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Adenosine receptors enhance the ATP-induced odontoblastic differentiation of human dental pulp cells

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ABSTRACT

Purinergic signaling regulates various biological processes through the activation of adenosine receptors (ARs) and P2 receptors. ATP induces the odontoblastic differentiation of human dental pulp cells (HDPCs) via P2 receptors. However, there is no information available about the roles of ARs in HDPC odontoblastic differentiation induced by ATP. Here, we found that HDPCs treated with ATP showed higher activity of *ADORA1* (A_1R), *ADORA2B* ($A_{2B}R$), and *ADORA3* (A_3R). Inhibition of A_1R and $A_{2B}R$ attenuated ATP-induced odontoblastic differentiation of HDPCs, whereas activation of the two receptors enhanced the odontoblastic differentiation induced by ATP. However, activation of ARs by adenosine did not induce the odontoblastic differentiation of HDPCs independently without induction of ATP. Our study indicates a positive role for ARs in ATP-induced odontoblastic differentiation of HDPCs, and demonstrates that ATP-induced odontoblastic differentiation of HDPCs may be due to the combined administration of ARs and P2 receptors. This study provides new insights into the molecular mechanisms of pulpal injury repair induced by ATP.

1. Introduction

Human dental pulp cells (HDPCs) have attracted the interest of researchers in the field of tissue regeneration because of their accessibility and abundance of stem/progenitor cells [1,2]. Recent studies have reported that HDPCs can differentiate not only into odontoblasts for dentin regeneration, but also into osteoblasts, which can repair bone defects under appropriate conditions [3,4]. The differentiation of mesenchymal cells in HDPCs into odontoblasts is induced by multiple cytokines such as four and a half LIM domains 2 (FHL2) [5], bone morphogenetic protein (BMP2) [6], and ID1 (a downstream target of BMP2 signaling) [7].

Purinergic signaling can regulate the proliferation [8], differentiation [9], and death [8] of different types of stem cells. As critical signaling molecules in this pathway, adenosine triphosphate (ATP) and its hydrolysates act through purinergic receptors, which are classified into P1 (adenosine receptors [ARs], e.g. A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R) and P2 (P2XR, e.g. $P2X_{1-7}R$; P2YR, e.g. $P2Y_{1,2,4,6,11-14}R$) receptors. P1 receptors are primarily activated by adenosine, whereas those in the P2 category are mainly regulated by purines such as ATP and adenosine diphosphate (ADP) [10]. Cutarelli et al. [11] were the first to report the biphasic effects of ATP on differentiation and mineralization in human osteoblasts, showing an increase in these processes in response to low concentrations (<100 μM), whereas high concentrations (>100 μM) led to a decrease in these processes, which they suggested was due to the combined activation of P2 receptors and ATP hydrolysis products (e.g. ADP, AMP, adenosine, the mineralization inhibitor PP_i, and the mineralization promoter inorganic phosphate (P_i]). In contrast, the osteogenic effects of ATP on human bone marrow mesenchymal stem cells was due to adenosine stimulation of the AR subtype, $A_{2B}R$ [12].

Extracellular ATP and downstream purinergic signaling have also been proposed to contribute to dental pulp tissue healing and dentin regeneration. Mechanical and thermal stimulation of external dentin can induce ATP release in dental pulp through pannexins [13]. Cold stimulation was also reported to induce ATP release from human odontoblast-like cells [14]. Our previous study demonstrated that high concentrations of ATP (800 μM) can induce odontoblastic differentiation of HDPCs, whereas low

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concentrations (<400 μM) promoted cell proliferation, and that P2 receptors and the ERK/MAPK signaling pathway were involved in this ATP-induced odontoblastic differentiation [15]. These investigations indicated positive roles for ATP and P2 receptors in dental pulp wound healing and dentin formation. However, the specific role of ARs in ATP-induced odontoblastic differentiation of HDPCs, and the effects of adenosine, the hydrolysate of ATP, on HDPC odontoblastic differentiation remain unknown.

All four AR subtypes were demonstrated to be expressed in dental pulp stem cells (DPSCs), and stimulation of A_1R might enhance the osteogenic differentiation of DPSCs, moreover, the progress of dentinogenesis is similar to that of osteogenesis, to some extent [16]. We conducted this study to identify the role of ARs in ATP-induced odontoblastic differentiation of HDPCs, and to determine whether the ARs activation by adenosine can induce HDPC odontoblastic differentiation independently, without the induction of ATP.

2. Methods

2.1. Cell culture

This study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (IRB-2013055), and informed consent was obtained. HDPCs were isolated according to a previous report [15]. Cells were cultured in proliferation medium (PM) containing α-minimum essential medium (α-MEM) (Gibco, Grand Island, NY) with 10% fetal bovine serum (Corning Cellgro, NY), 1% L-glutamine (Sigma-Aldrich, St. Louis, MO), and 1% penicillin/streptomycin (Gibco, BRL). For ATP-induced odontoblastic differentiation, cells were cultured in standard PM containing additional 600 μM ATP (ATP medium). Every experiment was repeated three times with HDPCs at the third passage from five different donors (three males and two females; 19–29 years old).

2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA isolation and RT-PCR were performed as described in the earlier study [15]. *GAPDH* was measured as a reference. The primers used in this process are listed in Table 1.

2.3. Quantitative real-time PCR

Real-time PCR was performed using an ABI 7500 real-time PCR machine (Applied Biosystems, Foster City, CA) and SYBR Green chemistry, according to the manufacturer's protocol. Expression of *DMP1* and *DSPP* (both specific markers in odontoblastic differentiation) in HDPCs cultured in PM, ATP medium (presence or absence of selective inhibitors/agonists of A_1R, $A_{2B}R$, and A_3R), and adenosine medium (0, 0.1, 1, 10, 100, 200, 400, 600, 800, 1000 μM) was detected at 24 h, 48 h, and 7 days. These time points were chosen for mRNA analysis, as they are the critical windows in the differentiation of HDPCs into odontoblasts. Adenosine and the selective antagonists of A_1R (8-cyclopentyl-1,3-dipropylxanthine (DPCPX)), $A_{2B}R$ (MRS-1754), and A_3R (PSB-11 hydrochloride), as well as the selective agonists of A_1R (2-Chloro-N^6-cyclopentyladenosine (CCPA)), $A_{2B}R$ (NECA), and A_3R (IB-MECA) were all purchased from Abcam (Cambridge, MA).

2.4. Western blotting

Western blotting analysis of A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R in HDPCs cultured in ATP medium was performed at 14 days, as described in a previous study [16]. The protein expression of DMP1 and DSPP in cells cultured in ATP medium (presence or absence of selective inhibitor/agonist of A_1R, $A_{2B}R$, and A_3R) and adenosine medium was detected at 48 h and 7 days. We chose the 7-day time point for western blotting because DMP1 and DSPP protein levels showed a visible response to the odontoblastic differentiation around 7 days [17–19]. The primary antibodies used were anti-GAPDH, anti-DMP1, anti-DSPP (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA); anti-A_1R, anti-$A_{2A}R$, anti-$A_{2B}R$, anti-A_3R (1:1000, Abcam, Cambridge, MA); GAPDH was evaluated as an internal control.

2.5. Alizarin Red S staining and quantification

After 21 days of odontoblastic induction, mineralization was analyzed using Alizarin Red staining. The 21-day time point is frequently used in Alizarin Red S staining in osteo/odontogenic differentiation research, as mineralized nodules are obviously visible around 21 days [16]. For quantification of mineralization, the stained mineralized nodules were dissolved in 10% (w/v) cetylpyridinium chloride (Sigma-Aldrich, St. Louis, MO) for 1 h, and the concentration was measured at 562 nm using a spectrophotometer (BioTek Instruments, Winooski, VT).

2.6. Statistical analyses

Data were presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using Student's *t*-test and one-way ANOVA, and differences were considered significant at $P < 0.05$. Experimental data were analyzed using Prism 6 software (GraphPad, San Diego, CA).

Table 1
Primers for adenosine-receptor-related and odontoblast-related gene expression analysis.

Gene	Accession Number	Primer	Sequences (5′-3′)	Fragment Size (bp)
ADORA1 (RT-PCR and Real-time PCR)	NM_000674	Forward	CAAGATCCCTCTCCGGTACAA	109
		Reverse	GCCAAACATAGGGGTCAGTCC	
ADORA2A (RT-PCR and Real-time PCR)	NM_000675	Forward	CATGCTAGGTTGGAACAACTGC	185
		Reverse	AGATCCGCAAATAGACACCCA	
ADORA2B (RT-PCR and Real-time PCR)	NM_000676	Forward	CTGTCACATGCCAATTCAGTTG	134
		Reverse	GCCTGACCATTCCCACTCTTG	
ADORA3 (RT-PCR and Real-time PCR)	NM_000677	Forward	GTGCTGGTCATGCCTTTGG	100
		Reverse	CGTGGGTAAAGATAAGCAGTAGG	
DMP1 (Real-time PCR)	NM_001079911	Forward	GTGAGTGAGTCCAGGGGAGATAA	111
		Reverse	TTTTGAGTGGGAGAGTGTGTGC	
DSPP (Real-time PCR)	NM_014208	Forward	TGGAGCCACAAACAGAAGCAA	127
		Reverse	TCCAGCTACTTGAGGTCCATC	
GAPDH (RT-PCR and Real-time PCR)	NM_001256799	Forward	ATGGGGAAGGTGAAGGTCG	108
		Reverse	GGGGTCATTGATGGCAACAATA	

3. Results

3.1. Expression of adenosine receptor subtypes in HDPCs

To study the expression of ARs in HDPCs, RT-PCR and real-time PCR were performed. The results showed that all four AR subtypes, *A_1R*, *$A_{2A}R$*, *$A_{2B}R$*, and *A_3R*, were expressed in HDPCs (Fig. 1A). However, the levels of *$A_{2A}R$* and *$A_{2B}R$* were much higher than those of *A_1R* and *A_3R*. *$A_{2B}R$* exhibited the highest and *A_3R* the lowest expression levels (Fig. 1B). These results were consistent with those of a previous study [16].

3.2. Adenosine receptor subtypes were activated in HDPCs exposed to ATP

To investigate the response of ARs to ATP exposure, the mRNA and protein expression levels of ARs in ATP-treated HDPCs were measured. The mRNA expression levels of *A_1R*, *$A_{2B}R$*, and *A_3R* increased 48 h after ATP treatment, whereas those of *$A_{2A}R$* slightly decreased (Fig. 1C). The western blotting experiments showed that protein expression of all ARs (except $A_{2A}R$) was up-regulated 14 days after ATP treatment, similar to the PCR results at 48 h (Fig. 1D and E).

3.3. Adenosine receptor inhibitors attenuated ATP-induced odontoblastic differentiation of HDPCs

The response of $A_{2A}R$ to ATP treatment was not as obvious as that of the other three receptors, especially at the protein level. In addition, A_1R and $A_{2B}R$ have been reported to enhance osteogenic differentiation [16,20]; nevertheless, their roles in odontoblastic differentiation have not been often studied. Therefore, we focused on the roles of A_1R, $A_{2B}R$, and A_3R (especially A_1R and $A_{2B}R$) in our study, rather than $A_{2A}R$.

To investigate the effects of A_1R, $A_{2B}R$, and A_3R on ATP-induced odontoblastic differentiation of HDPCs, cells were pretreated with 10 μM AR-selective antagonists (DPCPX, MRS-1754, and PSB-11 hydrochloride) 1 h prior to ATP exposure. Results showed that the mRNA expression of *DMP1* increased substantially at 24 h, 48 h, and 7 days after single ATP treatment, whereas that of *DSPP* only increased at 48 h and 7 days after ATP treatment (Fig. 2A). ATP treatment significantly increased the protein expression of both DMP1 and DSPP at 7 days (Fig. 2C). Compared to the single ATP-treated group, A_1R and $A_{2B}R$ antagonist pretreatment decreased *DMP1* mRNA expression significantly at 24 h, 48 h, and 7 days, and decreased protein expression at 7 days (Fig. 2A and C). A_1R and $A_{2B}R$ antagonist pretreatment also decreased *DSPP* mRNA expression at 48 h (Fig. 2A). However, on day 7, the mRNA and protein expression of DSPP both decreased in groups pretreated with $A_{2B}R$ and A_3R antagonists, with no change in the A_1R antagonist-pretreated group (Fig. 2A and C). However, at 48 h, the protein expression levels of DMP1 and DSPP showed no obvious changes in the single ATP-treated or antagonist + ATP-treated groups (Fig. 2B), which was different from the results of the mRNA analysis at 48 h. This inconsistency might be because 48 h is too short a time for a protein response to ATP. Moreover, 21 days after induction, A_1R and $A_{2B}R$ inhibitors attenuated the ATP-induced mineralization, whereas A_3R inhibitor rarely had such an effect (Fig. 2D). These results suggested that the inhibition of ARs could decrease the ATP-induced odontoblastic differentiation of HDPCs.

3.4. Adenosine receptor agonists enhanced the ATP-induced odontoblastic differentiation of HDPCs

To verify this speculation, cells were treated with ATP medium with AR-selective agonists (30 nM CCPA, 1 μM NECA, and 30 nM MECA). Compared to the results in single ATP-treated groups, A_1R and $A_{2B}R$ agonists enhanced the mRNA expression of *DMP1*

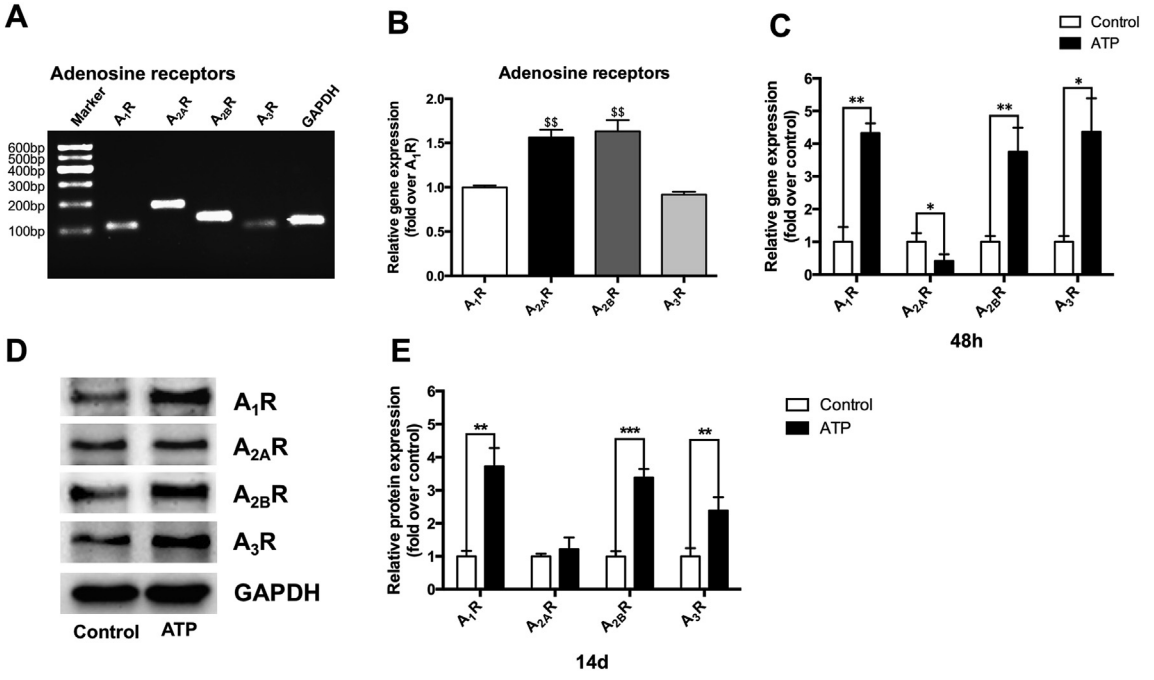

Fig. 1. Expression of ARs in HDPCs cultured in PM and ATP medium. (A) RT-PCR analysis of ARs in HDPCs cultured in PM. (B) RT-qPCR analysis of ARs in HDPCs cultured in PM when cells converged 80%. (C) RT-qPCR analysis of ARs in HDPCs cultured in ATP medium at 48 h. (D, E) Western blotting (D) and quantitative measurements (E) of A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R expression in HDPCs, 14 days after ATP treatment. Data are presented as the mean ± SEM (n = 5). $^{\$\$}P < 0.01$, vs. *$A_1R$*; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. control. Cells in the control group were cultured in PM.

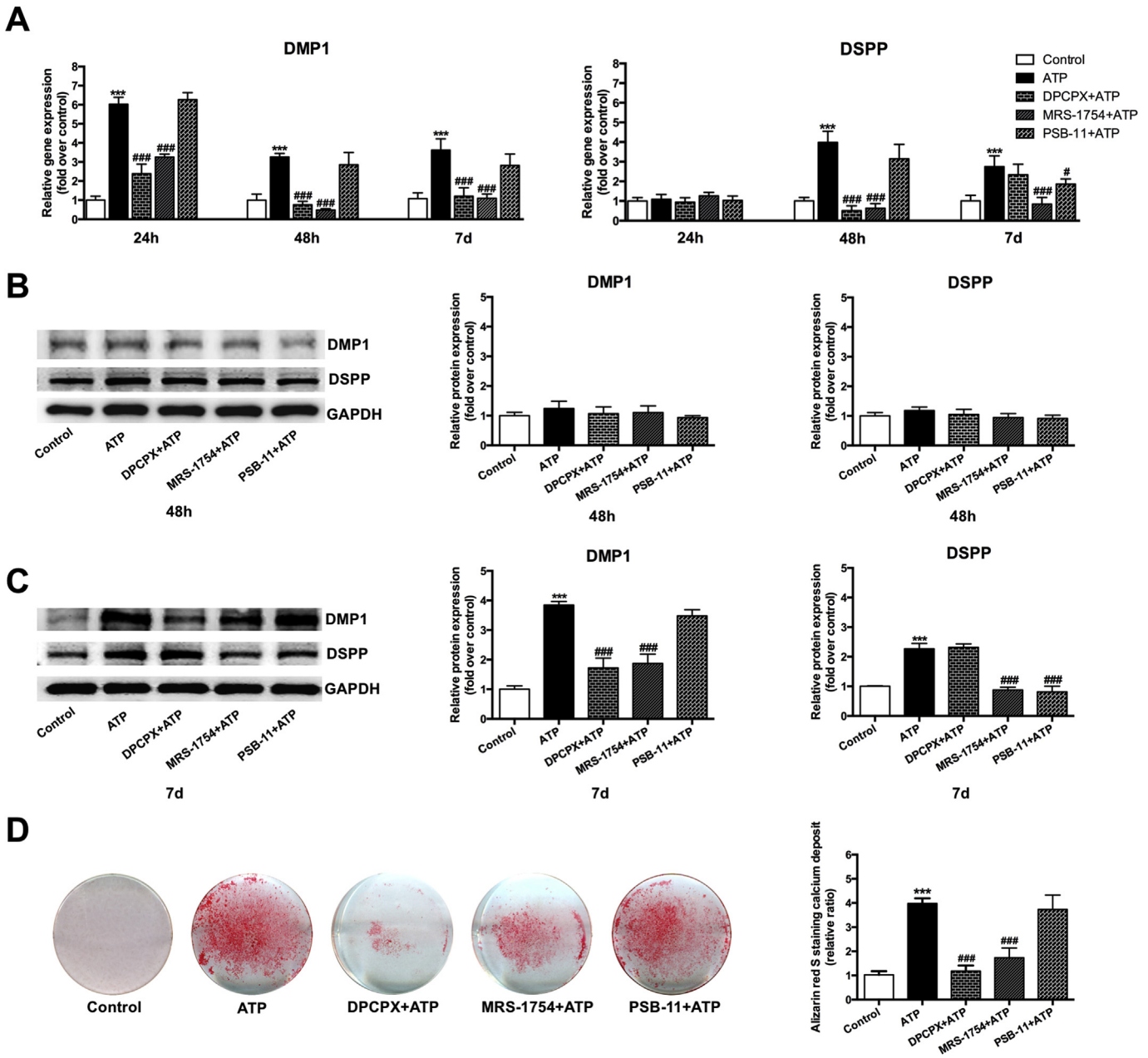

Fig. 2. Selective inhibition of ARs decreased the ATP-induced odontoblastic differentiation of HDPCs. (A) RT-qPCR analysis of *DMP1* and *DSPP* expression in HDPCs pretreated with 10 μM selective antagonists of A_1R (DPCPX), $A_{2B}R$ (MRS-1754), and A_3R (PSB-11) 1 h prior to ATP treatment for 24 h, 48 h, and 7 days. (B, C) Western blotting and quantification of DMP1 and DSPP expression in HDPCs pretreated with ARs selective antagonists 1 h prior to ATP treatment for 48 h (B) and 7 days (C). (D) Alizarin Red staining and quantification 21 days after cells were pretreated with 10 μM selective antagonists of A_1R, $A_{2B}R$, and A_3R 1 h prior to ATP exposure. Values are expressed as the mean ± SEM (n = 5). $^{***}P < 0.001$, vs. control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, vs. ATP.

significantly at both 24 h and 7 days (Fig. 3A), and enhanced the protein expression at 7 days (Fig. 3C). Furthermore, A_1R and $A_{2B}R$ agonists increased the *DSPP* mRNA level at 48 h (Fig. 3A), whereas $A_{2B}R$ and A_3R agonists increased the mRNA and protein expression at 7 days (Fig. 3A and C). The mRNA detection time of *DMP1* differed from that of *DSPP*, because ATP increased *DMP1* mRNA levels obviously at 24 h, whereas it increased *DSPP* mRNA levels obviously at 48 h (Fig. 2A). Similar to the results of the studies with inhibitors, the protein expression levels of both DMP1 and DSPP showed no obvious changes at 48 h in the single ATP-treated or the agonist + ATP-treated groups (Fig. 3B). A_1R and $A_{2B}R$ agonists enhanced ATP-induced mineralization, whereas the A_3R agonist had no effect (Fig. 3D).

Experiments with AR agonists further indicated that the activation of ARs might enhance the ATP-induced odontoblastic differentiation of HDPCs.

3.5. Effects of adenosine on the odontoblastic differentiation of HDPCs

As it is known that adenosine is a hydrolysate of ATP, and ARs are mainly activated by adenosine, we assumed that the effects of ARs may be due to the accumulation of adenosine in the culture medium from the hydrolysis of ATP. To determine the independent effects of AR activation by adenosine on the odontoblastic differentiation of HDPCs with the absence of ATP, different

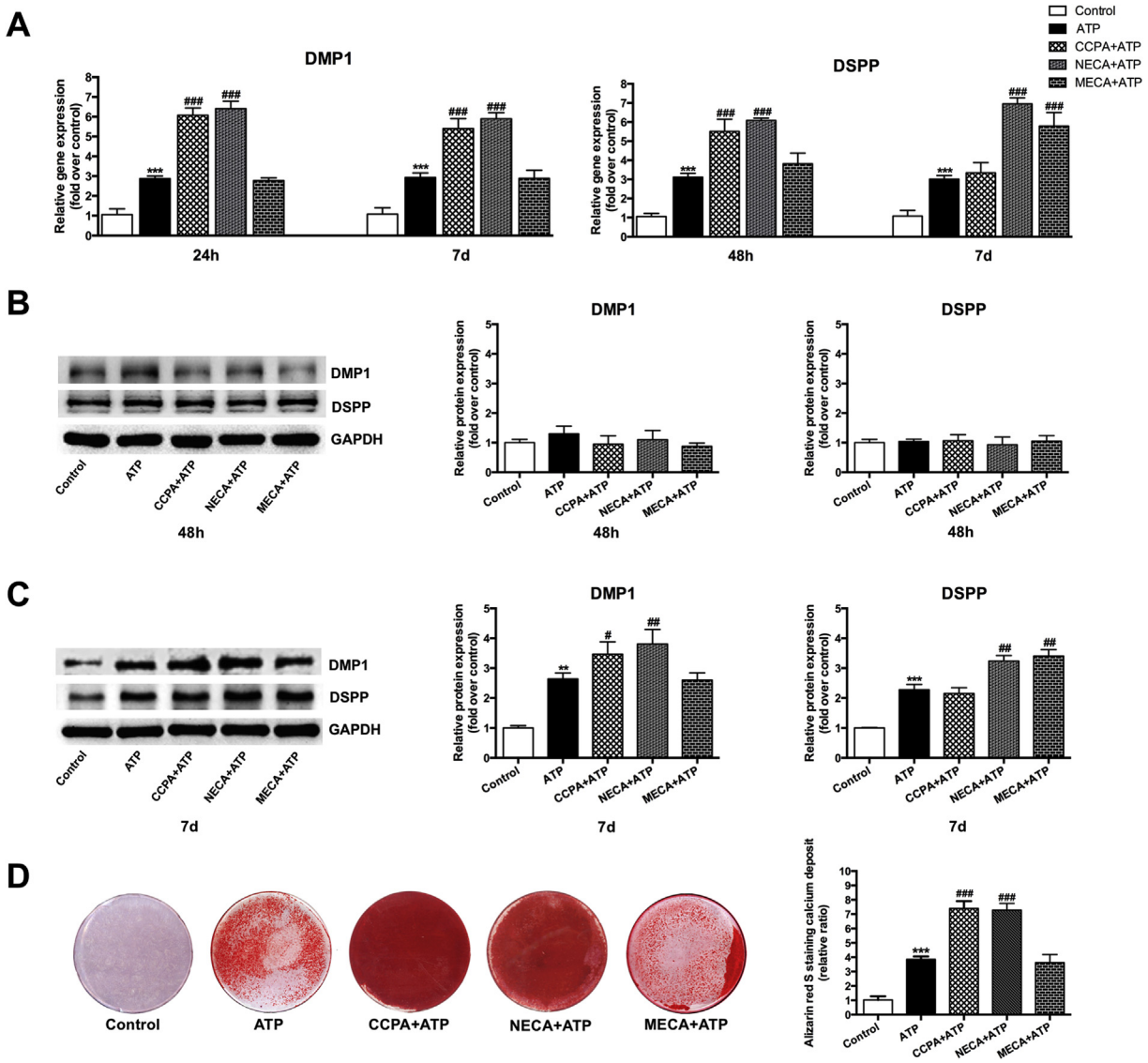

Fig. 3. Selective activation of ARs enhanced the ATP-induced odontoblastic differentiation of HDPCs. (A) RT-qPCR analysis of *DMP1* expression at 24 h and 7 days, and *DSPP* expression at 48 h and 7 days, in HDPCs cultured in ATP medium with selective agonists of A_1R (30 nM CCPA), $A_{2B}R$ (1 μM NECA), and A_3R (30 nM MECA). (B, C) Western blotting and quantification of DMP1 and DSPP expression in HDPCs cultured in ATP medium with ARs selective agonists for 48 h (B) and 7 days (C). (D) Alizarin Red staining and quantification after cells were cultured in ATP medium with selective agonists of A_1R, $A_{2B}R$, and A_3R for 21 days. Data are presented as the mean ± SEM (n = 5). $^{**}P < 0.01$, $^{***}P < 0.001$, vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, vs. ATP.

concentrations (0, 0.1, 1, 10, 100, 200, 400, 600, 800, and 1000 μM) of adenosine were used. As shown in Fig. 4A, adenosine increased the *DMP1* mRNA level at low concentrations (0.1, 1, and 10 μM) at 24 h, but not on day 7. At the same concentrations, it increased *DSPP* at 48 h; however, it was only effective at 0.1 and 1 μM on day 7. In western blotting assays, only DSPP was slightly up-regulated at the concentrations of 0.1 and 1 μM on day 7; DMP1 showed a decrease at 200, 400, 600, and 1000 μM (Fig. 4B and C). Unexpectedly, exogenous adenosine had no effect on the mineralization of HDPCs (Fig. 4D). Thus, our results indicated that the activation of ARs by adenosine could not significantly induce the odontoblastic differentiation of HDPCs independently, without the induction of ATP.

4. Discussion

Purinergic signaling affects various physiological processes, including osteogenesis [11], neurogenesis [9], inflammation [21], and pain [22]. Receptors involved in this signaling are classified into two main groups: P1 (adenosine receptors [ARs]) and P2 receptors. In our previous study, we had demonstrated that ATP could induce odontoblastic differentiation of HDPCs by analyzing the key events in this process, including DMP1 and DSPP expression, as well as the mineralization capacity of HDPCs *in vitro*. We found that this effect was mediated by the activation of P2 receptors [15]. In the present study, we first verified that ARs activation could enhance the ATP-induced odontoblastic differentiation of HDPCs. However, ARs activation by exogenous adenosine could not induce the odontoblastic differentiation of HDPCs independently, with the absence of ATP. The results suggested that the odontoblastic differentiation of HDPCs induced by ATP was probably due to the combined regulation of ARs and P2 receptors, and between these two receptors, P2 receptors might play a main role.

In the present study, we found that the expression of A_1R, $A_{2B}R$, and A_3R increased after ATP treatment, indicating that the three ARs

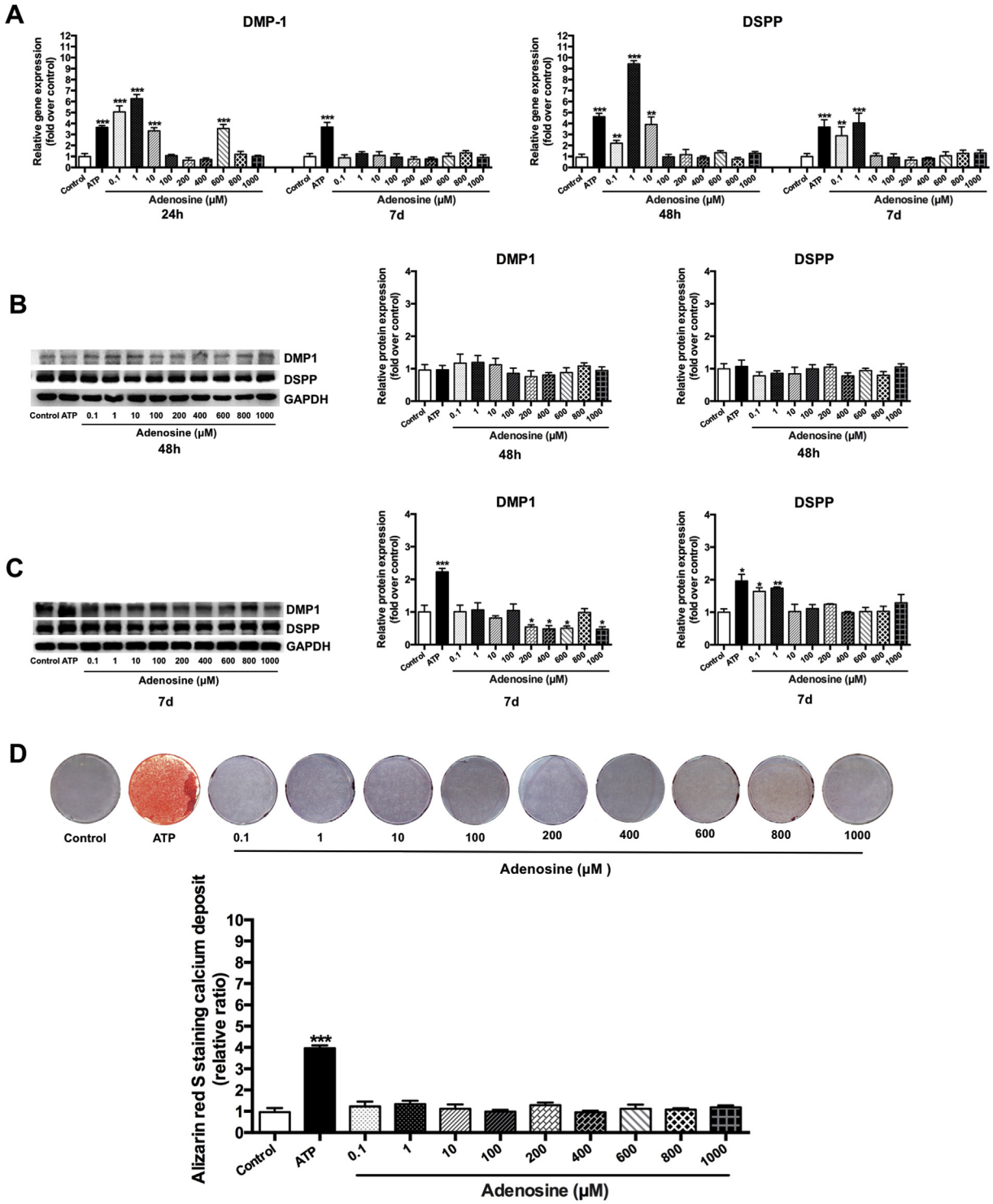

Fig. 4. Effects of adenosine on the odontoblastic differentiation of HDPCs. (A) RT-qPCR analysis of *DMP1* expression at 24 h and 7 days, and *DSPP* expression at 48 h and 7 days, in HDPCs cultured in adenosine medium. (B, C) Western blotting and quantification of DMP1 and DSPP expression in HDPCs cultured in adenosine medium for 48 h (B) and 7 days (C). (D) Alizarin Red staining and quantification after cells were cultured in adenosine medium for 21 days. Data are presented as the mean ± SEM (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. control.

in HDPCs were possibly activated after ATP exposure. It has been established that ARs are primarily activated by adenosine [10], which is generated by the hydrolysis of ATP [23]. Therefore, we speculated that the activation of ARs identified in this study may be a consequence of increased levels of adenosine generated by the hydrolysis of extracellular ATP.

Then, we verified that 600 μM ATP could induce the odontoblastic differentiation of HDPCs, and we thus chose 600 μM ATP in this study rather than 800 μM used in the earlier study because of the comparatively small impact on cell proliferation [15]. In our previous study, the maximum expression of *DSPP* mRNA was seen at 24 h [15], whereas it was 48 h in this study; this might be due to the hysteresis effect of a relatively low concentration (600 μM) of ATP, compared to 800 μM. However, this difference in

the level of *DSPP* mRNA at the times tested did not affect the differentiation of HDPCs.

To determine whether the observed activation of ARs was directly related to odontoblastic differentiation of HDPCs, cells were pretreated with selective antagonists of A_1R, $A_{2B}R$, and A_3R 1 h prior to ATP treatment. We found that inhibition of A_1R and $A_{2B}R$ attenuated the ATP-induced up-regulation of DMP1 and mineralization, whereas inhibition of $A_{2B}R$ and A_3R reduced DSPP; however, A_3R inhibition had no effect on the mineralization. The difference in the chosen time points (48 h vs. 7 days) could be one reason for the discrepancies between DSPP mRNA and protein expression at these time points. These results indicated that ARs might contribute to ATP-meditated induction of odontoblastic differentiation of HDPCs. To further verify this speculation, selective agonists of A_1R, $A_{2B}R$, and A_3R were used to analyze the effects on the ATP-mediated odontoblastic differentiation of HDPCs. The results showed that the activation of A_1R and $A_{2B}R$ enhanced the ATP-induced up-regulation of DMP1 and mineralization, whereas $A_{2B}R$ and A_3R activation enhanced that of DSPP; however, A_3R activation had no effect on the mineralization. Thus, we conclude that A_1R, and especially $A_{2B}R$, might enhance ATP-induced odontoblastic differentiation of HDPCs, although the two receptors activate cAMPs through opposite mechanisms. This could be attributed to other molecular pathways known to be coupled to these receptors, such as MAPK, PI3K/Akt, and Wnt signaling, which are also associated with the differentiation of MSCs into osteoblasts [16,24,25]. In addition, adenosine receptors have been demonstrated to regulate the levels of inflammatory cytokines (eg., IL-6) which could also affect osteogenic differentiation [23,26]. Thus, in our study, we speculated that A_1R and $A_{2B}R$ could influence the ATP-induced odontoblastic differentiation of HDPCs through one or more of these pathways. However, further studies are required to confirm this speculation.

When added to the culture medium, extracellular ATP is metabolized into adenosine by ectonucleotidases, and ARs are then activated by adenosine with concentrations in the micromolar range [27]. However, our study showed that activation of ARs by exogenous adenosine could not induce the odontoblastic differentiation of HDPCs independently, without induction of ATP. This may be because P2 receptors, activated by ATP/ADP, played a more important role in this process [15]. Therefore, ARs and P2 receptors may be co-regulators in the HDPC odontoblastic differentiation induced by ATP.

In summary, to the best of our knowledge, our study is the first to demonstrate the positive impact of ARs on ATP-induced odontogenetic differentiation. Among the four AR subtypes, A_1R, and particularly $A_{2B}R$, may enhance the ATP-induced HDPC odontoblastic differentiation. However, the activation of ARs by adenosine, a hydrolysate of ATP, cannot induce the odontoblastic differentiation of HDPCs independently, without the induction of ATP. Therefore, the ATP-induced odontoblastic differentiation of HDPCs was probably due to the combined administration of ARs and P2 receptors, and between these two receptors, P2 receptors may play a main role.

Conflict of interest

The authors declare no conflicts of interest.

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Transparency document

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