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# TNFα promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-κB signaling pathway

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ABSTRACT

Mesenchymal stem cells are multipotent cells able to differentiate into different mesenchymal lineages. Studies in the past had suggested that two of these mesenchymal differentiation directions, the chondrogenic and the myogenic differentiation, are negatively regulated by the transcription factor NF-κB. Although osteogenic differentiation has been extensively studied, the influence of NF-κB on this differentiation lineage was not subject of detailed analyses in the past. We have analyzed the consequences of TNF-α treatment and genetic manipulation of the NF-κB pathway for osteogenic differentiation of hMSCs. Treatment of hMSCs during differentiation with TNF-α activates NF-κB and this results in enhanced expression of osteogenetic proteins like bone morphogenetic protein2 (BMP-2) and alkaline phosphatase (ALP). In addition, enhanced matrix mineralization was observed. The direct contribution of the NF-κB pathway was confirmed in cells that express a constitutively active version of the NF-κB-inducing kinase IKK2 (CA-IKK2). The IKK2/NF-κB-induced BMP-2 up-regulation results in the enhancement of RUNX2 and Osterix expression, two critical regulators of the osteogenic differentiation program. Interestingly, a genetic block of the NF-κB pathway did not interfere with osteogenic differentiation. We conclude that TNFα mediated NF-κB activation, although not absolutely required for BMP-2 expression and matrix mineralization nevertheless supports osteogenic differentiation and matrix mineralization by increasing BMP-2 expression. Our results therefore suggest that NF-κB activation may function in lineage selection during differentiation of hMSCs by fostering osteogenic differentiation at the expense of other differentiation lineages.



## Introduction

Adult mesenchymal stem cells (MSC) are derived from bone marrow stroma or connective tissue and can differentiate into various lineages including fibroblasts, osteoblasts, adipocytes, and chondrocytes [11,15,57]. The multi-lineage differentiation potential of MSC populations has been extensively studied and culturing conditions for in vitro differentiation have been established. Although much progress has been made regarding the distinct differentiation processes in the last years, the signaling pathways involved in differentiation, are not yet completely understood.

The NF-κB signaling pathway is long known to play an important role in inflammation and control of the immune system [9,24,47]. In addition, it regulates the transcription of genes involved in cell growth and cell death [5,35]. NF-κB belongs to the Rel family of transcription factors and in mammals is encoded by five genes named *relA*, *relB*, *c-rel*, *nf-κb1*, and *nf-κb2*. All NF-κB proteins contain a conserved Rel homology domain, which is responsible for DNA binding, dimerization, and interaction with IκB proteins. In their inactive state, NF-κB proteins are located in the cytoplasm as homo- or heterodimers, bound to IκB family proteins, most importantly the IκBα protein. Upon stimulation by e.g. pro-inflammatory cytokines, such as TNF-α, the classical pathway is induced resulting in activation of the IκB-kinase-complex (IKK). This IKK-complex consists of two protein kinases (IKK1/α and IKK2/β) and a regulatory protein NEMO/IKKγ. IKK2 is largely responsible for the IκBα phosphorylation in the classical pathway. This phosphorylation triggers poly-ubiquitination and subsequent degradation of IκBα by the proteasome. NF-κB then translocates into the nucleus where it binds to specific sequences in the regulatory regions of target genes. Through a negative feedback loop newly synthesized IκBα binds to nuclear NF-κB and exports it back to the cytoplasm [9]. In addition to the classical pathway there is also an alternative pathway, which plays a central role in expression of genes involved in development and maintenance of secondary lymphoid organs. This alternative pathway is mainly stimulated via LTβR, BAFFR and CD40 leading to activation of NIK and subsequent activation of IKK1 [67].

A specific role of NF-κB in differentiation of MSC derivatives had been implicated in the past. It was shown that NF-κB induces degradation of the mRNA encoding the myogenic transcription factor MyoD in TNF-α treated myocytes [29,60]. In addition, NF-κB is responsible for TNF-α-induced muscle protein degradation in differentiated muscle cells

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[41,43,46]. Furthermore, NF-κB influences the chondrogenic differentiation as TNF-α-induced NF-κB was shown to down-regulate mRNA levels of the chondrogenic transcription factor Sox9, thereby inhibiting differentiation of chondrocytes [53,60]. However, there is also evidence that NF-κB can function as a positive regulator of mesenchymal cell differentiation as it was shown that NF-κB p65 expressed in growth plate chondrocytes facilitates growth plate chondrogenesis and longitudinal bone growth by inducing BMP-2 expression and activity [66].

Less is known about the role of NF-κB signaling in osteoblast differentiation. A negative regulation of osteoblast differentiation by NF-κB was suggested as inhibition of NF-κB signaling activity in osteosarcoma cells (Saos2) results in induction of several osteogenic markers, like BMP-4 and 7, Cbfa1, alkaline phosphatase, osteopontin and osteocalcin [4]. This negative role of NF-κB was also demonstrated by the $H_2O_2$-induced, NF-κB-dependent reduction of osteogenic differentiation markers, like alkaline phosphatase, collagen I, and Cbfa1 in rabbit bone marrow stromal cells and calvarial osteoblasts [6]. However, NF-κB was not analyzed directly in these cases, but rather the consequences of stimuli were studied, which amongst other pathways also induce NF-κB. Indeed some inhibiting effects of TNF-α on differentiation processes were documented to be independent of NF-κB signaling. Furthermore, previous studies demonstrated that TNF-α-induced inhibition of the terminal adipogenic differentiation of pre-adipocytes [68] and inhibition of osteogenic differentiation of pre-osteoblastic cells [25,49] is NF-κB-independent.

To elucidate the role of NF-κB in osteogenesis, we have analyzed its influence on osteogenic differentiation by infecting human mesenchymal stem cells with different NF-κB modulators. Our findings indicate that enhanced NF-κB activity in human mesenchymal stem cells increases osteogenic differentiation, whereas decreased NF-κB signaling does not impede the osteogenic differentiation.

## Materials and methods

### *Cell culture*

Human mesenchymal stem cells (hMSCs) were established from bone marrow samples with informed consent of the donors and following the guidelines of the ethics committee of the University of Ulm as described previously [22]. hMSCs were cultured in DMEM (GibcoBRL Life Technologies) supplemented with 10% heat inactivated fetal bovine serum (Biochrom AG) (FBS), 1% L-Glutamin (Biochrom AG) and 1% Pen/Strep (Biochrom AG) (growth medium) in a humidified atmosphere of 5% $CO_2$ in air. The technique of cell isolation and cultivation preserves the progenitor phenotype indicated by very low or absent expression of alkaline phosphatase and osteocalcin, and the potential to differentiate into various mesenchymal lineages. The cell populations were further characterized by positive staining with anti-CD9, CD54, CD90, CD166 and STRO-1 and the absence of CD34 and CD45 [20,21]. For osteogenic differentiation, the cells were plated in a density of $3\times10^3$ cells/cm$^2$ and cultured in growth medium, supplemented with 50 μg/ml ascorbic acid (Fluka) and 200 μg/ml β-glycerophosphate (Sigma) (osteogenic differentiation medium). hMSCs were differentiated for 21 days in absence or presence of 20 ng/ml tumor necrosis factor alpha (TNF-α) (kind gift of Dr. Adolf, Boehringer Ingelheim, Vienna). Differentiation medium, including TNF-α, was changed every third day.

The MTT assay was used to measure cell proliferation. hMSC were plated on 6-well plate at concentration $3\times10^3$ cells pro cm$^2$. TNF-α (20 ng/ml) was added and osteogenic differentiation was induced using 50 μg/ml ascorbic acid and 200 μg/ml of β-glycerophosphate. At the indicated time points the proliferation rate of the cells was assessed by MTT (Methylthiazolyldiphenyl-tetrazolium bromide) test. The culture medium was replaced with 5 mg/ml MTT solution in PBS and the plates were incubated for 5 h at 37 °C and 5% $CO_2$. The precipitate was extracted with DMSO and optical density was measured at wavelength 550 nm.

### *In vitro mineralization assay*

Mineralization of the extracellular matrix was examined by von Kossa staining. Cell layers were washed with warm phosphate-buffered saline (PBS), fixed for 15 min with neutral formalin (Lillie) and washed again with water. Silver nitrate (Fluka) was added to the dish and cells were incubated for 60 min. Dishes were rinsed with water and cells were incubated for 10 min in pyrogallol (Merck) to develop silver–calcium precipitations in the matrix. Cells were again rinsed with water, precipitates were fixed for 5 min with sodium thiosulfate (Merck) and following washing with water terminated the reaction. Lime precipitates were dark colored. Cells were then counterstained with nuclear fast red (Fluka) for 10 min and examined under a light microscope.

### *Calcium assay*

Mineralization of the extracellular matrix was quantified by measuring the acid-soluble calcium content in the cell/matrix layer using an o-cresolphtalein colorimetric assay (Calcium deposition assay for in vitro osteogenesis — Cambrex Bio Science). Cells were washed twice with phosphate-buffered saline (PBS) and decalcified for 24 h at 4 °C with 0.5 N HCl. The concentration of calcium in the HCl supernatant was determined colorimetrically following the instructions provided in the Stanbio Laboratory Calcium (CPC) Liquicolor kit (Stanbio Laboratory), which contains ortho-cresolphthalein complexone to form stable color reactions. Absorbance at 550 nm was determined after addition of the color reagent.

### *BMP-2 ELISA*

BMP-2 concentration in the supernatants of retrovirally-transduced cells was assessed using the Quantikine BMP-2 immunoassay (R and D Systens, Inc., Minneapolis, USA) according to the manufacturer's instructions.

### *Electrophoretic mobility shift assay*

For electrophoretic mobility shift assay (EMSA) preparation of nuclear extracts were performed from hMSCs. Cells were washed with PBS, suspended in an appropriate volume Dignam A buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF) [17] and incubated for 15 min on ice. Nonidet P-40 was added to a final concentration of 0.05%. Following incubation of 3–5 min at room temperature, cells were lysed by aspiration with a syringe for 10 times (26G needle). Nuclei were pelleted by microcentrifugation and washed twice with Dignam A. For preparation of nuclear extracts nuclei were incubated for 1 h on ice with Dignam C (20 mM HEPES pH 7.9, 25% glycerol,420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF) [17] followed by microcentrifugation (10 min) and collecting the supernatant.

For EMSA, the following oligonucleotides were annealed and subsequently labeled using [α-$^{32}$P]dCTP in a fill in reaction: NF-κB (5′-GCC TGG GAA AGT CCC CTC AA-3′); Oct (5′- ACC TGG GTA ATT TGC ATT TCT AAA AT-3′). 5 μg of nuclear extracts were used for EMSA as described earlier [50]. DNA–protein complexes were resolved on native (5%) polyacrylamide gels that were subsequently dried and autoradiographed.

### *RNA-isolation and RT-PCR*

Total RNA was isolated using the QIAzol lysis reagent (QIAGEN) according to the manufacturer's protocol. 1 μg of total RNA was reverse transcribed with AMV reverse transcriptase (Roche) and PCR was performed using the Taq DNA polymerase (Amersham Pharmacia Biotech).