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On the Role of the “Purinome” in Human Osteo/Chondrogenic Differentiation: Rethinking Stem Cell-Based Therapies to Rehabilitate the Osteochondral Diseases

Rui Pinto Cardoso

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Rui Miguel Pinto Cardoso

Instituto Ciências Biomédicas Abel Salazar



Rui Miguel Pinto Cardoso

On the Role of the “Purinome” in Human Osteo/Chondrogenic Differentiation: Rethinking Stem Cell-Based Therapies to Rehabilitate Osteochondral Diseases

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Orientador – Professor Doutor Paulo Correia-de-Sá

Categoria – Professor Catedrático

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Co-orientador – Professor Doutor José Bernardo Noronha-Matos

Categoria – Professor Auxiliar

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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“Yesterday I was clever, so I wanted to change the world.

Today I am wise, so I am changing myself.”

Rumi

“One day, in retrospect, the years of struggle will strike you as the most beautiful.”

Sigmund Freud

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Resumo

As articulações sinoviais são estruturas complexas compostas por osso, cartilagem articular, revestimento sinovial, tendões e ligamentos, formando a unidade osteocondral. A idade, doenças (como Osteoporose) ou traumas (que podem levar a uma Osteoartrite) estão muitas vezes associados a dano nessas estruturas, prejudicando a função articular e causando dor. As terapias atuais incidem sobretudo no alívio da sintomatologia mas são ineficazes na recuperação de tecidos, como osso e cartilagem. Vários estudos têm vindo a ser desenvolvidos com o intuito de utilizar células estromais mesenquimatosas derivadas de medula osso com capacidade de regenerar estes tecidos. Contudo, também aqui o factor idade tem sido limitador, reduzindo o seu potencial terapêutico. Em mulheres pós-menopáusicas (Pm), a capacidade destas células de se diferenciarem em osteoblastos e condrócitos parece estar comprometida devido a alterações hormonais, à senescência celular e a modificações no microambiente local, como a hipóxia prolongada. A hipóxia, via fator 1α induzido por hipóxia (HIF- 1α), desempenha um papel significativo na homeostasia da cartilagem e do osso, influenciando a expressão de enzimas envolvidas na sinalização purinérgica como ectonucleotidase3 (NTPDase3) ou CD73/ecto-5'-nucleotidase. De facto, a sinalização envolvendo principalmente o ATP e a adenosina desempenha um papel chave na diferenciação de BM-MSCs e na homeostasia dos tecidos. As BM-MSCs são capazes de libertar e metabolizar nucleótidos de adenina em adenosina, que se ligam aos recetores purinérgicos para regular a função celular. Em mulheres Pm, a metabolização do ATP e a atividade dos recetores sensíveis a nucleótidos de adenina estão alteradas, afetando tanto a osteogénese como a condrogénese.

Este estudo explora em que medida as alterações na sinalização purinérgica relacionadas com a idade influenciam a diferenciação das BM-MSCs em osteoblastos (**Artigo 1**) e em condrócitos (**Artigo 2**), bem como a possibilidade desses defeitos serem ultrapassados. O objectivo foi perceber de que forma a manipulação do purinoma em BM-MSCs poderá restaurar o potencial reparador destas células e com isso restaurar o osso e a cartilagem perdidos em condições patológicas ou traumáticas.

Neste trabalho, comparou-se a capacidade de diferenciação osteogénica das BM-MSCs isoladas da medula óssea de mulheres Pm ($n=30$; 69 ± 2 anos) e de mulheres mais jovens ($n=8$; 22 ± 4 anos) (**Artigo 1**; aprovação ética nº 137/2018-2). Após 7 e 21 dias de cultura constatou-se que células do grupo Pm apresentavam uma reduzida capacidade de diferenciação (atividade da fosfatase alcalina e níveis de Osterix, factor de diferenciação osteoindutor) e mineralização (ensaio de alizarina vermelha) após 35 dias quando comparado ao grupo de mulheres jovens. Dados de imunofluorescência sugerem que

imunorreatividade contra a NTPDase3 foi 5,6 vezes maior nas BM-MSCs de mulheres Pm do que nas mais jovens ao 21º dia de cultura. A confirmar isto, a atividade enzimática da NTPDase3 aumentou nas BM-MSCs de mulheres Pm comparando com as isoladas de mulheres mais jovens quando o ATP e o UDP foram usados como substratos, respectivamente. Paralelamente, estas células foram incubadas na presença de inibidores da NTPDase3 (PSB 06126 e anticorpo monoclonal hN3-B3_s); o pré-tratamento com um RNA de interferência lentiviral (Lenti-shRNA) foi utilizado para silenciar a expressão do gene que codifica a NTPDase3. Os inibidores farmacológicos da NTPDase3, provocaram aumentos na semivida extracelular do ATP e do UDP. Ambos os compostos, PSB 06126 (3 µM) e hN3-B3_s (0,5 µg/ml) promoveram aumentos na mineralização das culturas em diferenciação osteogénica aos 35 dias. Contudo, apenas o hN3-B3_s (0,5 µg/ml) aumentou a atividade da ALP nas culturas de BM-MSCs de mulheres Pm em diferenciação osteogénica nos dias 7 e 21, respetivamente. Os efeitos osteogénicos da inibição da NTPDase3 foram completamente anulados pelo bloqueio dos receptores P2X₇ e P2Y₆ com A438079 (3 µM) e MRS 2578 (100 nM), respectivamente. O silenciamento génico seletivo com um lenti-shRNA diminuiu transitoriamente a imunorreatividade da NTPDase3 nas BM-MSCs causando aumentos da atividade da ALP aos dias 7 e 21 e da mineralização ao dia 35. A inibição farmacológica e o silenciamento génico da NTPDase3 nas BM-MSCs isoladas de mulheres Pm reabilitaram os níveis do marcador de osteogénese, osterix, para níveis semelhantes aos observados em mulheres mais jovens.

Seguidamente, comparou-se a capacidade de diferenciação condrogénica em culturas de BM-MSCs provenientes de 27 mulheres Pm (75±2 anos) (**Artigo 2**; aprovação ética nº 137/2018-2) realizadas na ausência ou na presença do inibidor da prolyl-4-hidroxilase, DMOG, que é capaz de promover a acumulação intracelular do fator de transcrição, HIF-1α. O DMOG (30 µM) aumentou a transcrição do gene que codifica o HIF-1α, bem como o aparecimento de transcritos associados à degenerescência da cartilagem, como a MMP13 e o colagénio do tipo X, e ao processo inflamatório. In paralelo, DMOG (30 µM) reduziu a translocação nuclear do fator de transcrição SOX-9, bem como a produção de colagénio do tipo II. A densidade da enzima metabolizadora de ATP, CD39, foi reduzida cerca de 10-20% nas BM-MSCs em diferenciação condrogénica, bem como a sua atividade com indica os dados de bioluminescência e actividade enzimática. Do mesmo modo, após 14 dias de cultura, a densidade da enzima formadora de ADO, CD73, encontrava-se também diminuída comparativamente com a situação controlo. A acumulação intracelular de HIF-1α causada pelo DMOG (30 µM) alterou a relação entre a densidade A_{2A}R e A_{2B}R favorecendo a sobre-expressão do recetor A_{2A}R sensível ao SCH 442416 (10 nM) com maior afinidade para a ADO nas BM-MSCs diferenciadas (dia 14) de mulheres Pm.

Em conclusão, demonstrou-se neste trabalho que a sobreexpressão da NTPDase3 nas BM-MSCs pode ser um indicador clínico e, simultaneamente, um alvo terapêutico a ter em atenção em futuras abordagens no tratamento de deficiências da osteogénese em mulheres Pm para aumentar a massa óssea e reduzir o risco de fraturas. Por outro lado, mostrou-se que a acumulação sustentada de HIF-1 α associada à hipoxia prejudica a diferenciação condrogénica das BM-MSCs provenientes de mulheres Pm. Esta situação deve-se, em parte, à perda da capacidade de formar ADO a partir do catabolismo extracelular dos nucleótidos de adenina pela CD39/CD73, e também à perturbação do balanço A_{2A}R e A_{2B}R em favor do primeiro.

Abstract

Synovial joints are complex structures composed of bone, articular cartilage, synovial lining, tendons, and ligaments, forming the osteochondral unit. Aging, disease (e.g., osteoporosis), and trauma (which can lead to osteoarthritis) are frequently associated with damage to these structures, impairing joint function and causing pain. Current therapies primarily focus on symptomatic relief and are largely ineffective in promoting the regeneration of damaged tissues such as bone and cartilage. In recent years, several studies have explored the therapeutic potential of bone marrow-derived mesenchymal stromal cells (BM-MSCs) for tissue regeneration. However, the regenerative capacity of these cells is significantly limited by aging. In postmenopausal (Pm) women, the osteogenic and chondrogenic differentiation potential of BM-MSCs appears to be compromised due to hormonal changes, increased cellular senescence, and alterations in the local microenvironment, including sustained hypoxia. Hypoxia, through the hypoxia-inducible factor 1 alpha (HIF-1 α), plays a crucial role in maintaining bone and cartilage homeostasis, partly by regulating the expression of purinergic signaling enzymes such as ectonucleoside triphosphate diphosphohydrolase 3 (NTPDase3) and CD73 (ecto-5'-nucleotidase). Indeed, purinergic signaling, particularly involving ATP and adenosine (ADO), is a key regulator of BM-MSC differentiation and tissue homeostasis. BM-MSCs can release and metabolize adenine nucleotides into adenosine, which then interacts with purinergic receptors to modulate cellular function. In BM-MSCs derived from Pm women, both ATP metabolism and receptor-mediated signaling are dysregulated, negatively affecting osteogenesis and chondrogenesis.

This study investigates how age-related alterations in purinergic signaling impact the differentiation of BM-MSCs into osteoblasts (**Paper 1**) and chondrocytes (**Paper 2**), and whether these deficits can be pharmacologically or genetically overcome. The aim was to determine whether modulation of the purinome in BM-MSCs could restore their reparative potential and consequently support the regeneration of bone and cartilage in pathological or trauma-induced conditions.

Here, we compared the osteogenic differentiation capacity of BM-MSCs isolated from the bone marrow of Pm women (n=30; 69 $\pm$ 2 years) and younger women (n=8; 22 $\pm$ 4 years) (**Paper 1**; ethical approval no. 137/2018-2). After 7 and 21 days in culture, BM-MSCs from Pm donors exhibited significantly reduced differentiation potential, as evidenced by lower alkaline phosphatase (ALP) activity and reduced expression of Osterix, a key osteoinductive transcription factor. After 35 days, mineralization was also diminished (Alizarin Red assay) compared to BM-MSCs from younger donors. Immunofluorescence data showed a 5.6-fold higher NTPDase3 immunoreactivity in BM-MSCs from Pm women at day 21. Enzymatic

activity assays confirmed increased NTPDase3 activity in Pm-derived BM-MSCs compared to younger counterparts when ATP and UDP were used as substrates. Cells were then treated with NTPDase3 inhibitors (PSB 06126 and monoclonal antibody hN3-B3_s), and a lentiviral shRNA (Lenti-shRNA) was used to silence the NTPDase3 gene. Pharmacological inhibition of NTPDase3 led to increased extracellular half-life of ATP and UDP. Both PSB 06126 (3 μ M) and hN3-B3_s (0.5 μ g/ml) enhanced matrix mineralization at day 35 of osteogenic culture. Notably, only hN3-B3_s significantly increased ALP activity at days 7 and 21 in BM-MSCs from Pm donors. The osteogenic effects of NTPDase3 inhibition were abolished by blockade of P2X₇ and P2Y₆ receptors using A438079 (3 μ M) and MRS 2578 (100 nM), respectively. Selective gene silencing of NTPDase3 via Lenti-shRNA transiently reduced NTPDase3 immunoreactivity and resulted in increased ALP activity at days 7 and 21, as well as increased mineralization at day 35. Both pharmacological inhibition and gene silencing of NTPDase3 in Pm-derived BM-MSCs restored osterix expression to levels comparable to those of younger donors.

Subsequently, chondrogenic differentiation capacity was assessed in BM-MSC cultures derived from 27 Pm women (75 $\pm$ 2 years) (**Paper 2**; ethical approval no. 137/2018-2), with or without treatment with the prolyl-4-hydroxylase inhibitor DMOG, known to stabilize intracellular HIF-1 α . DMOG (30 μ M) increased transcription of the HIF-1 α gene and upregulated transcripts associated with cartilage degeneration (e.g., MMP13 and collagen type X) and inflammatory processes. Concurrently, DMOG reduced nuclear translocation of the SOX-9 transcription factor and decreased production of collagen type II. CD39 expression, an ATP-metabolizing enzyme, was reduced by 10–20% in chondrogenic BM-MSC cultures, confirmed by the bioluminescence and enzymatic activity assays. Similarly, CD73 expression (the enzyme responsible for adenosine formation) was also decreased at day 14 compared to control conditions. DMOG-induced HIF-1 α accumulation disrupted the balance between A_{2A} and A_{2B} adenosine receptors, favoring A_{2A} receptor overexpression (high affinity, pro-osteogenic), as indicated by increased sensitivity to the selective antagonist SCH 442416 (10 nM) in differentiated BM-MSCs from Pm donors.

In conclusion, this study demonstrates that overexpression of NTPDase3 in BM-MSCs may serve both as a clinical biomarker and a therapeutic target for enhancing osteogenesis in Pm women, potentially increasing bone mass and reducing fracture risk. Additionally, sustained accumulation of HIF-1 α under hypoxic conditions impairs chondrogenic differentiation of BM-MSCs from Pm women. This impairment is partially due to a diminished capacity to form adenosine through CD39/CD73-mediated extracellular nucleotide catabolism and to a shift in the A_{2A}/A_{2B} receptor ratio favoring the A_{2A} receptor.

ABBREVIATION LIST

3D, tridimensional;

α,β -meATP, α,β -methyleneadenosine 5'-triphosphate trisodium salt;

α -MEM, α -Minimal essential medium;

A438079 3-[[5-(2,3-Dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride;

AC, articular cartilage;

ACANs, aggrecans;

ACI, autologous chondrocyte implantation;

ADA, adenosine deaminase;

ADAMTS-4/5, ADAM metalloproteinase with thrombospondin type 1 motif 4/5;

ADP, adenosine 5'-diphosphate;

ADO, adenosine

ADSCs, adipose-derived mesenchymal stromal cells;

ADK, adenosine kinase;

Akt, protein kinase B;

ALF, acute liver failure;

Alizarin red S, 3,4-Dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt;

ALP, alkaline phosphatase;

AMP, adenosine 5'-monophosphate;

AP, apoprotein;

APCP, adenosine 5'-(α,β -methylene)diphosphate;

ARL 67156, 6-N,N-Diethyl-D- β,γ -dibromomethyleneATP

ATP, adenosine 5'-triphosphate;

A.u. Arbitrary units

BAE, bovine aortic endothelia;

BAY 60-6583, 2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide;

5-BDBD, 5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one;

BLC, B lymphocyte chemoattractant;

BM-MSCs, bone marrow-derived mesenchymal stem cells;

BMU, bone multicellular unit;

BMP, bone morphogenic protein;

BzATP, 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt;

Ca²⁺, calcium ion;

CAMKII, Ca²⁺/calmodulin-dependent protein kinase II;

CaCl₂, calcium chloride;

[Ca²⁺]_i, intracellular calcium concentration;

cAMP, cyclic adenosine 5'-monophosphate;

CD73, ecto-5'-nucleotidase;

CF101, 1-deoxy-1-[6-[[[(3-iodophenyl)methyl]amino]-9H-9-yl]-N-methyl-β-D-ribofuranuronamide;

CFU-Fs, colony-forming unit fibroblasts;

CGS 15943, 9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine;

CGS21680, 2-p-(2-Carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride;

CIA, collagen-induced arthritis;

CKD, chronic kidney disease;

CM, conditioned media;

CNT, concentrative nucleoside transporters;

CO₂, carbon dioxide;

CoCl₂, cobalt chloride;

COL2A1, Collagen Type II Alpha 1;

CPCs, chondrogenic progenitor cells;

CSCs, chondroblast stem cells;

Cx43, connexin 43;

DCs, dendritic cells;

DDR2, discoidin domain-containing receptor 2;

DFO, desferoxamine;

DJD, degenerative joint disease

Dkk1 / Dkk2, dickkopf family members 1 and 1;

D-MEM, Dulbecco's Modified Eagle Medium;

DMOG, dimethyloxalylglycine;

DMPX, 3,7-dimethyl-1-propargylxanthine
 DNA, deoxyribonucleic acid;
 DPMA, N(6)-methyladenosine;
 DZNep, 3-Deazaneplanocin A;
 EA, electroacupuncture;
 ECM, extracellular matrix;
 EHNA, erythro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride
 E-NNPs, ecto-nucleotide pyrophosphatase/phosphodiesterase;
 ENT-1/-2, equilibrative nucleoside transports 1/2;
 ERK 1/2, extracellular signal-regulated kinase 1/2;
 ESWT, extracorporeal shock wave therapy;
 EVs, extracellular vesicles;
 FBS , fetal bovine serum
 FoxO, forkhead box O;
 FiO₂, fraction of inspired oxygen;
 GAG, glycosaminoglycans;
 GAPDH, Glyceraldehyde-3-phosphate dehydrogenase;
 GJIC, gap junctional intercellular communication;
 GO, gene ontology
 GSK-3 β , glycogen synthase kinase-3 beta;
 GTP, guanosine 5'-triphosphate;
 HCAECs, human coronary artery endothelial cells;
 HCC, hepatocellular carcinoma;
 HI, hypoxic-ischemic insult;
 HIF-1 α /2 α , hypoxia-inducible factor-1 α /2 α ;
 HLFs, human lung fibroblasts;
 HLMVECs, human lung microvascular endothelial cells;
 HMEC-1, human microvascular endothelial cells;
 HPAECs, human pulmonary artery endothelial cells;
 HPLC, High-performance liquid chromatography;
 HRE, hypoxia responsive region

HUVEC, human umbilical vein endothelial cells;

Hx, Hypoxanthine;

IL-1 α , interleukin-1 α ;

IL-1 β , interleukin-1 β ;

IL-6, interleukin-6;

IL-10, interleukin-10;

IL-18, interleukin-18;

IGF-I, insulin-like growth factor I;

INO, Inosine

iNOS, inducible nitric oxide synthase;

JNK1, c-jun N-terminal protein kinase 1;

KO, knockout;

LASSBio-1359, N-[(E)-(3,4-dimethoxyphenyl)methylideneamino]-N-methylbenzamide;

LDH, Lactate dehydrogenase;

Lenti-shRNA, Lentiviral short hairpin RNA;

LIF, leukaemia inhibitory factor;

LIPUS, low-intensity pulsed ultrasound;

LPS, lipopolysaccharide;

MACI, matrix-induced autologous chondrocyte implantation;

MAPK, mitogen-activated protein kinase;

MCP3, monocyte specific chemokine 3;

MEK, mitogen-activated protein kinase kinase;

MgCl₂, magnesium chloride;

MIF, macrophage migration inhibitory factor

miRNAs, microRNAs;

MMP-3, matrix metalloproteinase-3;

MMP-13, matrix metalloproteinase-13;

mRNA, messenger RNA;

MRS 1220, N-[9-Chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-yl]benzene acetamide;

MRS1754, N-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide;

MRS2179, 2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt;
MRS 2578, N,N"-1,4-butanediylbis[N'-(3-isothiocyanatophenyl) thiourea];
MSCs, mesenchymal stem cells;
mTORC2, mechanistic target of rapamycin complex 2;
MTT 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide;
N₂, nitrogen;
NBTI, S-(4-Nitrobenzyl)-6-thioinosine;
NECA, 5'-N-ethylcarboxamidoadenosine;
NF-κB, nuclear factor kappa B;
NLRP3, NOD- LRR- and pyrin domain-containing protein 3;
NO, nitric oxide;
NOS, nitric oxide synthase;
NP, Nanoparticles;
NPPs, ecto-nucleotide pyrophosphatase/phosphodiesterases;
NTPDases, ecto-nucleoside triphosphate diphosphohydrolases;
NURR1, nuclear receptor related-1;
O₂, oxygen;
OA, osteoarthritis;
OGD, oxygen-glucose deprivation;
OPCs, oligodendrocyte precursor cells;
OPG, osteoprotegerin;
PAD, protein arginine deiminase;
Panx, pannexins;
PASCs, pulmonary artery smooth muscle cells;
PBMCs, peripheral blood mononuclear cells;
PBS, phosphate-buffered saline;
PDE4, phosphodiesterase-4 inhibitor;
PDGF, platelet derived growth factor;
PEMF, pulsed electromagnetic fields;
PFA, paraformaldehyde
PGE₂, prostaglandin E₂;

PHD, prolyl hydroxylase;
 PLA-PEG, poly(lactic acid)-poly(ethylene glycol);
 PLD, phospholipase D;
 Pi, inorganic phosphate;
 pO₂, pressure of oxygen;
 PPi, inorganic pyrophosphate;
 PI3K, phosphatidylinositol-3-kinase;
 PKA, protein kinase A;
 PKC, protein kinase C;
 Pm, post-menopausal;
 PMN, polymorphonuclear leukocytes;
 PNP, p-Nitrophenyl phosphate;
 PPi, pyrophosphate;
 PTH, parathyroid hormone;
 PSB 0603, 8-[4-[4-(4-Chlorophenyl)piperazide-1-sulfonyl]phenyl]]-1-propylxanthine;
 PSB 06126, 1-Amino-4-(1-naphthyl)aminoanthraquinone-2-sulfonic acid sodium salt;
 PSB 1115, 4-(2,3,6,7-Tetrahydro-2,6-dioxo-1-propyl-1H-purin-8-yl)-benzenesulfonic acid;
 PSB-12062, 10-[(4-Methylphenyl)sulfonyl]-10H-phenoxazine;
 RA, rheumatoid arthritis;
 RANKL, receptor activator of nuclear factor kappa β ligand;
 RNA, ribonucleic acid;
 ROI, Regions of interest;
 ROS, reactive oxygen species;
 ROCK, Rho-associated protein kinase;
 RT-PCR, reverse transcription polymerase chain reaction;
 Runx-2, Runt-related transcription factor 2;
 SAHH, S-Adenosyl-L-Homocysteine Hydrolase;
 SaO₂, peripheral saturation;
 SASP, senescence-associated secretory phenotype;
 SCH 58261, 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine;

SCH 442416, 2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine

SDS-PAGE, Sodium dodecyl sulphate–polyacrylamide gel electrophoresis;

Sfrps, secreted frizzled-related proteins;

shRNA, Short hairpin RNA;

siRNA, small interference RNA;

SOST, sclerostin;

Sox-9, sex-determining region Y (SRY)-box transcription factor 9;

TAA, thioacetamide;

TERC-deficient mice, telomerase-deficient mice;

TFG- β 1, tumour growth factor-beta 1;

TNBC, triple-negative breast cancer;

TNBS, 2,4,6-Trinitrobenzene sulfonic acid;

TNF- α , tumour necrosis factor- α ;

TRPV, transient receptor potential cation channel subfamily V member 4;

UDP, uridine 5'diphosphate;

UTP, uridine 5'-triphosphate;

VCAM-1, vascular cell adhesion molecule 1;

VEGF, vascular endothelial growth factor;

VVECs, vasa vasorum endothelial cells;

WJSCs-CM, Wharton's jelly derived stem cell conditioned medium;

Wnt, wingless-type mouse mammary virus integration site;

YO-PRO dye, 4-[(3-methyl-1,3-benzoxazol-2(3H)-ylidene)methyl]-1-[3-(trimethylammonio)propyl]quinolinium diiodide;

ZM 241385, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol.

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Pinto-Cardoso R, Pereira-Costa F, Pedro Faria J, Bandarrinha P, Bessa-Andrês C, Correia-de-Sá P, Noronha-Matos JB. Adenosinergic signalling in chondrogenesis and cartilage homeostasis: Friend or foe? *Biochem Pharmacol*. 2020 Apr;174:113784. doi: 10.1016/j.bcp.2019.113784. Epub 2019 Dec 26. PMID: 31884043.

Introduction

1. The osteochondral unit complex: the pathophysiological interaction between bone, cartilage and other players

Human synovial joints are complex structures comprising the articulating bones, the articular cartilage covering adjacent bone surfaces, the synovial lining of the joint cavity, the tendons and the ligaments (Gary Firestein, 2013; Goldring & Goldring, 2016). The subchondral bone located underneath the articular cartilage is of paramount importance to absorb cartilage mechanical stress (Zhu et al., 2020a). The osteochondral boundary is formed by a thin layer of calcified cartilage (Goldring & Goldring, 2016). The structure formed by the articular cartilage (90%), subchondral bone (5%) and calcified cartilage (5%) is known as the osteochondral unit (Oliveira Silva et al., 2020). This unit has the unique capability of transferring loads during weight-bearing and joint motion. A synovial membrane covers the intra-articular space of diarthrodial joints. Synoviocytes are the main cell type present on the synovial membrane. These cells contribute to synovial fluid production, thus providing articular cartilage nutrition and minimizing joint wear risks. Articular trauma usually affects synoviocytes, resulting in changes in their secretome and in the production of inflammatory infiltrates, which can further damage the articular cartilage and cause bone erosion (Mathiessen & Conaghan, 2017; Smith, 2011; Thoenen et al., 2022). Alterations in the composition and/or structure of individual components of the joint result in disruption of the articular integrity and loss of function. Such alterations may lead to degenerative osteoarthritis (OA) (Hunter et al., 2014; Johnson & Hunter, 2014).

1.1 Osteochondral unit: the articular cartilage

The articular cartilage is an avascular and nerveless tissue containing two major organic components entrapped in a watery fluid (>70%): type II collagen and aggrecan (ACAN), which is also known as cartilage-specific proteoglycan core protein or chondroitin sulfate proteoglycan (Goldring & Goldring, 2016). The fibrillary collagen network provides tensile strength, while entrapped proteoglycan aggregates provide compressive resilience. The organization and charge of both structural proteins dictate the physical properties of the cartilage (Andriacchi & Favre, 2014; Goldring & Goldring, 2016; Guo et al., 2015). These properties are important during joint motion and mechanical loading to facilitate the pumping and diffusion of molecules from the underlying subchondral bone to nourish the cartilage. This mechanism is particularly relevant for deeper layers of the articular cartilage where the income of nutrients is restricted (Goldring & Goldring, 2016; Imhof et al., 2000; Lyons et al., 2006; Pan et al., 2009). Alterations of these properties can lead to various disease

conditions, like chondrodysplasias, in which mutations or deficiencies in cartilage-encoding genes result in altered skeletal growth and cartilage structure (Basit et al., 2008; Goldring & Goldring, 2016; Olsen, 1995; Warman et al., 1993).

The cartilage is also responsible for providing painless joint movement and impact absorption. These abilities are facilitated by a boundary layer of lubricants, such as lubricin and hyaluronic acid, which are produced by chondrocytes and synovial cells (Greene et al., 2011). Chondrocytes are the major cell component of the cartilage, yet they only represent 1–2% of the total cartilage volume (Goldring & Goldring, 2016; Hunziker et al., 2014). Remarkably, these cells exhibit no detectable mitotic activity and display low oxygen consumption (Mobasheri et al., 2008; Pfander & Gelse, 2007; Thoms et al., 2013); their metabolic activity is low resulting from a steady-state equilibrium established between anabolic and catabolic processes (Goldring & Goldring, 2016; Hunziker et al., 2007). The pericellular matrix that surrounds individual chondrocytes contains essentially hyaluron, perlecan, biglycan, and aggrecan as monomers or small aggregates, as well as type IX collagen (Goldring & Goldring, 2016; Wilusz et al., 2014). This structural organization prevents resting chondrocytes from interacting abnormally with the extracellular milieu via cell-surface receptors, such as integrins, CD44, annexins, syndecans and discoidin domain-containing receptor 2 (DDR2), thereby helping them to maintain a differentiated phenotype, survival and low-turnover synthetic activity (Goldring & Goldring, 2016; Loeser, 2014; Pap & Bertrand, 2013). Differentiated chondrocytes survive in low-oxygen environmental conditions (around 10% oxygen tension at surface zones and < 1% in the deeper zones) and exhibit high levels of the hypoxia-inducible factor 1 α (HIF-1 α) transcription factor. Overexpression of HIF-1 α in the growth plate and articular cartilage helps chondrocytes to adapt to environmental changes and to maintain their physiological activities, linked to cartilage anabolism and chondrocyte differentiation (Goldring & Goldring, 2016; Maes et al., 2012). Chondrocytes also possess unique organelles called primary cilia. These plasma membrane specializations exist along with mechanosensitive receptors (such as integrins), transient receptor potential cation channels of the subfamily V member 4 (TRPV4, a Ca²⁺ channel), and ATP-releasing hemichannels containing connexin 43 (Cx43) (Goldring & Goldring, 2016; Loeser, 2014; Ruhlen & Marberry, 2014; Wann et al., 2012), which enable chondrocytes to sense mechanical alterations in their local environment. A variety of P1 and P2 purinoceptors allow chondrocytes to sense the energy status of neighbouring cells reflected by the release of adenine nucleotides (ATP) and nucleosides (adenosine) to the cartilage microenvironment (Corciulo & Cronstein, 2019). Stimuli, like mechanical loading and/or hypoxia, prompt chondrocytes to release ATP, which can act on its receptors and/or give rise to enzymatic breakdown products, like ADP and adenosine, which also possess

important metabolic and signalling properties in cartilage and bone formation (Graff et al., 2000; Hatori et al., 1995; Larranaga-Vera et al., 2021).

1.2 Osteochondral unit: the subchondral bone

The subchondral bone is located beneath the calcified cartilage, forming a plate-like structure very similar to the cortical bone at other skeletal locations (Goldring & Goldring, 2016). This plate-like structure comprises both a subchondral cortical plate and a subchondral trabecular bone structure (cancellous bone). Trabecular bone is porous and exhibits higher metabolic activity than cortical bone (Burr, 2004; Goldring & Goldring, 2016). The subchondral bone organization possesses unique architectural, biological and mechanical properties (Burr, 2004; Goldring & Goldring, 2010; Goldring, 2009). Physical and biological properties of the subchondral bone are influenced by its structural organization, organic matrix composition and mineralization status (Burr, 2004; Day et al., 2001; Meunier & Boivin, 1997). In physiological conditions, the subchondral bone adapts its structure and functional properties through plastic cellular processes of remodelling (Burr & Gallant, 2012; Goldring & Goldring, 2016), according to Wolff's hypothesis, which states that bone structure and function adapt to the loads under which it is submitted (Frost, 2001; Goldring & Goldring, 2016). These biological processes involve the balance between bone formation and resorption resulting from the coordinated activity of osteoblasts and osteoclasts within the bone multicellular unit (BMU) (Smith et al., 2012). Bone remodelling is undertaken in two stages: osteoclast-mediated bone resorption followed by osteoblast-mediated bone formation (Eriksen, 2010; Goldring & Goldring, 2016). Both phenomena are triggered by tissue damage/injury (such as in osteoarthritis) due to biomechanical factors and local soluble mediators, such as glucocorticoids, interleukins (IL-1 β , IL-1 α) or tumour necrosis factor- α (TNF- α), which are released and act synergistically with circulating hormones, such as oestrogen and 1,25(OH) $_2$ vitamin D $_3$.

Adding or removing bone may be directly and independently regulated by osteoblasts and/or osteoclasts, respectively, without the local coupling of resorption and formation (Goldring & Goldring, 2016). Besides osteoblasts and osteoclasts, osteocytes are also found in the subchondral bone. Osteocytes are widely distributed throughout the cortical and trabecular bone and they form an interconnected network with each other in the bone matrix and with other cells on the bone surface. Osteocytes are major bone mechanosensors, as these cells respond to biomechanical stimuli and release soluble mediators, like the Wingless-type mouse mammary virus integration site (Wnt) and sclerostin. Cell-to-cell interactions between osteocytes and osteoclasts or osteoblasts are responsible for bone remodelling (Burnstock et al., 2013; Dallas et al., 2013; Goldring &

Goldring, 2016). Indeed, osteocytes may counteract osteoclast differentiation and bone resorption through increased secretion of the anti-osteoclastogenic factor, osteoprotegerin (OPG). Osteocytes express OPG at levels comparable to or exceeding those exhibited by osteoblasts (Gu et al., 2005; Kramer et al., 2010). Several protein families can negatively modulate the canonical Wnt signalling, such as Dickkopf family members (Dkk1 and Dkk2) and secreted frizzled-related proteins (Sfrps) (Kramer et al., 2010). Another example is sclerostin, a glycoprotein encoded by the sclerostin (*SOST*) gene that is secreted by osteocytes; this glycoprotein binds to plasma membrane-bound receptors in osteoblasts triggering downstream intracellular signalling cascades, which ultimately result in the inhibition of bone morphogenetic protein (BMP)- and Wnt/ β -catenin-stimulated bone formation (Krause et al., 2010; Lewiecki, 2014). Articular chondrocytes may also express the *SOST* gene, thus suggesting that modulation of this pathway may have effects on both articular cartilage and the subchondral bone (Lewiecki, 2014; Roudier et al., 2013).

Osteoblasts are responsible for producing the bone extracellular matrix, named osteoid, whose unique organic properties undergo progressive mineral deposition (Goldring & Goldring, 2016). Bone turnover is responsible for controlling the stiffness of the osteoid. During rapid bone remodelling, the increased bone turnover leads to a state of relative hypomineralization (reduction of bone stiffness). Contrariwise, continued deposition of minerals during low bone turnover rate conditions leads to increased bone stiffness (Goldring & Goldring, 2016). Thus, bone is a highly dynamic tissue requiring continuous homeostatic control. Active osteoclasts secrete the leukaemia inhibitory factor (LIF) that is responsible for suppressing sclerostin expression while promoting bone formation (Koide et al., 2020).

1.3 Osteochondral unit: the calcified cartilage

Between the cartilage and the subchondral bone, there is a thin tissue layer of calcified cartilage resulting from endochondral ossification and growth plate closure. It is denser and more mineralized than the adjacent subchondral bone (Burr, 2004; Lyons et al., 2006; Simkin, 2012). Its composition includes a mixture of components of the two adjacent layers. Like cartilage, type II collagen (about 20% of the dry weight) is the most abundant collagen type. Hydroxyapatite is also present, even though at lower quantities (roughly 65% of the dry weight) when compared to subchondral bone (Zhang et al., 2012). Due to its composition, the calcified cartilage is designed to transfer and distribute mechanical forces to the bone during loading; it also serves to transfer nutrients and/or matrix components (Buckwalter & Mankin, 1998b; Zhang et al., 2012). The combination of efforts between the articular cartilage, the calcified cartilage, and the underlying cortical plate is responsible for

transforming shear stress into compressive and tensile stress during joint loading and motion (Goldring & Goldring, 2016). In addition, the calcified cartilage is responsible for maintaining the physiological microenvironment within the hyaline cartilage (Arkill & Winlove, 2008; Buckwalter & Mankin, 1998b), thus playing a relevant role in trauma. Calcified cartilage is denser and highly mineralized and, thereby, less resistant to traumatic injuries than the surrounding tissues (Buckwalter & Mankin, 1998b). Nevertheless, the remodelling process at the calcified cartilage is very active. Therefore, pathological changes in this equilibrium may result in musculoskeletal diseases, as verified in degenerative osteoarthritis (Ea et al., 2011; Oegema et al., 1997; Zhang et al., 2012).

2. Main challenges of bone and cartilage tissue regeneration

Homeostasis between cartilage and the underlying subchondral bone is paramount for healthy joint motion (Lepage et al., 2019; Prakash & Learmonth, 2002). Bone loss related to ageing (like osteoporosis), cartilage breakdown, subchondral bone sclerosis, osteophyte formation and synovial inflammation, leading to joint pain and physical disability in elderly people are common features of degenerative osteoarthritis (Berenbaum, 2013; Grässel, 2014; Guermazi et al., 2012; Larranaga-Vera et al., 2021; Lepage et al., 2019). Growing data suggests that ageing is associated, in part, to an accumulation of cell damage mechanisms affecting DNA (deoxyribonucleic acid), proteins, membranes, and organelles, which ultimately may affect cell replacement and tissue repair (Ju & Lenhard Rudolph, 2008). Recent studies have associated oestrogen decline with telomere shortening. The authors reported that oestrogen deficiency in mice results in a significant inhibition of telomerase maintenance of telomeres in mouse ovaries in a tissue-specific manner (Bayne et al., 2011). Since oestrogens are important regulators of bone remodelling, their lack during menopause may compromise the osteogenic commitment of MSCs due to telomerase deficiency. Furthermore, others have shown that TERC (the telomerase RNA component) mutant mice have a low bone mass phenotype and that age-related osteoporosis is, in part, the result of impaired osteoblast differentiation in parallel to intact osteoclasts differentiation (Pignolo et al., 2008). Furthermore, data suggested that the lifespan of osteoblasts is also compromised in elderly people; it was reported an age-related increase in cell apoptosis associated with increased oxidative stress (Almeida et al., 2007). Moreover, *in vitro* and *in vivo* studies show that free radicals lead to impaired osteoblastic function and bone formation during ageing (Manolagas, 2010), an effect that seems to be reverted upon treatment with oestrogens or androgens. In parallel, an age-related decrease in osteoblasts function may be associated with a decrease in the osteoblastic response to

the calciotropic hormones and growth factors. Osteoblasts proliferation and function depend on adequate response to such factors in order to control bone formation, leading to pathological conditions (e.g., osteoporosis). Osteoporosis placed in the 10th position among civilization diseases of the contemporary world, affecting 200 million women worldwide (Janiszewska et al., 2017). In 2019, 25 million European women and 6.5 million European men were estimated to suffer from osteoporosis. In Portugal, this number is 681,000 individuals, being that 80% are women (Kanis et al., 2021). It is expected that adults aging 50 or older will experience an osteoporotic fracture, with high prevalence in women (about 50%) when compared to men (20%) (LeBoff et al., 2022). Therefore, search for new options to prevent and effectively treat osteoporotic fractures and other bone disorders is necessary.

Concerning cartilage, tissue regeneration is even more complex and challenging. Since cartilage is an avascular tissue with no innervation, it depends entirely on chondrocytes for the production of extracellular matrix components like proteoglycans and collagen (Alford & Cole, 2005; Sophia Fox et al., 2009). The production of such matrix is important to maintain the homeostasis and structure necessary to provide adequate tissue compressive strength and to enable frictionless motion during routine loading (Pearle et al., 2005). Due to its load-bearing function, cartilage is highly sensitive to damage (Madry et al., 2011). As previously reported, cartilage has poor blood supply and low metabolic activity which results in a limited ability to heal. When left untreated, small traumas and defects may progress to cartilage degeneration, ultimately resulting in subsequent bone destruction (Hunter, 1995; Jiang & Tuan, 2015). Thus, it is worth considering cartilage and subchondral bone as coupled entities when searching for new therapeutic strategies for OA (Schuiling et al., 2011).

The clinical management of chondral, subchondral, or osteochondral changes has so far been purely symptomatic, primarily tackling inflammation and/or pain, rather than modifying disease progression (Lepage et al., 2019; Steinmeyer et al., 2018). Drugs, like methotrexate (Kingsbury et al., 2015; Zhu et al., 2020b) and sulfasalazine (Kim et al., 2018), are interesting in this context since they can reduce inflammation and prevent disease progression, as observed in rheumatoid arthritis (Singh, 2022). Likewise, clinical practices to treat bone disorders are based on a pharmacological approach. Current treatments are mainly anti-resorptive (such as bisphosphonates, that inhibit osteoclast activity); others include anabolic approaches such as teriparatide (parathyroid hormone [PTH]); others may have both anabolic and anti-resorptive actions, such as romosozumab – reviewed in (Langdahl & Harslof, 2011). Either way, these drugs cannot revert the underlying damage and idiosyncratic drug reactions are common (Lepage et al., 2019; Steinmeyer et al., 2018).

This unmet medical need often leads to surgical interventions as the last clinical option to ameliorate patients' compliance (Lepage et al., 2019). Total joint replacement (arthroplasty)

is considered a final option for patients with extreme articular disabilities (Gossec et al., 2011; Tiku & Sabaawy, 2015). Other surgical techniques have been attempted over the years, focusing particular on preventing the progression of bone and cartilage defects. Nonetheless, there have been only minor advancements concerning the regeneration of bone and cartilage. These include (1) mosaicplasty, the replacement of lost cartilage by an autologous transplant harvested from a non-weight-bearing area of articular cartilage; (2) microfracture, the disruption of subchondral bone to promote the recruitment of multi-potent bone-marrow-derived stromal cells to the bone and cartilage defect; (3) autologous chondrocyte implantation (ACI), with previous *in vitro* expansion of autologous chondrocytes harvested from a non-weight-bearing area of the articular cartilage, with subsequent local injection of these cells into the defect; and (4) matrix-induced autologous chondrocyte implantation (MACI), the transplantation of a commercial scaffold containing autologous chondrocytes (Medvedeva et al., 2018).

Still, therapeutic limitations to implantation methods include tissue/cell availability, risk of donor site morbidity and lack of cells differentiation into desired lineages (Abbott & Holtzer, 1966; Brittberg et al., 1994; Davies & Kuiper, 2019; Huey et al., 2012; Lepage et al., 2019; Pinto-Cardoso et al., 2020). Mature chondrocytes are not prone to proliferate and these cells may even dedifferentiate *in vitro* when removed from their original matrix (Mao et al., 2018; Medvedeva et al., 2018). Dedifferentiated chondrocytes produce less cartilage-specific type II, IX, and XI collagens, ACANs (Duan et al., 2015; Medvedeva et al., 2018) and glycosaminoglycans (GAGs) while showing an increased ability to synthesize non-specific type I collagen (Darling & Athanasiou, 2005; Medvedeva et al., 2018). Expanded dedifferentiated chondrocytes often develop into fibrocartilage rather than hyaline cartilage (Madry et al., 2011; Medvedeva et al., 2018). To avoid these issues, stem cell-based therapies have gained strength for osteochondral repair, despite optimal conditions to promote osteo- and chondrogenic differentiation are still unresolved (Boeuf & Richter, 2010; Djouad et al., 2009; Johnson et al., 2012; Tiku & Sabaawy, 2015).

3. Mesenchymal Stem Cells (MSCs) and osteo- / chondrogenic differentiation

Mesenchymal stem cells (MSCs) are multipotent self-renewable cells that can be found in the bone marrow and in all postnatal organs and tissues, namely adipose tissue, umbilical cord blood and compact bone (Porada et al., 2006). This rare population of non-hematopoietic stromal cells can differentiate into mesenchymal tissues, such as bone, cartilage, adipose tissue and muscle (Augello & De Bari, 2010; Augello et al., 2010). They are also able to differentiate into non-mesenchymal cells, such as neurons (Kopen et al., 1999). MSCs have a high proliferative capability while retaining their undifferentiated state

(Banfi et al., 2002; Bruder et al., 1997). MSC positively mark for CD105 (SH2), CD90, CD73 (or ecto 5'-nucleotidase), stromal antigen 1, CD44, CD166 (vascular cell adhesion molecule), CD54/CD102 (intracellular adhesion molecule), CD49 and c-Kit (or CD117) and negatively for hematopoietic markers such as CD14 and CD45 (Baddoo et al., 2003; Boiret et al., 2005; Conget & Minguell, 1999; Dennis et al., 2002; Gronthos et al., 2003; Pittenger et al., 1999; Sivasubramanian et al., 2012).

Variations of these cell markers may depend on species, originating tissue and *ex vivo* manipulations (Augello & De Bari, 2010; Augello et al., 2010; Jones et al., 2002). MSCs can leave their bone marrow niche and circulate through the bloodstream, assisting in the repair of tissues when required (Bobis et al., 2006). Based on that, MSCs are good candidates for new therapeutic approaches, including bone tissue bioengineering (BTE) techniques (Volarevic et al., 2011).

However, MSCs are also prone to cell senescence (Fehrer & Lepperdinger, 2005; Martin et al., 2019; Turinetto et al., 2016), which may be associated with a series of phenotypic and functional changes. These modifications include morphological and biological changes, loss of proliferative and differentiation ability, alterations in their characteristic biomarkers, as well as qualitative and quantitative changes in the secretome (Herranz & Gil, 2018; Weng et al., 2022), as previously mentioned.

In fact, aging cells acquire a senescence-associated secretory phenotype (SASP). This phenotype is characterized by the secretion of proteins that can affect the behaviour of neighboring cells via autocrine/paracrine mechanisms (Borodkina et al., 2018; Campisi et al., 2019). MSCs have potent anti-inflammatory and immunosuppressive functions and, thus, have therapeutic potential for inflammation-related diseases. The induction of SASP in these cells leads to the release of chemokines/cytokines with a pro-inflammatory profile, instead of the anti-inflammatory profile observed in cells from younger patients or in cells with a lower number of passages (Gnani et al., 2019). As aforementioned, the secretome of these cells comprises cytokines, chemokines, growth factors, and anti-inflammatory factors. Among them, molecules such as adenine-based purines (i.e. ATP and adenosine) are included (Carluccio et al., 2020). Age-related changes (e.g., in the purinergic signalling cascade) may affect the differentiation potential, immunomodulatory ability, and migration of both MSCs and differentiated cells (Martin et al., 2019; Wang et al., 2016). This will be further explained below.

Thus, strategies allowing the generation of large numbers of MSCs retaining their stemness are needed for clinical applications. Recent studies strive to find alternatives to enhance stem cell or osteoblast/chondrocyte availability and activity at lesion sites, which gains even more significance in elderly patients. One such possibility is the use of gene-therapy (Grol

& Lee, 2018). The delivery of target mediators is implemented through direct intra-articular injection of a plasmid/vector (*in vivo* gene therapy) or of transduced cells (*ex vivo* gene therapy) (Fahy et al., 2015; Grol & Lee, 2018). Currently, strategies regarding osteoarthritis treatment involve intra-articular delivery of genes coding the soluble IL-1 receptor, IL-10, transforming growth factor- β 1 (TGF- β 1), and SRY-box transcription factor 9 (Sox-9), which might reduce the inflammatory process and promote the regeneration of cartilage and subchondral bone (Fahy et al., 2015; Tao et al., 2016). Promising phase II and III clinical trials demonstrated that intra-articular delivery of genes encoding for TGF- β 1 is safe and effectively improves pain and motor scores of patients with moderate-to-severe disease compared to a placebo-controlled group (Bellavia et al., 2018; Cherian et al., 2015; Grol & Lee, 2018; Ha et al., 2015). Other strategies involve the intra-articular delivery of small regulatory nucleic acids, such as microRNAs (miRNAs) (Grol & Lee, 2018) with anti-inflammatory and pro-anabolic properties (Karlsen et al., 2016). Although MSC-based therapies have shown promising results in bone and cartilage renewal, they failed to improve long-term OA outcomes (Lee & Wang, 2017).

To this end, one strategy could be the combination of local factors present in bone and cartilage microenvironments, which are also able to modulate stem cell proliferation and differentiation. Such compounds must not be harmful to the host and cause minimal side effects. These criteria are fully met by purines and pyrimidines, which have also the advantage of being readily washable and/or degradable in the organism (Noronha-Matos & Correia-de-Sá, 2016).

4. The purinome

Nucleotides (e.g. ATP and UTP) are important local regulators of osteo-/chondrogenic differentiation of MSCs. These molecules are constitutively released by cells under basal and/or stressful conditions and bind to multiple P2 purinoceptors (P2Y and P2X) on MSCs, acting as autocrine/paracrine regulators of bone and cartilage remodelling – reviewed in (Noronha-Matos & Correia-de-Sá, 2016; Pinto-Cardoso et al., 2023; Pinto-Cardoso et al., 2020). Purinergic signals are influenced by several players besides the receptor subtype, which, altogether, are termed as the purinome complex; these include nucleotide-releasing sites and ectonucleotidases as key regulators of extracellular nucleotide levels.

Nucleotides are constitutively released by three major mechanisms: 1) cytosolic ATP release upon tissue and cell damage; 2) exocytic release, and 3) via plasma membrane channels or pores, which includes ligand-gated channels and hemichannels controlled

release (Novak, 2003). Exuberant ATP release in response to external stimuli, namely hypoxia (Orriss et al., 2009), mechanical stress (Bessa-Andr es et al., 2024; Hecht et al., 2013), fluid-flow (Genetos et al., 2005; Romanello et al., 2001; Rumney et al., 2012) has been previously demonstrated.

Once in the extracellular milieu, nucleotides might trigger the activation of several sensitive purinoceptors. The role of nucleotide-sensitive P2 purinoceptor subtypes in bone and cartilage cells is far from elucidated. P2 receptors are subdivided into seven ligand-gated ionotropic P2X (P2X1-7) and eight G protein-coupled metabotropic P2Y (P2Y_{1,2,4,6,11-14}) receptor subtypes (Abbracchio & Burnstock, 1994; Burnstock, 2007). Concise reviews summarizing major findings on the role of P2 purinoceptors in bone and cartilage have recently emerged (Corciulo & Cronstein, 2019; Noronha-Matos & Correia-de-S a, 2016; Pinto-Cardoso et al., 2020).

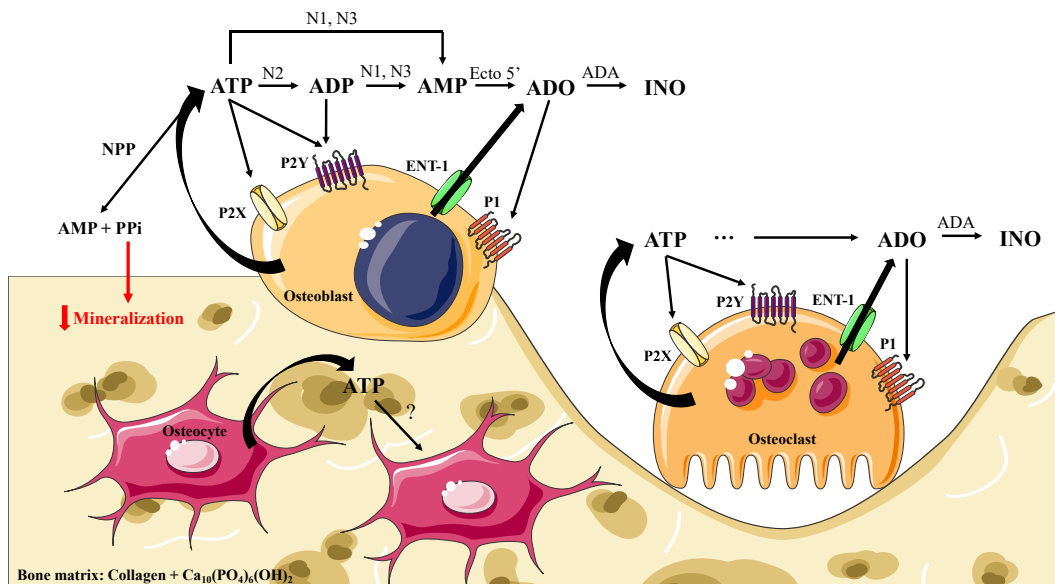


Figure 1 – Schematic overview of the purinergic signalling cascade in the bone niche. Bold black arrows indicate the cellular release of ATP and adenosine. Thin black arrows represent the activation of plasma membrane-bound P1 and P2 purinoceptors. Dashed arrows represent the enzymatic breakdown of adenine nucleotides and nucleosides. The red arrow represents the effect of PPI in bone mineralization. NTPDase1, N1; NTPDase2, N2; NTPDase3, N3; ecto 5'-nucleotidase, Ecto 5'; nucleotide pyrophosphatase/phosphodiesterase, NPP; pyrophosphate, PPI. For the sake of clarity, only adenine nucleotides are represented; missing UTP and UDP are important extracellular signalling molecules sharing the same metabolic fate as adenine-based compounds (see text for details). This figure was adapted from (Burnstock et al., 2013) and uses elements from Servier Medical Art (<http://smart.servier.com>). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Intracellular ATP levels can reach 2.5 mM, while extracellular levels of adenine (and uracil) nucleotides are kept at low concentrations, mainly due to the activity of ubiquitous ectonucleotidases and other ecto-phosphatases that rapidly breakdown nucleotides to their respective nucleotide 5'-di- and monophosphates, nucleosides and free phosphates or pyrophosphates (Burnstock et al., 2013; Picher et al., 2003) (Figure 1). This highly efficient enzymatic cascade controls the accumulation of nucleotides and nucleosides near their

plasma membrane receptors, which includes members of 1) ecto-NTPDases (the membrane-linked E-NTPDase1, 2, 3 and 8, and the soluble E-NTPDase4, 5, 6 and 7), 2) ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPPs), 3) ecto-alkaline phosphatases, and 4) ecto-5'-nucleotidase/CD73 (Kukulski et al., 2005). The tandem activity of NTPDase1/CD39, which converts ATP into nucleoside 5'-di- and monophosphates and free Pi or PPi, and ecto-5'-nucleotidase/CD73, which is responsible for dephosphorylating AMP into adenosine (Colgan et al., 2006; Zimmermann, 1992), exerts a pivotal role on multiple biological systems. Together with CD105 and CD90 antigens, ecto-5'-nucleotidase/CD73 (EC 3.1.3.5) is a membrane cell marker of pluripotent MSCs (Costa et al., 2011; Noronha-Matos et al., 2014; Noronha-Matos et al., 2012). The presence of this enzyme warrants the ability of these cells to generate significant amounts of adenosine from the extracellular catabolism of released adenine nucleotides (Figure 1). Ecto-5'-nucleotidase/CD73 catalysing AMP dephosphorylation into adenosine is the end enzyme of the ectonucleotides cascade in human BM-MSCs (Costa et al., 2011). This enzyme allows cell signalling via adenosine-sensitive P1 receptors (Noronha-Matos & Correia-de-Sá, 2016; Zimmermann et al., 2012). In human BM-MSCs, the kinetics of the extracellular ATP catabolism through members of overexpressed NTPDase1 and NPP families may explain why the conversion of AMP into adenosine by ecto-5'-nucleotidase/CD73 is faster than the formation of AMP from the catabolism of ATP; this may also elucidate why the concentration of the adenosine precursor in the extracellular milieu is kept at low levels compared to adenosine accumulation (Costa et al., 2011). This enzymatic feature is different from many other biological tissues where ecto-5'-nucleotidase/CD73 is the rate limiting enzyme for adenosine formation from adenine nucleotides – see e.g., (Cardoso et al., 2015; Cunha et al., 1996; Magalhães-Cardoso et al., 2003; Noronha-Matos & Correia-de-Sá, 2016; Vieira et al., 2014). Another interesting finding in human BM-MSC cultures is the low ADA activity detected; in fact, reduced amounts of inosine and hypoxanthine were found in the extracellular fluid of these cultures when adenosine was used as substrate, which might also contribute to significant extracellular adenosine accumulation and to the regulation of MSCs growth and differentiation via P1 receptors activation (Costa et al., 2011). Previous findings from our group show that high-affinity A_{2A} receptors are preferentially activated by adenosine formed from the catabolism of released adenine nucleotides in rat hippocampal, neuromuscular and myenteric synapses (Cunha et al., 1996; Duarte-Araújo et al., 2009). The tight association between ecto-5'-nucleotidase/CD73 and A_{2A} receptors has been elegantly and definitively documented by co-immunoprecipitation and proximity ligation assays in the striatum (Augusto et al., 2013) and, later on, by immunofluorescence confocal microscopy in astrocytes of the human hippocampus (Barros-Barbosa et al., 2016). Overall, ecto-5'-nucleotidase/CD73 seems to be positioned ideally to promote A_{2A} receptors

activation after the conversion of released adenine nucleotides into adenosine. Moreover, the expression of ecto-5'-nucleotidase/CD73 and the A_{2A} adenosine receptor seems to be mutually regulated. In this context, it has been demonstrated that the A_{2A} receptor favours the expression of the ecto-5'-nucleotidase/CD73, and vice versa, in murine MSCs and human B-cells (Katebi et al., 2009; Napieralski et al., 2003). A_{2A} receptor KO mice have lower amounts of ecto-5'-nucleotidase/CD73 compared to control animals (Katebi et al., 2009). In fact, the adenosine A_{2A} receptor and ecto-5'-nucleotidase/CD73 are both upregulated in hippocampal astrocytes of human patients with drug-resistant mesial temporal lobe epilepsy (Barros-Barbosa et al., 2016) and in terminally differentiating fibroblasts of the human subcutaneous tissue (Herman-de-Sousa et al., 2020).

Data in the literature show that P1 receptor subtypes are all present in osteoblasts, osteoclasts and chondrocytes. Adenosine-sensitive P1 receptors are G-protein coupled receptors known as A₁, A_{2A}, A_{2B} and A₃ (Strazzulla & Cronstein, 2016). A₁ and A_{2A} show high affinity while the A_{2B} and A₃ show relatively lower affinity for adenosine. Additionally, A₁ and A₃ are coupled to G_{i/o} proteins, while A_{2A} and A_{2B} are coupled to G_{s/olf} proteins. For that reason, A_{2A} and A_{2B} mediate cyclic AMP production, resulting in activation of protein kinase A (PKA) and phosphorylation of the cyclic AMP response element binding protein (CREB). On the other hand, A₁ and A₃ receptors lead to the inhibition of cyclic AMP production and decrease PKA activity and CREB phosphorylation (Cunha, 2001; Fredholm et al., 2011; Paes-De-Carvalho, 2002). Major findings and functions are well documented in two systematic reviews (Noronha-Matos & Correia-de-Sá, 2016; Pinto-Cardoso et al., 2020) which highlight that a fine-tuning regulation of the A_{2A} versus A_{2B} receptor tone in MSCs may be important for cartilage and bone repair. Some of these major findings will be further explored in the following sections.

4.1 The role of purinergic signalling in bone cells

4.1.1 Nucleotide-releasing mechanisms

Several reports point towards exocytosis as the primary mechanism of ATP release in osteoblasts (Romanello et al., 2005). Nevertheless, Cx43 is the most abundant gap junctional protein expressed in bone cells, thus playing a central role in cell-to-cell communication in the skeleton (Plotkin & Bellido, 2013). In addition to its role in the formation of ATP-releasing membrane hemichannels, Cx43 interacts with intracellular structural proteins and signalling molecules (Giepmans, 2006). For instance, Cx43

positively modulates the osteogenic differentiation of MSCs by up-regulating glycogen synthase kinase-3 β (GSK-3 β)/ β -catenin signalling pathways, whereas lentiviral knockdown of Cx43 suppresses osteogenesis (Lin et al., 2018). Several osteoblast cell markers, including osteocalcin, type I collagen, osteopontin and Runx-2, are downregulated in cells from Cx43 knockout (KO) mice (Lecanda et al., 2000). The bones of Cx43-deficient mice show features resembling those of ageing rodents and humans; these include elevated osteocyte apoptosis, increased endosteal resorption and periosteal expansion of long bones (Qiu et al., 2002).

Mechanical stimulation has marked effects on Cx43-mediated signalling in osteoblasts. The Cx43 expression is enhanced in stress-loaded bones *in vivo*, as well as in mechanically stimulated cells in culture (Cheng et al., 2001). Mechanical strain also induces the opening of Cx43 hemichannels in osteocytic cells, which is required for the release of adenine nucleotides and prostaglandin E₂ (PGE₂) (Cherian et al., 2005). Both carbenoxolone, a nonspecific gap-junction inhibitor, and meclofenamic acid, a Cx43 inhibitor, attenuate mechanically induced ATP release in osteoprogenitors of the human periodontal ligament. Knockdown of the Cx43 expression through small interfering RNA reduces the amount of ATP released upon mechanical stimulation (Luckprom et al., 2011). These findings suggest that Cx43 is a critical component of the mechanotransduction machinery by which the skeleton responds to mechanical forces. Other studies report the relevance of Cx43 on the number and activity of osteoclasts (Ilvesaro et al., 2000).

In addition to connexins, pannexins (Panx) are also implicated in nucleotide release. Panx comprises three gene products, Panx1, Panx2, and Panx3. Although Panx is not genetically related to connexins, these proteins share similar predicted four transmembrane spanning domains topology (Scemes et al., 2009). Like in connexin-containing hemichannels, six Panx subunits are required to form a channel (pannexon) in the cell membrane; the opening of pannexons may be induced by hypoxia, mechanical stress, cell depolarization and increases in intracellular Ca²⁺ (Li et al., 2018). These authors demonstrated that blockage of Panx1 with carbenoxolone inhibits the osteogenic differentiation of MSCs induced by mechanical stimulation, most probably via the regulation of p38 MAPK phosphorylation (Li et al., 2018). In osteoblasts, Panx1 is coupled to the P2X7 receptor, forming a complex hemichannel that is involved in ATP release. This theory is strengthened by others showing that ATP-induced YO-PRO dye uptake can be attenuated by incubation of wild-type cells with P2X7 receptor or Panx1 channel blockers (Thi et al., 2012). Other mechanisms involve facilitated diffusion or translocation via intrinsic plasma membrane ATP-binding cassette (ABC), which may be reviewed elsewhere (Sabirov & Okada, 2009; Wagstaff et al., 1998).

4.1.2 P2 purinoceptors expression and function during osteogenic differentiation

Osteoblasts

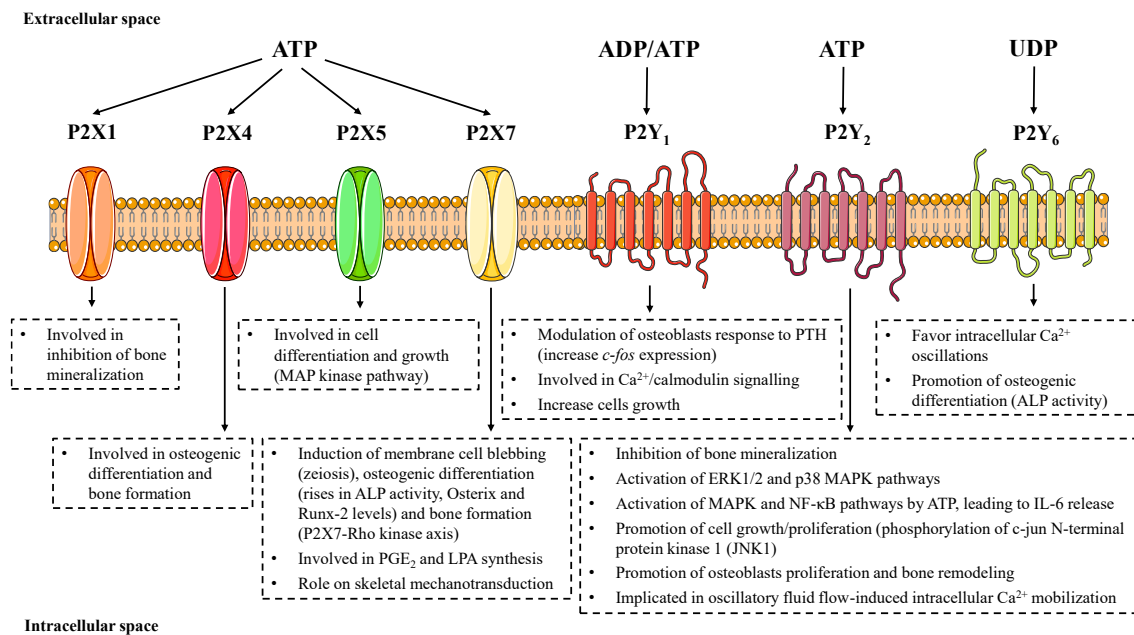


Figure 2 – The main role of P2 purinoceptors on osteoblast cell function. See text for details. This figure uses elements from Servier Medical Art (<http://smart.servier.com>).

Once released, nucleotides such as ATP and their derivatives may activate several P2 receptors (Figure 2). P2X1 and P2X3 receptors, for example, seem to be involved in the prevention of bone nodule mineralization of cultured osteoblasts from neonatal Sprague–Dawley rats (Orriss et al., 2012). The P2X2 receptor, which is mainly expressed in osteoclasts, also seems to have a role in bone resorption (King et al., 1997; Morrison et al., 1998). The P2X4 receptor, similarly to the P2X7 receptor, is also expressed in osteoblasts and seems to be involved in membrane pore formation. BzATP induces the incorporation of YO-PRO 1 fluorescent dye (a strong indicator of pore formation) via activation of the P2X7 receptor in human osteoblast-like cells; this effect was not fully prevented by P2X7 receptor antagonists, thus suggesting that the P2X4 receptor may also participate in this mechanism (Alqallaf et al., 2009). Dose-dependent reductions in bone formation (by 60–100%), alkaline phosphatase activity (by about 70%) and osteoblast cells growth ($\leq 40\%$) were also obtained with P2X4 antagonists, like 5-BDBD and PSB-12062, in the rat. Higher doses of both antagonists were also associated with reductions (by about 90–95%) in the formation of osteoclasts and their resorptive activity. Overall data suggest that the P2X4 receptor plays a relevant role in bone formation by favouring differentiation of the osteogenic lineage (Orriss et al., 2022). Regarding the P2X5 receptor, authors showed that extracellular ATP increases [3H]-thymidine incorporation and, so, cell proliferation in human MG-63 cells

(osteoblast-like); this mechanism was shown to be dependent on the mitogen-activated protein kinase (MAP kinase) pathway (Katz et al., 2008; Nakamura et al., 2000). The P2X5 receptor has been involved in human osteoblast-like cell differentiation and osteoclast cell function; this is assumed because *in vitro* blockage of this receptor reduces osteoclast maturation. Additionally, the P2X5 receptor is required for ATP-mediated inflammasome activation and IL-1 β production by osteoclasts (Hoeberitz et al., 2000; Ihara et al., 2005; Kim et al., 2017; Nakamura et al., 2000; Noronha-Matos & Correia-de-Sá, 2016). The P2X6 receptor has been already detected in rat and human (SaM-1) osteoblast-like cells, but its role in bone formation is largely unknown (Ihara et al., 2005). The P2X7 preferential agonist, BzATP, favours the osteogenic differentiation and mineralization of BM-MSCs by a mechanism involving reversible plasma membrane blebbing and cytoskeleton rearrangements associated with the P2X7 receptor/Rho-kinase axis (Noronha-Matos et al., 2014). Activation of the P2X7 receptor in cultured MSCs from Pm women increases the expression of Runx-2 and Osterix transcription factors, promotes ALP activity and increases mineralization in a Rho-kinase-dependent manner. Interestingly, mineralization observed in MSC cultures from Pm women challenged with an activator of the P2X7 receptor surpassed that observed in MSC cultures from younger females (Noronha-Matos et al., 2014). High ATP levels favour the activation of low-affinity P2X7 receptors to promote apoptosis of osteoclasts (Morrison et al., 1998); despite this, others have shown that the P2X7 receptor may favour nuclear factor kappa B (NF- κ B) activation and osteoclast resorptive activity (Korcok et al., 2004). ATP release and activation of the P2X7/Panx1 axis enhances bone resorption (Kopp et al., 2019; Mousseau et al., 2018) by (1) increasing RANKL expression in neighbouring osteocytes and osteoblasts, (2) inducing macrophage recruitment and (3) rising osteoclasts number in adjacent bone surface (Kong et al., 1999; Nakashima et al., 2011). Recently, our group showed the P2X7 receptor tonus may be severely affected in postmenopausal MSC cultures when compared to young females. This may be related to faster extracellular hydrolysis observed in BM-MSCs from postmenopausal women when compared to young females (Noronha-Matos et al., 2014).

Metabotropic P2Y receptors have also been associated with important roles in bone remodelling. In osteoblasts, P2Y receptors activation triggers phospholipase D (PLD) stimulation in a Ca²⁺/calmodulin-dependent manner without the involvement of PKC or the GTP binding protein (Suzuki et al., 1995). The ATP/ADP-sensitive P2Y₁ receptor modulates the response of human osteoblasts to systemic factors, like PTH; ATP and PTH synergize to induce immediate-early genes, like *c-fos*, which foster the responses to ubiquitous growth/differentiating factors (Bowler et al., 1999). Furthermore, low-intensity pulsed ultrasound (LIPUS) increases ATP release and promotes osteoblast proliferation via the activation of P2Y₁ receptors sensitive to blockage by MRS 2179 (Alvarenga et al., 2010).

Application of ADP in an acidified medium resulted in a 3-fold increase in osteoclasts formation and bone resorption; both effects were mimicked by selective activation of the P2Y₁ receptor and the opposite was observed with its antagonist MRS 2179 (Hoebertz et al., 2001). Stimulation of P2Y₂ receptors in osteoblasts involves activation of ERK1/2 and p38 MAPK pathways (Katz et al., 2006). Activation of MAPK and NF-κB pathways by ATP has been observed in human osteoblasts (Qi et al., 2009), which also promotes the release of cytokines, such as IL-6, by these cells (Katz et al., 2011). Additionally, activation of the P2Y₂ receptor induces the phosphorylation of c-jun N-terminal protein kinase 1 (JNK1) in osteoblast-like cells, which can stimulate osteoblast proliferation and bone remodelling (Katz et al., 2008). This proliferative effect of ATP implicates the participation of the PI3K/Protein kinase B (Akt) signalling pathway in rat osteoblasts (Katz et al., 2011), which is also involved in their differentiation via P2Y₂ receptors (Ayala-Pena et al., 2013). This receptor is implicated in oscillatory fluid flow-induced intracellular Ca²⁺ mobilization in osteoblasts (You et al., 2002). Others have found that the P2Y₂ receptor downregulates osteoblasts differentiation and matrix mineralization while inducing osteoclasts-mediated bone resorption (Hoebertz et al., 2002; Li et al., 2016; Orriss et al., 2017), but this contention has yet to be resolved. Data from our group suggest that activation of the metabotropic P2Y₆ receptor promotes bone formation by increasing intracellular Ca²⁺ accumulation in human BM-MSC bone progenitors, which may be advantageous to reduce osteoporotic fractures since this receptor expression is conserved in post-menopausal women (Noronha-Matos et al., 2012). Similar to P2X₇, this receptor is tonically compromised in BM-MSCs from postmenopausal women when compared to young females. Once again, the most prominent explanation might be related to the extracellular hydrolysis of UDP in these cultures (Noronha-Matos et al., 2012).

The P2Y₆ receptor has also been located in rat osteoclasts, where it is involved in cell survival via the activation of the NF-κB pathway (Korcok et al., 2005). Decreased bone resorption was observed in P2Y₆ knockout mice, thus strengthening the importance of this receptor in bone remodelling (Orriss et al., 2011). The literature is scarce regarding the role of the P2Y₁₁ receptor in bone. Despite the identification of the P2Y₁₁ receptor in human osteoclasts, its role in these cells remains unclear. Recently, the P2Y₁₁ receptor has been associated with breast cancer bone metastasis; most likely, ATP inhibits the bone tropism of breast cancer cells by downregulating the P2Y₁₁ receptor and CXCR4 expression (Liu et al., 2021). Regarding the ADP-sensitive P2Y₁₂ receptor, it was found that the clinical use of clopidogrel (Plavix®), a selective P2Y₁₂ receptor antagonist that is widely prescribed to reduce the risk of heart attack and stroke acting via the inhibition of platelet aggregation, is able to inhibit *in vitro* mineralization in a concentration-dependent manner. It has been

reported that this drug slows down rat osteoblast proliferation and cell viability, coupled to a reduction in ALP activity and collagen formation. *In vivo* treatment of mice with clopidogrel decreases the trabecular bone volume in tibiae and femur (Syberg et al., 2012), when compared to control littermates.

Another ADP-sensitive P2Y₁₃ receptor has also been found to affect bone remodelling; this assumption came from the fact that P2Y₁₃ knockout mice exhibit decreased bone formation and bone resorption when compared to control littermates (Orriss et al., 2011). ADP activation of P2Y₁₃^{+/+} (but not P2Y₁₃^{-/-}) in mice BM-MSCs increases the number of ALP-colony-forming units (CFU-ALP) and the expression of osteoblast-cell markers (e.g., Osterix, ALP and type I collagen) (Biver et al., 2013). BM-MSCs are more promptly differentiated into adipocytes than into osteoblasts in P2Y₁₃ knockout mice, coupled to an increase in the expression of PPARγ2 and adipsin (Biver et al., 2013). Interestingly, ongoing research from our group has highlighted the role of both receptors in human osteogenesis. Data suggest that P2Y₁₂ and P2Y₁₃ receptors are overexpressed in aged BM-MSCs compared to those from young females, leading to a diminished activation of the pro-osteogenic P2Y₁ receptor. In this context, pharmacological inhibition or silencing of P2Y₁₂ and P2Y₁₃, or disrupting their interactions with P2Y₁, may help restore the osteogenic potential of BM-MSCs from Pm women by reactivating P2Y₁ signalling (unpublished data from Catarina Bessa-Andr s' PhD thesis).

The role of the P2Y₁₄ receptor in bone remodelling remains elusive, but some authors suggest that it may control MSCs commitment to both adipogenic and osteogenic differentiation of human adipose tissue-derived stem cells; they found that the expression of this receptor decreases from undifferentiated to osteoblast-differentiated cells, suggesting a major role at early differentiation stages (Zippel et al., 2012).

Thus, as previously reported several of our results point towards a lack of P2X₇ and P2Y₆ receptors tone in Pm MSCs (Noronha-Matos et al., 2014; Noronha-Matos et al., 2012), which is not observed in younger females (Noronha-Matos et al., 2014). This led us to propose that age-related changes in the expression and activity of one or several NTPDases might have an impact on bone formation in Pm women, as previously suggested by our group (Noronha-Matos, 2014).

4.1.3 Nucleotide catabolism

As previously mentioned, the presence of NTPDase subtypes in the plasma membrane differs significantly amongst proliferating MSC osteoprogenitors and fully differentiated osteoblasts; the extracellular breakdown of adenine and uracil nucleotides is more evident in less proliferative / more differentiated cell populations (Noronha-Matos & Correia-de-Sá, 2016; Noronha-Matos et al., 2012). Ectonucleotidases, like CD39 and CD73, also regulate the production and/or breakdown of calcification inhibitors (e.g. inorganic pyrophosphate, PPI) and stimulators (e.g. inorganic phosphate, Pi) from extracellular nucleotides. This balance is critical for normal bone formation and remodelling but can be deleterious considering the calcification of blood vessels, cardiac valves and soft tissues (Opdebeeck et al., 2020). The accumulation of calcium-phosphate crystals in the arterial wall, as well as in cardiac valves, has a significant impact on the morbidity and mortality of several diseases, such as chronic kidney disease (CKD), osteoporosis, and diabetes by provoking severe cardiovascular events (Bourne et al., 2021; Zhu et al., 2012). In oestrogen-deficient ovariectomized mice, bone marrow-derived osteoblast precursors show reduced expression of NTPDase1/CD39, ecto-5'-nucleotidase/CD73, and extracellular adenosine levels. Notably, targeting the adenosine A_{2B} receptor with an exogenous agonist can mitigate osteoporotic bone loss (Shih et al., 2019). Overexpression of NTPDase1/CD39 in gingiva-derived MSCs can restore osteogenesis in ovariectomized female mice, as well as in a model of autoimmune arthritis, by stimulating the Wnt/ β -catenin signalling pathway (Wu et al., 2020) and/or by increasing the availability of adenosine to target its receptor (Luo et al., 2019), respectively. Accordingly, decreases in osteogenic differentiation due to deficits in adenosine formation owed to CD39 knock-down were attenuated upon blocking the cellular uptake of the nucleoside (He et al., 2013). Human adipose-derived MSCs attenuate experimental collagen-induced arthritis by inhibiting RANKL-induced osteoclasts genesis via a CD39-dependent pathway (Chang et al., 2020).

Bone marrow-derived MSC osteoprogenitors exhibit increasing amounts of ecto-5'-nucleotidase/CD73 as these cells differentiate into mature osteoblasts, both in rats (Ode et al., 2013) and in humans (Costa et al., 2011; Noronha-Matos et al., 2012). Deficits in AMP dephosphorylation into adenosine by inhibition of ecto-5'-nucleotidase/CD73 decrease the osteogenic differentiation of MSCs in the rat (Ode et al., 2013). Our group showed that human MSCs lack adenosine deaminase (ADA) expression and activity, which makes ecto-5'-nucleotidase/CD73 the rate-limiting enzyme to control the extracellular levels of adenosine and the activation of P1 receptors contributing to the osteogenic differentiation of these cells (Costa et al., 2011). Deficits in the activation of the most abundant A_{2B} receptor subtype are particularly relevant in this situation, given that this receptor has a low affinity

for adenosine and exerts a critical role in the osteogenic differentiation of MSCs (Costa et al., 2011). In keeping with this hypothesis, mice lacking ecto-5'-nucleotidase/CD73 show deficits in osteoblast differentiation and reduced bone formation, thus leading to osteopenia (Takedachi et al., 2012). Ecto-5'-nucleotidase/CD73 is also important for bone repair following injury in aged mice (Bradaschia-Correa et al., 2017). Mechanical straining of MSCs during 3-days with cyclic-compressive loading decreases ecto-5'-nucleotidase/CD73 at both mRNA and protein levels (Kang et al., 2012; Ode et al., 2011) and this effect persists for weeks even in the absence of a new set of stimulus (Ode et al., 2011). Similar results were obtained with umbilical cord-derived MSCs under uniaxial stretching for ten days (Kang et al., 2012). These findings agree with the theory that mechanically stressed MSCs release high amounts of ATP, which ultimately may generate adenosine as an end product (e.g., (Tan et al., 2017)). Likewise, it has been shown that shockwave therapy inhibits chondrogenic differentiation of human MSC in 3D culture conditions at least partially by generating excessive amounts of adenosine in the extracellular milieu, which eventually may activate low-affinity A_{2B} receptors.

4.1.4 Adenosine-sensitive P1 receptors

The role of adenosine sensitive receptors on bone homeostasis remains unsolved. Previous findings from our group demonstrate that high-affinity A_{2A} receptors are preferentially activated by adenosine formed from the catabolism of released adenine nucleotides in rat hippocampal, neuromuscular and myenteric synapses (Cunha et al., 1996; Duarte-Araújo et al., 2009). The tight association between ecto-5'-nucleotidase/CD73 and A_{2A} receptors has been elegantly and definitively documented by co-immunoprecipitation and proximity ligation assays in the striatum (Augusto et al., 2013) and, later on, by immunofluorescence confocal microscopy in astrocytes of the human hippocampus (Barros-Barbosa et al., 2016). Overall, ecto-5'-nucleotidase/CD73 seems to be positioned ideally to promote A_{2A} receptors activation after the conversion of released adenine nucleotides into adenosine. Moreover, the expression of ecto-5'-nucleotidase/CD73 and the A_{2A} adenosine receptor seems to be mutually regulated. In this context, it has been demonstrated that the A_{2A} receptor favours the expression of the ecto-5'-nucleotidase/CD73, and vice versa, in murine MSCs and human B-cells (Katebi et al., 2009; Napieralski et al., 2003). A_{2A} receptor KO mice have lower amounts of ecto-5'-nucleotidase/CD73 compared to control animals (Katebi et al., 2009). In fact, the adenosine A_{2A} receptor and ecto-5'-nucleotidase/CD73 are both upregulated in hippocampal astrocytes of human patients with drug-resistant mesial

temporal lobe epilepsy (Barros-Barbosa et al., 2016) and in terminally differentiating fibroblasts of the human subcutaneous tissue (Herman-de-Sousa et al., 2020).

Still, the low-affinity A_{2B} adenosine receptor is the most abundant receptor subtype in MSCs osteogenic precursors, where it exerts critical roles in cell proliferation and differentiation indicated by increases in the ALP activity; the pro-osteogenic effect of the adenosine A_{2B} receptor subtype was verified in bone marrow-derived MSCs undergoing osteogenic differentiation of post-menopausal women (Costa et al., 2011), as well as in the reversal of bone loss in ovariectomized female mice (Shih et al., 2019). The A_{2B} receptor-mediated effects are balanced through the activation of A₁ and A_{2A} receptors depending on the proliferation status of the cells (Costa et al., 2011). Incubation of mouse bone marrow-derived MSCs with the adenosine A_{2A} receptor agonist, CGS21680, increased the production of procollagen I (Katebi et al., 2009). CGS21680 also inhibited osteoclast differentiation and function, in part by decreasing IL-1 β and TNF- α secretions (Mediero et al., 2012b). Using an osteoblastic cell line, MG-63, it was shown that A_{2A} receptors activation regulates IL-6 secretion induced by lipopolysaccharide (LPS), thus directly implicating this adenosine receptor subtype in inflammation, osteoclast genesis and bone resorption (Russell et al., 2007). Other authors reported that A₁ receptor activation induced cell proliferation or promoted adipocyte differentiation (Costa et al., 2011; Gharibi et al., 2011, 2012). Regarding the A₃ receptor, literature suggests a role in cell proliferation. However, the mechanisms still elusive (Costa et al., 2011; Gharibi et al., 2011, 2012; Katebi et al., 2009).

4.2 The role of purinergic signalling in cartilage cells

4.2.1 Nucleotide-releasing mechanisms

In the cartilage, Cx43 is expressed in chondrocytes and its levels increase in OA (Knight et al., 2009), coinciding with the surplus ATP release in this pathological condition. Cx43 is expressed in bovine and human chondrocytes of the cartilage superficial region. Cx43 primarily mediates mechanotransduction in the superficial/middle region of the articular cartilage through distinct signalling pathways from those operating in cells of the deeper layers (Larranaga-Vera et al., 2021). Cx43 overactivation contributes to the maintenance of the immature phenotype of stem cells of OA cartilage. This feature can be reversed by downregulation of Cx43 using the CRISPR/Cas9 technology or by prevention of Cx43-mediated gap junctional intercellular communication (GJIC) using carbenoxolone; both

treatments can trigger re-differentiation of OA chondrocytes into a more differentiated state. Overall, data suggest that Cx43 may act as a positive regulator of chondrocyte-mesenchymal transition (Varela-Eirin et al., 2018).

Pannexins, namely Panx3, are expressed in chondrocyte precursors and hypertrophic chondrocytes, besides their presence in mature osteoblasts (Iwamoto et al., 2010). Panx3 promotes chondrogenic differentiation by regulating the intracellular ATP/cAMP levels (Iwamoto et al., 2010). In the growth plate, Panx3 expression precedes chondrocyte mineralization and co-localizes with bone development markers, such as type X collagen and osteopontin (Iwamoto et al., 2010). Contradictory results show that *in vivo* deletion of Panx3 leads to abnormal differentiation of hypertrophic chondrocytes in mice. On the other hand, Panx3 is overexpressed in damaged cartilage of mice and human OA (Moon et al., 2015). Although the mechanisms underlying nucleotide release from chondrocytes are not fully elucidated, it was suggested that ATP release causes articular cartilage mineralization (Golub, 2009; Hsu, 1992; Ryan et al., 1992) and tear (Brown et al., 1997). However, opposite effects were also observed when exogenous ATP was added to chondrocyte cultures (Brown et al., 1997; Millward-Sadler et al., 2004). Contrariwise, ATP and UTP stimulate proteoglycan and collagen accumulation in bovine chondrocyte cultures (Croucher et al., 2000), suppress nitric oxide (NO) release and stimulate proteoglycan synthesis in chondrocytes/agarose constructs (Chowdhury & Knight, 2006; Pingguan-Murphy et al., 2006). Moreover, ATP promotes the production and release of cytokines, like IL-1 β and TNF- α , by chondrocytes (Leong et al., 1993, 1994; Savio et al., 2018). This adenine nucleotide also increases the intracellular Ca²⁺ concentration ([Ca²⁺]_i), which is required for pre-chondrogenic condensation and chondrocyte differentiation (Fodor et al., 2009; Kwon, 2012). Others have shown that the extracellular accumulation of ATP activates P2 receptors and downstream effectors, such as extracellular signal-regulated kinase 1/2 (ERK1/2). This signalling cascade is associated with a self-sustained deleterious cycle with Runx-2, Panx3, and MMP13 activation, hence resulting in articular cartilage and joint destruction (Bond et al., 2011; Moon et al., 2015).

4.2.2 Nucleotide-sensitive P2 receptors

Figure 3 shows the main findings concerning P2 receptors activation in chondrocyte or cartilage cells. The P2X1 receptor activation increases NO and PGE₂ release in bovine chondrocytes, thus leading to a pro-inflammatory environment and ultimately cartilage destruction (Varani et al., 2008b; Varani et al., 2010). Extracellular ATP, via the P2X4

receptor and cAMP/PKA signalling, plays a significant role in skeletal development by operating ATP oscillations, which are critical for the differentiation of chondrogenic precursors (Kwon, 2012). The role of the ATP-sensitive P2X5 receptor in chondrocytes remains elusive. Rabbit articular chondrocytes express the low-affinity P2X7 receptor, which is responsible for ATP-induced inflammatory and apoptotic signals resulting in chondrocyte cell death (Tanigawa et al., 2018). Activation of the P2X7 receptor promotes extracellular matrix degradation and inflammatory changes in chondrocytes through NF- κ B/NLRP3 crosstalk (Li et al., 2021). High intracellular Ca^{2+} transients were observed in chondrocytes upon fluid flow stimulation (Yellowley et al., 1999) and/or pressure-induced strain (Millward-Sadler et al., 2004). These effects are similar to those seen in bone cells under the same conditions and may be mediated by the release of ATP and P2X4 receptors activation.

Chondroblasts

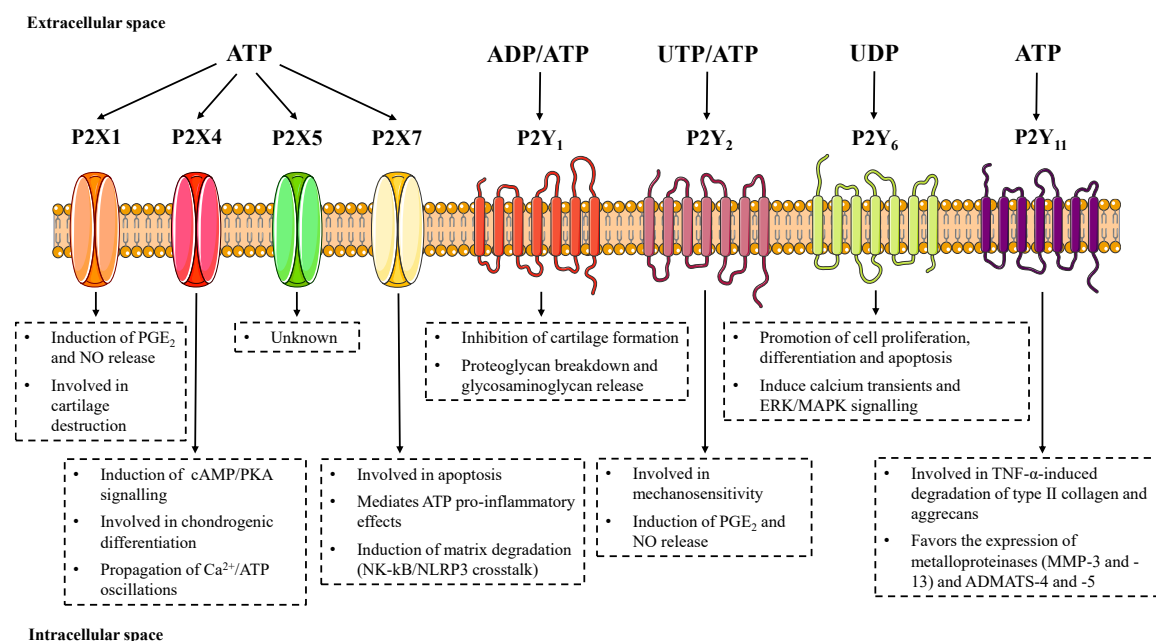


Figure 3 – The main roles of P2 purinoceptors on chondroblast cells function. See text for details. This figure uses elements from Servier Medical Art (<http://smart.servier.com>).

Through a positive feedback mechanism, ATP stimulation of metabotropic P2Y receptors contributes to further increase intracellular Ca^{2+} mobilization and surplus ATP release from adjacent cells, thus fostering the occurrence of intracellular Ca^{2+} waves within the chondrocyte cell network (Kono et al., 2006). Activation of the P2Y₁ receptor subtype is associated (1) with inhibition of cartilage formation in mesenchymal cell cultures originating from the chick limb (Meyer et al., 2001), and (2) with proteoglycan breakdown and glycosaminoglycan release in bovine nasal cartilage (Brown et al., 1997). Activation of the P2Y₂ receptor by ATP increases the production of inflammatory mediators, such as NO and

PGE₂, by chondrocytes (Berenbaum et al., 2003; Koolpe et al., 1999). A detailed link between purinoceptors and Ca²⁺ homeostasis in chondrogenic progenitor cells (CPC) was recently provided. Several P2 receptor subtypes were identified in CPC cells; these include the ADP-sensitive P2Y₁ and the UTP-sensitive-P2Y₄ receptor. Additionally, ATP can promote intracellular Ca²⁺ transients via the activation of several P2X receptor subtypes (P2X₁, P2X₄, P2X₅, P2X₆ and P2X₇), as well as via the P2Y₂ receptor (Matta et al., 2015). Both ADP and UDP can promote mature chondrocyte migration that may be critical for tissue regeneration (Szustak & Gendaszewska-Darmach, 2020). Interestingly, chondrocytes isolated from normal and OA cartilage show similar P2Y₂ mRNA levels. This observation may be relevant considering that the P2Y₂ receptor exerts a major role in chondrocytes' mechanosensitivity (Millward-Sadler et al., 2004; Xing et al., 2011; Zhang et al., 2021). Rat articular chondrocytes express the UDP-sensitive P2Y₆ receptor, which is known to regulate proliferation, differentiation, and apoptosis of these cells, while controlling the amount of matrix metalloproteinases (MMPs), via intracellular Ca²⁺ transients and activation of MAPK/ERK pathways (Kudirka et al., 2007).

Human primary chondrocytes express the P2Y₁₁ receptor subtype. This receptor is overexpressed in OA chondrocytes by a mechanism linked to the TNF-α signalling pathway. Blockage of the P2Y₁₁ significantly reduces TNF-α-induced degradation of type II collagen and aggrecans, and the expression of MMP-3, MMP-13 and ADAM metalloproteinase with thrombospondin type 1 motif 4/5 (ADAMTS-4 and ADAMTS-5) (Wang et al., 2019).

4.2.3 Nucleotide catabolism

In cartilage cells, studies concerning the presence, activity and/or influence of ectonucleotidases in cell biology are very scarce. Only a few studies have been conducted regarding the adenosine-forming enzyme, ecto-5'-nucleotidase/CD73. Here, CD73 KO mice spontaneously develop arthritis, suggesting that ecto-5'-nucleotidase/CD73 and the nucleoside adenosine are somehow involved in cartilage homeostasis (Chrobak et al., 2015). Different studies indicate that ecto-5'-nucleotidase/CD73 expression decreases during chondrogenic differentiation of MSCs (Song et al., 2006), a situation that goes in the opposite direction to that verified in MSCs undergoing osteogenic differentiation, both in humans (Costa et al., 2011) and rats (Ode et al., 2013). Inhibition of ecto-5'-nucleotidase/CD73 with adenosine 5'-(α,β-methylene)diphosphate (APCP) enhances the chondrogenic differentiation of rat MSCs, a situation that was mimicked by exposing the cells to mechanical loading (20% strain at 1 Hz for 72h); the opposite situation was observed when the same cells were cultured in an osteogenic-inducible medium (Ode et al., 2013).

Altogether, these findings point towards ecto-5'-nucleotidase/CD73 exerting a key role to fine-tuning regulate extracellular adenosine levels and, thereby, to determine cells differentiation outcome, either driving cells to an osteogenic (via low affinity A_{2B} receptors) or to a chondrogenic (via high affinity A_{2A} receptors) fate. However, this hypothesis has a major drawback, since ecto-5'-nucleotidase/CD73 tends to be down-regulated during chondrogenic differentiation (Song et al., 2006), whereas the activity and expression of this enzyme is upregulated in MSCs cultured in osteogenic-inducing media (Costa et al., 2011). Still, under prolonged hypoxic conditions or during shockwave trauma, HIF-1 α -induced overexpression of ecto-5'-nucleotidase/CD73 in parallel to a concomitant A_{2B} gene transcription might favour extracellular adenosine formation from released ATP breakdown to levels high enough to activate more abundant low affinity A_{2B} receptors, which is detrimental to chondrogenesis and cartilage homeostasis, while favouring marginal bone growth (spurs or osteophytes), a well-known characteristic of arthritic joints. Therefore, exploring adenosine release and formation, as well as the differential expression of P1 adenosine receptors throughout chondrogenic commitment of MSCs under standardized experimental conditions, will certainly contribute to clarify these controversies.

4.2.4 Adenosine-sensitive P1 receptors

Regarding this topic, the available information in the literature restricts the conclusions on the influence of adenosine receptors on cartilage homeostasis (Pinto-Cardoso et al., 2020). The role of the adenosine A_1 receptor on cartilage homeostasis remains unsolved. Several authors claim that the A_1 receptor is absent from chondrocyte-like MC615 cells (Mistry et al., 2006) and from human articular chondrocytes (Koolpe et al., 1999). Others have shown that this P1 receptor is present in BM-MSCs from Lewis rats undergoing chondrogenic differentiation (Ode et al., 2013), in the rat articular cartilage (Corciulo et al., 2017), in bovine chondrocytes (Varani et al., 2008a), in articular chondrocytes of adult pigs (Rosenthal et al., 2010) and in chondrogenic differentiating MSCs of healthy humans (Tan et al., 2017), but not in cell monocultures from RA, degenerative joint disease (DJD) and OA patients. Although it has been proposed that the adenosine A_1 receptor may be involved in Ca^{2+} transients in OA human CPCs (Matta et al., 2015) and in the detrimental effects of caffeine on chondrogenesis (Choi et al., 2017), no obvious association has been made at this point.

Increasing evidence indicates a major role for the A_{2A} adenosine receptor subtype in cartilage, as it promotes *in vitro* chondrocyte homeostasis while reducing cell senescence (Friedman et al., 2023). Direct involvement of endogenously generated adenosine in

cartilage homeostasis has been addressed using ADA, the enzyme that converts adenosine into inosine. In full-thickness articular cartilage dissected from fresh equine cadavers, ADA concentration-dependently increased GAG release and the production of MMPs, namely MMP-3 and MMP-13. These metalloproteinases usually participate in the breakdown of the cartilaginous extracellular matrix. Interestingly, these features occurred in parallel to increases in the extracellular levels of pro-inflammatory mediators, namely NO and PGE₂ (Tesch et al., 2004). The detrimental effect of adenosine inactivation by ADA in the equine articular cartilage was mimicked upon the exposure of this tissue to the selective A_{2A} receptor antagonist, ZM 241385 (Tesch et al., 2004). Data suggest that the A_{2A} receptor exerts anti-inflammatory and anti-apoptotic effects and, therefore, has chondroprotective effects (Bitto et al., 2011; Campo et al., 2015; Pinto-Cardoso et al., 2020).

Intra-articular injections of liposomal suspensions incorporating adenosine prevented the development of post-traumatic OA in rats and this effect was abrogated by co-application of ZM 241385, but not of the selective A_{2B} receptor antagonist, PSB 1115 (Corciulo et al., 2017). Contrarily, increasing evidence supports the deleterious effects of the A_{2B} receptor on cartilage (Pinto-Cardoso et al., 2020; Tan et al., 2017). Extracorporeal shock wave therapy (ESWT) significantly increases adenosine outflow from cells in parallel to A_{2B} receptor overexpression in 3-D cultures of human MSCs (Sun et al., 2013). Both adenosine and the A_{2B} receptor agonist, BAY60-6583, suppressed the chondrogenic differentiation of these cells by downregulating the expression of aggrecan, type I alpha 2 and type II alpha 1 collagen subtypes, as well as of SOX9 and SOX6 transcription factors; contrariwise, the selective A_{2B} receptor antagonists favoured the chondrogenic differentiation human MSCs (Tan et al., 2017).

Activation of A₃ receptors counteract the development of OA in a chemically-induced rat model (Bar-Yehuda et al., 2009). Authors used a highly selective A₃ receptor agonist, CF101, previously defined as a potent anti-inflammatory agent for the treatment of RA (Silverman et al., 2008). They found that CF101 induces a marked decrease in OA knee diameter (Jacobson et al., 1997). CF101 also prevented cartilage damage, osteoclast/osteophyte formation and subchondral bone destruction, while reducing pannus formation and lymphocyte infiltration in the OA animal model. The A₃ receptor agonist favoured apoptosis of inflammatory cells infiltrating OA knee joints while preventing chondrocyte apoptosis (Bar-Yehuda et al., 2009). The mechanism underlying CF101 anti-inflammatory effects probably implicates the deregulation of the nuclear factor kappa B (NF-κB) signalling pathway, thus resulting in the reduction of TNF-α production. In aged mice, ablation of the A₃ receptor results in OA development in a similar manner to that observed

in A_{2A}- and CD73-KO mice (Corciulo et al., 2017; Mediero et al., 2012a). It has also been suggested that the A₃ receptor-mediated signalling downregulates pro-catabolic pathways in human chondrocytes resulting in the prevention of cartilage degeneration (Shkhyan et al., 2018). The mechanism underlying the A₃ receptor-mediated effects includes the downregulation of (1) the Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) pathway, which is known to promote matrix degradation and inflammation, as well as (2) the RUNX2 activity (Li et al., 2011). Selective A₃ receptor activation is also known to protect human chondrocytes from cell apoptosis caused by pro-inflammatory cytokines or hypo-osmotic stress (Shkhyan et al., 2018). In addition, it seems that this receptor might be important in cell proliferation since the A₃ receptor agonist 2-CI-IB-MECA was able to increase cell growth of human BM-MSCs (Costa et al., 2011), with little effect on osteogenic differentiation.

After a critical analysis of available data, one may easily conclude that findings are sparse and difficult the necessary comparisons required to translate preclinical knowledge to clinical trials. This conclusion is mainly attributed to major limitations in studies design, as follows: (1) target species spun from rodents to human, passing through bovine and porcine, without a clear rationale about usage of subjects' age and gender; (2) mesenchymal stem cells undergoing osteogenic and chondrogenic differentiation and osteoblasts / chondrocyte progenitors are often compared with bone and articular chondrocytes derived from explants originated from healthy and/or diseased joints; (3) no attempt has been made to normalize the number of cell passages allowed before cultured cells lose their original phenotype, as this is particularly relevant when using cells with more than 1 or 2 passages. Given these limitations, these gathered findings, although scientifically relevant, must be interpreted with caution and contradictory results should be adequately confirmed in future studies – further discussed in (Pinto-Cardoso et al., 2020).

4.3 Adenosine and inflammation: how much is enough?

Extracellular basal levels of adenosine (roughly 100 nM) may increase under stressful conditions, namely during hypoxia and cell injury (Corciulo & Cronstein, 2019). Extracellular adenosine depends on ATP/adenosine catabolism but also on adenosine release and uptake. Adenosine may provide a protective cellular environment through its interaction with P1 purinoceptors, which may induce the production of anti-inflammatory mediators (Borowiec et al., 2006). Interestingly, the anti-inflammatory/immunomodulatory properties of low doses of the folate antagonist, methotrexate, are mediated by increases in adenosine

release at inflamed sites (Cronstein et al., 1993). This mechanism may be reverted by the non-selective adenosine receptor antagonist, caffeine (Nesher et al., 2003). Coffee consumption was also pointed out as a risk factor for RA, a mechanism that is probably associated to the production of the rheumatoid factor (Heliövaara et al., 2000). Following this theory, Nesher and co-workers have confirmed a reduction in methotrexate efficacy due to caffeine consumption in patients with RA (Nesher et al., 2003). Interestingly, prenatal caffeine exposure (120 mg/Kg/day, equivalent to 1–2 cups of coffee per day) associated with high-fat diet induces a poor quality of articular cartilage in male adult offspring (Luo et al., 2015). Yet, surprisingly, this effect was related to abnormal cholesterol accumulation in cartilage and downregulation of cartilage functional genes and components of the insulin growth factor-1 (IGF-1), without any mention of the fact that caffeine at this dosing regimen is a powerful adenosine receptor antagonist. More recently, there was a reduction in synovial expression of the orphan nuclear receptor related-1 (NURR1) in patients treated with methotrexate and these effects have been associated with an excess of adenosine acting on high-affinity A_{2A} receptors (Ralph et al., 2005). In addition to methotrexate, other anti-rheumatic immuno-modulatory agents have been associated with adenosine signalling. The adhesion of stimulated neutrophils to endothelial cells was inhibited by sulfasalazine in a dose-dependent manner. Elimination of extracellular adenosine by adenosine deaminase or blockade of adenosine receptors with the adenosine A_2 receptor antagonist 3,7-dimethyl-1-propargylxanthine (DMPX) completely reversed the anti-inflammatory effect of sulfasalazine (Gadangi et al., 1996), through a mechanism proposed to be similar to methotrexate (Cronstein, 1995). Other examples include the commonly used immunosuppressant tacrolimus (also known as FK506), which was shown to promote adenosine release from endothelial cells via inhibition of intracellular adenosine kinase (Hwang et al., 2001). Others have shown that the anti-inflammatory effects of aspirin and sodium salicylate, but not glucocorticoids, are largely mediated by promoting the extracellular accumulation of adenosine (Cronstein et al., 1999). Reinforcing these observations, it was found that in RA and systemic lupus erythematosus patients, ADA1 and ADA2 enzymes accumulate in the synovial fluid and synovial fibroblasts (Nakamachi et al., 2003; Sari et al., 2002). It is also worth noting that ADA levels are correlated with disease severity in RA (Tesch et al., 2004). Work from the same group showed that the adenosine kinase inhibitor, iodotubercidin, results in higher extracellular levels of adenosine in equine articular chondrocytes, which lead to lower levels of PGE_2 and NO, in parallel to a lower glycosaminoglycan (GAG) release, a known marker of proteoglycan degradation and cartilage damage (Tesch et al., 2002).

In parallel to the well-known anti-inflammatory role of adenosine via the A_{2A} receptor activation, the nucleoside may also exert pro-inflammatory effects by activating A₁ receptors; chemotaxis of stimulated neutrophils to the endothelium seems to be dependent of adenosine A₁ receptor activation, whereas the A_{2A} receptor subtype suppresses neutrophil adhesion and generation of oxygen free radicals (Cronstein et al., 1992). As a matter of fact, adenosine binding to the A_{2A} receptor counteracts the stimulation of neutrophil CD49d integrin expression and, thus, the binding of these cells to the vascular cell adhesion molecule 1 (VCAM-1) via a 3'-5'-cyclic adenosine monophosphate/protein kinase A (cAMP/PKA)-mediated pathway (Sullivan et al., 2004). Other studies have shown that (1) transmigration of the neutrophil through the endothelium, and that (2) vascular endothelial growth factor (VEGF) release from neutrophils, are both dependent on the activation of the low affinity A_{2B} adenosine receptor by surplus adenosine accumulation in the extracellular milieu (Wakai et al., 2001).

Recently, Aury-Landas and co-workers (2017) have found that 3-deazaneplanocin A (DZNep), a carbocyclic adenosine analogue that derives from the antibiotic neplanocin A, has anti-inflammatory effects. DZNep is, in fact, a potent inhibitor of S-adenosyl-L-homocysteine hydrolase (SAHH), which is a key component of the methionine cycle. These authors found that DZNep represses interleukin-1 β (IL-1 β)-induced expression of MMP enzymes, known to promote cartilage breakdown, and IL-1 β -induced NO and PGE₂ production. DZNep was also able to increase the expression of chondrogenic factors COL2A1 and SOX-9 (Aury-Landas et al., 2017), thus suggesting that inhibitors of S-adenosylmethionine-dependent methyltransferases could be used for OA treatment.

Data available so far indicates that there is a major inflammatory component involving adenosine underlying bothersome cartilage disorders. Given this, targeting inflammation pathways, which also includes the adenosinergic system, may be a promising therapeutic strategy for cartilage repair in particular as growing evidence exists showing that adenosine may have beneficial effects on cartilage cells growth and differentiation via preferential activation of the A_{2A} receptor subtype, usually connected to anti-inflammatory and immunosuppressive effects.

Keeping adenosine levels within a physiological range may be beneficial for bone and cartilage homeostasis since adenosine has anti-inflammatory properties (Chiu & Freund, 2014) and is responsible for the modulation of MSCs' proliferation and differentiation – reviewed in (Noronha-Matos & Correia-de-Sá, 2016; Pinto-Cardoso et al., 2020). Adenosine is ubiquitously produced as a physiological regulator. As mentioned in previous sections, extracellular adenosine originates mainly from the extracellular hydrolysis of ATP, a process chiefly facilitated by the tandem activity of CD39 and CD73 (ecto-5'-nucleotidase)

ectoenzymes. The activation time course of G-protein-coupled adenosine receptors, A₁, A_{2A}, A_{2B} and A₃, is highly regulated by the nucleoside inactivation systems, namely its enzymatic breakdown into inosine by adenosine deaminase (ADA) and the nucleoside cellular uptake by both equilibrative (ENT) and/or concentrative (CNT) nucleoside transporters – for details see (Hasko et al., 2018; Resta et al., 1998; Strazzulla & Cronstein, 2016). The equilibrative nucleoside transporter, ENT-1, is ubiquitously expressed both in humans and rodents (Hinton et al., 2014). Interestingly, ENT-1 null mice present ectopic bone mineralization, decreased bone density in the lower half of the spinal cord and femur, increased markers of osteoclast activity in the femur, and increased bone density in the cervical and upper thoracic vertebrae (Hinton et al., 2014). These researchers speculated that dysregulation of bone density could be secondary to the disruption of adenosine signalling. Nonetheless, changes in bone density observed in ENT-1 null mice might be related to age, as aberrant bone density was only found in older ENT-1 null mice (Hinton et al., 2014). Another plausible explanation for this phenomenon is that high adenosine levels desensitize adenosine receptors involved in the regulation of osteoclast and osteoblast function (Hinton et al., 2014; Strazzulla & Cronstein, 2016).

5. Hypoxia: the “unexpected” reconciler

Cartilage is an avascular tissue and, thus, has a reduced oxygen supply. For that reason, one can easily understand that hypoxia plays an important role in cartilage homeostasis (Gibson et al., 2008; Lennon et al., 2001; Murphy & Polak, 2004). Upon ageing, chondrocytes become less resistant to extrinsic stress leading to changes in proliferation activity, energy metabolism and response to growth factors (Martin et al., 2004). This feature increases the risk of degenerative osteoarthritic diseases (Martin & Buckwalter, 2001), which are characterized by surface fibrillation and/or microfractures of the subchondral bone. The oxygen tension gradient in the cartilaginous growth plate varies between 10% at the surface to about 1% in the deepest layers (Brighton & Heppenstall, 1971; Silver, 1975; Treuhaft & DJ, 1971), which fosters the maintenance of the specialized tissue function (Strobel et al., 2010). Controversy exists regarding the influence of oxygen tension gradients on chondrocyte function, most probably due to diverse experimental conditions concerning cells source, number of passages, age of donors and stage of cartilage degeneration, oxygen tension used, culture conditions and status of cells differentiation. For instance, low oxygen percentages (1 to 8%) have been associated to increases (Egli et al., 2008; Hansen et al., 2001; Malda et al., 2004b; Nevo et al., 1988), decreases (Lane et al., 1977; Malda et al., 2004a) and, even, no effects on chondrocyte proliferation (Grimshaw & Mason, 2000;

Marcus, 1973; Saini & Wick, 2004). Likewise, the expression of cartilage-specific genes and/or the extent of matrix protein synthesis/deposition may be either up-regulated (Domm et al., 2002; Egli et al., 2008; Hansen et al., 2001; Kurz et al., 2004; Malda et al., 2004a; Martinez et al., 2008; Murphy & Polak, 2004; Murphy & Sambanis, 2001a, 2001b; Nevo et al., 1972; Saini & Wick, 2004; Scherer et al., 2004), down-regulated (Clark et al., 1991; Grimshaw & Mason, 2000; Nevo et al., 1988) or non-modified (Malda et al., 2004b) in cells cultured under low oxygen tension compared to normoxic (20%) conditions.

Among the three HIF- α isotypes, HIF-1 α and HIF-2 α are the most studied. HIF-1 α is responsible for the primary response to hypoxia and its effects after acute hypoxic stimuli, whereas HIF-2 α plays an essential role in coordinating the hypoxic response during long exposure periods (Calejo et al., 2021; Holmquist-Mengelbier et al., 2006; Koh et al., 2011; Noguera et al., 2009). HIF-1 α and HIF-2 α are key transcription factors that respond to hypoxia changes and mediate important functions in chondrocytes (Semenza, 2012), such as proliferation, differentiation, and growth arrest (Schipani et al., 2001; Stegen et al., 2019). HIF-1 α is a positive regulator of SOX9, a transcription factor that plays a pivotal role in chondrocyte differentiation and cartilage formation (Robins et al., 2005). Moreover, *in vitro* and *in vivo* mice models show that shockwave treatment promotes the expression of HIF-1 α (Tan et al., 2017).

Interestingly, HIF-1 α can bind to the human A_{2B} receptor promotor (Kong et al., 2006; Tan et al., 2017). Others have shown that hypoxia is also a major promotor of the ecto-5'-nucleotidase/CD73 expression (Synnestvedt et al., 2002). Thus, we hypothesise that HIF-1 α -induced A_{2B} receptor overexpression may be part of a deleterious vicious cycle leading to increases in adenosine accumulation and to this receptor activation in the osteoarthritic cartilage microenvironment (see below). In this sense, overexpression of ectoenzymes, namely ecto-5'-nucleotidase, and of the adenosine A_{2B} receptor might be correlated to HIF-1 α cell amounts in osteoarthritic joints (Pinto-Cardoso et al., 2020; Synnestvedt et al., 2002; Tan et al., 2017; Zeng et al., 2022). Bone aspirates revealed an oxygen tension of about 6.6–8.6% (Harrison et al., 2002). Osteoblasts exposure to 2% O₂ leads to a 10-fold decrease in bone formation, which was fully abrogated when the O₂ tension reached 0.2%. Hypoxia may delay both osteoblast growth and differentiation, limiting overall bone formation (Utting et al., 2006). This delay in bone formation may be due to reduced Runx-2 gene transcription and protein activity in hypoxia, thus decreasing the osteogenic differentiation of multipotent MSCs (Ontiveros et al., 2004; Park et al., 2002; Salim et al., 2004). The osteoblast matrix mineralization is also inhibited during hypoxia, which results from decreased ALP, prolyl hydroxylase (PHD) and lysyl oxidase expression and activity

(Utting et al., 2006). Upon hypoxia, the osteoblasts leak huge ATP amounts, which controls the bone formation and stimulates osteoclast genesis (Morrison et al., 1998; Orriss et al., 2009). VEGF transcription upregulation during hypoxia improves the local vascularization, nutrient delivery, and overactivation of target genes in progenitor cells, which partially counteracts bone loss (Schipani et al., 2009; Wang et al., 2007).

Regarding osteoclasts, exposure to low O₂ tension (2%) increases cell growth and activity (Arnett et al., 2003; Knowles & Athanasou, 2009; Muzylak et al., 2006; Utting et al., 2010); these effects were fully abrogated by HIF-1 α silencing using a small-interference RNA (siRNA) (Hulley et al., 2017; Knowles & Athanasou, 2009). Despite the aforementioned experimental evidence, contradictory results also exist in the literature concerning the role of hypoxia/HIF-1 α on bone remodelling. Several papers report both an increase and decrease in osteoblast differentiation subsequently to HIF-1 α stimulation (Bozec et al., 2008; Leger et al., 2010). Intriguingly, HIF-1 α stabilization with cobalt and L-mimosine reduced osteoclast differentiation and bone resorption, but the same did not occur in cells exposed to low O₂ tension (2%). This discrepancy may be because hypoxia leads to PHD inhibition and HIF-1 α stabilizers might act via an alternative mechanism (Hulley et al., 2017; Knowles et al., 2010).

Hypoxia affects the energy demand and metabolism of bone and cartilage cells. Osteoclasts are mostly glycolytic cells exhibiting higher expression amounts of enzymes involved in the tricarboxylic acid cycle than in oxidative phosphorylation (Lemma et al., 2016). During bone resorption, glucose is the primary energy source (Indo et al., 2013; Williams et al., 1997). HIF stimulation leads to the upregulation of glucose transporters and glycolytic genes (Knowles & Athanasou, 2008), which subsequently increases glucose consumption in osteoclasts (Indo et al., 2013; Lemma et al., 2016; Williams et al., 1997). Contrariwise, osteoblasts predominantly convert glucose into lactate, even in the presence of sufficient oxygen (Esen & Long, 2014) and of their oxidative phosphorylation capability (Klein et al., 1993; Komarova et al., 2000; Passi-Even et al., 1993). In agreement with this, drug-induced HIF-1 α stabilization in osteoblasts stimulates the glycolytic rate and enzyme production *in vivo* (Regan et al., 2014). Moreover, the glucose uptake transporter 1 (GLUT1) is overexpressed in osteoblasts, where it plays a critical feed-forward activity in osteogenic differentiation (Wei et al., 2015). Curiously, the parathyroid hormone (PTH) can stimulate aerobic glycolysis in osteoblasts via transcriptional induction of IGF-1 (Esen et al., 2015).

6. Hypoxia-induced changes in the purinergic signalling cascade

6.1 Hypoxia-induced nucleotide release

Chondrocytes, osteoblasts and osteoclasts constitutively secrete ATP and/or adenosine under physiological conditions (Boeuf & Richter, 2010; Orriss et al., 2013). Nevertheless, the release of ATP and/or adenosine is boosted under stressful conditions, such as in joint motion, inflammation and hypoxia (Biddlestone et al., 2015), when the extracellular levels of adenine nucleotides and nucleosides may reach the millimolar concentration range (Phatarpekar et al., 2010). The literature is scarce concerning the effect of hypoxia on purinergic signalling in osteoblasts and chondrocytes. Our recent review gathers controversial information available in the literature about the role of hypoxia in the release of adenine nucleotides and nucleosides (Pinto-Cardoso et al., 2023). It seems that physiological hypoxia promotes ATP exocytosis from human osteoblasts (and umbilical vein endothelial cells, HUVEC), via a mechanism involving $[Ca^{2+}]_i$, PI3K and ROCK pathways (Lim To et al., 2015; Orriss et al., 2009). Hypoxia decreases Cx43 protein expression in rat H9C2 cardiomyocytes through a mechanism dependent on MMP activity (namely MMP-9) and ERK1/2 signalling (Wu et al., 2013). Contrariwise, chronic hypoxia increased the expression of Cx43 in rat pulmonary artery smooth muscle cells (PASMCs) via a HIF-1 α -dependent transcription activation (Han et al., 2021).

6.2 Hypoxia and P2 receptors

Hypoxia modulation of P2 purinoceptors expression and function in musculoskeletal tissues remains largely unexplored. All the aforementioned P2 purinoceptors may be affected by hypoxia, as previously demonstrated in other systems (reviewed in Pinto-Cardoso et al., 2023). Despite no changes were observed in the expression of P2X1, P2X4, and P2X7 receptor subtypes in the brainstem microglia when adult Sprague Dawley rats were exposed to hypoxia, the ATP analogue, BzATP, failed to increase the transcription of iNOS, IL-6, or TNF- α genes when compared to normoxia exposed animals (Smith et al., 2013). A different situation was observed in human breast cancer cells exposed to hypoxia for 48h; these cells had lower P2X4, P2X5 and P2X7 mRNA levels and displayed smaller ATP-induced Ca^{2+} transients (Azimi et al., 2016). A reduction in the P2X7 receptor expression was also reported in oligodendrocyte precursor cell (OPC) cultures from the forebrain of postnatal rats following oxygen-glucose deprivation (OGD) (Wang et al., 2009), but the opposite was detected in breast carcinoma cells exposed to hypoxia from 8 to 24h (Tafari et al., 2011). *In vivo*, hypoxia-ischemia also decreased the P2X7 receptor immunostaining in the lateral corpus callosum, adjacent cortex, and hippocampus (Wang et al., 2009).

Regarding the role of hypoxia in metabotropic P2Y receptors expression and function, hypoxia rapidly increases the density of the ADP-sensitive P2Y₁ receptor in endothelial cells of the mouse brain microvasculature; the newly formed P2Y₁ receptors are promptly mobilized to the cell surface (Raghavan et al., 2022). The ATP/UTP-sensitive P2Y₂ receptor is upregulated in human hepatocytes (HCCs) and HepG2 cells upon hypoxia; this feature is associated with increased cell viability (Tak et al., 2016). On the other hand, hypoxia downregulates the expression of the P2Y₁₁ receptor leading to the loss of immunosuppressive activity of dendritic cells (DCs) originating from human peripheral blood mononuclear cells (PBMCs), via a mechanism dependent on HIF-1 α transcriptional regulation (Chadet et al., 2015). Hypoxia-induced downregulation of P2Y₁ and P2Y₁₁ gene transcription was also observed in human breast cancer cells, whereas the opposite was verified regarding the P2Y₆ receptor subtype (Azimi et al., 2016).

6.3 Hypoxia and nucleotide-metabolizing enzymes

Despite the increasing evidence suggesting that hypoxia may modulate the extracellular breakdown of nucleotides and the formation of biologically active nucleosides in several systems (reviewed in Pinto-Cardoso et al., 2023), the role of hypoxia in the expression and activity of ectonucleotidases in cells of the osteochondral unit remains largely underexplored. In a recent paper, Knowles showed that osteoclasts exposed to hypoxia (2% O₂ for 24h) overexpress NTPDase1/CD39 and ecto-5'-nucleotidase/CD73 (Knowles, 2019). Overexpression of NTPDases and ecto-5'-nucleotidase/CD73 was also observed in the cerebral cortex of neonatal rats after hypoxic-ischemic (HI) insults. Increased ATP, ADP and AMP turnover immediately after the HI insult favours adenosine formation, thus contributing to the anti-inflammatory profile in this context (Losenkova et al., 2018; Pimentel et al., 2013). Whether increases in the ecto-ADA activity impair adenosine signalling after the HI insult remains to be fully elucidated (Decking et al., 1997; Pimentel et al., 2013). Curiously, hypoxia depleted intracellular ATP and adenosine uptake and phosphorylation in HUVEC cells (Baldissera et al., 2018; Losenkova et al., 2018).

The expression of ecto-5'-nucleotidase/CD73 is tightly regulated by the Wnt- β -catenin signalling pathway, which is one of the most important pathways contributing to bone homeostasis (Kang et al., 2012). The cellular amount of ecto-5'-nucleotidase/CD73 is also controlled by cytokines and growth factors, namely the IL-1 β and TNF- α (Kalsi et al., 2002; Niemela et al., 2004), as well as by the hypoxia/HIF-1 α pathway at an early stage of the bone healing process (Thompson et al., 2004). Interestingly, hypoxia is a major ecto-5'-nucleotidase/CD73 expression regulator (Thompson et al., 2004) and a known promotor of chondrogenic differentiation – for a review, see (Kanichai et al., 2008). The mechanism

underlying the effect of hypoxia (2% oxygen) on MSCs seems to involve translocation of HIF-1 α from the cytosol to the nucleus and subsequent binding to its target deoxyribonucleic acid (DNA) consensus sequence (Kanichai et al., 2008). Others have shown that hypoxia activates the phosphoinositide 3-kinase/protein kinase B/forkhead box O (PI3K/Akt/FoxO) pathway, which suppresses caspase-8 and caspase-3 activation and, thereby, prevents apoptosis of human BM-MSCs (Lee et al., 2013). Thus, hypoxic conditions improve cell growth, while maintaining MSC homogeneity, differentiation potential and genetic stability – reviewed in (Haque et al., 2015).

The stabilization of HIF-1 α is associated with the overexpression of NTPDase1/CD39 in human microvascular endothelial cells (HMEC-1) (Eltzschig et al., 2009) and with the increase in ecto-5'-nucleotidase/CD73 expression in gastric carcinoma cells (Eltzschig et al., 2009; Lu et al., 2013). Increases in NTPDase1/CD39 gene transcription and protein synthesis were also obtained *ex vivo* upon exposure of HMEC-1 to hypoxia for 8 to 24h. The same was observed *in vivo* in the ischemic myocardium of C57BL/6 mice treated with DMOG, a cell-permeable and competitive inhibitor of HIF prolyl hydroxylase (PHD) that results in HIF-1 α stabilisation and accumulation (Eltzschig et al., 2009). Experiments performed in CD39-deficient mice exposed to hypoxia (8%O₂ / 92%N₂ for 4h) supported the hypothesis that NTPDase1/CD39 is critical for permeability changes associated with the catabolism of nucleotides after hypoxia in the vasculature. Likewise, inhibition of ecto-5'-nucleotidase/CD73 prevented the ATP-mediated changes in endothelial permeability of HMEC-1 submitted to hypoxia (Eltzschig et al., 2003). Deletion of the putative HIF-1 α binding site abolished hypoxia-induced ecto-5'-nucleotidase/CD73 in bovine aortic endothelia (BAE) monolayers (Lu et al., 2013; Petruk et al., 2021; Synnestvedt et al., 2002). Furthermore, hypoxia-induced time-dependent increases in NTPDase1/CD39 and ecto-5'-nucleotidase/CD73 activity, mRNA expression and protein levels in the human metastatic colon carcinoma T84 cell line; likewise, ecto-5'-nucleotidase/CD73 overexpression was observed in the hypoxic mouse small intestine. Hypoxic conditions enhance NTPDase1/CD39 and ecto-5'-nucleotidase/CD73 density in HepaRG and Chang liver cell lines in a HIF-1 α -dependent manner, and the same occurs in liver cells of patients with acute liver failure (ALF) (Tak et al., 2017).

Experimental results pointing towards the opposite direction can also be found in the literature. For instance, chronic exposure to hypoxia delayed the extracellular ATP breakdown by fibroblasts and endothelial cells, thus increasing the tonic receptor-mediated effect of the nucleotide on DNA synthesis (Gerasimovskaya et al., 2002). Likewise, hypoxia of the *vasa vasorum* endothelial cells (VVEC) of pulmonary arteries display reduced

NTPDase and CD73 activities, without changing the density of these enzymes at the protein level (Yegutkin et al., 2011). These changes are associated with increases in the permeability and loss of the vascular control mediated by adenosine in pulmonary arteries, besides augmentation of pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNF- α) in the plasma (Gerasimovskaya et al., 2002; Yegutkin et al., 2011) triggered by ATP release/accumulation and superfluous P2X7 receptors activation (Doleski et al., 2016).

Overall, these findings suggest that upregulation of NTPDases and ecto-5'-nucleotidase/CD73 has beneficial effects against vascular inflammation, as it promotes ATP hydrolysis and adenosine formation (Chiu & Freund, 2014). Whether HIF-1 α -operated hypoxic-induced changes in ectonucleotidases transcription and function occur in the osteochondral unit in health and disease conditions deserves to be explored shortly. This is justified taking into consideration that (1) chondrocytes, osteoblasts and MSCs undergoing chondrogenic/osteogenic differentiation express various amounts of NTPDases and ecto-5'-nucleotidase/CD73 (Caswell & Russell, 1985; Roszek et al., 2015), and (2) chondrocyte cells normally subsist in a low O₂ microenvironment which fosters transcription of hypoxia-sensitive genes, namely those of the purinergic signalling cascade.

6.4 Hypoxia modulation of the adenosinergic system

Regarding this topic, hypoxia was found to decrease the expression and activity of ENT-1/2 in epithelial and endothelial cells (Casanello et al., 2005; Eltzschig et al., 2005), which may lead to increases in the extracellular levels of the nucleoside. This result was confirmed *in vivo* in the DMOG-treated ischemic myocardium mice model, where HIF-1 α directly downregulated the expression of ENT-1 (Eltzschig et al., 2005). In keeping with these findings, short-term hypoxia augmented the adenosine content in the spleen and bloodstream of mice and humans (Kiers et al., 2018).

Besides P2 purinoceptors, hypoxia may also affect the adenosine-sensitive P1 receptors' expression and function. Available data point towards a major impact of adenosine A_{2A} and A_{2B} receptor subtypes in the chondrogenic (Pinto-Cardoso et al., 2020) and osteogenic (Noronha-Matos & Correia-de-Sá, 2016) differentiation of MSCs, respectively. However, there is a gap in our knowledge concerning the underlying mechanism of action of these receptors in the osteochondral unit.

It was recently demonstrated that hypoxia increases the A_{2B} receptor expression by a mechanism associated with HIF-1 α and HIF-2 α in osteoclasts; superfluous A_{2B} receptors

activation triggers an intracellular feedforward loop to increase HIF-1 α accumulation leading to increased glycolysis and mitochondrial reductase activities, which are intrinsically linked to bone resorption by osteoclasts (Knowles, 2019). Remarkably, the A_{2B} receptor antagonism does not affect bone resorption under normoxia conditions, most likely because tonic activation of this low-affinity receptor requires high extracellular adenosine amounts which are only achieved under hypoxic conditions (Hannah et al., 2021).

Using other cellular systems to extrapolate data concerning the modulation of adenosine receptors expression and function by hypoxia, it has been demonstrated that chemically-induced hypoxia increases adenosine A_{2A} receptor transcription and activity in human lung microvascular endothelial cells (HLMVECs) and pulmonary artery endothelial cells (HPAECs) via a mechanism associated to HIF-2 α (Ahmad et al., 2009). Others have found the opposite in HUVECs, where hypoxia downregulated the activity of the high-affinity A_{2A} receptor in a time-dependent manner, while low-affinity A_{2B} receptors were upregulated. The A_{2B}/A_{2A} receptors mRNA ratio in HUVECs switched from a predominant A_{2A} receptor subtype in normoxia to a dominant A_{2B} receptor subtype upon hypoxia (Feoktistov et al., 2004). These findings strengthen our hypothesis that the low-affinity A_{2B} receptor is probably more relevant under hypoxic conditions when higher levels of adenosine accumulate in the extracellular milieu as a consequence of the upregulation of the nucleoside forming enzymes, NTPDase1/CD39 and ecto-5'-nucleotidase/CD73 activity (Eltzschig et al., 2003; Feoktistov et al., 2004). Hypoxia-induced overexpression of the A_{2B} receptor was also observed in human lung fibroblasts resulting in the myofibroblast differentiation of these cells (Zhong et al., 2005). The same occurred in HMEC-1 and in the mucosa of the colon of mice with TNBS-induced colitis, where the implication of HIF-1 α was also demonstrated (Kong et al., 2006).

Despite the extensive studies on osteo-/chondrogenesis discussed above, many questions remain unanswered, particularly regarding the role of purinergic signalling in human osteo-/chondrogenesis. Key variables such as culture conditions, differentiation cocktails, cell source, patient age, gender, and disease status must be carefully considered. To address some of these uncertainties, the following objectives and studies were designed to provide further insight into these critical aspects.

Goals

This thesis aimed at investigating new molecular targets for the therapeutic management of bone (**Paper 1**) and cartilage (**Paper 2**) defects focusing on the role of endogenously released purines in the growth and differentiation of human BM-MSCs, which are often used for autotransplantation. Modifications of the “purinome signature”, which encompasses ATP/UDP and adenosine release sites, metabolizing enzymes and purinoceptors, were determined according to patients’ age, gender and disease condition. Experiments were divided into osteogenic (**Paper 1**) and chondrogenic (**Paper 2**) *in vitro* studies:

Paper 1: Our working hypothesis was that age- (and/or pathophysiological)-related changes in the expression and activity of substrate-specific NTPDases might have a significant impact on Pm bone formation. Here, we tested whether changes in the expression and/or activity of NTPDase subtypes contribute to the osteogenic differentiation deficits of BM-MSCs obtained from Pm women compared to those isolated from younger females.

Paper 2: A scarcity of data exists on the putative interplay between the hypoxia-related transcription factor, HIF-1 α , and coexistent high-affinity A_{2A} and low-affinity A_{2B} receptors, as well as with other ectonucleotidases enzymes forming adenosine, on BM-MSCs from aged women submitted chondrogenic differentiation. So, our work hypothesis considered that HIF-1 α -operated hypoxic-induced changes in ectonucleotidases and receptors’ transcription and function occur in the osteochondral unit both in health and disease conditions, which may be associated with cartilage disorders such as osteoarthritis (OA). Here, we used the cell-permeable prolyl-4-hydroxylase inhibitor, DMOG (30 μ M), to promote the intracellular accumulation of HIF-1 α and, therefore, its transcription activity under normoxic conditions.

These tasks were undertaken to unravel the possibility of using a new stem cell-based therapy applied during “in vitro” MSCs expansion in the frame of tissue engineering to treat bone and cartilage defects. Hopefully this will provide new insights for the development of novel therapeutic strategies for bone and cartilage disorders such as osteoporosis and osteoarthritis, in which targeting both receptors and ectonucleotidases may prove beneficial.

Original Research Papers

The results obtained in this thesis originated two original research papers:

Paper 1: Noronha-Matos JB, Pinto-Cardoso R, Bessa-Andrês C, Magalhães-Cardoso MT, Ferreira F, Costa MA, Marinho J, Freitas R, Lemos R, Vilaça A, Oliveira A, Pelletier J, Sévigny J & Correia-de-Sá P. Silencing NTPDase3 activity rehabilitates the osteogenic commitment of post-menopausal stem cell bone progenitors. *Stem Cell Res Ther.* 2023 Apr 19;14(1):97. doi: 10.1186/s13287-023-03315-6. PMID: 37076930; PMCID: PMC10116749.

Paper 2: Pinto-Cardoso R, Bessa-Andrês C, Pereira-Costa F, Neiva R, Martins-Ferreira R, Guerra-Leal B, Catelas D, Vilaça A, Oliveira A, Pelletier J, Sévigny J, Noronha-Matos JB & Correia-de-Sá P. HIF-1 α stabilisation hampers the chondrogenic differentiation of aged bone marrow mesenchymal stem cells by adenosine A_{2A}/A_{2B} receptors imbalance. *ACS Pharmacol Transl Sci.* 2025 Jun 24;8(7):2075-2092. doi: 10.1021/acsptsci.5c00190. PMID: 40672664; PMCID: PMC12261222.

Paper 1

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Silencing NTPDase3 activity rehabilitates the osteogenic commitment of post-menopausal stem cell bone progenitors

José Bernardo Noronha-Matos^{1,2}, Rui Pinto-Cardoso^{1,2}, Catarina Bessa-Andrês^{1,2}, Maria Teresa Magalhães-Cardoso^{1,2}, Fátima Ferreirinha^{1,2}, Maria Adelina Costa^{1,2,3}, José Marinhos⁴, Rolando Freitas⁴, Rui Lemos⁴, Adélio Vilaça⁵, António Oliveira⁵, Julie Pelletier⁶, Jean Sévigny⁶, and Paulo Correia-de-Sá^{1,2}

¹Laboratório de Farmacologia e Neurobiologia, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS) – Universidade do Porto (UP), R. Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal; ²Center for Drug Discovery and Innovative Medicines (MedInUP), Porto, Portugal; ³Departamento de Química, Instituto de Ciências Biomédicas Abel Salazar - Universidade Do Porto (ICBAS-UP), 4050-313 Porto, Portugal; ⁴Serviço de Ortopedia e Traumatologia, Centro Hospitalar de Gaia - Espinho, 4434-502 Vila Nova de Gaia, Portugal ⁵Serviço de Ortopedia, Centro Hospitalar Universitário de Santo António, 4099-001 Porto, Portugal; ⁶Centre de Recherche en Rhumatologie et Immunologie, University Laval, 2325, rue de l'Université Québec, Québec, G1V 0A6 Canada

Abstract

Background: Endogenously released adenine and uracil nucleotides favour the osteogenic commitment of bone marrow-derived mesenchymal stromal cells (BM-MSCs) through the activation of ATP-sensitive P2X₇ and UDP-sensitive P2Y₆ receptors. Yet, these nucleotides have their osteogenic potential compromised in post-menopausal (Pm) women due to overexpression of nucleotide metabolizing enzymes, namely NTPDase3. This prompted us to investigate whether NTPDase3 gene silencing or inhibition of its enzymatic activity could rehabilitate the osteogenic potential of Pm BM-MSCs.

Methods: MSCs were harvested from the bone marrow of Pm women (69 ± 2 years old) and younger female controls (22 ± 4 years old). The cells were allowed to grow for 35 days in an osteogenic-inducing medium in either the absence or the presence of NTPDase3 inhibitors (PSB 06126 and hN3-B3_s antibody); pre-treatment with a lentiviral short hairpin RNA (Lenti-shRNA) was used to silence the NTPDase3 gene expression. Immunofluorescence confocal microscopy was used to monitor protein cell densities. The osteogenic commitment of BM-MSCs was assessed by increases in the alkaline phosphatase (ALP) activity, the amount of the osteogenic transcription factor Osterix and the alizarin red-stained bone nodule formation. ATP was measured with the luciferin-luciferase bioluminescence assay. The kinetics of the extracellular ATP (100 μM) and UDP (100 μM) catabolism was assessed by HPLC-ED.

RESULTS: The extracellular catabolism of ATP and UDP was faster in BM-MSCs from Pm women compared to younger females. The immunoreactivity against NTPDase3 increased 5.6-fold in BM-MSCs from Pm women vs. younger females. Selective inhibition or transient NTPDase3 gene silencing increased the extracellular accumulation of adenine and uracil nucleotides in cultured Pm BM-MSCs. Downregulation of NTPDase3 expression or activity rehabilitated the osteogenic commitment of Pm BM-MSCs measured as increases in ALP activity, Osterix protein cellular content and bone nodule formation; blockage of P2X₇ and P2Y₆ purinoceptors prevented this effect.

Conclusions: Data suggest that NTPDase3 overexpression in BM-MSCs may be a clinical surrogate of the osteogenic differentiation impairment in Pm women. Thus, besides P2X₇ and P2Y₆ receptors activation, targeting NTPDase3 may represent a novel therapeutic strategy to increase bone mass and reduce the osteoporotic risk of fractures in Pm women.

Keywords: Ectonucleotidases; Mesenchymal stem cells; Post-menopausal osteogenesis; Purinergic signalling.

Introduction

Bone marrow-derived mesenchymal stem cells (BM-MSCs) exhibit extensive proliferative ability in an uncommitted state, while retaining the great potential to differentiate into various cell lineages (e.g. osteoblasts, adipocytes and chondrocytes) (Bobis et al., 2006; Conget & Minguell, 1999; Costa et al., 2011; Pittenger et al., 1999). BM-MSCs retain their proliferative potential throughout life, yet their ability to differentiate into bone-forming osteoblasts is compromised in post-menopausal (Pm) women (Noronha-Matos et al., 2014; Noronha-Matos et al., 2012), leading to unbalanced bone resorption by osteoclasts.

Extracellular adenine and uracil nucleotides play important roles in the regulation of bone formation (Noronha-Matos & Correia-de-Sá, 2016). Nucleotides are constitutively released from BM-MSCs and differentiated osteoblasts when submitted to mechanical load, low-intensity pulsed ultrasound, or in the presence of chemical stimulants, like 1,25(OH)₂ vitamin D3 and bisphosphonates (Alvarenga et al., 2010; Biswas & Zanello, 2009; Hayton et al., 2005; Riddle et al., 2007; Romanello et al., 2006; Rumney et al., 2012), which may explain their relevance in bone remodelling. The extracellular nucleotide levels increase dramatically during bone injury, which is paramount to bone remodelling and fracture healing (Alvarenga et al., 2010; Brandao-Burch et al., 2012; Orriss et al., 2009). Epidemiological, pharmacological and molecular data suggest that the loss of function of the ATP-sensitive P2X7 receptor reduces bone mineral density and increases the risk of fractures in Pm women (Jorgensen et al., 2012; Noronha-Matos et al., 2014; Ohlendorff et al., 2007). Besides ATP, also uracil nucleotides are important regulators of osteogenic differentiation of Pm BM-MSCs through the activation of UDP-sensitive P2Y₆ receptors (Noronha-Matos et al., 2012). Conflicting results, however, exist implicating the influence of pyrimidine-sensitive P2Y₂ and P2Y₄ receptors in bone human formation (Bowler et al., 1995; Hoebertz et al., 2002; Maier et al., 1997; Orriss et al., 2007).

The effects of endogenously released adenine and uracil nucleotides by BM-MSCs are normally balanced by the activity of several plasma membrane-bound NTPDases, which catalyse their extracellular breakdown and, thereby, determine whether osteoblast progenitors are driven into proliferation or differentiation. Previous findings from our group showed that BM-MSCs from Pm women exhibit deficits in the activation of P2X7 and P2Y₆ receptors (Noronha-Matos et al., 2014; Noronha-Matos et al., 2012), but this was not observed in cells obtained from younger females (Noronha-Matos et al., 2014). Membrane compartmentalization of ectoenzymes, nucleotide-releasing sites and purinoceptors (Yegutkin, 2008) emphasizes the value of gathering information concerning the kinetics of the extracellular catabolism of adenine and uracil nucleotides by NTPDase subtypes, along

with the subsequent delivery of their metabolites to specific P2 purinoceptors, to predict their relevance in bone remodelling.

Our working hypothesis is that age- (and/or pathophysiological)-related changes in the expression and activity of substrate-specific NTPDases might have a significant impact on Pm bone formation. Increasing evidence demonstrates that targeting the activity of NTPDases may be therapeutically valuable in blood clotting, vascular inflammation, immune reactions, and certain types of cancer (Gendron et al., 2002). Here, we tested whether changes in the expression and/or activity of NTPDase subtypes contribute to the osteogenic differentiation deficits of BM-MSCs obtained from Pm women compared to those isolated from younger females. Data show that NTPDase3 overexpression emerges as a distinctive surrogate of osteogenic impairment of BM-MSCs in Pm women compared to other NTPDase subtypes. Thus, normalization of NTPDase3 expression levels and/or inhibition of its activity may represent novel therapeutic approaches to promote osteogenesis and increase bone formation in Pm women.

Materials and Methods

Reagents, lenti-shRNAs and antibodies

ATP, quinacrine mustard dihydrochloride, 3,4-dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt (Alizarin red S), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrasodium bromide (MTT), 4-nitrophenyl phosphate and UDP were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride (A438079), N,N''-1,4-butanediylbis[N'-(3-isothiocyanate phenyl) thiourea (MRS 2578) and 1-amino-4-(1-naphthyl)aminoanthraquinone-2-sulfonic acid sodium salt (PSB 06126) were obtained from Tocris Cookson Inc. (Bristol, UK). All primary anti-human and secondary conjugated antibodies used in this study have been previously validated (Munkonda et al., 2009; Noronha-Matos et al., 2012; Pelletier et al., 2017; Pinheiro et al., 2013). Anti- β -actin antibody (rabbit), anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; mouse), anti-Osterix (rabbit), horseradish-peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse) were obtained from Abcam (Cambridge, UK); anti-IgG2a (mouse) and anti-IgG2b (mouse) were purchased from BioLegend (San Diego, CA, USA); anti-human ecto-NTPDases antibodies, CD73 antibody and pre-immune rabbit and guinea pig serums were developed by JP and JS at the Centre de Recherche en Rhumatologie et Immunologie, University of Laval, Québec, QC, Canada (see <http://ectonucleotidases-ab.com> for further

details); primary antibodies included: h5'NT-2LI5 (rabbit), hN1-1cI₅ (guinea pig), hN2-kw3I4 (rabbit), hN3-1cI₄ (guinea pig), hN3-B3_s (mouse) and hN8-C5_s (mouse). Alexa Fluor 488-labelled anti-rabbit, Alexa Fluor 568-labelled anti-mouse and Alexa Fluor 649-labelled anti-guinea pig were supplied by Molecular Probes (Invitrogen, Carlsbad, CA, USA). TRITC 568-labelled anti-guinea pig was purchased from Jackson ImmunoResearch (Newmarket, Suffolk, UK). pGFP-C-shLenti Vectors were supplied from ORIGENE (Rockville, MD, USA).

Cell culture conditions and phenotypic characterization of the cells

Human cancellous bone marrow samples were obtained (i) from the neck of the femur of thirty consecutive female patients (69 ± 2 years old, Pm group) undergoing total hip arthroplasty as a result of non-inflammatory degenerative osteoarthritis, and (ii) from the iliac crest or sacrum of eight younger female patients (22 ± 4 years old, control group) requiring bone engraftment for spinal fusion to correct scoliosis or fracture osteosynthesis. For comparison purposes, cancellous bone marrow samples were also obtained from two men, one matching the age of the younger female group and the other pairing with the Pm group. Handling of bone marrow samples and culture of adherent cells was performed until near confluence for 10–15 days, as previously described (Noronha-Matos et al., 2014; Noronha-Matos et al., 2012). To avoid the influence of in vitro cells senescence and phenotypic modifications, we used only the first subcultures made with 2.5×10^4 cells/mL density, which were maintained for 35 days in standard culture medium (α -minimal essential medium, α -MEM) plus 10% foetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin and 2.5 μ g/mL amphotericin B supplemented with 50 μ g/mL ascorbic acid, 10 mM β -glycerophosphate and 10 nM dexamethasone to promote osteogenic differentiation. Bone marrow cell cultures were carried out for 35 days in either the absence (control) or the presence of the test drugs, which were added to the culture medium from day 1 onwards (Costa et al., 2011; Noronha-Matos et al., 2014; Noronha-Matos et al., 2012). Drugs were renewed in the culture at each medium change, i.e. twice a week. No pooled samples obtained from different individuals were used for any of the protocols described in this study.

Phenotypic characterization of the cells (first subculture) was performed by flow cytometry (Noronha-Matos et al., 2012). These cells exhibited positive immunoreactivity against CD105 (SH2), CD29 (integrin β 1) and CD117 (tyrosine-protein kinase Kit), which have been identified as surface markers of BM-MSCs (Baddoo et al., 2003; Bobis et al., 2006; Boiret et al., 2005; Conget & Minguell, 1999; Dennis et al., 2002; Gronthos et al., 2003; Pittenger et al., 1999). Conversely, the cells were negative for haematopoietic surface markers, like CD14 and CD45, which have been extensively used as a good argument to distinguish bone marrow haematopoietic cells from MSCs (Baddoo et al., 2003; Pittenger et al., 1999).

Thus, first passage plastic-adherent human bone marrow cells obtained under the present experimental conditions are highly enriched in multipotent MSCs. See e.g. Ref (Noronha-Matos et al., 2012).

NTPDase3 gene silencing using a lentivirus-coupled shRNA

For NTPDase3 gene silencing, the first sub-cultures were treated with pGFP-C-shLenti Vectors (ORIGENE – code TL313202V) against NTPDase3; 4 specific sequences were used, namely: TL313202VA (CTCTACACACACAGCTTCCAGTGCTATGG), TL313202VB (ATTTCTGGAGAAGAACCTGTGGCACATG), TL313202VC (CGCTCTTACTGCTTCTCAGCCAACTACAT) and TL313202VD (GCAGGATTCTACTACACAGCCAGTGCTTT). A mismatch sequence was used as a negative control (scramble, TR30021V). Cells were incubated with the lenti-shRNAs for 24 h; to promote more extensive cell transduction, half the volume of the cells medium was used during the incubation period for all 4 specific and scrambled sequences, as well as for control cultures (no treatment) (Lin et al., 2012). During validation experiments, we used increasing multiplicities of infection (MOI), namely 1, 3 and 10 (see Supplementary figure 2); we found that sequence TL313202VD (MOI 3) was the most promising NTPDase3 gene silencing condition, given that (i) it was able to significantly decrease NTPDase3 protein levels; (ii) the scramble sequence was unable to change NTPDase3 protein levels (see Supplementary figure 2); (iii) no difference was found in the number of viable cells between scramble and shRNA treated cells (data not shown). After a 24h incubation period, cell cultures were washed three times with phosphate-buffered saline (PBS, 1x) and then maintained for 35 days in supplemented cell medium as indicated above (Lin et al., 2012).

Viability/proliferation and osteogenic differentiation of BM-MSCs

Cell viability/proliferation was evaluated by the MTT assay (Costa et al., 2011; Noronha-Matos et al., 2014; Noronha-Matos et al., 2012). Data from the MTT assay correlates positively with the results measuring cell proliferation from total DNA quantification per culture well (Noronha-Matos et al., 2012). Osteogenic differentiation of BM-MSCs was inferred as increases in alkaline phosphatase (ALP) activity and the expression of the osteogenic transcription factor Osterix. ALP activity was determined in cell lysates by colorimetric determination of p-nitrophenyl phosphate (PNP) hydrolysis, as previously described (Costa et al., 2011; Hoemann et al., 2009; Noronha-Matos et al., 2014; Noronha-Matos et al., 2012); obtained values were expressed in nmol of PNP per min normalized by the MTT value ($\text{nmol min}^{-1} \text{MTT}^{-1}$) (Noronha-Matos et al., 2014) and presented as percentage of controls (100%).

Total amounts of Osterix protein were determined by Western blot analysis at culture day 21 (Noronha-Matos et al., 2014). Equal protein amounts (25 µg) were loaded into sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (10%) gels and transferred onto a polyvinylidene fluoride membrane using a Mini-Protean Tetra Cell coupled to a Mini-Trans-Blot module (Bio-Rad, Hercules, CA, USA). Blocked membranes were incubated with the anti-human primary antibody (1:200) anti-Osterix (rabbit); anti-β-Actin (1:1000, rabbit) or anti-GAPDH (1:200, mouse) were used for normalization purposes (i.e. Osterix/β-Actin and Osterix/GAPDH). The peroxidase detection system (1.25 mM luminol, 0.2 mM coumaric acid, 0.1 M Tris pH 8.5, 0.032% hydrogen peroxide) was used for visualization of the immunoreactivity, using the horseradish-peroxidase-conjugated secondary antibodies (1:70000). Gels were analysed using a gel blot imaging system (ChemiDoc MP, Bio-Rad, Hercules, CA, USA).

At culture day 35, calcium deposition in mineralized nodules was revealed by the Alizarin Red staining and photographed using an optic microscope (Olympus CKX41, Tokyo, Japan) equipped with a digital camera (Olympus SC30, Tokyo, Japan), running an image acquisition software (Olympus Analysis GetIT 5.1, Tokyo, Japan) (Noronha-Matos et al., 2014); calibrated images were exported to Image J 1.37c software (NIH, Bethesda, MD, USA) for quantification of total bone-nodule areas. Results are expressed as percentages relative to controls (100%) (Brandao-Burch et al., 2005; Noronha-Matos et al., 2014).

Immunofluorescence staining

Human BM-MSCs were allowed to grow in chamber slides for 7 or 21 days. Paraformaldehyde fixed cells were incubated in the dark for 2 h with the following primary antibodies: ecto-5'-nucleotidase 1:1000 (h5'NT-2LI5, rabbit), NTPDase1 1:1000 (hN1-1cI₅, guinea pig), NTPDase2 1:200 (hN2-Kw3I₄, rabbit), NTPDase3 1:200 (hN3-B3_s, mouse; hN3-1cI₄, guinea pig) and NTPDase8 1:1000 (hN8-C5_s, mouse). Alexa Fluor 488 1:1500 (anti-rabbit), Alexa Fluor 568 1:1500 (anti-mouse), Alexa-Fluor 649 1:1500 (anti-guinea pig) and TRITC 1:150 (anti-guinea pig) were applied as secondary antibodies for 1 h in the dark. Glass slides were mounted with VectaShield mounting medium and stored at 4 °C. Observations were made using a laser-scanning spectral confocal microscope (Olympus FV1000, Tokyo, Japan) built on an IX81SF-3 inverted motorized microscope with four laser lines controlled by an AOTF laser combiner. Both multi-argon laser and diode laser 405 lines, filtered by barrier filters Ion Coating for OLYMPUS UIS-2 optics, through a UPLSAPO40xOI / NA 0.5–1.0 WD 0.12 mm objective lens (Olympus, Tokyo, Japan), were used to acquire images unless otherwise stated. The Fluoview FV1000 Advanced Software (4.0.3.4 version; Olympus, Tokyo, Japan) was used to analyse data and to control image

acquisition parameters, which were set to one-way XY repeat scanning mode at 12.5 s/pixel speed with the pinhole set to 250 μm at an image resolution of 640×640 pixel ($317.583 \times 317.583 \mu\text{m}$ given that 1 pixel = $0.497 \mu\text{m}$). Acquired micrographs were stored in the Olympus Multi TIFF format (Tokyo, Japan) (Alqallaf et al., 2009; Costa et al., 2011; Noronha-Matos et al., 2014; Noronha-Matos et al., 2012). For comparison purposes, confocal microscope settings and image acquisition parameters were kept unaltered throughout parallel documentation procedures. Negative controls were performed in the absence of the primary antibodies; replacement of primary antibodies by pre-immune sera (rabbit, guinea-pig) or by corresponding IgG2a and IgG2b antibodies, was also made in certain conditions (see Supplementary figure 3). Five microscopic fields (area of approx. $93,000 \mu\text{m}^2$ each) were photographed per well. Standardization of XY image coordinates was as follows: the first image was taken at the centre of the well ($X = 0$; $Y = 0$) and the next four images were obtained sequentially from each corner of a hypothetical square enclosed in a circumference of 0.275 cm radius. The obtained five independent images were exported to Image J 1.37c software (NIH, Bethesda, MD, USA) for quantification analysis. Regions of interest (ROIs) outlining complete individual cells were done manually and the average intensity of the pixels inside each cell was calculated per micrograph. The background fluorescence estimated from outlined ROIs drawn without transecting any cell was subtracted from all monitored ROIs. The computed analysis of the five individual images was expressed as the average fluorescence intensity (arbitrary units, a.u.) for each experimental condition. Shown in the figures is typical immunofluorescence images for each experimental condition, taken from a single representative microscopic field without juxtaposition. When necessary, software adjustments were applied to the entire image.

Kinetic experiments and HPLC analysis

The kinetics of the extracellular catabolism of ATP and UDP in human primary BM-MSC cultures was evaluated, at 37°C , on days 7 and 21 (Costa et al., 2011; Noronha-Matos et al., 2012). After a 30-min equilibration period, the cells were incubated with gassed (95% O_2 plus 5% CO_2) Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 0.4 mM NaH_2PO_4 , 11.9 mM NaHCO_3 , and 11.2 mM glucose, pH 7.4) supplemented with 100 μM ATP or UDP (zero time). When testing the effect of NTPDase inhibitors, PSB 06126 (3 μM) or hN3-B3s (0.5 $\mu\text{g/ml}$), these drugs were added to the incubation solution 15 min before application of ATP and UDP. Samples (75 μl) were collected from each well at different times up to 30 min for HPLC analysis (LaChrom Elite, Merck, Frankfurt, Germany) of the variation of substrate disappearance and product formation; 20- μl injection volumes were used for the analysis. The concentrations of the substrates and their respective

metabolites were plotted as a function of time (progress curves, see e.g. Figure 7). The following parameters were analysed for each progress curve: half-life time ($t_{1/2}$) of the initial substrate, time of appearance of the different concentrations of the products, the concentration of the substrate or any product remaining at the end of the experiment, and the slope variation of ATP and UDP catabolism (rate of disappearance) obtained by linear regression between 0 and 15 min (linear region of the curve; Figure 7). The enzymatic activity was normalized to the number of viable cells given by the MTT assay (Figure 5; cf (Noronha-Matos et al., 2012)). The spontaneous degradation of ATP and UDP, at 37°C, was negligible over a 30-min time in the absence of the cells. At the end of the experiments, the remaining incubation medium was collected and used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity (Keiding et al., 1974). The negligible activity of LDH in the samples collected at the end of the experiments is an indication of the integrity of the cells during the experimental procedure.

Quinacrine-stained intracellular ATP deposits and extracellular ATP bioluminescence

We used quinacrine fluorescence staining (ex: 476 nm / em: 500–540 nm) to visualize ATP intracellular stores. To this end, cells were allowed to grow for 7 days in an osteogenic-inducible medium. After removing the incubation medium, the cells were washed three times with phosphate-buffered saline (PBS, 1x) and subsequently incubated for 1h with quinacrine (30 μ M), at 37°C (Pinheiro et al., 2013). Images were acquired using an epifluorescence microscope equipped with an XBO 75W Xenon arc lamp (Achromplan; Zeiss, Oberkochen, Germany). The light path included ET460/30 $\times$ excitation / ET520/40 m emission filters (Chroma Technology Corp, Bellows Falls, VT, USA) and a LUMPLFLN40XW/0.80NA/3.3WD water dipping objective lens (Olympus, Tokyo, Japan). A high-resolution cooled CCD camera (CoolSnap HQ, Roper Scientific Photometrics, Tucson, AZ, USA) connected to a computer running a digital image acquisition software (MetaFluor 6.3; Molecular Devices Inc., Sunnyvale, CA, USA) was used to record images in the TIFF format. The CCD exposure time was set to 100 ms, binning was adjusted to 2 and gain to 1.

Extracellular ATP was quantified using the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana, USA) in a multi-detection microplate reader (Synergy HT, BioTek Instruments, Vermont, USA), as described elsewhere (Pinheiro et al., 2013). Briefly, cells were seeded onto 96-well microplates, at a density of 2.5×10^4 cells/mL, for 7 days (2–4 replicas were performed per individual experiment). At the beginning of the experiment, cells were washed twice with Tyrode's solution and allowed to equilibrate for 30 min, at 37°C. After equilibrium, cells were washed again and incubated

with Tyrode's solution in the absence or the presence of test drugs; 30 min later, the incubation fluid was removed and snap-frozen in liquid nitrogen. Before adding the luciferin-luciferase mixture, collected samples were defrosted until 25°C according to the manufacturer's instructions. Sample bioluminescence was compared to external high-purity ATP standards; these were prepared daily within the same concentration range; all samples were run in duplicate. The remaining incubation medium was used to quantify the LDH (EC 1.1.1.27) activity (Keiding et al., 1974), to evaluate cell integrity during the experimental period (see e.g. Ref. (Pinheiro et al., 2013)). The LDH activity was negligible (< 0.1 mU/mL) in all measured samples indicating the integrity of the cells during the experimental period.

Presentation of data and statistical analysis

Data are expressed as mean $\pm$ S.E.M. from an n number of individuals. Due to restricted access to similar human samples and a limited pool of initial cell density, we were unable to perform all the indicated assays in all collected human samples. Therefore, immunoblot data are expressed as individual bars to show inter-individual variability and the lack of influence of the protein used for normalization purposes. Normality tests included D'Agostino & Pearson and Shapiro–Wilk, depending on sample size. According to normality test results, statistical analyses included parametric (one-way ANOVA with Sidak's test for multiple comparisons or the two-tailed unpaired t-test for double comparisons) or nonparametric tests (the Wilcoxon signed rank test for comparing the median of a set of values against a hypothetical median), besides, with a confidence level of 0.05 (95% confidence interval). Values of $P < 0.05$ were considered to represent significant differences.

Results

Age-related differences in the expression of NTPDases and ecto-5'-nucleotidase/CD73 in human BM-MSCs undergoing osteogenic differentiation

Using the MTT assay and DNA quantification methods, we showed that cultured human BM-MSCs proliferate in osteogenic-inducing media until near confluence is reached at culture day 21–28. While BM-MSCs retain their proliferative potential throughout life, the cells originating from Pm women have decreased ability to differentiate into bone-forming osteoblasts compared to those from younger females (Noronha-Matos et al., 2012). Although usage of different collection bone sites among the two women groups may be a limitation, the observed changes can hardly be explained solely by this bioethical constraint because care was taken to strictly use cancellous bone samples outside the subchondral bone region that would otherwise be influenced by the non-inflammatory degenerative joint disease process affecting Pm women samples, but not those from younger females. Here, we questioned whether the lack of tonic activity of P2X₇ and P2Y₆ receptors and, thereby, the purinergic-induced osteogenic commitment of Pm BM-MSC could be due to changes in the expression and activity of extracellular nucleotide metabolizing enzymes, namely NTPDases (Noronha-Matos et al., 2012), compared to cells of younger females.

Pm BM-MSCs show immunoreactivity against NTPDase1, -2, and -3 (Figure 4A, see also (Noronha-Matos et al., 2012)); while NTPDase1 and NTPDase2 enzymes were more expressed at early culture stages (day 7), NTPDase3 becomes the most represented enzyme at culture day 21 (Figure 4A and B). This scenario differs from that observed in osteogenic differentiating cells obtained from younger females, in which NTPDase1 and -2 protein amounts remain high throughout the culture period (7 = 21 days); the Pm/young females ratio ranged from 0.53 to 0.72 concerning NTPDase1 and -2 protein amounts at culture day 21, respectively (Figure 4A and B). The most striking difference relates to the expression of NTPDase3. While NTPDase3 protein amounts progressively increased with time in cells from Pm women (7 < 21 days), this enzyme almost disappeared from differentiated BM-MSCs of younger females (7 > 21 days); BM-MSCs from Pm women display 5.6-fold higher NTPDase3 amounts compared to the cells from younger females at culture day 21 (Figure 4A and Biii). We confirmed this phenotypic divergence using two distinct NTPDase3 antibodies, including the previously available hN3-1c1₄ polyclonal antibody (Noronha-Matos et al., 2012) and a newer highly-specific hN3-B3_s monoclonal antibody (Figure 4A) which is also instrumental as an enzyme inhibitor (Munkonda et al., 2009). The absence of NTPDase3 immunoreactivity in BM-MSCs from age-matched males,

while keeping a similar density pattern regarding other ectonucleotidases (see Supplementary figure 1), suggests

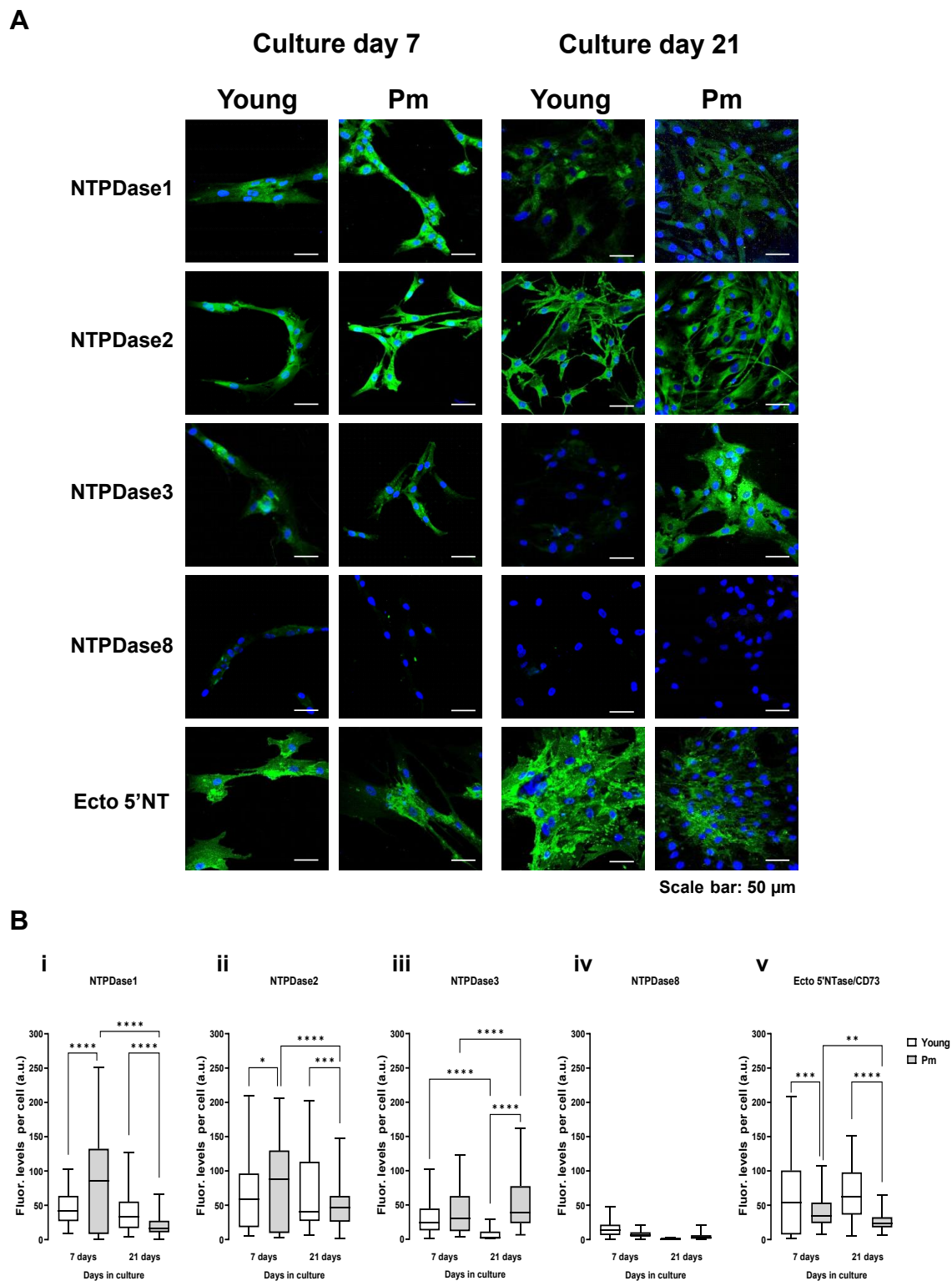


Figure 4 – The pattern of NTPDases expression in human BM-MSCs. Panel A presents representative images of the immunocytochemical detection of NTPDase1, -2, -3, -8 and ecto-5'-nucleotidase (Ecto 5'NTase/CD73) in cultured BM-MSCs (first subculture) obtained from a young female and a Pm woman. Shown is the time-related immunoreactivity (green) detected by confocal microscopy in BM-MSCs allowed to grow for 7 and 21

days in an osteogenic-inducing medium. Blue dots represent nuclei stained with DAPI. Experiments were performed in parallel keeping unaltered the settings of the confocal microscope throughout the documentation procedure (see Materials and Methods). The scale bar is 50 μm . In panel B, shown are immunofluorescence intensity graphs computed from confocal microscopy images acquired as in panel A. Ordinates represent fluorescence intensity per cell (arbitrary units, a.u.) of enzymes immunoreactivity as a function of the (continue)

(continue) number of days in culture (day 7 and 21). Regions of interest (ROIs, corresponding to individual BM-MSCs) were manually and the average intensity of the pixels inside this area was calculated. Background fluorescence was estimated from outlined regions (with no cells) and subtracted from all monitored ROIs. A total of 58–234 cells were analysed from three young females (21 ± 7 years old) and six Pm women (63 ± 4 years old). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ (one-way ANOVA with Sidák's multiple comparison test, single pooled variance) represent significant differences.

that overexpression of NTPDase3 may be a hallmark of osteogenic deficiency in Pm women. Figure 4 also shows that NTPDase8 is the least represented ectoenzyme of this series in BM-MSCs from both women groups, strengthening the idea that it might have a limited role in human osteogenesis.

Ecto-5'-nucleotidase/CD73 is expressed by more than 95% of osteoprogenitor cells (Lu et al., 2010). Even though we show that ecto-5'-nucleotidase/CD73 immunoreactivity is present in BM-MSCs at all differentiation stages (Figure 4A; see also Ref (Noronha-Matos et al., 2012)), the density of this adenosine-forming enzyme is consistently reduced (by 30–60%) in the cells from Pm women compared to those obtained from younger females (Figure 4Bv).

Age-related variation in the global NTPDase activity of human BM-MSCs undergoing osteogenic differentiation

Figure 5 illustrates the global NTPDase activity in osteogenic differentiating BM-MSC cultures from Pm women and younger females normalized by cells growth/viability (MTT assay) using ATP (Figure 5A) and UDP (Figure 5B) as substrates (100 μM , for 30 min). Data show that BM-MSCs from Pm women hydrolyze ATP (100 μM) 1.16 and 2.63 times faster than cells from younger females considering culture days 7 and 21, respectively (Figure 5A). The extracellular hydrolysis of UDP (100 μM) was slower in less differentiated cells (7-day cultures), but it exceeded the rate of ATP (100 μM) catabolism by Pm BM-MSC cultures at culture day 21 (Figure 5B); at this time point, the total NTPDase activity was 3.64 times faster than that achieved in cells from younger females (Figure 5B).

Inhibition of NTPDase3 activity increases the extracellular accumulation of ATP and UDP in osteogenic differentiating BM-MSCs from Pm women

So far, we showed that NTPDase3 expression increases over time in cultured BM-MSCs obtained from Pm women (Figure 4Biii), which correlates with faster kinetics of ATP and UDP breakdown in these cells compared to those originating from younger females (Figure

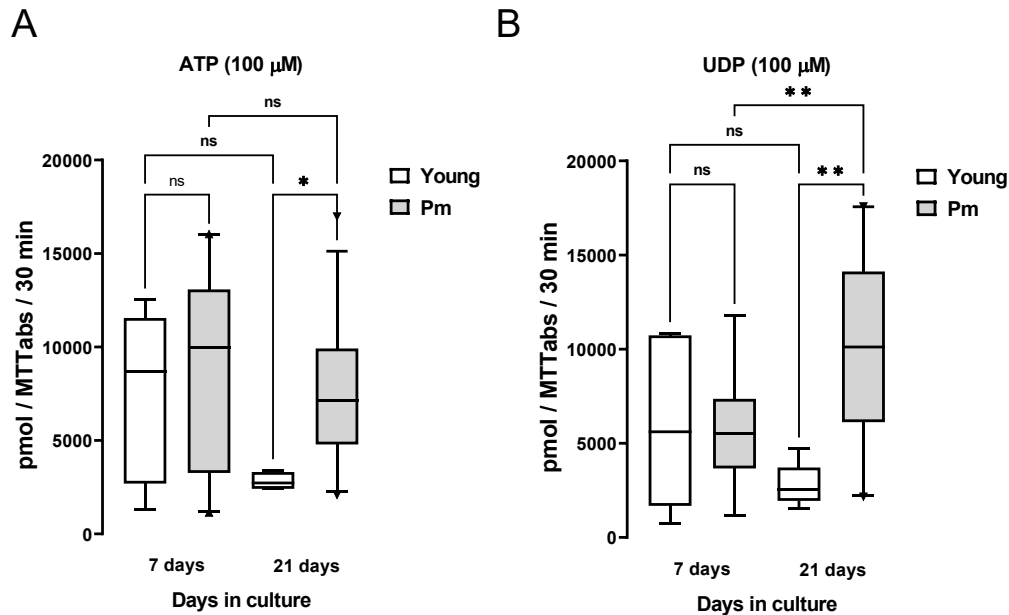


Figure 5 – The extracellular catabolism of ATP and UDP is faster in BM-MSCs from Pm women than in cells from younger females. Represented is the total NTPDase activity measured in BM-MSCs (first subculture) from young females and Pm women normalized by the number of viable cells given by the MTT assay when ATP (**A**) and UDP (**B**) were used as substrates. Cells were allowed to grow for 7 and 21 days in an osteogenic-inducing medium. Nucleotides (100 μ M) were added to the culture medium at zero time. Samples (75 μ l) were collected from each well 30 min after incubation with the nucleotides. Each collected sample was analysed by HPLC to measure the amounts of ATP or UDP remaining in the incubation fluid. Boxes and whiskers represent pooled data from three young females (19 ± 6 years old) and twelve Pm women (69 ± 3 years old); each individual was tested in triplicate. * $P < 0.05$ and ** $P < 0.01$ (one-way ANOVA with Sidák's multiple comparison test, single pooled variance) represent significant differences

5). Next, we questioned whether the age-related upsurge in the expression and/or activity of NTPDase3 contributes to impair the osteogenic commitment of Pm BM-MSCs owed to tonic P2X₇ and P2Y₆ purinoceptors activation by endogenously released ATP and UDP, respectively. Figure 6A shows that the selective inhibition of NTPDase3 with two chemically distinct compounds, the anthraquinone derivative PSB 06126 (3 μ M) (Baqi et al., 2009) and the monoclonal antibody hN3-B3_s (0.5 μ g/ml) (Munkonda et al., 2009), significantly increased ATP levels in the incubation medium of Pm BM-MSC cultures, but the same was not observed when cultured cells were obtained from younger females. To rule out the possibility that loss of the purinergic tone in cultured BM-MSCs from Pm women could be due to the depletion of ATP-containing intracellular reservoirs, we labelled the cells with quinacrine, a fluorescent dye normally staining ATP-containing granules. Figure 6B shows that quinacrine fluorescence exists scattered all over the cytoplasm of cultured Pm BM-MSCs in significant amounts, thus reproducing the staining pattern obtained by other groups in differentiated osteoblasts (Orriss et al., 2009).

Selective inhibition of NTPDase3 with PSB 06126 (3 μ M) and hN3-B3_s (0.5 μ g/ml) decreased the extracellular catabolism of ATP (100 μ M; Figure 7A) and UDP (100 μ M; Figure 7B) in osteogenic differentiating Pm BM-MSC cultures. While PSB 06126 (3 μ M) had

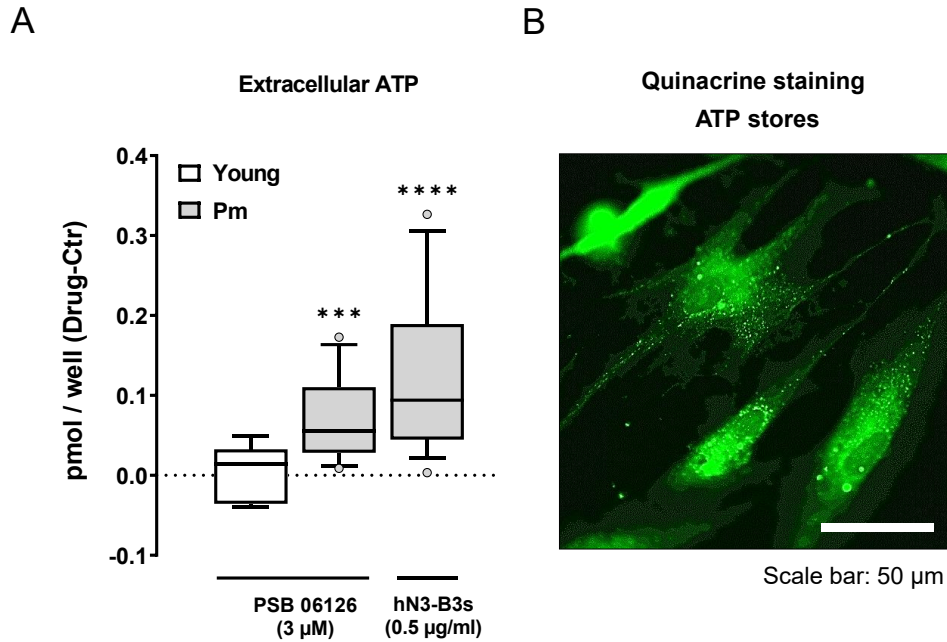
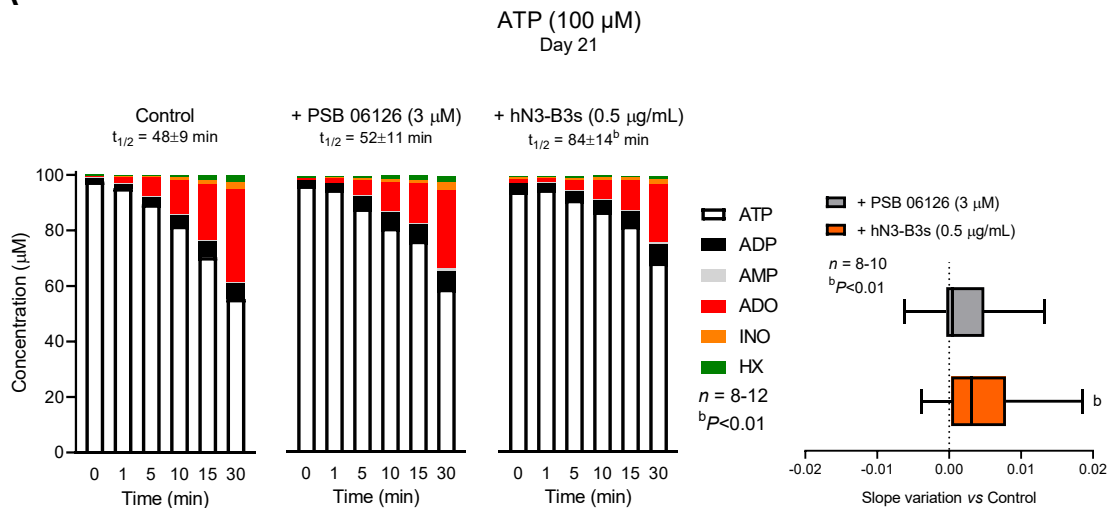


Figure 6 – Selective inhibition of NTPDase3 with PSB 06126 (3 μM) or the monoclonal antibody hN3-B3s (0.5 μg/ml) increases the extracellular ATP accumulation in Pm BM-MSCs cultures, but not in those obtained from younger females, when cells were allowed to grow for 7 days in an osteogenic-inducing medium (first subculture). In **panel A**, ordinates are the difference between endogenously released ATP during 30 min by cells of the same individual incubated in the presence and the absence of NTPDase3 inhibitors (Drug-Ctr, respectively); zero represents the identity between ATP values obtained in the absence and the presence of the inhibitors (horizontal dashed line). The ATP content of the samples was measured using the luciferin-luciferase bioluminescence assay (75 μl/well; see Materials and Methods for details). Boxes and whiskers represent pooled data from three younger females (21 ± 7 years old; ATP release in Ctr: 90 ± 20 fmol/well) and six Pm women (68 ± 5 years old; ATP release in Ctr: 180 ± 40 fmol/well); two to four replicates were performed per individual. ***P < 0.001 and ****P < 0.0001 (Wilcoxon signed rank test for comparing medians with a hypothetical null variation) represent significant differences. **Panel B** shows that BM-MSCs from a Pm woman undergoing osteogenic differentiation exhibit high amounts of ATP inside intracellular granules stained with quinacrine. The scale bar is 50 μm.

only a minor increasing effect on the extracellular ATP (100 μM) half-life, inhibition of NTPDase3 with the monoclonal antibody hN3-B3s (0.5 μg/ml) increased the half-life of this nucleotide by 1.75 times (Figure 7A). ATP (100 μM) was sequentially metabolized into ADP, AMP, adenosine (ADO), inosine (INO) and hypoxanthine (HX); at the end of the 30-min incubation period, adenosine was the more represented ATP metabolite, but its levels significantly decreased in the presence of NTPDase3 inhibitors (Figure 7A). Overall, these findings, together with the negligible accumulation of AMP, INO and HX in the cultures, are in keeping with the concept that human BM-MSCs usually exhibit high ecto-5'-nucleotidase/CD73 and low adenosine deaminase (ADA) activities, thus favouring the accumulation of adenosine as the main extracellular ATP metabolite – see e.g. Ref (Costa et al., 2011).

Likewise, both PSB 06126 (3 μM) and hN3-B3s (0.5 μg/ml) increased the extracellular half-life of UDP (100 μM) by 1.68 and 3.14 times, respectively (Figure 7B). Comparing the slope variation of extracellular ATP and UDP disappearance from culture media, data indicate that

A



B

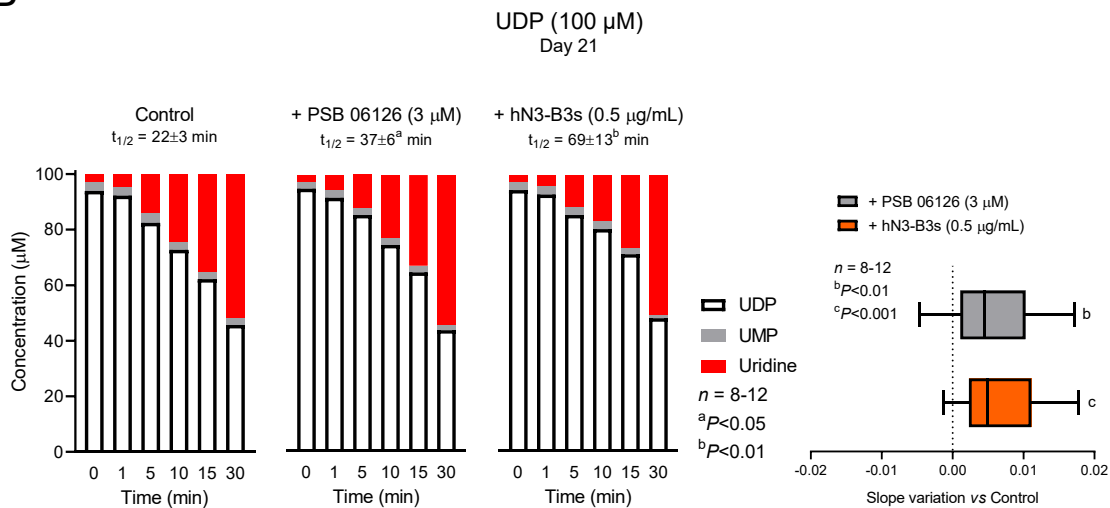


Figure 7 – Selective inhibition of NTPDase3 attenuates the extracellular catabolism of (A) ATP and (B) UDP in BM-MSCs from Pm women allowed growing for 21 days in an osteogenic-inducing medium. ATP (100 μ M) or UDP (100 μ M) were added to the culture medium at time zero either in the absence (left-hand-side panels) or in the presence of PSB 06126 (3 μ M; middle panels) or hN3-B3s (0.5 μ g/mL; right-hand-side panels). 75- μ l samples were collected at the indicated times for HPLC analysis to quantify the substrates ATP or UDP (white bars) and their metabolites, namely ADP (black), AMP (grey), adenosine (ADO, red), inosine (INO, orange), hypoxanthine (HX, green) or UMP (grey) and uridine (red). The calculated half-life time ($t_{1/2}$) for each initial substrate is shown for comparison; $^aP < 0.05$ and $^bP < 0.01$ (two-tailed unpaired t-test) represent significant differences. Right-hand-side box and whiskers graphs represent the effect of PSB 06126 (3 μ M) and hN3-B3s (0.5 μ g/mL), assessed by comparing the slope variation of ATP or UDP disappearance in the presence of NTPDase3 inhibitors or under control conditions (with no added drugs); zero represents identity between obtained values in the absence and the presence of the inhibitors. Box and whiskers represent pooled data from eight to twelve Pm women (69 ± 3 years old); cells from each individual were tested in triplicate. $^bP < 0.01$ and $^cP < 0.001$ (one-way ANOVA with Sidák's multiple comparison test, single pooled variance) represent significant differences.

selective NTPDase3 inhibition was more effective towards inhibition of extracellular UDP catabolism than that of ATP (Figure 7A and B, right-hand-side panels). Accumulation of UMP was almost negligible throughout the enzymatic assay, which may be explained considering that these cells exhibit significant ecto-5'-nucleotidase/CD73 activity converting very rapidly

adenine/uracil monophosphates into their nucleoside metabolites, either adenosine (Figure 7A) or uridine (Figure 7B).

Inhibition of NTPDase3 activity fosters osteogenic differentiation and matrix mineralization of cultured BM-MSCs from Pm women

Selective inhibition of NTPDase3 with the monoclonal antibody hN3-B3_s (0.5 µg/ml; Figure 8D), but not with the anthraquinone derivative PSB 06126 (3 µM; Figure 8A), increased the ALP activity of osteogenic differentiating Pm BM-MSCs by $237 \pm 63\%$ and $329 \pm 129\%$ above control levels at culture days 7 and 21, respectively. None of these compounds changed ($P > 0.05$) cells growth/viability predicted by the MTT assay obtained in the presence of PSB 06126 (3 µM; 0.22 ± 0.02 vs. control of 0.23 ± 0.02 at culture day 7, and 0.56 ± 0.07 vs. control of 0.63 ± 0.08 at culture day 21) or of hN3-B3_s (0.5 µg/ml; 0.33 ± 0.06 vs control of 0.34 ± 0.06 at culture day 7, and 0.63 ± 0.09 vs control of 0.62 ± 0.09 at culture day 21). Notwithstanding this disparity, both PSB 06126 (3 µM) and hN3-B3_s (0.5 µg/ml), significantly ($P < 0.05$) increased matrix mineralization on Pm BM-MSC culture day 35 by $243 \pm 35\%$ (Figure 8B) and $842 \pm 282\%$ (Figure 8E) above control values, respectively; typical Alizarin Red histoenzymatic culture staining is shown in panels C and F of Figure 8, respectively.

Activation of P2X₇ and P2Y₆ purinoceptors are most likely implicated in the promotor effects of PSB 06126 (3 µM) and hN3-B3_s (0.5 µg/ml) on matrix mineralization of Pm BM-MSC cultures (Noronha-Matos et al., 2014; Noronha-Matos et al., 2012; Panupinthu et al., 2007), considering that these effects were prevented by A438079 (3 µM; Figure 8B and C) and by MRS 2578 (100 nM; Figure 8E and F), which selectively antagonize P2X₇ and P2Y₆ receptors, respectively. On their own, these two purinoceptor antagonists did not change the total mineralized area of the cultures. These findings indicate that endogenously produced adenine and uracil nucleotides by Pm BM-MSCs might not reach high enough concentrations required to activate P2X₇ and P2Y₆ receptors unless the NTPDase3 activity is inhibited to recapitulate the lack of expression/activity of this enzyme (see Figure 4 and Figure 5) and the mineralization potential of BM-MSCs from younger females (Noronha-Matos et al., 2014; Noronha-Matos et al., 2012).

Transient NTPDase3 gene silencing using a short hairpin RNA rescues the osteogenic differentiation potential of cultured BM-MSCs from Pm women

To avoid the negative impact on osteogenic differentiation and matrix mineralization of NTPDase3 overexpression in BM-MSCs from Pm women, we infected the cells with a series

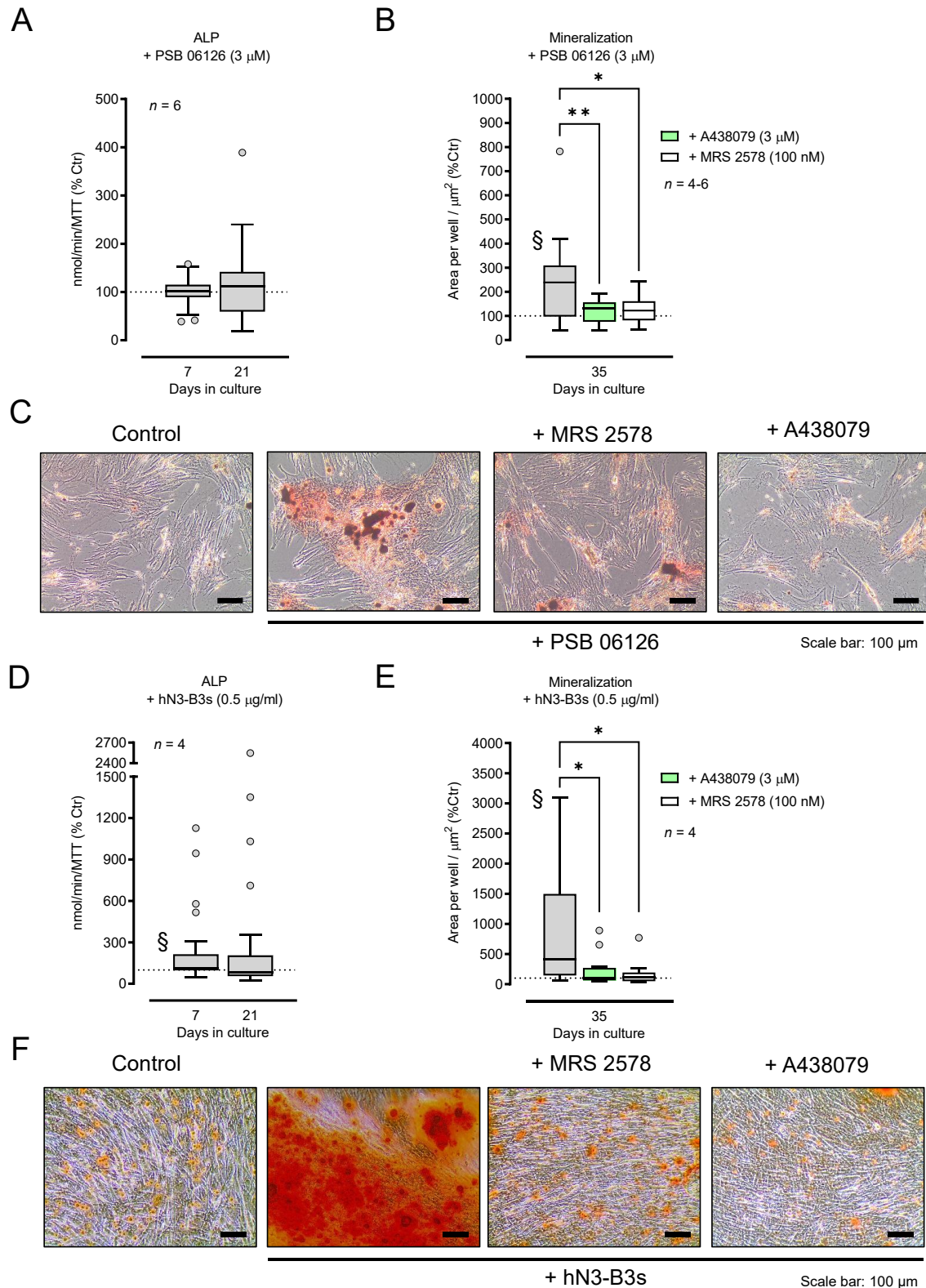


Figure 8 – Selective inhibition of NTPDase3 activity with the pharmacological inhibitor, PSB 06126 (3 μ M; **panels A–C**), or with the monoclonal antibody, hN3-B3s (0.5 μ g/ml; **panels D–F**), facilitates osteogenic differentiation and mineralization of cultured BM-MSCs from Pm women via the activation of P2X7 and/or P2Y₆ receptors. Each inhibitor was added to osteogenic-inducing media at culture day 0 and remained throughout the assay, i.e. until day 35. **Panels A and D** show the per cent variation of the ALP activity of Pm BM-MSC cultures on days 7 and 21 corrected for cells growth/viability (MTT assay) determined in the presence of NTPDase3 inhibitors versus the control situation with no added drugs (100%; 9.6 ± 2.6 and 18.3 ± 4.0 nmol/min/MTT at days 7 and 21, respectively). **Panels B and E** show the per cent variation of the total mineralized area of Pm BM-MSC at culture day 35 in the presence of NTPDase3 inhibitors compared to the control situation with (*continue*)

(continue) no added drugs (100%; $10,079.2 \pm 4210.5 \mu\text{m}^2$). $^{\S}P < 0.05$ (Wilcoxon signed rank test for comparing medians with a hypothetical null variation, 100%) represent significant differences. Involvement of P2X₇ and P2Y₆ purinoceptors activation in extracellular matrix mineralization was confirmed using selective antagonists, A438079 (3 μM) and MRS 2578 (100 nM), respectively, which were added to culture media synchronously to NTPDase3 inhibitors. **Panels C and F** show Alizarin Red staining indicating extracellular matrix mineralization and bone nodule formation of BM-MSC cultures (red-brownish spots) in two Pm women; the scale bar is 100 μm . Boxes and whiskers represent pooled data from four to six Pm women (70 ± 3 years old); three to eight replicas were made per individual experiment. $^*P < 0.05$ and $^{**}P < 0.01$ (one-way ANOVA with Sidák's multiple comparison test, single pooled variance) represent significant differences.

of lentiviruses carrying information to short hairpin RNAs (shRNAs) designed to silence NTPDase3 gene expression. The most effective sequence (TL313202VD from ORIGENE) was selected among four blocking sequences tested together with a scramble sequence (negative control) unable to associate with the target mRNA (see Materials and Methods; validation experiments are shown in Supplementary figure 2). Treatment of Pm BM-MSC cultures with the lenti-shRNA encoding to the TL313202VD sequence at a multiplicity of infection 3 (MOI 3) transiently decreased ($P < 0.05$) the NTPDase3 immunoreactivity of the cells at culture day 7 (23 ± 1 a.u.) compared to the situation where the scramble sequence was used (51 ± 2 a.u.); full recovery of NTPDase3 protein amounts was observed at culture day 21 (Figure 9A). Even though NTPDase3 gene silencing transiently decreased protein production for only a week under these experimental conditions, it had an outstanding effect to promote osteogenic commitment (increase in ALP activity at culture days 7 and 21; Figure 9Bi) and matrix mineralization (Alizarin Red bone nodules increased by $297 \pm 32\%$ at day 35; Figure 9Bii and 9Biii) of cultured BM-MSCs from Pm women, without significantly modifying cells growth/viability (MTT assay; data not shown).

Inhibition of NTPDase3 expression/activity rehabilitates age-dependent deficits of the osteogenic transcription factor, Osterix, in cultured Pm BM-MSCs

Osterix is a zinc finger-containing transcription factor that is essential for osteoblast differentiation and bone formation (Nakashima et al., 2002). Osterix deficiency has been associated with age-related osteoporosis – reviewed in (Liu et al., 2020). The immunoblots depicted in Figure 10A show that 21-day BM-MSC cultures from two younger females express higher amounts of Osterix normalized by the housekeeping protein β -actin than the cells from Pm women grown under similar experimental conditions (Figure 10B). Pharmacological inhibition of NTPDase3 enzymatic activity of Pm BM-MSCs with PSB

06126 (3 μ M) recapitulates the Osterix/ β -actin ratio to levels observed in younger females (Figure 10B). A similar effect was also observed using a different protein normalizer, GAPDH, in cells from a distinct Pm woman (Figure 10B).

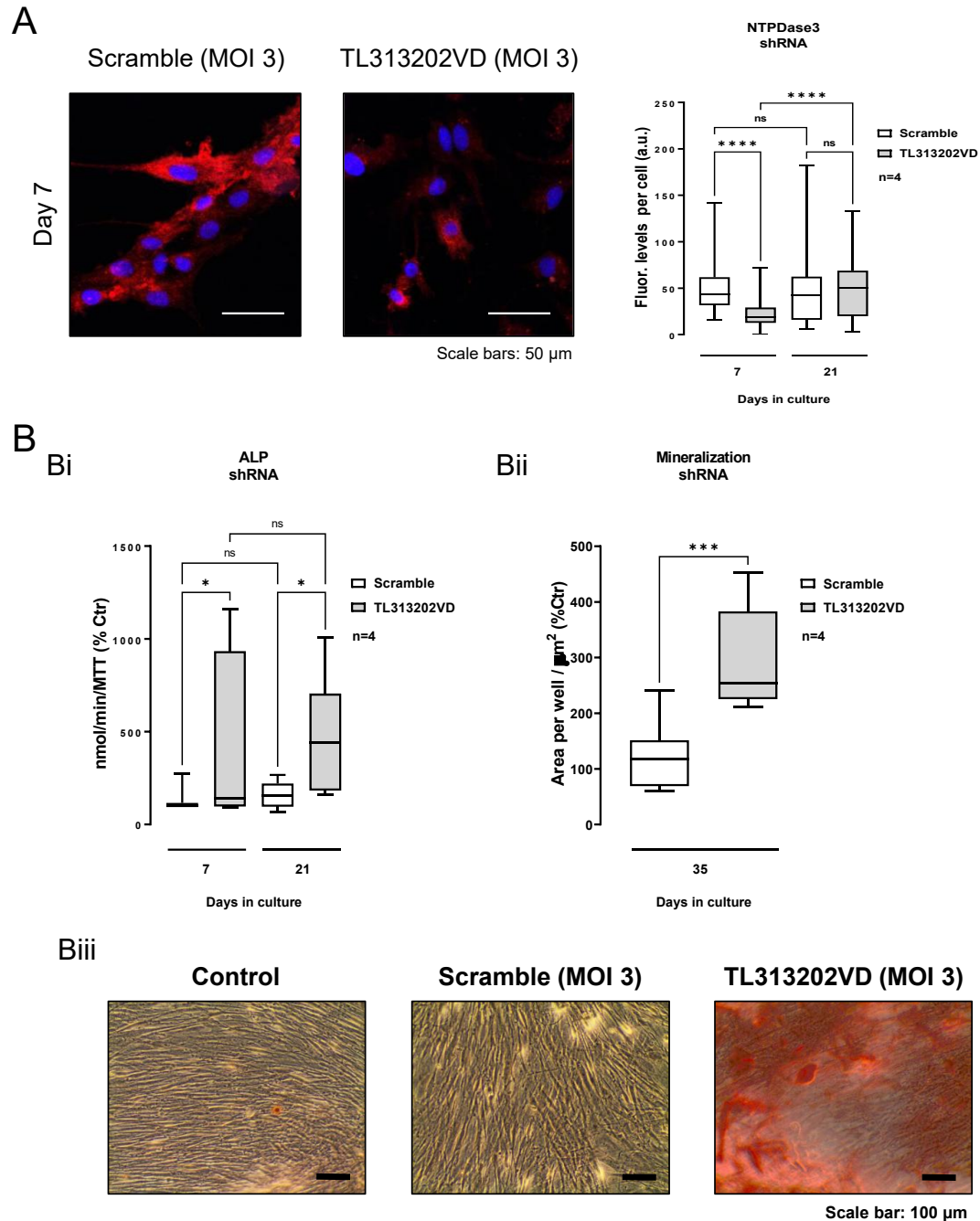


Figure 9 – NTPDase3 gene silencing using lenti-shRNA promotes mineralization of Pm BM-MSC cultures allowed to grow for 35 days in an osteogenic-inducing medium. Cells were exposed for 24h to a lenti-shRNA encoding to a sequence (TL313202VD) designed to silence NTPDase3 or to a scramble sequence applied at multiplicities of infection 3 (MOI 3). Confocal microscopy images shown in **panel A** confirm that NTPDase3 immunoreactivity transiently decreases 7-days after exposure of Pm BM-MSC cultures to a lenti-shRNA encoding to the TL313202VD sequence, but not when the cells were treated with the scramble sequence. Blue dots represent nuclei stained with DAPI. Experiments were performed in parallel keeping unaltered the settings of the confocal microscope throughout the documentation procedure (see Materials and Methods). The scale bar is 50 μ m. **Panel Bi** shows the per cent variation of the ALP activity of Pm BM-MSC cultures on days 7 and 21 (*continue*)

(*continue*) corrected for cells growth/viability (MTT assay) obtained after treatment with the lenti-shRNA encoding to TL313202VD or the scramble sequence (MOI 3) (100%; 0.6 ± 0.1 and 7.1 ± 3.7 nmol/min/MTT at days 7 and 21, respectively). **Panel Bii** shows the per cent variation of the total mineralized area of Pm BM-MSCs at culture day 35 after treatment with the lenti-shRNA encoding to TL313202VD or the scramble sequence (MOI 3) (100%; $13,714.1 \pm 3657.6 \mu\text{m}^2$). **Panel Biii** shows the Alizarin Red staining denoting extracellular matrix mineralization and bone nodule formation of BM-MSC cultures (red-brownish spots) in a Pm woman; the scale bar is 50 μm . Boxes and whiskers represent pooled data from four Pm women (69 ± 7 years old); three to four replicas were made per individual. In **panel Bi**, * $P < 0.05$ (one-way ANOVA with Sidák's multiple comparison test, single pooled variance) represents significant differences; in **panel Bii**, *** $P < 0.01$ (two-tailed unpaired t-test) represent significant differences.

Similarly, NTPDase3 gene silencing in Pm BM-MSCs using lenti-shRNAs encoding to TL313202VC (Seq C; MOI 3; Figure 10C) and TL313202VD (Seq D; MOI 3; Figure 10D) sequences also increased the relative amount of the Osterix transcription factor independently of the normalizer protein used, β -actin or GAPDH. Uncropped full-length gel blots may be found in Supplementary figure 4. Altogether, these findings suggest that curbing the expression and/or activity of the nucleotide metabolizing enzyme, NTPDase3, may recapitulate the osteogenic potential of Pm BM-MSCs to the levels observed in younger females

Discussion

Previous accounts from our group suggest that the osteogenic differentiation potential of BM-MSCs is compromised in Pm women compared to that of younger females (Noronha-Matos et al., 2014; Noronha-Matos et al., 2012); this situation may be in part attributed to impairment of the P2X7 and P2Y₆ purinoceptors tonic activation by released adenine and uracil nucleotides, such as ATP and UDP, respectively (Noronha-Matos et al., 2014; Noronha-Matos et al., 2012); for a review, see e.g. Ref (Noronha-Matos & Correia-de-Sá, 2016). Here, we provide new evidence that the purinergic signalling deficiency in Pm women is caused by outstanding hydrolysis of extracellular nucleotides due to NTPDase3 overexpression, which turns out to be the dominant ecto-nucleotidase subtype at this age and gender group. This contrasts with the minute amounts of the NTPDase3 enzyme found in osteogenic differentiating BM-MSCs of younger females and age-matched males. More importantly, our data show for the first time that transient NTPDase3 gene silencing with a lenti-shRNA and/or inhibition of its enzymatic activity with the anthraquinone derivative PSB 06126 (Baqi et al., 2009) or the monoclonal antibody hN3-B3_s (Munkonda et al., 2009) rescues the P2X7 and P2Y₆ receptors-induced osteogenic commitment of Pm BM-MSC. Consequently, this results in extensive matrix mineralization of Pm BM-MSC cultures to the levels found in younger females. These findings lead us to propose that inhibition of NTPDase3 expression and/or activity may be a novel therapeutic strategy to rehabilitate the

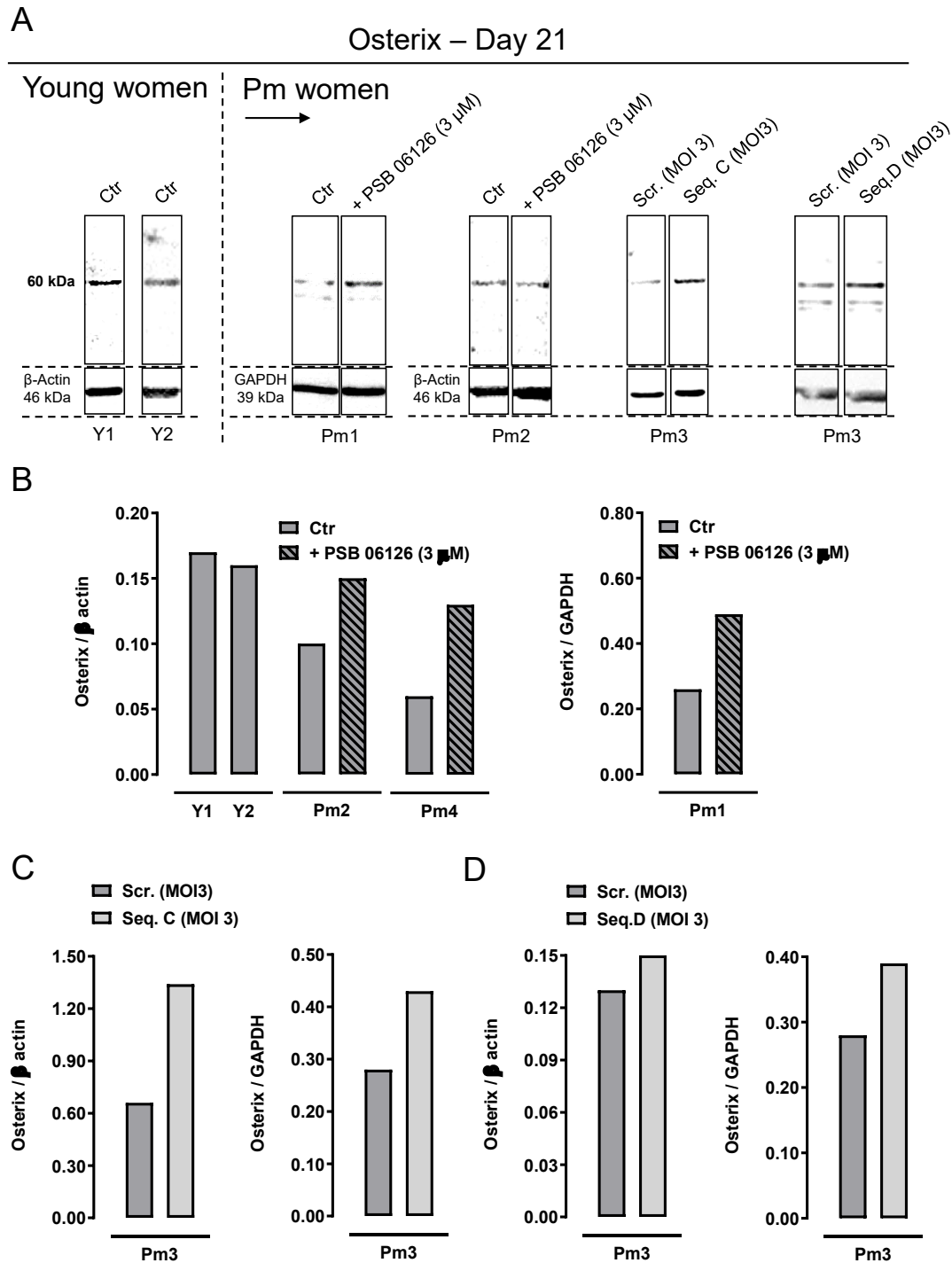


Figure 10 – Inhibition or silencing of NTPDase3 increases the amount of osteogenic transcription factor Osterix in 21-day BM-MSC cultures (first subculture) obtained from Pm women to the levels found in younger females. In **panel A**, shown are typical immunoblots stained for Osterix (52–61 kDa) transcription factor in BM-MSCs from two young (Y1 and 2) and three Pm (Pm1, 2 and 3) women cultured in an osteogenic-inducing medium (i) in the absence or the presence of the NTPDase3 inhibitor, PSB 06126 (3 μM), or (ii) after NTPDase3 gene silencing with lenti-shRNAs encoding for TL313202VD (Seq D; MOI 3) and TL313202VC (Seq C; MOI 3) inhibitory vs. control scramble sequences; either β -Actin (46 kDa) or GAPDH (39 kDa) protein amounts were used as house-keeping gene product standards for normalization purposes. Uncropped full-length gel blots can be found in Additional file 4: Fig. S4. **Panels B-D** shows the relative expression of Osterix in BM-MSCs from young females and Pm women submitted or not to NTPDase inhibition with PSB 06126 (3 μM) or to gene silencing with lenti-shRNAs encoding for TL313202VD (Seq D; MOI 3) and TL313202VC (Seq C; MOI 3). Each bar corresponds to the results from a single patient, thus reflecting some inter-individual variability

osteogenic potential of senescent BM-MSCs that is required to increase bone formation in Pm women. Human osteoprogenitor MSCs and osteoblast-like cells constitutively release ATP without cell damage; the estimated intracellular ATP levels are between 2 and 5 mM (Brandao-Burch et al., 2012; Buckley et al., 2003; Genetos et al., 2005; Riddle et al., 2007; Romanello et al., 2001), most of which can be easily identified in vesicular/granular organelles stained with fluorescent dyes, like quinacrine (Orriss et al., 2009).

Here, we found that quinacrine-stained ATP-containing granules were scattered abundantly in the cytoplasm of Pm BM-MSCs, meaning that loss of intracellular ATP reservoirs does not seem to account significantly for age-dependent purinergic signalling deficits and, thus, the shortage of the osteogenic commitment of Pm BM-MSCs.

Like ATP, the ability of various cell types (e.g. murine airway epithelial cells, human cardiomyocytes) to release UTP has been directly confirmed; using a laborious enzymatic assay, the release of UTP is estimated to be approximately 10 to 30% of all detected nucleotides including its breakdown products, like UDP (Lazarowski et al., 2003; Lazarowski & Harden, 1999). Likewise, UDP-sugars, like the P2Y₁₄ receptor activator UDP-glucose, may be released from cells together with ATP under certain experimental conditions (Kreda et al., 2007; Kreda et al., 2008; Lazarowski et al., 2003). UDP itself is an end product of glycogen synthesis that may be released into the extracellular fluid (Adeva-Andany et al., 2016; Lazarowski et al., 1997). Yet, the extracellular accumulation of uracil nucleotides, such as UTP and UDP, does not necessarily depend on their outflow from intracellular pools via dedicated transport systems; these nucleotides may accumulate in the extracellular fluid as a consequence of the metabolism (hydrolysis or synthesis) of nucleotide precursors that are directly released by cells under various physiological and pathological conditions (Burrell et al., 2005; Buxton et al., 2001; Joseph et al., 2004; Yegutkin et al., 2001; Yegutkin et al., 2002). There are two main mechanisms underlying the non-lytic release of nucleotides from cells: (a) exocytotic release, specifically concentrated within secretory granules or vesicles, and (b) controlled release of cytosolic nucleotides via intrinsic plasma membrane channels or pores, which includes ABC transporters, connexin hemichannels, voltage-dependent anion channels and the P2X₇ receptor channel itself (Donahue, 2000; Orriss et al., 2009; Riddle et al., 2007); reviewed in (Burnstock, 2006). Increasing evidence demonstrates that all stimuli that foster bone formation and accelerated fracture healing (e.g. mechanical loading, low-intensity pulsed ultrasound, 1,25(OH)₂ vitamin D₃, bisphosphonates) favour ATP release from osteoblast-like cells (Alvarenga et al., 2010; Biswas & Zanello, 2009; Hayton et al., 2005; Riddle et al., 2007; Romanello et al., 2006; Rumney et al., 2012), which levels depend on the differentiation status of the cells (Orriss

et al., 2009); using rat osteoblasts, these authors demonstrated that mature bone-forming cells release up to sevenfold more ATP than highly-proliferative immature cells. In this study, we failed to demonstrate any tonic activity of the P2X7 receptor in Pm BM-MSCs using its selective antagonist, A438079, which was applied in a concentration (3 μ M) that almost prevented the ALP activity in cells from younger females grown under similar experimental conditions (Noronha-Matos et al., 2014), and the same occurred regarding the P2Y₆ receptor (Noronha-Matos et al., 2012).

Considering that no differences were observed in the expression of osteogenic-inducing P2X7 and P2Y₆ receptors between young and aged women groups (Noronha-Matos et al., 2014; Noronha-Matos et al., 2012), we hypothesized that deficient activity of these two receptors may exist because their endogenous ligands (ATP and UDP, respectively) cannot reach high enough levels in cells from Pm women unless NTPDases (namely NTPDase3) expression and/or activity are inhibited. NTPDase3 is overexpressed in Pm BM-MSCs compared to the cells originating from younger females and age-matched males, thus contributing to accelerating the breakdown of ATP and UDP released in the proximity of ionotropic P2X7 and metabotropic P2Y₆ receptors. Moreover, we proved that (i) endogenously released ATP accumulates in culture media when Pm BM-MSCs were incubated with the two selective NTPDase3 inhibitors, PSB 06126 and hN3-B3_s, and that (ii) the kinetics of the extracellular ATP and UDP catabolism was delayed in the presence of either of these two NTPDase3 inhibitors. Moreover, transient NTPDase3 gene silencing with the lenti-shRNA encoding for the TL313202VD sequence and enzyme inhibition with PSB 06126, both rescued the expression of the osteogenic transcription factor Osterix, the ALP activity and matrix mineralization of Pm BM-MSC cultures grown in osteogenic-inducing media to the levels observed in cells from younger females. Osterix is known to trigger BM-MSCs differentiation into mature osteoblasts leading to the subsequent production of extracellular matrix proteins involved in mineralization, namely bone sialoprotein, osteocalcin and osteopontin, making it an ideal primary marker of the osteogenic commitment of these cells – see e.g. Ref (Nakashima et al., 2002).

Species and cell maturation differences in the expression and activity of certain nucleotide metabolizing enzymes, like NTPDases, (Noronha-Matos et al., 2012) may at least partially explain divergent findings in the literature regarding the involvement of P2 purinoceptors in osteogenesis – reviewed in (Noronha-Matos & Correia-de-Sá, 2016). The age-dependent extent of the extracellular catabolism of most commonly used P2X7 receptor agonists, like BzATP, may also account for these differences if one does not take into account the expression and activity of NTPDases (Noronha-Matos et al., 2014). Likewise, the

preferential P2Y₆ receptor activation vis a vis other pyrimidine-sensitive P2 receptor subtypes may be explained because distinctively from ATP catabolism that can either yield ADP or AMP, all membrane-bound NTPDases dephosphorylate UTP with a transient formation of the diphosphate metabolite, UDP (Kukulski et al., 2005). One cannot however discount the fact that P2Y₂ and P2Y₄ receptors recognizing both ATP and UTP have also been localized in human bone-forming cells (Bowler et al., 1995; Maier et al., 1997). However, controversy still exists regarding the fact that these receptors have been involved in the inhibition of bone mineralization, particularly when nucleotides were used in low concentrations (Hoebertz et al., 2002; Orriss et al., 2007).

In contrast to data obtained regarding the kinetics of ATP catabolism, which mostly depends on the number of viable cells at a given cell maturation stage (Figure 5A), normalization of BM-MSCs' ability to hydrolyze UDP per viable cell (MTT assay) showed that the net enzymatic activity of Pm BM-MSCs, but not those from younger females, substantially increases as cultures progress from day 7 to 21 (Figure 5B). Since the number of viable cells did not increase proportionally in the same period, the results suggest that mature BM-MSCs from Pm women exhibit higher NTPDase activity implicated in the extracellular inactivation of UDP. The dominant presence of NTPDase3 in differentiated Pm BM-MSCs and its low expression in cells from younger females may give a good explanation for the discrepancy between the two age groups concerning the ability to hydrolyze UDP (and ATP) with a direct impact on the osteogenic commitment of the cells.

BM-MSCs from Pm women and younger females express relatively high amounts of NTPDase1 and 2 – see Ref. (Noronha-Matos et al., 2012). NTPDase1 (nucleotide diphosphohydrolase or apyrase) is expected to terminate the actions of both ATP and UDP on P2X₇ and P2Y₆ receptors, respectively, by their conversion directly into inactive nucleotide monophosphate derivatives (AMP and UMP). Conversely, the presence of NTPDase2 (a preferential nucleotide triphosphatase) may promote the accumulation of nucleotide diphosphates, like UDP, and subsequent P2Y₆ receptors activation (Noronha-Matos et al., 2012). The virtual absence of NTPDase8 from BM-MSC membranes of both women groups suggests that this enzyme does not play a role in the catabolism of extracellular adenine and uracil nucleotides and, thus, in the osteogenic commitment of these cells.

The decreased osteogenic ability of BM-MSCs from Pm women as a consequence of the presence of high NTPDase amounts converting ATP into AMP could be partially compensated if subsequent dephosphorylation of AMP into adenosine by ecto-5'-nucleotidase/CD73 is endorsed to allow activation of osteogenic-inducing P1 receptors

(Costa et al., 2011). Regrettably, the density of this adenosine-forming enzyme is diminished in BM-MSCs from Pm women compared to that found in the cells of younger females. These findings comply with those obtained in animals with osteoporotic bone loss (Shih et al., 2019) also considering that ecto-5'-nucleotidase/CD73 is expressed in more than 95% of the cells exhibiting osteogenic capability (Lu et al., 2010). Among the adenosine-sensitive P1 receptors, the most abundant A_{2B} receptor subtype has been implicated in the differentiation of BM-MSCs from Pm women, which action is normally balanced through the activation of coexisting A_1 or A_{2A} receptors setting whether osteoprogenitor cells are driven into proliferation or differentiation (Costa et al., 2011). Despite that adenosine formation was detected as a consequence of the extracellular ATP hydrolysis in mature Pm BM-MSCs cultured in the presence of NTPDase3 inhibitors, PSB 06126 and hN3-B3s (see Figure 8), rescuing of matrix mineralization under such conditions was fully prevented by selective blockage of P2X7 and P2Y₆ receptors, ruling out the putative contribution of P1 adenosine receptors in this context.

Interestingly, the monoclonal antibody, hN3-B3s, proved to be more potent than the pharmacological NTPDase3 inhibitor, PSB 06126, resulting in powerful increases in P2X7 and P2Y₆ receptor-operated osteogenic differentiation and matrix mineralization of Pm BM-MSC cultures. Anthraquinone derivatives, such as PSB 06126, inhibit NTPDase isoenzymes via multiple mechanisms depending on the chemical nature of substituent groups, NTPDase enzyme subtypes and animal species (Baqi et al., 2009; Zebisch et al., 2014). Inhibition of NTPDases by monoclonal antibodies seems to be more consistent and does not vary with the concentration of substrates. This suggests that enzyme inhibition by monoclonal antibodies does not depend on the steric hindrance of the substrate for the active site, but may be due to the restriction of enzyme lobe movements or deformation of the active site (Munkonda et al., 2009). Moreover, monoclonal antibodies may have additional benefits, including fewer off-target adverse effects, lesser drug-drug interactions, higher specificity, and potentially increased efficacy through targeted therapy (Castelli et al., 2019).

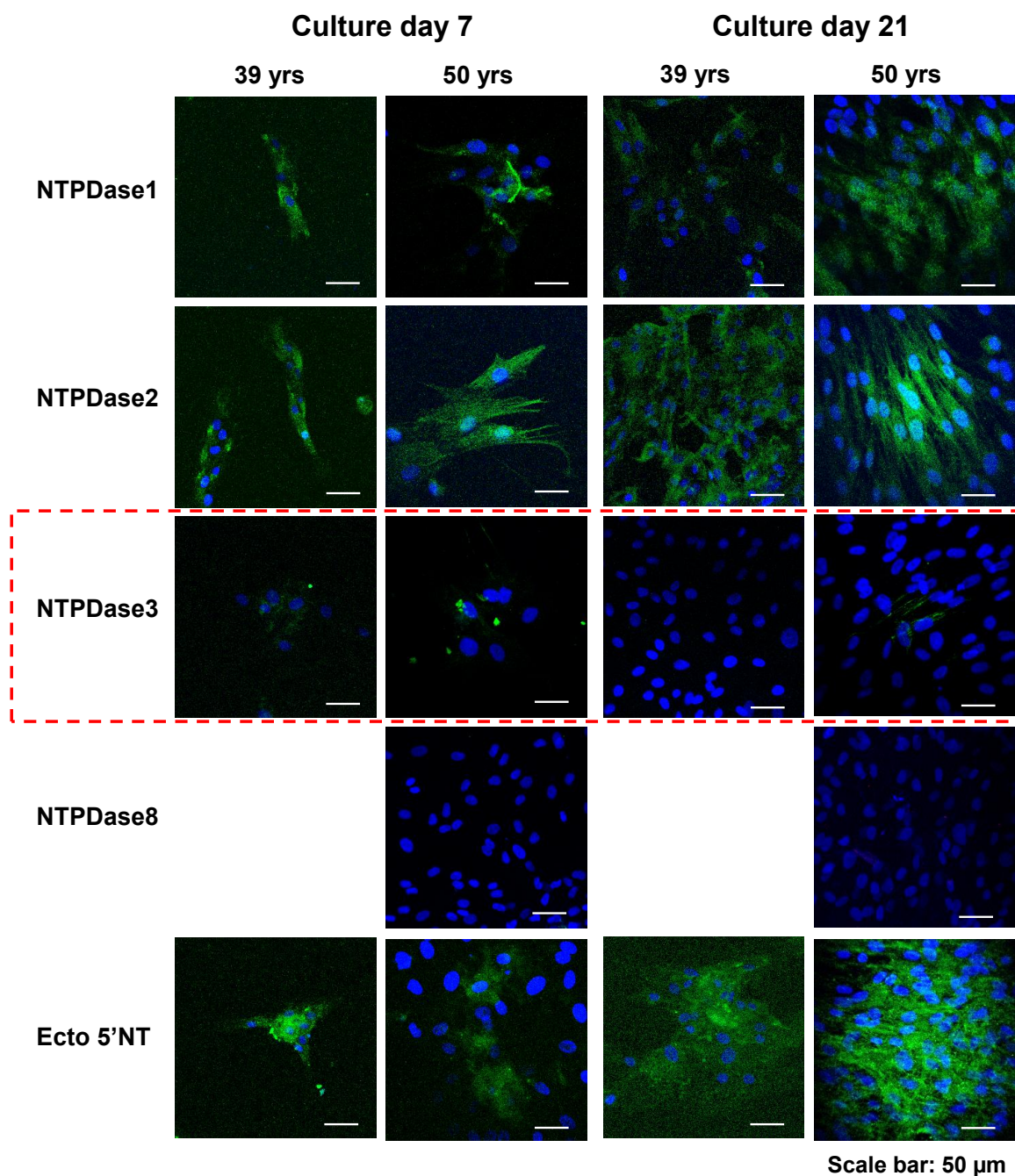
However, it is surprising that albeit NTPDase3 gene silencing with the lenti-shRNA was curtailed 7 days after infection of the cells, this procedure was sufficient to increase Osterix transcription, ALP activity and matrix mineralization of Pm BM-MSCs to levels comparable to those found in younger females. The exact trigger and the timing associated with age-related expression of NTPDase3 in Pm BM-MSCs remains uncertain. Data provided in Supplementary figure 1 show that NTPDase3 immunoreactivity is virtually absent in cultured BM-MSCs from a man requiring bone engraftment due to traumatic fracture, as well as from

an older male undergoing total hip replacement due to degenerative osteoarthritis, besides the lack of NTPDase3 in differentiating cells from young females. One may speculate about the relevance of oestrogen deficiency as a trigger concerning bone loss linked to NTPDase3 overexpression and P2 purinoceptors activation impairment in Pm women, an aspect worth investigating in a near future. Other putative mechanisms involved in the up-regulation of ecto-nucleotidase enzymes, like those comprising the hypoxia-inducible factor-1 α (Lee et al., 2015; Palomaki et al., 2013; Pinto-Cardoso et al., 2020; Tomlinson & Silva, 2015; Wagegg et al., 2012; Zhou et al., 2015) and microRNAs (Li, 2018; Liu et al., 2017; Liu et al., 2015), are also worth pursuing in this context.

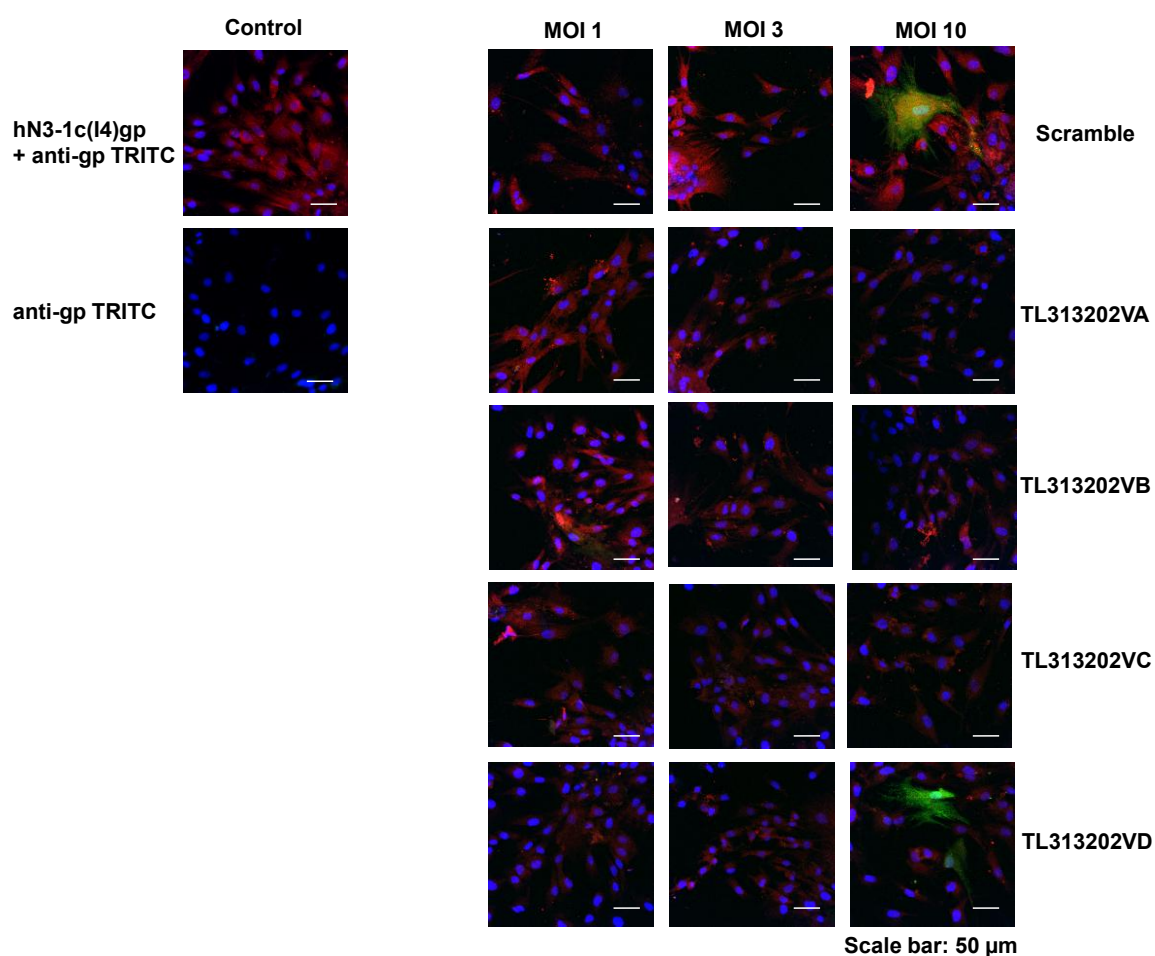
Conclusion

Data show that deficits in the osteogenic differentiation commitment of BM-MSCs may be due to overexpression of the NTPDase3 enzyme leading to excessive extracellular adenine and uracil nucleotides breakdown in bone niches of Pm women. We provide here compelling evidence that the osteogenic potential of these cells can be rehabilitated by NTPDase3 gene silencing (with a lenti-shRNA) or by inhibiting its enzymatic activity with small molecules, like the anthraquinone PSB 06126, or with highly-specific monoclonal antibodies, like hN3-B3_s. The aforementioned gaps in our knowledge do not invalidate the putative clinical impact of our findings concerning the therapeutic benefit of NTPDase3 gene silencing and/or enzymatic inhibition to increase bone formation in Pm women, as well as in other situations where bone destruction exceeds bone formation (e.g. osteoporosis, rheumatoid arthritis, osteogenesis imperfecta, fracture mal-union). The literature is relatively scarce concerning NTPDase3 expression and function in other tissues. NTPDase3 is relatively abundant in pancreatic β -cells where it may function as a regulator of glucose-induced insulin secretion (Sandhu et al., 2021; Syed et al., 2013). It was also found in hypocretin-1/orexin-A positive axon terminals of hypothalamic regions controlling feeding, circadian rhythm and reproduction behaviour (Belcher et al., 2006; Kiss et al., 2009). Obesity, metabolic syndrome and osteoporosis are major global health problems with increasing prevalence. The interactions between these conditions turn out to be more complex than previously anticipated, opening new opportunities for a central role of mechanisms involving the purinergic cascade in all these pathological conditions. Finally, the marked differences found between young vs. Pm women (and age-matched males) may suggest NTPDase3 overexpression being considered a novel clinical surrogate for the identification of osteogenic differentiation impairment of BM-MSCs among Pm women with high osteoporotic fracture risk. Whether this feature also applies to MSCs originating from other sources easier to deal with in clinical settings, requires further investigations.

Supplementary information



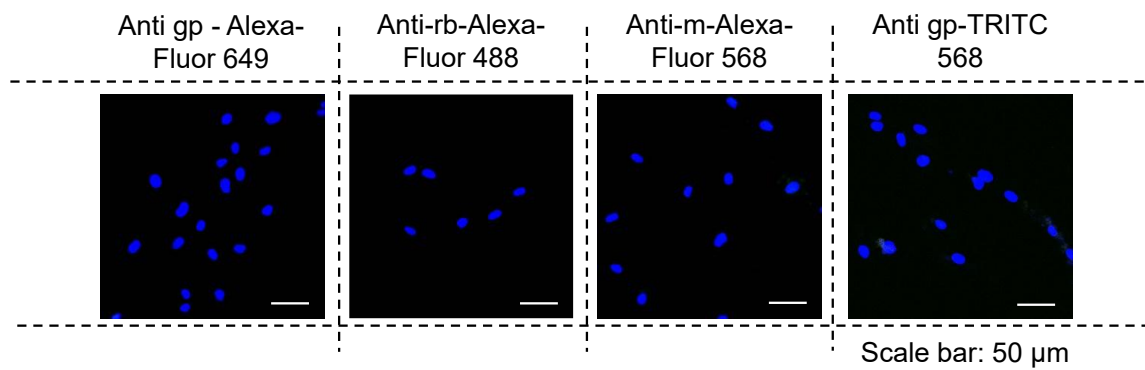
Supplementary Figure 1 – Immunocytochemical detection of NTPDase1, -2, -3, -8 and ecto-5'-nucleotidase (Ecto 5'-NT) in cultured BM-MSCs (first subculture) from young (Y) and older (O) males, which were allowed to grow for 7 and 21 days in an osteogenic-inducing medium. Blue dots represent nuclei stained with DAPI. These experiments were performed in parallel to those using women's samples shown in Figure 1 while keeping unaltered the settings of the confocal microscope throughout the experimental procedure (see Materials and Methods). The scale bar is 50 μm.



Supplementary Figure 2 – Efficacy of NTPDase3 gene silencing in 7-day BM-MSC cultures (first subculture) from a Pm woman undergoing osteogenic differentiation infected with several lenti-shRNAs encoding for four inhibitory and one scramble (negative control) sequences at increasing multiplicities of infection (MOI: 1, 3, 10). Left-hand-side micrographs show positive NTPDase3 immunostaining (red) in non-treated cells and the corresponding negative control (where no primary antibody was added). The positive transduction marker GFP (green) is detectable in some of the cells; blue dots represent nuclei stained with DAPI. All experiments were performed in parallel keeping unaltered the settings of the confocal microscope throughout the procedure (see Materials and Methods). The scale bar is 50 μ m.

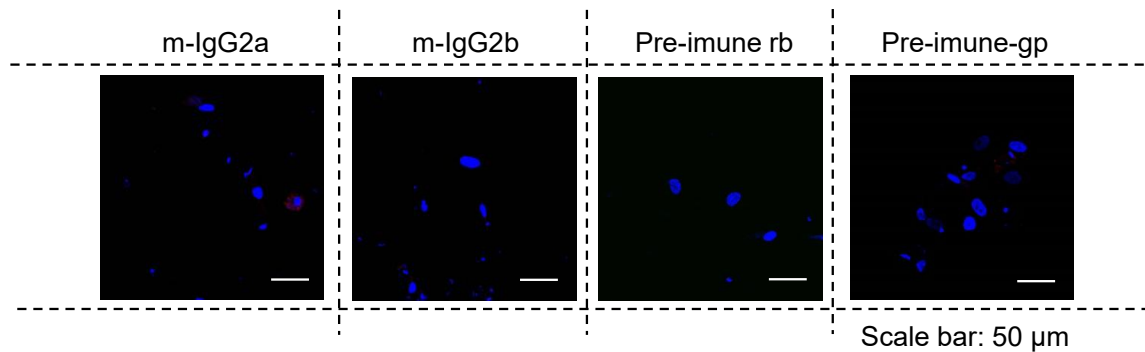
A

Negative controls (no primary antibody) - day 7

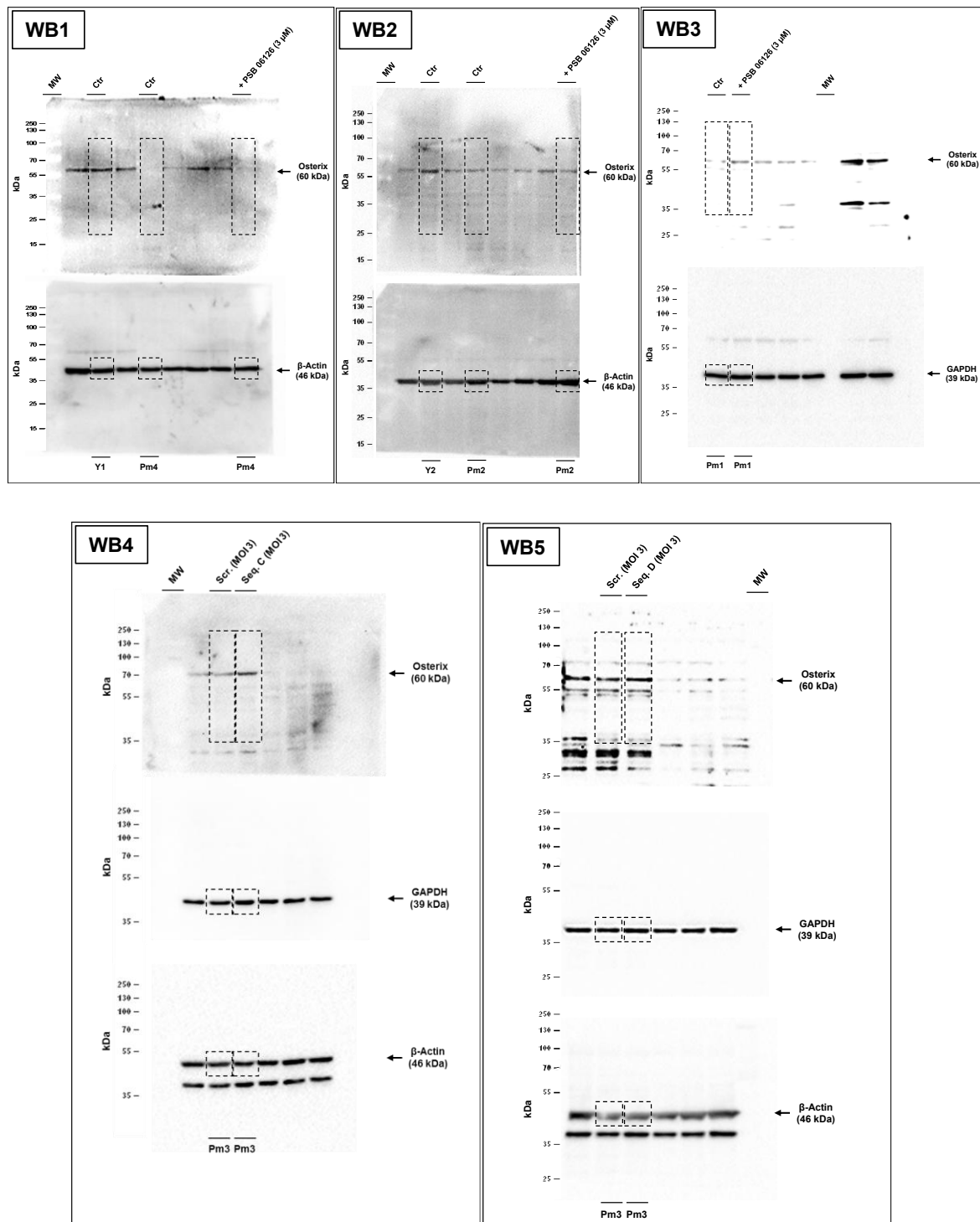


B

Negative controls (Pre-immune serum or IgG) – day 7



Supplementary Figure 3 – Negative controls of immunofluorescence staining using human BM-MSC cultures allowed growing for 7 days in an osteogenic-inducing medium (first subculture). **Panel A**, shown is the immunofluorescence staining detected in cells incubated with the secondary antibodies, but where primary antibodies were omitted. Data in **panel B** show that no immunoreactivity was obtained when primary antibodies were substituted by the corresponding IgG antibodies or by pre-immune sera followed by respective secondary antibodies (anti-mouse, anti-rabbit or anti-guinea pig). Blue dots represent nuclei stained with DAPI contained in the VectaShield mounting medium. The scale bar is 50 μ m. gp, guinea pig; m, mouse; rb, rabbit.



Supplementary Figure 4 – Uncropped full-length Western-blot gels depicted in Figure 7A (WB1-5). Shown are typical immunoblots stained for Osterix (~60 kDa) transcription factor in BM-MSCs from two young (Y1 and 2) and three Pm (Pm1, 2 and 3) women cultured in an osteogenic-inducing medium (i) in the absence or the presence of the NTPDase3 inhibitor, PSB 06126 (3 μM), or (ii) after NTPDase3 gene silencing with lenti-shRNAs encoding for TL313202VD (Seq D; MOI 3) and TL313202VC (Seq C; MOI 3) inhibitory vs. control scramble sequences; either β-Actin (46 kDa) or GAPDH (39 kDa) protein amounts were used as house-keeping gene product comparison standards for normalization purposes. Dashed boxes indicate where blots were cropped for comparison purposes.

Paper 2

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HIF-1 α stabilisation hampers the chondrogenic differentiation of aged bone marrow mesenchymal stem cells by adenosine A_{2A}/A_{2B} receptors imbalance

Rui Pinto-Cardoso^{1,2}, Catarina Bessa-Andr s^{1,2}, Fl vio Pereira-Costa^{1,2}, Rafael Neiva^{1,2}, Ricardo Martins-Ferreira^{3,4}, B rbara Guerra-Leal^{3,4}, Diogo N brega Catelas⁵, Ad lio Vila a⁵, Ant nio Oliveira⁵, Julie Pelletier⁶, Jean S vigny⁶ Jos  Bernardo Noronha-Matos^{1,2}, & Paulo Correia-de-S ^{1,2*}

¹Laborat rio de Farmacologia e Neurobiologia, ²Center for Drug Discovery and Innovative Medicines (MedInUP)/RISE-Health: Health Research Network, ³Laborat rio de Imunogen tica, Departamento de Patologia e Imunologia Molecular, and ⁴Unit for Multidisciplinary Research in Biomedicine (UMIB)/ITR – Laboratory for Integrative and Translational Research in Population Health, Instituto de Ci ncias Biom dicas Abel Salazar - Universidade do Porto (ICBAS-UP), 4050-313, Porto, Portugal; ⁵Servi o de Ortopedia, Centro Hospitalar Universit rio de Santo Ant nio, 4099-001, Porto, Portugal; and ⁶Centre de Recherche en Rhumatologie et Immunologie, University Laval, 2325, rue de l'Universit  Qu bec (Qu bec) G1V 0A6, Canada.

Abstract

Trauma and excessive motion are deleterious to cartilage by triggering HIF-1 α stabilisation, leading to changes in extracellular adenosine formation via CD73. How these changes affect adenosine A_{2A}/A_{2B} receptors activation balance in chondrogenesis is unclear. We used bone marrow mesenchymal stem cells (BM-MSCs) from aged women to investigate the impact of HIF-1 α overactivation on adenosine formation from ATP breakdown and subsequent A_{2A}/A_{2B} receptors tone-regulating chondrogenesis.

The chondrogenic differentiation of BM-MSCs from 27 post-menopausal (Pm) women was induced for 14 days with or without DMOG, a prolyl-4-hydroxylase (PHD) inhibitor that increases HIF-1 α intracellular accumulation. Chondrogenesis was ascertained by the nuclear translocation of SOX9, type II/X collagen and MMP13 production and by transcriptomic RNAseq analysis. Changes in the density of adenosine receptors, ecto-NTPDases and CD73 were assessed by immunofluorescence microscopy. The kinetics of ATP hydrolysis and adenosine formation was performed by HPLC.

DMOG-induced HIF-1 α transcriptional activity increased cartilage damage biomarkers (MMP13 and type X collagen), while decreasing cell viability, SOX9 nuclear translocation, and type II collagen production. DMOG upregulated pro-inflammatory genes and down-regulated chondrogenic gene transcripts determined by RNAseq. HIF-1 α stabilisation decreased CD39/CD73 amounts and activity, thus reducing adenosine formation. Hence, changes in the A_{2A}/A_{2B} receptor tone resulted in preferential activation of the SCH 442416-sensitive A_{2A} receptor which is deleterious to the cartilage.

In conclusion, HIF-1 α overactivation hampers the chondrogenic differentiation of aged BM-MSCs by decreasing adenosine formation from ATP hydrolysis via CD39/CD73 leading to a preferential activation of the high-affinity A_{2A} versus the chondroprotective A_{2B} receptor.

Keywords: Mesenchymal stem cells; HIF-1 α ; chondrogenesis; adenosine-sensitive receptors; ecto-nucleotidases; ENT-1 transporter.

Introduction

Cartilage, as an avascular tissue, has a limited oxygen supply. It is now widely accepted that hypoxia plays a critical (yet unresolved) role in cartilage homeostasis (Gibson et al., 2008; Lennon et al., 2001; Murphy & Polak, 2004). However, oxygen tension-associated mechanisms and their impact on chondrogenesis are still a matter of debate, mainly due to erratic experimental conditions. Confounding factors include cell origin, passage numbers, donors' age, cartilage lesion scores, oxygen tension, culture media, and cell differentiation status on isolation (Pinto-Cardoso et al., 2023; Vieira et al., 2010). For instance, aged chondrocytes react differently to extrinsic stressors (e.g. hypoxia), thus resulting in distinct proliferation rates; the energy metabolism, response to growth factors and autophagy protective mechanisms of aged chondrocytes are also affected (Martin et al., 2004), ultimately resulting in degenerative osteoarthritic diseases (Martin & Buckwalter, 2001).

The autologous transplantation of chondrocytes is used worldwide to tackle cartilage defects, yet unsolved questions remain regarding the ability of these cells to grow and differentiate in transplanted foci. Moreover, limitations to cartilage auto-transplantation also involve donor sites' accessibility, surgical risk and tissue/cell yield, in addition to age- and pathology-associated impairments of cell differentiation (Abbott & Holtzer, 1966; Brittberg et al., 1994; Lepage et al., 2019). New approaches have emerged to overcome part of these constraints considering the use of multipotent mesenchymal stromal cells (MSCs) from different sources (Augello & De Bari, 2010), although consensus in the literature regarding the ideal conditions to promote chondrogenic vs. osteogenic differentiation, is still missing (Djouad et al., 2009; Johnson et al., 2012). Difficulties in translating preclinical studies into clinical practice concerning osteochondral repair are related, in part, to the lack of standardized experimental procedures as previously mentioned.

During joint motion, thus favouring hypoxia and mechanical stress, both cartilage resident cells and transplanted MSCs release huge amounts of adenine nucleotides, including ATP, to the microenvironment (Bessa-Andr es et al., 2024). The peak and permanence of adenine nucleotides and adenosine formation in the extracellular milieu is balanced by the ability of the cells to release and inactivate purines by high-affinity NTPDases, ecto-5'-nucleotidase/CD73 and adenosine deaminase. These paths also determine activation of nucleoside- vs. nucleotide-specific P1 and P2 purinoceptors in the vicinity, respectively (Corciulo & Cronstein, 2019; Noronha-Matos et al., 2012; Phatarpekar et al., 2010). Our group recently reviewed data from the literature gathering information about the putative interplay between hypoxia and the purinergic signalling cascade to osteochondral homeostasis (Pinto-Cardoso et al., 2023).

Increasing evidence shows that endogenously generated adenosine is paramount to control both the chondrogenic (Pinto-Cardoso et al., 2020) and/or the osteogenic (Noronha-Matos & Correia-de-Sá, 2016) fate of human bone marrow-derived MSCs (BM-MSCs). The low-affinity A_{2B} receptor is involved in the osteogenic differentiation of BM-MSCs, since its activation promotes cell growth and differentiation translated into increases in alkaline phosphatase (ALP) activity and bone nodule formation. In osteogenic differentiating cells, the A_{2B} receptor signalling tone is balanced by the activation of co-localized A_1 and A_{2A} receptors depending on cells differentiation stage (Costa et al., 2011). Conversely, the high-affinity A_{2A} receptor subtype seems to promote cartilage homeostasis, while reducing chondrocyte cells senescence (Friedman et al., 2023) via its anti-inflammatory and anti-apoptotic properties (Bitto et al., 2011; Campo et al., 2015). The A_{2B} receptor activation normally suppresses the chondrogenic commitment and is deleterious to cartilage cells (Tan et al., 2017). Regrettably, most of this information derives from animals' research or the use of immortalized human cells lines, while pending information is still necessary in non-modified human BM-MSCs undergoing chondrogenic differentiation.

The hypoxia-inducible factor (HIF)-1 α is a key transcription factor that responds to low oxygen tension to regulate growth and differentiation of multiple cell types, including chondrocytes (Calejo et al., 2021; Koh et al., 2011; Noguera et al., 2009). HIF-1 α modulates important chondrogenic transcription factors, as for instance the sex-determining region Y (SRY)-box transcription factor 9 (SOX9) (Robins et al., 2005). Hypoxia, putatively via HIF-1 α , may also regulate the transcription of several players of the purinergic signalling cascade, such as ecto-nucleotidases and P1 purinoceptors, in non-cartilaginous and bone tissues – for a review, see e.g. (Pinto-Cardoso et al., 2023). Yet, the link between HIF-1 α transcriptional activation by hypoxia and the expression and function of purinergic signalling players in the osteochondral unit is still missing in both health and disease conditions. This prompted us to explore the impact of HIF-1 α overactivation under normoxic conditions using a cell-permeable prolyl-4-hydroxylase inhibitor, dimethyloxallyl glycine (DMOG) (Zhang et al., 2016; Zhdanov et al., 2015), on the expression and function of high-affinity A_{2A} and low-affinity A_{2B} receptors in chondrogenic-differentiating aged BM-MSCs from post-menopausal (Pm) women. Given its relevance in this endeavour, we also investigated the putative changes operated by DMOG in the extracellular ATP breakdown and adenosine formation by NTPDases.

Results

The upregulation of HIF-1 α in aged BM-MSCs favours the transcription of genes contributing to deficits in chondrogenesis and cartilage damage

Mounting evidence in the literature shows that hypoxia influences chondrogenesis and that HIF-1 α transcriptional activation by hypoxia may alter the expression and function of several players of the purinergic signalling cascade in the osteochondral unit (Pinto-Cardoso et al., 2023). Here, we used a cell-permeable prolyl-4-hydroxylase competitive inhibitor, DMOG (30 μ M), to explore the impact of HIF-1 α stabilisation and activation on chondrogenesis under normoxic conditions, focusing on the expression and function of high-affinity A_{2A} and low-affinity A_{2B} receptors, as well as on the extracellular ATP breakdown and adenosine formation by NTPDases in aged BM-MSCs from Pm women undergoing chondrogenic differentiation.

Figure 11A shows that following DMOG (30 μ M) application the HIF-1 α immunoreactivity increases in the cytoplasm, which subsequently translocates to the nucleus of BM-MSCs undergoing chondrogenic differentiation, without affecting cells' morphology. DMOG (30 μ M)-induced stabilisation of HIF-1 α at culture days 7 and 14 favours gene transcription of *COL10A1* (Figure 11B), with a similar (yet not significant) tendency regarding the *MMP13* gene transcription in some experiments; curiously, *COL10A1* and *MMP13* are both associated with deleterious cartilage hypertrophy (D'Angelo et al., 2000; Zuscik et al., 2008).

The RNA sequencing analysis of both control and DMOG (30 μ M)-treated chondrogenic-differentiating BM-MSCs revealed significant changes in gene expression patterns between the two studied groups (Figure 11C). The unsupervised clustering of DEGs (raw $P < 0.05$, total of 799 genes; see Supplementary Table 1) from all pairwise comparisons provided six different clusters (1-6) (see Figure 11C and 11D, and Supplementary Table 2). Data show that stem cell differentiation-related genes (Cluster 1) decrease in chondrogenic differentiating BM-MSCs from culture day 7 to 14, while the opposite is observed regarding gene transcripts related to inflammatory responses (Cluster 3), bone development (Cluster 5) and purinergic signalling (Cluster 6). The upregulation of HIF-1 α transcriptional activity by DMOG (30 μ M) increased DEGs concerning Clusters 4, 5 and 6 at culture day 7, while decreasing Cluster 2 genes at the same time point (Figure 11C); genes from Clusters 1 and 3 were unaffected by DMOG (30 μ M) throughout the culture period.

From GO analysis (Figure 11D; see Supplementary Table 3), the most prominent results include the enrichment of hypoxia-related terms in Clusters 4 and 6, purinergic signalling-related terms in Cluster 6, and stem cells differentiation-related terms in Cluster 2. It is noteworthy that, besides the expression of genes related to hypoxia, the upregulation of

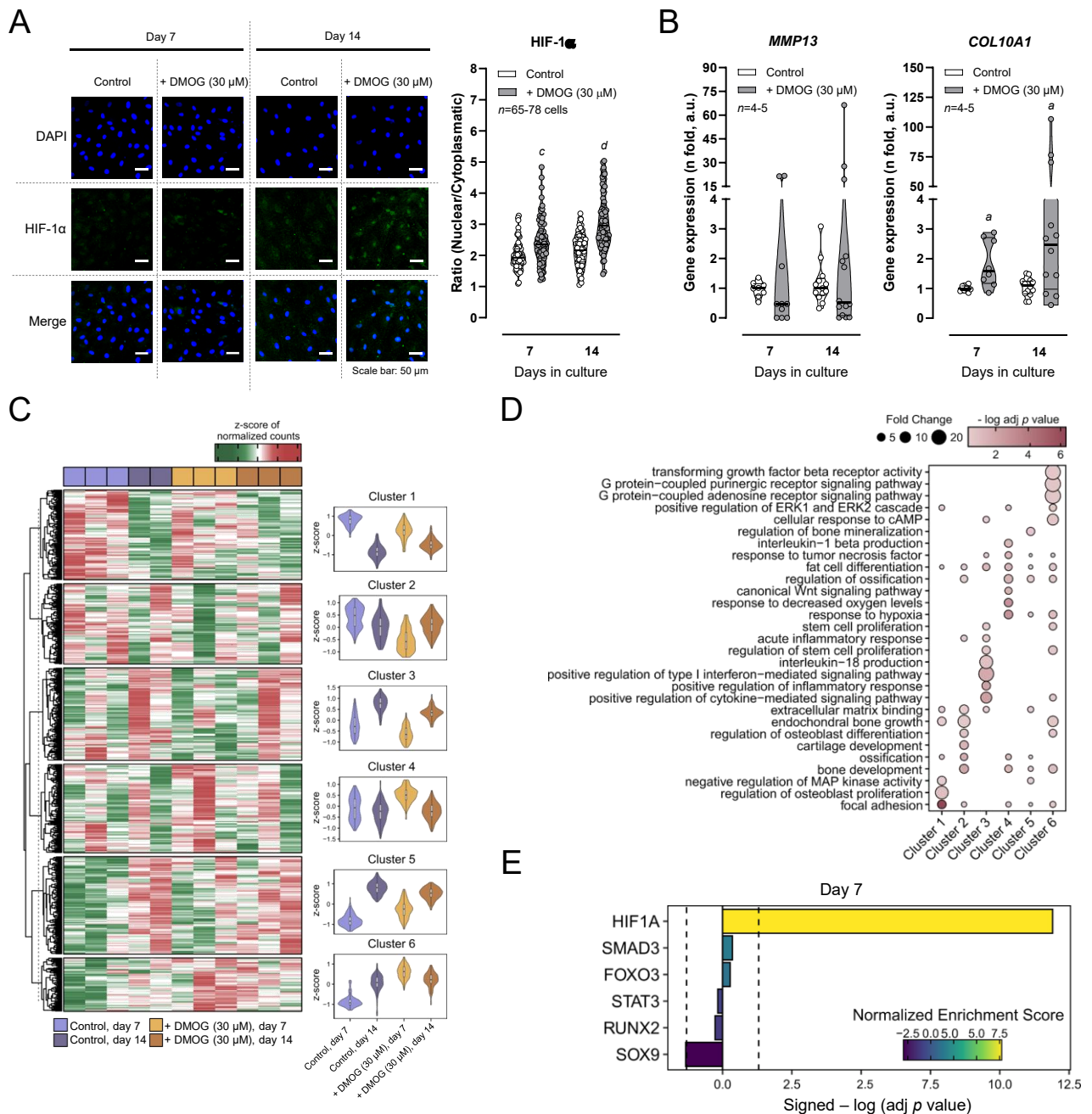


Figure 11 – Upregulation of HIF-1 α transcriptional activity impacts the chondrogenic differentiation of human bone marrow mesenchymal stem cells (BM-MSCs). The cells were allowed to grow for 7 and 14 days in a chondrogenic-inducing medium in either the absence or presence of DMOG (30 μ M). In **Panel A** are representative images of the immunocytochemical detection of HIF-1 α protein levels (green) by confocal microscopy. Blue dots represent nuclei staining with DAPI. The scale bar is 50 μ m; The right hand side panel shows immunofluorescence intensity graphs computed from acquired confocal microscopy images. Ordinates represent fluorescence intensity per cell (arbitrary units, a.u.) of the HIF-1 α immunoreactivity as a function of the number of days in culture (7 and 14 days). Plotted data correspond to 65-78 cells from a 79-year-old woman. $^{\circ}$ P<0.001 and ^{d} P<0.0001 (Kruskal-Wallis test with uncorrected Dunn's test) represent significant differences. **Panel B** shows the relative expression of matrix metalloproteinase-13 (MMP13) and type X collagen (COL10A1) mRNA levels detected by RT-PCR. Ordinates represent the fold-change (arbitrary units, a.u.) of the indicated mRNA levels normalized to ubiquitin C (UBC) determined in the same samples. Represented are triplicates from four to five Pm women (68 $\pm$ 6 years old). ^{a} P<0.05 (Kruskal-Wallis test with uncorrected Dunn's test) represent significant differences. **Panel C** presents a heat map of the differentially expressed genes (DEGs) obtained from the pairwise comparison between all studied conditions. Gene expression is represented as the z-score of the normalized counts. The significance cutoff was raw p-value < 0.01 and absolute log2FoldChange > 0.5. Unsupervised clustering divided the DEGs in six clusters (1-6). Represented are violin plots of the mean z-score of each DEG cluster for the indicated experimental conditions. **Panel D** represents a selected list of enriched gene ontology (GO) terms from indicated clusters (raw p-value < 0.05). The significance of enrichment is represented by the negative of the log of the adjusted p-value and the enrichment fold change. **Panel E** presents a bar plot showing the translational activity of relevant transcription factors (TF) involved in BM-MSC differentiation; the enrichment (*continue*)

(continue) is represented by the positive or negative Normalized Enrichment Score (NES) and the statistical significance by the adjusted p-value (FDR). The ladder is presented as the signed - log (adjusted p-value), which is calculated as the $-\log_{10}(\text{adjusted p-value}/(\text{NES}/\text{absolute}(\text{NES})))$. Zero represents the identity between the TF activities obtained in the absence or presence of DMOG (30 μM) at culture day 7. Differential expression analysis was performed by using the DESeq2 R package (see the Materials and Methods section for further details).

HIF-1 α favoured the transcription of genes encoding for pro-inflammatory cytokines (Cluster 4) and G protein-coupled purinoceptors (Cluster 6) in aged BM-MSCs (Figure 11D), while stem cell differentiation-related and cartilage development transcripts were significantly decreased (Cluster 2). The analysis of the regulon enrichment based on gene expression of transcription factor targets confirmed the increased activity of HIF-1 α and the decreased activity of SOX9 in the presence of DMOG (30 μM) (Figure 11E).

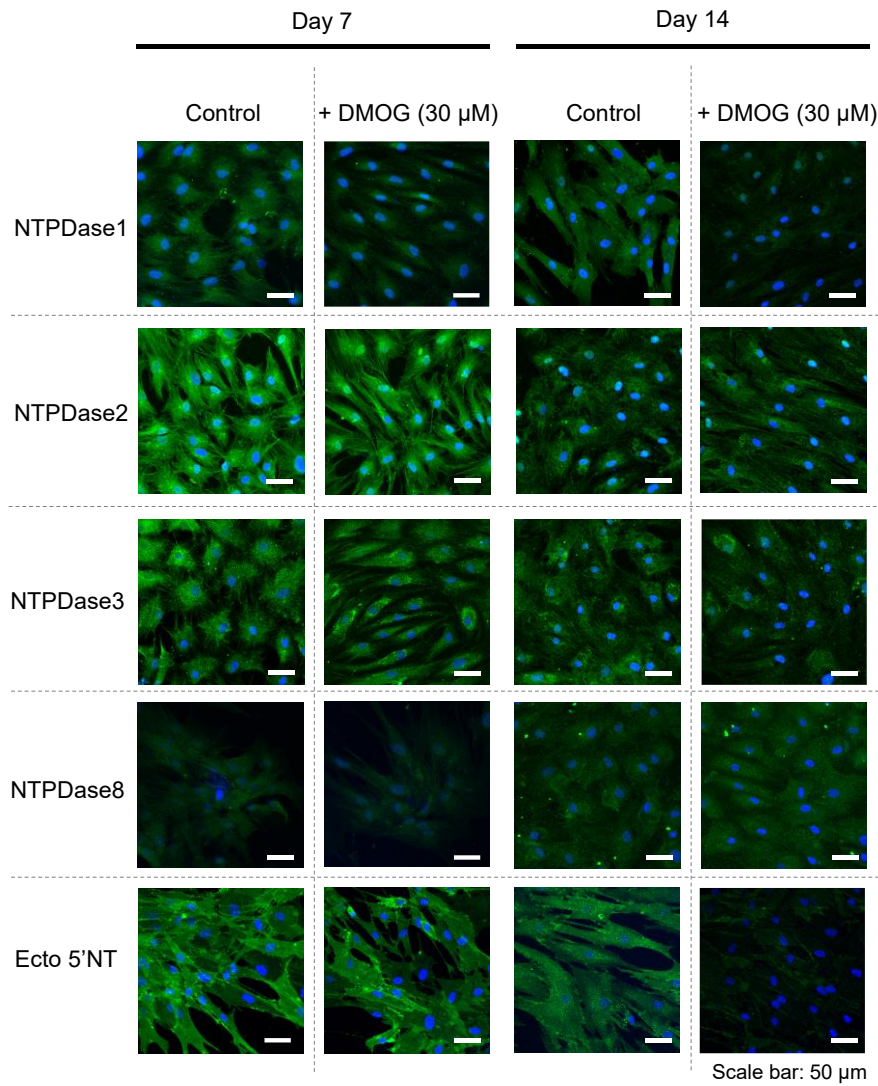
Overall, these results suggest that DMOG-induced stabilisation of HIF-1 α may contribute to hamper the chondrogenic differentiation of aged BM-MSCs.

DMOG-induced HIF-1 α stabilisation downregulates NTPDase1 and ecto-5'-nucleotidase/CD73 immunoreactivity in chondrogenic-differentiating BM-MSCs isolated from Pm women

Previous studies from our group showed that the extracellular breakdown of ATP and adenosine formation is impaired in aged Pm BM-MSCs, thus limiting the osteogenic differentiation capability of these cells (Noronha-Matos et al., 2023). This prompted us to explore whether the same occurred when these cells were challenged by chondrogenic differentiating conditions, given the putative role of these purines to the osteochondral unit homeostasis (Pinto-Cardoso et al., 2023).

Human BM-MSCs undergoing osteogenic differentiation exhibit immunoreactivity against ecto-NTPDase1, -2 and -3, and ecto-5'-nucleotidase/CD73 on the plasma membrane; the density of these nucleotide-metabolizing enzymes typically increases upon differentiation of the cells into mature osteoblasts (Noronha-Matos et al., 2012; Noronha-Matos et al., 2023). Figure 12 shows that chondrogenic-differentiating BM-MSCs also express all of these enzymes, yet their relative abundance slightly differ from that of the osteogenic differentiating lineage. While osteogenic-differentiating BM-MSCs exhibit high levels of NTPDase1 and -3 at all differentiation stages, the NTPDase2 immunoreactivity only becomes evident in mature osteoblasts (Noronha-Matos et al., 2012). A distinct pattern was evident in chondrogenic-differentiating BM-MSCs, given that at early culture stages (day 7) the cells predominantly express NTPDase2 (ATPase or CD39L1; EC 3.6.1.3), followed by NTPDase3 and NTPDase1, with very small amounts of NTPDase8 (Figure 12A). In contrast to that observed in osteogenic differentiating cells, NTPDase2 (but also NTPDase3) was

A



B

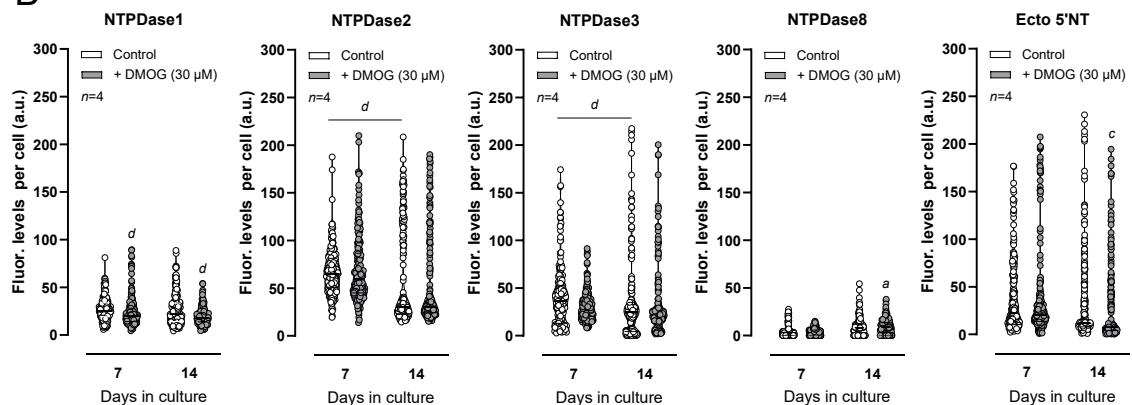


Figure 12 – Changes in the pattern of ecto-nucleotidase protein amounts in chondrogenic-differentiating BM-MSCs from Pm women upon exposure to the prolyl hydroxylase inhibitor, DMOG. **Panel A** shows representative images of the immunocytochemical detection of NTPDase1, -2, -3, -8 and ecto-5'-nucleotidase/CD73 (ecto-5'NT). Shown is the time-related immunoreactivity (green) detected by confocal microscopy. Blue dots represent nuclei staining with DAPI. The scale bar is 50 μ m. **Panel B** shows immunofluorescence intensity graphs computed from confocal microscopy images acquired as in **Panel A**. Ordinates represent fluorescence intensity per cell (arbitrary units, a.u.) of the enzymes' immunoreactivity as a function of the number of days in culture (day 7 and 14). A total of 134-240 cells were analysed from four Pm women (84 $\pm$ 3 years old). ^aP<0.05, ^cP<0.001 and ^dP<0.0001 (Kruskal-Wallis test with uncorrected Dunn's test) represent significant differences.

downregulated ($P<0.05$) during the chondrogenic differentiation of the cells from culture day 7 to 14 (Figure 12B). Typically, pluripotent BM-MSCs exhibit immunoreactivity against ecto-5'-nucleotidase/CD73, yet while the density of this adenosine-forming enzyme progressively increases to become a hallmark of osteoprogenitor cells – see e.g. (Liu et al., 2009a), its level remained roughly constant in cells cultured with the chondrogenic differentiation cocktail (Figure 12A and 12B).

In the presence of DMOG (30 μ M), the immunoreactivity against NTPDase1 (the enzyme that dephosphorylates ATP directly to AMP, also called CD39 or apyrase, EC 3.6.1.5), significantly ($P<0.05$) decreased when the chondrogenic-differentiating cells were exposed to this drug for 7 and 14 days compared to non-exposed cells (Figure 12B). The upregulation of HIF-1 α transcriptional activity by DMOG (30 μ M) for 14 days also slightly decreased the density of the ecto-5'-nucleotidase/CD73 immunoreactivity in these cells (Figure 12B).

DMOG-induced HIF-1 α stabilisation decreases both ATP-derived extracellular adenosine formation and the amount of ENT-1 transporters in chondrogenic-differentiating BM-MSCs from Pm women

Figure 13 shows the kinetics of the extracellular catabolism of ATP (10 μ M) and AMP (10 μ M) and metabolites formation in chondrogenic-differentiating BM-MSCs from Pm women cultured for 7 and 14 days in the absence or presence of DMOG (30 μ M). ATP (10 μ M) was sequentially dephosphorylated into ADP, AMP, adenosine (ADO), inosine (INO) and hypoxanthine (HX) in chondrogenic-differentiating BM-MSCs; at the end of the 30-min incubation period, ADO was the most represented ATP metabolite. Co-localization of NTPDase1, 2 and 3 ensures the sequential dephosphorylation of ATP and ADP directly to AMP, and justifies the appearance of small amounts of ADP in culture media. Despite the downregulation of NTPDase2 and 3 in chondrogenic differentiating cells (culture day 7 vs. 14; see Figure 12B), such modifications are probably not enough to influence the kinetics of the extracellular ATP breakdown and metabolites formation (Figure 13A-D). The negligible accumulation of AMP, INO and HX in the cultures also sustains the idea that pluripotent human BM-MSCs possess high adenosine-forming capability from AMP dephosphorylation, via ecto-5'-nucleotidase/CD73, but low adenosine deaminase (ADA) activity. This feature ensures fast AMP dephosphorylation and ADO accumulation, as the main extracellular ATP metabolite (Costa et al., 2011; Noronha-Matos et al., 2023).

Exposure of chondrogenic-differentiating BM-MSCs to DMOG (30 μ M) increased the half-life of extracellular ATP from 43 ± 4 min to 63 ± 8 min and from 38 ± 6 min to 78 ± 13 min at culture days 7 and 14, respectively (Figure 13A and 13C). Downregulation of NTPDase1 in

chondrogenic-differentiating BM-MSCs treated with DMOG (30 μ M) functionally translates into a reduction in the ATP metabolizing capacity and AMP formation by these cells, which was more evident at 14-day cell cultures (Figure 13Di). At this time point, we found that the incubation medium collected from DMOG (30 μ M)-treated chondrogenic-differentiating BM-MSCs had higher ($P<0.05$) levels of ATP compared to non-treated cells, providing that proliferation/viability of the cells were normalized by CCK-8 values and the incubation time was kept at 30 min (Figure 13Dii).

Using AMP (10 μ M) as the substrate of ecto-5'-nucleotidase/CD73, we found that treatment of chondrogenic-differentiating BM-MSCs with DMOG (30 μ M) resulted in the reduction of ADO formation at culture day 7 compared with non-treated cells; ADO amounts remained constant thereafter (Figure 13E-H). This finding may be explained since DMOG (30 μ M)-induced a partial loss of ecto-5'-nucleotidase/CD73 in the plasma membrane of chondrogenic-differentiating BM-MSCs (see Figure 12A and 12B). Moreover, high extracellular ATP accumulation can feed-forwardly inhibit ecto-5'-nucleotidase/CD73 by binding to its catalytic site and, thus, further reduce ADO formation from AMP in the cultures (Magalhães-Cardoso et al., 2003; Vieira et al., 2014) (see Figure 13B and 13D).

Besides, ADO originated from the extracellular catabolism of adenine nucleotides, the nucleoside is released as such from human BM-MSCs, via a dipyridamole-sensitive type 1 equilibrative nucleoside transporter (ENT-1) (Costa et al., 2011). This ADO release path may be susceptible to modulation by hypoxia (Eltzschig et al., 2005), yet no data is available regarding the effect of HIF-1 α stabilisation caused by DMOG on ENT-1 amounts in chondrogenic-differentiating BM-MSCs. Here, we used the intrinsic fluorescent photophysical properties of dipyridamole (an ENT-1 inhibitor) to tag the ENT-1 transporter on the plasma membrane of these cells (Joiner et al., 2001). Supplementary Figure 7A shows that ENT-1 transporters fluorescently labelled with dipyridamole (0.5 μ M; excitation overlap with DAPI at 405 nm) were significantly ($P<0.05$) downregulated in chondrogenic-differentiating BM-MSCs exposed to DMOG (30 μ M) for 7 and 14 days compared to non-exposed cells. Using a non-fluorescent ENT-1 inhibitor, S-(4-nitrobenzyl)-6-thioinosine (NBTI, 50 μ M), we discarded any effect of ENT-1 inhibition on cell proliferation/viability (Supplementary Figure 7B). To further validate this finding, ENT-1 was labelled using a specific antibody, and immunoreactivity analysis revealed a significant decline in ENT-1 expression in DMOG-treated cells when compared to controls on both culture day 7 and 14 (Supplementary Figure 7C).

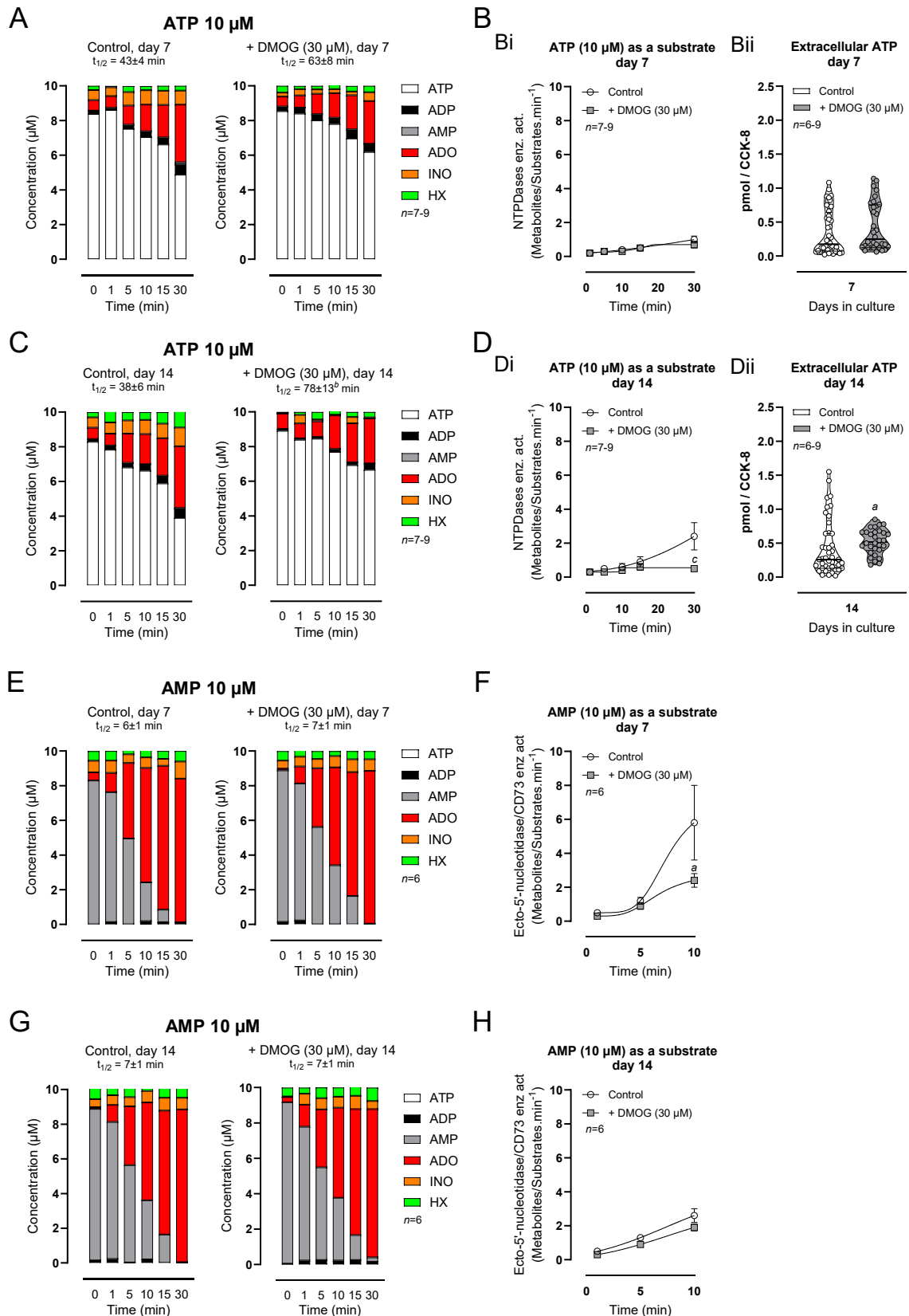


Figure 13 – Upregulation of HIF-1 α transcriptional activity attenuates adenosine formation from the extracellular catabolism of ATP (**Panels A and C**) and AMP (**Panels E and G**) in chondrogenic-differentiating BM-MSCs from Pm women grown in culture for 7 and 14 days. ATP (10 μ M) and AMP (10 μ M) were added to the incubation medium (Tyrode's solution) at time zero to BM-MSCs cultures exposed or not to DMOG (30 μ M), for 7 (*continue*)

(*continue*) and 14 days. 75- μ L samples were collected at the indicated times for HPLC analysis to quantify the kinetics of substrates disappearance and formation of metabolites. ATP (white bar), ADP (black), AMP (grey bar), adenosine (ADO, red), inosine (INO, orange), hypoxanthine (HX, green). The half-life time ($t_{1/2}$) for each initial substrate is shown for comparison. **Panels Bi and Di** represent the enzymatic activity of NTPDases; **panels F and H** represent the enzymatic activity of ecto-5'-nucleotidase/CD73. Values were obtained by quantifying the ratio [Metabolites]:[Substrates] per min from seven to nine Pm women (74 ± 4 years old); cells from each individual were tested in duplicate. In **panels Bii and Dii**, ordinates represent the extracellular ATP accumulation (pmol/CCK-8) during 30 min in Tyrode's solution (no drugs); values were corrected for cell viability/proliferation (CCK-8 assay). The ATP content of the samples was measured using the luciferin-luciferase bioluminescence assay (75 μ L/well; see Materials and Methods for details). Dots in the violin plots are triplicates from six to nine Pm women (72 ± 4 years old). ^a $P < 0.01$ and ^c $P < 0.001$ (Kruskal-Wallis test with uncorrected Dunn's test) represent significant differences.

The density of adenosine A_{2A} receptors overcomes that of the chondroprotective A_{2B} subtype in DMOG-treated chondrogenic-differentiating BM-MSCs from Pm women

ADO-sensitive receptors are present in chondrocytes and chondrocyte-like cells isolated from rodents and bovine (Mistry et al., 2006; Ode et al., 2013; Varani et al., 2008a); yet, much less information is available concerning the expression and function of ADO receptor subtypes in human cells undergoing chondrogenic differentiation (Pinto-Cardoso et al., 2020). Nonetheless, increasing evidence points towards endogenous ADO accumulation resulting from the extracellular catabolism of adenine nucleotides, via ecto-5'-nucleotidase/CD73, as a major determinant of osteogenic differentiation of human BM-MSCs through activation of most abundant low-affinity A_{2B} receptor subtype compared to co-localized high-affinity A_{2A} receptor (Costa et al., 2011; Noronha-Matos et al., 2012; Noronha-Matos et al., 2023).

Using immunofluorescence confocal microscopy, we show in Figure 14 (panels A and B) that both A_{2A} and A_{2B} receptor subtypes are expressed evenly in chondrogenic-differentiating human BM-MSCs at all culture stages (7 and 14 days; Figure 4B). Treatment with DMOG (30 μ M) transiently downregulated ($P < 0.05$) the A_{2B} receptor density at culture day 7, while disproportionally increasing the A_{2A} receptor amount when this drug treatment was prolonged to 14 days (Figure 14A and 14B). Overall, DMOG (30 μ M) exposure favours A_{2A} receptors' overexpression in human BM-MSCs undergoing chondrogenic differentiation leading to an increase in the A_{2A} *versus* A_{2B} receptor tone, which functional impact may be strengthened given the higher affinity of the nucleoside for the A_{2A} receptor (Figure 14B).

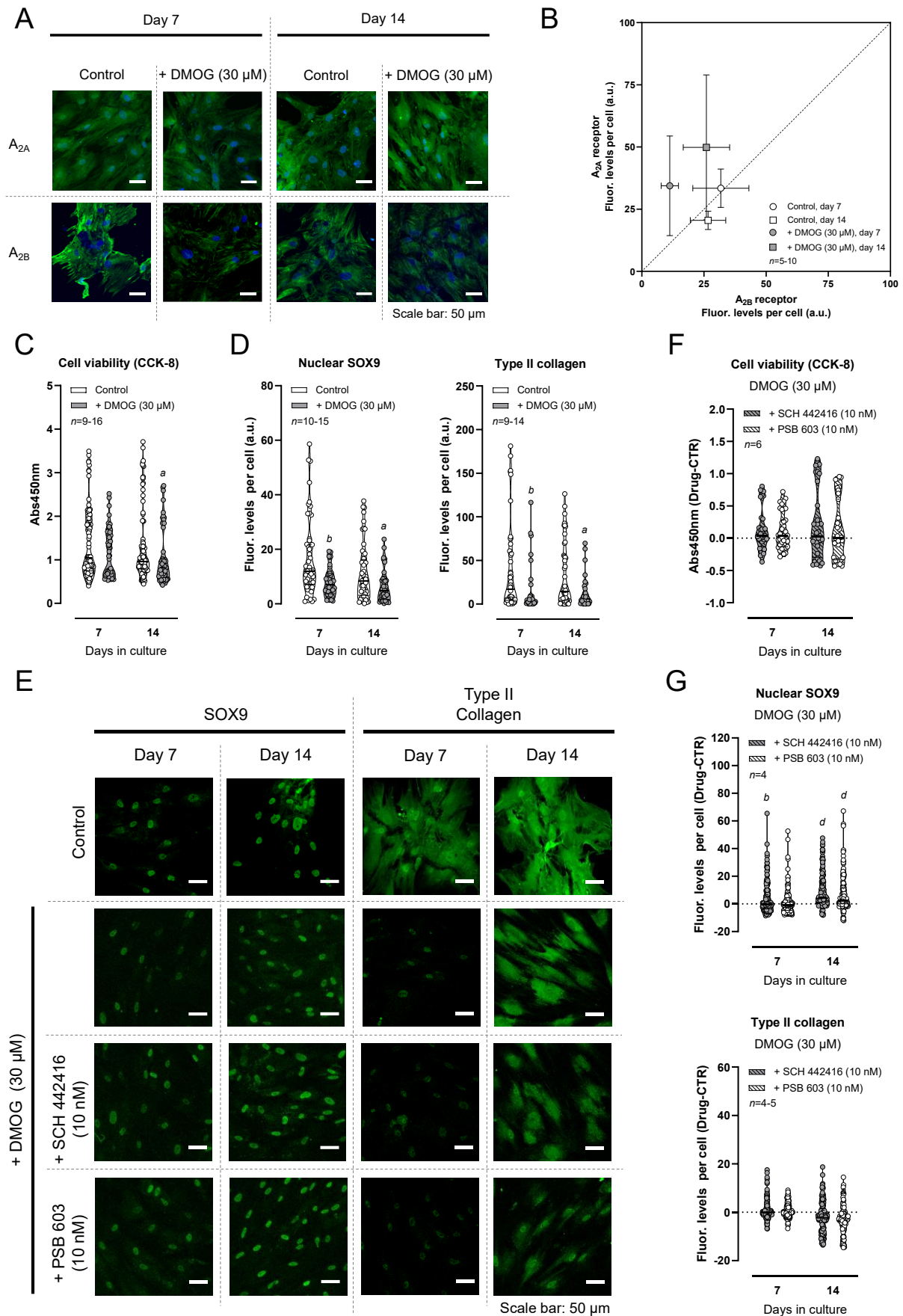


Figure 14 – Upregulation of HIF-1 α transcriptional activity promotes the tonic activation of high-affinity A_{2A} adenosine receptors in human BM-MSCs undergoing chondrogenic differentiation. Chondrogenic-differentiated BM-MSCs were allowed to grow for 7 and 14 days in the absence or presence of DMOG (30 μ M) and/or of selective adenosine A_{2A} (SCH 442416, 10 nM) and A_{2B} (PSB 603, 10 nM) receptor antagonists. **Panel A** shows representative images of the (continue)

(continue) immunocytochemical detection (green) of the indicated adenosine-sensitive receptors by confocal microscopy. Blue dots represent nuclei staining with DAPI. The scale bar is 50 μ m. **Panel B** shows the relative immunoreactivity of adenosine A_{2A} (ordinates) and A_{2B} (abscissas) receptors in chondrogenic-differentiating BM-MSCs in control conditions and after exposure to DMOG (30 μ M) for 7 and 14 days. The dashed line represents equal immunoreactivity for both receptors. Dots represent mean $\pm$ S.E.M. A total of 166-424 cells were analysed from five to ten Pm women (76 $\pm$ 3 years old). **Panel C** represents cell viability/proliferation data using the CCK-8 assay; ordinates represent absorbance values measured at 450 nm. Truncated violin plots represent pooled data from nine to sixteen Pm women (75 $\pm$ 2 years old); four to eight replicates were made per individual. **Panel D** shows the immunofluorescence intensity graphs computed from acquired confocal microscopy images stained against nuclear SOX9 and type II collagen chondrogenic markers. Ordinates represent fluorescence intensity per cell (arbitrary units, a.u.). A total of 340-729 cells were analysed from nine to fifteen Pm women (75 $\pm$ 3 years old). **Panel E** shows representative micrographs of the immunocytochemical detection (green) of nuclear SOX9 and type II collagen by confocal microscopy at all aforementioned experimental conditions. The scale bar is 50 μ m. **Panels F and G** show data from the cell viability/proliferation CCK-8 assay (**F**) and immunofluorescence confocal microscopy to detect nuclear SOX9 and type II collagen (**G**). Zero represents the identity between CCK-8 values, nuclear SOX9 and type II collagen levels in the absence and presence of the DMOG (30 μ M) (horizontal dashed line). A total of 160-458 cells were analysed from four to six Pm women (80 $\pm$ 5 years old). ^aP<0.05, ^bP<0.01, ^cP<0.001 and ^dP<0.0001 (Kruskal-Wallis test with uncorrected Dunn's test) represent significant differences.

Besides DMOG (30 μ M)-induced upregulation of genes, like *MMP13* and *COL10A1*, that are detrimental to cartilage (D'Angelo et al., 2000; Zuscik et al., 2008) (Figure 11B), we show here that it also significantly (P <0.05) attenuated the nuclear translocation of SOX9, a transcription factor that is essential for early chondrogenic differentiation of BM-MSCs. This finding occurred along with decreases in cell proliferation/viability and type II collagen production (a late chondrogenic marker comprising 90% of the collagen in hyaline articular cartilage (Eyre, 2002)) at all culture stages in the presence of DMOG (30 μ M) (Figure 14C-E).

DMOG (30 μ M)-induced inhibition of SOX9 nuclear translocation was mimicked by the selective A_{2A} receptor agonist, CGS21680 (10 nM, n =5), and by inhibition of ATP breakdown into adenosine by NTPDases with ARL67156 (50 μ M, n =5) in chondrogenic-differentiating BM-MSCs allowed to grow in culture for 7 days (data not shown). These preliminary findings strengthen the hypothesis that HIF-1 α stabilization favours the tonic activation of high-affinity A_{2A} receptors by low adenosine amounts. Moreover, selective blockage of the A_{2A} receptor with SCH 442416 (10 nM) partially rehabilitated the nuclear translocation of SOX9 in chondrogenic differentiating BM-MSCs exposed to DMOG (30 μ M) at all evaluated time points (culture days 7 and 14; Figure 14E and 14G). SCH 442416 (10 nM) did not change proliferation/viability (Figure 14F) and the production of the late chondrogenic marker, type II collagen, by these cells (Figure 14E and 14G; see also Supplementary Figure 8). The selective A_{2B} receptor antagonist, PSB 603 (10 nM), had only a minor blocking effect on DMOG (30 μ M)-induced decrease of the nuclear translocation of SOX9 when BM-MSCs were cultured for 14 days in the chondrogenic-enrichment medium, without repercussions

on the other evaluated parameters (Figure 14F-G). Blockage of the low-affinity A_{2B} receptor with PSB 603 (10 nM) had only a minor decreasing effect on type II collagen production when the cells were exposed for 14 days to the chondrogenic-inducing medium without DMOG (30 µM; Supplementary Figure 8).

Discussion

The articular cartilage is an avascular tissue and, thus, evolves under low oxygen tension conditions; this microenvironment may become harsher after trauma and during chronic inflammatory conditions (e.g. osteoarthritis) (Pinto-Cardoso et al., 2023; Zeng et al., 2022). The HIF transcription factor is the master regulator of cellular responses to hypoxia (Harris, 2002; Semenza, 2003). HIF is a heterodimer formed by tuneable HIF-α subunits (1α, 2α and 3α) and constitutively expressed HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator or ARNT)(Huang & Bunn, 2003; Wang et al., 1995). Upon activation by hypoxia (low O₂), HIF-1α resists to degradation and translocates to the nucleus, where it dimerizes with HIF-1β (Chilov et al., 1999), and this complex induces the expression of several transcriptional targets by binding to hypoxia-responsive elements (HRE)(Harris, 2002; Semenza, 2003). Stabilisation of HIF-1α may also be achieved in normoxia by exposure of the cells to the α-ketoglutarate analogue, DMOG (Zhang et al., 2016; Zhdanov et al., 2015). This compound competitively inhibits HIF-1α hydroxylation by prolyl hydroxylase (PHD) among other minor effects on NF-κβ, TNF-α, AMPK or histone demethylase systems (Cloos et al., 2008; Cummins et al., 2008; Hamada et al., 2009; Hindryckx et al., 2010; Takeda et al., 2009; Yan et al., 2012).

Besides the characteristic hypoxic microenvironment of articular cartilage, further increases in the endogenous levels of HIF-1α are frequent in osteoarthritic joints (Guo et al., 2022). Interestingly, the RNAseq analysis performed in DMOG-treated chondrogenic-differentiating BM-MSCs in this study showed significant increases in the expression of pro-inflammatory cytokines (e.g. *IL-18*, *TNF-α*, *IFN*, *IL-1β*; see Supplementary Tables 1 and 3). Overexpression of chondrocyte hypertrophy maturation markers, such as *VEGFA*, *MMP13* and *COL10A1* (D'Angelo et al., 2000; Zuscik et al., 2008), was also observed in DMOG-treated cells (Supplementary Table 1). As expected, exposure of BM-MSCs to DMOG dramatically increased HIF-1α transcriptional activity, which negatively correlated with the transcription of early chondrogenic gene markers, like *SOX9* (Figure 11E). The translocation of the *SOX9* transcription factor to the nucleus was also diminished in DMOG-treated chondrogenic-differentiating BM-MSCs and these cells produced less type II collagen amounts. Overall, these findings suggest that DMOG-induced persistent upregulation of

HIF-1 α transcriptional activity might be detrimental to chondrogenesis and may eventually result in progressive cartilage deterioration in osteoarthritic joints (Qing et al., 2017; Zeng et al., 2022). This is, however, paradoxical considering that the putative promotion of autophagy by DMOG should be chondroprotective (Carames et al., 2015; Carames et al., 2010; Carames et al., 2012), thus prompting to investigate alternative hypotheses.

The influence of hypoxia on the purinergic signalling cascade in cartilage and bone is scarce and the underlying mechanisms are controversial. Inspired on available data from other cell systems, we hypothesized that stabilisation of HIF-1 α by persistent hypoxia (or DMOG under normoxic conditions) could differentially affect the expression and activity of ATP metabolizing and ADO forming enzymes in osteogenic- and chondrogenic-differentiating BM-MSCs (Pinto-Cardoso et al., 2023). In fact, the intracellular stabilisation of HIF-1 α transcriptional activity is associated with NTPDase1/CD39 and/or ecto-5'-nucleotidase/CD73 overexpression in human endothelial cells (Eltzschig et al., 2009), gastric carcinoma cells (Eltzschig et al., 2009; Lu et al., 2013), human metastatic colon carcinoma T84 cells, and HepaRG and Chang liver cell lines (Tak et al., 2017). NTPDase1/CD39 gene transcription and protein synthesis were also upregulated *ex vivo* by exposure of human microvascular epithelial cells (HMEC-1) to hypoxia for 8 and 24h; a similar outcome was obtained in the ischemic myocardium of C57BL/6 mice upon treatment with DMOG (Lu et al., 2013). However, these findings are not consensual, since chronic exposure to hypoxia delayed the extracellular breakdown of ATP by fibroblasts and endothelial cells, thus increasing the tonic activation of ATP-sensitive receptors (Gerasimovskaya et al., 2002). Moreover, hypoxia reduced the activity of NTPDases and ecto-5'-nucleotidase/CD73 without affecting their density in endothelial cells of *vasa vasorum* (Yegutkin et al., 2011) by a mechanism associated to the production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF- α (Gerasimovskaya et al., 2002; Yegutkin et al., 2011).

Data obtained in the present study show that chondrogenic-differentiating BM-MSCs from Pm women are able to metabolize adenine nucleotides (e.g. ATP and AMP) and to generate ADO in the extracellular milieu. This competence is also shared by BM-MSCs undergoing osteogenic differentiation, as previously demonstrated (Caswell & Russell, 1985; Costa et al., 2011; Noronha-Matos et al., 2012; Noronha-Matos et al., 2023), but the plasma membrane density of nucleotide-metabolizing enzyme subtypes differs when the cells are exposed to chondrogenic- vs. osteogenic-differentiating conditions. Whilst osteogenic-differentiating BM-MSCs show high NTPDase1 and NTPDase3 protein amounts at all differentiation stages, the NTPDase2 (ATPase or CD39L1; EC 3.6.1.3) only became evident in mature osteoblasts (Noronha-Matos et al., 2012). This pattern is distinct from that

observed in BM-MSCs undergoing chondrogenic differentiation (this study). During chondrogenic differentiation of BM-MSCs, the NTPDase2 enzyme clearly predominates at early culture stages, followed by NTPDase3 and NTPDase1, with very little amounts of NTPDase8 being observed at culture day 7 (Figure 12A). The relative abundance of NTPDase2, an ATPase that converts ATP into ADP in chondrogenic-differentiating BM-MSCs could anticipate a putative role of ADP-sensitive receptors in chondrogenesis (Pinto-Cardoso et al., 2023). Notwithstanding this, our enzymatic kinetic experiments did not support this hypothesis, since we detected only small amounts of ADP in culture media of chondrogenic-differentiating BM-MSCs when ATP was the substrate (Figure 13). These results may be biased by the simultaneous presence of NTPDase1 and NTPDase3 isoforms in the plasma membrane of these cells (Figure 12), which may contribute to bypass ADP formation/accumulation by ATP (and ADP) dephosphorylation directly to AMP. These results agree with previous findings showing that NTPDase1 and NTPDase3 gene transcripts are upregulated in mature chondrocytes compared to undifferentiated cells (Roszek et al., 2015). DMOG-induced extracellular ATP accumulation emphasizes a role mediated by P2 purinoceptors upon hypoxia in human chondrogenesis (Corciulo & Cronstein, 2019; Noronha-Matos et al., 2012; Phatarpekar et al., 2010). Although the level of P2X₄ and P2Y₁₁ receptor gene transcripts increase under hypoxia conditions, the influence of these receptors in the chondrogenic differentiation of aged BM-MSCs has never been explored and, thus, warrant further investigation.

Another important difference amongst osteogenic- vs. chondrogenic-differentiating BM-MSCs is that the density of nucleotide-metabolizing enzymes tends to increase upon osteoblasts maturation (7<14 day), while both NTPDase2 and NTPDase3 were downregulated during chondrogenic differentiation of the same cells (7>14 day). A similar feature occurred concerning the density of the ADO-forming enzyme, ecto-5'-nucleotidase/CD73, that is responsible for the extracellular catabolism of AMP. The upregulation of this enzyme is a hallmark of osteoprogenitor cells, see, e.g. (Costa et al., 2011; Liu et al., 2009a). Yet, the immunoreactivity of ecto-5'-nucleotidase/CD73 in chondrogenic-differentiating BM-MSCs remained roughly constant throughout the entire culture period (Figure 12) and the same happened considering the extracellular formation of ADO when AMP was used as substrate (Figure 13). In fact, ADO was the most represented extracellular ATP metabolite in BM-MSCs undergoing chondrogenic differentiation. This finding is explained given that these cells exhibit high ecto-5'-nucleotidase/CD73 protein amounts and lack ADA activity, yielding very small amounts of ADO metabolites, like INO and HX (Costa et al., 2011).

Microenvironment changes, such as variations in oxygen tension, mechanical stress, and inflammation, have a tremendous impact on the release, extracellular metabolism and activity of purinergic signalling players involved in osteogenic and chondrogenic differentiation of BM-MSCs, making them putative candidates for pharmacological manipulation (Pinto-Cardoso et al., 2023; Pinto-Cardoso et al., 2020). In this study, we investigated whether impairment of the chondrogenic differentiation capability of aged BM-MSCs exposed to DMOG could be associated to changes in the extracellular ADO accumulation and activity, as previously predicted in other cell types (e.g. fibroblasts, endothelial cells) submitted to hypoxia (Gerasimovskaya et al., 2002; Yegutkin et al., 2011). Data show that chondrogenic-differentiating BM-MSC cultures accumulate higher extracellular ATP amounts when exposed to DMOG and this drug dramatically increased the HIF-1 α transcriptional activity under normoxic conditions. This feature may result specifically from the downregulation of NTPDase1/CD39 in the plasma membrane of aged BM-MSCs, whilst the subsequent reduction of extracellular ADO accumulation may be a consequence of multiple concurring mechanisms. First, DMOG also reduced the density of the ADO-forming enzyme, ecto-5'-nucleotidase/CD73, in chondrogenic-differentiating BM-MSCs. Second, the DMOG-induced extracellular ATP accumulation may contribute to feed-forwardly inhibiting the catalytic activity of ecto-5'-nucleotidase/CD73, further reducing ADO formation from AMP dephosphorylation (Magalhães-Cardoso et al., 2003; Vieira et al., 2014). Finally, the density of the ENT-1 nucleoside transporter, which was fluorescently-tagged with dipyrindamole and by a specific antibody, significantly decreased after exposure of BM-MSCs undergoing chondrogenic differentiation to DMOG, which eventually may limit the outflow of ADO from affected cells. The latter effect does not depend on changes in BM-MSCs viability/proliferation, because the non-fluorescent ENT-1 inhibitor, NBTI, had no measurable effect. Unfortunately, due to methodological constraints (lack of chromatographic detection limit for such small ADO amounts), we were unable to quantify the endogenous accumulation of ADO in the samples used to measure ATP through the high-sensitive luciferin-luciferase bioluminescence kit.

Modifications in ADO extracellular amounts resulting from deficits in the catabolism of released ATP, as well as in the nucleoside release via ENT-1 transporters, may be critical to determine whether ADO activates preferentially high-affinity (A_{2A}), low-affinity (A_{2B}), or both, receptor subtypes driving pluripotent BM-MSCs to proliferation and/or differentiation (Costa et al., 2011; Pinto-Cardoso et al., 2020). In aged BM-MSCs, ADO accumulation in the extracellular fluid facilitates activation of the most abundant low-affinity A_{2B} receptor subtype to promote the osteogenic differentiation of the cells (Costa et al., 2011; Noronha-Matos et al., 2012; Noronha-Matos et al., 2023). Although ADO-sensitive receptors are

present in chondrocytes and chondrocyte-like cells from rodents and bovine (Mistry et al., 2006; Ode et al., 2013; Varani et al., 2008a), their expression and function in human chondrocyte progenitors has been insufficiently explored (Pinto-Cardoso et al., 2020). We show here that A_{2A} and A_{2B} receptors immunoreactivity is evenly distributed in human BM-MSCs undergoing chondrogenic differentiation and it remains roughly constant at all culture stages (7 and 14 days; Figure 14B). Interestingly, the persistent transcriptional activity of HIF-1 α induced by DMOG in normoxic conditions transiently downregulated the A_{2B} receptor subtype at culture day 7 and disproportionally upregulated the A_{2A} receptor in more differentiated cells resulting from prolongation of DMOG treatment until day 14 (Figure 14A and 14B). DMOG-induced overexpression of the A_{2A} receptor in chondrogenic-differentiating BM-MSCs may affect the A_{2A}/A_{2B} receptors balance towards the preferential activation of high-affinity A_{2A} receptors by low ADO amounts (see above). We confirmed this trend given that one could partially rehabilitate chondrogenesis of DMOG-exposed BM-MSCs, which was indicated by restoration of SOX9 translocation to the nucleus (an early chondrogenic marker) upon blocking selectively the A_{2A} receptor with SCH 442416 at all culture time points. Although the rehabilitation effect of SCH 442416 was only partial, since it failed to affect BM-MSCs proliferation/viability and type II collagen production (a late chondrogenic marker), selective blockage of the A_{2B} receptor with PSB 603 had even lesser effects. Indeed, SOX9 plays a pivotal role in the regulation of type II collagen gene expression by binding to its promoter during chondrogenic differentiation (Bell et al., 1997). Yet, no positive correlation between SOX9 and type II collagen expression has been observed in adult articular chondrocytes (Aigner et al., 2003). This feature has been attributed to the time dynamics of chondrogenesis, where early increases in SOX9 transcriptional levels are followed (yet non-coincidentally) by type II collagen production (Herlofsen et al., 2011; Wu et al., 2021a; Yi et al., 2018).

Upregulation of the A_{2A} receptor transcription and activity has also been observed in endothelial cells of the human lung microvasculature (HLMVECs) and pulmonary artery (HPAECs) submitted to hypoxia, involving a mechanism related to HIF-2 α transcriptional activity (Ahmad et al., 2009). However, the opposite was also verified in human umbilical vein endothelial cells (HUVECs), where hypoxia switched the A_{2A}/A_{2B} receptor transcriptional ratio from a predominant A_{2A} receptor subtype under normoxia conditions to a dominant A_{2B} “angiogenic” receptor phenotype upon 3 hours of hypoxia (4.6% O₂), via a mechanism involving the expression of VEGF independently of HIF-1 α (Feoktistov et al., 2004). Likewise, hypoxia-induced A_{2B} receptor overexpression was also observed in human lung fibroblasts (Zhong et al., 2005), as well as in HMEC-1 and in the colonic mucosa of mice with TNBS-induced colitis (Kong et al., 2006). Thus, it seems that controversial results

concerning the impact of hypoxia on A_{2A}/A_{2B} receptors balance may emerge depending on the experimental conditions, namely the origin of the cells/tissue, the duration of the hypoxic stimulus, and on whether the receptors are activated by the natural ADO ligand or enzymatically-stable agonists. This study was designed to explore the impact of HIF-1 α transcriptional activation by DMOG under normoxic conditions in the expression and function of ATP metabolizing enzymes and ADO formation in chondrogenic-differentiating BM-MSCs obtained from aged (Pm) women. The influence of DMOG-induced transcriptional HIF-1 α activation was also assessed concerning the density and function of high-affinity A_{2A} and low-affinity A_{2B} receptors in these cells. Overall, data suggest that DMOG-induced HIF-1 α transcriptional activity hampers the chondrogenic differentiation of Pm BM-MSCs. The mechanism(s) underlying the impairment of the chondrogenic fate may be owed to deficient extracellular ADO formation and/or release from aged BM-MSCs via the CD39/CD73 cascade and ENT-1 transporters, respectively. The functional repercussion of ADO deficiency is highlighted as consequence of DMOG-induced disruption of the A_{2A}/A_{2B} receptors equilibrium leading to a preferential activation of the high-affinity A_{2A} receptor subtype in aged BM-MSCs from early chondrogenic commitment stages. Notwithstanding the fact that HIF-1 α stabilization influences A_{2A}/A_{2B} receptors expression and/or activation balance, we did not explore in this study the downstream molecular pathways responsible for their differential effects. This limitation deserves investigation in a dedicated study also considering that both A_{2A} and A_{2B} receptors share overlapping intracellular signalling pathways and/or act as “biased” receptors (see, e.g. (Bessa-Goncalves et al., 2018)).

Age-related changes in BM-MSCs compromises their clinical potential for both bone and cartilage regeneration. In aged women, BM-MSCs' ability to differentiate into osteoblasts and chondrocytes may be impaired due to hormonal decline, cell senescence and alterations in the local microenvironment, such as prolonged/exacerbated hypoxia. Aging cells acquire a senescence-associated secretory phenotype (SASP). This phenotype is characterized by the secretion of proteins, such as cytokines, chemokines and growth factors, as well as anti-inflammatory factors and/or adenine-based purines (i.e. ATP and adenosine), which may affect the communication with neighbouring cells via autocrine/paracrine mechanisms (Borodkina et al., 2018). Unfortunately, due to clinical limitations of bone marrow sampling, we were unable to evaluate the impact of HIF-1 α stabilisation in BM-MSCs from younger females undergoing chondrogenic differentiation. Yet, preliminary data show that the chondrogenic differentiating of younger BM-MSCs (47 $\pm$ 1 years old, n=3) express higher levels of chondrogenic biomarkers, namely SOX9 and type II collagen, compared to cells from aged women (unpublished results). Notwithstanding this limitation may deserve further research efforts, evaluation of the chondrogenic

differentiation of putatively affected BM-MSCs from PM women may open new avenues to restore chondrogenesis in the population most affected by degenerative osteochondral lesions.

In summary, this study gathers compelling evidence on the interplay between HIF-1 α and the purinergic signalling cascade in chondrogenic differentiation of aged human BM-MSCs while paving the way for novel therapeutic strategies to ameliorate and/or prevent osteochondral damage in osteoarthritic joints in aged Pm women.

Materials and Methods

Human bone marrow-derived mesenchymal stem cells (BM-MSCs) isolation and culture

Bone marrow samples were isolated from the neck of the femur of twenty-seven female patients (75 $\pm$ 2 years old) undergoing total hip arthroplasty owe to non-inflammatory degenerative osteoarthritis. Handling of bone marrow samples and cell culturing were performed as previously described (Bessa-Andr s et al., 2024; Noronha-Matos et al., 2023). To avoid *in vitro* phenotypic changes and senescence of the cells, we limited their usage to first subcultures. No pooled samples from different individuals were carried out at any circumstance.

Before differentiation of the cells into interest lineages, they were plated onto plastic bottom wells at an initial density of 2.5x10⁴ cells/mL and allowed to expand until 80% confluence in a humidified atmosphere of 95% air plus 5% CO₂, at 37°C, for 15 days. The expansion medium consisted of α -minimal essential medium (α -MEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin and 2.5 μ g/mL amphotericin B. Plastic non-adherent cells were discarded 5 days after plating; from this time point onwards, we started culture medium changes twice a week.

Multipotent capability of BM-MSCs and viability/proliferation assays

Under the aforementioned experimental conditions, cultured cell yields were highly enriched in multipotent BM-MSCs – see, e.g.(Noronha-Matos et al., 2012). The Supplementary Figure 11 shows that plastic-adherent BM-MSCs isolated under appropriate condition media can differentiate into chondrocyte, adipocyte and osteocyte cell lineages – see below, *cf.*(Muraglia et al., 2000; Noronha-Matos et al., 2023; Pittenger et al., 1999).

The osteogenic differentiation of BM-MSCs was performed by incubating the cells with α -MEM supplemented with ascorbic acid (50 $\mu\text{g/mL}$), β -glycerophosphate (10 mM) and dexamethasone (10 nM), during 35 days. BM-MSCs' osteogenic differentiation was confirmed by increases in (1) the activity of alkaline phosphatase (ALP), (2) the amount of the osterix transcription factor (Western blot analysis at 21-day cultures), and (3) the formation of mineralized bone nodules (Alizarin Red staining at 35-day cultures) (Bessa-Andr es et al., 2024; Noronha-Matos et al., 2023), as shown in Supplementary Figure 5A.

For adipogenic differentiation and assessment, expanded cells were allowed to grow for 21 days in Dulbecco's Modified Eagle's Medium (DMEM) - high glucose supplemented with FBS (10%), insulin (10 $\mu\text{L/mL}$), dexamethasone (100 nM), indomethacin (0.5 mM), 3-isobutyl-1-methylxanthine (IBMX, 60 μM), penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), and amphotericin B (2.5 $\mu\text{g/ml}$). We used Western blot analysis to assess increases in the density of the adipogenic marker, peroxisome proliferator-activated receptor γ (PPAR- γ) (James, 2013). Identification of intracellular lipid droplets was performed at culture day 21. This was done after washing the cells three times with phosphate-buffered saline (PBS) 1x, fixation with 4% paraformaldehyde (PFA) (Yang et al., 2018), and incubation with the Oil Red dye, for 2h. Cells documentation was made in an Olympus CKX41 microscope (Olympus, Tokyo, Japan) equipped with a digital camera (Olympus SC3, Tokyo, Japan) running the Olympus Analysis GetIT 5.1 imaging software (Olympus, Tokyo, Japan). Incubation of BM-MSCs with the adipogenic cocktail decreased their fibroblast-like morphology and favoured progressive accumulation of round lipid droplets in the cytoplasm of the cells (see Supplementary Figure 5A). Increases in PPAR- γ amounts in cell homogenates, also confirmed the adipogenic differentiation of cultured BM-MSCs (Supplementary Figure 5A).

For the chondrogenic differentiation, once BM-MSCs reached 80% confluence the expansion medium was changed by a chondrogenic differentiation cocktail. The latter consisted of a DMEM - high glucose medium supplemented with FBS (2.5%), transforming growth factor- β 3 (TGF- β 3, 10 ng/mL), insulin-transferrin-selenium (ITS) supplement (100x, 1%), ascorbic acid (50 $\mu\text{g/mL}$), L-proline (40 $\mu\text{g/ml}$), dexamethasone (100 nM), penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), and amphotericin B (2.5 $\mu\text{g/ml}$) (Garcia-Alvarez et al., 2011; Solchaga et al., 2011). To ascertain the chondrogenic differentiation of BM-MSCs, we evaluated the amount of the SOX9 transcription factor, a master regulator of chondrogenesis, in total cell homogenates by Western blot analysis at culture days 7 and 21 (Supplementary Figure 5B). We used the Toluidine Blue O assay to evaluate the cell matrix deposition of polysaccharides (Bergholt et al., 2019). To this end, the cells were fixed with 4% PFA at room temperature for 10 min. Then, the cells were washed out with sterile

PBS 1x and, subsequently, exposed to 1% acetic acid, for 10-15 seconds. Finally, the cells were stained with 1% Toluidine Blue O at room temperature for 5 min, and then washed out with PBS 1x three times. Cells documentation was made in an Olympus CKX41 microscope (Olympus, Tokyo, Japan) equipped with a digital camera (Olympus SC3, Tokyo, Japan) running the Olympus Analysis GetIT 5.1 imaging software (Olympus, Tokyo, Japan). Incubation of BM-MSCs with the chondrogenic cocktail increased cell matrix deposition of polysaccharides (see Supplementary Figure 5A). Using immunofluorescence confocal microscopy at culture days 7 and 14, Supplementary Figure 5C shows parallel increases in the nuclear translocation (DNA binding) of SOX9 and type II collagen production by BM-MSCs undergoing chondrogenic differentiation compared to cells cultured in the standard expansion medium.

To monitor cells viability/proliferation, we used in this study the premixed ready-to-use Cell Counting Kit-8 (CCK-8) high-sensitivity assay, according to the manufacturer's instructions (Liu et al., 2011). Absorbance was measured at 450 nm using a Synergy HT microplate reader (RRID: SCR_020536; BioTek Instruments, Vermont, USA).

Induction of HIF-1 α transcriptional activity in chondrogenic-differentiating BM-MSCs under normoxic conditions

Chondrogenic-differentiating BM-MSCs were allowed to grow, for 14 days, in 95% air plus 5% CO₂ humidified atmosphere (normoxic conditions) in the absence (control) or presence of the cell permeable competitive inhibitor of prolyl hydroxylase, dimethyloxallyl glycine (DMOG, 30 μ M), which promotes stabilisation and intracellular accumulation of HIF-1 α protein (Zhang et al., 2016; Zhdanov et al., 2015). The concentration of DMOG (30 μ M) was chosen as it gave the best results compared to other concentrations ranging from 10 to 300 μ M appraised during the optimization protocols using chondrogenic-differentiating BM-MSCs isolated from adult female Wistar rats (5.5 $\pm$ 2.0 months old, n=4; *Rattus norvegicus*; Charles River, RGD Cat. No. 13508588, RRID:RGD_13508588). These animals, which were euthanized for other research purposes, were used in order to reduce the number of experimental animals, as determined by the ARRIVE 2.0 guidelines. BM-MSCs were harvested by flushing rat femoral diaphysis with the expansion α -MEM medium (see above) onto culture plates, via a 18-G needle coupled to a 5-mL syringe (Ohgushi et al., 1989). The procedures to induce chondrogenesis and the phenotypic characterization of rat BM-MSCs were similar to those mentioned above for the Human cells, unless otherwise stated (see Supplementary Figure 6).

In some experiments, the human BM-MSCs undergoing chondrogenic differentiation were also incubated with adenosine modifying drugs, like selective A_{2A} and A_{2B} receptor antagonists, SCH 442416 (10 nM) (Todde et al., 2000) and PSB 603 (10 nM) (Borrmann et al., 2009), respectively, and ENT-1 transport inhibitors, namely dipyridamole (0.5 µM) or NBTI (50 µM). When used, these drugs were included in culture media from day 0 onwards and, thus, they were renovated whenever changing culture media, *i.e.* twice a week.

Immunofluorescence staining and confocal microscopy imaging

For imaging purposes, chondrogenic-differentiating BM-MSCs were plated and allowed to grow in glass-bottom chamber slides for 7 or 14 days in the conditions described above for induction of the HIF-1α transcriptional activity under normoxic conditions. For cell fixation, we used 4% PFA in PBS, with subsequent 1.5 h incubation with a blocking buffer I containing FBS (10%), bovine serum albumin (BSA, 1%), Triton X (0.1%) and NaN₃ (0.05%). Fixed cells were incubated for 2 hours, in the dark, with epitope-specific primary antibodies diluted in the blocking buffer II with an identical composition to blocking buffer I, except concerning FBS (5%). In this study, we used HIF-1α 1:200 (ab1 [H1alpha67], mouse), SOX9 5 µg/mL (ab185966, rabbit), type II collagen 1:100 (ab34712, rabbit), ecto-5'-nucleotidase 1:1000 (h5'NT-2L15, rabbit), NTPDase1 1:1000 (hN1-1c15, guinea pig), NTPDase2 1:200 (hN2-Kw314, rabbit), NTPDase3 1:200 (hN3-1c14, guinea pig), NTPDase8 1:1000 (hN8-C5s, mouse), A_{2A} receptor 1:100 (A2aR21-A, rabbit), A_{2B} receptor 1:50 (AAR-003, rabbit) and ENT-1 1:50 (sc-377283, mouse). After washout of the primary antibodies, the cells were incubated with secondary antibodies diluted in the blocking buffer II, namely Alexa Fluor 488 1:1500 (anti-rabbit), Alexa Fluor 568 1:1500 (anti-mouse), Alexa-Fluor 647 1:150 (anti-guinea pig) and/or TRITC 1:150 (anti-guinea pig), for 1 hour in the dark. Negative controls were performed in the absence of the primary antibodies as previously described (Supplementary figure 9) (Noronha-Matos et al., 2023). Glass slides mounted with Vectashield® antifade mounting medium with DAPI (H-1200-10; Vector Laboratories, Inc.; Newark, CA 94560, USA) were stored, at 4°C, until observation using an Olympus FV1000 laser-scanning confocal microscope running the FluoView Advanced Software for FV1000 (v. 4.0.3.4; Olympus, Tokyo, Japan). The microscope settings and acquisition parameters were kept unaltered during documentation to allow comparisons between acquired micrographs. The technical details about immunofluorescence staining, confocal microscopy observation and standardization procedures for imaged comparisons were extensively described in previously published papers (Bessa-Andr s et al., 2024; Noronha-Matos et al., 2023). Image analyses to assess the average fluorescence intensity per cell (outlined as a region of interest, ROI) and background subtraction were made with the

Image J 1.37c software (NIH, Bethesda, MD, USA) after importing confocal micrograph files stored in the Olympus Multi TIFF format (Tokyo, Japan). Manuscript figures depict typical immunofluorescence micrographs for each experimental condition.

Quantification of extracellular ATP and kinetics of adenosine formation from adenine nucleotides

Measurements of extracellular ATP in culture media were performed by bioluminescence using the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana, USA) in a multi-detection microplate reader (Synergy HT, BioTek Instruments, Vermont, USA), according to the manufacturer's instructions (Noronha-Matos et al., 2023; Pinheiro et al., 2013). Briefly, chondrogenic-differentiating BM-MSCs cultured for 7 and 14 days in the absence or presence of DMOG (30 μ M) were washed twice with gassed (95% O₂ plus 5% CO₂) Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.4 mM NaH₂PO₄, 11.9 mM NaHCO₃, and 11.2 mM glucose, pH 7.4). After maintaining the cells in the gassed Tyrode's solution for 30 min, at 37°C, the incubation fluid was removed, snap-frozen in liquid nitrogen and kept at -80°C, until ATP quantifications. The ATP measurements were made in duplicate against freshly prepared high-purity ATP external standards; we made two to four replicas per individual experiment. The amount of ATP per culture well (in pmol) was normalized for cell viability/proliferation using the CCK-8 result obtained in the same cell population. We found a negligible (< 0.1 mU/mL) lactate dehydrogenase (LDH; EC 1.1.1.27) activity in the samples used for extracellular ATP quantification (Keiding et al., 1974). This ensures the integrity of cells throughout the experimental period, which otherwise would complicate data interpretation due to intracellular ATP contamination of the analysed fluid.

The time course of the extracellular catabolism of ATP and AMP and metabolites formation was evaluated by High-Performance Liquid Chromatography (HPLC) in chondrogenic-differentiating BM-MSCs cultured for 7 and 14 days in the absence or presence of DMOG (30 μ M), following a methodology previously described by our group (Costa et al., 2011; Noronha-Matos et al., 2023). In brief, after washing out the culture medium, we incubated the cells for 30 min, at 37°C, with ATP or AMP (100 μ M) made in gassed Tyrode's solution (zero time). At predefined time points, we collected 75 μ L-samples of the incubation medium for HPLC analysis (LaChrom Elite, Merck, Frankfurt, Germany) of substrate disappearance and formation of metabolites. We plotted the variations in the concentration of substrates and formation of metabolites as a function of time (progress curves). The parameters analysed were the half-life time ($t_{1/2}$) of the initial substrate, the kinetic of metabolites

appearance, and the concentration of the substrate or any product remaining at the end of the experiment. The activity of NTPDases and ecto-5'-nucleotidase/CD73 was calculated by the [Metabolites]:[Substrate] ratio divided by the time point in minutes. Adenine nucleotides, namely ATP or AMP (100 μ M), did not suffer from spontaneous degradation over time during 30 min in the absence of the cells. The incubation fluid remaining at the end of the protocol had a negligible (<0.1 mU/mL) lactate dehydrogenase (LDH; EC 1.1.1.27) activity demonstrating that cell integrity was maintained during the whole procedure (Keiding et al., 1974).

RNA extraction, reverse transcription and real-time PCR

Chondrogenic-differentiating BM-MSCs were detached from culture plates at days 7 and 14 by incubating the cells with trypsin-EDTA (0.04%) and type I collagenase (0.025%) in PBS (pH 7.4) for 10 min. The resulting cell suspensions were centrifuged (5000 rpm) for 10 minutes, at 4°C; after removing the supernatants, the pellets were snap-frozen at -80°C. The total RNA was extracted using the RNAeasy Mini kit (Qiagen, Germany) and, subsequently, quantified using a Nanodrop 1000 Spectrophotometer (RRID:SCR_016517, Thermo Fisher Scientific, USA); the RNA samples with 260/280 nm absorbance ratios of 1.5–2.0, were considered eligible for processing. The cDNA synthesis of eligible samples was performed using the Nzy First-Strand cDNA Synthesis Kit (Nzy First-Strand cDNA Synthesis Kit). Matrix metalloproteinase 13 (*MMP13*; Hs00942584_m1), type X collagen (*COL10A1*; Hs00166657_m1), and Ubiquitin C (*UBC*, reference gene; Hs00824723_m1) gene transcripts were quantified by Real-Time PCR using commercially-available Taqman® probes (Taqman® Kits, Applied Biosystems, USA) in a Corbett Rotor Gene 600 Thermocycler (Corbett Research, UK). Each reaction was performed in triplicate per individual and the average Ct value was used in the analysis. The relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method (Guerra Leal et al., 2022).

Library preparation for transcriptome sequencing (RNAseq) and analysis

RNA sequencing libraries of human BM-MSC samples (RIN greater than or equal to 7.7) undergoing chondrogenic differentiation were prepared and sequenced by an outsourcing company Novogene (Cambridge) using 150-bp paired-end reads on the Illumina NovaSeq 6000 platform (two to three biological replicates per group). Each sample yielded over 40 million reads. Fastq files were aligned to the GRCh38/hg38 transcriptome using HISAT2 (2.0.5) (Mortazavi et al., 2008) with default settings, *nd* gene-specific read counts were

assigned using featureCounts (v1.5.0-p3) (Liao et al., 2014). Differentially expressed genes (DEGs) were calculated using DESeq2 (Anders & Huber, 2010; Love et al., 2014). Differential expression was analysed using DESeq2 (v1.20.0) for four groups, considering genes with at least ten raw counts in a minimum of three samples (Anders & Huber, 2010; Love et al., 2014). Donor variation was controlled as a covariate. Genes with a raw $P < 0.01$ and absolute $\log_2\text{FoldChange} > 0.5$ were deemed significant. Heatmaps and individual gene expression levels were represented as normalized count values and FPKM, respectively. Heatmaps were created with z-scores using heatmap2 (gplots package) and refined in the Heatmap function of ComplexHeatmap (Gu et al., 2016; Warnes et al., 2024). Functional enrichment was analysed for transcription factor (TF) activity with DoRothEA (Garcia-Alonso et al., 2018) and for Gene Ontology (GO) using the enrichGO function in clusterProfiler (Wu et al., 2021b).

Ethics Approval, Humans Consent to Participate and Use of Experimental Animals

Informed consent to use the biological material that would be otherwise discarded was obtained. All procedures were approved by the Ethics Committees of Centro Hospitalar de Vila Nova de Gaia – Espinho (CHVNGE, registration nº 137/2018-2, endorsed on January 10, 2019) via the Gabinete Coordenador de Investigação / DEFI – Centro Hospitalar Universitário de Santo António (CHUdSA, registration nº 2021-002(001-DEFI-001-CE, endorsed on September 01, 2021), and of Instituto de Ciências Biomédicas Abel Salazar (Medical School) of the University of Porto. The investigation conforms to the principles outlined in the Declaration of Helsinki. The use of animals in the DMOG optimization protocol was done for obvious ethical and practical reasons *vis a vis* the Human alternative. Experimental procedures and care of the animals followed the recommendations of the European convention for the Protection of Vertebrate animals for experimental and other Scientific purposes (ETS 123), Directive 2010/63/EU, and the Portuguese rules (DL 113/2013). We obtained approval for all experiments involving animals by the national authority, Direção Geral de Alimentação e Veterinária, and the ICBAS Animal Ethical Committee (No. 224/2017).

Reagents and antibodies

α -Minimum Essential Media (MEM, Cat. No. 0894), adenosine (ADO; Cat. No. 9251), adenosine 5'-monophosphate (AMP) sodium salt (Cat. No. 1752), 4-[2-[[6-amino-9-(nethyl- β -D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzene propanoic acid

hydrochloride (CGS21680, Cat. No. C141), amphotericin B (Cat. No. A2942), ascorbic acid (Cat. No. A4034), adenosine-5'-triphosphate (ATP, Cat. No. 7699), β -glycerophosphate (Cat. No. G9891), dipyridamole (Cat. No. 9766), dexamethasone (Cat. No. D4902), Dulbecco's Modified Eagle Medium (DMEM) – high glucose (Cat. No. D7777), dimethyloxallyl glycine (DMOG, Cat. No. D3695), fetal bovine serum (FBS; Cat. No. F9665), 3-isobutyl-1-methylxanthine (IBMX, Cat. No. I5879), indomethacin (Cat. No. I7378), insulin (Cat. No. I9278), Insulin-Transferrin-Selenium x100 (Cat. No. I3146), L-Proline (A4034), phosphate buffered saline (PBS; Cat. No. D1408), Penicillin-Streptomycin (Cat. No. P0781), Transforming Growth Factor (TGF)- β 3 (Cat. No. SRP3171) were supplied by Sigma-Aldrich (St. Louis, MO, USA; RRID: SCR_008988). 6-N,N-Diethyl-D- β , γ -dibromomethyleneATP trisodium salt (ARL 67156, Cat. No. 1283), 2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 442416, Cat. No. 2463), 8-[4-[4-(4-chlorophenyl)piperazine-1-sulfonyl]phenyl]-1-propylxanthine (PSB 603, Cat. No. 3198) and 6-S-[(4-nitrophenyl)methyl]-6-thioinosine (NBTI, Cat. No. 2924) were purchased from Tocris Bioscience (Bristol, United Kingdom). The stock solutions of SCH 442416, PSB 603, dipyridamole and NBTI were prepared in dimethyl sulfoxide (DMSO; Merck, Germany); the maximal percentage of DMSO used in the cultures was 0.05% v/v, as it proved to be harmless to cells. We used distilled water or culture medium to dissolve all other drugs. Stock solutions were stored as frozen aliquots at -20°C .

Cell Counting Kit-8 (CCK-8; Cat. No. CK04) was purchased from Dojindo Laboratories (Tokyo, Japan). ATP bioluminescence assay kit HS II was purchased from Roche Applied Science (Indianapolis, Indiana, USA). RNAeasy Mini kit was purchased from Qiagen Pty Ltd (Hilden, Germany; RRID:SCR_008539). NZY First Strand cDNA Synthesis kit (Cat. No. MB12501) and NZYSpeedy One-step RT-qPCR Probe Master Mix (MB40501) were purchased from NZYTech, Lda – Genes & Enzymes (Lisbon, Portugal). TaqMan probes for matrix metalloproteinase 13 (MMP13, Hs00942584_m1), type X collagen (COL10A1, Hs00166657_m1) and Ubiquitin C (UBC, Hs00824723_m1) were purchased from Thermo Fisher Scientific (MA, USA; RRID:SCR_008452).

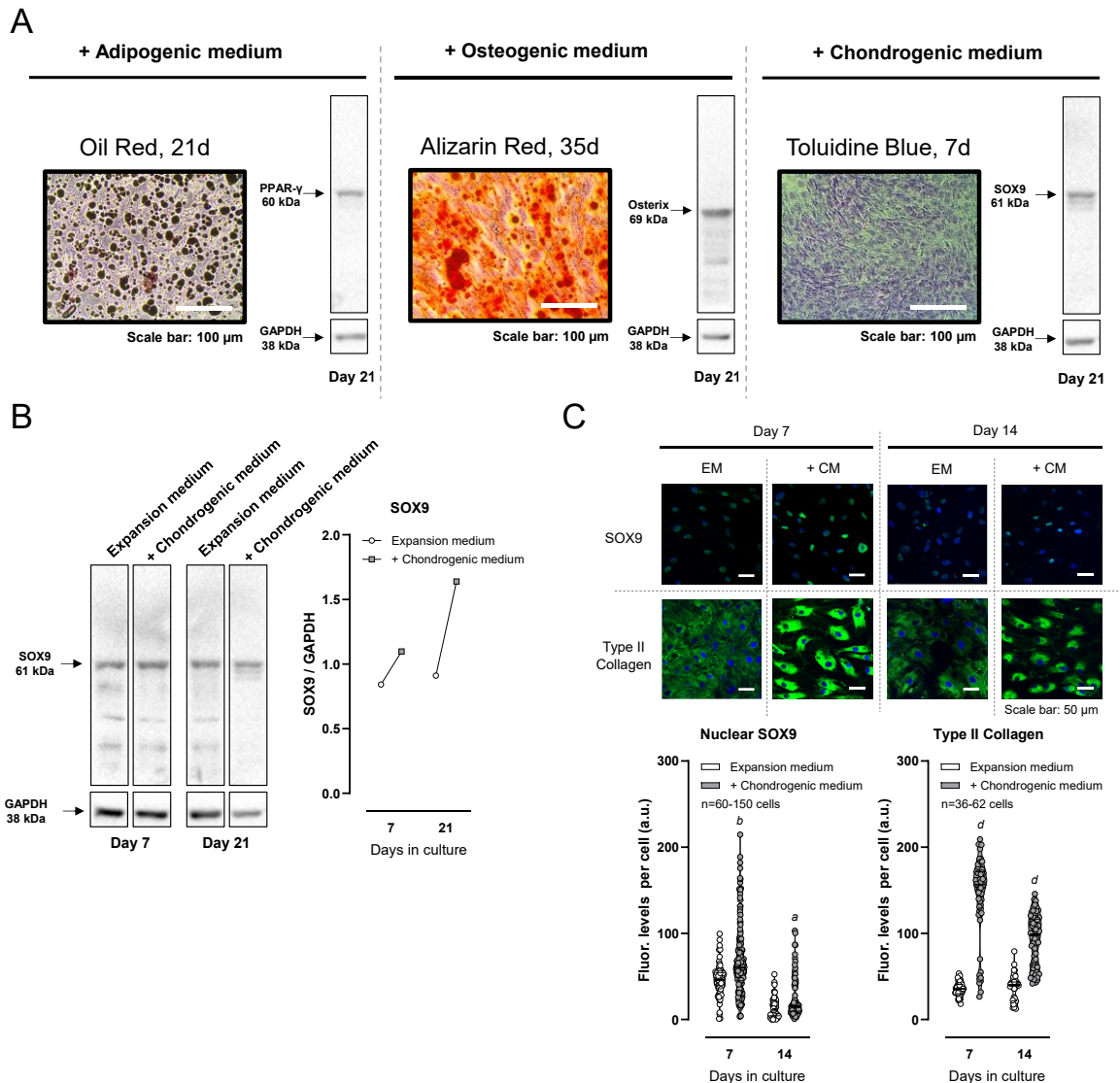
Primary anti-human and secondary conjugated antibodies used in this work were previously validated (Munkonda et al., 2009; Noronha-Matos et al., 2012; Pelletier et al., 2017; Pinheiro et al., 2013). Primary antibodies included: anti-A_{2A} receptor (rabbit, Alpha Diagnostic International Cat# A2AR21-A, RRID:AB_1609239) purchased from Alpha Diagnostics International (Texas, USA), anti-A_{2B} receptor (rabbit, Alomone Labs Cat# AAR-003, RRID:AB_2039709) purchased from Alomone Labs (Jerusalem, Israel; RRID:SCR_013570). The anti-HIF-1 α (mouse, Abcam Cat# ab1, RRID:AB_296474), anti-SOX9 (rabbit, Abcam Cat# ab185966, RRID:AB_2728660), anti-type II collagen (rabbit,

Abcam Cat# ab34712, RRID:AB_731688), and anti-OSX (rabbit, Abcam Cat# ab22552, RRID: AB_2194492) were purchased from Abcam (Cambridge, UK; RRID:SCR_012931). The anti-ENT-1 (mouse, Santa Cruz Biotechnology Cat# sc-377283) and anti-PPAR- γ (mouse, Santa Cruz Biotechnology Cat# sc-7273, RRID:AB_628115) were purchased from Santa Cruz Biotechnology (Texas, USA). The anti-human ecto-NTPDases and CD73 antibodies were developed and kindly supplied by JP and JS at the Centre de Recherche en Rhumatologie et Immunologie, University of Laval, Québec, QC, Canada (for additional information, see <http://ectonucleotidases-ab.com>); these included h5'NT-2_L1₅ (rabbit), hN1-1_{cl}5 (guinea pig), hN2-kw3I4 (rabbit), hN3-1_{cl}4 (guinea pig), and hN8-C5_s (mouse). The secondary antibodies Alexa Fluor 488 (anti-rabbit, Molecular Probes Cat# A-21206 (also A21206), RRID:AB_2535792) and Alexa Fluor 568 (anti-mouse, Thermo Fisher Scientific Cat# A10037, RRID:AB_2534013) were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA). The Alexa Fluor 647 (anti-guinea pig, Jackson ImmunoResearch Labs Cat# 706-605-148, RRID:AB_2340476) and Rhodamine (TRITC) (anti-guinea pig, Jackson ImmunoResearch Labs Cat# 706-025-148, RRID:AB_2340445) were purchased from Jackson ImmunoResearch (Newmarket, Suffolk, UK).

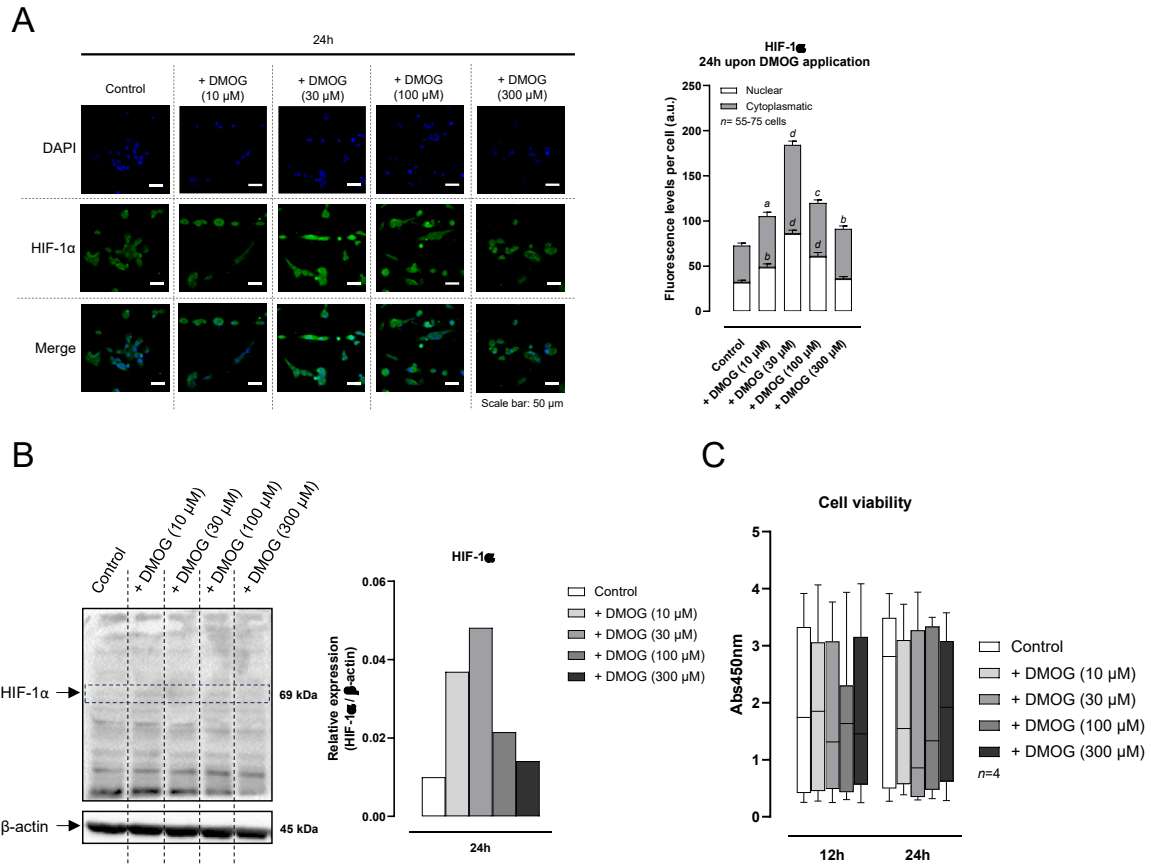
Presentation of data and statistical analysis

Data obtained in this study was expressed as dot plots from an n number of individuals. Shapiro-Wilk normality test was performed, and subsequent statistical data analyses included parametric (one-way ANOVA with Fisher's LSD test) or non-parametric (Kruskal-Wallis test with uncorrected Dunn's test) tests, with a confidence level of 0.05, i.e. a 95% confidence interval. Significant differences were considered for $P < 0.05$. Prism 10.0.2TM software (RRID: SCR_002798; GraphPad Software, CA, United States) was used for statistical data analysis.

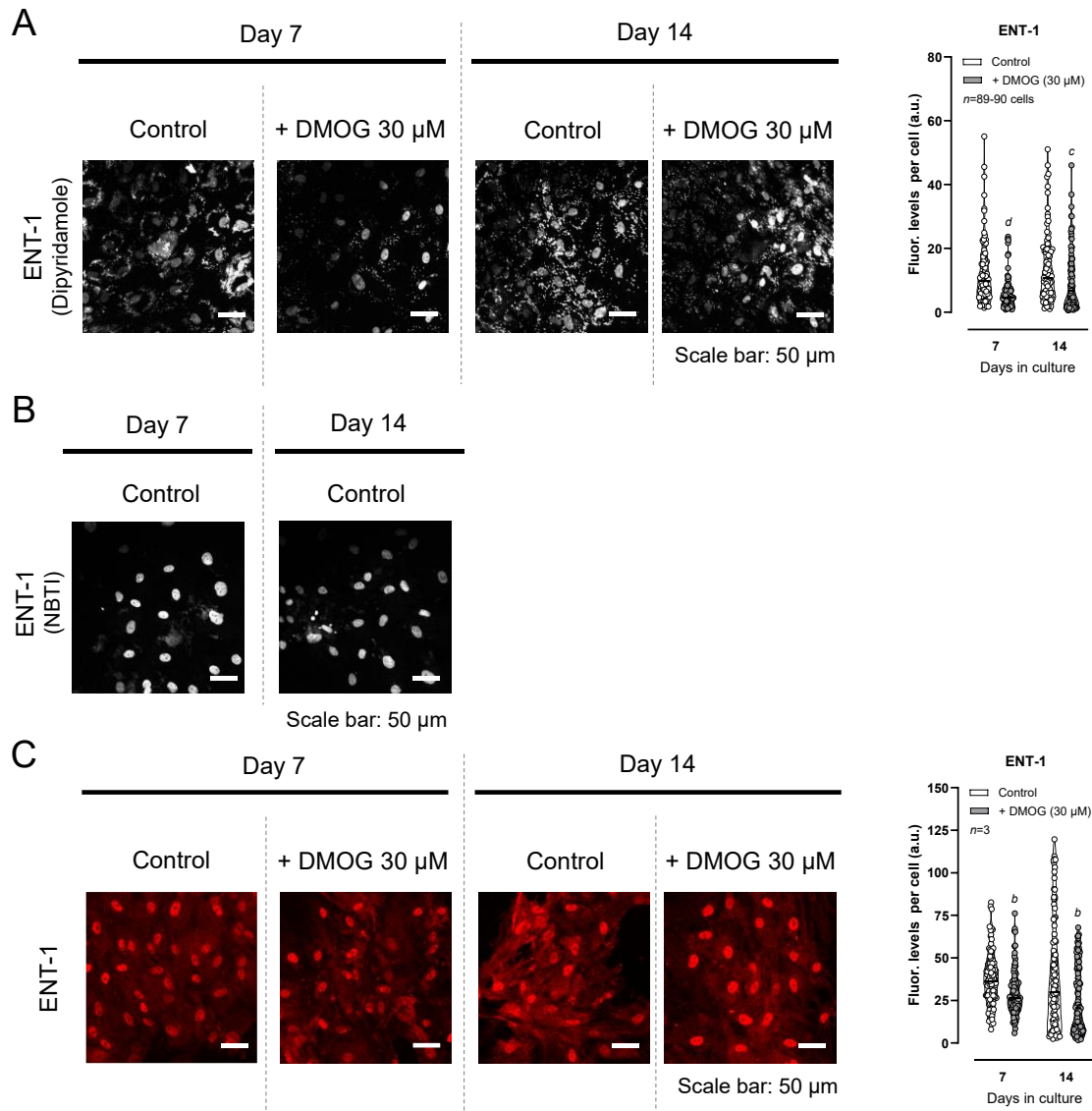
Supplementary information



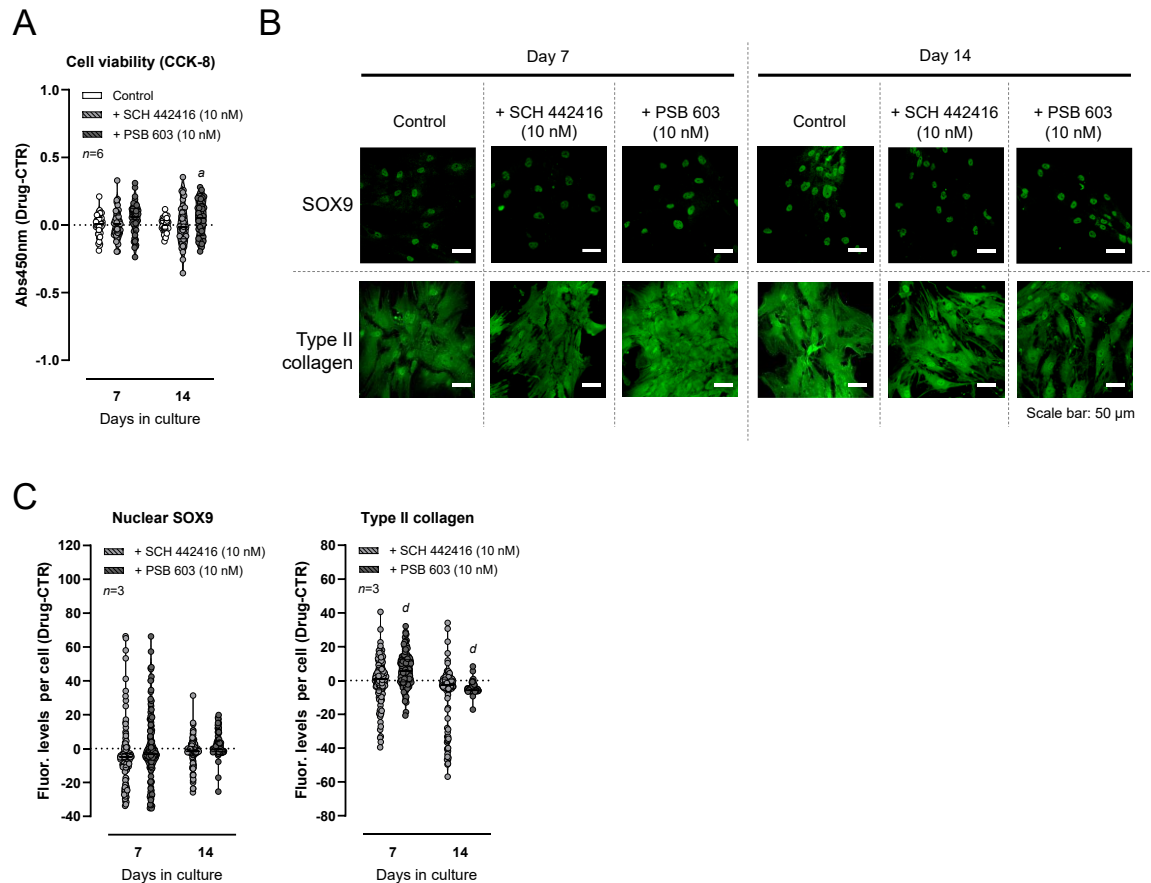
Supplementary Figure 5 – Evidence of the multipotent differentiation capability of plastic-adherent MSCs obtained from the bone marrow (BM) of a post-menopausal (Pm) woman (75-year-old). The cells were allowed to grow and differentiate using the adipogenic, osteogenic and chondrogenic cocktails for 7, 14, and/or 21 days. **Panel A** shows microscopic pictures of lipid droplets (oil red assay, day 21), matrix mineralization (alizarin red assay, day 35) and polysaccharide deposition (toluidine blue assay, day 7) pending the use of adipogenic, osteogenic and chondrogenic cocktails, respectively. The scale bar is 100 μm. Moreover, increases in the relative protein density of PPAR-γ (60 kDa), Osterix, (69 kDa) and SOX9 (61 kDa) transcription factor biomarkers of adipogenesis, osteogenesis and chondrogenesis were also obtained using lineage-specific differentiation cocktails; data was normalized by GAPDH (39 kDa) protein amounts. **Panel B** depicts immunoblots showing increases in the amounts of the SOX9 transcription factor (61 kDa) in BM-MSCs from a Pm 75-year-old woman treated with the chondrogenic-inducing medium compared to the incubation with expansion cocktail, both at culture day 7 and 21; GAPDH (39 kDa) immunoreactivity was used as normalizer. **Panel C** shows representative confocal micrographs of SOX9 and type II collagen in BM-MSCs from a Pm women grown in culture for 7 and 14 days in the presence of the expansion medium (EM) or the chondrogenic-differentiation (CM) cocktail. Blue dots represent nuclei staining with DAPI. Scale bar is 50 μm. Box and whiskers plots show the immunofluorescence intensity computed from acquired confocal microscopy images. Ordinates represent fluorescence intensity per cell (arbitrary units, a.u.) of the chondrogenic markers, SOX9 and type II collagen, obtained in 7- and 14-day cultures. A total of 36-150 cells were analysed from two to three Pm women (71±2 years old). ^a*P*<0.05, ^b*P*<0.01 and ^d*P*<0.0001 (Kruskal-Wallis test with uncorrected Dunn's test) represent significant differences.



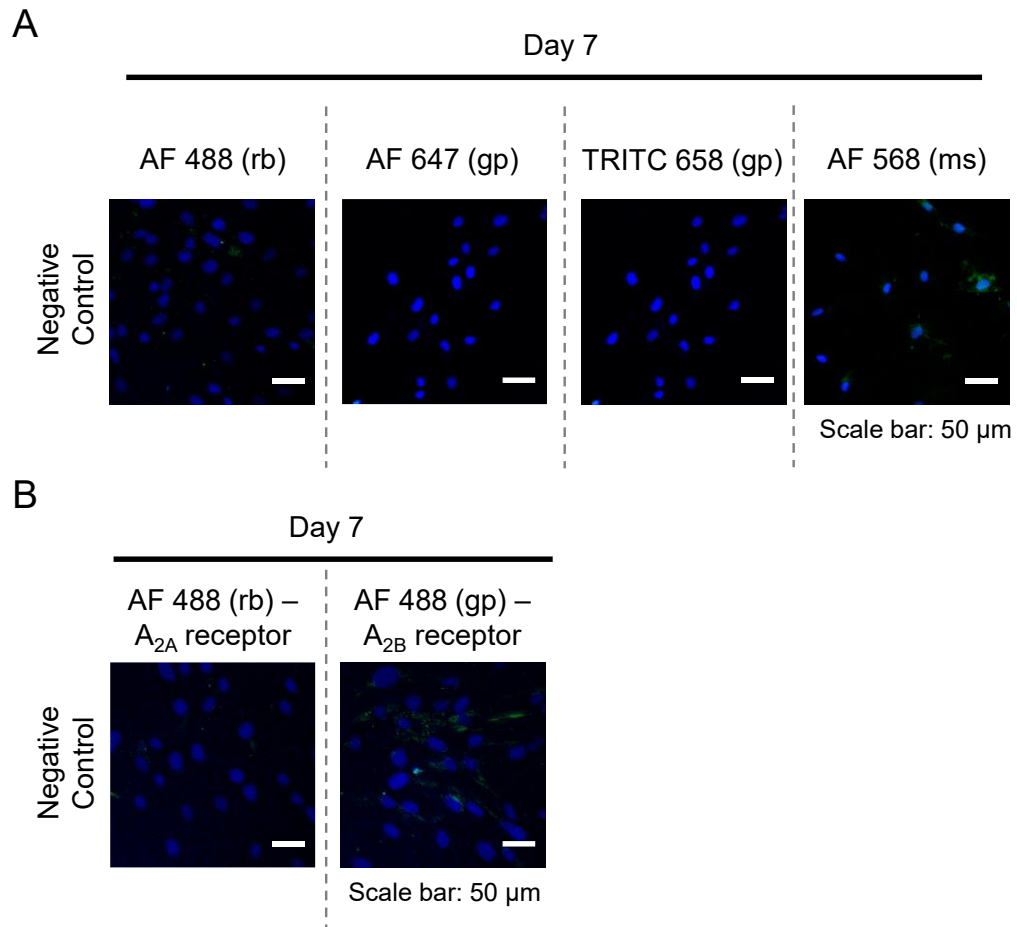
Supplementary Figure 6 – Experiments designed to optimize DMOG-induced upregulation of the HIF-1α transcriptional activity in BM-MSCs from adult female rats. Chondrogenic-differentiating rat BM-MSCs grew for 24 h in culture in either absence (control) or presence of DMOG used in concentrations ranging from 10 to 300 μM. Panel A shows confocal micrographs illustrating the immunocytochemical detection (green) of HIF-1α protein density in cultured BM-MSCs from a 3-month old female Wistar rat. Blue dots represent nuclei stained with DAPI. The scale bar is 50 μm. The right-hand side graph shows the computed average HIF-1α immunofluorescence intensity (ordinates, arbitrary units, a.u.) in the nucleus (white bars) and cytoplasm (grey bars) of cultured rat BM-MSCs. We analysed 55-75 cells from four animals. The vertical bars represent mean±S.E.M. ^a*P*<0.05, ^b*P*<0.01, ^c*P*<0.001 and ^d*P*<0.0001 (Kruskal-Wallis test with uncorrected Dunn's test) represent significant differences as compared to control values. Panel B shows a typical uncropped immunoblot stained against the HIF-1α protein (69 kDa) from BM-MSC homogenates of a 3-month-old female rat; β-actin (45 kDa) protein amounts were used as housekeeping protein for normalization purposes. The relative amount of HIF-1α corrected by the β-actin content of cell homogenates treated with increasing concentrations of DMOG is shown the right hand-side graph (grey bars). Panel C shows that increasing concentrations of DMOG (10-300 μM) did not affect the viability and/or proliferation of BM-MSCs when applied for 12 to 24 h, as determined by the CCK-8 assay; ordinates represent absorbance values measured at 450 nm. Boxes and whiskers represent pooled data from four female Wistar rat (5.5±2 months old); four replicates were made per individual experiment.



Supplementary Figure 7 – Upregulation of HIF-1 α transcriptional activity reduces the amount of the equilibrative nucleoside transporter 1 (ENT-1) in chondrogenic-differentiating BM-MSCs from Pm women. **Panel A** shows representative confocal images of BM-MSCs fluorescently tagged with dipyridamole (0.5 μ M). Exposure to DMOG (30 μ M) significantly decreased the density of ENT1 transporters in the plasma membrane. Please note that the absorption spectra of dipyridamole at physiological pH (7.4) overlaps with that of DAPI at 405 nm and, therefore, the white dots in cells centre denotes nuclei stained with DAPI contained in the Vectashield® antifade mounting medium (H-1200-10; Vector Laboratories, Inc.). Scale bar is 50 μ m. The right-hand side graphs show the obtained fluorescence intensity computed from acquired confocal microscopy images. Ordinates represent fluorescence intensity per cell (arbitrary units, a.u.) of ENT-1 upon background (no cells) and nuclear DAPI fluorescence being subtracted from all ROIs. A total of 89-90 cells were analysed. ^c $P < 0.001$ and ^d $P < 0.0001$ (Kruskal-Wallis test with uncorrected Dunn's test) represent significant differences. **Panel B** shows representative confocal images of BM-MSCs incubated with NBTI (50 μ M), instead of dipyridamole (0.5 μ M); all other experimental conditions were alike. White dots in the cells centre represent nuclei staining with DAPI. Scale bar is 50 μ m. **Panel C** shows representative images of the immunocytochemical detection of the adenosine transporter ENT-1 (red staining) in cultured BM-MSCs undergoing chondrogenic differentiation in the absence and in the presence of DMOG (30 μ M). The white dots in cells centre denotes nuclei stained with DAPI. Scale bar is 50 μ m. The right-hand side graphs show the ENT-1 immunofluorescence intensity computed from confocal microscopy images. Ordinates represent fluorescence intensity per cell (arbitrary units, a.u.) of ENT-1 immunoreactivity as a function of the number of days in culture (day 7 and 14) after nuclear DAPI fluorescence subtraction from all ROIs. A total of 91-109 cells were analysed from three Pm women (86 $\pm$ 5 years old). ^b $P < 0.01$ (Kruskal-Wallis test with uncorrected Dunn's test) represent significant differences.



Supplementary Figure 8 – The effect of the selective A_{2A} and A_{2B} receptor antagonists on human chondrogenesis under control conditions. Human BM-MSCs were allowed to grow for 7 and 14 days of culture in a chondrogenic medium in the absence or presence of selective A_{2A} (SCH 442416, 10 nM) and A_{2B} (PSB 603, 10 nM) adenosine receptor antagonists. **Panel A** represents cell viability/proliferation data using the CCK-8 assay; ordinates represent absorbance values measured at 450 nm. Dots in the violin plots represent four to eight individual replicates from six Pm women (70±4 years old). **Panel B** shows representative images of the immunocytochemical detection (green) of nuclear SOX9 and type II collagen under control conditions for 7 and 14 days in the absence or presence of selective adenosine receptor antagonists. The scale bar is 50 μ m. **Panel C** shows the immunofluorescence intensity graphs computed from acquired confocal microscopy images stained against chondrogenic markers, nuclear SOX9 and type II collagen. Ordinates represent fluorescence intensity per cell (arbitrary units, a.u.). Zero represents the identity between CCK-8 values, nuclear SOX9 and type II collagen levels in the absence and presence of the selective adenosine receptor antagonists (horizontal dashed line). A total of 99-130 cells were analysed from three Pm women (72±5 years old). ^aP<0.05 and ^dP<0.0001 (Kruskal-Wallis test with uncorrected Dunn's test) represent significant differences.



Supplementary Figure 9 – Negative controls of immunofluorescence staining using human BM-MSC cultures allowed to grow for 7 days in a chondrogenic-inducing medium (first subculture). **Panel A and B** shows the immunofluorescence staining detected in cells incubated with the secondary antibodies without prior incubation with primary antibodies against ecto-nucleotidases (**Panel A**) and A_{2A} and A_{2B} receptors (**Panel B**). Blue dots represent nuclei stained with DAPI contained in the VectaShield mounting medium. The scale bar is 50 μ m. gp, guinea pig; ms, mouse; rb, rabbit.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

According to the United Nations, the world population will reach 8.6 billion in 2023, 9.8 billion in 2050 and 11.2 billion in 2100. This upward trend is expected to continue, despite the fertility levels are decreasing (United Nations, 2017). Furthermore, life expectancy will increase from 73.6 year of age to 78.1 years of age in 2050 (Collaborators, 2024), meaning the world population will age. Aging is associated with a rise in musculoskeletal disorders, with common conditions including intervertebral disc issues, osteoporosis, bone fractures, osteogenesis imperfecta, osteoarthritis, and rheumatoid arthritis (RA) (Goltzman, 2002). Osteoporosis, in particular, is a global disease that significantly affects the social life of elderly people, especially post-menopausal women mostly probably due to downregulation of oestrogen levels (Akkawi & Zmerly, 2018; Boschitsch et al., 2017; Cheng et al., 2022). Additionally, mechanical (e.g., trauma or overuse associated to repetitive micro-trauma) and biological (e.g., osteochondritis dissecans or osteonecrosis) factors can cause joint surface issues in young and elderly people by damaging the articular cartilage from joint surfaces, with or without bone affection (Grimm et al., 2014). Cartilage defects present a significant clinical challenge due to reduced cartilage's self-healing ability (Buckwalter & Mankin, 1998a). The existing possibilities of cartilage repair, such as mosaicplasty, microfracture, and autologous chondrocyte implantation (ACI) (Medvedeva et al., 2018), fail on long-term outcomes regarding evolution to osteoarthritis (Bentley et al., 2003; Knutsen et al., 2004; Kreuz et al., 2006). The new cartilage is more fibrous and do not recapitulate the mechanical properties of physiological hyaline cartilage. In more severe cases, partial or total joint replacement surgery (arthroplasty) is the last resource to restore joint mobility in elderly patients, but are extreme solutions in younger individuals.

Mounting biomedical and clinical research studies now consider multiple uses of MSCs-based tissue engineering for bone and cartilage repair, mostly because these cells possess multi-differentiation potential, immunomodulatory effects, vasculogenic potential and trophic functions – reviewed in (Haque et al., 2015). However, their overall amounts in the body is relatively scarce, despite their presence in multiple tissues (Vizoso et al., 2017). Cell therapy-based procedures require hundreds of millions of functional MSCs per treatment; consequently, clonal expansion of these cells during several weeks is normally necessary, which may be cumbersome due to *in vitro* senescence and phenotypic modification of the cells (Baker et al., 2015). Our group showed that cell senescence and loss of their differentiation capability with ageing may limit the clinical application of MSCs for autologous cells transplantation and tissue engineering (Noronha-Matos & Correia-de-Sá, 2016). Other issues, such as patients' age, gender and co-morbidities, may also influence the optimal

culture conditions for scale production. Researchers and clinicians are working together to overcome these and other challenges related to incomplete knowledge about the necessary components of cell culture media, the optimal cell expansion periods, and the number of cell passages allowed before detrimental phenotypic changes occur (Haque et al., 2015). Therefore, the use of modified autologous BM-MSCs represents an appealing substitute for bone and cartilage repair with promising results in osteochondral renewal in critical defects (Lee & Wang, 2017).

Growing evidence in literature strengthens the role of the purinome on bone and cartilage homeostasis and osteo/chondrogenic differentiation of BM-MSCs. Once in the extracellular space, nucleotides (e.g. ATP) may be metabolised by nucleotide-metabolizing enzymes and trigger the activation of a variety of nucleotide-sensitive P2 and adenosine P1 receptors present on the surface of BM-MSCs, osteoblasts and chondrocytes. Therefore, this study was designed to elucidate the molecular mechanisms acting upstream and downstream P2 and P1 receptors activation in the osteogenic and chondrogenic differentiation of aged human BM-MSC under physiological and pathological conditions to identify novel drug targets.

The expression and function of chief players of the adenosinergic signalling pathway, particularly the low affinity A_{2B} receptor and ecto-5'-nucleotidase/CD73, are fine tune regulated by environmental conditions, such as the oxygen tissue tension, mechanical stress, inflammatory cells and secreted factors. Thus, fluctuations of the extracellular adenosine levels depend on pro- or anti-inflammatory environmental conditions by the regulation of the expression and/or activity of the adenosine-forming enzyme, ecto-5'-nucleotidase/CD73. Osteogenic differentiating BM-MSC are characterized by expressing high ecto-5'-nucleotidase/CD73 amounts in both rats (Ode et al., 2013) and human (Costa et al., 2011; Noronha-Matos et al., 2012; Noronha-Matos et al., 2023) and the opposite is observed in cells driven to chondrogenic differentiation (Noronha-Matos et al., 2012; Noronha-Matos et al., 2023). Mice lacking ecto-5'-nucleotidase/CD73 exhibited impaired osteoblast differentiation and reduced bone formation leading to osteopenia (Takedachi et al., 2012). These animals also spontaneously developed arthritis, thus suggesting a role of ecto-5'-nucleotidase/CD73 in cartilage homeostasis (Chrobak et al., 2015). Both NTPDase1 and ecto-5'-nucleotidase/CD73 regulate the amounts of calcification inhibitors (e.g. inorganic pyrophosphate, PPI) and stimulators (e.g. inorganic phosphate, Pi) derived from the breakdown of extracellular nucleotides. Deficiencies in NTPDase1 and ecto-5'-nucleotidase/CD73 activity are associated with osteoporotic bone loss and reduced adenosine levels in ovariectomized mice (Shih et al., 2019). Conversely, NTPDase1

overexpression in gingiva-derived MSCs can restore osteogenesis and elevate adenosine levels (Wu et al., 2020). Here, we show that NTPDase3 overexpression in BM-MSCs from Pm women contribute to decrease the extracellular levels of ATP/UDP, thus impairing the osteogenic differentiation of these cells. Data also demonstrate that silencing the NTPDase3 gene or inhibiting its enzymatic activity can restore the osteogenic differentiation of aged BM-MSCs due to the rehabilitation of the osteogenic activity of ATP-sensitive P2X7 and UDP-sensitive P2Y₆ pro-osteogenic purinoceptors (**Paper 1**).

At the osteochondral unit, the avascular cartilage and the subchondral bone are typically exposed to distinct oxygen tensions. The oxygen gradients in this region depend on multiple factors, including the oxygen concentration in the synovial fluid, the distance from the articular surface, the thickness of the tissue, and the cellular density (Zhou et al., 2004), as well as any pathological conditions that might accelerate or hinder the oxygen delivery to target cells. According to our hypothesis, oxygen tension fluctuations in the microenvironment may dramatically affect cell functions in the osteochondral unit. *In vitro* studies show that hypoxic conditions favour the transcription of genes encoding constituents of cartilage matrix, like the SOX9 transcription factor (Lafont, 2010; Lafont et al., 2008). Hypoxia may enhance cartilage regeneration and promote the activity and differentiation of subchondral bone tissue under certain experimental conditions (Zhang et al., 2023), but this is still a matter of controversy in the literature.

Hypoxia-induced downregulation of ecto-nucleotidases increases the accumulation of released adenine nucleotides in the microenvironment, which contributes to balance the activity of pro-chondrogenic vs. pro-osteogenic P2 purinoceptor subtypes. Thus, the activity of fast-desensitizing ionotropic P2X receptors may be downregulated under hypoxic conditions, but this is not applicable to the low-affinity non-desensitizing P2X7 receptor subtype to fight inflammation and promote bone remodelling (Noronha-Matos et al., 2014). Due to this duality, the role of hypoxia in the P2X7 receptor expression and activity is controversial (Azimi et al., 2016; Tafani et al., 2011). In theory, hypoxia-induced downregulation of the P2X7 receptor gene transcription may be beneficial for chondrocytes expansion *ex vivo* assuming a plausible relationship between P2X7 receptors activation, extracellular matrix degradation and the production of inflammatory mediators in the cartilage (Li et al., 2021). Contrariwise, downregulation of P2X4 receptors under hypoxic conditions may destabilize bone formation/remodelling (Orriss et al., 2022) and chondrogenic differentiation (Kwon, 2012). Curiously, the P2Y₆ receptor is upregulated by hypoxia in both bone and cartilage. This UDP-sensitive receptor is involved in the osteogenic differentiation of BM-MSCs and bone remodelling (Noronha-Matos et al., 2012),

as well as in joint chondrocytes proliferation via a mechanism associated to intracellular Ca^{2+} oscillations and the MAPK/ERK pathway (Kudirka et al., 2007). Regarding the ATP/UTP-sensitive P2Y_2 receptor subtype, several studies demonstrated that it promotes osteoblasts proliferation and differentiation involving ERK1/2 and p38 MAPK pathways (Katz et al., 2006, 2008). On the other hand, activation of the P2Y_2 receptor may promote bone resorption and remodelling due to osteoclasts activation (Orriss et al., 2017). It can also increase the production of inflammatory mediators (e.g. NO and PGE_2) in chondrocyte cultures (Berenbaum et al., 2003; Koolpe et al., 1999). Thus, upregulation of this receptor upon hypoxia (e.g. in hepatocytes; (Tak et al., 2016)) may produce conflicting results in the subchondral bone and articular cartilage. This scenario becomes more complicated given that the P2Y_2 receptor synergizes with the P2X_5 receptor to promote DNA synthesis and ATP release in proliferating osteoblasts (Katz et al., 2008; Nakamura et al., 2000), which may be compromised by the hypoxic downregulation of the ionotropic receptor.

Hypoxia-induced overexpression of the ADP-sensitive P2Y_1 receptor (Raghavan et al., 2022) may have beneficial effects on osteogenesis since this receptor modulates the response of osteoblasts to PTH (Bowler et al., 1999). Controversial data, however, exists about the role of the P2Y_1 receptor in cartilage; it has been associated with the inhibition of cartilage formation and the promotion of cartilage breakdown in non-human animal models (Brown et al., 1997; Meyer et al., 2001). Nevertheless, it seems that long-term exposure to hypoxia contributes to the downregulation of this receptor in cancer cells (Azimi et al., 2016), which may be chondroprotective if the same occurs in the cartilage. OA cartilage degradation associated with $\text{TNF-}\alpha$ also involves the upregulation of the P2Y_{11} receptor (Wang et al., 2019), which may be attenuated upon re-establishing the hypoxic conditions and normalization of the P2Y_{11} receptor density by preventing vascular overgrowth in the affected joint.

Data in this thesis show that stabilization of the HIF-1 α transcription factor by DMOG hampers the chondrogenic differentiation of aged BM-MSCs by decreasing adenosine formation from ATP hydrolysis via CD39/CD73, leading to the preferential activation of the high-affinity $\text{A}_{2\text{A}}$ vs. the chondroprotective $\text{A}_{2\text{B}}$ receptor (**Paper 2**).

The extracellular accumulation of ATP resulting from HIF-1 α stabilization by DMOG strengthens a role for P2 receptors in human chondrogenesis. RNA sequencing demonstrated the expression of P2X_4 and P2Y_{11} receptors in aged BM-MSCs undergoing chondrogenic differentiation. HIF-1 α stabilization increased (although not significantly) the transcription of the P2Y_{11} gene at early culture stages (day 7), prompting us to speculate about the role of this receptor in chondrogenesis under the present experimental conditions.

Intriguingly, the P2Y₁₁ receptor is upregulated in osteoarthritic chondrocytes and favours cartilage matrix destruction by a mechanism linked to TNF- α signalling (Wang et al., 2019).

Overall, experimental data suggest that *ex vivo* expansion and differentiation of BM-MSCs for auto-transplantation would benefit from strategies designed to manipulate the amount of endogenous mediators, such as ATP and adenosine, as well as the activity of their receptors and downstream signalling pathways, in drug-free conditions for safety reasons. Pursuing our aim to target the purinergic cascade in order to achieve and/or readmit osteochondral homeostasis following joint injuries, we gathered data suggesting that changes in purinoceptors expression are balanced by changes in the kinetics of the extracellular ATP breakdown and adenosine formation by tandem NTPDase1/CD39, NTPDase3 and ecto-5'-nucleotidase/CD73 activity. Proving this, currently used anti-rheumatic medications, such as methotrexate (Cronstein, 1995; Cronstein et al., 1993), which induced adenosine release, seems to improve the patients' clinical features. This makes the players involved in the ecto-nucleotidase cascade promising targets for personalized pharmacological modulation for musculoskeletal diseases.

Both studies conducted in this thesis have inherent limitations that should be addressed for the sake of scientific rigour. The first study (**Paper 1**) explored the impact of NTPDase3 overexpression on the osteogenic differentiation of BM-MSCs from postmenopausal (Pm) women compared to cells obtained from younger females and age-matched males. Two strategic approaches were applied for that purpose: selective pharmacological inhibitors of NTPDase3, PSB 603 and hN3-B3_s; and gene silencing with a lentiviral short hairpin RNA (Lenti-shRNA). Amongst all these strategies, the monoclonal antibody against NTPDase3, hN3-B3_s, produced the best results. Usage of antibodies for the treatment of bone diseases is common. Denosumab, a monoclonal antibody designed to inhibit the receptor activator of nuclear factor kappa-B ligand (RANKL), is commonly indicated for osteoporosis, as it decreases bone resorption by inhibiting osteoclasts development (Pang et al., 2020). This situation, encourages us to propose druggable hN3-B3_s-like monoclonal antibodies to increase bone mass and fractures prevention in Pm women by promoting osteoblasts differentiation. We expect limited side effects with the systemic use of this antibody, since NTPDase3 is overexpressed in bone osteoprogenitor cells and only a few other cells/tissues, such as pancreatic beta-cells (Haas et al., 2021; Sandhu et al., 2021; Saunders et al., 2019). The second most effective approach, the lentiviral short hairpin RNA (Lenti-shRNA) reduced the *de novo* synthesis of NTPDase3 without affecting the pre-existing ecto-enzyme. The genetic material contained in this 3rd generation lenti-shRNA vector is randomly inserted into cells' DNA, which may result in unexpected effects.

However, when compared to other viral vectors, such as adenovirus, lentiviral short hairpin RNAs (Lenti-shRNA) seem to be the optimal choice for *ex vivo* approaches (Miyazaki et al., 2008), if one considers the use of rejuvenated cells for bone tissue engineering approaches. Nevertheless, the proof-of-concept of these strategies need to be conducted in pre-clinical tests using animal models with critical bone defects, as previously addressed by our group (Bessa-Andr es et al., 2024).

In the second study (**Paper 2**), we investigated the role of HIF-1 α stabilization by DMOG under normoxic conditions in the expression and function of ecto-nucleotidases and A_{2A}/A_{2B} receptors balance in the chondrogenic differentiation of BM-MSCs from Pm women. Despite, at this point, we were unable to test the influence of senescence and hormonal decline using cells from younger females and age-matched males under the same experimental conditions, we focused on the aged women population most afflicted by cartilage defects in the clinics. However, efforts are being made to overcome this limitation using more difficult to obtain BM-MSCs from younger orthopaedic female patients. The preliminary results are as follows. First, BM-MSCs from younger females exhibit higher levels of chondrogenic markers, namely SOX9 and type II collagen, compared to elderly women. Second, younger cells exhibit smaller NTPDase amounts (immunofluorescence confocal microscopy) and activity (HPLC assays) leading to higher ATP/ADO ratios compared to BM-MSCs from elderly women. Third, BM-MSCs from younger females possess less adenosine receptors compared to aged cells and the A_{2A}/A_{2B} equilibrium is deviated towards the high-affinity A_{2A} receptor. Accordingly, blockage of the A_{2A} receptor with SCH 442416 increased the accumulation of SOX9 and type II collagen inside BM-MSCs from younger females compared to the control situation. Despite these preliminary results are interesting, the statistical power is low and require further repetitions.

According to the literature, there are two strategies to enhance HIF-1 α transcriptional activity: the physiological hypoxia (physioxia) and chemical activation. Physioxia involves restriction of oxygen supply by reducing the oxygen concentration in the incubator (to 5% O₂ instead of 20%), which may also cause metabolic stress in the cells. Chemical activation of HIF-1 α transcriptional activity involves hypoxia-mimetic agents that induce hypoxic-like conditions, including desferrioxamine (DFO), dimethyloxaloylglycine (DMOG), 2,4-dinitrophenol (DNP), cobalt chloride (CoCl₂), and isoflurane (ISO). For a comprehensive review on these approaches, see (Nowak-Stepniowska et al., 2022). Maintaining a dedicated hypoxia chamber implies additional maintenance costs. On the other hand, since the room atmosphere remains normoxic, the oxygen levels fluctuate every time the chamber is opened. Thus, to ensure proper controls, a separate chamber is required to maintain the

normoxic conditions, further increasing the logistical complexity. In this sense, physioxia is more suitable for short-term cultures or cyclic hypoxic stimuli, while hypoxia-mimetic drugs offer a cost-effective alternative for prolonged experimental protocols (this study). Nevertheless, when using chemical agents under normoxic conditions, factors such as cellular pressure responses and underlying molecular mechanisms, are not normally considered. Despite these concerns, hypoxia mimetic drugs are widely used and appear to replicate hypoxic effects, particularly within specific signalling pathways.

DFO is a chelating agent primarily used to remove an excess of iron from the body (Bajbouj et al., 2018), which acts as a co-factor for PHD. By chelating iron, DFO inhibits PHD leading to HIF-1 α stabilization even under normoxic conditions (Wahl et al., 2016). DMOG is a competitive inhibitor of PHD, also able to regulate HIF-1 α expression and phosphorylation under normoxic conditions (Zhdanov et al., 2015). DNP increases oxygen consumption by enhancing the oxidative metabolism (Haneef et al., 2014). As a lipophilic weak acid, DNP disrupts mitochondrial function by uncoupling the oxidative phosphorylation, thereby reducing ATP production (Shavell et al., 2012). CoCl₂ mimics hypoxia by replacing iron ions with cobalt ions in PHD, thereby blocking its activity and preventing HIF-1 α degradation – review in (Munoz-Sanchez & Chanez-Cardenas, 2019). ISO is a volatile anaesthetic known to induce HIF-1 α accumulation, although its exact mechanism remains unclear. Some studies suggest that Ca²⁺ signalling via PLC- γ activation is involved in isoflurane-induced HIF-1 α stabilization (Chai et al., 2016). Despite their ability to induce HIF-1 α upregulation, these chemical hypoxia-mimetic agents may have cytotoxic effects at certain concentrations. DFO is commonly used in the 0.1–500 μ M range, with cytotoxicity being reported above 120 μ M (Matsunaga et al., 2019; Peyvandi et al., 2018; Zeng et al., 2011). Some studies even suggest that 10 μ M DFO significantly reduced BM-MSC viability (Fujisawa et al., 2018). Additionally, DFO is typically used in short-term protocols (Matsunaga et al., 2019). DMOG is non-toxic up to 5 mM (Liu et al., 2009b) and even promoted BM-MSC and HUVEC proliferation in co-culture experiments (Nguyen et al., 2020). In our hands, DMOG exhibited no toxicity in rat BM-MSCs up to 300 μ M (Supplementary Figure 6C). Up to 0.25 mM, DNP did not affect rat BM-MSC viability when co-cultured with cardiomyocytes providing that the treatment does not exceed 20 min (Haneef et al., 2014). CoCl₂ (100 μ M, for 24–48h) did not affect MSC viability (Yoo et al., 2016), but concentrations higher than 500 μ M led to significant decrease in cells viability (Liu et al., 2016). The use of volatile agents, like ISO, raises technical and safety concerns and, thus, are usually applied for short incubation periods (Sun et al., 2015). Taking this into consideration a careful selection of the dose and time of exposure may be crucial upon using hypoxia mimetics to study BM-MSCs growth and differentiation. Changes in the

application protocol and concentration of hypoxia-mimetic agents introduce substantial biases in the interpretation of pre-clinical results before translation to use in regenerative medicine applications. Based on previous studies and optimization experiments conducted in our lab, we selected the 30- μ M concentration of DMOG to avoid misleading conclusions.

Notwithstanding, we believe that both studies demonstrate that the purinergic signalling plays a crucial role in modulating both the osteogenic and the chondrogenic differentiation of BM-MSCs (Figure 15). Data suggest that targeting the purinergic signalling cascade may represent a promising therapeutic strategy for bone and cartilage disorders, including osteoporosis and OA. By elucidating the interplay between hypoxia, HIF-1 α transcriptional activity, and purinergic signalling, this work provides valuable insights into the mechanisms governing bone and cartilage regeneration. Future studies should focus on refining these approaches, optimizing the use of hypoxia-mimetic agents, and validating their therapeutic potential using pre-clinical disease models.

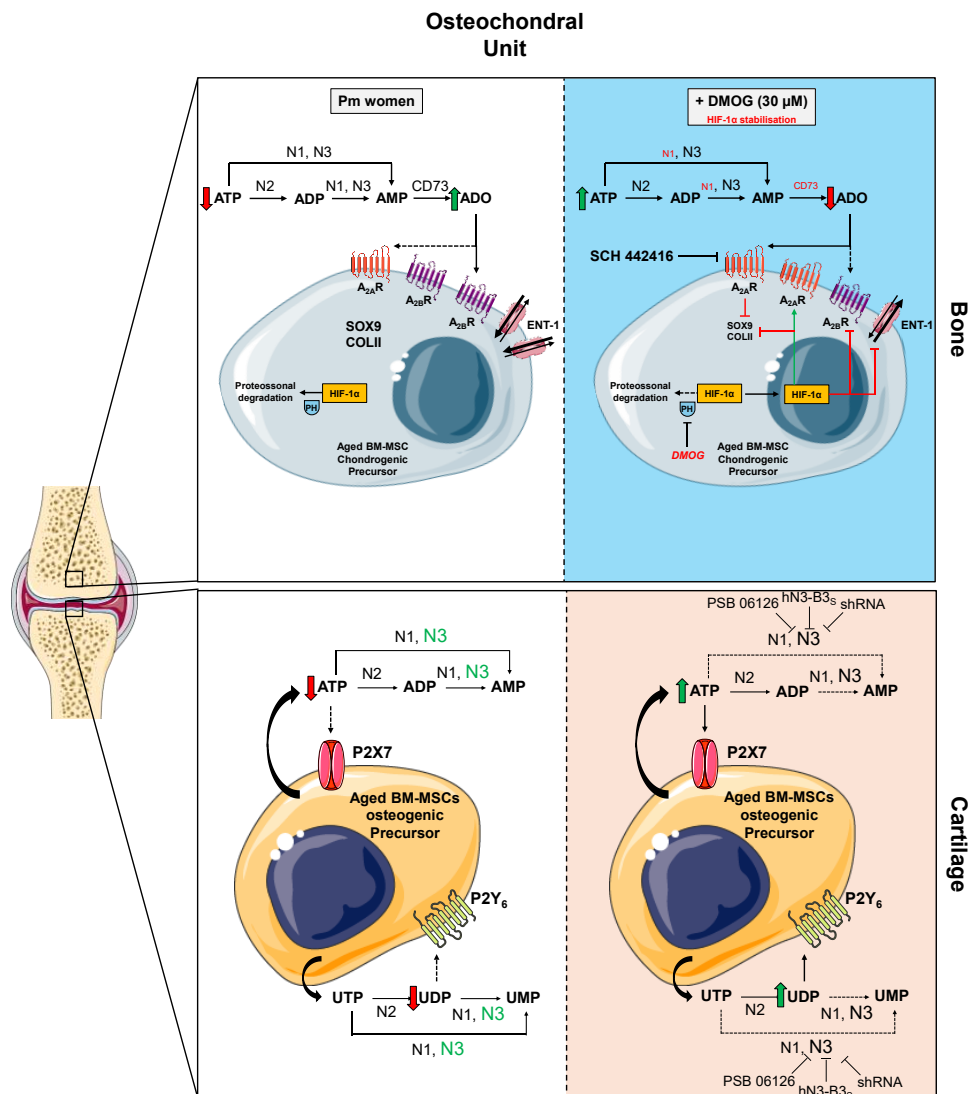


Figure 15 – Schematic representation of main findings presented in this thesis. This figure uses elements from Servier Medical Art (<http://smart.servier.com>).

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