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PLATELET-RELEASED SUPERNATANT INDUCES OSTEOBLASTIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS: POTENTIAL ROLE OF BMP-2

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Abstract

Platelet-rich preparations have recently gained popularity in maxillofacial and dental surgery, but their beneficial effect is still under debate. Furthermore, very little is known about the effect of platelet preparations at the cellular level, and the underlying mechanisms. In this study, we tested the effect of platelet-released supernatant (PRS) on human mesenchymal stem cell (MSC) differentiation towards an osteoblastic phenotype *in vitro*. Cultures of MSC were supplemented with PRS and typical osteoblastic markers were assessed at up to 28 days post-confluence. PRS showed an osteoinductive effect on MSC, as shown by an increased expression of typical osteoblastic marker genes such as collagen Iα1, bone sialoprotein II, BMP-2 and MMP-13, as well as by increased $^{45}Ca^{2+}$ incorporation. Our results suggest that the effect of PRS on human MSC could be at least partially mediated by BMP-2.

Activated autologous PRS could therefore provide an alternative to agents like recombinant bone growth factors by increasing osteoblastic differentiation of bone precursor cells at bone repair sites, although further studies are needed to fully support our observations.

Keywords: Mesenchymal stem cells, platelets, growth factors, differentiation, osteoblast.

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Introduction

Platelets play a pivotal role during wound healing. Upon being activated, they adhere to the exposed sub-endothelium and form a clot by binding to circulating fibrinogen molecules that cover the injured site and allow the healing process to begin. This clot is stabilized by a thick fibrin-mesh, which forms under the control of liver-produced thrombin. In addition, platelet activation and degranulation results in the release of a high number of biological factors (including Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Insulin-like Growth Factor (IGF), Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF) and Transforming Growth Factor (TGF)), which attract more platelets to the site of injury, but also other growth factors and cytokines that can target further cell types (Kiuru *et al*., 1991; Lowery *et al*., 1999). Many of those factors are known to play a direct role in normal bone turnover and in the events during early bone healing (Anitua *et al*., 2004). This led to the idea to use platelets for bone repair in the form of platelet-rich plasma (PRP) or similar highly concentrated platelet preparations (Whitman *et al*., 1997; Marx *et al*., 1998). PRP is autologous plasma that has a platelet concentration above baseline (~1Mio platelets/μL *vs*. ~0.2Mio platelets/μL), but a native fibrinogen concentration. While the gel-forming ability of fibrinogen-containing platelet preparations like PRP is highly beneficial in *in vivo* situations, these gel-forming abilities are hindering *in vitro,* because the addition of PRP to cell culture medium can result in gelling of the culture medium. This is why for *in vitro* applications platelet-rich preparations are often prepared in buffered systems like phosphate buffered saline (PBS), instead of autologous plasma, in order to avoid gel formation in the culture dishes. Such preparations are referred to as platelet-released supernatants (PRS) (Gruber *et al*., 2002a; Gruber *et al*., 2002b).

Today, platelet preparations are used routinely in oral and maxillofacial surgery in some centers in combination with bone grafts and sometimes with autologous mesenchymal stem cells. However, the many studies performed using platelet preparations in fracture repair resulted in very divergent outcomes. Many studies showed increased bone formation (Marx *et al*., 1998; Kitoh *et al*., 2004), some showed increased implant resorption (Yamada *et al*., 2004a; Yamada *et al*., 2004b) and others

have reported improved osseointegration (Lucarelli *et al*., 2005; Brodke *et al*., 2006) or increased vascularization (Yamada *et al*., 2004a; Yamada *et al*., 2004b; Kilian *et al*., 2005). Nevertheless, several studies have shown no beneficial influence of platelet preparations on bone healing (Roldan *et al*., 2004; Mooren *et al*., 2007). This divergence has to be considered carefully, since there is a great variance between the experimental setups used in these studies. There is a high variability in used species, carrier materials, cells, platelet concentration and preparation method, time points when measurements were taken, etc. But there are a few things that seem to be clear. The platelet concentration is essential and too low as well as too high concentrations are not beneficial (Graziani *et al*., 2006; Uggeri *et al*., 2007), and can even be disadvantageous (Choi *et al*., 2005). While it is undisputed that different carrier materials perform differently in combination with platelets (Lin *et al*., 2006), the presence of osteoblastic (precursor) cells seems to be highly beneficial on the effect of platelet preparations on bone healing (Kitoh *et al*., 2004; Yamada *et al*., 2004a; Yamada *et al*., 2004b). Despite the many efforts taken to use platelet preparations in *in vivo* bone repair, very little is known about their effect at the cellular level, and the underlying mechanisms. While it is generally accepted that highly concentrated platelet preparations (i.e. PRS) have a highly beneficial effect on bone cell proliferation (Gruber *et al*., 2002a; Gruber *et al*., 2002b; Gruber *et al*., 2003; Lucarelli *et al*., 2003; Arpornmaeklong *et al*., 2004; Choi *et al*., 2005; Graziani *et al*., 2006; Uggeri *et al*., 2007), its effect on bone cell differentiation is still controversial. Some studies have shown an increase in certain osteoblastic markers in bone cells upon exposure to platelet preparations (Lin *et al*., 2006; Uggeri *et al*., 2007), while others have reported no or even a negative influence of platelet preparations on osteoblastic differentiation (Arpornmaeklong *et al*., 2004).

In this study, we report an increase in osteoblastic differentiation of human mesenchymal stem cells (MSC) in long-term *in vitro* experiments upon exposure to platelet-released supernatant (PRS). Furthermore, we propose that this effect may at least in part, be due to increased BMP-2 levels in MSC, when exposed to PRS.

Materials and Methods

Origin of bone marrow and blood aspirates

Bone marrow (60mL) and blood (100mL) aspirates in CPDA (Citrate Phosphate Dextrose Adenine)-containing S-monovettes (Sarstedt, Nürnberg, Germany) were received from patients (28 to 79 years old, with the average age being 49 years: 12 males and 4 females) undergoing routine orthopedic surgery involving iliac crest exposure, after informed consent (KEK Bern 126/03). Blood aspirates were stored at room temperature (RT) under gentle agitation and bone marrow aspirates were stored at 4°C under gentle agitation until processed within 24 hours after harvesting. The platelets were counted using a Digitana Sysmex FS-3000 (Davos Hospital, Switzerland).

MSC isolation and culture

MSC were isolated from bone marrow aspirates as previously reported (Martin *et al*., 1997; Bianchi *et al*., 2003). In brief, after homogenization, bone marrow aspirates were diluted 1:4 with IMDM (Gibco, Paisley, UK) containing 5% (v/v) FBS (Gibco). After centrifugation (5 min 200g) and removal of the fatty top layer, samples were pipetted on the top of Ficoll (1 mL sample / 2.6 mL Ficoll) (Histopaque-1077, Sigma, St. Louis, MO, USA), then centrifuged at 800 g for 20 min at RT. The mononucleated cells interphases were collected and washed twice in 5 mL of IMDM/5%FBS followed by 15 min centrifugation at 400 g. Cells were seeded at densities of 8-10x10^6 mononucleated cells per 150 cm^2 T-flask in IMDM containing 10% FBS, nonessential amino acids (Gibco) and PenStrep (100 U/mL, Gibco). After 5 days, fresh medium containing 5 ng/mL basic-FGF (R&D Systems) was added (Martin *et al*., 1997; Bianchi *et al*., 2003). Medium was changed every 3 days and cells were subcultured 1:3. After the first passage, cells were termed mesenchymal stem cells (MSC). Cells between passages 2-4 were subsequently used.

PRS preparation

The blood aspirates were transferred from the CPDA-containing monovettes into 15 mL Falcon tubes, and were centrifuged at 200 g for 30 min at RT. The resulting plasma supernatants were pooled (donor specific), transferred into a new 15 mL Falcon tube, and centrifuged 5 min at 2,000 g (RT). The resulting supernatant was discarded, and the platelet pellet was resuspended in sterile phosphate-buffered saline (PBS) at 1/10^{th} of the initial blood volume. PRS was activated by freeze-thaw cycles according to the procedure described by Weibrich *et al*. (2002). The platelet activation efficiency was determined by measuring the release of PDGF-AB, -BB, and VEGF in the activated PRS using the DuoSet ELISA Development System (R&D Systems, Minneapolis, MN, USA) according the manufacturer instructions (Data not shown).

MSC culture supplementation with PRS

MSC were treated with 0.05% Typsin-EDTA, washed, counted and 30,000 cells/cm^2 were seeded in 24-well (Falcon BD, Franklin Lakes, NJ, USA) or 12-well plates (Falcon BD) in presence of IMDM/10%FCS. Culture medium during the experiments consisted of IMDM, 10% FBS, nonessential amino acids, 0.1 mM ascorbic acid-2-phosphate (Sigma) and 10 mM β-glycerophosphate (Sigma). In the experimental cultures, MSC were supplemented with 10% PRS (Lucarelli *et al*., 2003) or with 10 nM Dexamethasone (Dexa) (Sigma). Media were changed twice a week. Recombinant Noggin (R&D Systems) was added at concentrations of 100 ng/mL or 500 ng/mL (Abe *et al*., 2000).

$^{45}Ca^{2+}$ incorporation assay

1.25 µCi/mL of $^{45}Ca^{2+}$ isotope (Amersham CES3, Amersham, UK) were added to each well and the plates were incubated at 37°C o/n (Alini *et al*., 1994). After

Table 1. Primers and Probes for Real-Time RT-PCR

Target Gene	Sequence (5' → 3')
Collagen Iα1	
Forw	CCC TGG AAA GAA TGG AGA TGA T
Rev	ACT GAA ACC TCT GTG TCC CTT CA
Probe	CGG GCA ATC CTC GAG CAC CCT
Osteonectin	
Forw	ATC TTC CCT GTA CAC TGG CAG TTC
Rev	CTC GGT GTG GGA GAG GTA CC
Probe	CAG CTG GAC CAG CAC CCC ATT GAC
MMP-13	
Forw	CGG CCA CTC CTT AGG TCT TG
Rev	TTT TGC CGG TGT AGG TGT AGA TAG
Probe	CTC CAA GGA CCC TGG AGC ACT CAT GT
BMP-2	
Forw	AAC ACT GTG CGC AGC TTC C
Rev	CTC CGG GTT GTT TTC CCA C
Probe	CCA TGA AGA ATC TTT GGA AGA ACT ACC AGA AAC TG
Osteopontin	
Forw	CTC AGG CCA GTT GCA GCC
Rev	CAA AAG CAA ATC ACT GCA ATT CTC
Probe	AAA CGC CGA CCA AGG AAA ACT CAC TAC C
Runx2	
Forw	AGC AAG GTT CAA CGA TCT GAG AT
Rev	TTT GTG AAG ACG GTT ATG GTC AA
Probe	TGA AAC TCT TGC CTC GTC CAC TCC G
BSP II	
Forw	TGC CTT GAG CCT GCT TCC
Rev	GCA AAA TTA AAG CAG TCT TCA TTT TG
Probe	CTC CAG GAC TGC CAG AGG AAG CAA TCA
Taqman Gene Expression Assays (Applied Biosystems):	
Osx : Hs00541729_m1	
Wnt 7b : Hs00536497_m1	
ALP : Hs00758162_m1	

Probes were modified at the 5' end with the FAM fluorescent dye (6-carboxyfluorescein) and at the 3' end with the TAMRA fluorescent dye (6-carboxy-N,N,N',N'-tetramethylrhodamine).

medium removal and 3 washes in IMDM, 0.5 mL of 70% formic acid were added to each well and incubated at 65°C for 1 h. The formic acid solution was transferred to a scintillation tube containing 3.5 mL of scintillation liquid (OptiPhase HiSafe'3, Perkin Elmer, Waltham, MA, USA) and the radioactivity was measured at day 21 of culture using a Wallac 1414 WinSpectral Liquid scintillation counter (Perkin Elmer).

Von Kossa staining

MSC monolayers were rinsed with Tyrode's Balanced Salt Solution (TBSS) after 14 days in culture. Fresh silver nitrate solution (5%) was added and the cells were exposed to strong light for 20 min. After rinsing 3 times with distilled water (dH_2O), the cells were incubated in fresh 5% sodium thiosulfate for 10 min, before being rinsed 3 times with dH_2O. Then the cells were incubated in 0.1% nuclear fast red solution for 10 min, before being rinsed with dH_2O.

ALP activity assay

Samples for ALP activity measurement were harvested each 3rd day over a period of 18 days. After medium removal and a washing step with PBS, 500 µL of 0.1%Triton-X in 10 mM Tris-HCl (pH 7.4) were added to the MSC monolayers and incubated for 3 h at 4°C on a gyratory shaker (Alini *et al.*, 1994). ALP activity was assessed by measuring the p-nitrophenol production during 15 min incubation at 37°C with p-nitrophenyl phosphate as substrate (Sigma Kit No.104) on a Perkin Elmer Bio Assay Reader HTS 7000.

Gene expression analysis

Total RNA was extracted from cells monolayers at different time points as specified in the figure legends using TRI-reagent (Molecular Research Center, Cincinatti, OH. USA) according to the protocol from the manufacturer instructions with a modified precipitation method using High Salt Precipitation solution (Molecular Research Center). Reverse transcription was performed on 1 µg of sample's total RNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) and random hexamers to initiate transcription. Polymerase Chain Reaction (PCR) was performed on a 7500 real-time PCR system (Applied Biosystems).

Except for osterix, Wnt7b and 18S endogenous control which were from Applied Biosystems, oligonucleotide primers and TaqMan probes (Table 1) were all from Microsynth (Balgach, Switzerland) and were designed using the Primer Express Oligo Design software (Version1.5, Applied Biosystems). The nucleotide sequences were obtained from the GenBank database and the probes were designed to overlap an exon-exon junction in order to avoid amplification of genomic DNA. The PCR was performed under thermal conditions with TaqMan Universal PCR master mix (Applied Biosystems). Relative quantification of mRNA targets was performed according to the comparative C_T method with 18S ribosomal RNA as endogenous control (ABI PRISM 7700 Sequence Detector User Bulletin [2], PE Applied Biosystems 1997).

BMP-2 ELISA

MSCs were seeded in 12 well plates at the density of 10,000 cells/cm^2 and grown in unsupplemented culture medium (IMDM, 10% FCS, 10 mM beta-glycerophosphate, 0.1 mM ascorbic acid), in culture medium containing 10 nM Dexa, or in culture medium containing 10% PRS for 23 days. Culture supernatants were collected twice a week during media changes, were transferred to microcentrifuge tubes and stored at -20°C until the end of the experiment. After centrifugation at 18,000 g for 2 min to remove cellular debris, the concentration of BMP-2 in the collected supernatants was determined using a Quantikine BMP-2 ELISA Kit (R&D Systems). Recombinant human BMP-2 (R&D Systems) was used as a standard.

Statistics

All experiments were carried out using different donors (indicated by the n values) from which, the analyses were all done in triplicates. All results are shown as mean±SEM. Statistical analysis was performed by 1-way ANOVA or by *t*-test using Graphpad's Prism 4 (Graphpad, La Jolla, Ca, USA). $p<0.05$ was considered to be statistically significant.

Results

The bone marrow and blood samples used in this study were obtained from healthy donors undergoing hip surgery. Isolated MSCs and PRS were used in an autogenous setting only, and samples from different donors were not pooled. There were no obvious differences in the results obtained between different donor ages or gender. However, due to the high variability in the osteogenic potential of BMSC between donors, some data (Figs. 5 and 6) are shown from one representative experiment, although, the same trends were observed in all experiments, which are represented in Tables 2 and 3.

Platelet activation was estimated by measuring the release of PDGF-AB, PDGF-BB and VEGF proteins from platelets into the supernatant upon their activation by freeze-thaw cycles (data now shown).

PRS effect on MSC differentiation

mRNA levels by real-time RT-PCR: PRS increased the mRNA levels of collagen Iα1 ($p<0.05$) and bone sialoprotein II ($p<0.05$) in MSC supplemented with PRS

Table 2. Results all 3 donors. * = values of Donor 1 shown in Figure 5. Cells were treated with either of PRS, PRS + noggin 100 ng/mL or PRS + noggin 500 ng/mL. Results are expressed relative to PRS treatment.

Treatment condition / Gene expression rel to PRS	PRS	PRS+nog 100			PRS+nog 500		
		Donor 1*	Donor 2	Donor 3	Donor 1*	Donor 2	Donor 3
Coll1	1	0.77	0.58	1.53	0.68	0.63	0.90
OP	1	0.56	0.58	0.36	0.4	0.96	0.24
MMP13	1	0.50	0.69	1.16	0.31	0.69	0.80
BMP2	1	0.73	0.25	1.53	0.78	0.42	0.90
Runx2	1	0.85	0.98	0.57	0.99	0.75	0.46

Table 3. Results of all donors. *= Donor shown in Figure 6. Wnt7b gene expression was measured in MSC and in MSC + PRS. Results are presented relative to the corresponding 18S (2^-dCt) for different time points.

Donor / Time points	Donor 1* (2^-dCt)		Donor 2 (2^-dCt)	
	MSC	MSC + PRS	MSC	MSC + PRS
Day1	0.496	1.867	1.683	5.588
Day 7	1.564	3.426	1.573	5.032
Day 14	0.252	2.423	2.555	7.073
Day 21	-	-	-	-
Day28	0.240	2.426	0.412	1.855

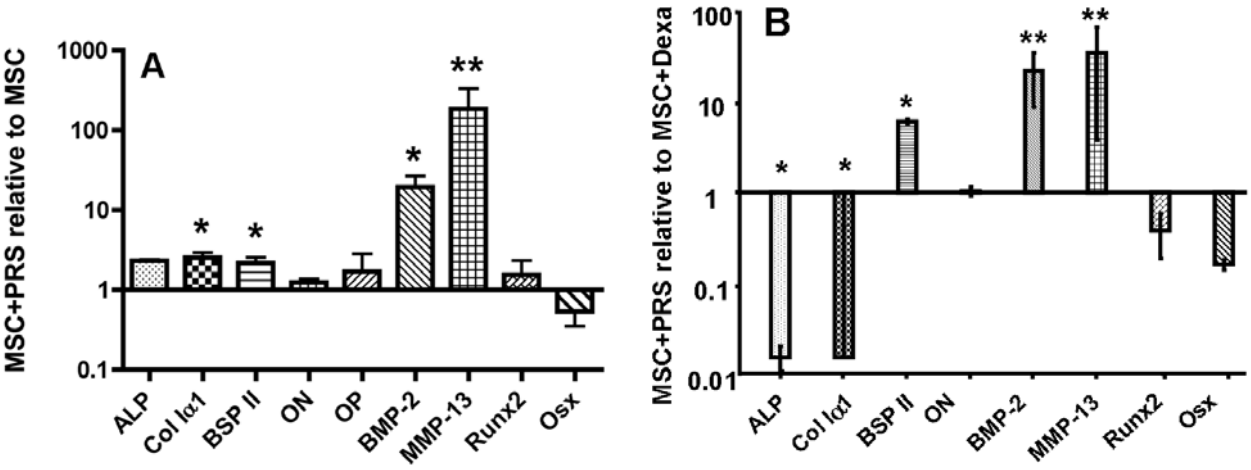


Fig. 1: Gene expression of typical osteoblastic marker genes by MSC at day 28 (n=3).
A: MSC + PRS relative to MSC only Type I collagen (Col Iα1), bone sialoprotein II (BSPII), BMP-2 and MMP-13 were significantly up-regulated. Alkaline phosphatase (ALP), osteonectin (ON), osteopontin (OP) and Runx2 were also slightly up-regulated while Osx was down-regulated. Data are expressed as mRNA levels in MSC+PRS relative to un-supplemented MSC.
B: MSC + PRS relative to MSC + Dexa. BMP-2, MMP-13 and BSP II were significantly up-regulated, while Runx2, OSX and ALP were down regulated in MSC cultures in presence of PRS when compared to Dexa treatment. (* $p<0.05$, ** $p<0.01$)

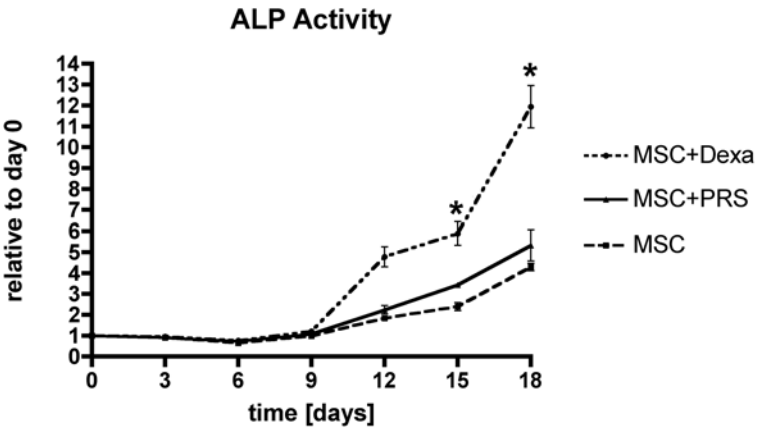
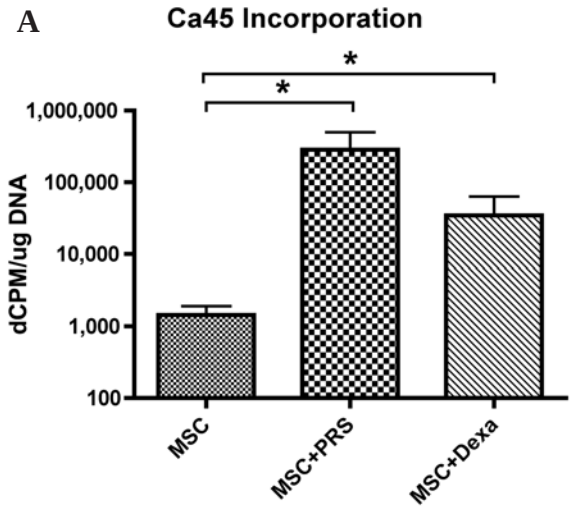


Fig. 2: ALP activity of MSC cultures (n=3): Supplementation of MSC with PRS resulted in a almost two-fold increase in alkaline phosphatase activity after 15 days of culture, when compared to un-supplemented MSC. However, stimulation of MSC with dexamethasone (Dexa) resulted in more than four-fold increase of ALP activity (* $p<0.01$, by 1 way ANOVA).

at 28 days after confluence when compared with non treated MSC (Fig. 1A). In addition, BMP-2 ($p<0.05$) and MMP-13 ($p<0.01$) were highly up-regulated by PRS. Other osteoblastic markers including ALP, osteonectin (ON), osteopontin (OP) and Runx2 showed a trend to up-regulation without reaching significance. The transcription factor Osterix (Osx) showed a tendency to be down-regulated in the presence of PRS (not significant). When compared to cell treated with Dexa (Fig. 1B), the same trends were observed, with high up-regulation of BMP-2, down regulation of ALP, and non significant changes of Runx2 and Osx.

ALP Activity: Alkaline phosphatase activity in MSC supplemented with PRS was by day 15 found higher when compared to MSC cultured without supplementation. This trend was observed up to 18 days post-confluence. We also measured ALP activity in MSC stimulated with Dexa, which resulted, as expected, in a highly significant ($p<0.01$) stimulation of ALP activity compared to PRS (Fig. 2).



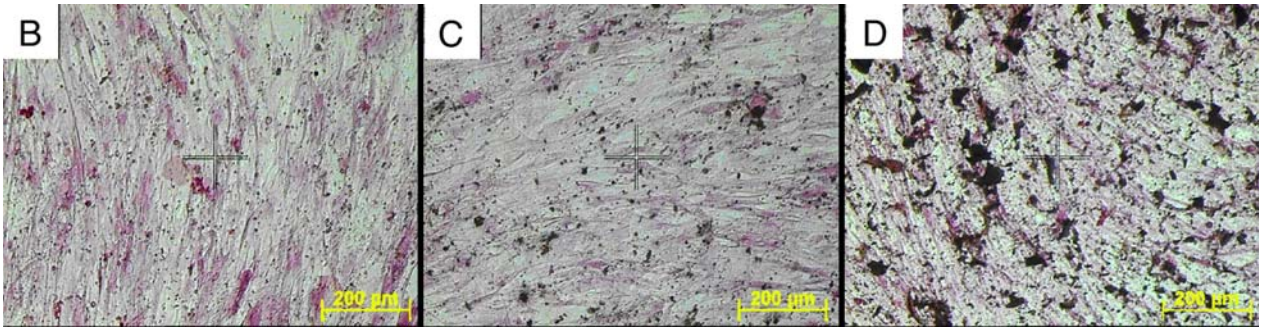


Fig. 3: A: $^{45}Ca^{2+}$ incorporation by MSC supplemented with PRS at day 21, relative to un-supplemented MSC (n=3). CPMs (counts per minute) were normalized to the DNA content. MSC supplemented with PRS and Dexa significantly (* $p<0.005$, by 1 way ANOVA) increased calcium deposition.
B, **C** and **D**: Von Kossa staining at day 14: Supplementation of MSC with PRS (**D**) resulted in significantly higher calcium incorporation than un-supplemented MSC (**B**). Dexa was also able to induce matrix mineralization in MSC (**C**).

Ca45 incorporation: The effect of PRS on matrix mineralization by MSC was estimated by measuring $^{45}Ca^{2+}$ incorporation into the extracellular matrix. MSC grown in medium supplemented with PRS showed an highly significant up-regulation of $^{45}Ca^{2+}$ incorporation by up to 400-fold compared to un-supplemented MSC after 21 days post-confluence (Fig. 3A). This was an approximately 10 fold higher increase than when MSC were supplemented with Dexa. This result was confirmed by Von Kossa staining of MSC monolayers at day 14: Supplementation with PRS resulted in highly increased calcium deposition by MSC (Fig. 3D) compared to unsupplemented MSC (Fig. 3B). Dexa, as expected, was also able to induce matrix mineralization in MSC (Fig. 3C).

BMP-2 gene expression and protein levels: Due to the very high up-regulation of BMP-2 mRNA levels by PRS, the time course of BMP-2 gene expression over two weeks was determined using real-time RT-PCR. While MSC control cultures showed no significant increase in BMP-2 gene expression over time, MSC cultures supplemented with PRS showed a clear increase of BMP-2 gene expression after only 24 h of culture, and an approximately 14-fold significant increase in BMP-2 after 14 days post-confluence (Fig. 4A). We also measured BMP-2 protein levels in supernatant of MSC alone and MSC supplemented with either PRS or Dexa using ELISA. While the levels of BMP-2 in unsupplemented MSC or treated with Dexa was under the ELISA detection limit (11 pg/mL) for the whole duration of the experiment, MSC supplemented with PRS showed a clear increase in BMP-2 protein levels over time (Fig. 4B). After 23 days of culture, BMP-2 levels in MSC stimulated with PRS were approximately 10 times higher than at the beginning of the culture.

Noggin
Due to the clear up-regulation of BMP-2 gene expression and protein levels by PRS, we evaluated the effect of the BMP-2 antagonist Noggin on PRS-supplemented MSC cultures at two different Noggin concentrations (100 and 500 ng/mL). After 28 days of culture, the addition of both concentrations of Noggin effectively down-regulated PRS-induced osteoblastic marker genes in MSC, including BMP-2, Col Iα1, MMP-13, BSP II, ON, OP and Runx2 (Fig. 5 and Table 2).

Wnt7b
To explain the discrepancy between up-regulation of many osteoblastic markers – in particular BMP-2 – and the down-regulation of Osterix, we studied the involvement of wnt-ligands in the effect of PRS on MSC. Interestingly, Wnt7b was always up-regulated in MSC supplemented with PRS over the whole culture period (Fig. 6 and Table 3). We could not detect elevated levels of Wnt3a or Wnt4 (data not shown).

Discussion

Due to the suspected osteoinductive effect of growth factors released by activated platelets, several forms of

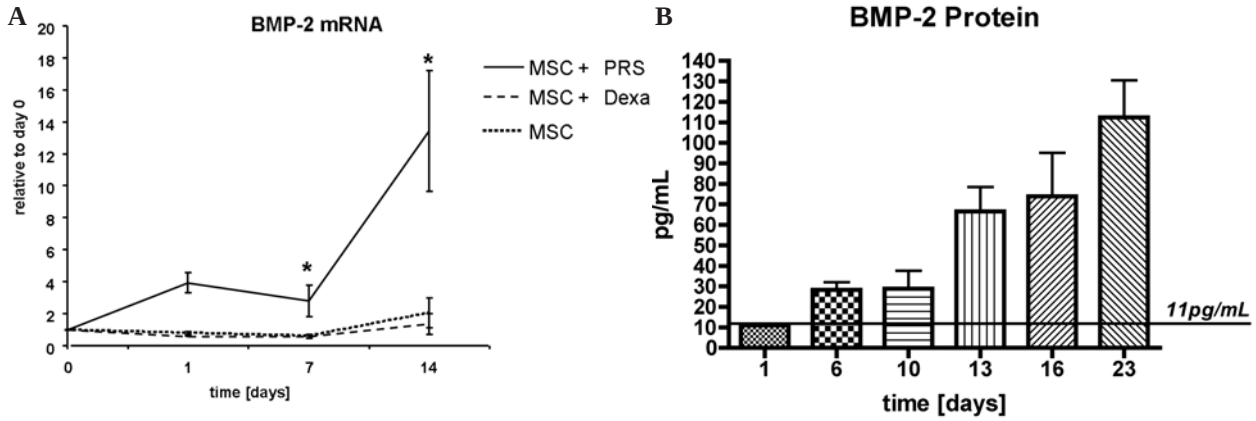


Fig. 4: A: BMP-2 mRNA levels in MSC compared to MSC+PRS (n=4): PRS significantly up-regulated BMP-2 levels (* $p<0.05$) at 14 days of culture, compared to un-supplemented MSC and Dexa.
B: BMP-2 protein levels in supernatants of MSC supplemented with PRS measured by ELISA (n=3): PRS significantly increased BMP-2 protein levels in MSC over the whole culture period. After 23 days of culture, BMP-2 levels in MSC stimulated with PRS were significantly up-regulated by 10-fold compared to day 1 ($p<0.05$). The BMP-2 levels in un-supplemented MSC and MSC stimulated with Dexa remained below the detection limit of the assay (11 pg/mL) for the whole duration of the experiment and are therefore not shown.

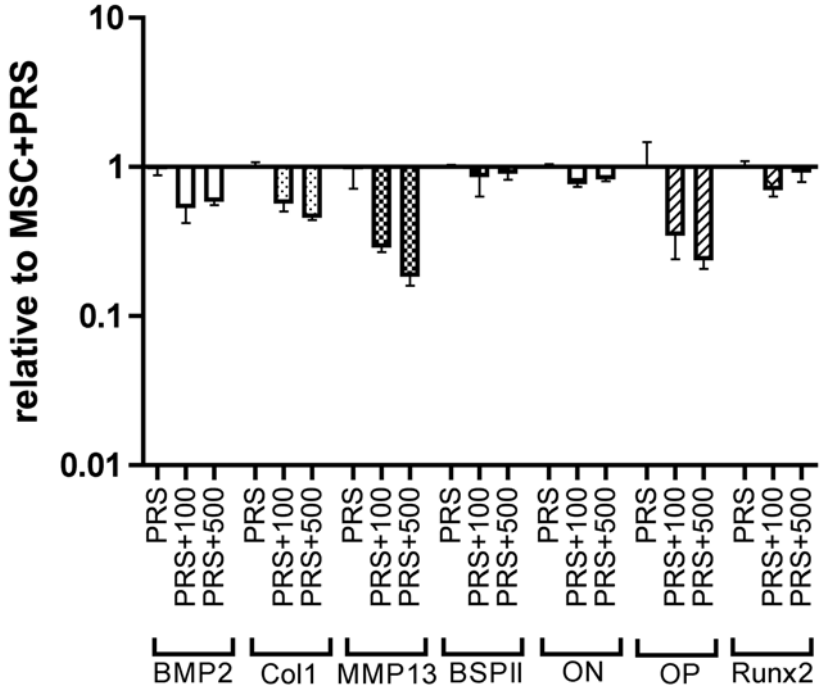


Fig. 5: Effect of the BMP-2 antagonist Noggin by real-time RT-PCR (triplicates of a representative experiment shown; see Table II for more results): MSC cultures supplemented with PRS and 100 ng/mL or 500 ng/mL of Noggin show a clear significant down-regulation of mRNA levels compared to MSC supplemented with PRS without Noggin, suggesting a regulatory effect of BMP-2. See also Figure 1 for comparison.

platelet preparations have recently gained popularity in maxillofacial and dental surgery, but their beneficial effect is still under debate (Kitoh *et al.*, 2004; Marx *et al.*, 1998; Mooren *et al.*, 2007; Roldan *et al.*, 2004).

In the present study, we investigated the effect of platelet preparations on the osteoblastic differentiation of human mesenchymal stem cells (MSC) by determining typical osteoblastic markers in a direct comparison with the effect of Dexa. Dexamethasone is a glucocorticoid with well established osteogenic properties *in vitro* (McCulloch and Tenenbaum, 1986) and potential beneficial effect *in vivo* (Miller *et al.*, 2010). To date, as it is easily available and a reliable inducer of MSC osteogenic differentiation, Dexa is the most used osteogenic factor. It has been shown to stimulate most of the osteoblastic phenotype features (but not all of them) (Chen *et al.*, 1986; Cheng *et al.*, 1994; Cheng *et al.*, 1996) such as increase of ALP activity and promote *in vitro* mineralization (Cheng *et al.*, 1994; Cheng *et al.*, 1996; Jorgensen *et al.*, 2004). However, even if glucocorticoids have been shown to increase the number and size of bone nodule formation in primary cell culture, the adverse effect of increased circulating concentration

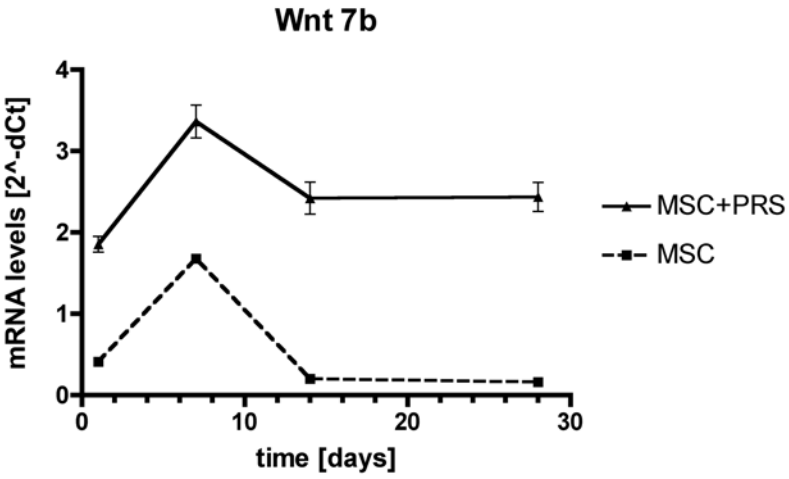


Fig. 6: Wnt7b mRNA levels measured by real-time RT-PCR (triplicates of a representative experiment shown; see Table III for more results): During the whole 28-days duration of the experiment, Wnt7b mRNA levels are always higher in MSC supplemented with PRS compared to MSC alone. This suggests involvement of the Wnt signaling system.

of such factors has also been described (Cooper *et al*., 1999; Cooper, 2004), and this could have an adverse effect if used *in vivo*.

In maxillofacial and dental surgery, platelet-preparations are usually administered in the form of PRP, which basically is a high concentration of platelets suspended in autologous plasma. One of the benefits of such preparations is the high gel forming ability, which *in vivo* helps to keep the PRP from diffusing out of the repair site. In *in vitro* experiments however, this gel forming capability is a problem, since it impedes proper oxygen and nutrient transport to the cells, and waste products away from the cells. We therefore used platelets resuspended in PBS instead of plasma to avoid clot formation. We validated this approach my measuring the level of several known growth factors released by activated platelets (PDGF-AB, -BB, VEGF) and were able to show that there was no significant difference between the released levels in PRP and PRS preparations (data not shown).

Our MSC preparations were prepared from bone marrow samples harvested at the iliac crest. This is a highly established procedure, which has been in use for many decades (Bolano and Kopta, 1991). In MSC supplemented with PRS, alkaline phosphatase was slightly up-regulated (approximately 2-fold at day 15) when compared to un-supplemented MSC. The differentiation effect of PRS on human MSC however was confirmed by $^{45}Ca^{2+}$-incorporation and Von Kossa staining. Interestingly, Dexa was able to induce ALP activity to a clearly higher level than PRS (> 3-fold higher than PRS at day 18). Measurements at the mRNA level of MSC exposed to PRS showed a significant up-regulation of collagen Iα1, bone sialoprotein II, MMP-13 and BMP-2 when compared to MSC alone. In contrast, osteonectin, osteopontin, Runx2 and Osx were not affected by PRS. Interestingly, of all the osteoblastic markers of MSC determined by real-time RT-PCR, two stuck out by being very strongly up-regulated by PRS: MMP-13 and BMP-2, this strong up-regulation was maintained when comparison was done with Dexa treated cultures. MMP-13 (collagenase-3) is a matrix metalloproteinase that has been found to be a major player during endochondral bone formation by efficiently degrading collagen types I- IV, as well as aggrecan. Furthermore, MMP-13 is under the control of the transcription factor Runx2 and is expressed as a late differentiation marker in osteoblasts (Jimenez *et al*., 1999). Factors released by platelets at a fracture site are known to act as chemoattractants for neutrophil granulocytes, followed by monocytic phagocytes (which differentiate into macrophages at the fracture site), fibroblasts, osteoblasts, vascular endothelial cells and for various precursor cells (Anderson, 1999). By degrading bone matrix components, MMP-13 can play an active role in support of the chemoattractive role of platelet-released factors. The other highly up-regulated gene was BMP-2. BMPs have the exquisite ability to induce ectopic bone formation (Wozney and Rosen, 1998). BMPs induce the expression of typical osteoblastic markers, including type I collagen, osteocalcin, MMP-13, ALP (Yamaguchi *et al*., 2000), and they stimulate the formation of mineralized bone-like nodules *in vitro* (Chen *et al*., 1997). These facts led us to the hypothesis that the stimulatory effect of our PRS preparations on human MSC could act at least partially through BMP-2 up-regulation. We therefore studied BMP-2 mRNA levels at earlier time points and were able to show, that BMP-2 is already 4-fold up-regulated after 24h of MSC exposure to PRS, and this up-regulation rises over time. This up-regulation was confirmed at the protein level by ELISA. Noggin is a well-known BMP antagonist and acts by binding to BMP-2, -4 and -7 receptors and therefore inhibits the BMPs from binding to their respective receptors (Zimmerman *et al*., 1996). We used Noggin at two different concentrations in our system and showed that most of the genes that were up-regulated by PRS were down-regulated upon exposure to PRS+Noggin. This further suggests that PRS could act through BMP-2 autocrine loop, as we showed an up-regulation of BMP-2 at both gene and protein level under PRS stimulation. It is well known that the "classical" *in vitro* pathway of osteoblastic differentiation especially in the murine system involves the sequential up-regulation of Runx2, Osx and ALP leading to matrix mineralization (Komori *et al*., 1997; Nakashima *et al*., 2002). This pattern has been observed in mice and rat MSC cultured either with Dexa or BMP-2 (Lee *et al*., 2000; Igarashi *et al*., 2004). This "classical" pathway also takes place when human MSC are stimulated

with Dexa, however it has been shown that Dexa treatment does not induce up-regulation of BMP-2 gene expression in human MSC (Meury *et al*., 2006; Zhou *et al*., 2006). In contrast, human MSC treated with BMP-2 have been shown to not follow the above "classical" pathway (Diefenderfer *et al.*, 2003; Osyczka *et al.*, 2004; Osyczka and Leboy, 2005; Zhou *et al*., 2006). In human cells, BMP-2 can induce osteoblastic differentiation independently of the transcription factors Runx2, its downstream partner Osx and ALP (Jorgensen *et al*., 2004; Osyczka *et al*., 2004; Osyczka and Leboy, 2005). This Runx2 and Osx independent pathway has also been shown in rodent cells as an alternative to the classical pathway, and it apparently involves the Wnt signaling cascade (Rawadi *et al*., 2003; Mbalaviele *et al*., 2005). Indeed, we did measure significantly elevated levels of Wnt7b in our system and this could explain our finding, that PRS did not up-regulate Runx2 and Osx. In the murine system, this alternative Runx2- and Osx-independent pathway seems to involve an increase in ALP activity (Rawadi *et al*., 2003; Mbalaviele *et al*., 2005). In contrast, in the human system, ALP activity has been shown not to be necessarily increased by BMP-2 (Jorgensen *et al*., 2004; Osyczka *et al*., 2004; Osyczka and Leboy, 2005; Kim *et al*., 2008). The long-term study of Jorgensen (Jorgensen *et al*., 2004) clearly showed that ALP, in BMP-2 treated cultures, was not increased compared to the control for up to 6 weeks, while Dexa strongly induced ALP activity. In contrast, Kim showed a 2-3 fold increase in ALP activity, when human MSC were exposed to BMP-2 (Kim *et al*., 2008). The increase in ALP activity in MSC+PRS cultures in our study is very low compared to the increase in ALP activity obtained by exposure to Dexa (10-15 fold increase), so whether this relatively small increase in ALP activity as seen in our study and reported by Kim can be considered biologically relevant, is presently not possible to be concluded from our results and from the above literature.

In summary, we have shown that addition of 10% of our PRS preparation induces osteoblastic differentiation and matrix mineralization in human MSC, in a comparable way to cell treatment with Dexa but the mechanisms involved clearly differ. These findings are in correlation with recent studies that reported similar effects (Lin et *al.*, 2006; Uggeri *et al*., 2007). In addition, we propose that the effect of PRS on the differentiation of human MSC could at least be partially mediated by BMP-2, although other biological factors present within the PRS could also play important roles. These findings are in correspondence with recent works showing that platelet preparations potentate BMP-dependent osteoblastic differentiation (Tomoyasu *et al*., 2007).. Furthermore, our observations suggest an involvement of the Wnt-signaling cascade, but further studies need to be performed to fully examine and characterize this last hypothesis.

In this study we have shown, that highly concentrated platelet preparations do positively influence osteoblastic differentiation of MSC and have therefore the potential to play an important role in bone healing, if applied appropriately. Besides their osteogenic effect, PRS preparations present the major advantage of being autologous. As they can be prepared intra-operatively, they constitute an easy and in a cost-effective way to obtain high concentrations of various cytokine and growth factors. The variety of the factors present in the platelet released supernatant also present the advantage of their variety, in the way that, in an *in vivo* situation, they can attract, proliferate and differentiate different cell types in a synergetic or sequential manner.

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Discussion with Reviewers

Reviewer I: Based on the data you presented in the paper, what is the significance of BMP and Wnt signaling pathways in the osteoblastic differentiation of MSCs after the treatment with PRS?

Authors: Unlike Dexamethasone treatment, PRS seems to induce human MSC differentiation and matrix mineralization through a non-classical pathway which does not involve activation of runx, osx and ALP up-regulation. MSC differentiation under PRS treatment appears to involve BMP2 protein up-regulation. When compared to cell differentiation under recombinant BMP2 treatment, similarities could be observed. The exact involvement of the Wnt pathway is currently under investigation and will be the object of a later publication.

Reviewer I: What are the pros and cons of using recombinant BMPs, autologous PRS or dexamethasone to induce osteoblastic differentiation in clinical settings?

Authors: BMPs are well known to induce osteogenic differentiation of MSC *in vitro* and to induce bone formation in numerous animal models. BMP-2 and BMP-7 are approved for clinical use in open fractures of long bones, non-unions and spinal fusion. However, despite significant evidence of their potential benefit on bone repair and regeneration in animal and preclinical studies, there is, to date, a lack of convincing clinical outcomes. The short life span of recombinant proteins and the inefficient delivery to target cells is also of a problem. Recombinant factors are expensive and as high doses might be required to reach the wanted therapeutic effect. An increasing number of publications questioning the efficiency-cost ratio have been published in the past few years. As compared with dexamethasone, PRS preparations have the strong advantage of being composed by a cocktail of different factors in physiological concentrations that can act in parallel, or in a sequential manner. Moreover the possibility to prepare PRS in an autologous way also reduces the risk of immunogenic reaction or disease transmission. Using other stimulating factors, like BMP or dexamethasone, the questions concerning the

appropriate dosage for *in vivo* application, linked to the "un-controlled" release problem are still an issue. Furthermore, the patient inter-individual variation is still poorly understood. PRS might constitute a good alternative, as it can be prepared intra-operatively in a cost effective way and thus provides the surgeon with an autologous high concentration cocktail of growth factors and cytokines, although further research evaluating the PRS growth factors composition need to be performed, in order to avoid inter-patient variation.

Reviewer III: Platelet released factors are proposed as a potential alternative to classic MSC differentiation methods. Looking at the different features of MSC stimulated by PRS and dexamethasone (e.g., increased matrix mineralization *vs*. alkaline phosphatase activity), could it be that MSC triggered by PRS reach a different osteoblastic phenotype/lineage as compared to the one typically obtained using conventional differentiation factors?

Authors: This is certainly a possibility. Alternatively, one could also speculate that due to the heterogeneous cell populations present within the MSC pool (which are at different differentiation stages and with different lineage progression potential) each different trigger (PRS, Dexa and BMPs) could act preferentially on a cellular subset of that pool.