Journal of Bone Marrow Research
ISSN: 2329-8820

Thorfve et al., J Bone Marrow Res 2014, 2:3
DOI: 10.4172/2329-8820.1000151

Research Article Open Access

Madindoline A Affects the Osteogenic Potential and the Wnt Signaling Pathways during Osteogenic Differentiation of Human Mesenchymal Stem Cells *in vitro*

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Abstract

Background: Human mesenchymal stem cells (hMSCs) have the potential to differentiate at least into adipocytes, chondrocytes and osteoblasts. The differentiation capacity can be modulated by drugs, or chemical substances that affect diverse mechanisms essential for e.g. bone formation. The aim of this study was to investigate the osteoinductive capacity of the interleukin-6 (IL-6) inhibitor Madindoline A (MadA) and its relation to the bone-inducing Wnt signaling pathways.

Methods: After stimulation with MadA of hMSC from 4 donors (aged 13-33 years) in an *in vitro* culture, alkaline phosphatase (ALP) activity and extracellular matrix (ECM) mineralization of hMSCs were quantified and calcification visualized by von Kossa staining. The expression of bone- and Wnt related markers was further studied at gene and protein levels. In addition, stimulation with the non-canonical Wnt5a ligand was added as a positive control, and the effect of MadA on *IL-6* gene expression and STAT3 phosphorylation was evaluated.

Results: Stimulation with MadA induced increased ECM mineralization and upregulated the expression of the bone related genes *RUNX2*, *COL1A1* and *Osteocalcin*, although large donor-to-donor differences were observed. Further, MadA affected both the canonical and non-canonical Wnt signaling pathways and displayed a superior osteoinducing property compared to Wnt5a in some cases.

Conclusion: In summary, all donors displayed higher gene expression of *IL-6* and reduced STAT3 phosphorylation after MadA stimulation. The present results provide for the first time indications of an *in vitro* osteoinduction potential of the IL-6 inhibitor MadA.

Keywords: IL-6 inhibitor; Madindoline A; Osteogenic differentiation; Wnt signaling pathway; Human mesenchymal stem cells

Abbreviations: ALP: Alkaline Phosphatase; CAMK2A: Calcium/Calmodulin-Dependent Protein Kinase II Alpha; CCND1: Cyclin D1; COL1A1: Collagen, Type I, Alpha 1; DMEM-LG: Dulbecco's Modified Eagles Medium-Low Glucose; ECM: Extracellular Matrix; FZD: Frizzled; hMSCs: Human Mesenchymal Stem Cells; IL-6: Interleukin-6; JAK: Janus Kinase; LDH: Lactate Dehydrogenase; LRP5/6: LDL-Related Proteins 5 and 6; MadA: Madindoline A; NFAT5: Nuclear Factor of Activated T-Cells 5; OPN: Osteopontin; RUNX2: Runt-Related Transcription Factor 2; STAT: Signal Transducer and Activator of Transcription; Wnt: Wingless-Type MMTV Integration Site Family

Introduction

Mesenchymal stem cells (MSCs) differentiate to mature osteoblasts during a process named osteogenesis, which is characterized by upregulated alkaline phosphatase (ALP) activity and formation of a calcium-rich mineralized extracellular matrix (ECM). Several signaling pathways are involved in regulation of osteogenesis and direct MSCs into the osteogenic lineage, where the runt-related transcription factor 2 (RUNX2) is the master switch for osteogenesis [1]. Also the Wnt signaling pathways are essential for the guidance of MSCs towards the osteoblastic lineage [2,3]. The canonical Wnt signaling pathway is implicated as the dominant mechanism in bone biology. Several Wnt proteins bind to the frizzled (FZD) receptor and the co-receptors named LDL-related proteins 5 and 6 (LRP5/6). The activation of FZD and LRP5/6 triggers disruption of the intracellular β-catenin (CTNNB1) degradation complex, one part of which is glycogen synthase kinase 3β (GSK-3β). The complex normally degrades the key protein β-catenin via phosphorylation, but when Wnt ligands binds to FZDs, the β-catenin level becomes stabilized and is subsequently translocated into the nucleus, resulting in transcription of downstream canonical Wnt-related genes, such as FOS-like antigen 1 (*FOSL1*) and Cyclin D1 (*CCND1*) [4,5]. The non-canonical pathways, which function independently of β-catenin, are less well understood, but are gaining improved interest [6]. The FZD9 receptor is suggested as a positive regulator of intramembranous and endochondral ossification during fracture healing *in vivo* via non-canonical pathways [7,8], and the non-canonical ligand Wnt5a induces osteogenic differentiation of human adipose stem cells (hASCs) [9]. Wnt5a is further suggested to both activate or inhibit the canonical Wnt pathway depending on factors such as available

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Received September 27, 2014; **Accepted** October 15, 2014; **Published** October 17, 2014

Citation: Thorfve A, Svala E, Brisby H, Thomsen P, Lindahl A, et al. (2014) Madindoline A Affects the Osteogenic Potential and the Wnt Signaling Pathways during Osteogenic Differentiation of Human Mesenchymal Stem Cells *in vitro*. J Bone Marrow Res 2: 151. doi: 10.4172/2329-8820.1000151

receptors, time and site of expression [10,11]. A previous study by Okamoto et al. suggested that Wnt5a regulates osteoblastogenesis through upregulation of the Wnt/β-catenin signaling pathway [12]. Since the critical role of Wnt signaling is well recognized also for bone mass in adults, Wnts are therefore attractive growth factors in e.g. bone tissue engineering. Hence, in order to improve bone formation the Wnt signaling pathways are a promising target for modifications [13], and improvement of bone healing around bone-anchoraged implants [14]. Bone homeostasis is also regulated and maintained by interleukin-6 (IL-6) family of cytokines. These are known to induce the differentiation of osteoblasts and osteoclasts. It is a multifunctional cytokine produced by various cell types and participates in immune response, acute-phase reactions, hematopoiesis, and bone and cartilage metabolism [15,16]. Moreover, IL-6 seems to have a modulatory role in cartilage homeostasis, displaying both anabolic and catabolic activities [17-20], whereas excessive production of IL-6 plays a major role in the pathogenesis of cancer, multiple myeloma, rheumatoid arthritis and postmenopausal osteoporosis [21-23]. The action of IL-6 is mediated via the signal transducer glycoprotein 130 (gp130) that associates with the IL-6 receptor (IL-6R) to form a signal-transduction complex. The IL-6/IL-6R/gp130 complex leads to activation of the Janus kinase (JAK), which in turn phosphorylates and activates STATs (signal-transducer and activator of transcription), including STAT3. This results in STAT3 dimerization, translocation into the nucleus, and transcription of targets genes [24,25]. The canonical Wnt signaling pathway displays a more obscure role in cartilage and is implicated in the pathogenesis of osteoarthritis (OA) [26,27]. We demonstrated recently [28] that IL-6 stimulation suppresses the canonical Wnt pathway in cartilage, and may be linked to anti-inflammatory and pro-chondrogenic effects [18,29]. With the ying and yang parable of Wnt signaling in bone and cartilage in mind, the aim of this *in vitro* study was to investigate a possible positive osteoinduction capacity effect of suppressed IL-6 activity. Thus, we tested whether the IL-6 activity inhibitor Madindoline A (MadA) [30] activates the canonical Wnt signaling pathway and subsequently induce an improved osteogenic differentiation of hMSCs *in vitro*. Madindolines (A and B) are non-cytotoxic indole alkaloids, originally isolated from *Streptomyces nitrosporeus* K93-0711 [30], and MadA is the more potent compound. MadA have been reported to specifically inhibit the growth of IL-6 dependent cell lines, probably by interference with the homodimerization of gp130, resulting in inhibition of the JAK/STAT signaling pathway [31-33]. Since Wnt5a induces osteogenic differentiation of hASCs and is involved in osteoblastogenesis [9,12,34,35], we included it here as a positive control.

Subjects and Methods

Cell source and culture

Bone marrow from the iliac crest was obtained from patients undergoing spinal fusion surgery at the Sahlgrenska University Hospital, Gothenburg, Sweden. The study was approved by the local human ethics committee (ethical approval 532-04-T936-13) and all samples were collected with the informed consent of the patients. hMSCs from four different donors were used in this study; Male (M) aged 33yrs, Female (F) aged 28yrs, Male aged 13yrs and Female aged 15yrs and henceforth referred to as Don1-4. A mononuclear cell population was isolated from the bone marrow by gradient centrifugation, using Vacutainer CPT tubes (Becton, Dickinson and Company (BD); Franklin Lakes, NJ, USA) prefilled with Ficoll (GE Healthcare Bio-Sciences AB; Uppsala, Sweden), according to the manufacturer's instructions. The mononuclear cell fraction was subsequently seeded in Primaria tissue culture flasks at a density of ~ 2,40,000 cells/cm^2 in culture medium consisting of Dulbecco's modified Eagles medium-low glucose (DMEM-LG) (Thermo Fisher Scientific; Waltham, MA, USA) supplemented with 2mM L-glutamine (Life Technologies, Carlsbad, CA, USA), 1% penicillin–streptomycin (PEST, Thermo Fisher Scientific), 10 ng/mL of human recombinant fibroblast growth factor beta (FGF-β, Life Technologies) and 10% fetal calf serum (FCS, Sigma-Aldrich; St. Louis, MO, USA). After 24 hr, the flask was rinsed with DMEM-LG, unattached cells were discarded and the adherent cells were expanded in the cell culture medium as described above. During expansion, cells were passaged at 80% confluence using 0.05% trypsin with EDTA (Life Technologies) and reseeded at a density of 10,000 cells/cm^2 (method previously described in [36]).

Flow cytometry

In order to evaluate the purity of the isolated hMSC population, the cells were characterized by flow cytometry before stimulation towards osteogenic differentiation as previously described in [36,37]. The cells, in passage 4-5, were trypsinized, resuspended and washed twice with FACS buffer consisting of phosphate buffered saline (PBS) supplemented with 5% FCS, 1% bovine serum albumin (BSA; Sigma-Aldrich) and 2nM EDTA (Sigma-Aldrich). The cells were subsequently stained with the following antibodies; CD166-PE, CD105-APC (positive markers for hMSCs), CD34-FITC, CD45-PE-Cy7 (negative markers for hMSCs), 7-AAD (viability staining) and isotype controls (BioLegend; San Diego, CA, USA), and incubated in the dark on ice for 30 min. The cells were then washed twice in cold FACS buffer and kept on ice until acquisition. All samples were analyzed using the FACS ARIA III flow cytometer (BD), Comp Beads plus (BD) for compensation and the software FACS Diva (Tree Star; Ashland, OR, USA).

Osteogenic differentiation

hMSCs in passage 4-5, from the four donors, were trypsinized, counted and reseeded at a density of 5,000 cells/cm^2 in 24-well plates (10,000 cells/well, in triplicates for each individual donor) in culture medium (DMEM-LG supplemented with L-glutamin (2 mM), 1% PEST, 10% FCS, ascorbic acid (45 mM, Sigma-Aldrich)) and left to adhere for 24 hr. The cells were then cultured for up to 6 weeks in osteogenic differentiation medium containing DMEM-LG supplemented with 1% PEST, L-glutamin (2 mM), 10% FCS, dexamethasone (10 nM, Sigma-Aldrich), ascorbic acid (45 mM, Sigma-Aldrich) and β-glycerophosphate (20 mM, Calbiochem; Darmstadt, Germany) [36]. Fresh osteogenic medium was prepared every week, changed twice a week and in test series supplemented with 50 ng/mL recombinant Wnt5a (R&D Systems; Minneapolis, MN, USA; dissolved in PBS + 0.1% BSA) or Madindoline A (MadA, Enzo Life Sciences Inc.; Farmingdale, NY, USA; dissolved in dimetylsulfoxid (DMSO)) at 5 μM and 10 μM concentrations. The control groups (Ctrl) were treated with osteogenic medium where only PBS + 0.1% BSA or DMSO, respectively, was added.

Proliferation, LDH and ALP assays

After two weeks of osteogenic differentiation, the cells were permeabilized and stabilized before the nuclei were stained with propidium iodide in the NucleoCassette™ and counted by the NucleoCounter® (ChemoMetec; Allerød, Denmark) according to the manufacturer's protocol. The mean number of cells per well, for each treatment, was used for proliferation analysis and for ALP activity normalization. ALP and lactate dehydrogenase (LDH) was analyzed after 2 weeks of osteogenic differentiation. For ALP analysis the cells cultured in culture-well plate were rinsed twice with DMEM-LG and subsequently lysed using M-PER (Thermo Fisher Scientific). The ALP activity in the cell lysates was thereafter measured by using

ρ-nitrophenylphosphate as substrate. The quantity of ρ-nitrophenol produced, with an absorbance maximum at 405nm, was considered directly proportional to the ALP activity. The results are given in enzyme activity, μKAt per well. The LDH activity in the cell culture medium was used as an indication of toxicity of compounds to cells and measured after 2 weeks of culture using a Cytotoxicity Detection Kit. Briefly, the medium was collected and incubated with the substrate mixture from the kit. The LDH activity was subsequently determined in a coupled enzymatic reaction during which nicotinamideadenine dinucleotide (NAD+) is reduced to NADH. The formation rate of NADH is considered proportional to the catalytic activity of LDH and was measured photometrically at 340 nm. The above-described analyses were previously described in [36] and performed at the accredited laboratory at Sahlgrenska University hospital.

Calcium and phosphate assay

The amount bone matrix produced by the cells cultured under osteogenic differentiation was evaluated up to 6 weeks of culture. Briefly, samples were rinsed twice in DMEM-LG and subsequently fixed in Histofix™ (HistoLab Products AB, Gothenburg, Sweden) for 30 min. After rinsing with distilled H_2O, the samples were demineralized by incubation in HCL (0.6M) with agitation for 24 hr at room temperature. The calcium content was then measured using the orthocresolphthalein complexone (OCPC) method. This reagent forms, under alkaline conditions, a complex with calcium that can be detected at 600 nm and the absorbance is directly proportional to the calcium concentration. Further, the phosphate levels were measured by colormetry of phospho-vanadomolybdic acid. This reagent forms, under acidic conditions, a complex that can be detected at 340 nm and the absorbance is directly proportional to phosphate concentration. The analyses were performed at the accredited laboratory at Sahlgrenska University Hospital and previously described in [36].

von kossa staining

Cells were cultured for up to 6 weeks under osteogenic condition and the mineralization was assayed by von kossa staining, previously described in [36-37]. Briefly, cells were washed with DMEM-LG and then fixed in Histofix™ (HistoLab Products AB) for 30 min. After rinsing with distilled H_2O, a solution of $AgNO_3$ (2% w/v; Sigma-Aldrich) was added to samples and kept in dark for 10 min. The plates were then rinsed three times with distilled H_2O and subsequently exposed to bright light for 15 min. After washing with distilled H_2O, samples were quickly dehydrated adding ethanol (EtOH, 95%).

qPCR analysis

Cells were cultured for 2 weeks under osteogenic condition and total RNA from the cells was extracted using RNeasy Micro kit (QIAGEN GmbH; Hilden, Germany), according to the manufacturer's instructions. DNAse treatment was performed in order to eliminate any contamination from genomic DNA. The purity and concentration of RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific). cDNA was prepared from total RNA using the HighCapacity cDNA Reverse Transcription kit (Life Technologies). The 7900HT instrument used for real-time qPCR analysis, software and reagents were from Life Technologies, and the method was previously described in [38]. The commercially available assay-on-demand mixes of primers and TaqMan MGB probes (FAM dye-labeled) were used (Life Technologies; order number in parentheses): *ALPL* (Hs01029144_m1), *BGLAP* (Hs00609452_g1), *BMP2* (Hs00154192_m1), *CAMK2A* (Hs00211096_m1), *CCND1* (Hs00277039_m1), *COL1A1* (Hs00164004_m1), *CTNNB1* (Hs00355049_m1), *IL-6* (Hs00985639_m1), *NFAT5* (Hs00952011_m1) and *RUNX2* (Hs00231692_m1). *18s* (Hs9999901_s1) was used as endogenous control. cDNA corresponding to 2.5 ng total RNA were analyzed in duplicates for all samples. Raw data were analyzed by using SDS v2.2.2. Software (Life Technologies) and relative gene expression level (the amount of target, normalized to the endogenous control gene) was calculated using the $2^{-\Delta\Delta Ct}$ method [39] in GenEx Enterprise 5.2.3.13 (MultiD analyses; Gothenburg, Sweden).

Total β-catenin ELISA and STAT3 phosphorylation assay

In order to verify the inhibitory effect of MadA on STAT3 phosphorylation, and whether Wnt5a stimulation or IL-6 inhibition by MadA affected the canonical Wnt pathway, hMSCs were seeded at a density of 5000 cells/cm^2 in culture medium (DMEM-LG supplemented with L-glutamin, 1% PEST, 10% FCS, ascorbic acid (45 mM)) and left to adhere for 24 hr. The cell cultures were incubated for 3 hr with/without Wnt5a (50 ng/mL) and with/without MadA (5 μM or 10 μM) under osteogenic conditions, and then lysed using RIPA lysis buffer (50 mM Tris HCL pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (Sigma Aldrich)) with protease inhibitor (Roche Diagnostics Corporation; Indiana, USA) and phenylmethanesulfonyl fluoride (PMSF, Sigma Aldrich) added. Phosphorylated STAT3 was measured using the semi-quantitative RayBio® Phospho-Stat3 ELISA kit (RayBiotech; Norcross, GA, USA), and the concentration of total β-catenin was measured using a Total β-catenin enzyme immunometric assay kits (R&D Systems), previously described in [14]. Both kits were used according to the manufacturer's instructions. Briefly, cell lysate was added to the pre-coated plate. After 2 hr of incubation at room temperature, the plate was washed several times, and the detection antibody and the SA-HRP conjugate were added. Finally, the substrate solution was added, and the absorbance was measured at 450 nm (Tecan Infinite F50 plate reader; Tecan, Männedorf, Switerland). The total protein concentration was determined by the BCA assay (Thermo Fisher Scientific) according to the manufacturer's protocol, using bovine serum albumin (BSA) as a standard. The absorbance was measured at 562 nm (Tecan Infinite F50 plate reader; Tecan).

Statistics

Statistical analyses were performed with GenEx (MultiD analyses) and SPSS v19 (IBM Corp.; Armonk, NY, USA) software. Logarithmic values of the gene-expression data were used for the statistical calculations. Statistical significance was determined in Student's t-test (paired or un-paired). A significant difference was assumed at a p-value of ≤ 0.05. Unless otherwise stated, data are expressed as mean ± standard deviations.

Results

Flow cytometric analysis of hMSCs

Prior to the osteogenic differentiation of the hMSCs, a flow cytometric analysis was performed in order to verify the enrichment of hMSC phenotypic cells. Cell debris, aggregates and non-viable (7AAD positive) cells were excluded in the analysis, and in order to calculate the percentages of positively stained cells for each marker a maximum of 0.5% false positive cells stained with isotype control antibody was allowed. The result demonstrated that ~ 97-99% of the cells were positively stained for CD105 and CD166 whereas ~ 99% of the cells were negative for CD34 and CD45 (Figure 1).

Cell proliferation

After 2 weeks of osteogenic differentiation the average cell number

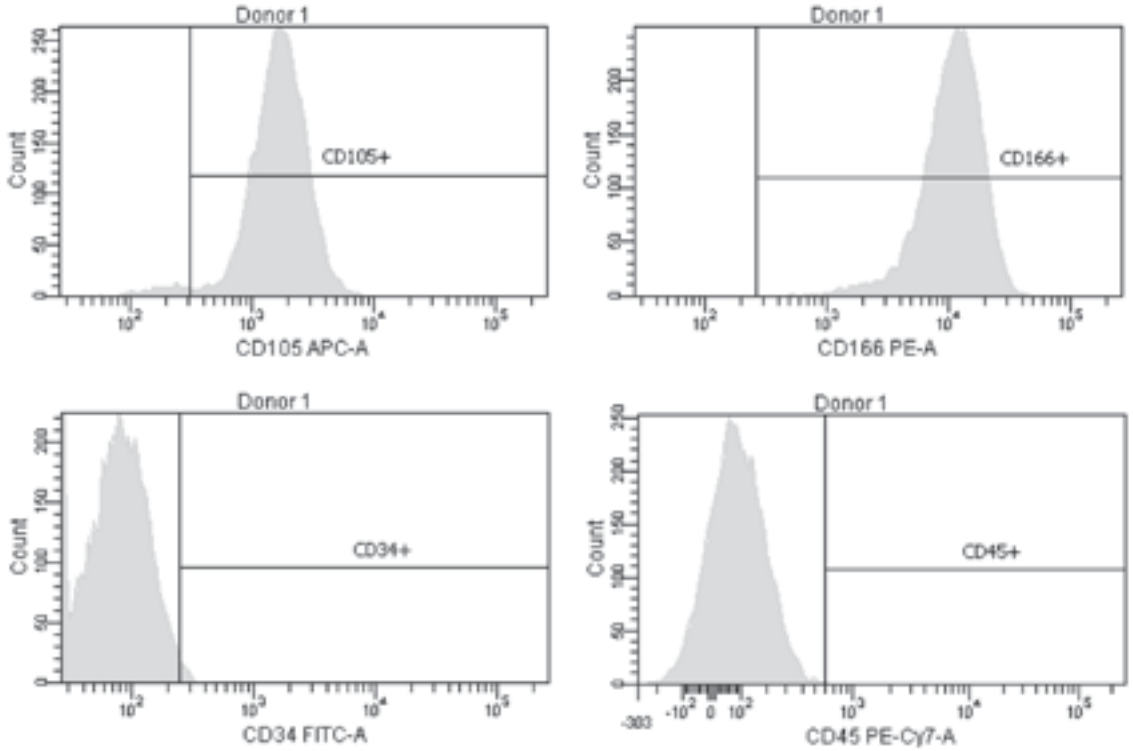

Figure 1: hMSC phenotype verification by flow cytometry.

Representative flow cytometry histograms of hMSCs in passage 4 from Don1 of surface markers CD105, CD166 (positive markers for hMSCs), CD34 and CD45 (negative markers for hMSCs)

per well increased from initially 10,000 seeded cells to approximately 50 000 cells. For donor 2 (Don2), a significant increase in cell number was observed for samples that were cultured with 5 μM MadA compared to Ctrl, 10 μM MadA or 50 ng/mL Wnt5a. In contrast, both Don3 and Don4 demonstrated significantly higher cell numbers when cultured in 10 μM MadA as compared to Ctrl. Don4 demonstrated also a significantly increased in cell number in the presence of Wnt5a compared to its control. No significant differences in cell proliferation were observed for Don1 (Figure 2A).

ALP and LDH assays

ALP and LDH activities of hMSCs were measured after 2 weeks of osteogenic differentiation. Don1 demonstrated overall the highest enzymatic activity among the donors and a significant 1.8-fold increase in ALP activity when stimulated with the two MadA concentrations, as compared to Ctrl. For Don2, stimulation with 5 μM MadA induced a significantly decreased enzymatic activity compared to Ctrl and 50 ng/mL Wnt5a. Further, Don3 showed after stimulation with 10 μM MadA a significantly decreased enzymatic activity compared to Ctrl and 5 μM MadA. There was also a significantly decreased ALP activity after stimulation with Wnt5a as compared to the 5 μM MadA. No significant differences between the different treatments were observed for Don4 (Figure 2B). Furthermore, the addition of MadA or Wnt5a showed only minor effects on LDH activity. No differences in any of the donors, except a significant decrease in LDH for 10 μM MadA compared to its Ctrl, were observed (Figure 2C).

Matrix mineralization

The calcium and phosphate contents in ECM were quantified after 4-6 weeks of osteogenic differentiation. Don1 demonstrated already after 4 weeks the overall highest calcium and phosphate levels among the donors, with significant increase upon the addition of Wnt5a or MadA. Around a significant 10-fold increase in calcium content was displayed for Don1 after stimulation with MadA compared to Ctrl, whereas stimulation with Wnt5a induced a significant 6-fold increase in calcium concentration compared to Ctrl. The phosphate levels followed similar trends with a significant 4-6 fold increase upon stimulation with Wnt5a or MadA. The same tendency in calcium and phosphate levels was observed for Don2, with significant increase in calcium after stimulated with 10 μM MadA, and a similar trend in phosphate concentration ($p=0.063$). In Don2, there was also a large increase in calcium after 10 μM MadA stimulation compared to the 5 μM MadA ($p=0.066$), whereas stimulation with 10 μM MadA concentration induced significantly higher phosphate level compared to 5 μM MadA. No significant differences in calcium were observed for Don3, whereas stimulation with 5 μM MadA demonstrated a significantly decreased phosphate level compared to unstimulated Ctrl. When compared to its Ctrl, Don4 displayed a significant decrease in calcium after stimulation with Wnt5a stimulation. Addition of 5 μM MadA led, on the other hand to a significant increase in phosphate (Figure 3A-B). Further, for Don1, all culture conditions resulted in positive von Kossa stainings as observed by light microscopy. No differences between the treatments were observed for the other donors (Figure 3C).

Gene expression analysis

After 2 weeks of culturing the expression of osteogenic and Wnt signaling related markers was evaluated by qPCR (Figure 4). For Don1, stimulation with 10 μM MadA led to a significantly higher

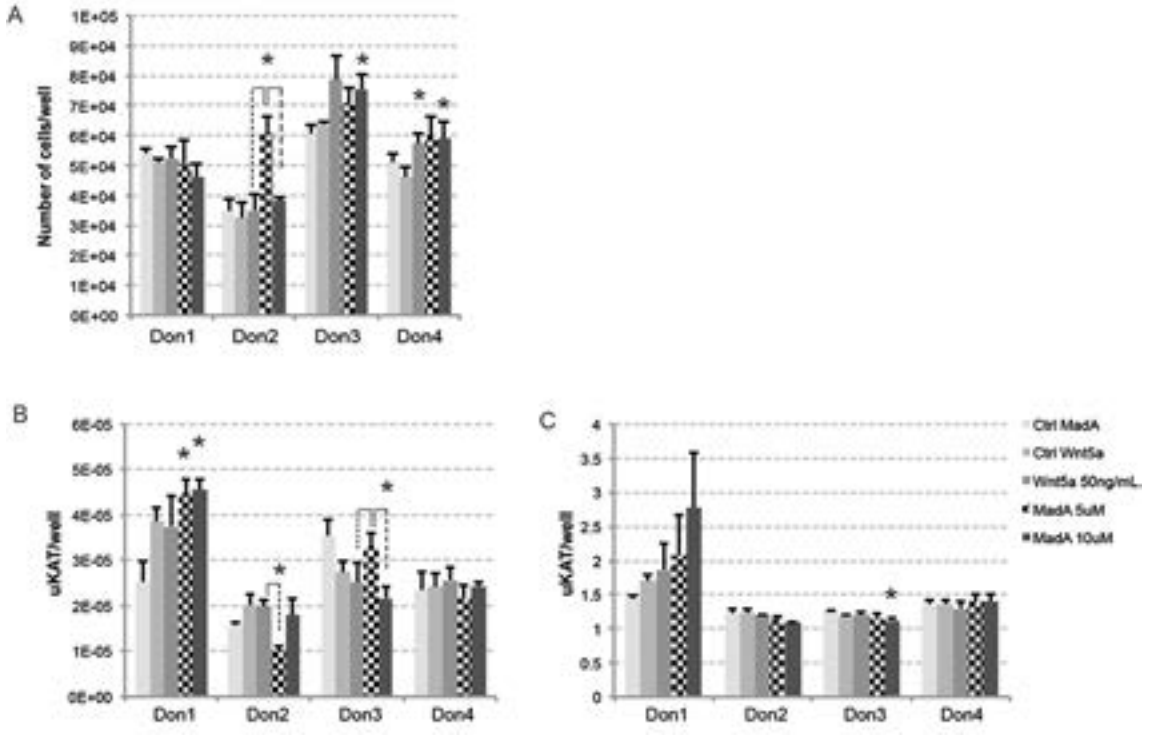

Figure 2: Proliferation, ALP- and LDH activity in the presence of Wnt5a or MadA

A) Cell proliferation of hMSCs in osteogenic medium with/without Wnt5a or MadA after 2 weeks of osteogenic differentiation. B) ALP activity and C) LDH activity of hMSCs after 2 weeks of osteogenic differentiation with/without Wnt5a or MadA. The diagram shows mean values and standard deviations. Paired student T-test was used for statistical analyses (n = 3), * indicates a p value < 0.05 compared to the Ctrl, whereas a dashed line indicates a p value < 0.05 compared between the treatments. Don1 is Male (M) aged 33yrs, Don2 is Female aged 28yrs, Don3 is Male aged 13yrs and Don4 is Female aged 15yrs.

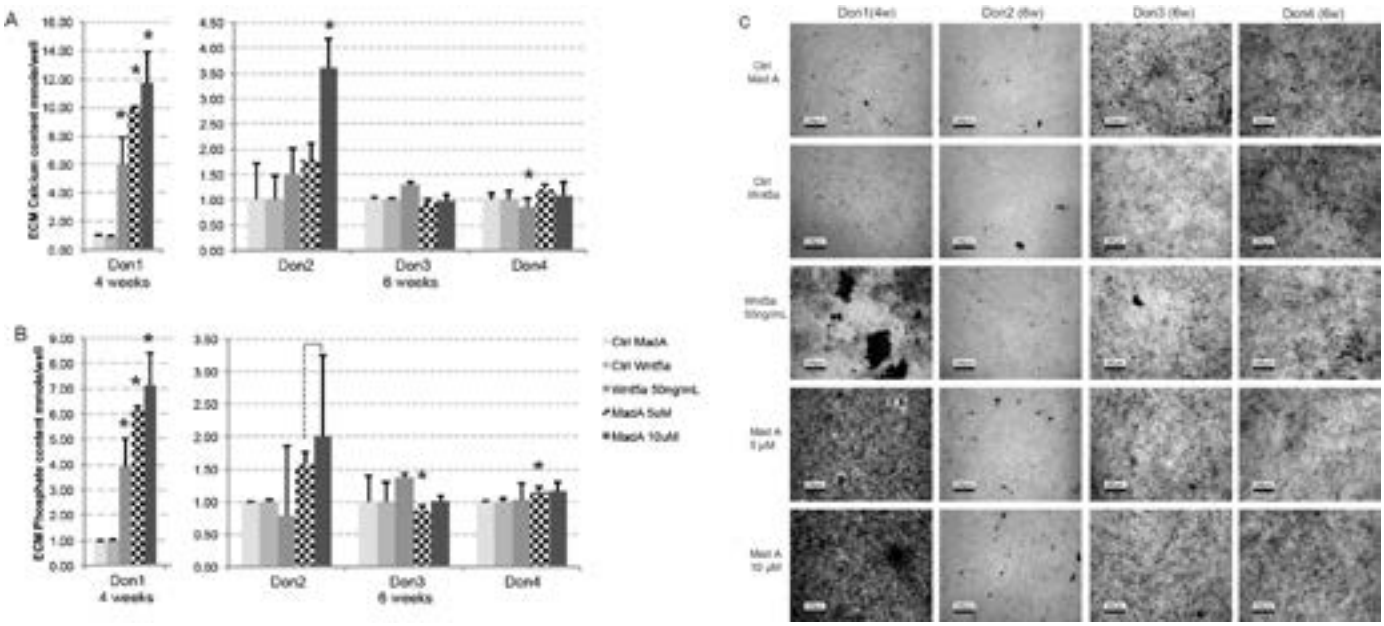

Figure 3: ECM mineralization during osteogenic differentiation in the presence of Wnt5a or MadA

A) Calcium and B) phosphate levels of the ECM of hMSCs after 4-6 weeks of osteogenic differentiation with/without Wnt5a or MadA C) Deposition of a mineralized matrix visualized by von Kossa staining after 4-6 weeks of osteogenic differentiation. Paired or unpaired student T-test was used for statisticl analyses (n = 2-3), * indicates a p value < 0.05 compared to the Ctrl, whereas a dashed line indicates a p value < 0.05 compared between the treatments.

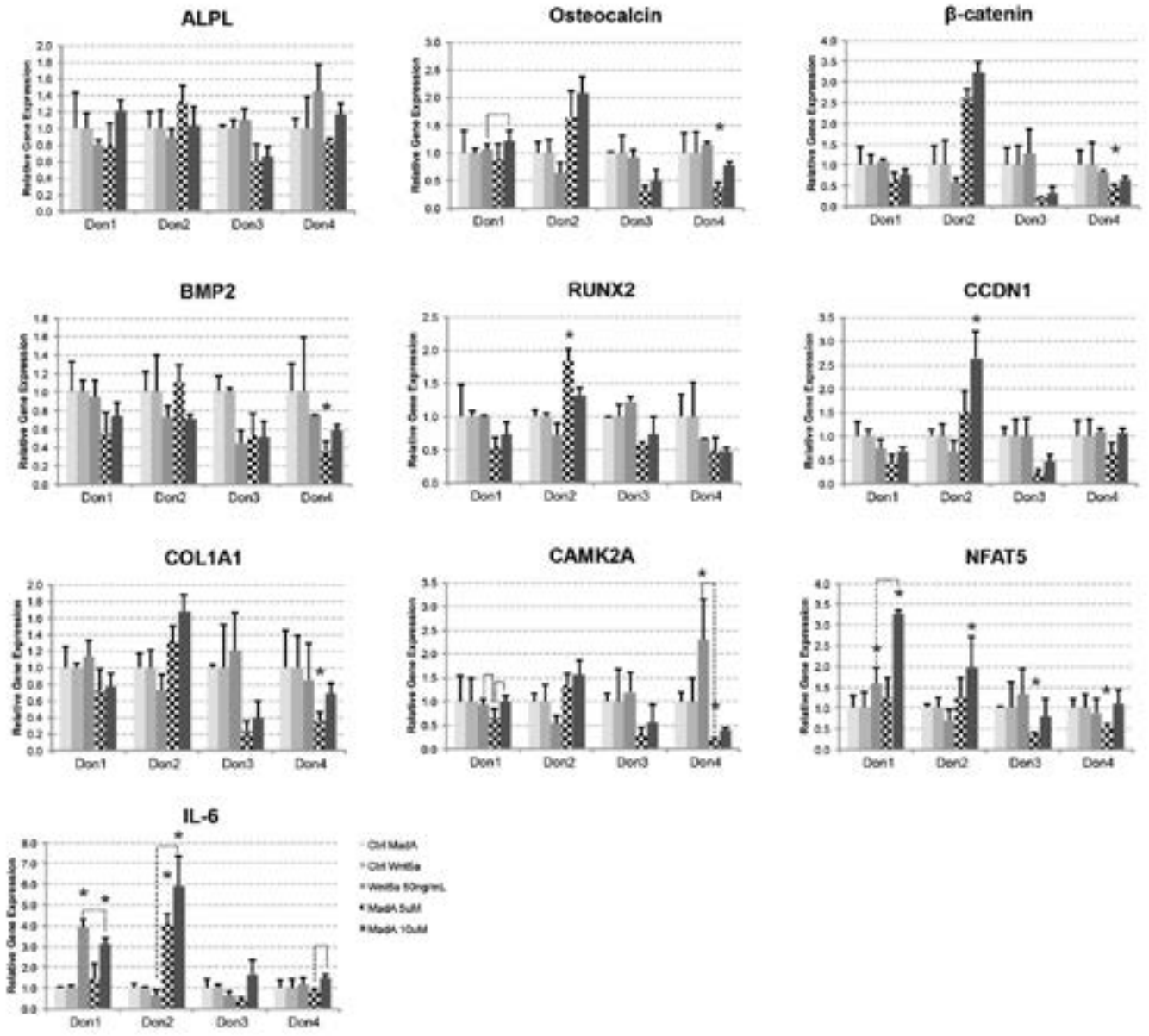

Figure 4: Gene expression analysis of osteogenic- and Wnt related markers

Relative gene expression quantified by qPCR for osteogenic- and Wnt related markers of hMSCs after 2 weeks of culture in osteogenic medium with/without Wnt5a or MadA. Paired student T-test was used for statistical analyses (n = 3), * indicates a p value < 0.05 compared to the Ctrl, whereas a dashed line indicates a p value < 0.05 compared between the treatments.

expression of *Osteocalcin* in comparison to added 50ng/mL Wnt5a. The expression of *CAMK2A* was significantly increased by 10 μM MadA and Wnt5a compared to 5 μM MadA. Both 50ng/mL Wnt5a and 10 μM MadA demonstrated significantly higher expression of *NFAT5* and *IL-6* compared to their Ctrls, as well as to each other. For Don2, a significantly increased expression of *RUNX2* was induced upon addition of 5 μM MadA. Similar trends were observed for *Osteocalcin* (p=0.055), *COL1A1* (p=0.068) and *β-catenin* (p=0.077). Stimulation with 10 μM MadA induced a significantly higher expression of *CCND1* and *NFAT5* compared to Ctrl, and a similar trend was observed for *β-catenin* (p=0.051) and *Osteocalcin* (p=0.067). *IL-6* was significantly upregulated after stimulation with either concentration of MadA as compared to unstimulated Ctrl, and significantly increased in 10 μM MadA compared to 50ng/mL Wnt5a. For Don3, stimulation with 5 μM MadA significantly decreased the expression of *NFAT5* as compared to Ctrl, and a similar trend was observed for *RUNX2* (p=0.068). Further, stimulation with 10 μM MadA decreased the expression of *ALPL* (p=0.052) and *CCND1* (p=0.058), whereas stimulation with Wnt5a induced increased *NFAT5* expression (p=0.072) compared to Ctrl. For Don4, 5 μM MadA concentration induced a significantly decreased expression of *Osteocalcin*, *BMP2*, *COL1A1*, *β-catenin*, *CAMK2A* and *NFAT5* compared to Ctrl. In contrast, stimulation with Wnt5a induced a significantly higher expression of *CAMK2A*, compared to the Ctrl and the 5 μM MadA concentration. The expression of *IL-6* was significantly higher after stimulation with 10 μM MadA than 5 μM MadA, and the expression of *Osteocalcin* displayed a similar trend (p=0.078).

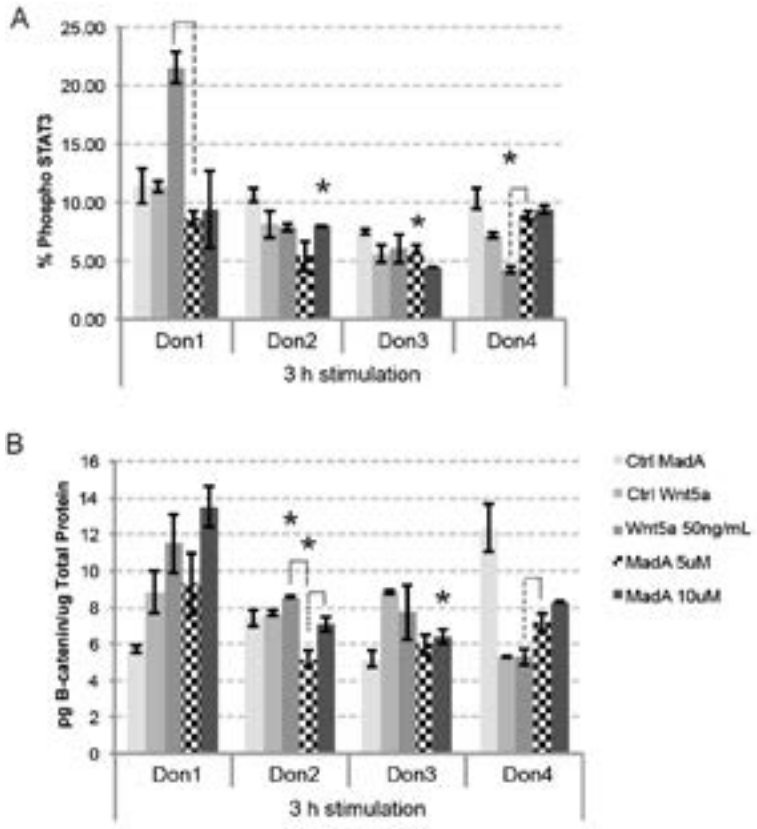

Figure 5: STAT3-phopho and β-catenin protein expression

A) Semi-quantiative STAT3-phospho and B) quantiative total β-catenin expression in hMSCs after 3 hours of culture in osteogenic medium with/without Wnt5a or MadA. Paired student T-test was used for statistical analyses (n = 2), * indicates a p value < 0.05 compared to the Ctrl, whereas a dashed line indicates a p value < 0.05 compared between the treatments.

Phospho-STAT3 ELISA

In order to verify the MadA induced suppression of STAT3 phosphorylation a semi-quantitative phospho-STAT3 ELISA was performed. After 3 hours of stimulation the average amount of phosphorylated STAT3 was ~ 10% for unstimulated Ctrls, compared to 7-8% for samples cultures with added MadA or Wnt5a. More in detail, in Don1, stimulation with 50ng/mL Wnt5a induced higher levels phosphorylated STAT3 than its Ctrl (p=0.083) and significantly higher levels compared to 5 μM MadA. Compared to the unstimulated Ctrls, both Don2 and Don3 showed a significantly decreased STAT3 phosphorylation upon stimulation with MadA. In Don4, 50ng/mL Wnt5a induced significantly decreased phosphorylated STAT3 compared to Ctrl and 5 μM MadA (Figure 5A).

Total β-catenin protein expression

Enhanced protein expression of total β-catenin was observed in Don1 after stimulation with 10 μM MadA (p = 0.053), and was also higher than that of the 5 μM MadA (p = 0.063). For Don2, stimulation with 50ng/mL Wnt5a significantly increased the total β-catenin expression, and the expression induced by Wnt5a was significantly higher than that of 5 μM MadA. When compared to Ctrl, the lower concentration of MadA significantly decreased the total β-catenin expression and there was also a statistically difference in total β-catenin expression after stimulation with 5 μM or 10 μM MadA. A significantly higher total β-catenin expression was observed for Don3 after stimulation with 10 μM MadA in comparison to its Ctrl. Stimulation with 5 μM MadA displayed the same trend (p = 0.064). For Don4, 5 μM MadA stimulation resulted in a significantly higher expression of total β-catenin compared to 50ng/mL Wnt5a (Figure 5B).

Discussion

In the present *in vitro* stem cell study, an in-depth screening of the MadA osteoinduction capacity and its relation to the bone-inducing Wnt signaling pathways was performed. Wnt5a was, due to its role during osteoblastogenesis, used as a positive control. We analyzed how the hMSC gene expression was affected when IL-6 activity was blocked during osteogenic differentiation, and quantitatively measured osteogenic markers such as matrix mineralization and ALP activity. Our results demonstrated, that the addition of MadA during osteogenic differentiation induced a significantly increased ECM mineralization, increased expression of several osteogenic and Wnt related genes and showed a significantly higher total β-catenin protein expression for some of the donors. This suggests affected activity in both the canonical and non-canonical Wnt signaling pathways upon suppression of the IL-6 dependent activities. Furthermore, MadA was occasionally a more potent osteogenic inducer of hMSCs than Wnt5a. Hitherto, many studies have reported on the synthesis of Madindolines [40-42]. Hayashi et al. reported that MadA inhibited osteoclastogenesis *in vitro* as well as bone resorption in an experimental osteoporosis model *in vivo* [31]. However, no previous study has comprehensively evaluated the response of osteoblast progenitors i.e. the hMSCs during MadA

exposure, aiming at the mechanisms of bone formation, in contrast to bone resorption as mediated by osteoclasts. In our study, the toxicity of the MadA during osteogenic differentiation was evaluated by analysis of LDH activity in cell cultures. MadA showed, within the range of concentrations used here, no toxic effects. The results demonstrate differences between the donors in cell growth rate, osteogenic potential, and how cells from the different donors respond to external stimuli after addition of recombinant Wnt5a or MadA into cell culture media. Donors 1 and 2 were adults and donors 3 and 4 teenagers, and represent thus two different age groups. Donor-to-donor variability and significant differences between hMSCs populations with regard to proliferation, ALP enzymatic activity and expression of bone-specific genes during osteogenic differentiation are well-known [43-46]. In our study, Don1 displayed higher kinetics and osteogenic potential already after 4 weeks of culture as compared to the other donors. However, no general trend with increased gene expression was observed for Don1 compared to other donors, and variations in hMSC gene expression is consistent with studies from other groups [46]. The results showed further in detail that for Don1, the stimulation with MadA induced an osteoinductive effect on hMSCs manifested by a significantly higher ALP activity, significantly higher calcium and phosphate levels in ECM, increased von Kossa staining, and higher expression of total β-catenin protein compared to unstimulated Ctrl. Surprisingly, for Don1 none of the osteogenic related markers *ALPL*, *RUNX2*, *BMP2* or *COL1A1* displayed increased expression after stimulation with MadA. This result did not correlate with the observed increase in ECM mineralization, and is probably a time-dependent factor in gene and protein expression. For Don1, also the addition of Wnt5a induced significantly higher calcium and phosphate levels, as compared to Ctrl, but no significant differences in expression of the above-mentioned bone-related markers were observed. Nevertheless, 10 μM MadA induced a significantly higher expression of the late bone marker *Osteocalcin* [47] than Wnt5a. The expression of the non-canonical Wnt5a-calcium-dependent pathway downstream effector C*AMK2A* [48] was significantly increased after addition of 10 μM MadA or 50ng/mL Wnt5a, as compared to 5 μM MadA. Both Wnt5a and 10 μM MadA induced significantly higher expression of the non-canonical transcription factor *NFAT5* [48] and *IL-6* compared to control, as well as to each other. Thus, this suggests that stimulation with Wnt5a or MadA is potential effectors of the non-canonical pathway. In addition, NFAT5 is also implicated in processes such as embryonic development, cell migration and proliferation [49]. Recent studies indicate that this non-canonical component is involved in the regulation of the canonical Wnt pathway by regulating the expression of Wnt ligands and their antagonists [50]. NFAT5 is also reported to directly interact with the key player β-catenin to attenuate the canonical Wnt signaling pathway [51]. For Don1, no significant differences in gene expression of the canonical Wnt genes *β-catenin* or *CCND1* were observed after addition of MadA or Wnt5a. However, for Don1 and Don3, MadA stimulation induced a higher total β-catenin protein expression, potentially indicating an affected canonical Wnt signaling pathway. Further analysis is needed here. In contrast to Don1, both Don2 and Don3 displayed significantly decreased ALP activity in the presence of MadA. However, this was not correlated to decreased ECM mineralization in Don2, that displayed a significantly higher calcium level after 6 weeks. The same trend was observed also for phosphate, but Don3 showed a significantly lower phosphate in ECM after stimulation with 5 μM MadA, and was in line with the lowered ALP activity. No significant difference in ALP activity was observed for Don4 that demonstrated significantly lower calcium after addition of Wnt5a and, on the contrary, significantly increased phosphate in the presence of MadA. These differences most likely represent the inter-donor variability related to age and gender as well as the fact that the measured ALP activity after 2 weeks of culturing is not an unambiguous indication for a mineralized ECM after prolonged culture time. Similar to Don1, also Don2 showed a mineralized matrix in terms of higher levels of calcium and phosphate after stimulation with MadA, although Don2 showed a different gene expression profile in comparison to Don1. After two weeks of osteogenic induction in the presence of MadA, Don2 displayed a dynamic gene profile with a significantly increased expression of the bone related marker *RUNX2*. Similar trends were observed for *Osteocalcin* and *COL1A1*. Interestingly, higher gene expression of *β-catenin* and its downstream target gene *CCND1* [52] was observed after addition of MadA, possibly indicating an active canonical Wnt pathway. A significantly higher expression of the non-canonical effector *NFAT5* that has been implicated in the regulation of the canonical Wnt pathway (mentioned above) was observed for Don2. However, the increased gene expression of the canonical Wnt markers was not paralleled by increased protein expression of total β-catenin, although Wnt5a induced in Don2, a significantly higher expression of total β-catenin than its Ctrl. Intriguingly, *CCND1* is also recognized as a downstream target gene promoted by STATs, and upregulation of *CCND1* has been reported in response to IL-6 stimulation [53,54]. Consequently, and in contrast with its reported inhibitory properties, the observed increase in both *CCND1* and *IL-6* gene expression could speculatively also indicate STAT activity by MadA. However, Don2 displayed a significantly lower percentage of phosphorylated STAT3 in the presence of MadA. The above-mentioned increased expression of *IL-6* and *CCND1* most likely reflect compensatory mechanisms at the gene level. In Don3 and Don4 (younger hMSC donors), MadA displayed an inhibitory osteogenic effect in all studied genes with significantly decreased expression of *NFAT5* in Don3 and significantly decreased expression of *Osteocalcin*, *BMP2*, *COL1A1*, *β-catenin*, *CAMK2A* and *NFAT5* in Don4. However, a significantly increased protein expression of total *β-catenin* was observed in Don3 after stimulation with MadA. The data from this study suggests that MadA in young, still growing individuals, rather suppresses the osteogenic potential at gene level, as well as the expression of some Wnt related markers. This is in line with the lack of any coincident improved bone differentiation with regard to ALP activity and ECM mineralization. All donors displayed a higher *IL-6* gene expression upon stimulation with MadA. This most likely reflects a compensatory mechanism for reduced IL-6 activity as induced by MadA. Further, and in line with the proposed inhibitory role of STAT3 phosphorylation by MadA [31], stimulation with MadA reduced the average percentage of phosphorylated STAT3 in all donors, and with significantly decreased levels observed for Don2 and Don3. This most likely verifies the inhibitory effect of the IL-6 activity as exerted by MadA. The collected results from this study indicate that stimulation with MadA during osteogenic differentiation *in vitro* increased matrix mineralization in terms of calcium and phosphate in hMSCs from adults donors but not from teenage donors. However, only in one of the two adults donors (Don2), did MadA induce an increased gene expression of bone- and Wnt related markers such as *RUNX2*, *Osteocalcin*, *COL1A1*, *β-catenin* and *CCND1*, whereas a decreased gene expression of several genes was observed for the young Don3 and Don4. In conclusion, our results suggests that stimulation with Wnt5a or MadA increased proliferation in teenage donors, whereas the more mature, adult donors, rather responded to the stimulation by increased differentiation and ECM mineralization. Cells from younger donors may be less sensitive to external stimuli (e.g. Wnt5a or MadA) since growing individuals are naturally exposed to a high degree of internal stimuli, such as growth factors and hormones that are excessive during adolescence. The dominant growth process likely overrule the potential

positive effect by Wnt5a or MadA in terms of differentiation/mineralization in young hMSCs, and this may explain the differences in response between the donor age groups. Nevertheless, no uniform picture regarding the effect of MadA was observed and further experiments with additional hMSCs donors are needed. However, all donors demonstrated in this preliminary study increased gene expression of *IL-6*, possibly indicating a compensatory mechanism due to a repressive effect of IL-6 activity exerted by MadA. Despite the varying data and although a profound donor-to-donor variability was observed, these results provide the first indication of the osteoinduction potential of MadA via osteoprogenitors such as hMSCs. The present observations open up possibilities to further study the osteogenic effect of MadA and its coupling to Wnt signaling pathways. Interestingly, large differences in hMSC responses can be expected between non-mature and mature individuals. Increased knowledge in this field may be of importance for the development of individualized *in vivo* stimulation of bone healing.

Acknowledgements

The authors gratefully acknowledge the financial support from the Swedish Research Council (grant number 621-2011-6037), the BIOMATCELL VINN Excellence Center of Biomaterials and Cell Therapy, The Hjalmar Svensson foundation, and Region Västra Götaland.

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