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Epidermal growth factor as a mechanosensitizer in human bone marrow stromal cells

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ABSTRACT

Epidermal growth factors (EGFs) e.g. EGF, heparin-binding EGF and transforming growth factor alpha and their receptors e.g. EGFR and ErbB2 control proinflammatory signaling and modulate proliferation in bone marrow stromal cells (BMSC). Interleukin-6 and interleukin-8 are EGF targets and participate in the inflammatory phase of bone regeneration via non-canonical wnt signaling. BMSC differentiation is also influenced by mechanical strain-related activation of ERK1/2 and AP-1, but the role of EGFR signaling in mechanotransduction is unclear. We investigated the effects of EGFR signaling in telomerase-immortalized BMSC, transfected with a luciferase reporter, comprising a mechanoresponsive AP1 element, using ligands, neutralizing antibodies and EGFR inhibitors on mechanotransduction and we found that EGF via EGFR increased the response to mechanical strain. Results were confirmed by qPCR analysis of mechanoresponsive genes. EGF-responsive interleukin-6 and interleukin-8 were synergistically enhanced by EGF stimulation and mechanical strain. We show here in immortalized and primary BMSC that EGFR signaling enhances mechanotransduction, indicating that the EGF system is a mechanosensitizer in BMSC. Alterations in mechanosensitivity and -adaptation are contributors to age-related diseases like osteoporosis and the identification of a suitable mechanosensitizer could be beneficial. The role of the synergism of these signaling cascades in physiology and disease remains to be unraveled.

1. Introduction

The family of epidermal growth factors (EGFs) describes a group of proteins with high structural and functional similarities. Family members besides EGF are heparin-binding epidermal growth factor (HbEGF), transforming growth factor alpha (TGFα), betacellulin, amphiregulin, epiregulin, epithelial mitogen and the neuregulins 1–4 (Abdallah et al., 2005). The proteins are ligands for members of the EGF receptor family of protein tyrosin kinases, which are ubiquitously expressed. The EGF receptor family consists of four members: EGFR (ErbB1, Her1), ErbB2 (Her2), ErbB3 (Her3) and ErbB4 (Her4), which differ in their ligand binding and phosphorylation capacities as ErbB2 is lacking the ligand-binding and ErbB3 the phosphorylation domain. After ligand binding receptor homo- or heterodimerization is induced, followed by subsequent phosphorylation and activation of signaling cascades (Ahmed et al., 2003). EGFR family signaling induces activation of RAS/MAPK, PLCγ/PKC, PI3K/AKT, and STAT signaling pathways, but also nuclear translocation and signaling have been reported (Alexander, 2001; Azizi et al., 2012). EGFR systems control proinflammatory signaling cascades and proliferation and are targeted for anticancer treatment, ErbB2 and EGFR targeting in breast (Brand et al., 2011) and colon cancer being prominent examples (Chandra et al., 2013; Cohen et al., 2015).

In recent years a role for EGF family members has been identified in bone biology: EGF enhances proliferation and migration in bone marrow stromal cells (BMSC) (Cruz-Lopez et al., 2011; De Luca et al., 2011), which can give rise to e.g. osteoblasts, chondrocytes and adipocytes and thus are the source of mesenchymal tissue regeneration. Stimulation of BMSC with EGFR ligands increases the production of growth and differentiation factors and cytokines like vascular endothelial growth factor (VEGF), angiopoetin-2, platelet-derived growth factor BB (PDGF-BB), granulocyte-colony stimulating factor (GCSF), hepatocyte growth factor (HGF), and interleukin-6 and -8 (IL-6, IL-8) in BMSCs and osteoblast precursors (Ebert et al., 2015; Eccles, 2011). Similar to other target tissues EGFR family stimulation in BMSCs and

Abbreviations: AP-1, activator protein 1; BMSC, bone marrow stromal cell; EEF1A1, eukaryotic translation elongation factor 1 alpha 1; EGF, epidermal growth factor; EGFR, EGF receptor,; ELISA, enzyme linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GCSF, granulocyte-colony stimulating factor; HbEGF, heparin-binding EGF-like growth factor; HGF, hepatocyte growth factor; IL6, interleukin 6; IL8, interleukin 8; hMSC-TERT, telomerase-immortalized human mesenchymal stem cells; PDGF-BB, platelet-derived growth factor BB; PI3K/Akt, Phosphatidylinositol 3-kinase/AKT Serine/Threonine kinase; PTGS2, prostaglandin-endoperoxide synthase 2; PTH, parathyroid hormone; PU, polyurethane; TGFα, tumor growth factor; VEGF, vascular endothelial growth factor; wnt, wingless-type MMTV integration site family.

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osteoblasts activates the above mentioned signaling cascades. Furthermore, extensive crosstalk to osteogenic signaling pathways such as wnt signaling, estrogen receptor(s), prostaglandins and PTH receptor type 1 signaling has been reported (Fedorchak et al., 2014). Proinflammatory genes like IL-6 and IL-8 are direct EGF targets and participate in the inflammatory phase of fracture healing/bone regeneration where they enhance osteogenic differentiation and mineralization via non-canonical wnt signaling via Wnt-5a and ROR2 (Hahn and Schwartz, 2009; Hedhli et al., 2014; Hess et al., 2004). EGFR knockout animals and dominant negative variants for EGFR indicated that EGFR plays an anabolic role in the skeleton, but recent work has also shown that EGFR signaling keeps osteoblasts in an undifferentiated stage, inhibits the expression of key osteogenic differentiation markers and is dispensable for the anabolic effect of intermittent PTH treatment (Huang et al., 2013; Humphrey et al., 2014; Iskratsch et al., 2014; Kerpedjieva et al., 2012).

Mesenchymal fate decision, lineage commitment and cell differentiation processes in mesodermal and mesenchymal precursors are also influenced by mechanotransduction following e.g. cyclic stretching or fluid flow, but the role of EGFR signaling in this context is unclear. Mechanical forces in the microenvironment are translated into biochemical cues by e.g. integrins and calcium channels that are associated to the cell membrane and the basis of the primary cilium (Kim et al., 2003; Klein-Nulend et al., 2012). Mechanoresponsive transcription is mediated via strain-related activation of e.g. ERK1/2 and nuclear translocation of transcription factors that bind to so called strain sensitive response elements (Kolar et al., 2010). AP1-response, SP1-response and other shear stress DNA-response elements have been described to mediate and modulate mechanoresponse (Lemmon, 2009; Liedert et al., 2006; Liedert et al., 2009; Liedert et al., 2010).

Alterations in mechanically orchestrated remodeling and adaptation are contributors to disease mechanisms and age-related tissue degeneration like in osteoporosis, a syndrome of dysadaptation caused by polygenetic susceptibility and lifestyle changes (Marie et al., 2014; Marmor et al., 2004; Mehta et al., 2010). The identification of a suitable "mechanosensitizer" could be of great benefit to these patients.

We have previously described a reporter gene system for mechanotransduction in BMSC using stably transfected telomerase-immortalized human bone marrow stromal cells (hMSC-TERT) cultured in polyurethane dishes where cyclic stretching can be applied with a homogenous stretching profile (Seefried et al., 2010). Here we show in hMSC-TERT cells that EGF is a mechanosensitizer that enables and amplifies the mechanoresponse of intrinsic AP-1 reporter gene system and downstream targets of mechanical stretch like Prostaglandin-Endoperoxide Synthase 2 (PTGS2) and Fos Proto-Oncogene, AP-1 Transcription Factor Subunit (FOS) (Liedert et al., 2009; Noriega et al., 2010). While strain alone does not significantly activate EGF-responsive genes like IL-6 and IL-8 on mRNA level, cyclic stretching is able to enhance EGF-responsive gene regulation in the presence of EGFR activating ligands. Since AP-1 transcription complexes are well described pro-osteogenic systems, the mechanotransduction via EGFR should drive osteogenic differentiation and regeneration and explain anabolic effects of EGFR on bone (Ozcivici et al., 2010). We identified EGFR and its ligands as powerful modulators of mechanosensitivity in early stages of mesenchymal commitment and differentiation.

2. Materials and methods

2.1. Cell culture

Media for cell culture were obtained from Thermo Fisher Scientific (Darmstadt, Germany), FCS was obtained from Biochrom GmbH (Berlin, Germany). Primary human bone marrow stromal cells (BMSC) were isolated from bone marrow from seven different donors (three male, four female, mean age 65.6 ± 10.0 SD) and cultivated up to four weeks by a standardized protocol (Hess et al., 2004). Bone marrow was obtained with informed consent from the femoral head of patients undergoing elective hip arthroplasty. The procedure was approved by the local Ethics Committee of the University of Würzburg. Briefly, bone marrow preparations were washed with Dulbecco's modified Eagle's medium, (DMEM/F12) supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μg/ml ascorbate (Sigma-Aldrich GmbH, Munich, Germany), and centrifuged at 1200 rpm for 5 min. The pellet was reconstituted in medium and washed four times, and the supernatants of the washing steps containing the released cells were collected. Cells were centrifuged and cultivated at a density of 1×10^9 cells per 175 cm^2 culture flask. Adherent cells were washed after 2 days and cultivated until confluence. Telomerase immortalized human bone marrow stromal cells (hMSC-TERT), established from a 33 year old male donor by the group of M. Kassem, and the stable hMSC-TERT-AP-1 cells generated therefrom, comprising a luciferase-based AP-1 reporter, were used as a tool to perform mechanistic studies. HMSC-TERT cells display a high proliferation capacity, while maintaining their mesenchymal differentiation capacity in vitro and in vivo (Liedert et al., 2010; Peake and El Haj, 2003; Pelaez et al., 2012). hMSC-TERT cells were cultivated in Earle's MEM containing 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin. All cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

2.2. Biochemical activation, cyclic stretching of hMSC-TERT cells and luciferase assay

3×10^4 cells per well were seeded on 24-well polyurethane (PU) plates where they adhere tightly after a few hours as shown before (Liedert et al., 2010) and stimulated on the following day with different concentrations of recombinant human EGF (PeproTech GmbH, Hamburg, Germany), Gefitinib (Biozol Diagnostics GmbH, Eching, Germany), Mubritinib (Sigma Aldrich GmbH), human EGF antibody (monoclonal mouse IgG clone #10825, R&D Systems, Wiesbaden, Germany) or human HbEGF antibody (monoclonal mouse IgG clone #406316, R&D Systems) as indicated. For cyclic stretching the same cell number was used, seeded on 24-well PU plates and cultivated for 24 h. After 24 h preincubation with different factors and inhibitors, PU dishes were placed in a bioreactor as previously described (Liedert et al., 2010) and cyclic stretching was performed twice for 30 min at 1 Hz and 1% extension with a 60 min pause in between. This stimulation regime resulted in a 2.5-fold higher induction of luciferase activity compared to the application of cyclic strain for 120 min without pausing in between (data not shown). Cells were harvested after another 24 h and lysed in 150 μl Reporter Lysis Buffer (Promega GmbH, Mannheim, Germany). 20 μl of each extract was analyzed for luciferase activity using the reporter gene assay provided by Promega GmbH in an Orion II Luminometer (Berthold Detection Systems, Pforzheim, Germany) in 96-well plates. Relative light units were normalized to protein content determined by using RotiQuant Protein Assay (Carl Roth GmbH, Karlsruhe, Germany) and the data from four wells were used to calculate the mean value.

2.3. Cyclic stretching of hMSC-TERT cells, RT-PCR and quantitative PCR

For cyclic stretching, 5×10^5 cells per well were seeded on 4-well PU plates and cultivated for 48 h overall. Stimulants were added 24 h hours before stretching or as indicated. PU dishes were placed in a bioreactor and cyclic strain was applied as previously described (Liedert et al., 2010). Immediately after stretching, cells were harvested and total RNA was isolated by using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed with MMLV reverse transcriptase (Promega GmbH) in a volume of 25 μl. For RT-PCR 1 μl of cDNA was used as a template in a volume of 50 μl. Taq DNA polymerase was obtained from Promega GmbH and primers were obtained

Table 1
PCR primers and conditions. Primer names, sequences, product lengths, annealing temperatures and Genbank accession numbers are shown.

Gene	Primer	Sequence 5′-3′	Product length (bp)	Annealing temp (°C)	Genbank accession number
Primers obtained from Biomers					
EEF1α1	EEF1α1 FOR	CTGTATTGGATTGCCACACG	368	55	NM_001402.5
	EEF1α1 REV	AGACCGTTCTTCCACCACTG			
EGFR	EGFR FOR	AAGGCACGAGTAACAAGC	183	60	NM_005228.3
	EGFR REV	AGGGCAATGAGGACATAA			
ERBB2	ERBB2 FOR	CCATCTGCACCATTGATGTC	102	59	NM_004448.3
	ERBB2 REV	ATGCGGGAGAATTCAGACAC			
Il-6	Il6 FOR	AAAGCAGCAAAGAGGCACTG	108	60	NM_000600
	Il6 REV	TTTTCACCAGGCAAGTCTCC			
IL-8	IL8 FOR	CATACTCCAAACCTTTCCAC	165	60	BT007067
	IL8 REV	TCAAAAACTTCTCCACAACC			
RPS27A	RPS27A FOR	TCGTGGTGGTGCTAAGAAAA	141	60	NM_001135592
	RPS27A REV	TCTCGACGAAGGCGACTAAT			
TGFα	TGFa FOR	TGATACACTGCTGCCAGGTC	142	57	NM_003236.3
	TGFa REV	ACTCCTCCTCTGGGCTCTTC			
Primers obtained from Qiagen					
EGF	Hs_EGF_1_SG	Qiagen sequence		60	NM_001963.5
FOS	Hs_FOS_1_SG	Qiagen sequence		57	NM_005252.3
HBEGF	Hs_HBEGF_1_SG	Qiagen sequence		57	NM_001945
PTGS2	Hs_PTGS2_1_SG	Qiagen sequence		59	NM_000963

from biomers GmbH, Ulm Germany (see Table 1 for primer sequences and PCR conditions). EEF1α1 was used as a housekeeping gene and PCR bands were analyzed by agarose gel electrophoresis. For quantitative PCR the cDNA was diluted 1:10 and qPCR was performed in 20 µl by using 2 µl of cDNA and 10 µl of KAPA SYBR FAST qPCR Universal Mix (Peqlab Biotechnologie GmbH, Erlangen, Germany) and 0.25 pmol of sequence specific primers obtained from biomers.net GmbH (Ulm, Germany) or Qiagen GmbH (Hilden, Germany) (see Table 1 for primer sequences and PCR conditions). Results were calculated with the efficiency-corrected Ct model (Pfaffl, 2001) with RPS27A as the housekeeping gene (Rauner et al., 2012). Significances were tested with Student's *t*-test.

2.4. Cyclic stretching of BMSC and hMSC-TERT cells and enzyme linked immunosorbent assay (ELISA)

For cyclic stretching, 5×10^5 cells per well were seeded on 4-well PU plates and cultivated for 48 h overall. Recombinant EGF was added 3 h hours before stretching. After stretching and cultivating the cells for 4 h (cs1) or 16 h (cs2) as indicated, supernatants were harvested, diluted 1:10 (hMSC-TERT) or 1:20 (BMSC) and IL-6 and IL-8 were determined in duplicates with ELISA (IL-6: 900-K16, IL-8: 900-K18, both PeproTech GmbH) according to the manufacturer's instructions. Four independent BMSC preparations were used and values were normalized to IL-6 and IL-8 standard curves. Significances were tested with Student's *t*-test.

2.5. Statistical analyses

Statistical analyses were calculated with Student's *t*-test, p values < 0.05 were considered to be significant. In terms of luciferase activity, data are expressed as the mean of four different measure points of four independent experiments as percent of controls ± SD (*$p < 0.05$, normalized to basal activity; #$p < 0.05$, normalized to untreated, but cyclic stretched values). Quantitative PCR data are expressed as the mean of three independent experiments ± SD, qPCRs were performed three times. Values were obtained with the ΔΔct method and significances were calculated with Student's *t*-test (*$p < 0.05$, normalized to basal activity; #$p < 0.05$, normalized to untreated, but cyclic stretched values). ELISA values were obtained from duplicates of six (hMSC-TERT) or four (primary hMSC) independent experiments and are shown as means ± SD. Significances were calculated with the Student's *t*-test test (*$p < 0.05$, normalized to basal activity; #$p < 0.05$, normalized to unstimulated, but cyclic stretched (cs) value).

3. Results

3.1. EGFR, ErbB2 and respective ligands are expressed in primary BMSC and in hMSC-TERT cells

To clarify if primary BMSC and human telomerase-immortalized BMSC (hMSC-TERT) cells express EGF receptors and their ligands, RT-PCR was performed. Primary BMSC from three different donors and hMSC-TERT cells from two different passages were used. Cells expressed the EGF receptors EGFR and ErbB2. ErbB3 was only weakly expressed, while ErbB4 was not present at all (data not shown). The EGFR ligand, which was most prominently expressed in BMSC and hMSC-TERT cells, was HbEGF. EGF and TGFα could also be detected, but to a much lesser extend (Fig. 1). EEF1α was amplified as housekeeping gene.

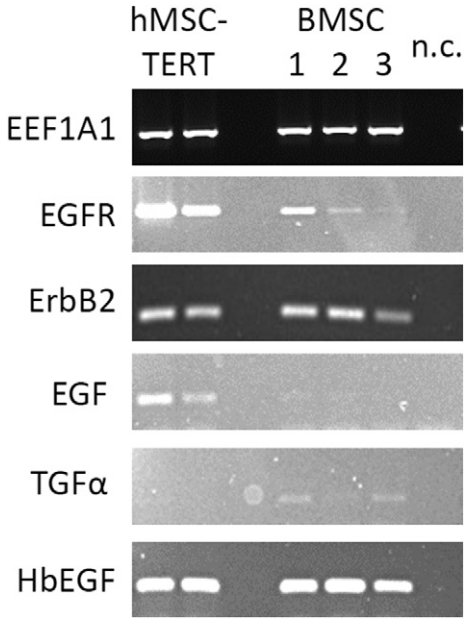

Fig. 1. Expression of EGF family receptors and ligands in hMSC-TERT and primary BMSC. RT-PCR detection of EGFR (epidermal growth factor receptor), ErbB2 (Erb-B2 receptor tyrosine kinase 2), EGF (epidermal growth factor), TGFα (transforming growth factor alpha) and EEF1A1 (eukaryotic translation elongation factor 1 alpha 1), which was amplified as housekeeping gene. RNA from two different passages of hMSC-TERT and three different donors of primary BMSC was used.

3.2. AP-1 transcriptional activity is modulated by signaling via EGF receptors

To elucidate the role of the EGF system, clones stably expressing AP-1 luciferase reporter constructs were incubated with the specific EGFR tyrosine receptor kinase inhibitors gefitinib (EGFR-tyrosine kinase phosphorylation inhibitor) and mubritinib (ErbB2-phosphorylation inhibitor). The basal reporter activity was significantly reduced in a dose dependent manner in both cases, indicating an involvement of EGFR and ErbB2 in the AP-1 mediated activation of luciferase in hMSC-TERT cells (Fig. 2A and B). Basal reporter activity was not stimulated upon additional exogenous treatment with EGF, possibly indicating that the auto-/paracrine stimulation by the culture media was already at its maximum (Fig. 2C). This assumption was supported by the results of treatment with an EGF specific antibody, which significantly reduced basal reporter activity (Fig. 2D) whereas an HbEGF specific antibody showed no effect (data not shown). All data are expressed as the mean of four different measure points of three to five independent experiments as percent of controls ± SD. Significances were calculated with Student's *t*-test (*$p < 0.05$).

3.3. EGF-ligands and EGFR kinase inhibitors modulate the AP-1 reporter gene response to cyclic stretching

As reported earlier cyclic stretching stimulates AP-1 reporter gene activity to approx. 150% compared to basal activity in unstretched cells (Liedert et al., 2010). In the presence of the EGFR-specific inhibitor gefitinib (5 μM) AP-1 mediated activity was not inducible by mechanical strain in hMSC-TERT cells. The ErbB2-specific inhibitor mubritinib (0.1 nM) showed no effect, AP-1 was still inducible by mechanical strain (Fig. 3A). These data show, that for mechanotransduction the EGFR plays a fundamental role.

In the presence of EGF (100 ng/ml) the AP-1 response to cyclic stretching was enhanced to 230% of basal values, indicating a sensitizing effect of EGF for mechanotransduction (Fig. 3B). Consistent with these data the primary response to mechanotransduction is markedly reduced in the presence of a neutralizing antibody against EGF (0.1 μg/ml anti-EGF, Fig. 3B), indicating that ligand-mediated EGFR-signaling is essentially necessary and is the main modulator for AP-1 stimulation through mechanoresponse in this cellular context. All data are expressed as the mean of four different measure points of four independent experiments as percent of controls ± SD. Significances were calculated with Student's *t*-test (*$p < 0.05$, normalized to basal activity; #$p < 0.05$, normalized to untreated but cyclic stretched value).

3.4. EGF enhances transcription of distinct mechanoresponsive genes in the presence of mechanical strain

In order to analyze, if EGF enhances the expression of the mechanoresponsive genes PTGS2 and FOS after cyclic stretching, hMSC-TERT cells were stimulated with 100 ng/ml EGF for 0.5 to 24 h and cyclic stretching was applied. qPCRs revealed that the mRNAs of PTGS2 and FOS were upregulated approx. 2-fold upon cyclic stretching (Fig. 4A and B). Basal expression of PTGS2 transiently and significantly increased upon EGF stimulation 30-fold very early after 15 and 30 min and declined to initial levels after 24 h (white bars). However, basal FOS expression was only influenced by EGF treatment after longer stimulation periods (1–2 h) and declined after 24 h. This might be a secondary effect as FOS seems not primarily EGF responsive in shorter stimulation regimes. After pretreatment with EGF the response to the application of mechanical stress by cyclic stretching was significantly enhanced in terms of PTGS2 expression. For non-EGF responsive genes like FOS, this synergistic effect could not be shown (Fig. 4A and B, black bars). All data are expressed as the mean of three independent experiments ± SD, qPCRs were performed three times. RPS27A was amplified as housekeeping gene. Values were obtained with the ΔΔct method and significances were calculated with Student's *t*-test (*$p < 0.05$, normalized to basal activity; #$p < 0.05$, normalized to untreated but cyclic stretched value).

3.5. EGF enables and amplifies mechanical strain effects on the expression of the proinflammatory genes IL-6 and IL-8 on mRNA and protein levels

The mRNA expression of the proinflammatory genes IL-6 and IL-8 were significantly stimulated after pretreatment with EGF after 15 and 30 min, respectively in hMSC-TERT cells and declined to initial levels after 24 h (Fig. 4C and D, white bars). This response was significantly enhanced after cyclic stretching (black bars). The synergistic enhancement

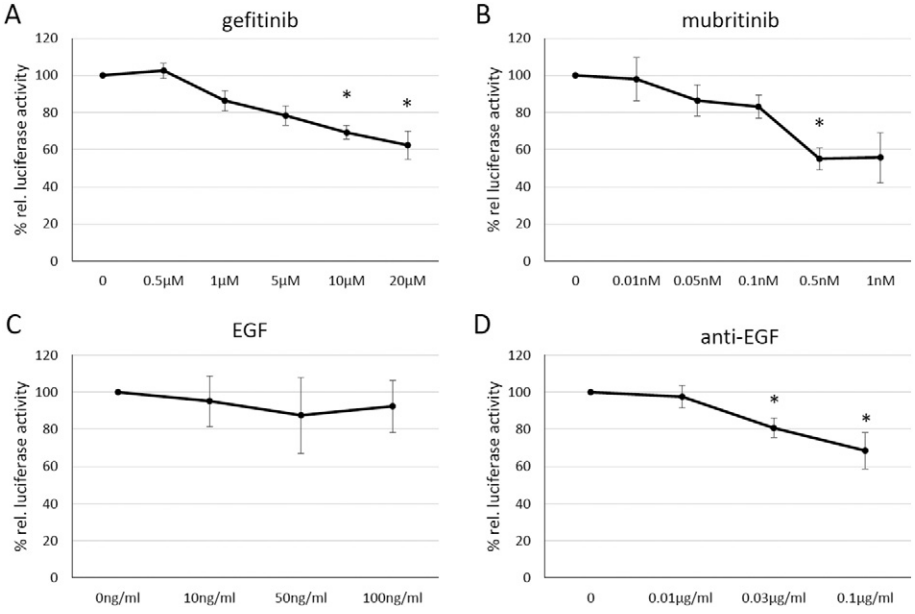

Fig. 2. AP-1 transcriptional activity is modulated by signaling via EGF receptors. hMSC-TERT cells stably expressing AP-1 luciferase reporter constructs were incubated with A) the EGFR inhibitor gefitinib (0.5 to 20 μM), B) the ErbB2 inhibitor mubritinib (0.01 to 1 nM), C) EGF (10 to 100 ng/ml) and D) anti-EGF (0.01 to 0.1 μg/ml) and luciferase activity was determined. Relative Light Units were normalized to the protein content. All data are expressed as the mean of four different measure points of three to five independent experiments as percent of controls ± SD. Significances were calculated with Student's *t*-test (*$p < 0.05$).

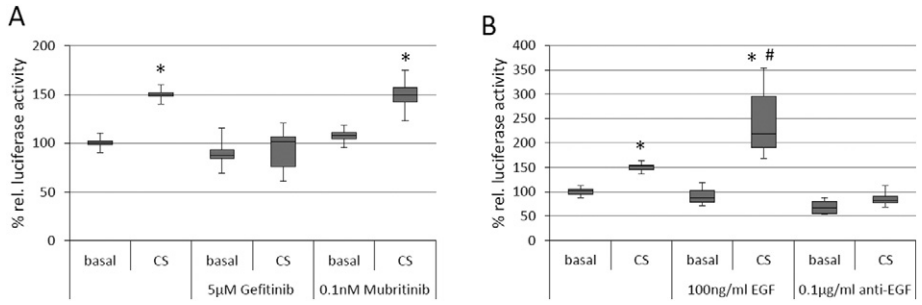

Fig. 3. Modulation of AP-1 reporter gene response to cyclic stretching by EGFR kinase inhibitors and EGF-ligands. hMSC-TERT cells stably expressing a mechanoresponsive, AP-1 driven luciferase reporter were stimulated with A) the EGFR inhibitor gefitinib (5 μM) and the ErbB2 inhibitor mubritinib (0.1 nM) as well as with B) EGF (100 ng/ml) and anti-EGF (0.1 μg/ml) and cyclic stretching was applied. Luciferase activity was determined and normalized to the protein content. All data are expressed as the mean of four different measure points of four independent experiments as percent of controls ± SD. Significances were calculated with Student's *t*-test (*p < 0.05, normalized to basal activity; #p < 0.05, normalized to untreated but cyclic stretched value).

of transcription was especially seen in the time frame of early transcription and mRNA levels were back to baseline after 24 h. All data are expressed as the mean of three independent experiments ± SD, qPCRs were performed three times. RPS27A was amplified as housekeeping gene. Values were obtained with the ΔΔct method and significances were calculated with Student's *t*-test (*p < 0.05, normalized to basal activity; #p < 0.05, normalized to untreated but cyclic stretched value).

IL-6 and IL-8 were also quantified in supernatants of EGF stimulated and cyclic stretched hMSC-TERT and in four different preparations of primary BMSC by ELISA. While IL-6 levels in supernatants of hMSC-TERT cells were not influenced by cyclic stretching (Fig. 5A), IL-6 protein production in BMSC and IL-8 protein production in hMSC-TERT cells and primary BMSC were significantly enhanced (Fig. 5A, B and D). EGF synergistically increased IL-6 and IL-8 secretion in hMSC-TERT and primary BMSCs after 4 h pre-treatment. Values were obtained from duplicates of four to five independent experiments and are shown as means ± SD. Significances were calculated with the Student's *t*-test test (*p < 0.05, normalized to basal activity; #p < 0.05, normalized to unstimulated but cyclic stretched value).

4. Discussion

Bone is a classically mechanoresponsive tissue and this is the basis for adaptation both in the micro- and macro-environment, e.g., the adaptation of bone structure and bone mass. In osteoporosis the lack of sufficient adaptation to environmental needs is clearly key for the development of fragility fractures. This might be one of the crucial issues in early onset osteoporosis but is also part of aging phenomena in that the adaptive response deteriorates with aging. Hence, both genetic and epigenetic events may be underlying to maintain bone mass and quality on a reasonable and sufficient adaptation threshold. This is also evident in recent clinical trials where the increase in bone mass and structure after osteoporosis treatment can get lost within one years time in dimensions of up to 10% and more of bone mass gained during 2–3 years of treatment (Robling, 2012). This indicates that having unraveled molecular mechanisms of bone formation and bone resorption, the knowledge about the molecular mechanisms for its mechano-adaptive maintenance is a missing link in bone biology. However the basis of data in the literature is increasing and both cytosolic and nuclear mechanisms of mechanotransduction and their therapeutic

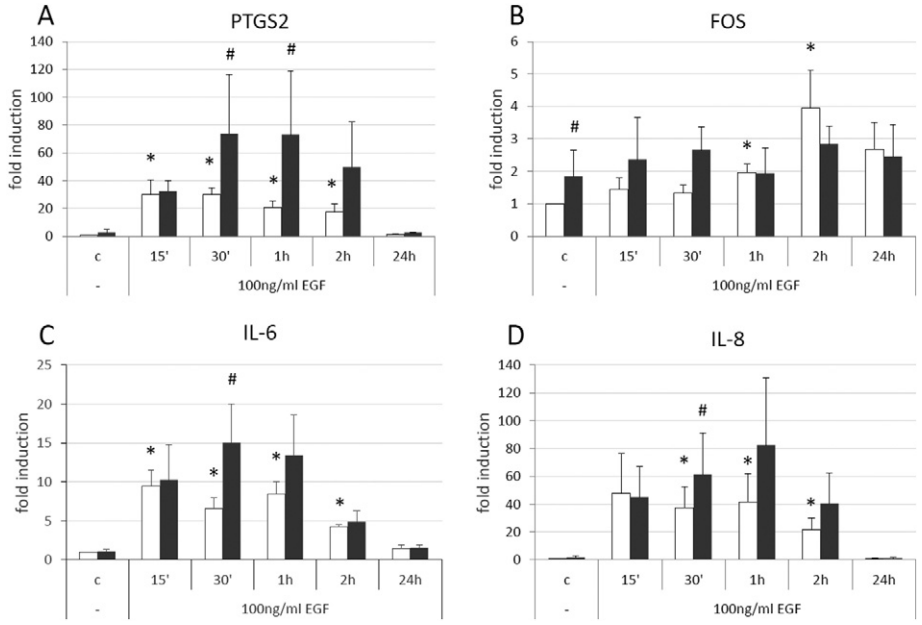

Fig. 4. Expression of mechanoresponsive genes after cyclic stretching and EGF stimulation. hMSC-TERT cells were stimulated with 100 ng/ml EGF (white bars) or left untreated (c) and cyclic stretching was applied (black bars). Cells were harvested after 15, 30, 60, 120 min and 24 h. A) PTGS2 (Prostaglandin-Endoperoxide Synthase 2), B) FOS (Fos Proto-Oncogene, AP-1 Transcription Factor Subunit), C) IL-6 (interleukin 6) and D) IL-8 (interleukin 8) were quantified by qPCR. RPS27A was amplified as housekeeping gene. All data are expressed as the mean of three independent experiments ± SD, qPCRs were performed three times. White bars represent the basal levels, black bars the stretched samples. Values were obtained with the ΔΔct method and significances were calculated with Student's *t*-test (*p < 0.05, normalized to basal activity; #p < 0.05, normalized to untreated but cyclic stretched value).

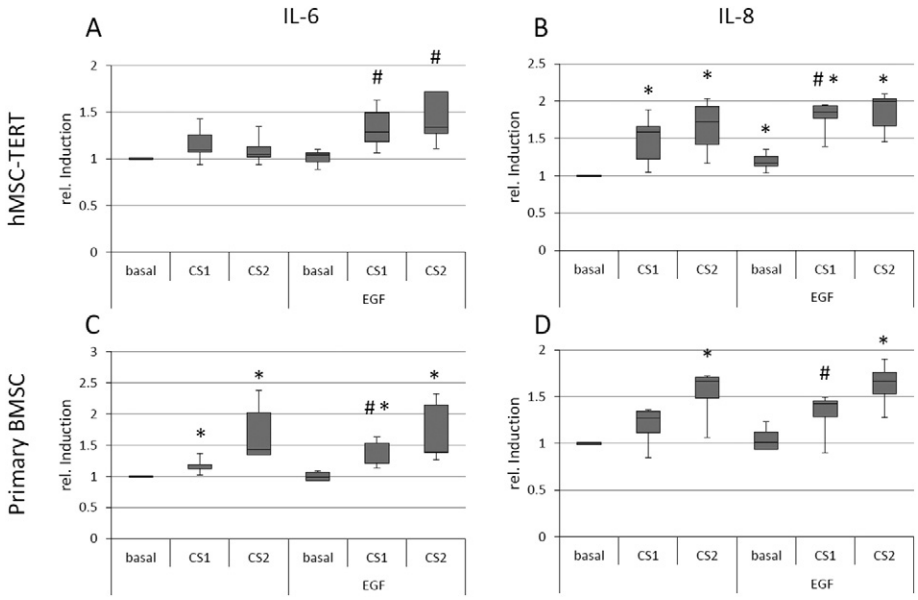

Fig. 5. Protein expression of IL-6 and IL-8 after cyclic stretching and stimulation with EGF. hMSC-TERT cells and primary BMSC were stimulated with 100 ng/ml EGF and cyclic stretching was applied. IL-6 and IL-8 were quantified in cell culture supernatants of hMSC-TERT (A and B) and primary BMSC (C and D) after 4 h (cs1) or 16 h (cs2). Values were obtained from duplicates of six (hMSC-TERT) or four (primary hMSC) independent experiments and are shown as means ± SD. Significances were calculated with the Student's *t*-test test (*$p < 0.05$, normalized to basal activity; #$p < 0.05$, normalized to unstimulated but cyclic stretched (cs) value).

potential are issues of intensive research (Roskoski, 2014; Sato et al., 2005; Schneider et al., 2012; Seefried et al., 2010).

In recent years, an important role for EGF family members has been identified in bone biology. EGF enhances proliferation and migration in BMSCs as well as in preosteoclasts along with reduced differentiation. EGFR signaling is associated with enhanced bone mass as it was shown in mice with deleted EGFR signaling (Huang et al., 2013; Simonsen et al., 2002). The emerging picture is that EGFR signaling controls the early phase of BMSC expansion ahead of osteogenic commitment. EGFR signaling suppresses expression of the key osteogenic factors RUNX2 and osterix (Iskratsch et al., 2014). Furthermore, enhanced survival and antiapoptotic effects were described in osteoprogenitors by EGFR signaling via stimulation of the transcription factor EGR-2 (Cruz-Lopez et al., 2011). Interestingly, TGFα as a ligand shows somehow different and subtle effects on bone formation as it controls endochondral bone formation and survival of hypertrophic chondrocytes (Srinivasan et al., 2012). Here we report for the first time that EGF via EGFR is a mechanosensitizer as it increases the expression of known mechanoresponsive genes and enhances the AP-1 activity of a luciferase reporter after cyclic stretching in skeletal precursors. Mechanical strain apart from mechanotransduction via fluid flow in mature osteocytic cells may be especially important in the early phase of bone healing where the amplification of precursors and hence the callus volume depends on the mechanical stability of a fracture/fracture fixation (Tamama and Kerpedjieva, 2012).

The rational of using an AP-1 reporter gene system to test mechanotransduction effects in the presence and absence of EGFR signaling is an accumulating body of literature that describes the AP-1 signaling pathway as an important mechanotransduction system, which drives the mechanoresponsive transcription of genes like HB-GAM, FOS and PTGS2 (Liedert et al., 2006; Liedert et al., 2009; Liedert et al., 2010; Noriega et al., 2010; Usmani et al., 2012). Furthermore the AP-1 transcription factor family provides multiple links to bone formation as for example FOS itself is associated with enhanced bone formation (Ozcivici et al., 2010). Hence although screening for molecular cues to regulate mechanoperceptive adaptation may not represent the whole spectrum of mechanosensitive regulators, we believe that this system is very useful to mechanistically analyze segments of mechanotransduction, given that the effects that are observed can be translated into regulation of transcription and translation (Liedert et al., 2010).

A key mechanism of BMSC-mediated healing and tissue regeneration is the paracrine secretion of various growth factors and cytokines (Wagner, 2010). EGF stimulates the gene expression of growth factors and cytokines such as IL-6 and IL-8 (Fig. 4) as well as EGFR ligands in a PKC and/or MAPK/ERK-dependent manner (Eccles, 2011). In hMSC-TERT cells EGF-responsive IL-6 and IL-8 gene and protein expression is clearly enhanced by mechanical stretching. The expression of the mechanoresponsive gene PTGS2 is enhanced upon pretreatment with EGF indicating, that EGF acts as a mechanosensitizer. However, this synergistic effect of mechanotransduction and EGF stimulation was not seen in a gene that is not primarily stimulated by EGF as FOS, which is, however, immediately induced after mechanical loading. Nevertheless, we have observed that basal FOS expression was influenced by EGF treatment after longer stimulation periods, but this might be a secondary effect. Although AP-1 subunits are phosphorylated and activated by kinases as JNK and ERK (Wozniak and Chen, 2009), and EGFR signaling is mediated via MAPK, we were not able to detect an AP-1 driven increase in luciferase activity after EGF stimulation under basal conditions in the cellular system or the time-frames we have chosen. Mechanotransduction is a multifaceted process influenced by cellular adhesion, stiffness of the extracellular matrix, recruitment of receptors and their dimerization, ion channel activation and the activation of signaling cascades, resulting in the recruitment of transcription factors. A whole body of high-ranked reviews summarize the state of the art in this field (Zhang et al., 2011a; Zhang et al., 2011b). Crosstalk of the mentioned signaling cascades and transcription factors might be relevant in mediating the here reported effects. By using specific inhibitors or activators, one might unravel the underlying mechanisms. As a first attempt we used both, neutralizing antibodies against EGF as well as inhibitors of the EGFR receptor tyrosine kinase, which both abolished the effects of mechanical strain in hMSC-TERT-AP-1 cells. This reveals an unexpected dominance of EGFR signaling with respect to mechanoadaptation in this early skeletal precursor cellular context. We conclude that at least in this early phase of amplification of multipotent BMSC – e.g. in the course of fracture healing and bone regeneration – EGF signaling is crucial for mechanoadaptation at least via AP-1 and the downstream pattern of gene regulation. The general relevance of the EGF receptor

system in progenitor amplification and mechanoregulation remains to be unraveled.

In order to identify a more functional view on the events influenced by EGFR signaling in the early phase of regeneration the respective target genes IL-6 and IL-8 can be interpreted as mediators of the early pro-inflammatory phase of bone healing and regeneration as well as initiators of angiogenesis, which also has a pivotal role during bone healing and remodeling (Ebert et al., 2015). However while the current literature is dominated by reports about the angiogenic role of the EGFR system in cancer, its role in physiology is neglected. The initiation of sprouting angiogenesis from existing bone vasculature and the maintenance of newly formed vessels is regulated by skeletal precursors and later on osteoclasts, osteoblasts, and osteocytes. EGF, HbEGF and betacellulin significantly promote angiogenesis, human umbilical vein and tumor endothelial cell proliferation, cell migration and tube formation in vitro via PI3K and MAPK signaling pathways (Zhang et al., 2013). Neuregulin 1 is reported to be a mediator of angiogenesis in a cardiovascular setting (Zhu et al., 2011). TGFα induces a significant increase in the levels of secretion of VEGF and angiopoetin-2, GCSF, HGF, IL6, IL-8, and PDGF-BB in BMSCs and human fetal osteoblasts respectively via MEK/MAPK and the PI3K/AKT signaling (Ebert et al., 2015). The cytokines IL-6 and IL-8 in addition are part of proinflammatory signaling, which is active as a cascade triggered through TLR4 stimulation during the initial phase of osteogenic signaling and is capable of inducing non-canonical wnt-signaling via Wnt-5a and ROR2 signaling (Hedhli et al., 2014; Hess et al., 2004).

5. Conclusion

In summary we show here that mechanotransduction via AP-1 response elements initiated by cyclic stretching appears to be enabled and amplified by EGF via EGFR signaling as shown by neutralizing antibodies and receptor kinase inhibitors. Our data indicate that the EGF system is a mechanosensitizer for skeletal precursors, which may be relevant in healthy bone remodeling and fracture healing and which may also be impaired in pathology like in osteoporosis. The exact role of the synergism of these signaling cascades in physiology and disease on the basis of genetic or epigenetic events remains to be unraveled.

Conflict of interest

There are no conflicts of interest.

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