

# Nitric Oxide Activates Signaling by c-Raf, MEK, p-JNK, p38 MAPK and p53 in Human Mesenchymal Stromal Cells and inhibits their Osteogenic Differentiation by Blocking Expression of Runx2

Tino Felka<sup>1</sup>, Christine Ulrich<sup>1</sup>, Bernd Rolauffs<sup>2</sup>, Falk Mittag<sup>3</sup>, Torsten Kluba<sup>3</sup>, Peter DeZwart<sup>2</sup>, Gunnar Ochs<sup>2</sup>, Michael Bonin<sup>4</sup>, Kay Nieselt<sup>5</sup>, Melanie L Hart<sup>1,6</sup>, Kurt Geckeler<sup>7</sup> and Wilhelm K Aicher<sup>1,6\*</sup>

<sup>1</sup>ZMF Center for Medical Research, University of Tuebingen Medical Faculty, Tuebingen, Germany

<sup>2</sup>Department of Trauma Surgery, University of Tuebingen, Tuebingen, Germany

<sup>3</sup>Department of Orthopaedic Surgery, University of Tuebingen Hospital, Tuebingen, Germany

<sup>4</sup>Institute of Medical Genetics and Applied Genomics, University of Tuebingen, Tuebingen, Germany

<sup>5</sup>Integrative Transcriptomics, Center for Bioinformatics, University of Tuebingen, Tuebingen, Germany

<sup>6</sup>KFO273, Department of Urology, University of Tuebingen Hospital, Tuebingen, Germany

<sup>7</sup>School of Materials Science and Engineering, Gwangju Institute of Science & Technology (GIST), 1 Oryong-dong, Buk-gu, Gwangju 500-712, South Korea

## Abstract

**Introduction:** Mesenchymal stromal cells (MSC) are a promising therapy for wound healing and regeneration of inflamed tissues. They are used clinically for different symptoms and diseases and are being investigated in clinical trials world wide at an increasing rate. However, depending on the application protocol and site of treatment, MSC may face an inflammatory environment.

**Objective:** Nitric oxide (NO) is one of the soluble factors produced in acute and chronic inflammation and influences growth, apoptosis, proliferation and differentiation of cells. NO therefore may have an influence on MSC injected into inflamed sites. Thus we investigated the effects of NO radicals on human MSC.

**Methods:** Human MSC were expanded and characterized. Expression of the mesenchymal lineage markers was determined by flow cytometry and their tri-lineage differentiation was explored *in vitro*. MSC were incubated with the NO-donor sodium nitroprusside (SNP) at different concentrations (5  $\mu$ M-5 mM) and over different periods of time (15 min–24 hrs), and analyzed for their respiratory activity, gene expression responses, cell signalling pathways, and differentiation potential.

**Results:** Human MSC expressed the mesenchymal marker proteins CD73, CD90, CD105, CD146, but failed to express the hematopoietic markers CD11b, CD14, CD34, and CD45. Activation of the MSC *in vitro* by nitric oxide activated c-Raf-, p-38-MAPK, and p-JNK-mediated signalling in a dose dependent manner, and also significantly regulated genes involved in cellular proliferation (cyclin D1, GAS1), apoptosis (p53), and induced an intense nuclear factor E2-related factor (NRF2)-associated stress response. Moreover, NO inhibited the entry of MSC in the osteogenic differentiation pathway and NO-treated MSC expressed less of the transcription factor Runx2. In contrast, the expression of the adipogenic marker gene PPAR $\gamma$ 2 remained unchanged.

**Conclusion:** We conclude that NO modulates the metabolism of MSC and compromises their osteogenic differentiation potential, which may have detrimental consequences for bone remodelling or bone regeneration.

**Keywords:** Mesenchymal stromal cells; MSC; Nitric oxide; NO radicals; Cell differentiation; Osteogenesis; Runx2

## Introduction

Mesenchymal stromal cells (MSC), also referred to as mesenchymal stem cells, are attracting interest for different applications, including tissue engineering and regenerative cell-based therapies [1,2]. They have been applied clinically to control autoimmune and graft-versus-host diseases [3-6]. MSC are characterized by fibroblast-like morphology, high proliferation rate, attachment to cell culture dishes and the capacity to differentiate into different mesenchymal lineages [1]. MSC express a set of mesenchymal surface antigens such as CD73, CD90, CD105, and others, but lack expression of hematopoietic antigens CD14, CD34 and CD45 [7-9].

Possible clinical applications for MSC are wide ranging. They have been used to treat bone or chondral defects [10-15], facilitate muscular regeneration [16], modulate cancer development [17], vascularisation [18,19], and deliver anti-inflammatory effects *in vivo* [20]. However, in clinical applications to support wound healing and regeneration of inflamed tissues, MSC may face pro-inflammatory stimuli such

as interleukin-1 (IL1 $\beta$ ) or tumour necrosis factor- $\alpha$  (TNF $\alpha$ ), which influence their gene expression patterns [21,22].

Nitric oxide (NO) is a free radical that is synthesized in acute and chronic inflammation [20,23-26]. It is produced *in vivo* by oxidation of L-arginine to L-citrulline. This reaction is catalyzed by different NO synthases (NOS). The endothelial (eNOS) and neuronal (nNOS) NO synthases are expressed constitutively and produce low amounts of NO

**\*Corresponding author:** Prof. Dr. Wilhelm K. Aicher, KFO273, Department of Urology, University of Tuebingen Hospital (UKT), Paul-Ehrlich-Str. 15, 72076 Tuebingen, Germany, Tel: +49 7071 298 7020; Fax: +49 7071 292 5072; E-mail: [aicher@uni-tuebingen.de](mailto:aicher@uni-tuebingen.de)

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[27]. Higher amounts are produced by the inducible NOS (iNOS) after stimulation of the cells by IL1 $\beta$ , TNF $\alpha$ , or interferon- $\gamma$  (IFN $\gamma$ ) [28]. In MSC production of NO is elicited by activation of the cells by IFN $\gamma$  in the presence of either IL1 $\beta$  or TNF $\alpha$  [29]. *In vitro*, it has also been shown that activated macrophages produce up to 65 mM/10<sup>6</sup> cells of intracellular NO within 72 hrs [30], and this raise the extracellular nitrite concentrations to 80 mM [31]. In the inflamed colon, NO production rates of 2.3  $\pm$  0.6 pmol/sec were calculated from 10<sup>6</sup> cells, resulting in a cumulative dose of 560  $\mu$ M/min to nearby cells [25]. Cytokine-activated macrophages began to undergo apoptosis when NO concentrations exceeded 115 mM [32]. Hence, NO production at these concentrations or above is cytotoxic.

The small NO molecule can move freely across cell membranes, is quite stable under physiological conditions, and has a half-live time of approximately 2–3 sec. Therefore, within seconds NO may reach distances of 200  $\mu$ m in tissues [33]. Depending on the concentration and localization, NO is involved in numerous physiological processes [27]. At low concentrations (<1  $\mu$ M) NO affects different transcription factors. It also affects heme-containing enzymes such as guanylate cyclase [34]. Higher NO concentrations (>1  $\mu$ M) result in chemical reactions that modify proteins by nitrosylation or nitration [34]. Recent work suggests that physiological concentrations of NO may be in the pico- to nano-molar range, rather than in the micro-molar range [35]. This is a hundred fold below the concentration range considered physiological a few years ago [34]. Technical differences in either quantifying NO *in vivo* and *in vitro* or differences in delivering defined amounts of NO may account for the different concentrations reported in different experiments. But there is ample evidence that NO influences growth, apoptosis, proliferation and differentiation of different cell types, and may even promote tumorigenesis [13,36-41]. Lack of cardiac NO production during embryonic development delayed the maturation of the heart. The affected mice (eNOS<sup>-/-</sup>) often died soon after birth [42], suggesting an important role for NO in proliferation and differentiation of mesenchymal progenitor cells.

Activation of iNOS and elevated production of NO are observed in chronic degenerative processes [23], acute injury [43,44], and inflammation [20,24,45]. Thus, NO radicals may represent a serious burden for transplantation of MSC in inflamed areas. Herein we report effects of NO on respiratory activity, gene expression and differentiation of human MSC.

## Materials and Methods

### Isolation of human MSC

Human MSC were isolated using Ficoll density gradient fractionation from femoral bone marrow aspirates of 11 patients undergoing endoprosthetic surgery as described [46]. This study was approved by the local ethics committee. MSC were expanded in low-glucose DMEM (Lonza) supplemented with 5% human fresh frozen plasma (FFP), 5% platelet concentrate (10<sup>8</sup> platelets/ml medium), 2 mM glutamine (Lonza), 1000 IU heparin sodium (Roth), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (Invitrogen). The starvation expansion medium utilized in several sets of experiments consisted of low-glucose DMEM supplemented with 0.5% FFP and 0.5% platelet concentrate.

### Characterization of human MSC

The MSC were characterized according to the defined minimal criteria as described recently [7]. The expression of CD146, CD90,

CD11b (R&D Systems), CD105, CD73, CD45, CD34, and CD14 (BD Pharmingen) was analysed by flow cytometry [47]. The differentiation to osteogenic or adipogenic cells was performed as described recently, visualized by cytochemical staining [47], and confirmed by quantification of transcripts encoding the corresponding marker genes (see below).

### XTT-assay

To analyse the effect of NO radicals on MSC, cells were treated with NO-donor sodium nitroprusside (SNP, Calbiochem) at different concentrations (5  $\mu$ M to 5 mM). Under physiological conditions SNP breaks down by hydrolysis with a half-live time of approx. 10 min. and causes e.g., relaxation of smooth muscle cells and vasodilatation cyclin [48,49]. Mock-treated cells served as controls. After 24 hrs of incubation, the respiratory activity and viability of MSC were measured using a XTT- assay (EZ4U, Biomedica) according to the manufacturer's instructions. In this assay the water soluble dye (XTT) is reduced by mitochondrial dehydrogenases and NADH or NADPH to an insoluble formazan, which is detected by spectrophotometry. This assay therefore detects mitochondrial glycolysis (= respiratory activity) and NADH/NADPH content in viable cells. The data is expressed as normalized respiratory activity, and generation of formazan by mock-treated cells is set to a respiratory activity of 100%. (=1).

### Compensation of NO activity

To investigate the specificity of NO-mediated effects, the reactive oxygen species were neutralized by addition of the water soluble fullerene PNPAM-6C<sub>60</sub> as described before [50]. In brief, The MSC was incubated in the presence of 1  $\mu$ M to 100  $\mu$ M PNPAM-6C<sub>60</sub> and SNP was added ad 1.5 mM. Mock-treated cells (control 1) and cell incubated with 1.5 mM SNP w/o PNPAM-6C<sub>60</sub> (control 2) served as controls. After 24 hrs of incubation, metabolic activity / viability of the MSC was determined by XTT assay.

### Nitrite-nitrate assay

Nitrite concentration was measured in cell culture media (control) or supernatants of mock-treated and SNP-treated MSC (5  $\mu$ M to 5 mM) utilizing a colorimetric nitrite-nitrate assay kit (Cayman Chemical) according to the manufacturer's protocol.

### Microarray analysis

Sodium nitroprusside (add 1.5 mM) was added to MSC (n=3 donors) and the cells were incubated in starvation expansion medium for 24 hrs. MSC without SNP served as controls. Total RNA was isolated using the RN easy Kit (Qiagen) according to the manufacturer's protocol. The quality of the RNA was confirmed by chromatography and cRNA was produced. Gene expression analysis was performed by Affymetrix GeneChip<sup>®</sup> technology (Human U133+2.0 Genome). The gene expression data (Expression Console<sup>®</sup>, Affymetrix) was evaluated by GeneSpring GX 11.5 software (Agilent Technologies).

### Transcript analysis

To corroborate the NO-induced differences in expression of some representative genes in MSC, a quantitative RT-PCR (qRT-PCR) analysis was performed. SNP (add 1.5 mM) was added to MSC (n  $\leq$  8 donors) and the cells were incubated for 24 hrs as described above. MSC without SNP served as controls. Total RNA was isolated, reverse transcription was performed on 1  $\mu$ g total RNA using the Advantage RT for PCR Kit (Clontech) to generate cDNA. Transcripts

were enumerated by qRT-PCR (LightCycler, Roche) [51] using commercially available primer pairs for sequestosome 1 (SQSTM1), peroxiredoxin 1 (PRDX1) glutathione reductase (GSR), thioredoxin reductase 1 (TXNRD1), ATP-binding cassette transporter (ABCC1), NAD(P)H dehydrogenase (NQO1), glutamate cysteine ligase (GCLM), aldehyde oxidase (AOX1), stress induced phosphoprotein 1 (STIP1), protein tyrosine phosphatase (PTPLAD1), ER stress induced domain 1 (HERPUD), valosin containing protein (VCP), tRNA guanine transglycosylase (USP 14) and FOS-like antigen 1 (FOSL1) (all from Qiagen). Serial dilutions of recombinant DNA standards served as controls. GAPDH served as the reference gene in each PCR [51].

To investigate the effects of NO on differentiation of MSC *in vitro*, expression of the osteogenic differentiation markers runt related transcription factor 2 (Runx2), and osteocalcin, and the adipogenic marker peroxisome proliferator-activated receptor gamma-2 (PPAR $\gamma$ 2) (all from QIAGEN) was determined. Serial dilutions of recombinant DNA standards served as controls. GAPDH served as the reference gene in each PCR [51].

### Immunoblot analysis

For immunoblot analysis,  $1 \times 10^6$  MSC were incubated with 10  $\mu$ M or 1.5 mM SNP for different periods of time (15 min to 24 h). After washing with cold PBS, cells were harvested by mechanical detachment, collected by centrifugation and lysed in 100  $\mu$ l RIPA buffer (c-c-pro) containing proteinase inhibitors. The protein concentration was measured using the RCDC Kit (BioRad). Cellular protein extracts (100  $\mu$ g) were mixed with Laemmli sample buffer, denatured at 95°C and separated by electrophoresis in a 10% SDS-PAGE. After blotting, the nitrocellulose membrane was blocked with 5% milk, PBS, 0.1% Tween-20 (blocking buffer) and probed overnight at 4°C with mAb specific for phospho-c-Raf, phospho-MEK1/2, phospho-Erk1/2 (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), phospho-PI3K, phospho-p38, phospho-p53 (Ser15) and  $\beta$ -actin (Cell Signaling Technology). Secondary HRP-labelled goat-anti-rabbit-antibody (Jackson ImmunoResearch) was used in blocking buffer. Binding of antibodies was visualized by enhanced chemiluminescence (ECL), by exposure of the luminiscence on Amersham Hyperfilm™ECL (GE Healthcare) and, then the film was developed in a photo developer (Curix 60, AGFA).

### Statistics

Unless otherwise noted, experimental data are presented as mean values  $\pm$  standard deviations. Statistical analyses were performed using a two-sided Student's t-test. Differences in gene expression levels yielding *p*-values less than 0.05 were considered significant and marked accordingly (\**p*<0.05, \*\* *p*>0.01, \*\*\**p*<0.001).

## Results

### Characterization of MSC

The MSC were expanded and characterized as fibroblast-like cells that expressed the typical antigens CD73, CD90, CD105 and CD146. At the same time the expression of hematopoietic antigens CD11b, CD14, CD45 and CD34 was low or absent (Figure 1A). The MSC were able to differentiate to chondrogenic cells as visualized by Alcian Blue staining of proteoglycans in micro masses, to adipocytes as documented by Oil Red O staining of lipid vesicles, and to osteoblasts as documented by von Kossa staining of mineralized extracellular matrix (Figure 1B). Thus the cells employed in this study fulfilled the inclusion criteria defined for human bone marrow-derived MSC [7].

### Effect of NO radicals on respiratory activity of MSC

The treatment of MSC (n=3) with different concentrations of SNP revealed a dose dependent effect on MSC. Low SNP concentrations (10-25  $\mu$ M) yielded a slight increase in the respiratory activity of MSC (up to 120%) compared to untreated controls (100%) as depicted by the XTT assay. Higher SNP doses (1 mM and higher) resulted in a decrease in the respiratory activity of MSC (60%-20%, Figure 2A). As shown in our recent study with cell lines [50], addition of the fullerene-based water soluble radical scavenger PNIPAM-6C<sub>60</sub> rescued the respiratory activity on NO-treated human MSC (Figure 2B). This confirmed that the effects observed were caused by NO-radicals.

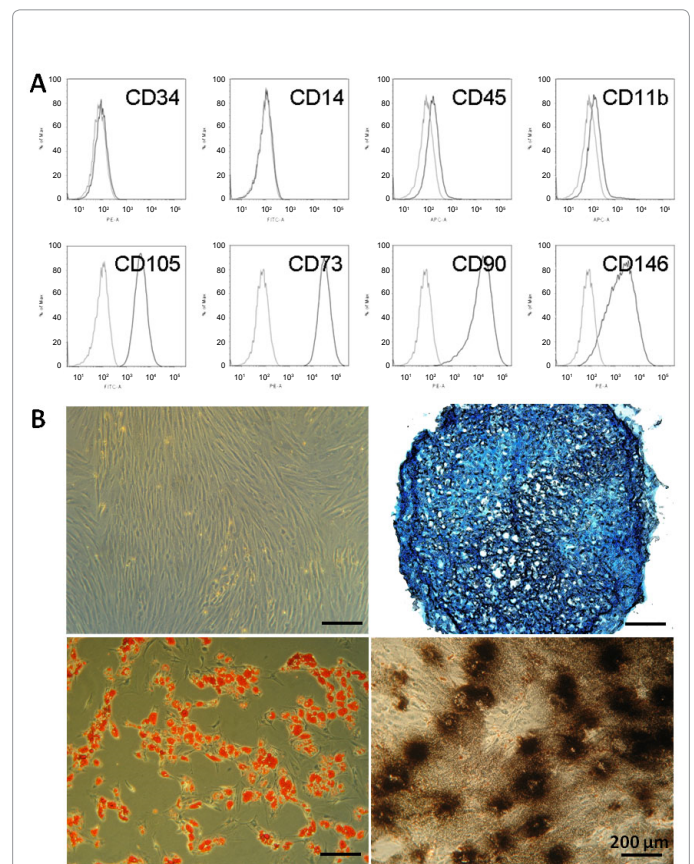
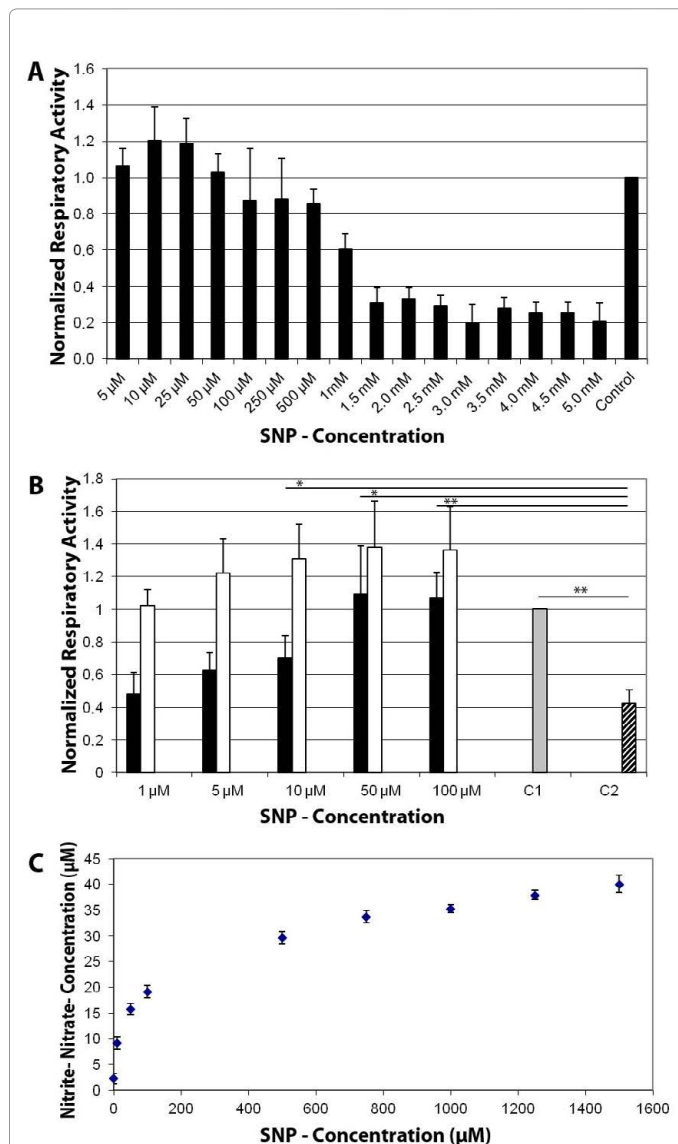


Figure 1: Characterization of MSC.

**A.** Expression of cells surface proteins on human MSC was investigated by flow cytometry [7]. Expression of CD34, the marker for hematopoietic stem and progenitor cells, CD14, the co-receptor for LPS on macrophages, CD45, the common leucocyte antigen, and CD11b, the complement receptor 3 (= Mac-1, ITGAM) were not detected or showed very low expression on MSC *in vitro*. MSC expressed the typical antigens including TGF- $\beta$  receptor CD105 (endoglin), CD73 (5'ecto-nuclease), CD90 (GPI-anchored glycoprotein, = Thy-1), and CD146 (adhesion molecule, = MUC18, MCAM).

**B.** The tri-lineage differentiation of MSC was investigated after 4 weeks of differentiation *in vitro*. Chondrogenesis was induced in micromasses and differentiation was documented by Alcian Blue staining (upper right). Adipogenesis (lower left) and osteogenesis (lower right) were induced in monolayer cultures and success of differentiation was investigated by Oil Red O and von Kossa staining, respectively. MSC in expansion medium maintained their fibroblastoid appearance and served as controls (upper left). Size bars measure 200  $\mu$ m.





**Figure 2:** Effects of nitric oxide radicals on cell viability and nitrite-nitrate formation in cell culture media.

**A** SNP was added to MSC in concentrations ranging from 5 μM to 5 mM and respiratory activity was measured by XTT assay. Low doses of SNP (10 μM and 25 μM) activated respiratory activity to some extent. SNP at 100–500 μM reduced the respiratory activity of MSC to approximately 80%. Dosages of 1 mM SNP or higher reduced the respiratory activity of MSC to 30% or 20%. Mock-treated MSC served as controls (= 100%).

**B** Human MSC was incubated for 24 h in medium without SNP (C1, striated column) or in presence of 1.5 mM SNP (C2, grey column). Addition of SNP reduced the normalized respiratory activity / viability of MSC to 42% compared to the mock-treated controls (= 100%). Addition of 1 μM and 5 μM PNIPAM-6C<sub>60</sub> elevated measurably but statistically not significant to 47% and 62%, respectively. Addition of 10 μM, 50 μM, or 100 μM PNIPAM-6C<sub>60</sub> elevated the respiratory activity / viability of SNP-treated human MSC significantly to 70% (± 13%; p<0.039), 101 (± 29%; p<0.02), and 101 (± 16%, p<0.004; black bars). Addition of PNIPAM-6C<sub>60</sub> only did not reduce the respiratory activity / viability of human MSC (white bars).

**C** The formation of nitrite / nitrate in MSC cultures was investigated following addition of SNP at concentrations between 5 μM and 1.5 mM and determined after 24 h of incubation. At lower SNP concentrations (up to 500 μM) a steep increase in nitrite / nitrate concentrations (0–35 μM) was measured. Higher SNP concentrations (500 to 1500 μM) resulted in a slight increase of nitrite / nitrate concentration (35 to 40 μM).

## Nitrite accumulation

S-nitrosylation by NO is a mechanism for post-translational modification of many proteins. However, at high concentrations of NO, nitrosylation may yield toxic effects and nitrite will accumulate. The accumulation of nitrite after 24 hrs of incubation of MSC (n=3) with different concentrations of SNP was measured by the nitrite-nitrate assay (Figure 2C). Incubation of MSC in medium containing 10 μM SNP resulted in a nitrite concentration of 9.2 μM (± 1.2 μM) in the supernatants. A steep increase in nitrite concentration was observed at low doses of SNP. Addition of 500 μM SNP or more yielded smaller increases in nitrite concentrations. Concentrations of 500 μM SNP to 1.5 mM SNP resulted in a significant drop of respiratory activity of MSC (Figure 2A) and in this range of SNP dosages an accumulation of 29 (± 1.5) mM to 40 μM (± 1.7 μM) nitrite (Figure 2C). Thus, the mitogenic effects measured with low SNP concentrations on MSC *in vitro* were possibly associated with NO activity itself, whereas toxic effects observed upon addition of SNP at concentrations of 500 μM or more may be caused mainly by nitrite.

## Effect of SNP on gene expression of MSC

The incubation of MSC with SNP (1.5 mM, 24 hrs) yielded for instance a significant elevation of transcripts encoding cyclin D1 (5.6-fold up, p<0.02) and growth arrest-specific 1 gene (GAS1, 10.9-fold down, p<0.01) was significantly reduced. Thus, depending on the conditions, NO may facilitate the G1/S transition of the cell cycle in MSC. Significant changes in mRNA expression patterns (factor ≥ 2) of more than 1000 genes were observed (gene array data not shown). These genes were compared to known regulatory networks and pathways and related to cellular and physiological functions, as well as diseases. A high number of NO-regulated factors were associated with regulation of proliferation, growth and cell death (Table 1). A comparison to known regulatory networks and pathways indicated a connection to tumorigenic processes as well. By gene array, the most significant changes were observed for the NRF2-mediated oxidative stress response pathway (p=4.73 E-06; Table 1). In this canonical pathway NO significantly induced for instance the expression of the modifier subunit of glutamate-cystein ligase (GCLM, 5.4-fold up, p<0.004)

Molecular and Cellular Functions	p-value	# Genes
Growth, Proliferation	8.47E-05–1.81E-02	172
Cell Death	4.76E-06–2.04E-02	253
Small Molecule Biochemistry	1.89E-05–1.89E-02	104
Amino Acid Metabolism	1.89E-05–1.43E-02	17
Lipid Metabolism	2.72E-05–1.89E-02	79
Diseases		
Cancer	6.52E-14–2.22E-02	448
Reproductive System Disease	3.10E-05–1.86E-02	151
Gastrointestinal Disease	1.47E-06–2.10E-02	126
Top Canonical Pathways		
NRF2-mediated oxidative stress response	1.65E-06	23
Pyrimidine Metabolism	5.35E-03	
Interferon Signaling	6.18E-03	
Glutathione Metabolism	5.02E-03	

List of the most significantly regulated pathways modulated by NO in MSC. The molecular and cellular functions of the annotated pathways are listed in the left column, the levels of significances (i.e., p-value) for changes in NO-dependent gene regulation in the respective pathways in the middle, and numbers of factors or genes regulated by NO in the right column (1.5 mM SNP, 24 hrs)

**Table 1:** Significant NO-regulated pathways in human MSC: a brief summary of microarray data.

Method: SNP Dosage: Genes:	Differences mRNA expression			Function
	Array	qRT-PCR		
	1.5 mM	1.5 mM	10 μM	
Sequestosome 1	<i>11.58</i>	2.3		Antioxidative protein
Peroxiredoxin 1	2.47	3.87	<b>-1.85</b>	
Glutathionereductase	3.37	3.31	<b>-1.06</b>	
Thioredoxinreductase 1	4.2	6.1	1.96	
ATP binding cassette transporter	3.28	1.35	1.02	Drug resistance
NAD(P)H- dehydrogenase		5.66	<b>-1.37</b>	De-toxification, cell survival- tumorigenesis
Glutamate cysteine ligase	5.38	6.84	<b>-1.72</b>	
Aldehyde oxidase	<b>-2.44</b>	<b>-3.13</b>	<b>-2</b>	
Stress induced phosphoprotein 1 Protein tyrosine phosphatase	2.52			Repair and removal of damaged proteins
ER stress induced domain 1		<b>-1.45</b>	<b>-2.63</b>	
Valosin containing protein	2.05	<b>-1.43</b>	<b>-2.63</b>	
tRNA guanine transglycosylase	2.09	1.07	2.17	
FOS like antigen 1	11.27	6.92	1.22	Modulation of malignant cells

Selected factors regulated by NO in human MSC in comparison to untreated cells showed x-fold differences dependent on the concentration of SNP added. Data were selected from the transcript array data or computed from qRT-PCR as indicated. Factors expressed at higher steady state mRNA levels upon incubation of MSC with SNP are denoted in *italics*, and factors expressed at lower levels in **bold** numbers

**Table 2:** NO-regulated relative mRNA expression of stress response genes in MSC.

and the microsomal glutathion S-transferase 1(MGST1, 3.74-fold up,  $p < 0.015$ ). The other NO-regulated canonical pathways significantly changed included the pyrimidine metabolic pathway ( $p = 5.35E-03$ ), interferon signalling ( $p = 6.18E-03$ ) and glutathione metabolic pathway ( $p = 7.91E-03$ ). The results indicated that NO provoked a significant change in the metabolism of human MSC.

### Analysis of the nuclear factor E2-related factor (NRF2)-mediated oxidative stress response pathway

Factors associated with the NRF2-mediated oxidative stress response were the most significantly altered pathways upon NO stimulation of MSC (Table 1). To validate these gene array experiments, qRT-PCR analyses were performed for some representative genes and the differences induced by high concentrations of NO (1.5 mM SNP) were enumerated in independent samples (Table 2). At the same time, the effects of low concentration of NO were tested (10 μM SNP; Table 2). High SNP concentration (1.5 mM) yielded an increase in gene expression encoding proteins associated with anti-oxidation (e.g., sequestosome 1, peroxiredoxin 1), drug resistance (e.g., ATP binding cassette transporter), detoxification, cell survival and tumorigenesis (e.g., NAD(P)H-dehydrogenase, glutamate cystein ligase, aldehyde oxidase), as well as repair and removal of damaged proteins (Table 2). However, low concentrations of SNP (10 μM) yielded either a slight increase or a decrease of mRNA expression of stress response genes as detected on the transcript levels by qRT-PCR.

The NRF2-mediated pathway includes activation of different mitogen activated protein kinases (MAP-kinases) [52], followed by changes of mRNA expression of stress response genes. Therefore the activation of MAP-kinases after incubation of MSC with SNP at a low dose (10 μM) and a high dose (1.5 mM) was investigated by analysis of the phosphorylation kinetics for up to 24 hrs after stimulation (Figure 3). At low dose SNP treatment, changes in phosphorylation of MEK1/2, ERK-1/2 p38, JNK or p53 were not observed, but a transiently enhanced phosphorylation of c-Raf was recorded thirty minutes to two hours after stimulation (Figure 3). At high dosage, SNP yielded the transient phosphorylation of c-Raf as observed with 10 mM SNP. In addition, 1.5 mM SNP resulted in a significant phosphorylation signal for MEK-1/2 and JNK eight and twelve hours after stimulation, and for p38 an increase in phosphorylation signal intensities was seen

one to twelve hours after induction (Figure 3). Furthermore, addition of 1.5 mM SNP caused a phosphorylation of serine at position 15 of the apoptosis regulating factor p53. Phosphorylation of p53 was not observed with 10 mM SNP (Figure 3), confirming the pro-apoptotic effects of higher concentrations of NO on MSC (Figure 2A).

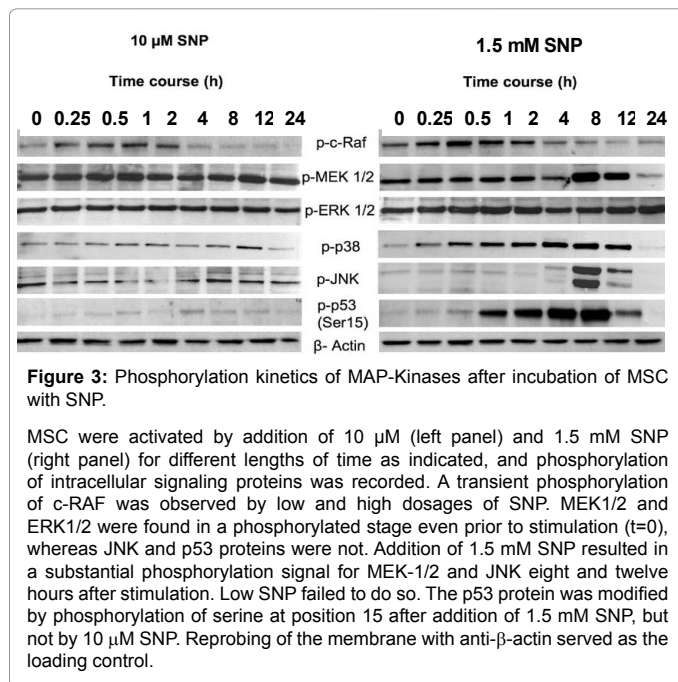
### Effect of NO on osteogenic differentiation of MSC

Regeneration of bone fractures and wound healing after bone surgery require osteogenic differentiation of MSC. Stimulation of MSC by NO caused an activation of c-Raf (Figure 3). Parathyroid hormone (PTH) is involved in regulation of both, growth and apoptosis of osteoblasts by an intracellular signaling cascade involving MAP kinases and c-Raf [53]. We therefore investigated the effects of NO on the osteogenic differentiation of MSC *in vitro* (Figure 4). Incubation of MSC for 7 days in osteogenic differentiation media in the absence of SNP elevated the expression of transcription factor Runx2, an early key regulator of osteogenic differentiation [54] (Figure 4A). Addition of SNP to cells in osteogenic differentiation medium (medium enriched by 1mM SNP changed daily for 7 days) completely blocked the elevated expression of Runx2 transcripts (Figure 4A), suggesting that NO affected the early stages of osteogenic differentiation. In contrast, induction of osteocalcin expression, a late marker of osteogenesis, was only slightly elevated (Figure 4B), suggesting that within this short period of induction time (i.e., 7 days) only entry in osteogenic differentiation was achieved.

In addition, induction of adipogenic differentiation was investigated, and PPARγ2, a marker gene for adipogenic differentiation of MSC, was significantly elevated after 7 days of induction (Figure 4C). But in contrast to the effects of NO on Runx2 expression (Figure 4A), a reduction of expression of PPARγ2 was not observed in MSC during adipogenesis in the presence of SNP (Figure 4C). We conclude that excess NO, as produced in chronic inflammatory processes, alleviates osteogenic differentiation of MSC and thus may contribute to reduced fracture healing and bone regeneration *in vivo*. Through blocking osteogenesis in MSC, NO may also facilitate osteoporosis, a condition affecting the elderly, whereas low NO concentrations may be beneficial, as this may stimulate proliferation of MSC *in situ*.

### Discussion

The treatment of MSC with the NO donor SNP at low



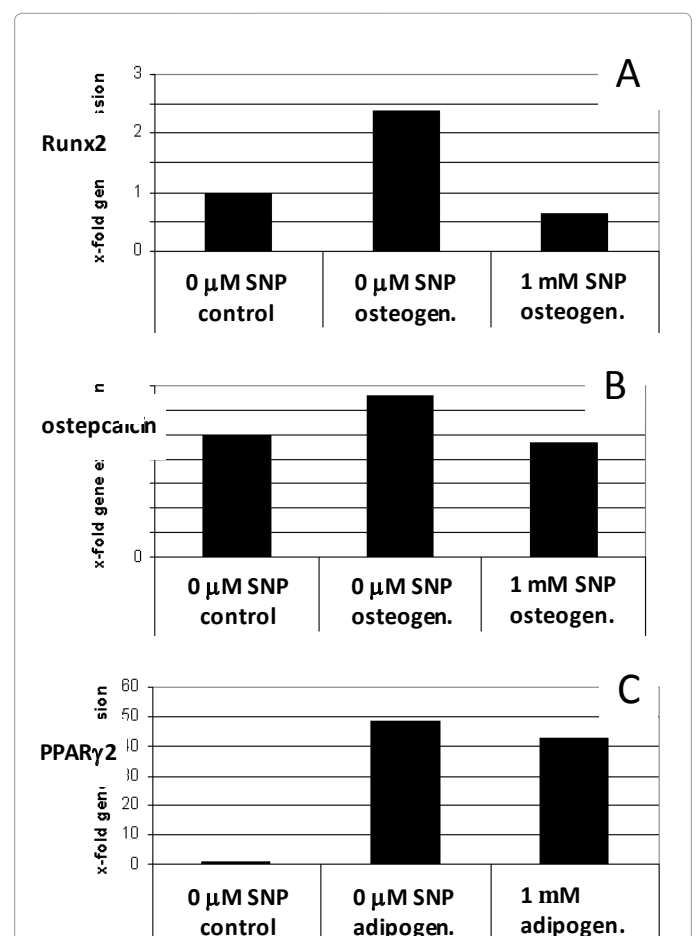
concentrations did not significantly affect the metabolic activity of MSC. The normalized respiratory activity and viability index was about 20% above the mock-treated controls. This slight increase in metabolic activity could be associated with the cell signalling effects reported for cytoplasmic NO. Nitric oxide binds to the heme moiety of soluble guanylate cyclase (sGC), triggering its enzymatic activity within seconds, and generates cGMP [55]. The various cGMP regulated signalling pathways include kinases, cGMP-gated ion channels, and phosphodiesterases. Moreover, activation of sGC inhibits apoptosis [56], which may cause the trend towards higher metabolic activity or cellular viability. A significant induction of D1 (5.6-fold up,  $p < 0.02$ ) and a more than tenfold reduction of GAS1 (10.9-fold down,  $p < 0.01$ ) by SNP were noted, thus facilitating cell cycle progression [57]. The activation of c-Raf by low doses of NO may contribute to proliferative activation of MSC as well [58].

Addition of SNP to MSC at higher concentrations (100  $\mu\text{M}$  to 1.5 mM) resulted in a concentration-dependent reduction of the respiratory activity of MSC. Needless to say that addition of SNP to cells in these concentrations does not deliver NO in comparable amounts over an extended time. In cell culture media containing serum, SNP has a half-life of about 30 minutes, and after 2 hours of incubation more than 90% of its activity is lost [59]. Therefore, the effective concentrations of NO in these experiments are considerably lower than one might assume from initial SNP input. However, specific measurement of NO in supernatants of cell culture media used for growth of somatic cells is a complex endeavour. A recent study publishing a spectrophotometric method to measure NO omitted glutamine, serum and phenol red in the cell culture medium [60]. Therefore this technique is not suitable as NO release from SNP may be different in medium containing glutamine, serum and phenol red. Others used chemiluminescence to detect reactive oxygen species (ROS) [61]. This method detects ROS in cell complete culture media, enriched with serum and glutamine, but the assay is sensitive to all reactive oxygen species, including OH, HOO, NO, O<sub>2</sub>, ONOO [61].

Besides that, the loss of metabolic activity in MSC at high SNP

dosage can be explained by activation of p53 and by an enhanced binding of NO to different heme-containing enzymes, including cytochrome C oxidase, catalase, and cytochrome P450. This affects the respiratory chain in mitochondria [62], peroxide detoxification by peroxisomes, and the oxidative degradation of many different compounds. Furthermore, macrophages displayed signs of cell death at 115  $\mu\text{M}$  NO [32], indicating that this concentration can cause toxic effects, which is in line with our results.

The GTPase Ras is activated by nitrosylation [63] and activation of Ras results in activation of the c-Raf-MEK1/2-ERK1/2 signalling pathway. The signal transduction of Ras to ERK 1/2 is part of the NRF2-mediated oxidative stress response pathway analysed in this study (Table 2) [64]. Immunoblot analyses revealed the phosphorylation



**Figure 4:** Effects of nitric oxide radicals on the differentiation of MSC.

MSC were expanded and osteogenic or adipogenic differentiation was induced in the absence or presence of SNP. MSC in expansion media served as the control.

**A** Incubation of MSC in osteogenic induction medium for 7 days resulted in an increase in expression of the early osteogenic marker Runx2. Addition of SNP to osteogenic induction (daily change of medium containing 1 mM SNP) blocked expression of Runx2.

**B** However, the expression of the late osteogenic marker osteocalcin was only slightly elevated and NO reduced this small increase to levels recorded in mock controls.

**C** Upon addition of adipogenic induction medium for 7 days to MSC, expression of the early adipogenic marker PPAR $\gamma$ 2 was drastically elevated, but a major NO effect was not recorded for adipogenesis (C).



of c-Raf as early as 15 minutes after addition of SNP to the MSC. The SNP concentration seems to have no influence on the kinetics of this process, since the pattern of phosphorylation of c-Raf was not different in MSC activated with 10  $\mu$ M or 1.5 mM SNP (Figure 3). In contrast, a basic phosphorylation of MEK1/2 and ERK1/2 was observed prior to stimulation of the cells by SNP, and at almost all time points investigated (Figure 3). This may be attributed to the cell culture conditions employed. Therefore some activated MEK and ERK seem to be available at all times. But in contrast to ERK, MEK was transiently activated by 1.5 mM by SNP, as were JNK and p38 MAP kinase. We therefore conclude that these two are the main MAP kinases involved in NO signaling downstream of Ras and Raf in MSC (Figure 5). Moreover, blocking JNK was shown to promote osteogenesis [65], supporting the working hypothesis that NO may influence the osteogenic differentiation of MSC via activation of JNK.

As discussed earlier, NO generates a complex pattern of cellular responses depending on targets and doses applied [27]. Recently a novel NO-dependent intracellular signalling pathway was described [66]. Here NO not only raises the cytoplasmic cGMP concentrations, but in addition generates a novel signalling molecule, the 8-NO<sub>2</sub>-cGMP. A direct, chemical non-enzymatical nitrosylation of cGMP was suggested [66]. This pathway is important especially at higher concentrations of NO, thus explaining in part the bi-phasic effects

observed with NO on MSC. The 8-NO<sub>2</sub>-cGMP then generates cGMP-modified proteins by S-guanylation [66]. This post-translational modification was described with the redox sensor protein, Keap1. The cGMP-modified Keap1 cannot interact with NRF-2, thus providing an additional regulatory pathway for the NRF-2-mediated oxidative stress responses, complementing the p38MAPK- modulated NRF-2 responses discussed earlier.

Oxidative stress plays an important role in aseptic loosening of orthopaedic endoprostheses [67,68] and high-output production of NO by CD68<sup>+</sup> cells in the interface membrane was recorded [69,70]. We provide evidence that the elevated production of NO blocks the osteogenic differentiation of MSC by reducing the expression of Runx2 [54,71]. Blocking osteogenesis of MSC may allusively contribute to the formation of the characteristic interface membrane, which contains activated fibroblasts that resorb the bone matrix [72]. Moreover, elevated NO contributes to loss of bone mass and therefore seems to promote osteoporosis [73-75]. However, the regulation of Runx2 is complex and both activatory and inhibitory effects were observed, depending on the experimental set-up [71,76] (Figure 5). In contrast, low doses of NO may facilitate osteogenic differentiation or NO facilitated MSC-mediated tissue repair *in vivo* [77,78]. Low NO thus may be beneficial to patients suffering from osteoporosis or bone defects.

## Conclusions

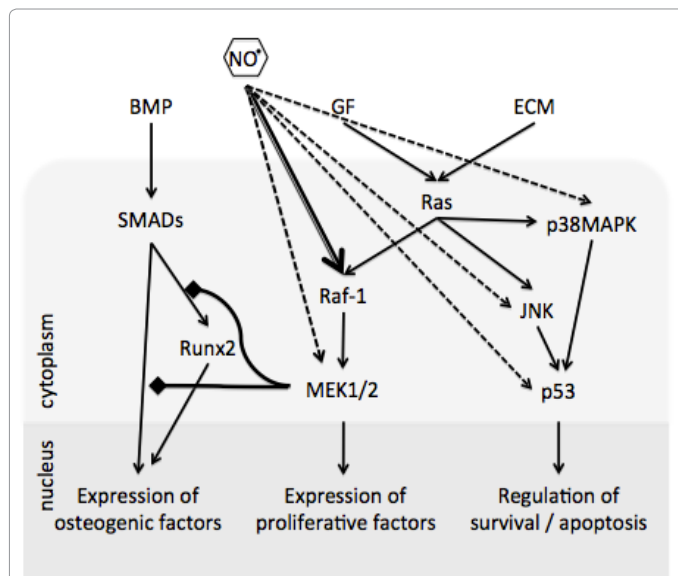
In summary, low dose NO does not reduce the viability or respiratory activity of MSC, nor activate the p38-, JNK- or p53-pathways, but activates c-Raf. This represents possibly the range of a rather beneficial NO dosage with regard to MSC proliferation or viability. In contrast, at higher SNP dosages, pathways associated with cell death and cancer is activated. In addition, the osteogenic differentiation of MSC is impaired by NO radicals, whereas adipogenesis is not affected. Therefore, at sites of tissue regeneration removal of pro-inflammatory and NO-producing cells or pharmacological control of NO production may increase the chances for efficient bone regeneration by MSC.

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**Figure 5:** Proposed role of NO in the osteogenic, proliferative and apoptotic pathways in MSC.

The bone morphogenetic proteins (BMP) induce the phosphorylation of SMADs and facilitate their translocation in the nucleus. The SMADs contribute to expression of the key osteogenic factor Runx2 (left side of the panel). Growth factors (GF) such as fibroblast growth factor and the extracellular matrix (ECM) activate, among other pathways, the small GTPase Ras and the Ras-Raf-pathway. Ras controls different signaling pathways, including different MAP-kinases and p53 (right side of the panel). BMPs, GFs and the ECM regulate the gene expression required for osteogenic differentiation, proliferation and survival or apoptosis of MSC (thin solid arrows). Depending on the cell, MEK1/2 block the activation and nuclear translocation of SMADs (thick line with rombus), thus inhibiting expression of Runx2 (71). Nitric oxide radicals cause a transiently elevated phosphorylation of MEK1/2, p38MAPK, JNK, and p53 (thin dashed arrows) involved in proliferation, survival and apoptosis. Low NO activates c-Raf (thick arrow), which facilitates the activation of MEK1/2, and may thus interfere with SMAD- and Runx2-dependent regulation of expression in MSC.

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