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Review

Expression of P2 purinergic receptors in mesenchymal stem cells and their roles in extracellular nucleotide regulation of cell functions

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Extracellular ATP and other nucleotides induce autocrine and/or paracrine purinergic signalling via activation of the P2 receptors on the cell surface, which represents one of the most common signalling mechanisms. Mesenchymal stem cells (MSC) are a type of multipotent adult stem cells that have many promising applications in regenerative medicine. There is increasing evidence to show that extracellular nucleotides regulate MSC functions and P2 receptor-mediated purinergic signalling plays an important role in such functional regulation. P2 receptors comprise ligand-gated ion channel P2X receptors and G-protein-coupled P2Y receptors. In this review, we provide an overview of the current understanding with respect to expression of the P2X and P2Y receptors in MSC and their roles in mediating extracellular nucleotide regulation of MSC proliferation, migration and differentiation.

Purinergic signalling, despite its unreceptive inception, represents one of the most common signalling mechanisms in cells (Burnstock, 2012; Burnstock and Verkhratsky, 2010). Extracellular nucleotides activate autocrine and/or paracrine purinergic signalling via the P2 family of purinergic receptors on the cell surface. Two structurally and functionally distinctive subfamilies of P2 receptors have been defined, namely, P2X and P2Y (Ralevic and Burnstock, 1998). The P2X receptors function as ligand-gated cationic channels that are assembled as homo/hetero-trimers from the seven P2X receptor subunits (P2X1-P2X7) (Browne et al., 2010; Hattori and Gouaux, 2012; Khakh and North, 2012; North, 2002). The P2X receptor ion channels are gated exclusively by extracellular ATP and form a transmembrane pathway for efflux of K^+ and influx of Na^+ and Ca^{2+}, leading to an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and/or membrane depolarization. The P2Y receptors are classical G-protein-coupled receptors. Mammalian cells express eight P2Y subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-$P2Y_{14}$) (Burnstock, 2012; Jacobson et al., 2012; Jacobson and Muller, 2016; Jacobson et al., 2015; von Kügelgen and Harden, 2011). Unlike the P2X receptors, the P2Y receptors are activated by a diversity of extracellular nucleotides, such as ATP, ADP, UTP, UDP, UDP-glucose and nicotinamide adenine dinucleotide (NAD), but each P2Y

receptor displays distinctive nucleotide preference. Activation of the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors triggers the $G_{\alpha,q/11}$-PLCβ-IP_3R signalling pathway, namely, stimulation of phospholipase C β (PLCβ), generation of inositol triphosphate (IP_3) and activation of the IP_3 receptors (IP_3R). This leads to Ca^{2+} release from the endoplasmic reticulum (ER) to elevate the $[Ca^{2+}]_i$. Reduction in the ER Ca^{2+} level in many cell types, mostly non-excitable cells, can activate the Ca^{2+} release activated Ca^{2+} (CRAC) channels on the cell surface to mediate the so-called store-operated Ca^{2+} entry (Amcheslavsky et al., 2015; Parekh, 2010). In contrast, the $P2Y_{12}$-$P2Y_{14}$ receptors are coupled to the $G_{\alpha,i}$-AC-cAMP signalling pathway, resulting in inhibition of the adenylyl cyclase (AC) activity and reduction in the generation of intracellular cyclic adenosine monophosphate (cAMP). Some P2Y receptors are known to link to additional downstream signalling pathways. For example, the $P2Y_{11}$ receptor can also associate with the $G_{\alpha,s}$-AC-cAMP signalling pathway to stimulate AC and elevate the intracellular cAMP level (von Kügelgen and Harden, 2011). Both P2X and P2Y receptors are widely expressed in excitable and non-excitable cells and play important roles in mediating extracellular nucleotide-initiated purinergic signalling in a plethora of physiological and pathological processes, as have been elegantly covered in numerous recent reviews (Burnstock and Ralevic, 2014; Burnstock and Ulrich, 2011; Burnstock and Verkhratsky, 2010; Jacobson et al., 2012; Jacobson and Muller, 2016; Jiang, 2012; North, 2002; Surprenant and North, 2009; von Kügelgen and Harden, 2011).

Mesenchymal stem cells (MSC) are a group of non-hematopoietic multipotent stem cells, which were firstly isolated from bone marrow and later also from other adult tissues including adipose tissue, umbilical cord, periodontal ligament and dental pulp. MSC are well-documented to differentiate into adipocyte, osteoblast and chondrocyte (Bianco, 2014; Dominici et al., 2006; Pittenger et al., 1999), the important cell types in adipose, bone and cartilaginous tissues, respectively. Emerging evidence suggests the potential of differentiation into other lineages, such as neuronal (Tu et al., 2014) and Schwann glial cells (Faroni et al., 2013; Martens et al., 2014). Preclinical studies and clinical tests have demonstrated a number of promising applications of MSC in regenerative medicine (Aurrekoetxea et al., 2015; Bianco, 2014; Caplan, 2007; Christ et al., 2015; Kim et al.,

2012; Levi and Longaker, 2011; Momin et al., 2010; Phinney and Prockop, 2007; Psaltis et al., 2008; Rastegar et al., 2010; Reiser et al., 2005; Wakitani et al., 2011).

Stem cells including MSC reside in a highly specialized microenvironment termed the stem cell niche (Scadden, 2006). The stem cell niche is hypoxic, a condition that is important in maintaining stem cells in an undifferentiated state (Mohyeldin et al., 2010). It is known that hypoxia stimulates ATP release (Dutta et al., 2004; Kahlin et al., 2014; Lim To et al., 2015; Mortensen et al., 2011; Orriss et al., 2009; Roger et al., 2015). An increasing number of studies show that under in vitro culture conditions and in vivo, MSC release ATP and other nucleotides constitutively or in response to mechanical or chemical stimulation, via vesicular exocytosis, connexin hemi-channel or other yet defined mechanisms (Biver et al., 2013; Coppi et al., 2007; Kawano et al., 2006; Kwon, 2012; Riddle et al., 2008; Riddle et al., 2007; Sun et al., 2013; Weihs et al., 2014). Extracellular nucleotides can impose significant regulation of MSC functions. It is worth pointing out that MSC also express ecto-nucleotidases on the cell surface that metabolize extracellular ATP and other nucleotides (Noronha-Matos and Correia-de-Sa, 2016; Roszek et al., 2015; Scarfi, 2014). Indeed, adenosine as an ATP metabolite has been proposed to contribute in ATP regulation of MSC functions via activating the adenosine P1 receptors (Carroll et al., 2012; Ciciarello et al., 2013; Gharibi et al., 2011; Shih et al., 2014). Several recent reviews have appeared that focus on various aspects of the purinergic signalling mediated by the adenosine P1, P2X and P2Y receptors and ecto-nucleotidases in MSC, particularly in osteogenic differentiation (Cavaliere et al., 2015; Lenertz et al., 2015; Noronha-Matos and Correia-de-Sa, 2016; Scarfi, 2014). In this review, we discuss the current understanding of the P2 receptor-mediated purinergic signalling in MSC, with respect to expression of the P2X and P2Y receptors and their roles in mediating extracellular nucleotide regulation of MSC proliferation, viability, migration and differentiation into adipocyte, osteoblast, chondrocytes, neuronal and glial cells. We aim this review to provide a more inclusive overview of the findings reported by recent studies, prompting the reader to bear in mind the noticeable discrepancies currently existing in the literature.

Expression of P2 receptors in MSC

Expression of the P2X and P2Y receptors in MSC has been examined at the mRNA level, using conventional or real time reverse transcription-polymerase chain reaction (RT-PCR), and at the protein level, using western blotting or immunocytochemistry. Their functional expression has been studied by measuring agonist-induced responses, often using fluorescent imaging to monitor agonist-evoked change in the $[Ca^{2+}]_i$, in conjunction with using selective antagonists to inhibit the receptor activity or small interference RNA (siRNA) to reduce the receptor expression. Table 1 summarizes the P2X and P2Y receptors that have been reported in MSC of various species and tissues, and the methods used to shown their expression, which gives a quick reference to the reader. However, studies using MSC from different species, particularly different tissues, have reported a considerably variable expression profile for both P2X and P2Y receptors, noticeably at the protein and functional expression levels. For simplicity, we discuss expression of the P2X and P2Y receptors according to the tissue origins of MSC used.

P2X receptors

BM-MSC. Expression of the P2X receptors in bone marrow derived MSC (BM-MSC) was firstly examined in two independent studies (Coppi et al., 2007; Riddle et al., 2007). Coppi et al showed, using patch-clamp recording, that ATP evoked an inwardly-rectifying current with a reversal potental close to 0 mV in a subset of human BM-MSC (Coppi et al., 2007). In addition, exposure of such cells to ATP caused membrane deploarization. Treatment with pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS), a generic P2 antagonist, reduced ATP-induced inward currents and led to membrane hyperpolarization. These results were interpreted to indicate functional expression of the P2X receptor(s) (Coppi et al., 2007), but the molecular identity of the receptor(s) mediating ATP-induced inward currents was not established. The second study showed P2X7 protein expression in human BM-MSC, using western blotting (Riddle et al., 2007). Ferrati et al were the first to systematically assess expression of the P2X receptors in human BM-MSC, and reported mRNA expression for all the P2X subunits, with the exception of P2X2, and protein expression for P2X1, P2X4 and P2X7 (Ferrari et al., 2011). The P2X7 protein expression in human BM-MSC has been further demonstrated in two

recent studies, using western blotting (Sun et al., 2013) and immunocytochemistry (Noronha-Matos et al., 2014) . Ferrati et al also studied, using Ca^{2+} imaging, the role of the P2X receptors in ATP-induced Ca^{2+} signalling in human BM-MSC (Ferrari et al., 2011). ATP induced a biphasic increase in the $[Ca^{2+}]_i$ in extracellular Ca^{2+}-containing solutions, composed of an initial transient increase and a second sustained increase. In extracellular Ca^{2+}-free solutions, the transient increase was attenuated and the sustained increase was absent, indicating major contribution of extracellular Ca^{2+} influx to the sustained increase in the $[Ca^{2+}]_i$. In the same study, α,β-methylene-ATP (αβ-meATP), an ATP synthetic analogue that activates the P2X receptors containing P2X1, P2X3 or P2X5 subunit, was shown to induce a measureable increase in the $[Ca^{2+}]_i$, suggesting expression of some or all of these P2X receptors. However, ATP-evoked sustained increase in the $[Ca^{2+}]_i$ was almost completely abolished by KN62, a human P2X7 selective antagonist, and oxidized ATP (oxATP), an irreversible P2X7 inhibitor (Ferrari et al., 2011), indicating ATP-induced Ca^{2+} influx is mainly mediated by the P2X7 receptor. In a more recent study, in addition to a sustained increase in the $[Ca^{2+}]_i$, ATP also evoked membrane blebbing and large pore formation (Noronha-Matos et al., 2014), which are characteristics of the P2X7 receptor activation (Virginio et al., 1999; Wei et al., 2016). Moreover, 2',3'-(benzoyl-4-benzoyl)-ATP (BzATP), another ATP synthetic analogue with a greater potency than ATP at the P2X7 receptor, was more potent than ATP in raising the $[Ca^{2+}]_i$, membrane blebbing and large pore formation. BzATP-induced effects were blocked by A-438079, a P2X7 selective antagonist (Noronha-Matos et al., 2014), providing further evidence to support functional expression of the P2X7 receptor in human BM-MSC. A recent study has shown P2X7 mRNA and protein expression in rat BM-MSC (Li et al., 2015). As discussed further below, this study has found that BzATP-induced regulation of adipogenic and osteogenic differentiation was attenuated by brilliant blue G (BBG), a P2X7 selective antagonist, and P2X7-specific siRNA, demonstrating functional expression of the P2X7 receptor and an important role in the regulation of differentiation (Li et al., 2015). Kwon reported oscillations in the extracellular ATP level in mouse BM-MSC, which are dependent of the intracellular Ca^{2+} level (Kwon, 2012). Such ATP oscillations were prevented by 5-BDBD, a P2X4 selective

antagonist. These observations have led to the notion that the functional P2X4 receptor mediates ATP-induced Ca^{2+} influx as part of an autocrine signaling mechanism resulting in the ATP oscillations (Kwon, 2012).

AT-MSC. Expression of the P2X receptors in human adipose tissue derived MSC (AT-MSC) was firstly exmained by Zippel and colleagues (Zippel et al., 2012). The study reported mRNA expression for P2X3-P2X7, but not P2X1 and P2X2, and protein expression for P2X5-P2X7, using western blotting. They showed that ATP elicited an increase in the $[Ca^{2+}]_i$, which was prevented by suramin, another P2 generic antagonist, and attenuated by NF279 at a concentration (100 μM), which is known to inhibit multiple P2X receptors including P2X1 (Rettinger et al., 2000). A recent study has evaluated expression of the P2X receptors in rat AT-MSC and observed mRNA expression for P2X3 and P2X4, but not any other P2X subunits (Faroni et al., 2013). The protein expression for P2X4 and P2X7 was examined, using western blotting, but not detectable. ATP increased the $[Ca^{2+}]_i$ in a concentration-dependent manner (10-1000 μM) with the concentration evoking half of the maximum (EC_{50}) of approximately 30 μM. In agreement with the lack of P2X7 mRNA expression, ATP-induced Ca^{2+} response was insensitive to inhibition by AZ10606120, a P2X7 selective antagonist (Faroni et al., 2013).

UC-MSC. In human umbilical cord derived MSC (UC-MSC), the mRNA transcript was detected for P2X1 and P2X4-P2X7, but not P2X2 and P2X3 (Tu et al., 2014) . As discussed further below, this study has shown that ATP stimulated neuronal differentiation, which was prevented by 2',3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) (Tu et al., 2014) at a concentration (30 μM), which is known to inhibit several P2X receptors including the P2X1 and P2X4 receptors (Virginio et al., 1998). These results suggest functional expression of the P2X receptor(s) in human UC-MSC, but the molecular identity of the P2X receptor(s) was not established.

PDL-MSC. It remains unclear whether the P2X receptors are expressed in human periodontal ligament derived MSC (PDL-MSC), with an exception of the P2X7 receptor.

The P2X7 expression has been documented at the mRNA and protein levels in human PDL-MSC (Trubiani et al., 2014). In addition, BzATP induced a monophasic increase in the $[Ca^{2+}]_i$ and large pore formation, which were inhibited by oxATP. BzATP also induced release of interleukin-8 (IL-8) and CC chemokine ligand 20 (CCL20), which was suppressed by A-740003, a P2X7 selective antagonist, as well as oxATP (Trubiani et al., 2014). There results support functional expression of the P2X7 receptor in human PDL-MSC and an important role in mediating the immunomodulatory property of MSC.

DP-MSC. Expression of the P2X receptors in human dental pulp derived MSC (DP-MSC) has been reently investigated by us (Peng et al., 2016). The mRNA transcript was detected for P2X4, P2X6 and P2X7, but not for P2X1-P2X3 and P2X5. A majority of human DP-MSC responded to ATP with a robust increase in the $[Ca^{2+}]_i$, albeit with a variable amplitude. ATP-induced Ca^{2+} responses were dependent of ATP concentration (3-300 μM) with an EC_{50} of 22 μM. BzATP also evoked an increase in the $[Ca^{2+}]_i$ concentration-dependently with an EC_{50} of approximately 90 μM, but there was no discernible Ca^{2+} response to αβmeATP, consistent with the lack of expression for P2X1, P2X3 and P2X5. ATP and BzATP also induced an increase in the $[Ca^{2+}]_i$ in extracellular Ca^{2+}-free solutions, which however were much more transient than those in extracellular Ca^{2+}-containing solutions, indicating extracellular Ca^{2+} influx in response to ATP and BzATP. ATP-induced increase in the $[Ca^{2+}]_i$ was insensitive to inhibition by the P2X4 antagonist 5-BDBD. In contrast, the increases in the $[Ca^{2+}]_i$ induced by both ATP and BzATP were attenuated by AZ11645373, a human P2X7 selective antagonist, and P2X7-specific siRNA, leading to the conclusion that functional expression of the P2X7 receptor contributes in mediating ATP-induced Ca^{2+} signaling in human DP-MSC (Peng et al., 2016).

P2Y receptors

BM-MSC. Ferrari et al performed RT-PCR analysis of the P2Y receptors in human BM-MSC and showed mRNA expression for all eight P2Y receptors (Ferrari et al., 2011). Several groups examined protein expressio of the P2Y receptors in human BM-MSC and

the results are not completely consistent. An early study, using western blotting, showed protein expression for $P2Y_2$, $P2Y_6$ and $P2Y_{11}$, but not $P2Y_1$, $P2Y_4$ and $P2Y_{12}$ (Riddle et al., 2007). Ferrari et al, also using western blotting, confirmed the protein expression for $P2Y_2$ and $P2Y_{11}$, and, additionally. they reported $P2Y_1$ protein expresssion (Ferrari et al., 2011). A more recent study, using immunocytochemistry, has demonstrated the $P2Y_1$ protein expression in human BM-MSC (Noronha-Matos et al., 2014).

There are several lines of evidence to support functional expression of the $P2Y_1$ receptor and its role in extracellular nucleotide-induced Ca^{2+} signaling in human BM-MSC. First of all, in a very early study, Kawano et al observed spontaneous Ca^{2+} oscillations in a subset of human BM-MSC (Kawano et al., 2002). In the follow-up study, they showed that Ca^{2+} oscillations promoted nuclear translocation of nuclear factor of activated T cells (NFAT) (Kawano et al., 2006). Moreover, such spontaneous Ca^{2+} oscillations were lost upon inhibition by U73122, a PLCβ inhibitor, overexpression of an IP_3-binding protein to remove intracellular IP_3, PPADS, BzATP or adenosine 3'-phosphate 5'-phosphosulfate. These results led the authors to propose an autocrine/paracrine Ca^{2+} signaling mechanism including ATP release and subsequent activation of the $P2Y_1$-$G_{\alpha,q}$-PLC-IP_3R signaling pathway (Kawano et al., 2006). Secondly, as shown in two separate studies, ADP, a $P2Y_1$ selective agonist, and its metabolically stable analogue, ADPβS, elicited an increase in the $[Ca^{2+}]_i$ (Ferrari et al., 2011; Noronha-Matos et al., 2014). Thirdly, in addition to the above-discussed inward currents, ATP also induced outward K^+ currents in a subset of human BM-MSC that exhibited strong dependence of the intracellular Ca^{2+} level as well as membrane potential (Coppi et al., 2007). Such Ca^{2+}-dependent K^+ outward currents were prevented by PPADS and MRS2179, a $P2Y_1$ selective antagonist, indicating that Ca^{2+} release after activaiton of the $P2Y_1$-$G_{\alpha,q}$-PLC-IP_3R signalling pathway triggers ATP-induced K^+ outward currents. Finally, as discussed further below, ATP-stimulated adipogenic differentiation of human BM-MSC was inhibited by MRS2279, another $P2Y_1$ specific antagonist (Ciciarello et al., 2013).

In addition to the $P2Y_1$ receptor, ATP also activates the $P2Y_2$ and $P2Y_{11}$ receptors. UTP prefers the $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors. Both ATP and UTP were potent in inducing an increase in the $[Ca^{2+}]_i$ in human BM-MSC in extracellular Ca^{2+}-free solution

as well as in extracellular Ca^{2+}-containing solutions (Ferrari et al., 2011), indicating functional expression of other P2Y receptor(s) besides the $P2Y_1$ receptor. Different studies, mainly using selective agonists and/or antagonists, support functional expression of the $P2Y_4$, $P2Y_6$ or $P2Y_{11}$ receptor in human BM-MSC. A recent study has shown that INS45973, an agonist at the $P2Y_2$ and $P2Y_4$ receptors, stimulated adipogenic differentiation, whereas MRS2768, a $P2Y_2$ agonist, was ineffective (Ciciarello et al., 2013). This finding is in support of functional expression of the $P2Y_4$ but not $P2Y_2$ receptor. The study by Ferrari et al showed that UDP, an agonist favoring the $P2Y_6$ receptor, induced an increase in the $[Ca^{2+}]_i$ in extracellular Ca^{2+}-containing solutions, indicating functional expression of the $P2Y_6$ receptor (Ferrari et al., 2011). Ferrari et al also showed that BzATP, which preferentially activates the $P2Y_{11}$ receptor among the P2Y receptors, elicited a transient increase in the $[Ca^{2+}]_i$ in extracellular Ca^{2+}-free solutions as well as in extracellular Ca^{2+}-containing solutions in the presence of KN62 (Ferrari et al., 2011). Functional expression of the $P2Y_{11}$ receptor in human BM-MSC has been further supported by the study investigating NAD-induced Ca^{2+} signalling (Fruscione et al., 2010). NAD induced a biphasic increase in the $[Ca^{2+}]_i$ in extracellular Ca^{2+}-containing solutions. Both transient and sustained components of NAD-induced Ca^{2+} response were abolished by NF157, a selective $P2Y_{11}$ antagonist, and $P2Y_{11}$-specific siRNA, but they were not affected with oxATP or KN62 (Fruscione et al., 2010). Further characterization suggests that NAD activates the $P2Y_{11}$-$G_{\alpha,s}$-AC-cAMP signalling pathway that leads to Ca^{2+} influx and ER Ca^{2+} release via the L-type voltage-gated Ca^{2+} channel and ryanodine receptor, respectively (Fruscione et al., 2010). UDP-glucose, a $P2Y_{14}$ selective agonist, induced an increase in the $[Ca^{2+}]_i$ in extracellular Ca^{2+}-containing solutions (Ferrari et al., 2011). As introduced above, the $P2Y_{14}$ receptor is coupled to the $G_{\alpha,i}$-AC-cAMP signalling pathway, and the downstream Ca^{2+} signalling mechanism underlying UDP-glucose-induced increase in the $[Ca^{2+}]_i$ remains unclear. Currently, there is no information regarding functional expression of the $P2Y_{12}$ and $P2Y_{13}$ receptors in human BM-MSC.

In contrast with in human BM-MSC, there is clear evidence to show functional expression of the $P2Y_2$ and $P2Y_{13}$ receptors in rodent BM-MSC. An early study demonstrated $P2Y_2$ mRNA and protein expression in rat BM-MSC (Ichikawa and Gemba,

2009). In the same study, ATP and UTP were shown to be equally potent in inducing an increase in the $[Ca^{2+}]_i$ in extracellular Ca^{2+}-containing and Ca^{2+}-free solutions, whereas ADP and UDP were ineffective. Furthermore, ATP-induced increase in the $[Ca^{2+}]_i$ was inhibited by suramin, but not PPADS. The overall pharmacological profile of the Ca^{2+} responses favors functional expression of the $P2Y_2$ receptor (Ichikawa and Gemba, 2009). Consistently, as discussed further below, a recent study has shown that UTP-induced regulation of osteogenic and adipogenic differentiation of rat BM-MSC was prevented by $P2Y_2$-specific siRNA, but not $P2Y_4$-specific siRNA or MRS2578, the $P2Y_6$ selective antagonist (Li et al., 2016). There were remarkable differences in adipogenic and osteogenic differentiation of BM-MSC from the wild-type (WT) and $P2Y_{13}$ knockout (KO) mice, indicating functional expression of the $P2Y_{13}$ receptor in mouse BM-MSC (Biver et al., 2013).

AT-MSC. Expression of the P2Y receptors in human AT-MSC was studied (Zippel et al., 2012). All the P2Y receptors were detected at the mRNA level, and the $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ were further demonstrated at the protein level, using western blotting. UTP and 2-methylthio-ADP, an agonist at the $P2Y_1$ and $P2Y_{11}$-$P2Y_{13}$ receptors, elevated the $[Ca^{2+}]_i$, whereas NF546, a $P2Y_{11}$ selective agonist, did not. ATP-induced increase in the $[Ca^{2+}]_i$ was reduced by the P2X receptor antagonist NF279 inhibitor as discussed above, and was also attenuated by pertussis toxin (PTX), the $G_{\alpha,i}$ inhibitor, supporting involvement of the $G_{\alpha,i}$-coupled P2Y receptors. Moreover, ATP and UDP-glucose suppressed adipogenic differentiation (Zippel et al., 2012). These results provide evidence to support functional expression of the P2Y receptors including $P2Y_{14}$, but not $P2Y_{11}$. However, the P2Y receptors that mediate ATP- and UTP-induced Ca^{2+} signaling in human AT-MSC remain not fully understood.

DP-MSC. The role of the $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ receptors in mediating ATP-induced Ca^{2+} signalling in human DP-MSC has been investigated in our recent study (Peng et al., 2016). While mRNA expression for $P2Y_1$ and $P2Y_{11}$ was consistently observed in human DP-MSC from different donors, $P2Y_2$ mRNA transcript was undetectable or at a very low

level (Peng et al., 2016). ATP, ADP and BzATP were all effective in evoking an increase in the $[Ca^{2+}]_i$ in extracellular Ca^{2+}-free solutions as well as in extracellular Ca^{2+}-containing solutions. Furthermore, ATP-induced increase in the $[Ca^{2+}]_i$ was attenuated by $P2Y_1$-specific siRNA or $P2Y_{11}$-specific siRNA. ADP-induced increase in the $[Ca^{2+}]_i$ was also diminished by $P2Y_1$-specific siRNA. These results support functional expression of the $P2Y_1$ and $P2Y_{11}$ receptors and their contribution in mediating ATP-induced Ca^{2+} signaling (Peng et al., 2016). Furthermore, our study has identified Orai1 and Stim1 as critical molecular constituents of the CRAC channel and demonstrated Orai1 channel-mediated Ca^{2+} influx is an important downstream mechanism in ATP-induced Ca^{2+} signalling in human DP-MSC (Peng et al., 2016).

In summary, all studies have consistently shown that extracellular ATP and other nucleotides evoke an increase in the $[Ca^{2+}]_i$ in MSC, regardless of species and tissues. In terms of the expression of P2X receptors, the results indicate no expression of the P2X2 receptor, and a majority of studies support functional expression of the P2X7 receptor as a mechanism mediating ATP-induced Ca^{2+} influx. It remains less understood with regards to the other P2X receptors. Emerging evidence also supports functional expression of multiple P2Y receptors in MSC, including $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{13}$ and $P2Y_{14}$, but it is important to point out that there are noticeable variations in the expression profile of the P2Y receptors and their contribution in extracellular nucleotide-evoked Ca^{2+} signaling described in the literature, which depend on the species, particularly the tissues, from which MSC were derived.

Extracellular nucleotide regulation of MSC functions and roles of the P2 receptors

As introduced above, extracellular nucleotides, particularly ATP, are highly likely to present in the stem cell niche, and released from MSC spontaneously or in response to mechanical or chemical stimulation under in vitro and in vivo conditions. Our knowledge is still limited regarding whether nucleotides act as extracellular signals in maintaining MSC in the self-renewal and undifferentiated state. However, recent studies show that ATP and other extracellular nucleotides exert prominent regulation of MSC proliferation,

viability, migration and differentiation into multiple lineages, and also accumulated clear evidence to further support important roles for the P2X and P2Y receptors in mediating such functional regulation.

Proliferation and viability

Several studies have evaluated the effects of ATP and NAD, released endogenously or applied exogenously, on BM-MSC proliferation and viability (Coppi et al., 2007; Ferrari et al., 2011; Fruscione et al., 2011; Li et al., 2015; Li et al., 2016; Riddle et al., 2007). Riddle et al showed that exposure to oscillatory fluid flow resulted in greater human BM-MSC proliferation, using 5-bromo-2´-deoxyuridine (BrdU) labelling assay (Riddle et al., 2007). Exposure of cells to fluid flow induced vesicular ATP release, an increase in the $[Ca^{2+}]_i$, activation of Ca^{2+}-sensitive calcineurin, and nuclear translocation of NFAT, particularly NFATc1, all of which were prevented by apyrase. Treatment of cells cultured under static conditions with ATP also gave rise to activation of calcineurin, nuclear translocation of NFATc1, and increased proliferation. In contrast, treatment with ATP metabolites, ADP, AMP and adenosine, or other nucleotides, including UTP, failed to affect cell proliferation. These results support a specific role for extracellular ATP in mediating fluid flow-induced increase in cell proliferation. As discussed above, this study demonstrated protein expression for the P2X7, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors. However, it was not established whether or which of these receptors mediated the increase in cell proliferation in response to fluid flow or ATP. A separate study, using luminescence assay, found that the ATP content was significantly higher in the culture medium in the presence of human BM-MSC, suggesting spontaneous release of ATP (Coppi et al., 2007). In addition, the number of cells after 5 days in culture was increased by inclusion of PPADS or the $P2Y_1$ antagonist MRS2179 in the culture medium, and decreased by addition of ATP (10 μM). These results suggest that ATP inhibits cell proliferation at least in part via activation of the $P2Y_1$ receptor. Ferrari et al profiled the gene expression in human BM-MSC with or without treatment with 1 mM ATP for 24 hours (Ferrari et al., 2011). ATP up-regulated the expression of growth arrest genes and cell cycle inhibitors, and down-regulated the expression of genes related to cell cycle, DNA replication and

repair, cell division and chromosome segregation. Consistently, they showed that pretreatment of human BM-MSC with 1 mM ATP for 24-72 hours inhibited cell proliferation, assessed after cells were cultured for 5 days, but pretreatment with ATP at lower concentrations (1-100 µM) was without effect (Ferrari et al., 2011). Fruscione et al showed, using thiazol blue tetrazolium bromide (MTT) cell viability assay, that the number of human BM-MSC was increased after they were cultured in the presence of NAD for 72 hours (Fruscione et al., 2011). Such effect was not observed for cells cultured in the presence of AMP, ADP-ribose and other NAD metabolites. The number of cells was decreased after they were treated with connexin43-specific siRNA to reduce connexin43-mediated NAD release or cultured in the presence of NADase to remove endogenously released NAD. Similar increase or decrease in the number of cells, induced by treatment with NAD and NADase respectively, was also observed, using ^{3}H-thymidine incorporation assay. The study also analyzed cell death, by combining annexin and/or propidium iodide staining with fluorescence activated cell sorting (Fruscione et al., 2011). Treatment with NAD reduced the percentage of apoptotic cell without changing the percentage of dead cells, whereas treatment with NADase increased the percentage of dead cells without altering the percentage of apoptotic cells. Taken together, these results suggest that NAD stimulates proliferation and reduces apoptotic death (Fruscione et al., 2011). Further investigation supports that the $P2Y_{11}$-$G_{\alpha,i}$-AC-cAMP signalling pathway is important in mediating NAD-induced stimulation of cell proliferation (Fruscione et al., 2011). The same study observed no effect of treating cells with ATP up to 50 µM on cell proliferation (Fruscione et al., 2011). As discussed above, ATP and UTP induced an increase in the $[Ca^{2+}]_i$ in rat BM-MSC via the $P2Y_2$ receptor (Ichikawa and Gemba, 2009). The sensitivity to UTP was dependent of the cell density with EC_{50} values of 28, 11 and 5 µM at low, medium and high density, respectively, and the amplitude of UTP-induced increase in the $[Ca^{2+}]_i$ was much greater in cells at high density. In addition, UTP induced Ca^{2+} oscillations in cells but only at medium density, which depended on the store-operated Ca^{2+} entry. As the cell density increased, the $P2Y_2$ mRNA level was increased. Furthermore, the percentage of proliferating cell nuclear antigen-positive cells, as identified using immunocytochemistry, was 93% at low density, which was reduced by

about half at medium density and further down to nearly zero at high density. These results were interpreted to suggest cell density-dependent changes in the $P2Y_2$ expression and intracellular Ca^{2+} mobilization that may relate to cell cycle progression. Two recent studies from the same group have reported that BzATP and UTP, both applied at 5-125 μM, did not alter the number of rat BM-MSC after they were cultured for 7 days, evaluated using cell counting kit-8 (CCK-8) assay (Li et al., 2015; Li et al., 2016).

Cell proliferation and viability of MSC from other tissues have also been examined. Human DP-MSC proliferated in the presence of ATP at 0.3-300 μM for 72 hours in a similar rate as they did in the absence of ATP, as shown in our recent study based on both cell counting and MTT assay (Peng et al., 2016). A recent study, using MTT assay, has documented that the human PDL-MSC viability was considerably reduced after cultured in the presence of BzATP at 30-300 μM for 48 hours (Trubiani et al., 2014). As discussed below, this study has shown functional expression of the P2X7 receptor, but it was not clear whether the P2X7 receptor is critical in BzATP-induced reduction in the cell viability.

In summary, studies have shown extracellular nucleotide regulation of MSC proliferation and viability and a significant role for the $P2Y_1$ and $P2Y_{11}$ receptor in the regulation of BM-MSC proliferation by ATP and NAD respectively. However, the effects of extracellular nucleotides on MSC proliferation and viability are highly variable, and even ATP-induced effects are noticeably different. These discrepancies may associate with MSC used, due to factors like the donor age and tissue origin, which as discussed above express the P2X and P2Y receptors at different levels, and may also relate to the experimental conditions, such as concentration, treatment duration and assays used.

Cell migration

Studies have drawn attention to whether extracellular nucleotides regulate MSC migration and their homing ability in vivo (Ferrari et al., 2011; Fruscione et al., 2011; Peng et al., 2016). Ferrari et al showed, using trans-well migration assay, that addition of ATP to the upper chamber enhanced human BM-MSC migration (Ferrari et al., 2011). When added to the lower chamber as a chemotactic stimulus, ATP had no effect but

increased the chemotactic activity of chemokine CXCL-12. In contrast, UTP induced no effect. In addition, pretreatment with 1 mM ATP enhanced the homing rate of human BM-MSC, assessed 16 hours after injected into immunocompromised mice (Ferrari et al., 2011). However, the P2 receptor(s) that mediate(s) ATP-induced stimulation of cell migration was (were) not identified. Fruscione et al examined NAD regulation of human BM-MSC migration, also using trans-well assay (Fruscione et al., 2011). Pretreatment with NAD stimulated cell migration, whereas pretreatment with NADase was without effect. NAD also enhanced cell migration when added to the lower chamber. NAD-induced increase in cell migration was prevented by the $P2Y_{11}$ antagonist NF157 and ddA, an inhibitor of membrane-associated AC, supporting a key role of the $P2Y_{11}$-$G_{\alpha,i}$-AC-cAMP signalling pathway (Fruscione et al., 2011). The same study also showed that pretreatment with ATP (1-10 μM) or addition of ATP in the lower chamber stimulated cell migration. Again, the molecular identity of the P2 receptor(s) involved in ATP-induced stimulation of cell migration was not clearly defined (Fruscione et al., 2011).

Our recent study has shown that human DP-MSC migration was accelerated in the presence of ATP at 30 μM but not 3 μM, assessed at 24, 48 and 72 hours mainly using wound healing assay (Peng et al., 2016). ATP-induced stimulation of cell migration was inhibited by PPADS, the human P2X7 antagonist AZ1164373, or specific siRNA targeting the P2X7, $P2Y_1$ or $P2Y_{11}$ receptor, but not CGS15943, a generic antagonist for adenosine receptors. These results suggest engagement of these three P2 receptors but not the adenosine receptors. Our study has also shown that activation of the Orai1 CRAC channel as an important downstream mechanism mediates ATP-induced stimulation of cell migration (Peng et al., 2016).

In summary, studies have gathered consistent evidence to demonstrate that both ATP and NAD stimulate human MSC migration. While activation of the $P2Y_{11}$-$G_{\alpha,i}$-AC-cAMP signalling pathway is critical for NAD-induced stimulation of human BM-MSC, several P2 receptors, including the P2X7, $P2Y_1$ and $P2Y_{11}$ receptors, participate in ATP-induced stimulation of human DP-MSC.

Adipogenic differentiation

MSC are well-documented to commit differentiation to adipocyte. There is evidence to show substantial changes in the expression of both P2X and P2Y receptors during adipogenic differentiation. Two recent studies from the same group have shown that after rat BM-MSC were cultured in adipogenic medium for 7 day, the P2X7 mRNA and protein expression was down-regulated (Li et al., 2015) and by contrast the $P2Y_2$ mRNA expression was up-regulated (Li et al., 2016). Zippel et al reported that the mRNA expression was increased for P2X6 and $P2Y_{11}$ and decreased for $P2Y_4$ and $P2Y_{14}$ after adipogenic differentiation of human AT-MSC (Zippel et al., 2012). The change in expression for P2X6, $P2Y_4$ and $P2Y_{11}$ was also demonstrated at the protein level, using western blotting. Furthermore, after adipogenic differentiation, the increases in the $[Ca^{2+}]_i$ induced by ATP and 2-MeSADP remained unchanged, but UTP-induced increase in the $[Ca^{2+}]_i$ was lost, which is consistent with down-regulation expression of the $P2Y_4$ receptor (Zippel et al., 2012).

Numerous genes are known to express or their expression is up-regulated during adipogenic differentiation, including peroxisome proliferator-activated receptor γ (PPARγ), fatty acid binding protein 4 (FABP4), adiponectin and adipsin. Increased expression of these adipogenic genes leads to generation of lipid droplets, which is characteristic of adipocytes and can be visualized by Oil red O staining. A recent study has shown that treatment of rat BM-MSC with 125 μM BzATP during 7 day culture in basal medium reduced the expression of PPARγ, FABP4 and adipsin, and lipid droplet formation. BzATP-induced decrease in the expression of these adipogenic genes was attenuated by the P2X7 selective antagonist BBG and P2X7-specific siRNA, demonstrating the importance of P2X7 receptor in the down-regulation of adipogenic differentiation (Li et al., 2015). BzATP also reduced expression of the aforementioned adipogenic genes and lipid droplet formation in rat BM-MSC under adipogenic differentiation conditions. The study has further shown that in ovariectomized mice, a model of osteoporosis, the number of bone marrow adipocytes was reduced by intraperitoneal administration of BzATP, suggesting that activation of the P2X7 receptor suppresses adipogenic differentiation of BM-MSC in vivo. In addition, the study has examined the role of mitogen-activated protein kinases (MAPK) signalling in BzATP-

induced down-regulation of adipogenic differentiation. Treatment with BzATP initially (5-10 min) stimulated and subsequently (20-60 min) inhibited the activity of extracellular signal-regulated kinase (ERK). The same treatment induced persistent activation of c-Jun NH_2-terminal kinase (JNK) but no effect on the p38 activity. Both BzATP-induced activation of ERK and JNK and reduction in the expression of PPARγ, FABP4 and adipsin were completely inhibited by UO126, an inhibitor of the ERK signalling, and SP600125, an inhibitor of the JNK signalling. These results suggest that the ERK and JNK MAPK signalling pathways play a crucial role in P2X7 receptor-mediated down-regulation of MSC adipogenic differentiation (Li et al., 2015).

Studies using agonists and antagonists provide evidence to show that various P2Y receptors play a significant role in extracellular nucleotide regulation of adipogenic gene expression and lipid droplet formation during adipogenic differentiation of MSC. Ciciarello et al showed that treatment of human BM-MSC with 1 mM ATP for 24 hours, prior to adipogenic differentiation, enhanced the PPARγ expression and also fat droplet formation, examined two and three weeks after adipogenic differentiation (Ciciarello et al., 2013). ATP-induced increase in the PPARγ expression was inhibited by the $P2Y_1$ antagonist MRS2279, but not by the $P2Y_6$ antagonist MRS2578, $P2Y_{11}$ antagonist NF340, $P2Y_{12}$ antagonist ARC66096 or P2X7 antagonist KN62. Pretreatment with UTP or INS45973, an agonist at the $P2Y_2$ and $P2Y_4$ receptors, resulted in a similar effect on the PPARγ expression, whereas the $P2Y_2$ selective agonist MRS2768 was ineffective. These results have been interpreted to support a critical role for the $P2Y_1$ and $P2Y_4$ receptors in regulation of adipogenic differentiation of human BM-MSC by ATP and UTP, respectively (Ciciarello et al., 2013). However, ATP-induced increase in the PPARγ expression was also inhibited by the $G_{\alpha,i}$ inhibitor PTX (Ciciarello et al., 2013) , suggesting involvement of the $G_{\alpha,i}$-coupled P2Y receptors. In support of no significant role for the $P2Y_{11}$ receptor in adipogenic differentiation, an earlier study showed that during adipogenic differentiation of human BM-MSC, activation or inhibition of NAD-induced $P2Y_{11}$-mediated signaling resulted in no effect on the expression of PPARγ, FABP4 and adiponectin, and lipid droplet formation (Fruscione et al., 2010). A recent study using BM-MSC derived from the WT and $P2Y_{13}$-KO mice has shown that $P2Y_{13}$

deficiency led to earlier expression of PPARγ isoform 2 (PPARγ2), an early adipogenic gene, elevated expression of adipsin, and lipid droplet formation (Biver et al., 2013). Furthermore, there was an increase in the number of bone marrow adipocytes in the $P2Y_{13}$-KO mice. These results have disclosed an important role for the $P2Y_{13}$ receptor in suppressing adipogenic differentiation of mouse BM-MSC (Biver et al., 2013). A more recent study has investigated the effect of UTP (5-125 μM) on adipogenic differentiation of rat BM-MSC (Li et al., 2016). Inclusion of UTP in adipogenic medium enhanced the mRNA expression of PPARγ, FABP4 and adipsin, and fat droplet formation. UTP-induced increase in the expression of adipogenic genes was inhibited by $P2Y_2$-specific siRNA, but not $P2Y_4$-specific siRNA or the $P2Y_6$ antagonist MRS2578, suggesting strong dependence of the $P2Y_2$ receptor (Li et al., 2016). Like BzATP as discussed above, initial treatment with UTP (5-10 min) activated, but prolonged treatment (20-60 min) inhibited, the ERK signaling. However, treatment with UTP resulted in no effect on the JNK and p38 MAPK signaling. UTP-induced ERK activation was prevented by $P2Y_2$-specific siRNA. The ERK activity in both control and UTP-treated cells was completely abolished upon treatment with the ERK signaling inhibitor UO126. When UO126 was added to adipogenic medium, UTP-induced increase in the expression of adipogenic genes was completely inhibited on the first day, but was persistently observed after treatment with UO126 for 3 and 7 days (Li et al., 2016). These results indicate that the ERK signalling pathway is involved in the early stage of UTP up-regulation of adipogenic differentiation.

Inclusion of PPADS during adipogenic differentiation of human AT-MSC attenuated lipid droplet formation, and such an inhibition was mitigated by co-application of exogenous UTP but not ATP, suggesting involvement of the $P2Y_4$ but not $P2Y_2$ receptor (Zippel et al., 2012), which is different from the finding from the above-discussed study of human BM-MSC by Ciciarello et al (Ciciarello et al., 2013). Application of UTP, BzATP or ADP alone was without effect on lipid droplet formation. However, application of ATP alone reduced and, in contrast, treatment with apyrase enhanced, lipid droplet formation, suggesting that endogenously released ATP or its metabolites inhibit adipogenic differentiation. Like ATP, UDP-glucose reduced lipid droplet formation.

These observations, together with down-regulated $P2Y_{14}$ expression during adipogenic differentiation discussed above, supports the notion that activation of the $P2Y_{14}$ receptor inhibits adipogenic differentiation of human AT-MSC (Zippel et al., 2012).

To summarize, studies provide evidence to show extracellular nucleotide regulation of adipogenic differentiation of MSC via activation of different P2 receptors. Activation of the P2X7, $P2Y_{13}$ or $P2Y_{14}$ receptor down-regulates, whereas activation of the $P2Y_1$, $P2Y_2$ or $P2Y_4$ receptor up-regulates, adipogenic differentiation.

Osteogenic differentiation

The bone-forming cell, osteoblast, is also differentiated from MSC. As during adipogenic differentiation discussed above, studies have documented changes in the expression of the P2X and P2Y receptors during osteogenic differentiation of MSC. Again, there are substantial variations in the reported results. During osteogenic differentiation of human BM-MSC, the protein expression was decreased for P2X7 and $P2Y_1$, increased for $P2Y_2$ and $P2Y_4$, and remained unaltered for $P2Y_6$ (Noronha-Matos et al., 2012). However, during osteogenic differentiation of rat BM-MSC, the P2X7 mRNA expression was elevated (Li et al., 2015) but the $P2Y_2$ mRNA expression was reduced (Li et al., 2016). The $P2Y_{13}$ mRNA expression was up-regulated during osteogenic differentiation of mouse BM-MSC (Biver et al., 2013). After osteogenic differentiation of human AT-MSC, the mRNA and protein expression was down-regulated for P2X6, P2X7, $P2Y_1$, $P2Y_2$ and $P2Y_4$ and up-regulated for P2X5, and the $P2Y_{14}$ mRNA expression was also decreased (Zippel et al., 2012).

It is well-documented that during osteogenic differentiation, a number of genes including alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), osterix, osteocalcin and osteopontin, are expressed or their expression is up-regulated. Osteoblast is responsible for bone extracellular matrix production and mineralization. These genotypes and phenotypes have been commonly used to indicate osteogenic differentiation of MSC. Treatment of human BM-MSC with ATP or shockwave, a mechanical stimulus inducing endogenous ATP release, increased the ALP activity, osteocalcin protein expression, and matrix mineralization (Sun et al., 2013). These effects

were strongly reduced or completely abolished by PPADS, the human P2X7 antagonist KN62 and P2X7-specific siRNA. Shockwave-induced effects were also prevented by apyrase, supporting a role for endogenous ATP release. In addition, both ATP and shockwave induced robust increases in the expression of c-Fos and c-Jun. Furthermore, all these effects were inhibited by SB203850, an inhibitor of the p38 MAPK signalling (Sun et al., 2013). These findings have led to the conclusion that activation of the P2X7 receptor triggers the p38 MARK signalling pathway to induce the expression of c-Fos and c-Jun, which together form the transcription factor activating protein-1 to up-regulate the expression of osteogenic genes (Sun et al., 2013). Similarly, treatment of human BM-MSC with BzATP enhanced the ALP activity, Runx2 expression, and matrix mineralization (Noronha-Matos et al., 2014). BzATP-induced up-regulation of osteogenic differentiation was inhibited by H1152, a Rho kinase inhibitor as well as the human P2X7 antagonist A438079 (Noronha-Matos et al., 2014). Treatment of rat BM-MSC with BzATP under basal and osteogenic differentiation conditions upregulated the mRNA and protein expression of Runx2, ALP and osteopontin (Li et al., 2015). These effects were inhibited by the P2X7 antagonist BBG. As discussed above, BzATP induced activation of the ERK and JNK but not p38 MAPK signalling pathways in rat BM-MSC, and BzATP-induced increase in the expression of the osteogenic genes was prevented by the ERK signalling inhibitor U0126 and the JNK signalling inhibitor SP600125. Taken together, these studies support the idea that activation of the P2X7 receptor triggers the Rho and/or MAPK downstream signalling pathways to up-regulate osteogenic differentiation of BM-MSC (Li et al., 2015).

There is evidence to support a role for the P2Y receptors in extracellular nucleotide regulation of osteogenic differentiation. UTP, UTPβS, the non-hydrolysable UTP analogue, UDP, and PSB0474, a metabolically stable $P2Y_6$ agonist, were potent in inducing an increase in the ALP activity in human BM-MSC (Noronha-Matos et al., 2012). Enhancement in osteogenic differentiation induced by these agonists was inhibited by PPADS and the $P2Y_6$ antagonist MRS2578 (Noronha-Matos et al., 2012). These results support a critical role for the $P2Y_6$ receptor. However, treatment with UTP of rat BM-MSC down-regulated the expression of Runx2, ALP and osteopontin (Li et al.,

2016). Such down-regulation in the expression of osteogenic genes was attenuated by $P2Y_2$-specific siRNA, but not $P2Y_4$-siRNA or the $P2Y_6$ antagonist MRS2578, indicating main involvement of the $P2Y_2$ receptor (Li et al., 2016). Furthermore, UTP-induced inhibition of osteogenic differentiation was prevented during the first 24 hour treatment with the ERK signalling inhibitor UO126, but was unabated after treatment with UO126 was extended to 3 and 7 days (Li et al., 2016). This result suggests that the ERK signalling pathway is mainly involved in the early stage of UTP down-regulation of adipogenic differentiation. Activation of the $P2Y_{13}$ receptor in mouse BM-MSC by ADP up-regulated the expression of ALP and osterix and, BM-MSC from the $P2Y_{13}$-KO mouse exhibited preferential adipogenic differentiation, consistently highlighting that activation of the $P2Y_{13}$ receptor is necessary for commitment of BM-MSC to osteogenic differentiation (Biver et al., 2013).

An early study by Zippel et al showed that treatment of human AT-MSC with suramin, PPADS or apyrase during osteogenic differentiation inhibited matrix mineralization (Zippel et al., 2012). Such an inhibition was reversed by co-application of ATP but not UTP, excluding a major role for the $P2Y_2$ or $P2Y_4$ receptor. However, it was not clearly established which P2 receptor(s) mediate(s) the inhibition of osteogenic differentiation by suramin and PPADS (Zippel et al., 2012).

In summary, it is evident that extracellular nucleotide regulation of osteogenic differentiation of MSC via activation of the P2X and/or P2Y receptors. More specifically, activation of the P2X7, $P2Y_6$ or $P2Y_{13}$ receptor up-regulates, and activation of the $P2Y_2$ receptor down-regulates, osteogenic differentiation. Of notice, activation of the P2X7, $P2Y_2$ and $P2Y_{13}$ receptors results in an opposite regulation of adipogenic and osteogenic differentiation, which is not surprising considering that MSC differentiation into adipocyte and osteoblast often shows mutual exclusion (Chen et al., 2016).

Chondrogenic differentiation

Chondrocyte represents the third lineage of MSC differentiation. Our current knowledge with regards to extracellular regulation of chondrogenic differentiation of MSC is limited. The expression of chondrogenic genes such as aggrecan, type II collagen and SOX9 is

up-regulated during chondrogenic differentiation. A previous study using ATD5, a pre-chondrogenic cell that can differentiate into chondrocytes, showed an important role for ATP oscillations in pre-chondrogenic condensation, which were driven by intracellular Ca^{2+} oscillations (Kwon, 2012). Similar ATP oscillations and increase in the expression of the above-described chondrogenic genes were observed in the micromass culture of mouse BM-MSC during chondrogenic differentiations, which were lost by treatment with the P2X4 antagonist 5-BDBD (Kwon, 2012). These results support ATP-induced P2X4 receptor-mediated Ca^{2+} influx is required in the ATP oscillations and chondrogenic differentiation of mouse MSC (Kwon, 2012).

Neuronal differentiation

Astrocyte, one type of glial cells in the central nervous system, is well-known to release ATP, which is critical for Ca^{2+} wave propagation among astrocytes and modulation of synaptic transmission (Haydon and Carmignoto, 2006). A recent study has examined the effects of ATP released from astrocytes or exogenously applied on neuronal differentiation of human BM-MSC (Tu et al., 2014). ATP release was induced by 470 mm light activation of astrocytes expressing the light-sensitive channelrhodopsin-2 (ChR2). After co-cultured with ChR2-expressing astrocytes in the neurobasal medium for 3 weeks, during which cells were exposed to light for 30 min every 3 days, the percentage of MSC expressing Tuj1 and neuN, two neuronal markers, identified by immunocytochemistry, was increased by approximately 3-fold and 4-fold, respectively. The increase in the percentage of Tuj1-positive cells was prevented by TNP-ATP at 30 μM, a concentration that as discussed above inhibits multiple P2X receptors. An increase in the mRNA expression of Tuj1 and Pax6, a transcriptional factor, was also observed in human BM-MSC after cultured for 3 weeks in the neurobasal medium that was either conditioned by light-activated ChR2-expressing astrocytes or supplemented with ATP (10 μM). ATP-induced increase in the expression of Tuj1 and Pax6 was inhibited by TNP-ATP. These results suggest that extracellular ATP promotes neuronal differentiation of human BM-MSC. ATP (10 μM) also increased the expression of β-catenin and FZD8, two target genes for the Wnt/β-catenin signalling, as well as inducing

nuclear translocation of β-catenin. ATP-induced increase in the expression of β-catenin and FZD8 was abolished by TNP-ATP. These results support that the P2X receptors and Wnt/β-catenin signalling are engaged in ATP-induced up-regulation of neuronal differentiation of human BM-MSC. However, the P2X receptors and the signalling pathway(s) linking the P2X receptor activation to the Wnt/β-catenin signalling remain unknown. This study has further examined neuronal differentiation of human BM-MSC in vivo and contribution in protecting brain damage resulting from middle cerebral artery occlusion (MCAO), a widely-used model of ischemic stroke. Rats were transplanted with human BM-MSC alone or together with astrocytes expressing ChR2 or YFP in striatum 2 days after MCAO, and received light stimulation one day later. Immunostaining confirmed the presence of human BM-MSC in the close vicinity of co-transplanted rat astrocytes in the ischemic brain. The percentage of Tuj1-positive cells determined 2 weeks after MCAO was much higher in the ischemic brain co-transplanted with human BM-MSC and rat ChR2-expressing astrocytes. Moreover, MCAO-induced infarct volume and neurological impairment were reduced in rats that were co-transplanted with human BM-MSC and rat ChR2-expressing astrocytes and received light stimulation. These results show that astrocyte derived ATP can stimulate neuronal differentiation of human BM-MSC in vivo and provide the proof of concept that ATP stimulation of neuronal differentiation of human BM-MSC is useful in treating ischemic stroke-induced neurological deficits.

Glial differentiation

There is evidence to suggest that rat AT-MSC can differentiate into cells with Schwann cell (SC) phenotypes, such as expression of glial markers and growth factors, and an ability to produce myelin and induce neurite outgrowth in vitro and enhance nerve regeneration in vivo, and a recent study has examined the expression of P2X receptors after rat AT-MSC were differentiated into SC-like cells (Faroni et al., 2013). As discussed above, in rat AT-MSC, P2X4 and P2X7 at the protein level were not detected but became readily detected after differentiation into SC-like cells. Consistently, ATP-induced increase in the $[Ca^{2+}]_i$ in SC-like cells were sensitive to inhibition by the P2X7 antagonist

AZ10606120, which was not observed in AT-MSC. ATP was also more potent, with EC_{50} reduced from ~100 µM in AT-MSC to below 30 µM in SC-like cells. Furthermore, in patch-clamp recording, both ATP and BzATP concentration-dependently induced non-sensitizing currents in SC-cells and BzATP was potent more than ATP (Faroni et al., 2013). ATP-induced currents were abolished by the P2X7 antagonist AZ10606120. All these results provide consistent evidence to indicate functional expression of the P2X7 receptor in SC-like cells. It is long known that the P2X7 receptor as a cytolytic receptor because sustained activation of the P2X7 receptor causes cell death (Jiang, 2009; Virginio et al., 1999). Exposure of SC-like cells to 5 mM ATP for 1 hour induced cell death as determined by lactate dehydrogenase release cytotoxicity assay and EthD-1 staining viability assay (Faroni et al., 2013). ATP-induced SC-like cell death was prevented by AZ10606120. These results further support up-regulation of the P2X7 receptor expression during Schwann cell differentiation of AT-MSC (Faroni et al., 2013). However, it remains unclear whether extracellular nucleotides regulate glial differentiation of MSC and whether up-regulation of the P2X7 receptor expression is causatively involved in the regulation of glial differentiation.

Concluding remarks and perspectives

It is evident from the discussion above that studies in the past few years have significantly enriched our knowledge with respect to expression of the P2X and P2Y receptors in MSC, and also provided evidence to support a role for these receptors in mediating the regulation of MSC viability, proliferation, migration and differentiation by extracellular nucleotides, particularly ATP and UTP. However, it is also obvious that substantial discrepancies exist in the findings from studies examining MSC from different species and tissues, even MSC from the same type species and tissue. More investigations are clearly required to gain a better understanding of extracellular nucleotide regulation of MSC functions and the role of the P2X and P2Y receptors in such functional regulation. Currently, it is completely unclear whether extracellular nucleotides are important in maintaining MSC stemness, namely, MSC in the self-renewal and undifferentiated state, and if they are, it is important to determine the role of the P2X and P2Y receptors.

Ca^{2+} is a ubiquitous intracellular messenger that plays a crucial role in a vast range of cell functions (Berridge et al., 2003). As discussed above, extracellular nucleotides, via activation of the P2X and P2Y receptors, induce extracellular Ca^{2+} influx and/or intracellular Ca^{2+} release, which can form specific Ca^{2+} signatures with distinct spatial and temporal dynamics. Further efforts are warranted to investigate whether the increase in the $[Ca^{2+}]_i$ or the Ca^{2+} signature is more critical in the regulation of MSC proliferation, migration and differentiation by extracellular nucleotides. Moreover, previous studies have shown that diverse Ca^{2+}-dependent downstream signaling pathways are important in driving cell functions (Agell et al., 2002; Argentaro et al., 2003; Preß et al., 2015; Stoeckl et al., 2013; Zayzafoon, 2006). Indeed, as discussed above, emerging evidence supports that activation of the P2X7 and P2Y receptors triggers the Rho and/or MAPK signaling pathways in the regulation of adipogenic and osteogenic differentiation of MSC. More remain to be learnt in terms of the Ca^{2+}-dependent signaling mechanisms in extracellular nucleotide regulation of MSC functions. A clear understanding of extracellular nucleotide-induced purinergic signaling in MSC functions should be rewarding in improving the applications of MSC in regenerative medicine.

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Conflict of interest

The authors declare no conflict of interest.

Table 1 Summary of expression of P2X and P2Y receptors in MSC and change in their expression during differentiation

Receptor	Cell preparations	Examination methods	Change in expression during differentiation	References
P2X1	Human BM-MSC	RT-PCR; WB		(Ferrari et al., 2011)
	Human UC-MSC	RT-PCR		(Tu et al., 2014)
P2X3	Human BM-MSC	RT-PCR		(Ferrari et al., 2011)
	Human AT-MSC	RT-PCR		(Zippel et al., 2012)
	Rat AT-MSC	RT-PCR		(Faroni et al., 2013)
P2X4	Human BM-MSC	RT-PCR; WB		(Ferrari et al., 2011)
	Human AT-MSC	RT-PCR		(Zippel et al., 2012)
	Human UC-MSC	RT-PCR		(Tu et al., 2014)
	Human DP-MSC	RT-PCR		(Peng et al., 2016)
	Rat AT-MSC	RT-PCR		(Faroni et al., 2013)
P2X5	Human BM-MSC	RT-PCR		(Ferrari et al., 2011)
	Human AT-MSC	RT-PCR; WB	↑ Osteogenesis	(Zippel et al., 2012)
	Human UC-MSC	RT-PCR		(Tu et al., 2014)
P2X6	Human BM-MSC	RT-PCR		(Ferrari et al., 2011)
	Human AT-MSC	RT-PCR; WB	↑ Adipogenesis; ↓ osteogenesis	(Zippel et al., 2012)
	Human UC-MSC	RT-PCR		(Tu et al., 2014)
	Human DP-MSC	RT-PCR		(Peng et al., 2016)
P2X7	Human BM-MSC*	WB		(Riddle et al., 2007)
		IS; Ca^{2+} imaging	↓ Osteogenesis	(Noronha-Matos et al., 2012)
		IS; Ca^{2+} imaging; dye uptake		(Noronha-Matos et al., 2014)
	Human BM-MSC	RT-PCR; WB; Ca^{2+} imaging		(Ferrari et al., 2011)
		RT-PCR; WB	↑ Osteogenesis	(Sun et al., 2013)

	Human AT-MSC	RT-PCR; WB	↓ Osteogenesis	(Zippel et al., 2012)
	Human UC-MSC	RT-PCR		(Tu et al., 2014)
	Human DP-MSC	RT-PCR; Ca^{2+} imaging		(Peng et al., 2016)
	Human PDL-MSC	RT-PCR; WB; IS; Ca^{2+} imaging		(Trubiani et al., 2014)
	Rat BM-MSC*	RT-PCR; WB; IS	↓Adipogenesis;↑osteogenesis	(Li et al., 2015)
P2Y1	Human BM-MSC	Ca^{2+} imaging		(Kawano et al., 2006)
		Patch-clamp current recording		(Coppi et al., 2007)
		RT-PCR; WB; Ca^{2+} imaging		(Ferrari et al., 2011)
	Human BM-MSC*	IS; Ca^{2+} imaging	↓ Osteogenesis	(Noronha-Matos et al., 2012)
		IS; Ca^{2+} imaging		(Noronha-Matos et al., 2014)
	Human AT-MSC	RT-PCR; WB; Ca^{2+} imaging	↓ Osteogenesis	(Zippel et al., 2012)
	Human DP-MSC	RT-PCR; Ca^{2+} imaging		(Peng et al., 2016)
P2Y2	Human BM-MSC*	WB		(Riddle et al., 2007)
		IS	↑ Osteogenesis	(Noronha-Matos et al., 2012)
	Human BM-MSC	RT-PCR; WB		(Ferrari et al., 2011)
	Human AT-MSC	RT-PCR; WB	↓ Osteogenesis	(Zippel et al., 2012)
	Rat BM-MSC*	RT-PCR; IS; Ca^{2+} imaging		(Ichikawa and Gemba, 2009)
		RT-PCR; IS	↑ Adipogenesis; ↓ osteogenesis	(Li et al., 2016)
P2Y4	Human BM-MSC*	IS	↑ Osteogenesis	(Noronha-Matos et al., 2012)
	Human BM-MSC	RT-PCR		(Ferrari et al., 2011)
	Human AT-MSC	RT-PCR; WB	↓ Adipogenesis;↓ osteogenesis	(Zippel et al., 2012)
P2Y6	Human BM-MSC*	WB		(Riddle et al., 2007)
	Human BM-MSC	IS; Ca^{2+} imaging		(Noronha-Matos et al., 2012)
		RT-PCR; Ca^{2+} imaging		(Ferrari et al., 2011)
	Human AT-MSC	RT-PCR		(Zippel et al., 2012)
P2Y11	Human BM-MSC*	WB		(Riddle et al., 2007)

	Human BM-MSC	RT-PCR; WB; Ca^{2+} imaging		(Ferrari et al., 2011)
		RT-PCR; Ca^{2+} imaging		(Fruscione et al., 2011)
	Human AT-MSC	RT-PCR; WB	↑ Adipogenesis	(Zippel et al., 2012)
	Human DP-MSC	RT-PCR; Ca^{2+} imaging		(Peng et al., 2016)
P2Y12	Human BM-MSC	RT-PCR		(Ferrari et al., 2011)
	Human AT-MSC	RT-PCR		(Zippel et al., 2012)
P2Y13	Human BM-MSC	RT-PCR		(Ferrari et al., 2011)
	Human AT-MSC	RT-PCR		(Zippel et al., 2012)
	Mouse BM-MSC	RT-PCR	↑ Osteogenesis	(Biver et al., 2013)
P2Y14	Human BM-MSC	RT-PCR		(Ferrari et al., 2011)
	Human AT-MSC	RT-PCR	↓ Adipogenesis; ↓osteogenesis	(Zippel et al., 2012)

Abbreviations: MSC, mesenchymal stem cells (*MSC, mesenchymal stromal cells used in the original studies); BM, bone marrow; AT, adipose tissues; UC, umbilical cord; DP, dental pulp; PDL, periodontal ligament; RT-PCR, reverse transcription-polymerase chain reaction; WB, western blotting; IS, immuno-staining; ↓, up-regulated expression; ↑, down-regulated expression.

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