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Role of the P2Y₁₃ receptor in the differentiation of bone marrow stromal cells into osteoblasts and adipocytes

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ABSTRACT

Accumulating evidence indicates that extracellular nucleotides, signaling through purinergic receptors, play a significant role in bone remodeling. Mesenchymal stem cells (MSC) express functional P2Y receptors whose expression level is regulated during osteoblast or adipocyte differentiation. P2Y₁₃-deficient mice were previously shown to exhibit a decreased bone turnover associated with a reduction in the number of both osteoblasts and osteoclasts on the bone surfaces. We therefore examined whether P2Y₁₃R activation was involved in the osteogenic differentiation of MSC. Our study demonstrated that ADP stimulation of P2Y₁₃R^{+/+} (but not P2Y₁₃R^{-/-}) adherent bone marrow stromal cells (BMSC) increased significantly the formation of alkaline phosphatase-colony forming units (CFU-ALP), as well as the expression of osteoblastic markers (Osterix, Alkaline Phosphatase, Collagen I) involved in the maturation of pre-osteoblasts into osteoblasts.

The number of CFU-ALP obtained from P2Y₁₃R^{-/-} BMSC and the level of osteoblastic gene expression after osteogenic stimulation were strongly reduced compared to those obtained in wild-type cell cultures. In contrast, when P2Y₁₃R^{-/-} BMSC were incubated in an adipogenic medium, the number of adipocytes generated and the level of adipogenic gene expression (PPAR γ 2 and Adipsin) were higher than those obtained in P2Y₁₃R^{+/+} MSC. Interestingly we observed a significant increase of the number of bone marrow adipocytes in tibia of P2Y₁₃R^{-/-} mice. In conclusion, our findings indicate that the P2Y₁₃R plays an important role in the balance of osteoblast and adipocyte terminal differentiation of bone marrow progenitors. Therefore, the P2Y₁₃ receptor can be considered as a new pharmacological target for the treatment of bone diseases like osteoporosis.

INTRODUCTION

Bone marrow is a heterogeneous tissue composed of differentiated cells, lineage restricted progenitors and stem cells. Among

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Corresponding author: Dr. Bernard Robaye, 10 Rue A.Bolland, Université Libre de Bruxelles, Gosselies 6041, Belgium. E-mail address: brobaye@ulb.ac.be. Tel: +3226509839; Abbreviations: ADP β S: adenosine-5'-O-(2-thiodiphosphate); ALP: alkaline phosphatase; BMSC: bone marrow stromal cells; CFU: colony forming unit; CFU-ALP: alkaline phosphatase-positive colony forming unit; CFU-F: colony forming unit-fibroblast; COL1: collagen of type I; HPRT: hypoxanthine-guanine phosphoribosyltransferase; MSC: mesenchymal stem cells; ns.: not significant; OSX: osterix; WT: wild-type. NS: non stimulated; DGAA: dexamethasone/ β -glycerophosphate/ ascorbic acid; DII: dexamethasone/insuline/3-isobutyl-methylxanthine.; Received October 02, 2012; accepted for publication March 26, 2013; 1066-5099/2013/\$30.00/0 doi: 10.1002/stem.1411

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these are the multipotent mesenchymal stem cells (MSCs) that are able to differentiate into a number of distinct cell lineages including osteoblasts, adipocytes, chondrocytes and myocytes [1, 2]. Osteoblasts and marrow adipocytes share a common mesenchymal precursor and their ability to differentiate along one of these two lineages is determined by growth factors, cytokines and various signaling molecules. Factors promoting osteoblast differentiation are generally considered as inhibitory for adipocyte maturation and vice versa [3-9]. In age-related osteoporosis or osteopenia, the decreased bone volume is at least partly due to the fact that bone marrow stromal cells have a reduced capacity to differentiate into osteoblasts but an increased capacity for adipocyte differentiation. This shift favouring the adipocyte over osteoblast lineage leads to progressive accumulation of fat cells in bones [4, 10]. Therefore the correction of such disrupted balance is a potential therapeutic target for osteoporosis treatment. Over the last decade, a new strategy involving MSCs transplantation has been developed to cure bone formation deficiency. Because osteoblasts and adipocytes share a common precursor among MSC population, the main issue of this strategy is to avoid the differentiation of these MSCs into adipocyte cell lineage. Therefore many studies focused on identifying factors promoting osteogenesis and inhibiting adipogenesis in order to direct the differentiation toward osteogenic differentiation *in vitro* before transplantation [11-13]

The differentiation into both osteoblasts and adipocytes is typically described as a two step process: determination and maturation. Firstly, MSC differentiate into pre-osteoblasts or pre-adipocytes and this requires the expression of the early transcription factors Runx2 in pre-osteoblasts [14-16] and CEBP/β and PPARγ1 in pre-adipocytes [3, 17]. An essential feature of these pre-differentiated progenitors is that they exhibit a plasticity which allows a process of trans-differentiation into the other (reciprocal) lineage [18, 19].

Up-regulation of Runx2 expression is not sufficient for triggering osteoblast

differentiation. Its transcription factor activity is regulated through the MAP kinases signaling pathway in cooperation with the small G protein Rho [14, 16, 20, 21]. Conversely, agents elevating intracellular cyclic AMP upregulate the expression and activity of the transcription factor, PPARγ [17, 22]. In mouse as well as in human, this nuclear receptor is present in two isoforms, PPARγ1 and PPARγ2, generated by alternative splicing. It is well established that they are both involved in the differentiation of MSCs into adipocytes, but several studies support that PPARγ2 is expressed later and more selectively in fat cell lineage. Moreover, PPARγ2 is known to have a greater specificity to induce adipogenesis [23-25].

Growing evidence suggests that extracellular nucleotides play important roles in the regulation of bone metabolism [26-32]. Nucleotides are mainly intracellular but can be released through various mechanisms (i.e. cell lysis, exocytosis, membrane channels)[33]. In the extracellular environment, they activate ionotropic P2X (PX1-7) and metabotropic G-protein coupled P2Y receptors (P2Y_{1, 2, 4, 6, 11, 12, 13, 14}) [34]. The P2Y₁₃R is activated by ADP and is coupled to Gα_i and *inter alia* adenylyl cyclase inhibition. Expression of P2Y₁₃R mRNA has been detected in brain, liver, spleen and bone marrow [35-37]. Several P2Y receptor subtypes are activated by ADP (P2Y₁, P2Y₁₂ and P2Y₁₃). Since the availability of specific agonists and antagonists for these receptors is limited, the P2Y-deficient mice strains constitute a powerful tool for the identification of their physiological roles.

Knockout mice have been generated for the P2Y_{1, 2, 6} and ₁₃ receptors and each strain displays a unique bone phenotype [38-41]. In particular, P2Y₁₃-deficient (P2Y₁₃R^{-/-}) mice exhibit a decreased bone turnover associated with a reduction in the number of both osteoblasts and osteoclasts [40]. We have used P2Y₁₃R^{-/-} mice to investigate the functional expression of the P2Y₁₃R in bone marrow stromal cells and its role in the differentiation of these cells into osteoblasts *versus* adipocytes. This study provides evidence that the P2Y₁₃R physiologically stimulates the

differentiation of osteoblasts and inhibits that of adipocytes.

MATERIALS AND METHODS

Animals

P2Y₁₃R^{-/-} mice that we previously generated were crossed with C57BL/6 mice for up to 10 generations on a C57BL/6 genetic background [35]. All of the mice used in this study were generated using heterozygous parents and housed in a conventional animal facility. All of the animal studies were authorized by the Animal Care Use and Review Committee of the Université Libre de Bruxelles (CEBEA-IBMM protocol 44).

Preparation and culture of mouse bone marrow cells

Mice were killed by cervical dislocation. Mouse BMSCs were obtained from femur and tibia of 8 month old female P2Y₁₃ wild type and knockout C57BL/6 mice. The two femurs and tibias were collected and bone marrow cavities were flushed with PBS or bones were centrifuged as previously described [42]. Cells were cultured in α MEM medium (Invitrogen, Carlsbad, NM) supplemented with 10% FBS, penicillin (50 units/ml) and streptomycin (50 μ g/ml) at 37°C in a humidified atmosphere containing 5% CO₂. Non adherent cells were removed after 24 h and 5 days by washing with PBS.

Flow cytometry analysis of cell surface markers

Cells collected by trypsin treatment (2.5mM in PBS/EDTA, 10 min, 37°C) were washed in FACS medium (0.5% BSA/0, 1% NaN₃ (w/v) in PBS). 10⁶ cells were incubated with antibody (2 μ g/ml) in 100 μ l of FACS medium. After washing, the cell labeling was analysed using a Cytomics FC500 cytometer (Beckman Coulter). For each antibody, gating was determined based on appropriately stained controls. Anti-CD45, -Sca1, -PDGFR α CD90 and -CD44 antibodies were purchased from eBiosciences.

Osteogenic and adipogenic differentiation

For osteogenic differentiation, cells were cultured with osteogenic differentiation media (10nM dexamethasone; 50 μ g/ml ascorbic acid; 5mM β -glycerophosphate (Sigma-Aldrich, St. Louis, MO)) for 7 days after the primary culture. For adipogenic differentiation, cells were cultured in the adipogenic differentiation media (0.5mM IBMX; 10 μ g/ml insulin; 10nM dexamethasone (Sigma-Aldrich, St. Louis, MO)) for 14 days after the primary culture. The media were replaced every 3 or 4 days.

Alkaline phosphatase (ALP) staining and measurement of CFU-ALP and CFU-F colonies

Osteoblasts were detected using Naphthol Fast Red (Sigma-Aldrich, St. Louis, MO) staining as described previously [43]. After 7 days in osteogenic differentiation media, cells were washed with PBS and incubated for 20 minutes in Naphthol AS-MX/Fast Red solution (0.5mg/ml Naphthol; 1mg/ml Fast Red in Tris 0,1 M pH 7.2) in order to detect the ALP-positive colonies (CFU-ALP). Cells were then counterstained with Mayer's hematoxyline in order to detect the total colonies formed (CFU-F). CFU were detected using binocular microscope.

Alizarin red staining

Alizarin red staining was performed after 14 days culture in osteogenic medium. Cells were washed with PBS and fixed with 4% formaldehyde for 30 minutes at room temperature. The cell layers were washed with deionized water and allowed to air dry. The fixed cells were stained with 2% Alizarin red S pH 4.2 in deionized water (Sigma-Aldrich, St. Louis, MO). After 20 minutes cells were washed with deionized water and observed under light microscope in order to detect mineralized matrix nodules.

Isolation of RNA, reverse transcription and quantitative PCR

Total RNA was extracted using TriPure reagent (Roche, Basel, Switzerland) and quantified on a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). After DNase I treatment (Fermentas, Hanover, Germany),

RNA was reverse-transcribed using the RevertAid H Minus M-MuLV reverse transcriptase kit following manufacture instructions (Fermentas). PCR was performed using the QiagenTaq Core Kit (Qiagen, Venlo, Netherlands). Quantitative RT-PCR was performed with the quantitative RT-PCR (qPCR) MasterMix for SYBR Green I (Biorad) on CFX86 Real time system. For qPCR, housekeeping genes were HPRT, RPL32 and RPL12. Primers were purchased from EUROGENTEC and their sequences are shown in Table I.

Oil red O staining

Lipid droplets accumulation was assessed by an Oil red O staining. After 14 days in adipogenic medium, culture dishes were washed in PBS and cells were fixed in 4% formaldehyde for 1 hour. Cells were then washed with deionized water followed by 60 % isopropanol. The cells were stained with 0.6% Oil Red O solution in isopropanol. After four washes with deionized water, Oil Red O dye was eluted with 100% isopropanol and quantified by measuring the optical absorbance at 490 nm.

Nile Red staining

Adipocytes were detected using Nile Red staining (Sigma-Aldrich, St. Louis, MO) as described previously [6]. Briefly, after 14 days in adipogenic differentiation media, cells were washed in PBS and fixed in 4% formaldehyde for 30 min. The cells were stained with 1 µg/ml Nile Red for 20 min at 4°C. Nile Red staining was detected using an inverted fluorescence microscope Zeiss Axio Observer Z1 with excitation at 485 nm and emission at 572 nm. The percentage of differentiated adipocytes was determined by counting cells stained by the lipid-specific dye Nile Red and normalizing them to the total cell number determined via nuclear 4,6-diamidino-2 phenylindole (DAPI) staining. An average value was determined by counting cells in at least 8 microscopic fields (50-70 cells/field).

ATP and ADP assay

Five days after primary culture, cells were incubated in HBSS supplemented or not with differentiation medium for 15 minutes. The

amount of ATP or ADP in cell cultures supernatants was determined using the ATP bioluminescence assay kit or the ADP colorimetric/fluorometric assay kit (Sigma-Aldrich, St. Louis, MO). Data are expressed as nmoles of ATP or ADP produced per mg of cell protein (determined by Lowry assay). The pannexin-1 mimetic blocking peptide 10panx1 (WRQAAFVDSY) was supplied by R&D Systems.

Histological analysis

Female WT and P2Y₁₃R^{-/-} mice were euthanized at age of 20 weeks. Left tibiae of mice were dissected free of soft tissue, fixed in 10% buffered formalin, and embedded in paraffin wax. Three µm sections were cut using a Leica Microsystems Microtome and followed by Haematoxylin& Eosin (H&E) staining [44]. The number of adipocytes (N.Adi) was counted in a 0.75 mm² field, starting 0.25mm from the growth plate and viewed on a DMRB microscope (Leica Microsystems), and normalized to the bone marrow area (Ma.Ar) measured.

Dual energy x-ray absorptiometry (DEXA) analysis of P2Y₁₃R knockout mice

Percentage fat content of P2Y₁₃R knockout mice and corresponding wild type animals was measured by DEXA using a small animal scanner (Lunar PIXImus, GE Healthcare, Chalfont St Giles, UK). Data were analysed using the manufacturer's software (version 1.8).

Statistical analysis

Non-parametric Mann Whitney tests were performed using Prism, version 5.00, for Windows (Graph Pad, San Diego, CA).

RESULTS

ADPBS stimulates MSC differentiation into osteoblasts through the P2Y₁₃R

To study the role of the P2Y₁₃R in osteoblast differentiation, we used the classical model of adherent bone marrow cells as a source of stem cells [2]. We first observed that the P2Y₁₃R mRNA is present in bone marrow cells as well as in 5 days cultured adherent cells (Fig 1A). In order to determine if ADP through P2Y₁₃R

activation could affect differentiation of MSC cells into osteoblasts, we stimulated both wild type (WT) and P2Y₁₃R^{-/-} bone marrow adherent cells with ADPβS (a relatively stable analogue of ADP) for 7 days and identified the presence of CFU-ALP. As shown in Fig 1B, we observed that after ADPβS treatment the number of WT CFU-ALP was more than doubled compared to untreated control condition. This ADPβS-induced increase of CFU-ALP was absent in P2Y₁₃R^{-/-} cultures. By quantitative RT-PCR, we observed that ADPβS stimulated osterix (OSX), ALP and collagen type I (COLI) mRNA expression in WT-derived cells; this effect was significantly reduced in P2Y₁₃R^{-/-} cell cultures (Fig 1C). In contrast, ADPβS had no effect on Runx2 mRNA expression. The Rho signaling pathway has been shown to be important in the process of MSC differentiation toward the osteoblastic lineage [21, 45]. As P2Y₁₃R have been shown to activate this pathway [46], we tested the effect of the Rho inhibitor Y27632. In the presence of this Rho inhibitor, the stimulatory action of ADPβS on the expression of OSX, ALP and COLI mRNA was reduced by 49 %, 35% and 36% respectively (Fig 1D). These data suggest that the activation of the P2Y₁₃R supports the differentiation of MSCs into osteoblasts in a Rho-dependent way and that this action occurs downstream of Runx2 upregulation.

P2Y₁₃R gene deletion inhibits osteoblastic differentiation in an osteogenic medium

To determine whether these effects of P2Y₁₃R activation have a physiological relevance, we compared the generation of CFU-ALP induced by an osteogenic medium containing dexamethasone-βglycerophosphate-ascorbic acid (DGAA) in P2Y₁₃R^{-/-} and WT MSCs cultured *in vitro*. We first observed that P2Y₁₃R mRNA expression was stable during osteoblast differentiation (Fig 2A). As expected, the average number of CFU-ALP detected was higher when WT cells were treated with osteogenic inducers (mean of 9.5 CFU-ALP per dish) than with ADPβS treatment (mean of 1.8 CFU-ALP per dish) (Fig 1A and 2B). The number of CFU-ALP formed in culture dishes of P2Y₁₃R^{-/-} bone marrow cells was significantly lower than that

counted in WT cultures. To confirm that the targeting of the P2Y₁₃ gene affected the differentiation of MSCs into osteoblasts, we studied another specific characteristic of these bone forming cells *i.e.* the formation of mineralized matrix nodules. As expected, the number of Alizarin red-S stained nodules formed in culture dishes of P2Y₁₃R^{-/-} culture dishes was also significantly lower than that counted in WT cultures (Fig 2C). Furthermore the DGAA-induced upregulation of OSX, ALP and COLI mRNA was reduced in cell cultures from P2Y₁₃R^{-/-} mice, whilst Runx2 expression was unchanged (Fig 2D).

This altered differentiation of MSCs toward the osteoblastic lineage could be due to an alteration in the differentiation capability of progenitor cells or to a decreased number of these progenitors. Both mechanisms seem to be involved since we observed that the total number of CFU (detected by a hematoxylin coloration) counted in P2Y₁₃R^{-/-} cultures was reduced by 44%, and the percentage of CFU-ALP among total CFU was also diminished by 54% in cultures of P2Y₁₃R^{-/-} MSCs (Fig 2E).

Among bone marrow cells, MSCs are very rare (45). The experimental protocol we followed allows isolating them by adherence. However the population we got remained heterogeneous as shown by flow cytometry. On a FFC/SSC dot plot (data not shown) we identified a low size/low granularity population (population A) and a medium size/medium granularity one (population B); the former is mainly CD45⁻ (95%) while the latter is for the most part (75% of cells) CD45⁺. These haematopoietic cells are predominantly of the myeloid lineage as they were mainly CD11b⁺ (data not shown). To identify MSCs, we studied the expression of Sca-1 (stem cell antigen1), CD90, CD44 and PDGFRα (Platelet-derived growth factor receptor α) among CD45⁻ cells (46). As expected, we detected Sca-1⁺/CD90⁺, Sca-1⁺/CD44⁺ and Sca-1⁺/PDGFRα⁺ cells but in a very low proportion (Table II). Among CD45⁻/Sca-1⁺ cells of both A and B subpopulations, we could not observe significant differences between WT and P2Y₁₃R^{-/-} cell cultures in terms of CD90 or CD44 expression. Interestingly, among Sca-1⁺ cells, we detected

a significantly reduced proportion of PDGFR α ⁺ cells in P2Y₁₃R^{-/-} cell cultures in the A population compared to WT cells.

ATP release and metabolism

These data suggested that maximal osteoblast differentiation requires P2Y₁₃R activation and implied that ADP may be released in the extracellular space constitutively or in a regulated way. Therefore, we measured the concentration of ATP, the precursor of ADP, in the culture medium and observed that it was increased following addition of the osteogenic inducers and that this stimulatory effect was affected by a ¹⁰Panx inhibitor of Pannexin channel (Fig 3A). Interestingly, we also noticed that the DGAA treatment induced upregulation of NTPDase2 (also known as CD39L1) mRNA (Fig 3B). This ectonucleotidase preferentially hydrolyzes ATP into ADP, thereby leading to the accumulation of extracellular ADP [47]. Indeed, osteogenic inducers stimulated the accumulation of ADP in the culture supernatant (Fig 3C).

P2Y₁₃ gene targeting increases adipogenic differentiation

As mentioned above, the osteoblast and adipocyte differentiations are linked and mutually exclusive. Therefore we wanted to ascertain the effect of P2Y₁₃R gene deletion on adipocyte differentiation of bone marrow adherent cells. The expression of the P2Y₁₃ messenger was stable during adipocyte differentiation induced by the dexamethasone-insulin-IBMX (DII) cocktail (Fig 4A). Culture of P2Y₁₃R^{+/+} MSCs in a medium containing the adipogenic inducers induced the accumulation of cells with lipid droplets that can be evaluated by Oil Red O and Nile Red staining. As shown in Fig 4B, the amount of Oil Red O dye eluted from P2Y₁₃R^{-/-} treated cells was higher than that eluted from WT treated cells. Moreover, the number of Nile Red positive cells was significantly increased in P2Y₁₃R^{-/-} MSC cultures (Fig 4C) while we did not find any difference in cell or droplet sizes (data not shown). Furthermore the percentage of MSCs that spontaneously differentiated into adipocytes was significantly increased in P2Y₁₃R^{-/-} cell cultures (Fig 4 D).

These data show that the deletion of the P2Y₁₃R favors the formation of new adipocytes from precursor cells but do not affect the fat cell size.

While the mRNA expressions of CEBP/ β and PPAR γ 1 were not modified in P2Y₁₃R^{-/-} cells, that of PPAR γ 2 was increased at an early time (Fig 4E). Indeed, in WT cultures the stimulation of PPAR γ 2 mRNA expression was detectable after 7 days of DII treatment, but in P2Y₁₃R^{-/-} cultures it was already observed at day 3. Moreover, the expression of adipsin (a marker of adipocyte differentiation [10, 48]) was higher in P2Y₁₃R^{-/-} cells compared to WT cells (Fig 4E). It is interesting to notice that adipogenic differentiation stimulates accumulation of ATP and ADP (Fig 4F,G). These data suggest that the deletion of the P2Y₁₃R favors the differentiation of MSCs into adipocytes by accelerating the expression of PPAR γ 2.

P2Y₁₃R deletion increases the accumulation of adipocytes in vivo

To see if the increased capability of P2Y₁₃R^{-/-} MSCs to differentiate into adipocytes observed *in vitro* translated to the *in vivo* situation, we performed histological analysis of the long bone cellular content. As illustrated in figure 5 (A,B and C), the number of adipocytes in tibial sections of 20 week old female mice was more than doubled in P2Y₁₃R^{-/-} mice compared to WT. There was however no difference in the percentage of total body fat measured by dual energy X-ray absorptiometry (DEXA) between WT and P2Y₁₃R^{-/-} mice (Fig 5D).

DISCUSSION

Growing evidence suggests a role for extracellular nucleotides and P2Y receptors in the differentiation of MSCs [26, 31, 49]. Zippel *et al* have recently shown that the mRNAs of each P2 receptor subtype are detectable in human MSC derived from adipose tissue or dental follicles. Moreover, the expression of P2Y₁, P2Y₂, P2Y₄ and P2Y₁₄ mRNAs is reduced after osteogenic differentiation while the expression of the P2Y₄ and P2Y₁₄ receptors is down-regulated after adipogenic

differentiation and that of the P2Y₁₁ receptor is up-regulated [49]. It was previously shown that P2Y₁₃R^{-/-} mice exhibit a decreased bone turnover in vivo and that this phenotype is associated with a reduction in the number of osteoblasts at the surface of bone [40]. These data suggested that the P2Y₁₃ receptor might be involved in the control of osteoblast and adipocyte differentiation from stem cells. Therefore, we have investigated the role of the P2Y₁₃R in the generation of osteoblasts from adherent MSCs isolated from bone marrow. We show that ADP triggers the generation of CFU-ALP and stimulates the expression of ALP, OSX and COLI mRNA, three osteoblast markers. These effects involve the activation of the P2Y₁₃R since the stimulatory effect of ADP is reduced in P2Y₁₃R^{-/-} MSCs cell culture. To determine at which level of the differentiation process the stimulation of the P2Y₁₃R favors the osteoblastic lineage, we tested the mRNA expression of the Runx2 transcription factor, which is one of the earliest determinants of osteoblast differentiation driving the differentiation of MSCs into pre-osteoblasts [14]. Its pro-osteogenic activity implies not only the up-regulation of its expression but also post-translational modifications like phosphorylation, which depend on the Rho and MAP kinase signaling pathways [16, 20]. ADP does not modulate Runx2 expression in these cultures, but increases the mRNA level of OSX, another important transcription factor involved in osteoblast differentiation that is under the control of Runx2. ATP, the precursor of ADP, has been shown to stimulate Runx2 DNA-binding activity in osteoblast-like HOBIT cell line in an Erk1, 2-dependent way [50]. Moreover, the RhoA/ROCK1 signaling pathway is linked to P2Y₁₃R activation in murine osteoblasts isolated from calvaria and ADP stimulation of human hepatocytes leads to RhoA/ROCK1 activation [40, 46]. In agreement with these observations, we show here that an inhibitor of the Rho G protein (the compound Y27632) reduced ADP-induced up-regulation of OSX, ALP and COLI mRNA expression in MSCs. Therefore, we suggest that ADP activates the differentiation of pre-osteoblasts into osteoblasts by stimulating the DNA binding activity of Runx2 through

RhoA/ROCK1 signaling pathways in a P2Y₁₃R-dependent way.

The capability of the P2Y₁₃R to modulate the differentiation of MSCs into the osteoblastic lineage is relevant at the physiological level, as the number of the CFU-ALP and mineralized nodules induced by the osteogenic cocktail DGAA is largely reduced in P2Y₁₃R^{-/-} cell cultures. This is due both to the reduction of total CFU obtained after culture of bone marrow stromal cells and to their decreased ability to differentiate into osteoblasts. The reduction of total CFU could be correlated to the decreased number of CD45⁻/Sca-1⁺/PDGFRα⁺ seen in a sub-population of P2Y₁₃R^{-/-} adherent cells. Indeed, it has been recently shown that CD45⁻/Sca-1⁺/PDGFRα⁺ cells isolated from the bone marrow have the highest capability to generate CFU-Fs after plating (46). Human MSCs isolated from the bone marrow or adipose tissue express the P2Y₁₃R [26, 49]. However, because the cell population we stimulated in this work was heterogeneous, we cannot exclude that the effect of the P2Y₁₃R on osteoblast differentiation is partly indirect involving other cells that stem cells. Among cells we obtained after 5 days of preculture, we identified CD45⁺/CD11b⁺ myeloid cells. In this context, it is interesting to refer to the work of Pirraco et al. showing that the co-culture of human macrophages with human bone marrow derived MSCs leads to increase differentiation into osteoblasts compared to monoculture of MSCs [51].

We confirm that the influence of the P2Y₁₃R on osteogenesis is downstream of the Runx2 up-regulation, as its expression is not altered by the P2Y₁₃R gene targeting. But the expression of osterix is significantly reduced in mutated MSCs. This suggests that the P2Y₁₃R does not control the initiation of MSCs differentiation, but the maturation of pre-osteoblasts into osteoblasts.

The fact that the differentiation of MSCs into osteoblasts requires P2Y₁₃R expression implies that the osteogenic cocktail may favor accumulation of ADP in the extracellular space. This is supported by the observation that the incubation of MSCs with apyrase, that

hydrolyses extracellular nucleotides, inhibits osteoblast differentiation [49]. We observed that in osteogenic conditions the concentration of extracellular ATP, as well as of ADP, is enhanced compared to control conditions. In human bone marrow stromal cells, it has been shown that ecto-nucleotidases are expressed and that the supplementation of culture medium with ATP leads to transient production of ADP [31]. We also detected the expression of the ectonucleotidases NTPDase2 (CD39L1) which preferentially hydrolyses ATP into ADP [47]. Therefore, we conclude that the DGAA-induced maturation of pre-osteoblasts into osteoblasts involves partially the release of ATP and its conversion through CD39L1 into ADP that activates the P2Y₁₃R. The release of nucleotides involves multiple mechanisms like cell lysis, secretory granules exocytosis, membrane transporter and hemi-channels like connexin and pannexin [33, 52]. DAAG-induced ATP release seems to result from the opening of pannexin hemichannels as DGAA-induced ATP accumulation is reduced by ¹⁰Panx, an inhibitory peptide of Pannexin 1.

The impaired ability of P2Y₁₃R^{-/-} MSCs to differentiate into osteoblasts *in vitro* may explain the decreased number of osteoblasts present in P2Y₁₃R^{-/-} bones *in vivo*, leading to reduced trabecular bone volume [40]. It is important to mention that the ADP effects that we observed were not completely abolished in P2Y₁₃R^{-/-} cell cultures suggesting implication of the other purinergic receptors as P2Y₁ or P2Y₁₂ or adenosine receptors. Several studies support that the P2Y₁ receptor promotes proliferation of undifferentiated cells and osteoblasts, but there are no indication to our knowledge regarding to a role of this receptor in MSCs differentiation [32, 53]. On the contrary, the expression of the P2Y₁ mRNA is down-regulated during osteogenic differentiation of human adipose-tissue derived stem cells [49]. Recently, it has been shown that the expression of the P2Y₁₂ receptor mRNA is up-regulated in a model of rat osteoblast differentiation of precursors isolated from calvarial bones [54]. Finally, adenosine that can accumulate into extracellular space after ADP hydrolysis, is well known to promote osteoblast differentiation [55, 56].

The differentiation of MSCs into osteoblastic or adipogenic lineages are not independent processes: molecular components promoting one cell fate inhibit the mechanisms governing the differentiation of the alternative lineage [4, 7]. This is also the case for the P2Y₁₃R. Its expression is required for full osteoblastic differentiation as discussed above, while the P2Y₁₃R gene targeting increases the potential of MSCs to generate adipocytes. Indeed, the number of adipocytes generated in P2Y₁₃R^{-/-} MSCs in response to the adipogenic cocktail and the expression of adipocyte markers like adiponectin are enhanced compared to that obtained in WT culture. This suggests that the activation of the P2Y₁₃R inhibits adipocyte differentiation and implies that the adipogenic stimulation stimulates the accumulation of ADP in the extracellular space, what we also observe. CEBP/β and PPARγ1 are transcription factors that regulate the first steps of adipocyte differentiation [23, 57]. The P2Y₁₃R gene targeting does not modulate their mRNA expression. However, the earlier expression of PPARγ2 observed in P2Y₁₃R^{-/-} cultures correlates with increased adipocyte formation and adiponectin expression, suggesting that the P2Y₁₃R controls negatively the maturation of pre-adipocytes into adipocytes. It could be linked to the control of the transcriptional expression of PPARγ2 by cAMP-linked signaling pathway [22]. Indeed the P2Y₁₃R belongs to the G protein-coupled receptor superfamily and activates Gα_{i/o} proteins inhibiting cAMP production [37]. The gene deletion of Gα_{i/o}-coupled type 1 cannabinoid receptor CB1 leads to an analogous phenotype in terms of MSCs differentiation: reduced production of osteoblasts but increased generation of adipocytes [3]. Moreover, it has been recently described that an impairment of Rho signaling upregulates PPARγ2 expression as well as adipocyte formation [45, 58]. Therefore a cooperation between cAMP inhibition and Rho signaling pathways may be the effector mechanism downstream the P2Y₁₃R stimulation to control negatively the differentiation of adipocytes. The fact that we observed a significant increase of adipocytes in non-induced P2Y₁₃R^{-/-} MSCs culture as well as in the bone marrow of P2Y₁₃R^{-/-} mice, but not

in the total body fat, underscores the relevance of our observations in relation with bone diseases. Osteoporosis, for example, is a disease in which resorption by osteoclasts is not sufficiently compensated by bone formation [59] and is associated with increased bone marrow adipogenesis [60, 61]. The identification of molecular components able to favor osteogenesis and reduce adipogenesis will help to design new drugs for bone diseases cure.

CONCLUSION AND SUMMARY

In conclusion, this study demonstrates that the P2Y₁₃R is a physiological determinant of MSCs differentiation. This receptor is one of the factors controlling the balance between osteogenesis and adipogenesis, at the level of the maturation of pre-committed progenitor cells. Our *in vitro* observations are consistent with our *in vivo* data showing a high marrow fat content in the bone of the P2Y₁₃ knockout mice and the previously reported decreased bone formation in these mice [40]. Agonists of the P2Y₁₃R might be used to favor osteoblast differentiation and bone formation, in particular in case of MSC transplantation.

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Figure 1. Osteogenic differentiation of BMSCs: Effect of ADPβS

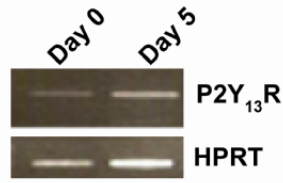
(A) Detection of P2Y₁₃ messenger: RT-PCR (29 cycles) was performed using cDNA synthesized from RNAs extracted from freshly isolated BMSCs or cells cultured for 5 days in MEM. HPRT gene was used to control cDNA quality.

(B) CFU-ALP formation: 5×10^6 marrow cells collected by flushing method were seeded on 6-wells plates and cultured for 5 days in MEM medium followed by 7 days in MEM medium supplemented or not with 50 μM ADPβS (replaced every 2 days). To identify CFU-ALP, cells were stained with Naphthol-FastRed as described in the material and methods section. Data are expressed as mean ±SD of 6 cell preparations; each point is the mean of 3 wells from each of these preparations. * p<0.05

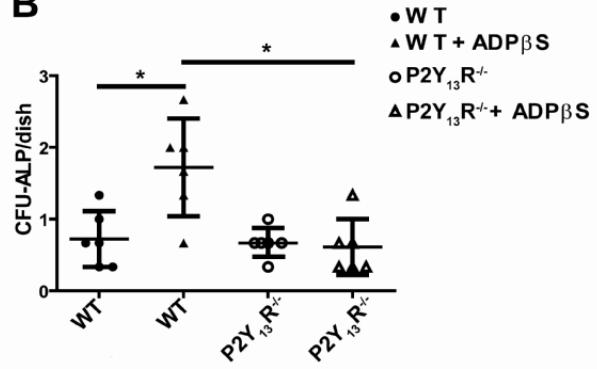
(C) Runx2, OSX, ALP and COL I expression: 7.5×10^6 cells collected by centrifugation were seeded on Petri dishes and cultured for 5 days in MEM medium followed by 1, 3 or 5 days in MEM supplemented or not with ADPβS (50 μM). RNAs are extracted 1, 3 or 5 days after ADPβS additions. Total RNAs were extracted as described in the material and methods section and mRNA expression was determined by RT-qPCR. Quantitative mRNA values were normalized by the amount of HPRT, RPL13 and RPL32, and results represent the ratio between ADPβS-stimulated and control cultures. Data are means ± SD of 6 cells preparations. Data were normalized to those of the housekeeping gene HPRT, RPL32 and RPL12. *p<0.05; **p<0.01

(D) Effect of Rho inhibitor Y27632 : 7.5×10^6 cells collected by centrifugation were seeded on Petri dishes and were cultured for 5 days in MEM medium followed by 1 or 3 days in MEM supplemented with ADPβS (50 μM) with or without Y7632 (10 μM). RNA extraction and mRNA quantification were performed as described above. Data are means ± SD of 5 (OSX and RUNX2), 6 (ALP) or 4 (COLI) cell preparations. Data were normalized to those of the housekeeping gene HPRT, RPL32 and RPL12. *p<0.05; **p<0.01

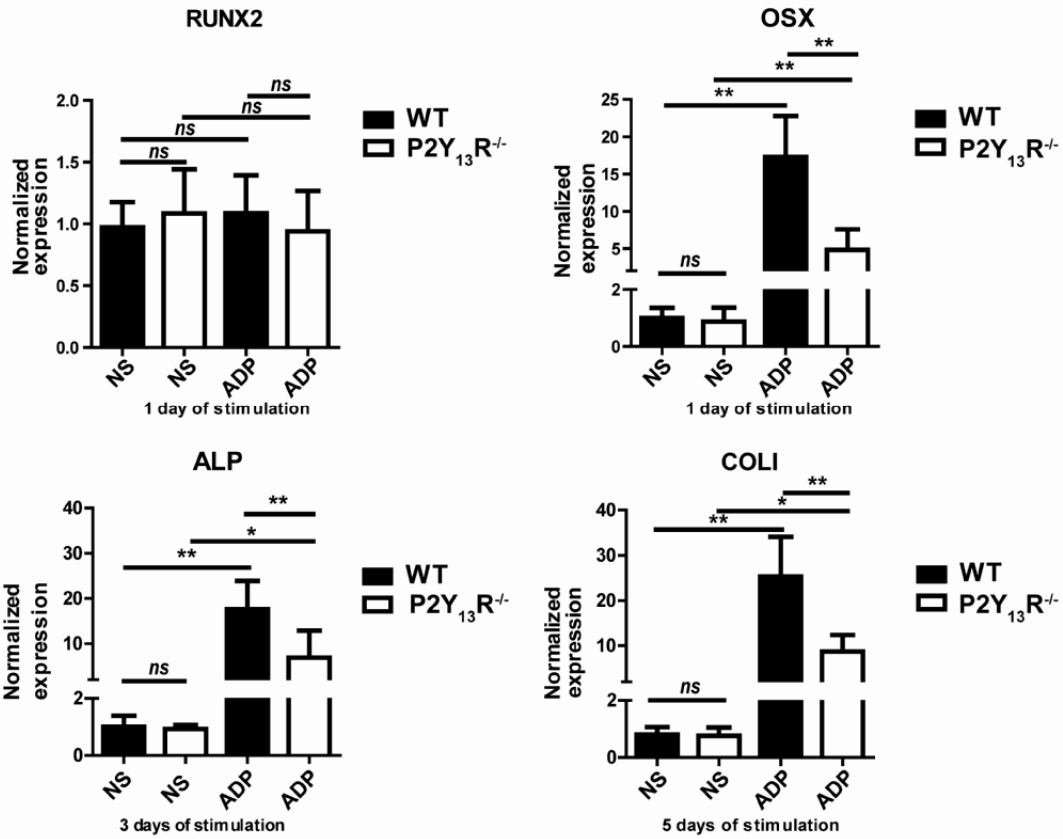
A



B



C



D

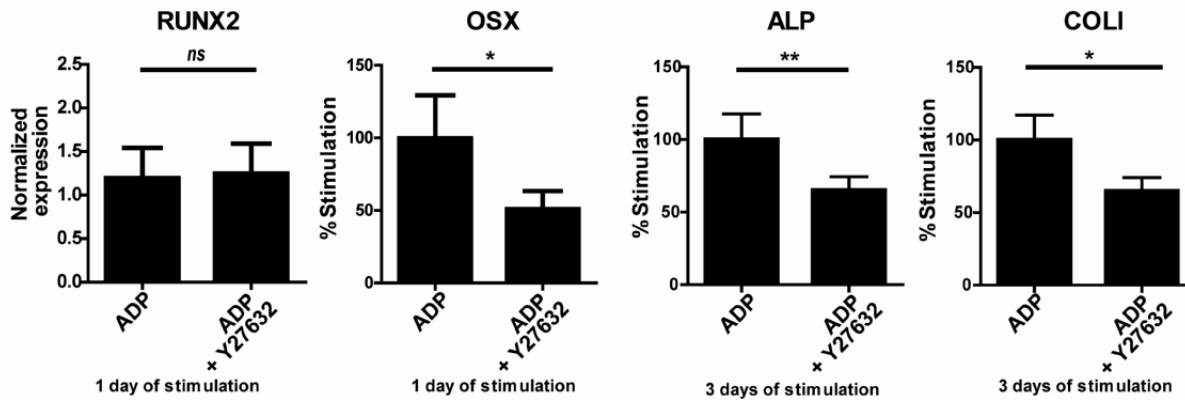


Figure 2. Osteogenic differentiation of BMSCs: comparison between P2Y₁₃R^{-/-} and WT mice

(A) P2Y₁₃R expression: quantitative RT-PCR was performed using cDNA synthesized from RNAs extracted from cells cultured for 5 days in MEM and cells cultured additionally for 1, 3, 5 and 7 days in MEM supplemented with 50µg/ml ascorbic acid, 5mM β-glycerophosphate and 10nM dexamethasone. Data are means ± SD of 4 cells preparation. Data were normalized to those of the housekeeping gene HPRT, RPL32 and RPL12.

(B) CFU-ALP formation: 5x10⁶ marrow cells collected by flushing method were seeded on 6-wells plates and cultured for 5 days in MEM followed by 7 days in MEM supplemented with 50µg/ml ascorbic acid, 5mM β-glycerophosphate and 10nM dexamethasone. Top panel: data are expressed as mean of CFU-ALP ± SD; nWT =14, n P2Y₁₃R^{-/-} = 16, ***p<0.001.

Bottom panel: picture of CFU-ALP in culture dishes.

(C) Mineralized nodule formation : 5x10⁶ marrow cells collected by flushing method were seeded on 6-wells plates and cultured for 5 days in MEM followed by 14 days in MEM supplemented with 50µg/ml ascorbic acid, 5mM β-glycerophosphate and 10nM dexamethasone. Top panel: data are expressed as mean ± SD; nWT =11, n P2Y₁₃R^{-/-} = 10, ***p<0.001.

Bottom panel: picture of mineralized nodules in culture dishes.

(D) RUNX2, OSX, ALP and COLI expression: quantitative RT-PCR for RUNX2, OSX, ALP, OSX was performed as in figure 1C, but cells were treated for 5 days in MEM, followed by 1 (RUNX2,OSX), 3(ALP) or 5 (COLI) days in MEM supplemented with 50µg/ml ascorbic acid, 5mM β glycerophosphate and 10nM dexamethasone. Data are means ± SD of 6 cell preparations. Data were normalized to those of the housekeeping gene HPRT, RPL32 and RPL12. *p<0.05;**p<0.01

(E) CFU formation: BMSCs were seeded and treated as described in Figure 2A. Total CFU (left) were detected using hematoxylin staining and the percentage of CFU-ALP among total CFU (right) was quantified. Data are expressed as mean of CFU± SD, nWT =14, nP2Y₁₃R^{-/-} = 16, ***p<0.001.

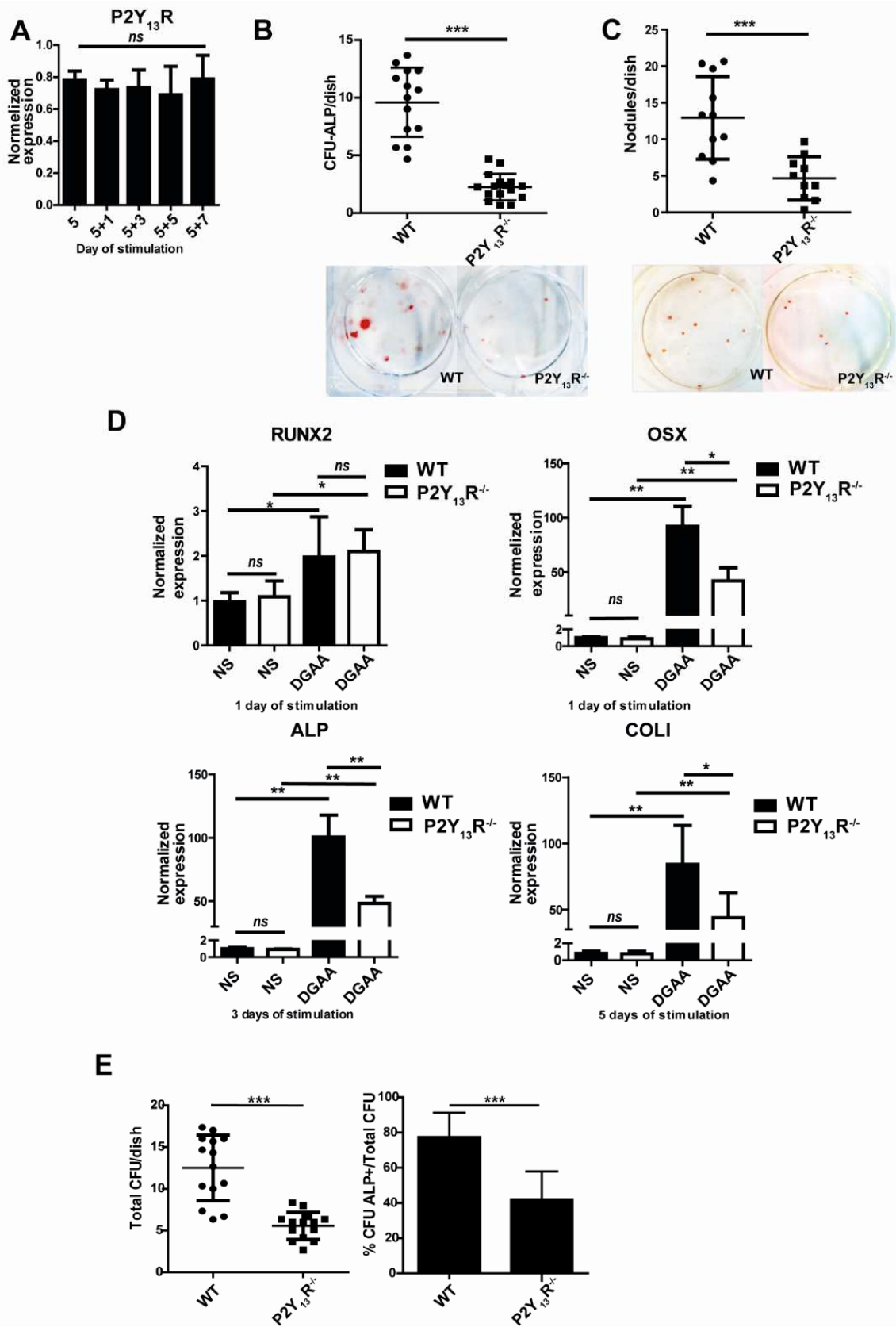


Figure 3. ATP release and metabolism

(A) ATP release: cells were cultured for 5 days in MEM medium and 15 min in MEM supplemented with 50 μ g/ml ascorbic acid, 5mM β glycerophosphate and 10nM dexamethasone in the presence or not of Pannexin 1 inhibitor, ¹⁰PANX (100 μ M). The concentration of ATP in the supernatant was determined by luminescence using an ATP assay kit and normalized to protein content as described in the material and methods section. Data are means \pm SD of 4 cells preparation. *p<0.05

(B) CD39L1 expression: quantitative RT-PCR for CD39L1 was performed as in figure 2D. Data are means \pm SD of 4 cells preparation. Data were normalized to those of the housekeeping genes HPRT, RPL32 and RPL12. *p<0.05

(C) ADP release: cells were cultured for 5 days in MEM medium and 15 min in HBSS supplemented with 50 μ g/ml ascorbic acid, 5mM β glycerophosphate and 10nM dexamethasone. The concentration of ADP in the supernatant was determined using the ADP Colorimetric/Fluorometric Assay Kit and normalized to protein content as described in the material and methods section. Data are means \pm SD of 5 cells preparation. *p<0.05

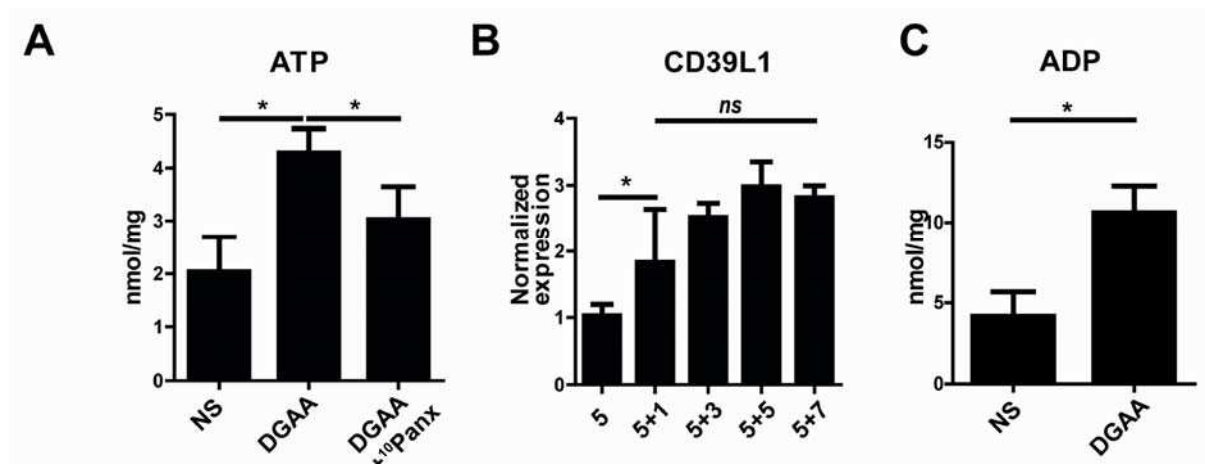


Figure 4. Adipogenic differentiation of BMSCs : comparison between P2Y₁₃R^{-/-} and WT mice

(A) P2Y₁₃R expression: quantitative RT-PCR was performed using cDNA synthesized from RNAs extracted from cells cultured for 5 days in MEM and cells cultured additionally for 1, 3, 5 and 7 days in MEM supplemented with 10nM dexamethasone, 0.5mM IBMX and 10µg/ml insulin. Data are means ± SD of 4 cell preparations. Data were normalized to those of the housekeeping gene HPRT, RPL32 and RPL12.

(B) Lipid droplet accumulation : 2.5x10⁶ marrow cells collected by the flushing method were seeded on 6-wells plates and cultured for 5 days in MEM medium followed by 14 days incubation in MEM medium supplemented with 10nM dexamethasone, 0.5mM IBMX and 10µg/ml insulin. The lipid droplets accumulation was assessed by Oil Red O assay as described in the material and method section. Data are means ± SD (nWT=12 and nKO=9). *p<0.05

(C) Adipocyte formation: 5x10⁵ marrow cells collected by the flushing method were seeded on Cellview™ dishes and cultured for 5 days in MEM medium followed by 14 days incubation in MEM medium supplemented with 10nM dexamethasone, 0.5mM IBMX and 10µg/ml insulin. The lipid droplets of adipocytes were identified by Nile Red staining as described in the material and methods section.

Left panel : quantification of Nile Red positive cells; data are expressed as mean ± SD, (nWT=6 and nP2Y₁₃R^{-/-}=6), **p<0.01.

Right panel : fluorescence microphotographs taken at ×20 of Nile Red staining.

(D) Adipocyte formation: 5x10⁵ marrow cells collected by the flushing method were seeded on Cellview™ dishes and cultured for 21 days in MEM medium. The lipid droplets of adipocytes were identified by Nile Red staining as described in the material and methods section.

Data are expressed as mean ± SD, (nWT=5 and nP2Y₁₃R^{-/-}=5) **p<0.01

(E) CEBPβ, PPARγ1, PPARγ2, and adipin expression: 7.5x10⁶ cells, collected by centrifugation, were seeded on Petri dishes and cultured for 5 days in MEM medium followed by 1 (CEBPβ), 3 (PPARγ1 and PPARγ2), 7 (PPARγ2) or 10 (ADIPSIN) days in MEM supplemented with 10 nM dexamethasone, 0.5mM IBMX and 10µg/ml insulin. Total RNA was extracted as described in the material and methods section and mRNA expression was determined by RT-qPCR. Quantitative mRNA values were normalized to the amount of HPRT, RPL13 and 32, and results are given as the ratio between adipogenic and control medium. Data are means ± SD of 6 cells preparation. Data were normalized to those of the housekeeping gene HPRT, RPL32 and RPL12. *p<0.05, **p<0.01

(F) ATP release: cells were cultured for 5 days in MEM medium and 15 min in MEM supplemented with 10 nM dexamethasone, 0.5mM IBMX and 10µg/ml insulin. The concentration of ATP in the supernatant was determined by luminescence using ATP assay kit and normalized to protein content as described in the material and methods section. Data are means ± SD of 4 cell preparations. *p<0.05

(G) ADP release: cells were cultured for 5 days in MEM medium and 15 minutes in HBSS supplemented with 10 nM dexamethasone, 0.5mM IBMX and 10µg/ml insulin. The concentration of ADP in the supernatant was determined by ADP Colorimetric/Fluorometric Assay Kit and normalized to protein content as described in the material and methods section. Data are means ± SD of 4 cell preparations. *p<0.05

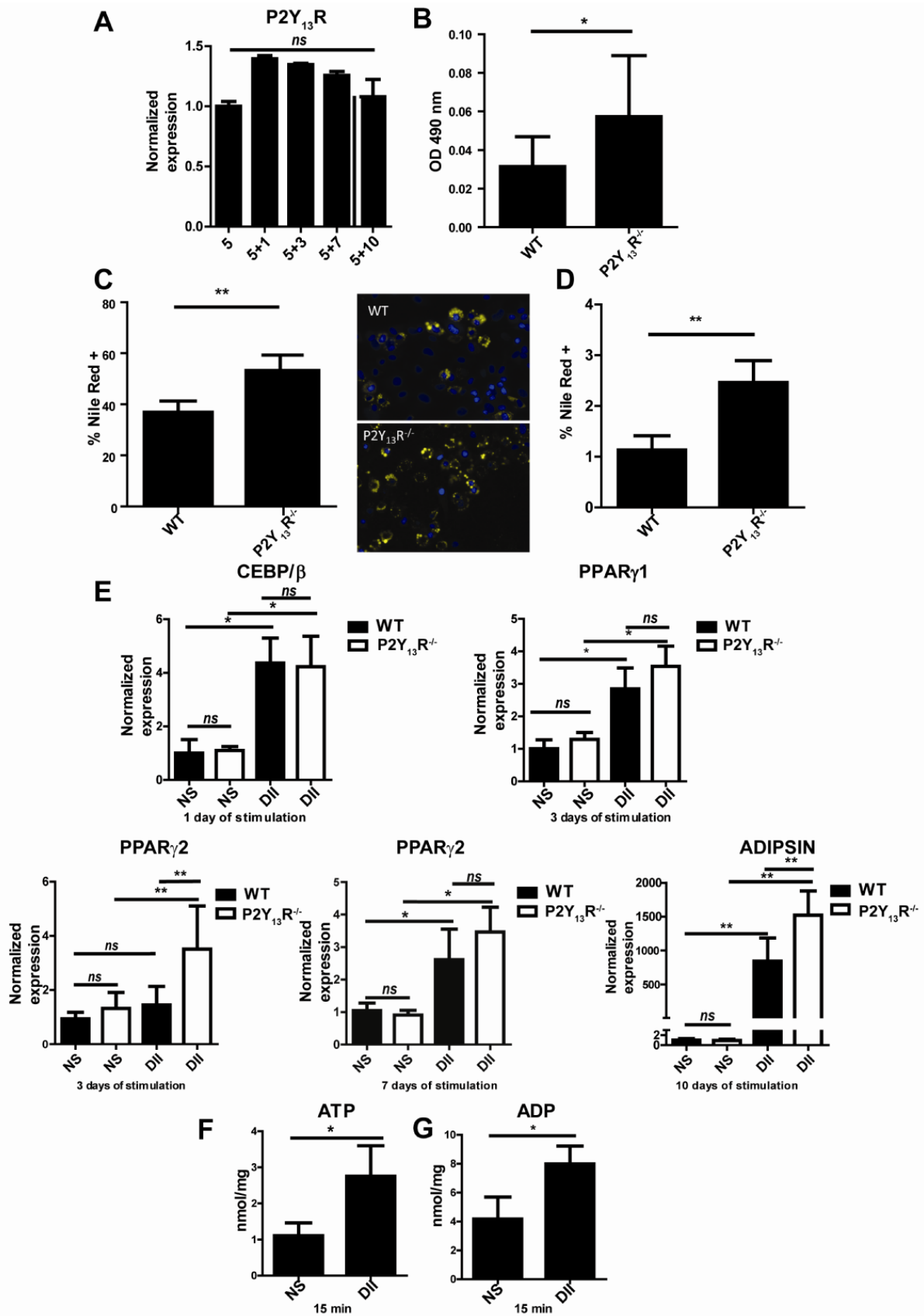


Figure 5. Fat content in bone marrow and total body : comparison between WT and P2Y₁₃R^{-/-} mice

(A) Transparent round adipocytes can be identified in the tibial proximal end bone marrow, scale bar = 0.5mm.

(B) Adipocytes were marked as 'a' in magnified view of bone marrow area, scale bar = 50 μ m.

(C) The number of adipocytes (N.Adi) normalized by area of bone marrow (Ma.Ar) was quantified for mice at 20 weeks of age, nWT=5, nP2Y₁₃R^{-/-}=6. Data are mean \pm SEM, ** p<0.01.

(D) DEXA analysis of P2Y₁₃R^{-/-} mice and their corresponding WT : results are expressed in % fat content (nWT=7 and nP2Y₁₃R^{-/-}=10).

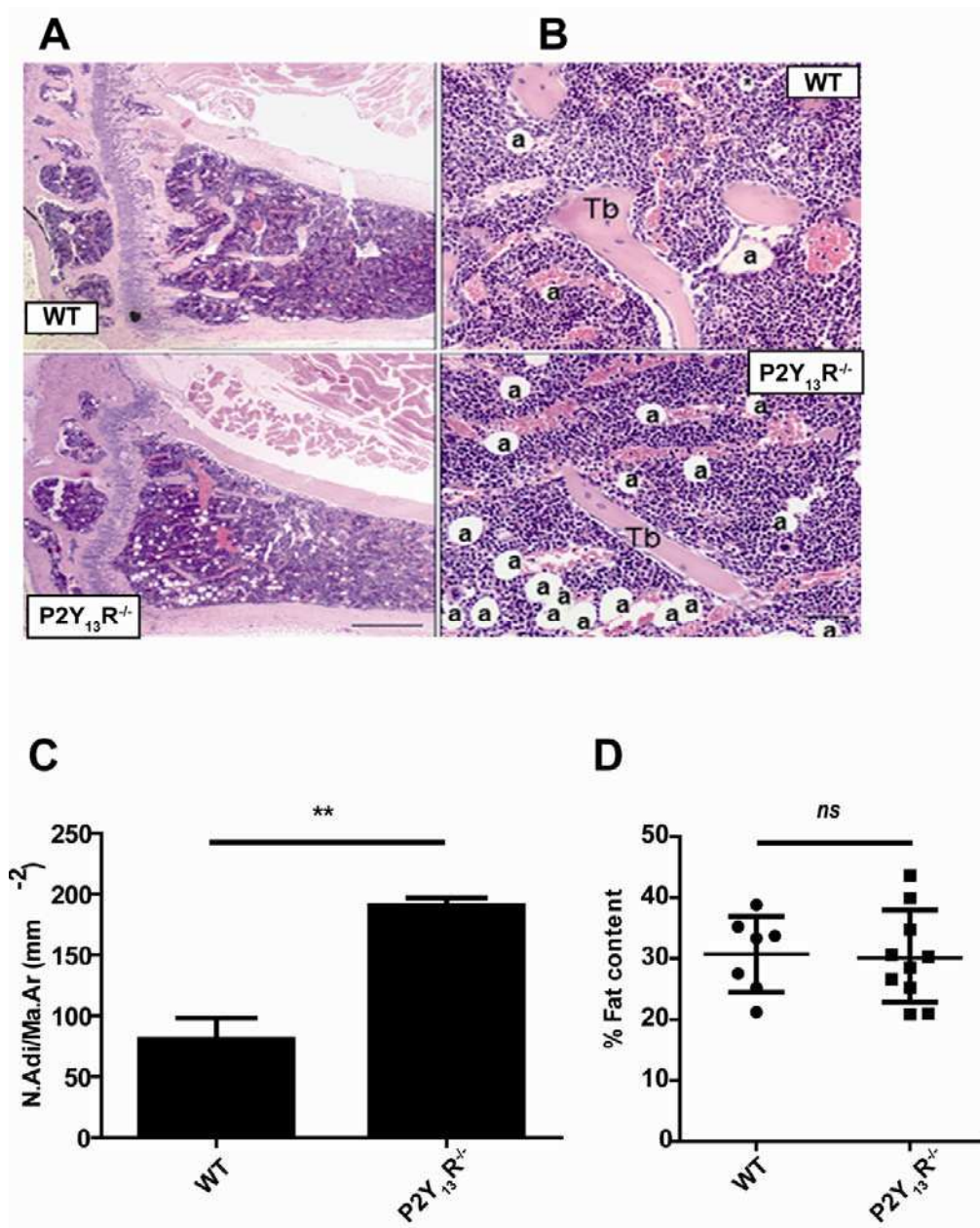


TABLE 1. List of specific primers used in this study

	Gene	Forward (5'–3')	Reverse (5'–3')
qPCR	P2Y ₁₃	CATATCCAGTGCCTGTGTGG	TGTTGCATGTGCCTCTC TTC
	RUNX2	CCCAGCCACCTTTACCTACA	TATGGAGTGCTGCTGGT CTG
	ALP	CCAGCAGGTTTCTCTCTTGG	CTGGGAGTCTCATCCTG AGC
	COLI	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGG TTC
	OSX	ACTCATCCCTATGGCTCGTG	GGTAGGGAGCTGGGTT AAGG
	PPAR α 1	TTTTCAAGGGTGCCAGTTTC	AATCCTTGGCCCTCTGA GAT
	PPAR α 2	CTTCGCTGATGCACTGCCTA	CATCACGGAGAGGTCC ACAG
	ADIPSIN	TGCACAGCTCCGTGTACTTC	CACCTGCACAGAGTCGT CAT
	CD39L 1	AACCAGTCCATCTGAAGATCC AGATAAT	AGTAGAAAGCAGAAAA GGCTATGAAGTT
	RPL12	ATGACATTGCCAAGGCTACC	CAAGACCGGTGTCTCAT CTGC
	RPL32	GGCACCAGTCAGACCGATAT	CAGGATCTGGCCCTTGA AC
	HPRT	AAGCTTGCTGGTGAAAAGGA	TTGCGCTCATCTTAGGC TTT
RT-PCR	P2Y ₁₃	ATTCGTGGGTTGAGCTAGTAA	ATCAGGGACCAGACGG AAAT
	HPRT	CCTGCTGGATTACATTAAAGC ACTG	GTCAAGGGCATATCCA ACAACAAAC

TABLE II: Immunophenotyping of adherent bone marrow cells

<i>Population A</i>	<u>WT</u>	<u>P2Y₁₃R^{-/-}</u>	
CD45 ⁺	4.76±1.72	4.00±2.66	<i>ns</i>
CD45 ⁻	95.57±1.46	93.45±4.61	<i>ns</i>
CD45 ⁻ Sca-1 ⁺ CD90 ⁺	0.08±0.05	0.07±0.07	<i>ns</i>
CD45 ⁻ Sca-1 ⁺ CD44 ⁺	0.14±0.05	0.16±0.07	<i>ns</i>
CD45 ⁻ Sca-1 ⁺ PDGFRα ⁺	0.18±0.05	0.10±0.05	<i>p<0,05</i>
<i>Population B</i>	<u>WT</u>	<u>P2Y₁₃R^{-/-}</u>	
CD45 ⁺	74.8±7.8	82.16±10.43	<i>ns</i>
CD45 ⁻	24.5±2.81	19.23±3.66	<i>ns</i>
CD45 ⁻ Sca-1 ⁺ CD90 ⁺	0.7±0.16	0.63±0.27	<i>ns</i>
CD45 ⁻ Sca-1 ⁺ CD44 ⁺	3.97±1.38	4.22±1.68	<i>ns</i>
CD45 ⁻ Sca-1 ⁺ PDGFRα ⁺	3.07±2.41	1.77±1,11	<i>ns</i>