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Evidence that TNF-β suppresses osteoblast differentiation of mesenchymal stem cells and resveratrol reverses it through modulation of NF-κB, Sirt1 and Runx2

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Abstract

It has been established that inflammation plays an important role in bone formation and bone loss. Although a lot is known about the role of TNF-α in bone health, very little is understood about TNF-β, also called lymphotoxin. In this report, we examine the effect of TNF-β on osteogenic differentiation of mesenchymal stem cells (MSCs) and its modulation by resveratrol. Monolayer and high-density cultures of MSCs were treated with osteogenic induction medium with/without TNF-β, Sirt1 inhibitor nicotinamide (NAM), antisense oligonucleotides against Sirt1 (ASO) and/or Sirt1 stimulator resveratrol. We found that TNF-β inhibits, in a similar way to NAM or Sirt1-ASO, the early stage of osteogenic differentiation of MSCs and this was accompanied with downregulation of bone-specific matrix, β1-integrin, Runx2 and with upregulation of NF-κB phosphorylation and NF-κB-regulated gene products involved in the inflammatory, degradative processes and apoptosis. However, resveratrol reversed TNF-β- and NAM-suppressed MSCs osteogenesis by activation of Sirt1 and Runx2 that led to osteoblast differentiation. Furthermore, downregulation of Sirt1 by mRNA inhibited the effect of resveratrol, highlighting the important impact of this enzyme in the TNF-β signaling pathway. Finally, resveratrol was able to manifest its effect both by suppression of TNF-β-induced NF-κB and through direct activation of the Sirt1 and Runx2 pathway. Thus, through these studies, we present a mechanism by which a T cell-derived cytokine, TNF-β can affect bone formation through modulation of MSCs differentiation that involves NF-κB, Sirt1, Runx2 and resveratrol reversed TNF-β-promoted impairments in MSCs osteogenesis.

Keywords Resveratrol · MSC · Osteogenesis · TNF-β (lymphotoxin-alpha) · NF-κB

Introduction

Mesenchymal stem cells (MSCs) are the basis for regeneration and repair of a large range of damaged tissue including bone and cartilage (De Bari and Roelofs 2018). However, in inflammatory joint pathologies, due to a persisting pro-inflammatory environment, multilineage differentiation of MSCs is impaired, resulting in inadequate cell and matrix synthesis, apoptosis and leading further to tissue extracellular matrix breakdown and cell dedifferentiation (Armiento et al. 2018; Kotake and Nanke 2014). Inflammatory joint disorders such as rheumatoide arthritis (RA) and osteoarthritis (OA) cause major physical and psychological impairment in patients associated with pain, subchondral bone loss, bone-cartilage remodeling and loss of bone integrity and stability (Harth and Nielson 2019; Lepage et al. 2019; Ramiro et al. 2011).

TNF-α has been identified as a key player to induce bone inflammation and osteoclastogenesis enhancing and stimulating diseases such as RA or OA (Corrado et al. 2017; Weitzmann

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2017; Zhao et al. 2012). Further, TNF-α markedly upregulates inflammation in bone disorders and suppresses the master osteogenic transcription factor Runx2 and thus blocks osteoblast differentiation and novel bone formation (Corrado et al. 2017; Gilbert et al. 2002). Underlining the importance of TNF-α-induced suppression of osteogenesis, an in vivo study on mice showed that pre-implantation treatment of umbilical cord-derived MSCs with anti-TNF-α resulted in better osteogenic differentiation and a lower arthritis score (Liu et al. 2018). Together with the pro-inflammatory cytokine TNF-α, the earliest member of the TNF superfamily to be identified in the early 1980s was TNF-α closest homolog TNF-β (lymphotoxin α) (Aggarwal 2003; Aggarwal et al. 2012; Aggarwal et al. 1985b; Aggarwal et al. 1984). Originally discovered in lymphocytes, TNF-β is produced by a large number of different cells (Aggarwal et al. 2012). Intriguingly, TNF-β has since its first discovery been described to induce inflammation with the same potency as TNF-α and can also stimulate an inflammatory environment in RA and OA (Buhrmann et al. 2017; Buhrmann et al. 2013; Calmon-Hamaty et al. 2011a, b). Similar to TNF-α, high TNF-β levels are linked with inflammatory joint disorders and clinical investigations have demonstrated that high levels of TNF-β are found in the synovium of RA patients (O'Rourke et al. 2008); its role in bone pathophysiology and the underlying mechanisms are poorly understood (Hirose et al. 2018).

Activation of nuclear factor-kappaB (NF-κB) plays a fundamental role in upregulating inflammatory cytokines and mediating inflammation also in RA and OA (Aggarwal 2003; Aggarwal et al. 2012; Jue et al. 1999). Interestingly, in previous in vitro studies, our group explored the role of TNF-β during chondrogenesis of MSCs and showed that TNF-β upregulates NF-κB and activates a pro-inflammatory signaling cascade inducing and self-maintaining an inflammatory microenvironment (Buhrmann et al. 2017; Buhrmann et al. 2013).

In vivo MSCs commitment to the osteoblast lineage involves at a very early stage activation of the master osteoblastic transcription factor Runx2 (alias Cbfa1) (Komori 2008) stimulating osteocalcin, osteopontin, collagen type I and alkaline phosphatase (ALP) expression (Komori 2010; Shui et al. 2003). Indeed several studies have shown that knockout of Runx2 suppresses skeleton mineralization and facilitates adipogenic lineage commitment of MSCs (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997). Further, during the early stage of osteoblastic lineage differentiation, MSCs produce pericellular matrix, which has an osteoinductive effect and stimulates further osteoblast differentiation (Ferreira et al. 2018). This cell-pericellular matrix interplay is mediated by interaction of the pericellular matrix components in the microenvironment with cell membrane receptors. Here, the most important membrane receptors are the integrin receptors, which may act as adhesion and bi-directional signaling molecules (Ferreira et al. 2018; Siebers et al. 2005).

In RA and OA, the natural polyphenol resveratrol shows anti-inflammatory properties and may enhance osteogenesis from MSCs under osteogenic culture conditions (Dosier et al. 2012; Safaeinejad et al. 2018; Shakibaei et al. 2008; Shakibaei et al. 2012). Interestingly, pre-treatment of MSCs with resveratrol induced more mineralization at earlier time points and overall produced more osteogenic cells (Erdman et al. 2012), highlighting the role of resveratrol in influencing the very early stage of osteogenic differentiation of MSCs. A major intracellular target molecule of resveratrol is Sirtuin-1 (Sirt1), a NAD+-dependent deacetylase that is involved in regulating a wide range of diseases including metabolic disorders, cancer and cardiovascular disorders (Mendes et al. 2017). Recent studies have shown that activation of Sirt1 positively regulates Runx2, promotes Runx2 downstream targets (Zainabadi et al. 2017a; Zainabadi et al. 2017b) and blocks adipogenic differentiation of MSCs during osteogenic treatment (Backesjo et al. 2006; Shakibaei et al. 2012). Additionally, Sirt1 down-modulates NF-κB thus helping to maintain bone homeostasis (Edwards et al. 2013) and controls NF-κB-induced upregulation of inflammatory pathways through antagonistic crosstalk by deacetylation of the p-65 subunit (Kauppinen et al. 2013). Resveratrol-induced Sirt1 activation has been shown to stimulate osteogenesis of MSCs by upregulating Runx2 (Shakibaei et al. 2012; Tseng et al. 2011), suppress osteoclastogenesis (Matsuda et al. 2018) and decrease adipocyte formation during osteoblastogenesis (Backesjo et al. 2006).

Given that TNF-β may act as a potent and unknown pro-inflammatory cytokine interfering with tissue engineering strategies and joint regeneration in RA and OA, this investigation was performed to examine the possible mechanism of TNF-β on osteogenic differentiation of MSCs and its modulation by resveratrol in vitro.

Material and methods

Antibodies, cytokines and reagents

Antibodies to CD90, CD105, CD34 and CD45 were derived from Acris Antibodies GmbH (Hiddenhausen, Germany). Polyclonal anti-collagen type I, polyclonal anti-osteocalcin and secondary antibodies for immunoblotting were purchased from Millipore (Schwalbach, Germany). Anti-phospho-specific p65 (NF-κB), anti-MMP-9 and anti-caspase-3 were obtained from R&D Systems (Heidelberg, Germany). Polyclonal anti-Runx2 was purchased from Alpha Diagnostics Int. (San Antonio, TX, USA). Anti-β-actin, anti-β1-integrin, resveratrol, nicotinamide and *Staphylococcus aureus* bacteria were purchased from Sigma-Aldrich Chemie (Munich, Germany). Cyclo-oxygenase-2 antibody was obtained from Cayman Chemical (Ann Arbor, MI, USA). Anti-Sirt1 was purchased from Abcam PLC

(Cambridge, UK). TNF-α, TNF-β and anti-TNF-β-receptor were purchased from eBiosciences (Frankfurt, Germany). Additionally, secondary rhodamine-coupled antibodies for immunofluorescence from Dianova (Hamburg, Germany) were applied.

Cell culture

Canine mesenchymal stem cells (MSCs) were isolated from adipose tissue and canine primary osteoblasts (POS) were isolated from femoral heads; both samples were obtained during total canine hip replacement surgeries as reported in detail (Buhrmann et al. 2010; Csaki et al. 2009; Csaki et al. 2007). The age of canine donors ranged from 5 to 9 years. It has been previously demonstrated that adipose tissue contains a population of multipotent mesenchymal stem cells, which can be differentiated into multiple lineage-specific cells (Gimble and Guilak 2003; Zuk et al. 2002; Zuk et al. 2001). Cells were cultured in normal cell growth medium containing 10% FCS and passaged when they reached around 80% confluency as described in detail (Buhrmann et al. 2014). All experiments were performed with POS or MSCs up to passage six.

Experimental design

Experiments were performed as monolayer or as three-dimensional high-density cultures as reported (Shakibaei et al. 2012). To investigate the effect of TNF-β and/or resveratrol on osteogenic differentiation, MSCs were cultured as monolayer or high-density cultures in normal cell growth medium (NM) or in osteogenic induction medium (OIM) and left untreated, or treated with resveratrol (5 μM), TNF-β, TNF-α (10 ng/ml), NAM (10 mM) and then transfected with Sirt1-SO (0.5 μM) or Sirt1-ASO (0.5 μM) in the presence of Lipofectin (10 μl/ml). Other MSCs cultures were pre-treated with resveratrol (5 μM) for 1 h followed by co-treatment with TNF-β or TNF-α (10 ng/ml), NAM (10 mM) and then were transfected with Sirt1-SO, or Sirt1-ASO (0.5 μM) in the presence of Lipofectin (10 μl/ml) for 21 days. In a second approach, MSCs were transferred to a 3D high-density environment and either left untreated, treated with 5 μM resveratrol, TNF-β (5, 10 ng/ml), TNF-α (5, 10 ng/ml), or a combination of 5 μM resveratrol with TNF-β (10 ng/ml) or TNF-α (10 ng/ml) in the presence of OIM for 21 days to evaluate the effects of TNF-β and/or resveratrol on osteogenic MSCs differentiation potential. Medium was changed every 3 days. Primary osteoblast cultures and the negative control of MSCs were cultured in NM containing 10% FCS. OIM was prepared as described before (Csaki et al. 2007; Pittenger et al. 1999). To further elucidate the potential mechanistic role of TNF-β/TNF-βR in MSCs, MSCs in monolayer cultures were left untreated, treated with various concentrations of TNF-β (1, 5, 10, 20 ng/ml), or co-treated with TNF-β (5 ng/ml) and various concentrations of resveratrol (1, 5, 10 μM) for 12 h.

Immunofluorescence microscopy

Immunofluorescence analysis was used for characterization of mesenchymal stem cells by applying a set of specific markers on untreated MSCs in monolayer culture as previously described (Buhrmann et al. 2014). For the negative control, MSCs were incubated with PBS alone instead of the primary antibodies, followed by incubation with secondary antibodies and DAPI. Additionally, MSCs treated as depicted above were investigated for expression of TNF-βR. Counterstaining was performed with 4′,6-diamidin-2-phenylindol (DAPI) (Sigma, Munich, Germany) to visualize cell nuclei.

Transient transfection with antisense oligonucleotides

Transient transfection with antisense oligonucleotides of Sirt1 mRNA nucleotide sequence (Sirt1-ASO) or control sense oligonucleotides (Sirt1-SO) was performed as described (Buhrmann et al. 2014). Briefly, cells in monolayer or high-density culture were transfected with 0.5 μM of Sirt1-ASO or Sirt1-SO in addition with 10 μl/ml Lipofectin transfection reagent (Life Technologies, Invitrogen, Darmstadt, Germany) and OIM for 21 days. Sirt1-ASO (5′-GTAT TCCACATGAAACAGACA-3′) and Sirt1-SO (5′-TGTC TGTTTCATGTGGA ATAC-3′) were purchased from Eurofins (MWG/Operon, Ebersberg, Germany) and were phosphorothioate modified to protect them from cell nucleases.

Von Kossa staining of mineralized matrix

Deposition of mineralized matrix was evaluated with von Kossa staining as reported (Csaki et al. 2007). Briefly, monolayer cultures treated as described above were fixated with methanol for 15 min, washed in aqua dest three times for 5 min and incubated for 2 h under UV light with sliver-nitrate solution (Merck-Sigma-Aldrich, Munich, Germany), followed by sodium thiosulfate solution. After additional washing, cultures were counterstained with nuclear fast red solution.

Ultrastructural investigations

Ultrastructural electron microscopy on high-density cultures was undertaken to study the effect of resveratrol and TNF-β on osteogenesis of MSCs as previously described (Buhrmann et al. 2014; Shakibaei et al. 2012). Briefly, high-density cultures were fixed for 2 h in Karnovsky solution, dehydrated in an ascending alcohol series, postfixated with OsO_4 and after

embedding in Epon (Plano, Marburg, Germany), ultrathin cuts were investigated with transmission electron microscope Jeol 1200 EXII (Akishima Tokyo, Japan).

Western blotting

Monolayer and high-density cultures were treated as described above and whole cell lysates were prepared for immunoblotting as previously reported (Buhrmann et al. 2014). After final separation of proteins under reducing conditions with SDS-PAGE electrophoresis and blotting onto nitrocellulose membranes (Transblotting apparatus, Bio-Rad, Munich, Germany), membranes were incubated with primary antibodies against Sirt1, collagen I, Runx2 target gene (osteocalcin), β1-integrin, Runx2, p-NF-κB, MMP-9, Cox2, Caspase-3 and TNF-βR and incubated with alkaline phosphatase conjugated secondary antibodies. β-actin was used as a loading control and to normalize samples. Western blots were semi-quantified with densitometry (Bio-rad Laboratories, USA).

Immunoprecipitation

Immunoprecipitation assay was performed as reported previously (Buhrmann et al. 2014). Briefly, whole cell lysates of MSCs high-density cultures, treated as described above, were precleared with normal rabbit IgG and protein A/G-Sepharose beads and incubated with anti-Sirt1 or anti-TNF-βR diluted in wash buffer (0.1% Tween 20, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM PMSF) for 2 h at 4 °C. After washing and additional incubation with *Staphylococcus aureus* bacteria, the obtained samples containing the immune complexes were subjected to SDS-PAGE and samples were blotted onto a nitrocellulose membrane. Finally, immunoblot analysis was performed with antibodies to β1-integrin, p-NF-κB, Runx2, and Sirt1.

Statistics

The experiments were performed as individual experiments and each repeated at least three times. A Wilcoxon-Mann-Whitney test was used to investigate statistical significance. The data are presented as mean values of ±SD or SEM and were compared by one-way, two-way, or three-way ANOVA using SPSS Statistics, if the normality test was passed (Kolmogorov-Smirnov test). A p value < 0.05 was considered to establish a statistically significant difference.

Results

In this paper, we investigated the impact of the TNF-β/TNF-βR-induced inhibition on osteogenic differentiation of MSCs and its modulation by resveratrol/Sirt1 signaling in an in vitro monolayer and 3D model of osteogenesis.

Characterization of adipose tissue-derived MSCs

MSCs featured in two-dimensional cultures a polymorphic fibroblast-like phenotype (Fig. 1a). To examine that the isolated cells are MSCs, immunofluorescence was performed to demonstrate the surface stem cell-specific marker profile ($CD90^+$ and $CD105^+$) and the hematopoietic specific marker profile ($CD34^-$ and $CD45^-$). As shown in (Fig. 1c, d), the MSCs showed a strong positive expression of the specific stem cell markers ($CD90^+$ and $CD105^+$) (Fig. 1c, d) and negative expression of the hematopoietic specific surface markers ($CD34^-$ and $CD45^-$) compared with controls (Fig. 1e, f). These results are confirmed in Fig. 1(g). The stem cell markers CD105 and CD90 are well expressed and no expression is seen with the hematopoietic cell markers (CD 34 and CD45). Functional analysis was also performed to confirm the multilineage differentiation capacity of these MSCs, to chondrogenic, adipogenic and osteogenic differentiation in vitro, as shown previously (Buhrmann et al. 2010; Csaki et al. 2007).

Resveratrol suppresses inhibition of osteogenic differentiation of MSCs promoted by TNF-β and NAM but not by knockdown of Sirt1 with ASO in monolayer and in 3D high-density cultures

When MSCs were incubated in monolayer cultures for 21 days in normal growth medium, they revealed a fibroblast-like morphology and no calcium deposition was observed after von Kossa staining (Fig. 2a). However, incubation of MSCs under the same condition but with osteogenic induction medium (OIM) over 21 days resulted in osteogenesis and von Kossa staining was positive with high amounts of calcium deposition (Fig. 2b). Resveratrol pretreatment in MSC cultures with the OIM induced osteogenesis and produced more mineralization (Fig. 2c). More interestingly, MSCs grown in OIM and treated with TNF-β, similar to TNF-α, revealed a more fibroblast-like morphology, compared with control cells incubated in normal medium and no calcium deposition was observed in von Kossa staining (Fig. 2d, e). In addition, pre-treatment of MSCs with resveratrol and co-treatment with TNF-β or TNF-α promoted osteogenic differentiation (Fig. 2f, g).

To test the pharmacological modulation effects of Sirt1 to control TNF-β-induced inflammation in osteogenic differentiation of MSCs, we examined the effects of targeting Sirt1 with ASO/SO (Sirt1-ASO, Sirt1-SO) or nicotinamide (NAM) with or without resveratrol. In MSCs that were transfected with SO against Sirt1 in the presence of Lipofectin, osteogenesis was observed and von Kossa staining was positive with

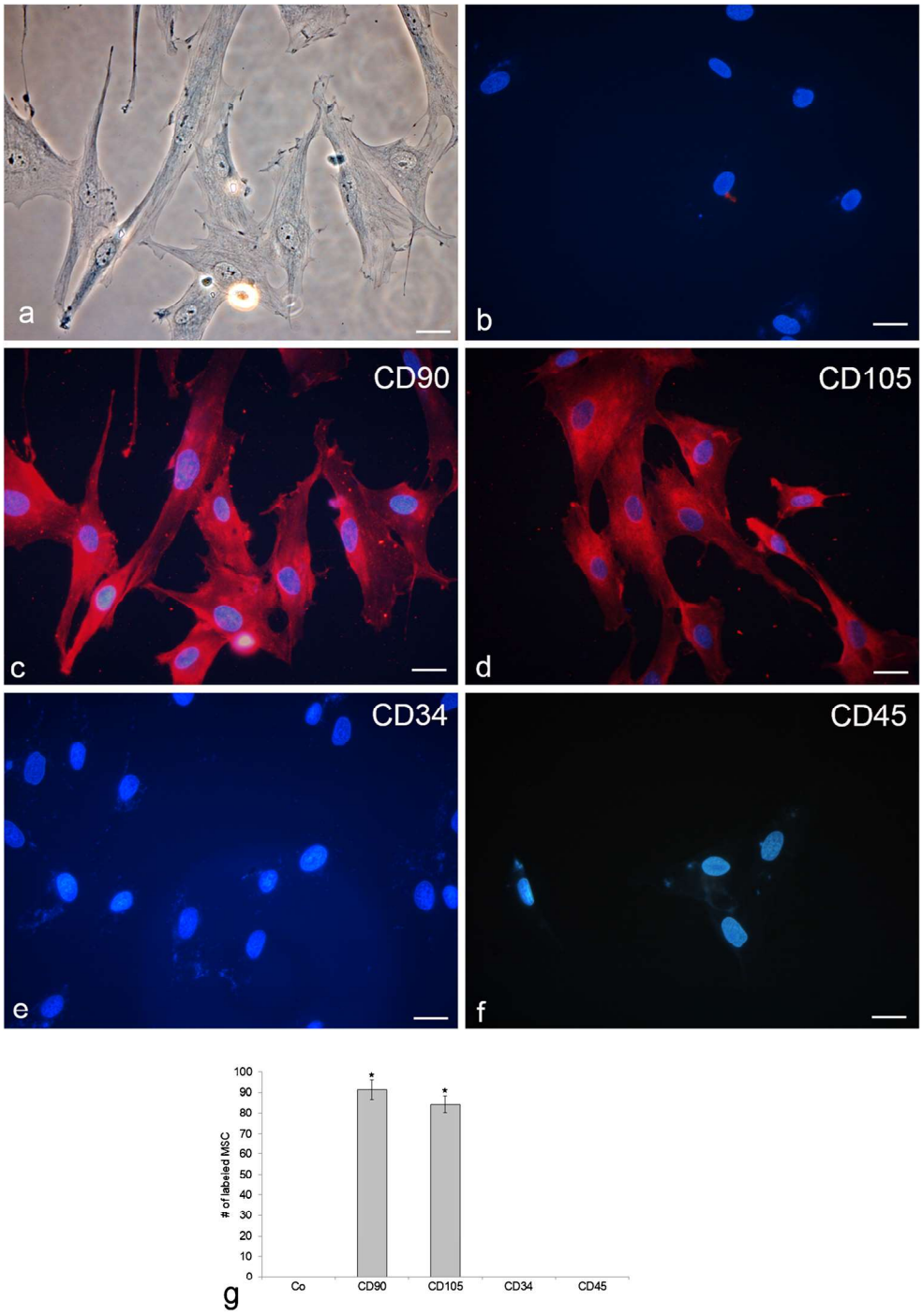

Fig. 1 **a–g** Characterization of MSCs. MSCs from adipose tissue exhibit in monolayer culture (**a**) a polymorphic, fibroblast-like phenotype. The MSCs either were left as controls (without primary antibody) (**b**), or are positive labeled for the stem cell-specific markers CD90 (**c**) and CD105 (**d**) and are negative for the hematopoietic stem cell markers CD34 (**e**) and CD45 (**f**). Magnification: ×400, Scale bar = 30 nm. To determine the stem cell marker-labeled cells, the stained monolayer cultures were evaluated by counting 100 cells from 5 microscopic fields (**g**). The evaluation was performed in triplicate. Values were compared with the control and statistically significant values with $p < 0.05$ are designated by a star. **b** Negative control (without primary antibodies), incubation with rhodamine-coupled secondary antibodies and counterstaining with DAPI to visualize the cell nuclei

calcium deposition (Fig. 2h). In contrast, in the presence of the sirtuin inhibitor nicotinamide or transfected with ASO against Sirt1 in the presence of Lipofectin, osteogenesis was not observed but the cells exhibited a fibroblast-like morphology (Fig. 2i, j). However, MSC cultures that were pre-treated with resveratrol and then co-treated with TNF-β and NAM, or Sirt1-SO, or Sirt1-ASO promoted osteogenic differentiation in all cells, formed nodules and were stained positive with von Kossa stain for mineral deposition, except in MSCs treated with Sirt1-ASO (Fig. 2k–m). Statistical examination, as revealed in Fig. 2(n), confirmed the results in Fig. 2(a–m).

To validate further, whether TNF-β, the specific knockdown of Sirt1 mRNA and protein expression by ASO, suppresses the differentiation of MSCs to osteoblasts and bone tissue formation, we analyzed ultrastructural cell morphology by transmission electron microscopy in 3D high-density cultures (Fig. 3). As positive control, primary osteoblasts (POS) were incubated in high-density culture and prepared for

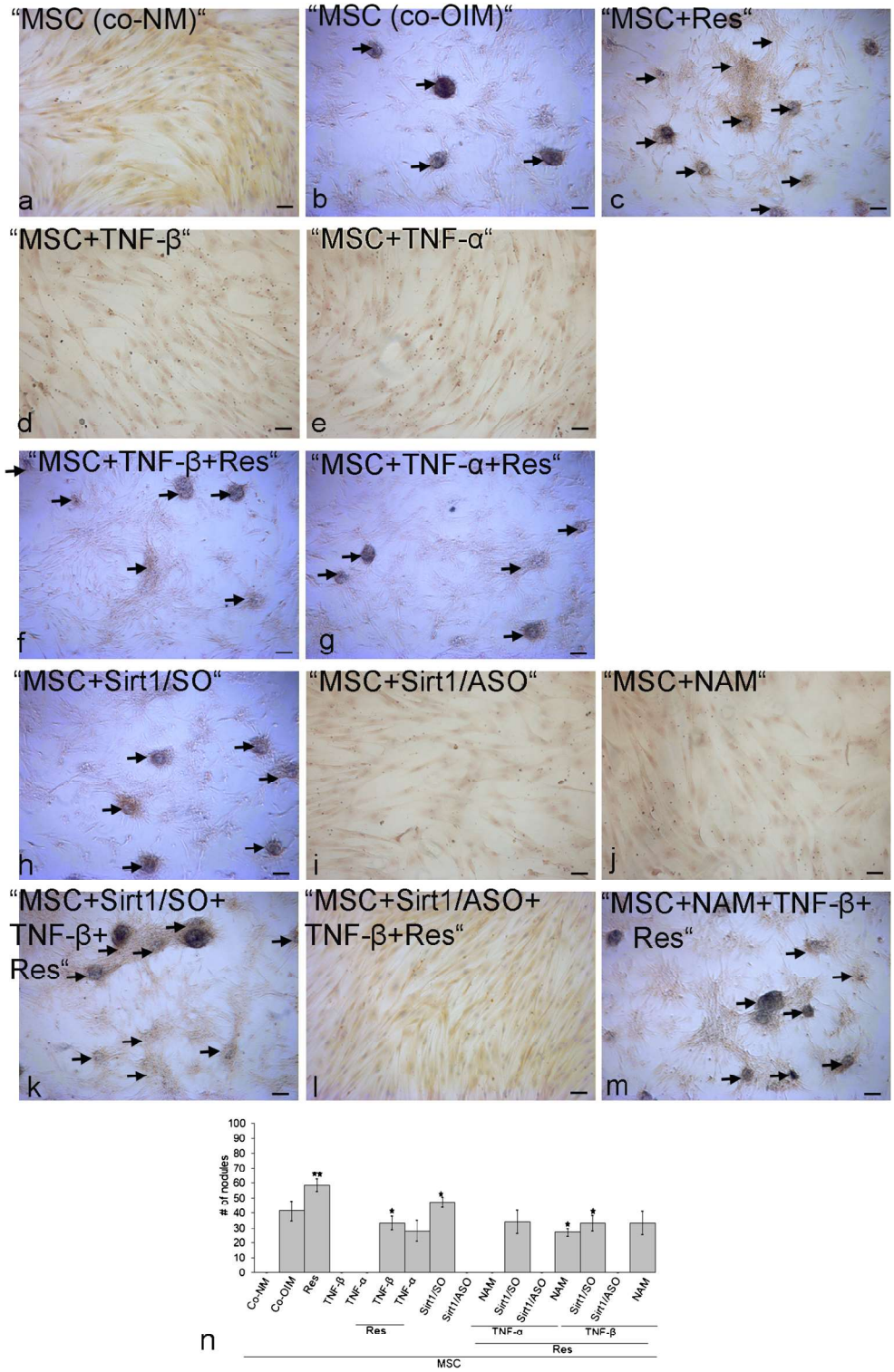

Fig. 2 Effects of resveratrol, TNF-β, TNF-α, ASO against Sirt1 and NAM on osteoblastic differentiation of MSCs in monolayer culture revealed by light microscopy. **a–m** MSCs, monolayer culture for 21 days. Light microscopy of von Kossa staining. MSCs showed capabilities of osteogenic differentiation. MSC cultures were grown in normal medium (co-NM) (**a**). MSCs cultures were grown in osteogenic induction medium (co-OIM) (**b**). MSC cultures were grown in OIM and treated with resveratrol (**c**), TNF-β (**d**), TNF-α (**e**), pre-treated with resveratrol and co-treated with TNF-β (**f**), pre-treated with resveratrol and co-treated with TNF-α (**g**), treated with Sirt1-SO (**h**), treated with Sirt1-ASO (**i**), treated with NAM (**j**), pre-treated with resveratrol and co-treated with TNF-β and Sirt1-SO (**k**), pre-treated with resveratrol and co-treated with TNF-β and Sirt1-ASO (**l**), pre-treated with resveratrol and co-treated with TNF-β and NAM (**m**). Arrows: nodule formation. Magnification: ×200, Scale bar = 30 μm. **n** Formation of nodules and labeled positive with von Kossa stain for mineral deposition in the cultures was evaluated by counting 100 cells from 25 different microscopic fields. Each investigation was performed in triplicate and significant values are shown with (*)

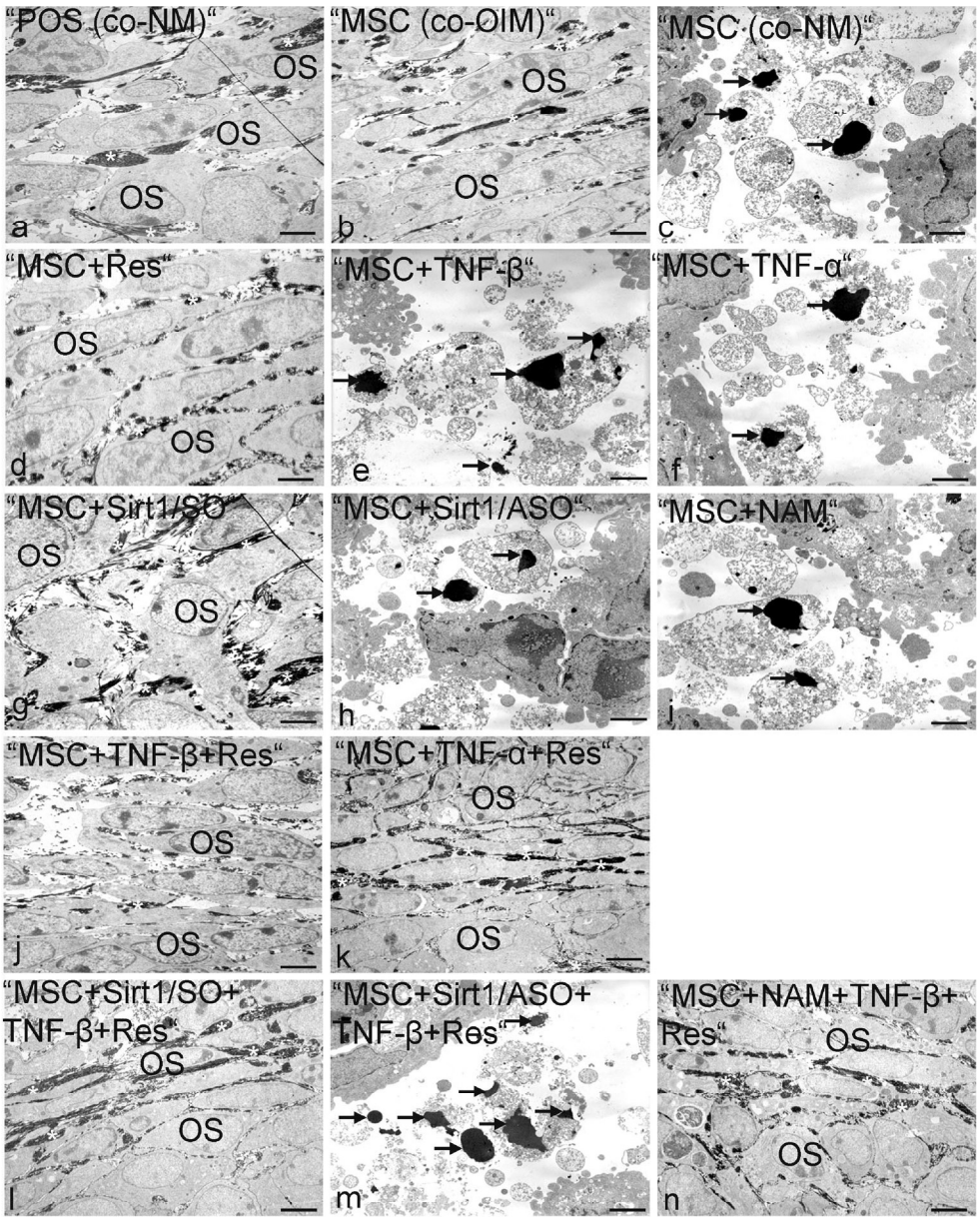

Fig. 3 Effects of resveratrol, TNF-β, TNF-α, ASO against Sirt1 and NAM on osteoblastic differentiation of MSCs in 3D high-density culture as shown by transmission electron microscopy. MSCs, high-density culture for 21 days. Transmission electron microscopy. Primary osteoblast (POS) cultures were grown in normal medium (co-NM) (**a**). MSC cultures were grown in OIM (co-OIM) (**b**). MSC cultures were grown in NM (co-NM) (**c**). MSC cultures were grown in OIM and treated with resveratrol (**d**), TNF-β (**e**), TNF-α (**f**), Sirt1-SO (**g**), Sirt1-ASO (**h**), NAM (**i**), pre-treated with resveratrol and co-treated with TNF-β (**j**), pre-treated with resveratrol and co-treated with TNF-α (**k**), pre-treated with resveratrol, co-treated with TNF-β and Sirt1-SO (**l**), pre-treated with resveratrol, co-treated with TNF-β and Sirt1-ASO (**m**), pre-treated with resveratrol, co-treated with TNF-β and NAM (**n**). Os, osteoblast; star, extracellular matrix; arrows, apoptotic cells; magnification, ×6000; scale bar = 1 μm

transmission electron microscopy to visualize bone formation. After 21 days in high-density cultures, POS or MSCs with or without resveratrol treatment in osteogenic induction medium revealed well-organized nodules and formed bone tissue with viable osteoblasts and well-developed extracellular proteins closely attached to the cell membrane and organized cell organelles (high amount of nuclear euchromatin and morphologically normal mitochondria, rough ER, Golgi apparatus) (Fig. 3a, b, d). However, no considerable differences in osteogenic differentiation could be observed at the ultrastructural level between resveratrol treated, Sirt1-SO treated and MSC cultures (Fig. 3b, d, g). In contrast, cultivation of MSCs in NM, or treatment of MSCs with TNF-β, or TNF-α, or NAM or with Sirt1-ASO, resulted clearly in suppression of osteogenesis and several MSCs underwent apoptosis, with degeneration of the cells, membrane blebbing, nuclear damage and formation of apoptotic bodies (Fig. 3c, e, f, h, i). Furthermore, pre-treatment of MSCs with resveratrol in the OIM and co-treated with TNF-β, or TNF-α, or NAM or Sirt1-ASO, or Sirt1-SO, promoted MSCs osteogenic differentiation in all treatment groups (Fig. 3j, k, l, n), except in the group treated with Sirt1-ASO (Fig. 3m). Taken together, these results confirmed the results from Fig. 2 and indicate that (1) Sirt1 is a downstream target protein for the TNF-β-induced signaling pathway, (2) suppression of Sirt1 on the protein level by TNF-β, NAM and on the mRNA level by Sirt1-ASO induces an identical inhibition of osteogenic differentiation of MSC, (3) knockdown of Sirt1 by TNF-β, similar to TNF-α, NAM and Sirt1-ASO enhances apoptosis of MSCs and (4) Sirt1 suppression on mRNA levels is not reversible by

resveratrol and shows the essential significant role of Sirt1 during osteogenic differentiation of MSCs in the TNF-β-promoted inflammatory environment.

Resveratrol down-modulates suppression of TNF-β-promoted Sirt1, matrix proteins, β1-integrin and Runx2 expression during osteogenic differentiation of MSCs in high-density cultures

MSCs in high-density cultures were either incubated only with normal growth medium, or with osteogenic induction medium with or without resveratrol or with osteogenic induction medium and TNF-β or TNF-α with different concentrations (5 and 10 ng/ml) or a combination of resveratrol with TNF-α or TNF-β (10 ng/ml) for 21 days. Whole cell lysates were fractionated and analyzed by immunoblotting using anti-Sirt1, anti-collagen type I, anti-bone-specific proteoglycans (osteocalcin), anti-β1-integrin and anti-osteogenic-specific transcription factor Runx2 (Fig. 4a–e). As revealed in immunoblotting analysis and densitometric quantification, treatment with normal growth medium and TNF-β, similar to TNF-α, clearly inhibited the expression of Sirt1, collagen type I, osteocalcin proteins, β1-integrin and Runx2 (Fig. 4a–e). In contrast, resveratrol alone or co-treatment with TNF-β, similar to TNF-α, induced in MSCs an increasing amount of Sirt1, osteogenic-specific ECM, β1-integrin and Runx2 compared with control cultures (Fig. 4a–e). Taken together, these data suggest that (1) Sirt1 protein is an important subcellular molecule for the TNF-β pathway and downregulation of Sirt1 by TNF-β, similar to NAM and Sirt1-ASO induces inhibition of osteogenic differentiation of MSCs, (2) apparently, there is a signaling link between resveratrol/Sirt1 protein, TNF-β, bone-specific transcription factor Runx2 and β1-integrin, which has the capacity to promote osteogenic differentiation of MSCs in high-density cultures, at least partially, by upregulation of Runx2.

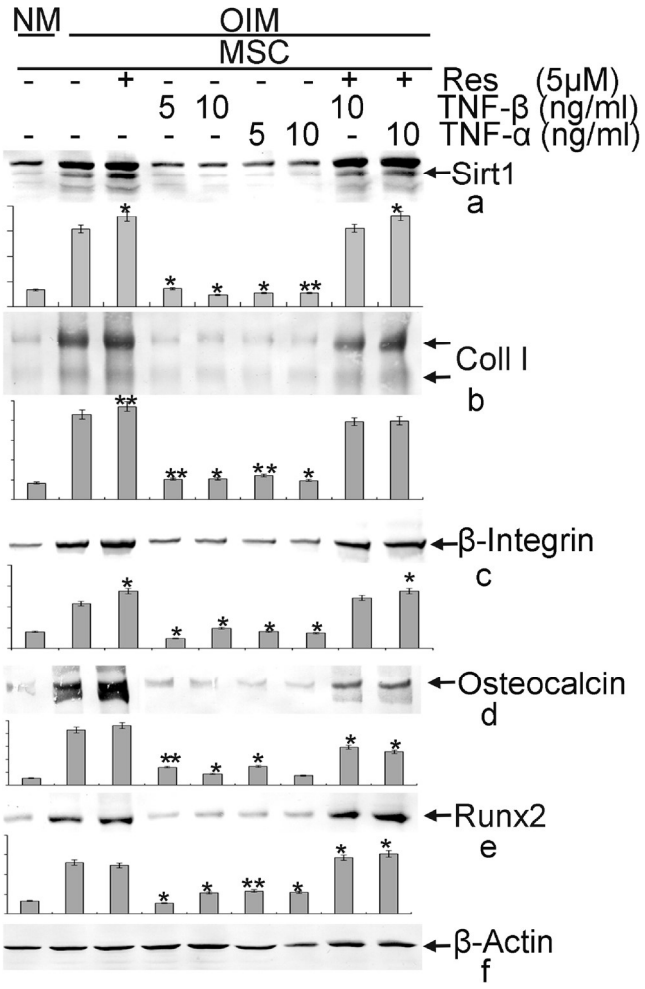

Fig. 4 Effects of resveratrol and/or TNF-β, TNF-α on Sirt1, matrix proteins, β1-integrin and Runx2 during osteogenesis of MSCs in high-density culture. MSCs were cultured in high density with normal medium (NM) or with osteogenic induction medium (OIM) and either left untreated or treated with resveratrol, TNF-β, or TNF-α or co-treatment with resveratrol and TNF-β or TNF-α for 21 days. Whole cell lysates were probed by immunoblotting with antibodies against Sirt1 (**a**), collagen type I (Coll I) (**b**), β1-integrin (**c**), bone-specific proteoglycans (osteocalcin) (**d**) and osteoblast-specific transcription factor Runx2 (**e**). The results shown are representative of three independent experiments. Housekeeping protein β-actin (**f**) served as a loading control in all experiments. Densitometric evaluation was performed for Sirt1 (**a**), collagen I (**b**), β1-Integrin (**c**), Osteocalcin (**d**), Runx2 (**e**). $*p < 0.05$, $**p < 0.01$ compared with control

Resveratrol promotes TNF-β, NAM down-modulation of matrix proteins, β1-integrin, Runx2 expression but not by Sirt1-ASO during osteogenesis of MSCs in high-density culture

MSCs in high-density culture were either left untreated or treated with resveratrol (5 μM), or TNF-β (10 ng/ml), NAM (10 mM), or were transfected with Sirt1-SO or Sirt1-ASO (0.5 μM in the presence of Lipofectin 10 μl/ml), or MSCs were pre-treated with resveratrol (5 μM) for 1 h followed by co-treatment with either TNF-β (10 ng/ml), NAM (10 mM), or transfected with 0.5 μM Sirt1-SO or Sirt1-ASO in osteogenic induction medium for 21 days. Whole cell lysates were fractionated and probed by immunoblotting with antibodies against collagen type I, osteocalcin, β1-integrin and Runx2. As shown in Fig. 5, incubation of MSCs in high-density cultures and treated with TNF-β, Sirt1 inhibitor (NAM), or Sirt1-ASO by itself markedly downregulated collagen type I (a), osteocalcin synthesis (b), β1-integrin (c) and Runx2 (d) expression. Furthermore, expression of the abovementioned proteins was significantly gained in MSC cultures treated with resveratrol alone or co-treated in all combinations of the mentioned agents comparable with control cultures but not in Sirt1-ASO-incubated MSCs (Fig. 5a–d). The control basal amount of the abovementioned protein expression was not significantly changed after treatment with Sirt1-SO (Fig. 5a–d). Taken together, these results indicate that resveratrol/Sirt1

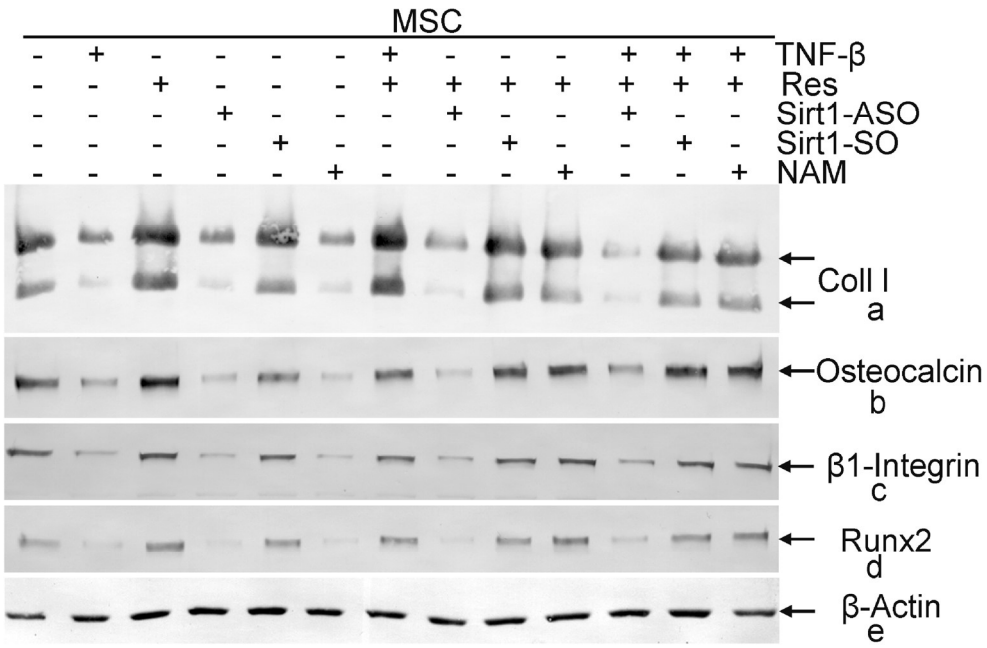

Fig. 5 Effects of resveratrol, TNF-β, ASO against Sirt1 and NAM on matrix proteins, β1-integrin and Runx2 during osteogenesis of MSCs in high-density culture. MSCs were cultured in high density with osteogenic induction medium and either left untreated or treated as described in detail in the "Material and methods" section. Whole cell lysates were fractionated and probed by immunoblotting with antibodies against collagen type I (**a**), osteocalcin (**b**), β1-integrin (**c**) and Runx2 (**d**). The results shown are representative of three independent experiments. Housekeeping protein β-actin (**e**) served as a loading control in all experiments

signaling suppressed TNF-β-promoted downregulation of bone matrix production, β1-integrin and Runx2 during osteogenesis of MSCs, suggesting a preventive chance of the resveratrol/Sirt1 pathway to provide a favorable protective microenvironment to enable the osteogenesis of MSCs in the TNF-β-induced inflammatory environment.

Resveratrol down-modulates inhibition of Sirt1 expression and upregulation of NF-κB phosphorylation, NF-κB-mediated pro-inflammatory, matrix-degrading gene products promoted by TNF-β or NAM but not by Sirt1-ASO

To further examine the regulatory mechanism of resveratrol-modulated TNF-β-promoted inflammation and suppression of osteogenic differentiation of MSCs, we next analyzed whether this modulation was linked additionally with the inhibition of NF-κB stimulation. Several lines of evidence have demonstrated that pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 promote specific NF-κB expression (Aggarwal 2003; Aggarwal et al. 2012; O'Rourke et al. 2008). MSCs in high-density culture were either left untreated or treated as described in the "Material and methods" section. As shown in Fig. 6, the expression of Sirt1 protein was substantially suppressed by treatment with TNF-β, NAM, or Sirt1-ASO, whereas in co-treated cultures, resveratrol enhanced Sirt1 protein expression in all combinations of the mentioned agents but not with Sirt1-ASO (a). Figure 6 showed further that TNF-β, NAM, or Sirt1-ASO stimulated NF-κB phosphorylation and NF-κB-mediated matrix-degrading gene products (Cox2 and MMP-9, cleavage of caspase-3) (b, c, d, f). However, treatment with resveratrol downregulated the expression of the mentioned proteins in all co-treatments, except with Sirt1-ASO (Fig. 6b–f), underlining the important role of Sirt1 for MSC osteoinduction and inhibition of Sirt1 on mRNA levels is not reversible by resveratrol in TNF-β-induced inflammatory environment. A similar basal level of the abovementioned proteins in control was also observed in Sirt1-SO-treated cultures (Fig. 6a–d, f). Taken together, these data suggest that (1) NF-κB is one of the major downstream signaling transcription factors for the TNF-β pathway and (2) resveratrol/Sirt1 signaling has a modulatory and protective effect against TNF-β-induced cellular degeneration and apoptosis during osteogenesis of MSCs by NF-κB inhibition.

TNF-β stimulates TNF-βR expression in MSCs and resveratrol reverses it

To examine whether MSCs express TNF-βR and whether TNF-β induces this expression, MSCs in monolayer cultures were either left untreated (Fig. 7a) or treated with resveratrol (5 μM) (Fig. 7b), or with 5 ng/ml TNF-β (Fig. 7c) for 4 h, or pre-treated with resveratrol (5 μM) for 1 h and then co-treated with TNF-β for 4 h (Fig. 7d) and subjected to immunolabeling with anti-TNF-βR, rhodamine-coupled secondary antibodies. DAPI counterstaining was performed to visualize cell nuclei. As shown in Fig. 7(a–d) untreated or resveratrol-treated MSCs had low basal expression of TNF-βR; however, inflammatory stimulation with TNF-β markedly promoted TNF-βR expression in MSCs. We found that co-treatment with resveratrol decreased clearly the expression of TNF-βR in MSCs. In an additional approach, MSCs were left untreated, or treated with various

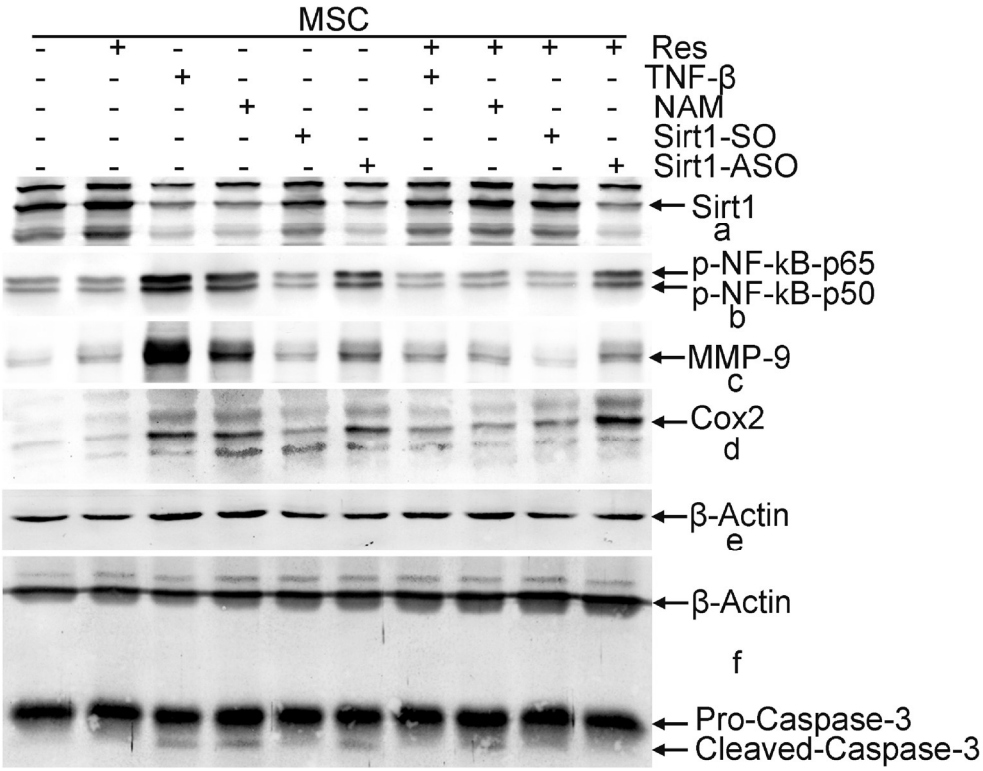

Fig. 6 Effects of resveratrol, TNF-β, ASO against Sirt1 and NAM on Sirt1, NF-κB phosphorylation and NF-κB signaling pathway during osteogenesis of MSCs in high-density culture. MSCs were cultured in high density with osteogenic induction medium and either left untreated or treated as described in detail in the "Material and methods" section. Whole cell lysates were fractionated and probed by immunoblotting with antibodies against Sirt1 (**a**), p-NF-κB (**b**), MMP-9 (**c**), Cox2 (**d**) and cleaved caspase-3 (**f**). The results shown are representative of three independent experiments. Housekeeping protein β-actin (**e**, **f**) served as a loading control in all experiments

concentrations of TNF-β for 12 h or treated with different concentrations of resveratrol (1, 5, 10 μM) and co-treated with 5 ng/ml TNF-β for 12 h (Fig. 7e, f). Western blotting of whole cell extracts showed a dose-dependent increase in TNF-βR expression in MSCs during TNF-β stimulation (Fig. 7e). Moreover, we found that co-treatment with resveratrol decreased clearly the expression of TNF-βR in MSCs dose-dependently (Fig. 7f). Taken together, these data suggest that TNF-βR expression is linked to TNF-β-promoted inflammation, osteogenic lineage suppression in MSCs and resveratrol suppresses specific TNF-β/TNF-βR signaling in MSCs.

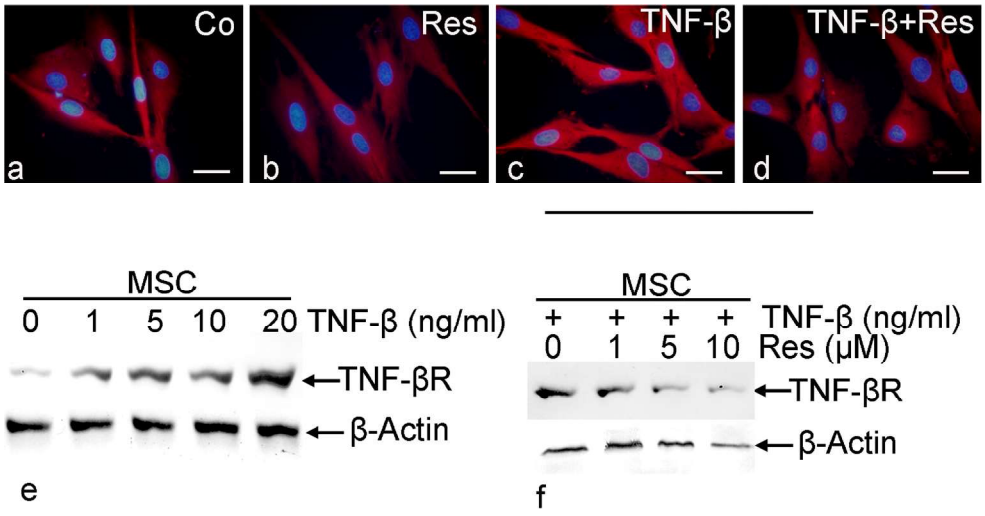

Fig. 7 Effects of TNF-β and/or resveratrol on TNF-βR expression in MSCs. **a–d** MSCs in monolayer culture were either left untreated (**a**) or treated with resveratrol (**b**), or with TNF-β (**c**) or pre-treated with resveratrol and then co-treated with TNF-β (**d**), followed by incubation with rhodamine-coupled secondary antibodies and counterstaining with DAPI to visualize cell nuclei. Magnification, ×600; scale bar = 1 μm. **e–f** MSCs in monolayer culture were either left untreated or treated with various concentrations of TNF-β (1, 5, 10, 20 ng/ml) (**e**) or treated with different concentrations of resveratrol (1, 5, 10 μM) and then co-treated with 5 ng/ml TNF-β for 12 h (**f**). Whole cell lysates were fractionated and probed by immunoblotting with antibodies against TNF-βR. Housekeeping protein β-actin (**e**, **f**) served as a loading control in all experiments

TNF-β suppresses interaction of Sirt1 with β1-integrin, Runx2 and promotes interaction with NF-κB and resveratrol can reverse it

To evaluate the downstream mechanism pathway of the TNF-β/TNF-βR-induced inflammatory environment during MSCs osteogenic differentiation and modulation effects of resveratrol/Sirt1 signaling in this pathway, we examined the hypothesis of an interaction between Sirt1 with NF-κB, β1-integrin and Runx2 in MSCs and subsequently promotion of MSC osteogenic differentiation. To this end, the MSCs in high-density culture were either left untreated or treated with TNF-β (10 ng/ml), resveratrol (5 μM), or pre-treated with resveratrol (5 μM) for 1 h followed by co-treatment with TNF-β (10 ng/ml) in osteogenic induction medium for 21 days (Fig. 8). The whole cell lysates were immunoprecipitated with anti-Sirt1 and immunoblotted with antibodies against p-NF-κB, β1-integrin and Runx2 (Fig. 8a–c). Interestingly, immunoprecipitates from MSCs in untreated cultures uncovered co-immunoprecipitation of Sirt1 with β1-integrin, NF-κB, and Runx2 (Fig. 8a–c). Treatment with TNF-β markedly increased the interaction between Sirt1 and p-NF-κB but was decreased between Sirt1 and β1-integrin or Runx2 (Fig. 8a–c). However, treatment with resveratrol by itself or co-treated with TNF-β clearly raised association of Sirt1 and β1-integrin or Runx2 and reduced interaction of Sirt1 and p-NF-κB, highlighting the crucial role of the resveratrol/Sirt1-signaling pathway in inhibiting the TNF-β-promoted pro-inflammatory microenvironment by the NF-κB pathway and promoting β1-integrin and Runx2, which may stimulate the osteogenic differentiation pathway in MSCs (Fig. 8a–c).

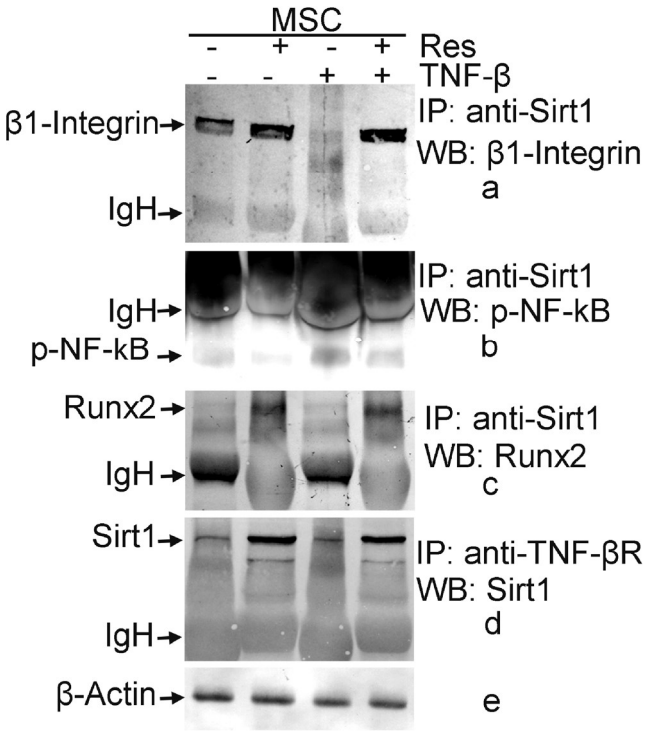

Fig. 8 Complex formation of Sirt1 protein with β1-integrin, p-NF-κB, Runx2 and TNF-βR during osteogenesis of MSCs in high-density culture. MSCs were cultured in high-density cultures with osteogenic induction medium and either left untreated or treated with resveratrol, TNF-β by itself or cells were pre-treated with resveratrol for 1 h followed by co-treatment with TNF-β. After 21 days, whole cell extracts were lysed and immunoprecipitated (IP) with anti-Sirt1 (**a–c**) or anti-TNF-βR (**d**); subsequently, the immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting (WB) using anti-β1-integrin (**a**), anti-p-NF-κB (**b**), anti-Runx2 (**c**) and anti-Sirt1 (**d**). The original samples were probed with an antibody to β-actin (**e**) as a loading control. IgH, immunglobulin heavy chain

TNF-β suppresses interaction of TNF-βR with Sirt1 and resveratrol can reverse it during MSCs osteogenesis in vitro

In a second approach, the abovementioned cell lysates were immunoprecipitated with anti-TNF-βR and immunoblotted with anti-Sirt1. Immunoprecipitates from MSCs in untreated controls revealed co-immunoprecipitation of TNF-βR with Sirt1. Treatment with TNF-β markedly decreased the amount of Sirt1 in this interaction. However, treatment with resveratrol by itself or co-treated with TNF-β clearly raised the amount of Sirt1 in this association (Fig. 8d). Taken together, these results show that Sirt1-crosstalk is actively involved in the TNF-β/TNF-βR signaling pathway at the molecular stage; the effects of resveratrol are strained through promotion of both the Sirt1 and β1-integrin receptor pathways, leading to activation of the master osteoblast transcription factor Runx2 and suppression of TNF-βR expression.

Discussion

Increased production of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-7, has been previously demonstrated in various inflammatory bone diseases, including OA, RA and osteoporosis (Pfeilschifter et al. 2002; Weitzmann and Pacifici 2006). Moreover, pro-inflammatory cytokines, like TNF-α or IL-1β, affect the multipotent MSCs differentiation to osteoblasts or chondroblasts through breakdown of extracellular matrix, cell death and suppression of osteogenesis or chondrogenesis (Buhrmann et al. 2014; Liu et al. 2011). Furthermore, it has been demonstrated that pivotal pro-inflammatory cytokines, like TNF-α, amplify the pathophysiology of OA and RA and suppression of these cytokines with an antibody is a successful therapeutic target for patients with OA and RA (Gartlehner et al. 2006; Hochberg et al. 2005; Kapoor et al. 2011). However, several lines of evidence indicate that a high percentage of patients treated with anti-TNF-α drugs (such as rituximab, anakinra and abatacept) show a significant increase of adverse side effects, serious infections, risk of malignancies in patients and up to 50% of patients

become resistant to TNF-α therapy compared with a placebo in OA and RA (Bongartz et al. 2006; Dixon et al. 2007; Leombruno et al. 2009; Salliot et al. 2009; Schneeweiss et al. 2007; Thompson et al. 2011; Westlake et al. 2011; Wolfe et al. 2006), underlining that other inflammatory cytokines of the TNF superfamily may be involved (Dinarello and Kim 2006). Indeed, it has been previously demonstrated that TNF-β, another member of the TNF family with high familiar homology to TNF-α (Aggarwal et al. 1985a; Aggarwal et al. 2012; Aggarwal et al. 1985b; Aggarwal et al. 1984; Calmon-Hamaty et al. 2011a; Gramaglia et al. 1999), can play an important and destructive role in OA and RA (Buhrmann et al. 2017; Buhrmann et al. 2013; Calmon-Hamaty et al. 2011a; O'Rourke et al. 2008). However, the impact of TNF-β in these diseases and the role of Sirt1 during MSCs osteogenesis in a TNF-β-mediated inflammatory environment are still poorly understood and require further investigation. Therefore, the aim of this study was to examine whether TNF-β affects inflammatory signaling in the osteogenic differentiation of MSCs in a high-density culture environment and to further investigate the inflammation preventing impact of resveratrol/Sirt1-induced signaling in this process.

We found that the pro-inflammatory cytokine TNF-β, similar to NAM and Sirt1-ASO, affected MSCs viability and markedly suppressed the multipotent MSCs differentiation to osteoblasts, inhibited extracellular matrix protein and surface receptor β1-integrin expression and induced apoptosis. Furthermore, the expression of Sirt1 protein and of the bone-specific transcription factor Runx2 was downregulated. In addition, TNF-β induced, in a similar way to NAM and Sirt1-ASO, NF-κB phosphorylation and NF-κB-promoted pro-inflammatory gene end products (MMP-9, Cox2, Caspase3) during osteogenic differentiation of MSCs in high-density culture. However, we found that pre-treatment of MSCs with resveratrol effectively down-modulated TNF-β- and NAM-promoted suppression of MSCs differentiation to osteoblasts, of extracellular matrix, β1-integrin and Runx2 expression and upregulated NF-κB and NF-κB-mediated pro-inflammatory proteins but not of Sirt1-ASO. These data indicate, for the first time that (1) the Sirt1-signaling pathway is involved in the TNF-β-mediated signaling pathway, which leads to inhibition of MSCs osteogenic differentiation in a similar way to NAM and Sirt1-ASO and (2) knockdown of Sirt1 protein by ASO abrogates the suppression potential of resveratrol on TNF-β-mediated inhibition of MSCs osteogenic differentiation.

In addition, these results are in agreement with previous reports that pro-inflammatory cytokines, such as TNF-α and IFN-γ, affect the multipotent and differentiation properties of MSCs through cell death and suppression of osteogenesis (Liu et al. 2011). It has been reported that resveratrol, as a multi-targeted natural polyphenol possesses anti-inflammatory properties (Estrov et al. 2003; Manna et al. 2000; Ren et al. 2013), promotes significantly the tissue engineering for bone (Kamath et al. 2014; Rutledge et al. 2016; Shakibaei et al. 2011; Shakibaei et al. 2012), cartilage (Buhrmann et al. 2014; Lei et al. 2008; Shakibaei et al. 2009) and tendon (Busch et al. 2012a; Busch et al. 2012b) formation and regeneration. In addition, it has been further demonstrated that resveratrol with downstream target Sirt1 has protective properties on MSCs viability and proliferation, thus for osteogenic differentiation of MSCs (Pillarisetti 2008; Shakibaei et al. 2012; Tseng et al. 2011) and cell regeneration in vivo (Okay et al. 2015; Pinarli et al. 2013). Indeed, we used resveratrol for this study; since resveratrol is an ingredient of a wide amount of vegetables and fruits actively consumed by people, it is desirable to evaluate the mechanism of action of resveratrol.

The high-density culture allows the undifferentiated mesenchymal cells to differentiate to osteoblasts and as we demonstrated by transmission electron microscopy, mesenchymal cells form first aggregates and synthesize an osteogenic-specific pericellular matrix composed of collagen type I and bone-specific proteoglycans. These data are in agreement with our previous findings and underline that in high-density cultures only cells with osteogenic potential can survive and these offer an ideal microenvironmental condition for MSCs differentiation compared with the monolayer cell culture (Csaki et al. 2009; Csaki et al. 2007; Shakibaei et al. 2011; Shakibaei et al. 2012). The pericellular matrix plays a crucial role for differentiation, survival of cells and the cell-matrix interaction on the cell surface is mediated by specific surface receptors. Integrin receptors such as adhesion and signaling molecules are one of the most important surface receptors mediating this cell-matrix adhesion, which is essential for differentiation, proliferation and survival of the cells (Cao et al. 1999; Luo et al. 2019; Rajshankar et al. 2017). Indeed, it has been reported that MSCs synthetize high amounts of the integrin receptor that mediates and binds to the corresponding extracellular matrix compounds (Frith et al. 2012; Gronthos et al. 2001). Interestingly, it has been shown that the resveratrol signal transduction pathway is one of the important signaling pathways that may promote MSCs osteogenic differentiation (Backesjo et al. 2006). However, the cellular mechanisms mediating extracellular matrix compounds with Sirt1 stimulation during osteogenic differentiation are not fully understood.

To gain more insight about the anti-inflammatory effects, protection and promotion role of the resveratrol/Sirt1 signaling pathway (s) during MSCs osteogenic differentiation in a TNF-β-mediated inflammatory environment, we hypothesized that Sirt1 mediated complex formation with Runx2, β1-integrin and NF-κB, thereby stimulating MSCs osteogenic differentiation with subsequent inhibition of pro-inflammatory master transcription factor NF-κB and activation of bone-specific transcription factor Runx2 and this contributes to the successive gain in osteoinduction. Indeed, we showed that TNF-β induced decreased expression of β1-integrin, extracellular matrix breakdown and inhibited MSCs

osteogenic differentiation. These effects of TNF-β might be a primary action in the early stages of MSCs osteogenic differentiation and thus reduce cell-matrix interactions, which at least in part may be an important mechanism for TNF-β-induced suppression of bone-specific transcription factor Runx2 and upregulation of NF-κB-regulated pro-inflammatory and pro-apoptotic proteins (MMP-9, Cox2 expression and caspase-3 cleavage) during osteogenic differentiation of MSCs. Most excitingly, we have also shown that Sirt1 was linked with β1-integrin, Runx2 as well as with NF-κB and that TNF-β treatment induced increased phosphorylation of NF-κB and suppression of Sirt1 and Runx2 expression but resveratrol could reverse these complex formations. In fact, these results are in agreement with previous findings that suppression of Sirt1 by NAM or knock down of Sirt1 led to Runx2 acetylation in MSCs and this was markedly reduced by pre-treatment with an activator of Sirt1 (resveratrol), suggesting that Runx2 protein might be a natural substrate for Sirt1 deacetylation (Shakibaei et al. 2012; Zainabadi et al. 2017a; b). Furthermore, it has been shown that resveratrol/Sirt1 signaling exerts anti-osteoclastogenic effects, which is regulated by the downregulation of RANKL-p300-NF-κB activation (Shakibaei et al. 2011).

We have shown that TNF-β dose-dependently increased its receptor expression in MSCs, whereas resveratrol treatment suppressed this. These results underline that TNF-β specifically induces a pro-inflammatory signaling cascade in MSCs by enhancing its own receptor. Additionally, this highlights the protective role of resveratrol in suppressing TNF-β-/TNF-βR-mediated pro-inflammatory signaling in MSCs providing a favorable environment to enable osteogenic differentiation of MSCs. Indeed, this is in agreement with previous studies reporting that TNF-α induces its own expression and receptor thereby up-keeping and self-stimulating an inflammatory environment (Aggarwal et al. 2012).

Most interestingly, we further found that TNF-βR was associated with Sirt1 and TNF-β downregulated Sirt1 expression and resveratrol could reverse this complex formation. Indeed, these results are further consistent with previous reports, which showed that resveratrol acts as a potent activator of Sirt1 and specific inhibitor of the NF-κB transcription factor (Gupta et al. 2010; Manna et al. 2000) and Sirt1 is able to suppress inflammation (Wellman et al. 2017). These data underline the protective role of resveratrol in the crosstalk among these signaling pathways, as it clearly increased co-immunoprecipitation of Sirt1 with β1-integrin or with

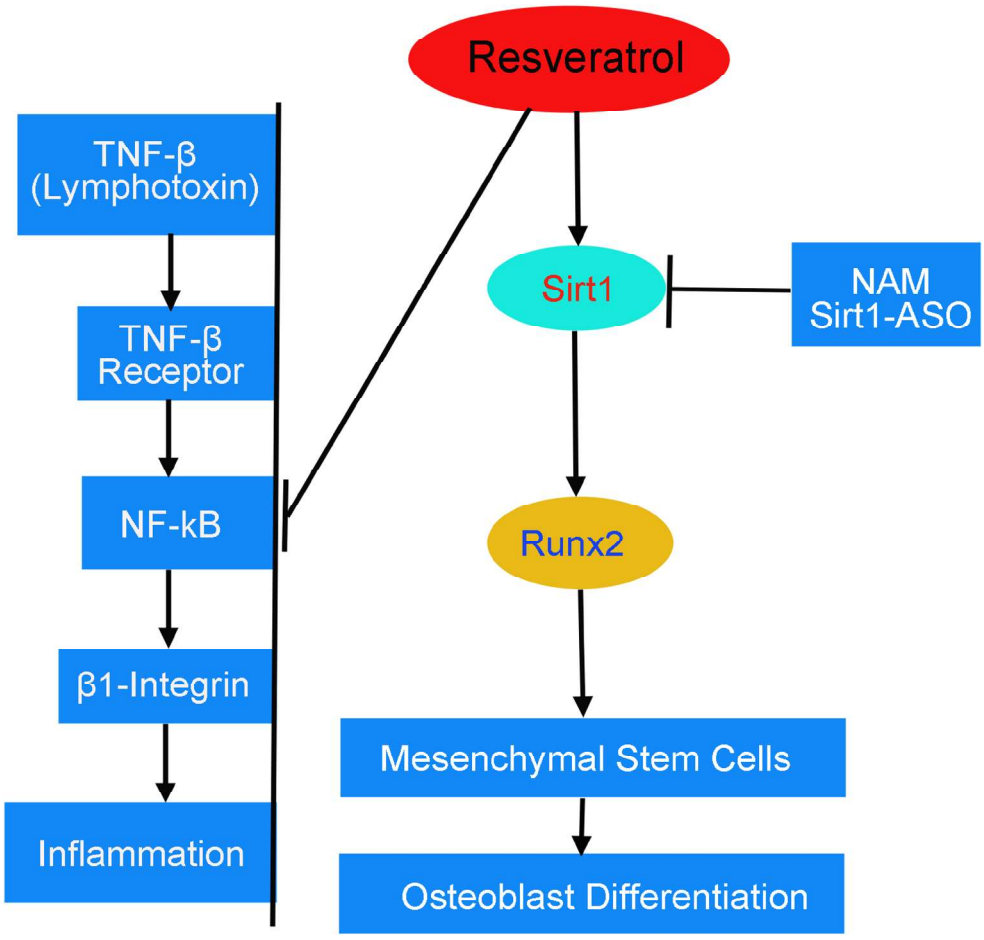

Fig. 9 Working model on the effect of TNF-β on osteogenic MSC differentiation and its modulation by resveratrol in vitro

Runx2 and decreased the complex formation between Sirt1 and NF-κB in MSCs during osteogenic differentiation in the TNF-β/TNF-βR-mediated inflammatory environment, which may activate the osteogenic differentiation pathway in MSCs.

In conclusion, in this study, we present a new mechanism by which a T cell-derived pro-inflammatory cytokine, TNF-β, has an impact on MSCs osteogenic differentiation by involving NF-κB, Sirt1, Runx2 and resveratrol protected MSCs osteogenesis against TNF-β-promoted impairments (Fig. 9). These findings suggest that TNF-β could be an important target, similar to TNF-α for the treatment of bone loss and osteoporosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All specimens (adipose tissue and canine primary osteoblasts) were waste products that occur during surgery from implantation of artificial hip joints in dogs.

Informed consent Not applicable.

References

Aggarwal BB (2003) Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 3:745–756

Aggarwal BB, Moffat B, Harkins RN (1984) Human lymphotoxin. Production by a lymphoblastoid cell line, purification, and initial characterization. J Biol Chem 259:686–691

Aggarwal BB, Eessalu TE, Hass PE (1985a) Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon. Nature 318:665–667

Aggarwal BB, Kohr WJ, Hass PE, Moffat B, Spencer SA, Henzel WJ, Bringman TS, Nedwin GE, Goeddel DV, Harkins RN (1985b) Human tumor necrosis factor. Production, purification, and characterization. J Biol Chem 260:2345–2354

Aggarwal BB, Gupta SC, Kim JH (2012) Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood 119:651–665

Armiento AR, Alini M, Stoddart MJ (2018) Articular fibrocartilage-why does hyaline cartilage fail to repair? Adv Drug Deliv Rev

Backesjo CM, Li Y, Lindgren U, Haldosen LA (2006) Activation of Sirt1 decreases adipocyte formation during osteoblast differentiation of mesenchymal stem cells. J Bone Mineral Res 21:993–1002

Bongartz T, Sutton AJ, Sweeting MJ, Buchan I, Matteson EL, Montori V (2006) Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. Jama 295:2275–2285

Buhrmann C, Mobasheri A, Matis U, Shakibaei M (2010) Curcumin mediated suppression of nuclear factor-kappaB promotes chondrogenic differentiation of mesenchymal stem cells in a high-density co-culture microenvironment. Arthritis Res Ther 12:R127

Buhrmann C, Shayan P, Aggarwal BB, Shakibaei M (2013) Evidence that TNF-beta (lymphotoxin alpha) can activate the inflammatory environment in human chondrocytes. Arthritis Res Ther 15:R202

Buhrmann C, Busch F, Shayan P, Shakibaei M (2014) Sirtuin-1 (SIRT1) is required for promoting chondrogenic differentiation of mesenchymal stem cells. J Biol Chem 289:22048–22062

Buhrmann C, Popper B, Aggarwal BB, Shakibaei M (2017) Resveratrol downregulates inflammatory pathway activated by lymphotoxin alpha (TNF-beta) in articular chondrocytes: comparison with TNF-alpha. PLoS One 12:e0186993

Busch F, Mobasheri A, Shayan P, Lueders C, Stahlmann R, Shakibaei M (2012a) Resveratrol modulates interleukin-1beta-induced phosphatidylinositol 3-kinase and nuclear factor kappaB signaling pathways in human tenocytes. J Biol Chem 287:38050–38063

Busch F, Mobasheri A, Shayan P, Stahlmann R, Shakibaei M (2012b) Sirt-1 is required for the inhibition of apoptosis and inflammatory responses in human tenocytes. J Biol Chem 287:25770–25781

Calmon-Hamaty F, Combe B, Hahne M, Morel J (2011a) Lymphotoxin alpha revisited: general features and implications in rheumatoid arthritis. Arthritis Res Ther 13:232

Calmon-Hamaty F, Combe B, Hahne M, Morel J (2011b) Lymphotoxin alpha stimulates proliferation and pro-inflammatory cytokine secretion of rheumatoid arthritis synovial fibroblasts. Cytokine 53:207–214

Cao L, Lee V, Adams ME, Kiani C, Zhang Y, Hu W, Yang BB (1999) Beta-integrin-collagen interaction reduces chondrocyte apoptosis. Matrix Biol 18:343–355

Corrado A, Maruotti N, Cantatore FP (2017) Osteoblast role in rheumatic diseases. Int J Mol Sci 18

Csaki C, Matis U, Mobasheri A, Ye H, Shakibaei M (2007) Chondrogenesis, osteogenesis and adipogenesis of canine mesenchymal stem cells: a biochemical, morphological and ultrastructural study. Histochem Cell Biol 128:507–520

Csaki C, Matis U, Mobasheri A, Shakibaei M (2009) Co-culture of canine mesenchymal stem cells with primary bone-derived osteoblasts promotes osteogenic differentiation. Histochem Cell Biol 131:251–266

De Bari C, Roelofs AJ (2018) Stem cell-based therapeutic strategies for cartilage defects and osteoarthritis. Curr Opin Pharmacol 40:74–80

Dinarello CA, Kim SH (2006) IL-32, a novel cytokine with a possible role in disease. Ann Rheum Dis 65(Suppl 3):iii61–iii64

Dixon WG, Symmons DP, Lunt M, Watson KD, Hyrich KL, Silman AJ (2007) Serious infection following anti-tumor necrosis factor alpha therapy in patients with rheumatoid arthritis: lessons from interpreting data from observational studies. Arthritis Rheum 56:2896–2904

Dosier CR, Erdman CP, Park JH, Schwartz Z, Boyan BD, Guldberg RE (2012) Resveratrol effect on osteogenic differentiation of rat and human adipose derived stem cells in a 3-D culture environment. J Mech Behav Biomed Mater 11:112–122

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89:747–754

Edwards JR, Perrien DS, Fleming N, Nyman JS, Ono K, Connelly L, Moore MM, Lwin ST, Yull FE, Mundy GR et al (2013) Silent information regulator (Sir)T1 inhibits NF-kappaB signaling to maintain normal skeletal remodeling. J Bone Mineral Res 28:960–969

Erdman CP, Dosier CR, Olivares-Navarrete R, Baile C, Guldberg RE, Schwartz Z, Boyan BD (2012) Effects of resveratrol on enrichment of adipose-derived stem cells and their differentiation to osteoblasts in two-and three-dimensional cultures. J Tissue Eng Regen Med 6(Suppl 3):s34–s46

Estrov Z, Shishodia S, Faderl S, Harris D, Van Q, Kantarjian HM, Talpaz M, Aggarwal BB (2003) Resveratrol blocks interleukin-1beta-induced activation of the nuclear transcription factor NF-kappaB, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. Blood 102:987–995

Ferreira SA, Motwani MS, Faull PA, Seymour AJ, Yu TTL, Enayati M, Taheem DK, Salzlechner C, Haghighi T, Kania EM et al (2018) Bi-directional cell-pericellular matrix interactions direct stem cell fate. Nat Commun 9:4049

Frith JE, Mills RJ, Hudson JE, Cooper-White JJ (2012) Tailored integrin-extracellular matrix interactions to direct human mesenchymal stem cell differentiation. Stem Cells Dev 21:2442–2456

Gartlehner G, Hansen RA, Jonas BL, Thieda P, Lohr KN (2006) The comparative efficacy and safety of biologics for the treatment of rheumatoid arthritis: a systematic review and metaanalysis. J Rheumatol 33:2398–2408

Gilbert L, He X, Farmer P, Rubin J, Drissi H, van Wijnen AJ, Lian JB, Stein GS, Nanes MS (2002) Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2alphaA) is inhibited by tumor necrosis factor-alpha. J Biol Chem 277:2695–2701

Gimble J, Guilak F (2003) Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy 5:362–369

Gramaglia I, Mauri DN, Miner KT, Ware CF, Croft M (1999) Lymphotoxin alphabeta is expressed on recently activated naive and Th1-like CD4 cells but is down-regulated by IL-4 during Th2 differentiation. J Immunol 162:1333–1338

Gronthos S, Simmons PJ, Graves SE, Robey PG (2001) Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. Bone 28:174–181

Gupta SC, Sundaram C, Reuter S, Aggarwal BB (2010) Inhibiting NF-kappaB activation by small molecules as a therapeutic strategy. Biochim Biophys Acta 1799:775–787

Harth, M., and Nielson, W.R. (2019). Pain and affective distress in arthritis: relationship to immunity and inflammation. Expert review of clinical immunology

Hirose, T., Fukuma, Y., Takeshita, A., and Nishida, K. (2018). The role of lymphotoxin-alpha in rheumatoid arthritis. Inflamm Res ... [et al.] 67, 495–501

Hochberg MC, Lebwohl MG, Plevy SE, Hobbs KF, Yocum DE (2005) The benefit/risk profile of TNF-blocking agents: findings of a consensus panel. Semin Arthritis Rheum 34:819–836

Jue DM, Jeon KI, Jeong JY (1999) Nuclear factor kappaB (NF-kappaB) pathway as a therapeutic target in rheumatoid arthritis. J Korean Med Sci 14:231–238

Kamath MS, Ahmed SS, Dhanasekaran M, Santosh SW (2014) Polycaprolactone scaffold engineered for sustained release of resveratrol: therapeutic enhancement in bone tissue engineering. Int J Nanomedicine 9:183–195

Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H (2011) Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 7:33–42

Kauppinen A, Suuronen T, Ojala J, Kaarniranta K, Salminen A (2013) Antagonistic crosstalk between NF-kappaB and SIRT1 in the regulation of inflammation and metabolic disorders. Cell Signal 25: 1939–1948

Komori T (2008) Regulation of bone development and maintenance by Runx2. Front Biosci 13:898–903

Komori T (2010) Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell Tissue Res 339:189–195

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M et al (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764

Kotake S, Nanke Y (2014) Effect of TNFalpha on osteoblastogenesis from mesenchymal stem cells. Biochim Biophys Acta 1840:1209–1213

Lei M, Liu SQ, Liu YL (2008) Resveratrol protects bone marrow mesenchymal stem cell derived chondrocytes cultured on chitosan-gelatin scaffolds from the inhibitory effect of interleukin-1beta. Acta Pharmacol Sin 29:1350–1356

Leombruno JP, Einarson TR, Keystone EC (2009) The safety of anti-tumour necrosis factor treatments in rheumatoid arthritis: meta and exposure-adjusted pooled analyses of serious adverse events. Ann Rheum Dis 68:1136–1145

Lepage S, Robson N, Gilmore H, Davis O, Hooper A, St John S, Kamesan V, Gelis P, Carvajal D, Hurtig M et al. (2019) Beyond cartilage repair: the role of the osteochondral unit in joint health and disease. Tissue engineering. Part B, Reviews

Liu Y, Wang L, Kikuiri T, Akiyama K, Chen C, Xu X, Yang R, Chen W, Wang S, Shi S (2011) Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes via IFN-gamma and TNF-alpha. Nat Med 17:1594–1601

Liu C, Zhang H, Tang X, Feng R, Yao G, Chen W, Li W, Liang J, Feng X, Sun L (2018) Mesenchymal stem cells promote the osteogenesis in collagen-induced arthritic mice through the inhibition of TNF-alpha. Stem Cells Int 2018:4069032

Luo K, Gao X, Gao Y, Li Y, Deng M, Tan J, Gou J, Liu C, Dou C, Li Z et al (2019) Multiple integrin ligands provide a highly adhesive and osteoinductive surface that improves selective cell retention technology. Acta Biomater 85:106–116

Manna SK, Mukhopadhyay A, Aggarwal BB (2000) Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. J Immunol 164:6509–6519

Matsuda Y, Minagawa T, Okui T, Yamazaki K (2018) Resveratrol suppresses the alveolar bone resorption induced by artificial trauma from occlusion in mice. Oral Dis 24:412–421

Mendes KL, Lelis DF, Santos SHS (2017) Nuclear sirtuins and inflammatory signaling pathways. Cytokine Growth Factor Rev 38:98–105

Okay E, Simsek T, Subasi C, Gunes A, Duruksu G, Gurbuz Y, Gacar G, Karaoz E (2015) Cross effects of resveratrol and mesenchymal stem cells on liver regeneration and homing in partially hepatectomized rats. Stem Cell Rev 11:322–331

O'Rourke KP, O'Donoghue G, Adams C, Mulcahy H, Molloy C, Silke C, Molloy M, Shanahan F, O'Gara F (2008) High levels of Lymphotoxin-Beta (LT-Beta) gene expression in rheumatoid arthritis synovium: clinical and cytokine correlations. Rheumatol Int 28: 979–986

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR et al (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771

Pfeilschifter J, Koditz R, Pfohl M, Schatz H (2002) Changes in proinflammatory cytokine activity after menopause. Endocr Rev 23:90–119

Pillarisetti S (2008) A review of Sirt1 and Sirt1 modulators in cardiovascular and metabolic diseases. Recent Pat Cardiovasc Drug Discov 3: 156–164

Pinarli FA, Turan NN, Pinarli FG, Okur A, Sonmez D, Ulus T, Oguz A, Karadeniz C, Delibasi T (2013) Resveratrol and adipose-derived mesenchymal stem cells are effective in the prevention and treatment of doxorubicin cardiotoxicity in rats. Pediatr Hematol Oncol 30:226–238

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Rajshankar D, Wang Y, McCulloch CA (2017) Osteogenesis requires FAK-dependent collagen synthesis by fibroblasts and osteoblasts. FASEB J 31:937–953

Ramiro S, Radner H, van der Heijde D, van Tubergen A, Buchbinder R, Aletaha D, Landewe RB (2011) Combination therapy for pain management in inflammatory arthritis (rheumatoid arthritis, ankylosing

spondylitis, psoriatic arthritis, other spondyloarthritis). Cochrane Database Syst Rev:Cd008886

Ren Z, Wang L, Cui J, Huoc Z, Xue J, Cui H, Mao Q, Yang R (2013) Resveratrol inhibits NF-kB signaling through suppression of p65 and IkappaB kinase activities. Die Pharmazie 68:689–694

Rutledge KE, Cheng Q, Jabbarzadeh E (2016) Modulation of Inflammatory Response and Induction of Bone Formation Based on Combinatorial Effects of Resveratrol. J Nanomedicine Nanotechnol:7

Safaeinejad Z, Kazeminasab F, Kiani-Esfahani A, Ghaedi K, Nasr-Esfahani MH (2018) Multi-effects of resveratrol on stem cell characteristics: effective dose, time, cell culture conditions and cell type-specific responses of stem cells to resveratrol. Eur J Med Chem 155: 651–657

Salliot C, Dougados M, Gossec L (2009) Risk of serious infections during rituximab, abatacept and anakinra treatments for rheumatoid arthritis: meta-analyses of randomised placebo-controlled trials. Ann Rheum Dis 68:25–32

Schneeweiss S, Setoguchi S, Weinblatt ME, Katz JN, Avorn J, Sax PE, Levin R, Solomon DH (2007) Anti-tumor necrosis factor alpha therapy and the risk of serious bacterial infections in elderly patients with rheumatoid arthritis. Arthritis Rheum 56:1754–1764

Shakibaei M, Csaki C, Nebrich S, Mobasheri A (2008) Resveratrol suppresses interleukin-1beta-induced inflammatory signaling and apoptosis in human articular chondrocytes: potential for use as a novel nutraceutical for the treatment of osteoarthritis. Biochem Pharmacol 76:1426–1439

Shakibaei M, Harikumar KB, Aggarwal BB (2009) Resveratrol addiction: to die or not to die. Mol Nutr Food Res 53:115–128

Shakibaei M, Buhrmann C, Mobasheri A (2011) Resveratrol-mediated SIRT-1 interactions with p300 modulate receptor activator of NF-kappaB ligand (RANKL) activation of NF-kappaB signaling and inhibit osteoclastogenesis in bone-derived cells. J Biol Chem 286: 11492–11505

Shakibaei M, Shayan P, Busch F, Aldinger C, Buhrmann C, Lueders C, Mobasheri A (2012) Resveratrol mediated modulation of Sirt-1/Runx2 promotes osteogenic differentiation of mesenchymal stem cells: potential role of Runx2 deacetylation. PLoS One 7:e35712

Shui C, Spelsberg TC, Riggs BL, Khosla S (2003) Changes in Runx2/Cbfa1 expression and activity during osteoblastic differentiation of human bone marrow stromal cells. J Bone Mineral Res 18:213–221

Siebers MC, ter Brugge PJ, Walboomers XF, Jansen JA (2005) Integrins as linker proteins between osteoblasts and bone replacing materials. A critical review. Biomaterials 26:137–146

Thompson AE, Rieder SW, Pope JE (2011) Tumor necrosis factor therapy and the risk of serious infection and malignancy in patients with early rheumatoid arthritis: a meta-analysis of randomized controlled trials. Arthritis Rheum 63:1479–1485

Tseng PC, Hou SM, Chen RJ, Peng HW, Hsieh CF, Kuo ML, Yen ML (2011) Resveratrol promotes osteogenesis of human mesenchymal stem cells by upregulating RUNX2 gene expression via the SIRT1/FOXO3A axis. J Bone Mineral Res 26:2552–2563

Weitzmann MN (2017) Bone and the immune system. Toxicol Pathol 45: 911–924

Weitzmann MN, Pacifici R (2006) Estrogen deficiency and bone loss: an inflammatory tale. J Clin Invest 116:1186–1194

Wellman AS, Metukuri MR, Kazgan N, Xu X, Xu Q, Ren NSX, Czopik A, Shanahan MT, Kang A, Chen W et al (2017) Intestinal epithelial sirtuin 1 regulates intestinal inflammation during aging in mice by altering the intestinal microbiota. Gastroenterology 153:772–786

Westlake SL, Colebatch AN, Baird J, Curzen N, Kiely P, Quinn M, Choy E, Ostor AJ, Edwards CJ (2011) Tumour necrosis factor antagonists and the risk of cardiovascular disease in patients with rheumatoid arthritis: a systematic literature review. Rheumatology 50:518–531

Wolfe F, Caplan L, Michaud K (2006) Treatment for rheumatoid arthritis and the risk of hospitalization for pneumonia: associations with prednisone, disease-modifying antirheumatic drugs, and anti-tumor necrosis factor therapy. Arthritis Rheum 54:628–634

Zainabadi K, Liu CJ, Caldwell ALM, Guarente L (2017a) SIRT1 is a positive regulator of in vivo bone mass and a therapeutic target for osteoporosis. PLoS One 12:e0185236

Zainabadi K, Liu CJ, Guarente L (2017b) SIRT1 is a positive regulator of the master osteoblast transcription factor, RUNX2. PLoS One 12: e0178520

Zhao B, Grimes SN, Li S, Hu X, Ivashkiv LB (2012) TNF-induced osteoclastogenesis and inflammatory bone resorption are inhibited by transcription factor RBP-J. J Exp Med 209:319–334

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7: 211–228

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13: 4279–4295