pointing to a role for the cAMP/PKA pathway in this balance. This data provides more insight in the stimuli required for osteogenic differentiation of hMSCs and, in addition, information on the underlying biology between osteo- and adipogenic differentiation may be valuable for the treatment of osteoporosis, where this balance is disturbed.

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Disclosure statement

No conflict of interest exists.

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Figure legends

Figure 1. db-cAMP and 8-br-cAMP have distinct effects on differentiation of hMSCs. (a) As demonstrated before, db-cAMP induced ALP expression in hMSCs after 5 days of culture, whereas 8-br-cAMP has no, or only slight effects. (b) When hMSCs were cultured in mineralization medium in the presence of db-cAMP, mineralization was not affected, whereas with 8-br-cAMP, the formation of lipid droplets was observed. (c) Quantification of the staining confirmed these observations. D147-D206; Donor numbers in laboratory databank, magnification in (b) = 40X. OD512; optical density at 512 nm, * $P<0.05$, ** $P<0.01$

Figure 2. 8-br-cAMP induces expression of PPARγ. Treatment of hMSCs with db- and 8-br-cAMP for 3, 5 and 10 days did not result in significant changes in runx2 expression. Expression of PPARγ however, showed a clear trend towards higher expression in 8-br-cAMP-treated cultures. Data is depicted as relative gene expression in either 8-br-cAMP- or db-cAMP-treated cells, relative to cells in basic medium. * $P<0.05$, ** $P<0.01$

Figure 3. 8-br-cAMP induces adipogenesis synergistically with dexamethasone. (a) Treatment of hMSCs with db-cAMP and 8-br-cAMP in mineralization medium with increasing amounts of dexamethasone demonstrated that, in long-term cultures, the amount of lipid droplets in 8-br-cAMP cultures increases with higher amounts of dexamethasone. (b) In addition, after 5 days of culture, 8-br-cAMP reduced runx2 expression with increasing amounts of dexamethasone, whereas runx2 expression was increased with db-cAMP, compared to basic medium. In contrast, PPARγ expression was significantly increased by treatment with 8-br-cAMP with synergistic effects of dexamethasone. Data is depicted as gene expression in treated cells, relative to cells in basic medium. Magnification in (a) = 100X, * $P<0.05$, ** $P<0.01$

Figure 4. db-cAMP and 8-br-cAMP have distinct activation mechanisms. (a) PKA activity assay after 2, 8, 24 and 48 hours of treatment with both cAMP analogues showed that 8-br-cAMP is a more potent stimulator of PKA. Data is depicted as PKA activity relative to basic medium. (b) Gene expression analysis of cAMP-responsive genes, after 6 hours of treatment with either 8-br-cAMP or db-cAMP showed a trend towards higher expression of cAMP-responsive genes after treatment with 8-br-cAMP. Data is depicted as gene expression in either 8-br-cAMP- or db-cAMP-treated cells relative to cells in basic medium. * $P<0.05$, ** $P<0.01$

Figure 5. 8-br-cAMP induces adipogenic gene expression. Whole genome expression analysis after 6 and 72 hours of treatment with 8-br-cAMP and db-cAMP. After 6 hours, expression of several adipogenic genes was increased in 8-br-cAMP-treated cultures, and this number increased further after 72 hours. In addition, expression of several osteogenic genes was increased after 6 hours, but, after 72 hours this profile had changed, and these genes were mostly decreased in 8-br-cAMP. Also of interest was the high number of Wnt-regulated genes. After 6 hours, a few negative regulators were increased in 8-br-cAMP cultures, whereas most positive regulators were decreased. However, after 72 hours, 8-br-cAMP had increased expression of a large number of positive Wnt regulators, as well as negative regulators, suggesting active Wnt signalling in 8-br-cAMP-treated cultures. In addition, expression of IGF binding proteins was differentially regulated. After 6 hours, expression of IGFBP5 and IGF2BP3 was increased

by treatment with 8-br-cAMP, whereas expression of IGFBP2, -3 and -7 was decreased. After 72 hours however, expression of all IGFBPs was increased by treatment with 8-br-cAMP. Data is depicted as gene expression in 8-br-cAMP cultures relative to db-cAMP cultures.

Figure 6. Schematic overview of signalling pathways and crosstalk. PKA is normally activated upon binding of a ligand to a G-protein coupled receptor, via adenylate cyclase and cAMP. PKA phosphorylates CREB, which activates transcription of target genes in the nucleus. In addition, cAMP can activate Epac, which activates Rap1 and -2. The cAMP analogues used in this study are non-specific, thus they directly activate PKA, but can also activate Epac. Treatment with 8-br-cAMP results in higher PKA activity than treatment with db-cAMP, and high levels of cAMP are thought to exert additional effects via Epac. In this model, the large arrow depicts relatively strong activation. Furthermore, PKA inhibits Rho kinase (inducer of osteogenic differentiation) which, together with Epac, has been shown to induce adipogenic differentiation of 3T3-L1 preadipocytes. On the other hand, PKA phosphorylates insulin receptor substrate-1 (IRS-1) at serine-789, thus inhibiting its activity and subsequently, inhibiting adipogenic differentiation.The exact mechanism and the role of the various components remains to be determined.

Tables

Table 1. Primer sequences

Gene	Forward primer	Reverse primer
h18S	5’ CGGCTACCACATCCAAGGAA	5’ GCTGGAATTACCGCGGCT
Runx2	5’ ATGGCGGGTAACGATGAAAAT	5’ ACGGCGGGGAAGACTGTGC
PPARγ	5' GATGTCTCATAATGCCATCAGGTT	5' GGATTCAGCTGGTCGATATCACT

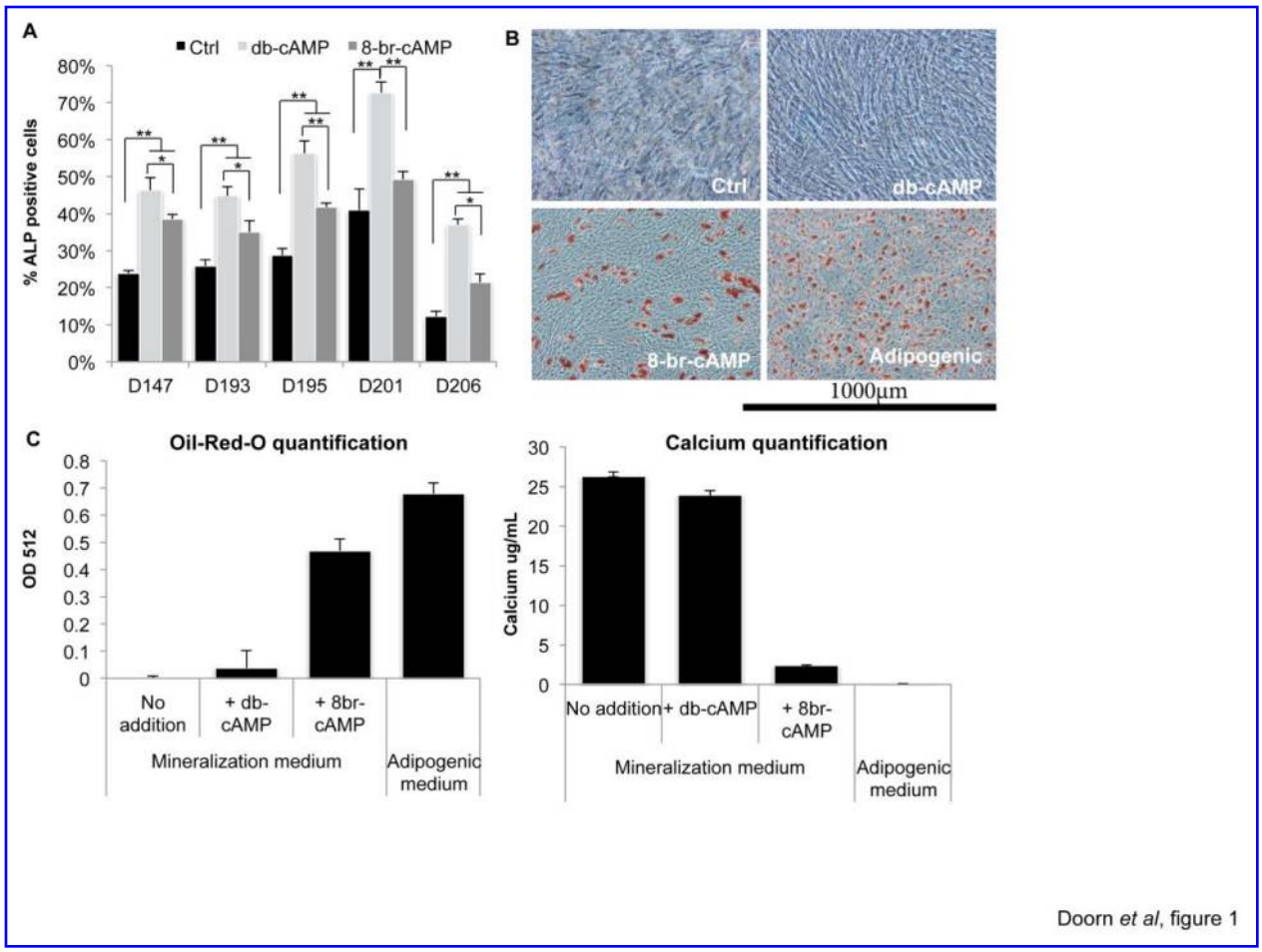
Table 2. Used donors (from laboratory donor bank).

Experiment	**Donor, passage**
ALP expression (figure 1a)	D147, P3
	D193, P3
	D195, P3
	D201, P3
	D206, P3
Mineralization (figure 1b, 1c)	D201, P2
qPCR on Runx2 / PPARg (figure 2)	D147, P3
	D174, P2
	D040, P2
	D195, P3
	D206, P3
	D2408, P2
	D193, P3
	D195, P3
	D201, P3
Mineralization / qPCR with dexamethasone (figure 3)	D192, P2
PKA activity (figure 4a)	D174, P2
Microarray (figure 4b, 5)	D174, P2

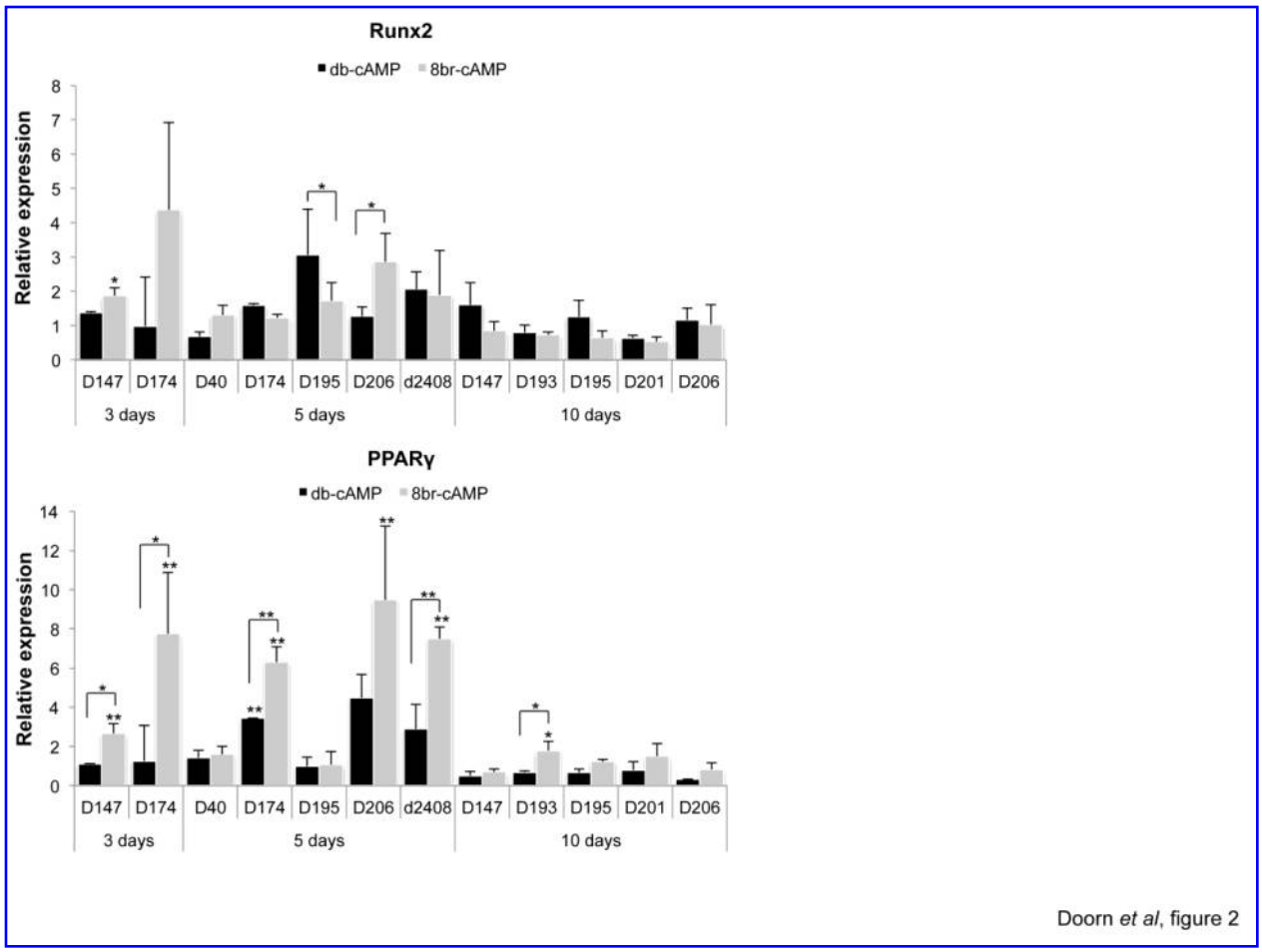
Table 3. Name and function of differentially expressed genes as identified by whole genome expression analysis.

Gene	**Name and function**	**References**
Adipogenesis		
LDLR	*Low density lipoprotein receptor*, ApoE receptor	[47]
sFRP1	*Secreted frizzled-1*, Wnt antagonist, induces maturation of adipocytes, inhibits osteoblast differentiation, stimulates adipocyte differentiation	[48, 49]
sFRP4	*Secreted frizzled-4*, Wnt antagonist, inhibits osteoblast proliferation, specific overexpression results in decreased bone mass, sFRP4 mRNA was gradually increased during adipogenic differentiation in hMSCs	[50, 51]
CEBP ß	*CCAAT-enhancer binding protein* ß, expressed during early stages of adipogenesis, induces expression of CEBPα and PPARγ	[52]
ApoE	*Apoelipoprotein E*, lipid transport / scavenger, secretion of ApoE can be induced by dexamethasone or adipogenic medium	[47]
LBP	*lipopolysaccharide binding protein*, circulating levels are associated with obesity	[53]
CEBP∂	*CCAAT-enhancer binding protein ∂*, expressed during early stages of adipogenesis, induces expression of CEBPα and PPARγ	[52]
PPARγC1A	*PPARγ coactivater 1a*, enhances lipogenesis, activates the fatty acid synthase (FAS) promoter	[54]
CEBPα	*CCAAT-enhancer binding protein* α, expression was increased during terminal stage of adipogenic differentiation	[52]

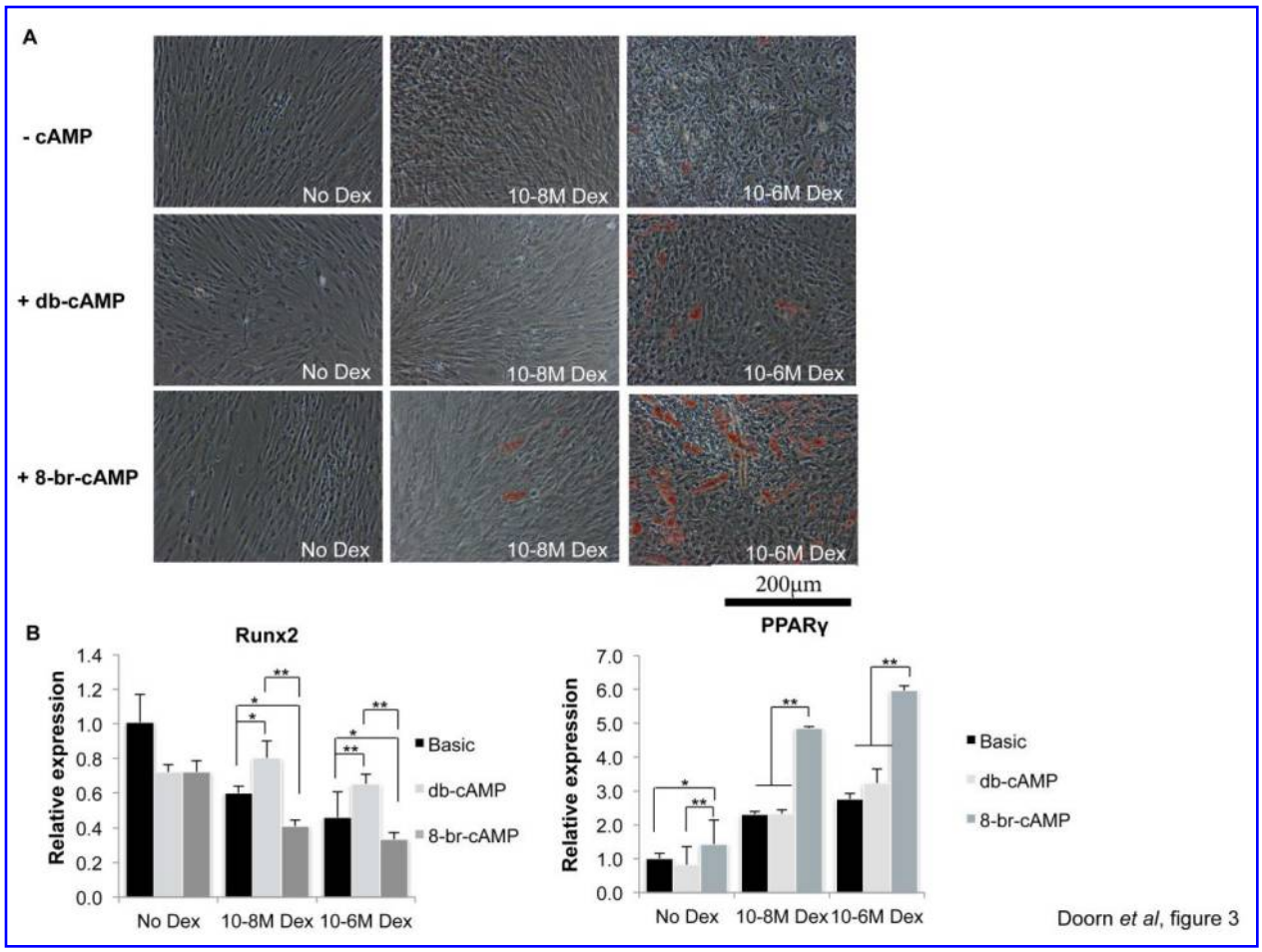
LRP1	*Low density lipoprotein receptor-related protein-1*, regulator of adipogenic differentiation and PPARγ expression	[55]
Osteogenesis		
STC1	*Stanniocalcin-1*, enhances maturation of osteoblasts	[56]
ID1	*Inhibitor of differentiation-1*, BMP target gene, promotes bone formation	[57, 58]
ID2	*Inhibitor of differentiation-2*, BMP target gene (mouse)	[59]
Il11	*Interleukin-11*, involved in both osteoblast and osteoclast signaling	[60]
SMAD6	*Mothers against decapentaplegic-6*, inhibitory smad	[61]
BMP6	*Bone-morphogenetic protein-6*, induces osteogenic differentiation of hMSCs	[62]
Msx	*Msh homeobox 1*, regulates frontal bone development	[15]
ALP	*Alkaline phosphatase*, early osteogenic marker, enzyme mediating mineralization	[63]
LRP5	*Low density lipoprotein receptor-related protein*, enhances bone formation	[64]
Runx2	*Runt-related transcription factor-2*, master transcription factor of osteogenesis	[15]
FOSB	*FBJ murine osteosarcoma viral oncogene homolog B*, osteogenic transcription factor, increases number of osteoblasts, inhibits adipogenic differentiation	[15]
DDIT3	*DNA damage inducible transcript 3*, induces osteoblast differentiation	[65]
Dlx5	*Distal-less homeobox 5*, stimulates osteoblast differentiation	[15, 66]
	Wnt signaling	
sFRP1	See above	
sFRP4	See above	
Dkk	*Dickkopf1*, Wnt inhibitor, expressed during early stages and decreased rapidly during late stages of adipogenesis in hMSCs, reported to promote osteogenic differentiation, overexpression reported to induce adipogenic differentiation	[48, 51, 67]
FZD1	*Frizzled-1*, expression was reduced during adipogenic differentiation of hMSCs	[51]
FZD2	*Frizzled-2*, Wnt receptor	
FZD4	*Frizzled-4*, Wnt receptor	
FZD7	*Frizzled-7*, expression was reduced during adipogenic differentiation of hMSCs	[51]
FZD8	*Frizzled-8*, Wnt receptor	
CTNNB1	*B-catenin*, Wnt stimulator	
CTNNBIP1	*B-catenin interacting protein 1*, Wnt inhibitor	
Wnt5a	*Wingless-type MMTV integration site family, member 5a*, inactivates PPARγ function/adipogenesis, induces osteogenesis in ST2 cells, associated with obesity in mice, induces mineralization and osteopontin expression in hMSCs	[48, 68, 69]
Wnt5b	*Wingless-type MMTV integration site family, member 5b*, induces PPARγ and AP2 in mouse 3T3-L1 preadipocytes	[70]
LRP1	See above	
LRP5	See above	
Src	*Tyrosine kinase*, binds Dishevelled2, Wnt stimulation	
Nkd2	*Naked2*, Wnt inhibitor	
Dvl2	*Dishevelled 2*, Wnt inhibitor	
Dvl3	*Dishevelled 3*, Wnt inhibitor, after an initial increase, gene expression decreases during adipogenic differentiation of 3T3-L1 cells	[71]
DACT1	*DAPPER/FRODO protein 1*, Wnt inhibitor, expression was decreased after early induction of adipogenesis in human preadipocytes, required for adipogenesis in 3T3-L1 preadipocytes	[71]
DACT3	*DAPPER/FRODO protein 3*, Wnt inhibitor	
WISP1	*WNT1-inducible-signaling pathway protein 1*, enhances effects of BMP-2 to induce osteogenic differentiation/bone formation of hMSCs	[72]
WISP2	*WNT1-inducible-signaling pathway protein 2*, in hMSCs expressed during the initial stage of adipogenic differentiation, followed by a gradual decrease	[73]

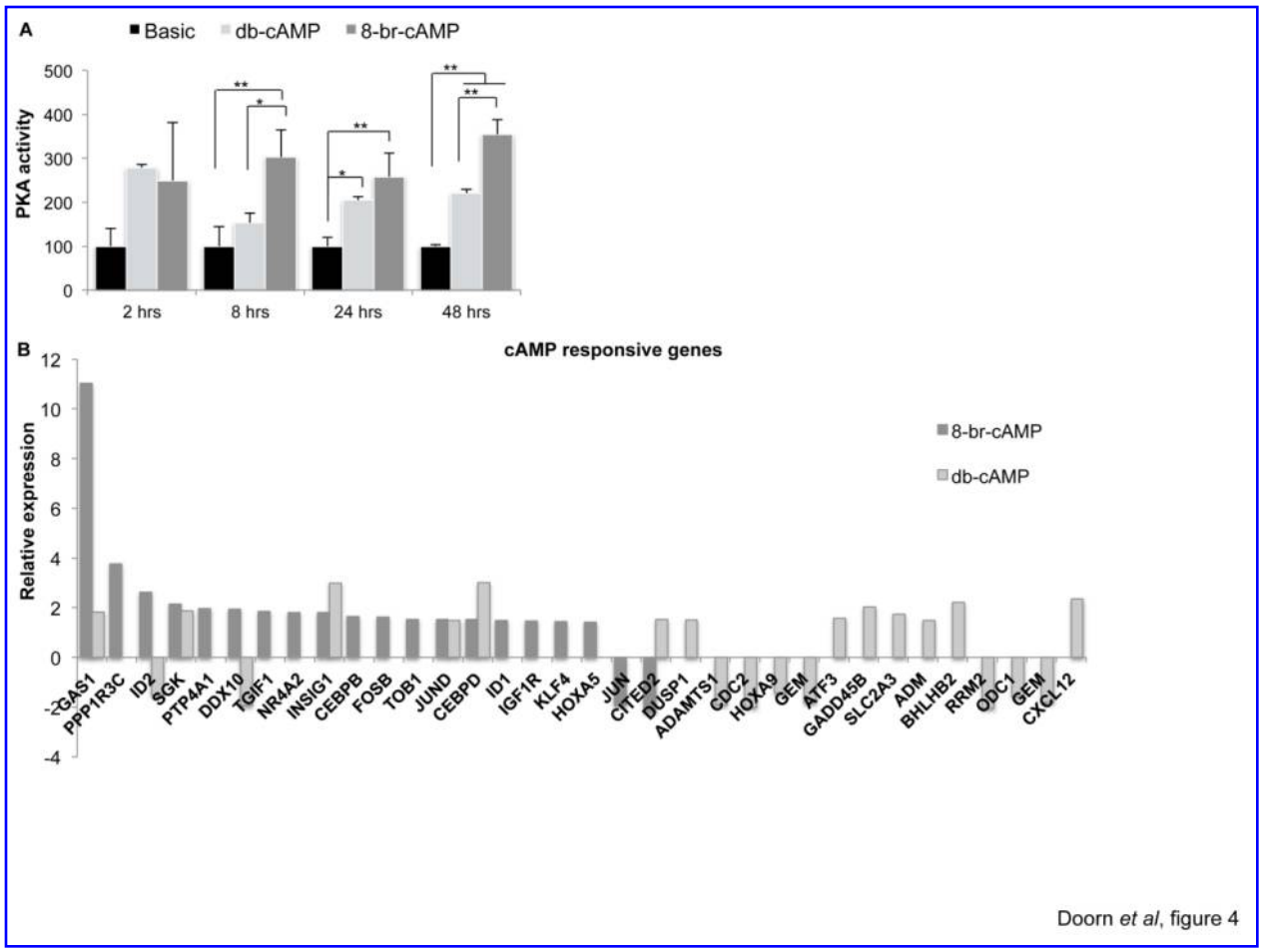


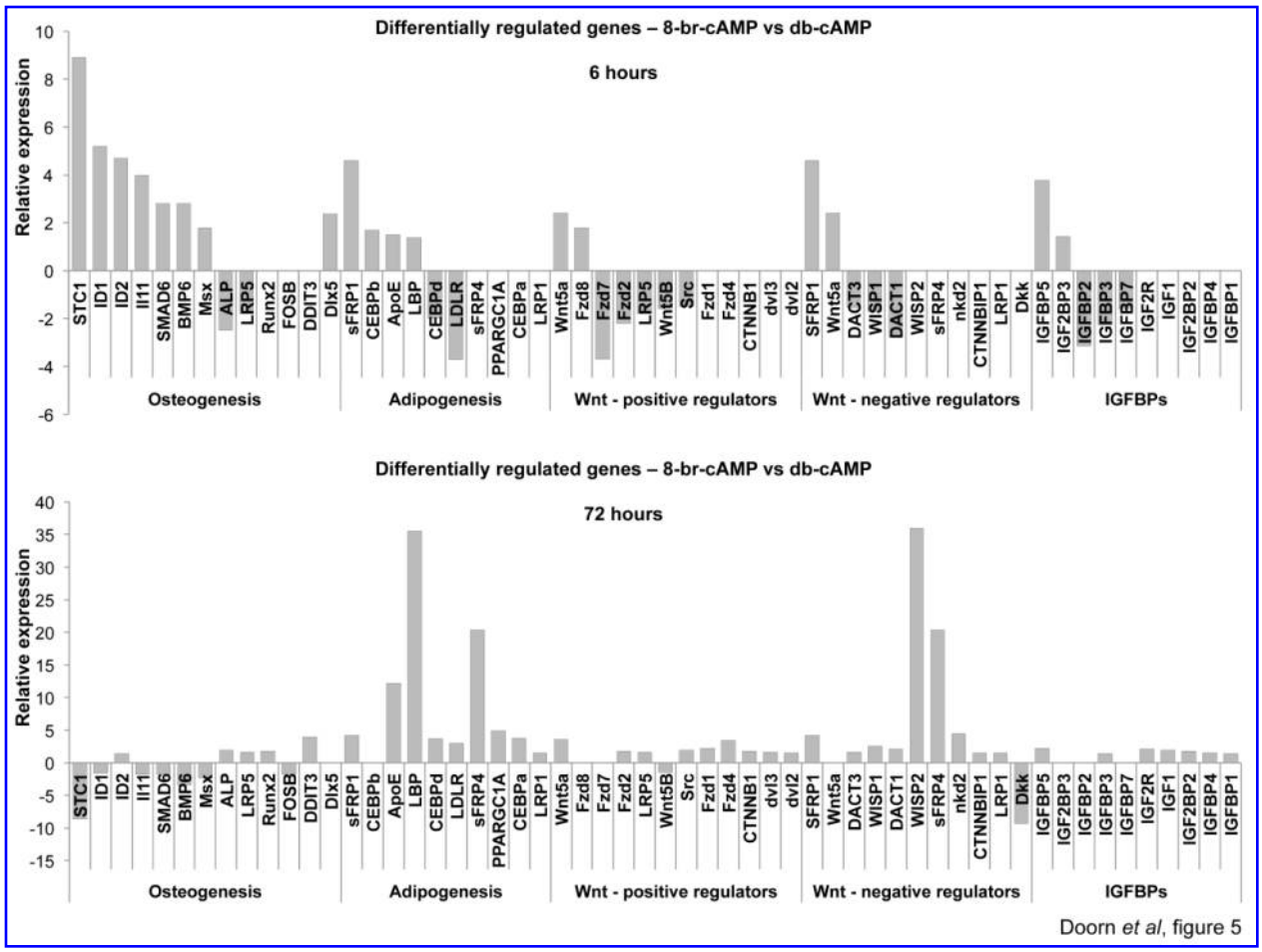
Doorn *et al*, figure 1

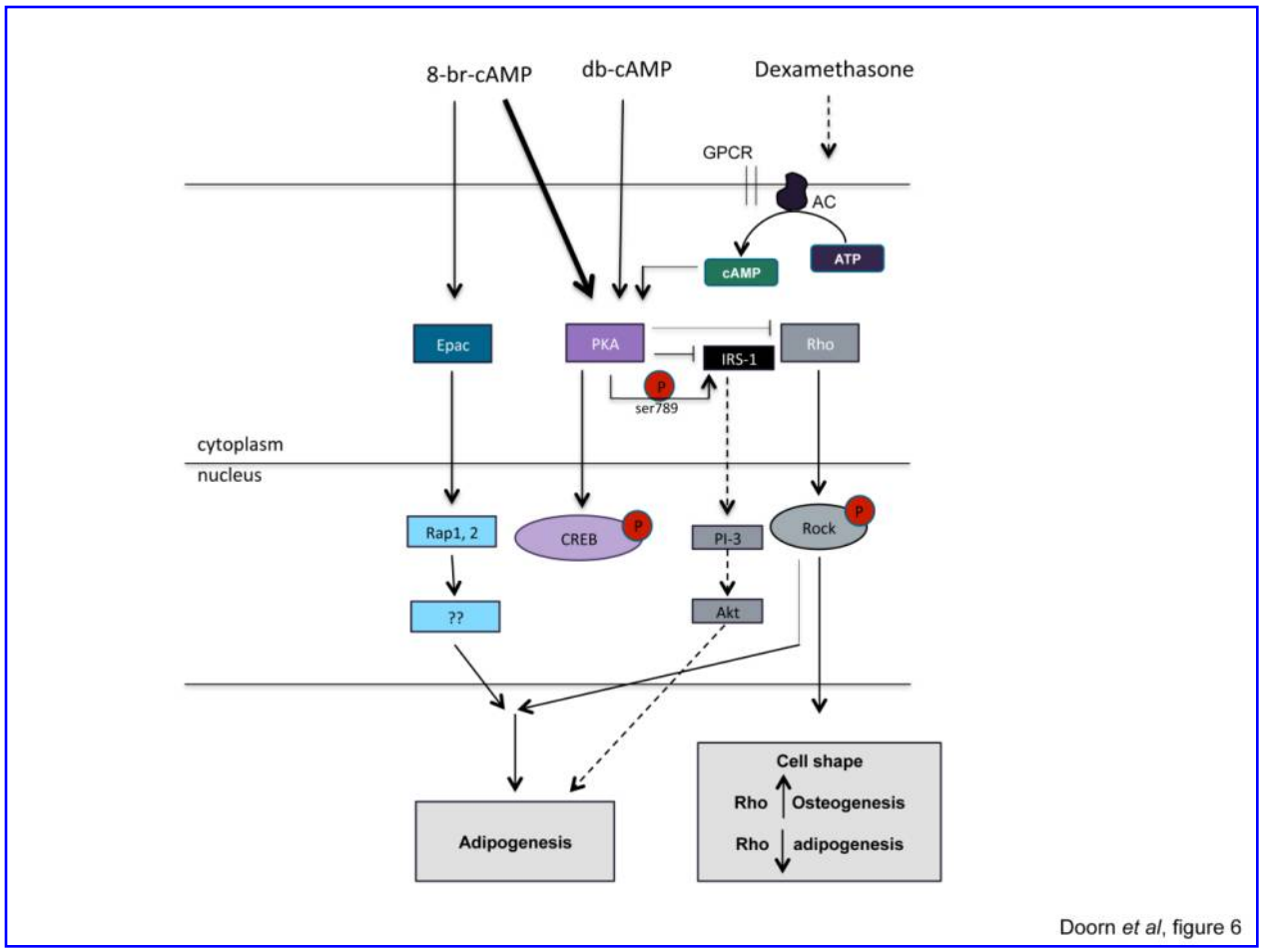


Doorn *et al*, figure 2









Doorn *et al*, figure 6