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Contact of myeloma cells induces a characteristic transcriptome signature in skeletal precursor cells –Implications for myeloma bone disease



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ABSTRACT

Physical interaction of skeletal precursors with multiple myeloma cells has been shown to suppress their osteogenic potential while favoring their tumor-promoting features. Although several transcriptome analyses of myeloma patient-derived mesenchymal stem cells have displayed differences compared to their healthy counterparts, these analyses insufficiently reflect the signatures mediated by tumor cell contact, vary due to different methodologies, and lack results in lineage-committed precursors.

To determine tumor cell contact-mediated changes on skeletal precursors, we performed transcriptome analyses of mesenchymal stem cells and osteogenic precursor cells cultured in contact with the myeloma cell line INA-6. Comparative analyses confirmed dysregulation of genes which code for known disease-relevant factors and additionally revealed upregulation of genes that are associated with plasma cell homing, adhesion, osteoclastogenesis, and angiogenesis. Osteoclast-derived coupling factors, a dysregulated adipogenic potential, and an imbalance in favor of anti-anabolic factors may play a role in the hampered osteoblast differentiation potential of mesenchymal stem cells. *Angiopoietin-Like 4 (ANGPTL4)* was selected from a list of differentially expressed genes as a myeloma cell contact-dependent target in skeletal precursor cells which warranted further functional analyses. Adhesion assays with full-length ANGPTL4-coated plates revealed a potential role of this protein in INA-6 cell attachment.

This study expands knowledge of the myeloma cell contact-induced signature in the stromal compartment of myelomatous bones and thus offers potential targets that may allow detection and treatment of myeloma bone disease at an early stage.

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Abbreviations: ABCA1, ATP-binding cassette, sub-family A (ABC1) member 1; ALP, alkaline phosphatase; ANGPTL4, angiopoietin-like 4; BSA, bovine serum albumin; CA9, carbonic anhydrase IX; C/EBP, CCAAT/enhancer binding protein; CMFDA, 5-chloromethylfluorescein diacetate; DKK-1, dickkopf-1; EEF1A1, eukaryotic translation elongation factor 1 alpha 1; FACS, fluorescence activated cell sorting; FCS, fetal calf serum; FC, fold change; FIGN, fidgetin; HBSS, hank's buffered salt solution; HILPDA, hypoxia inducible lipid droplet-associated; IL-6, interleukin-6; ITPR, inositol 1,4,5-trisphosphate receptor; JAM2, junctional adhesion molecule 2; KISS1R, Kiss1 receptor; LFA-1, leukocyte function-associated antigen-1; MACS, magnetic activated cell sorting; MEOX2, mesenchyme homeobox 2; MGUS, monoclonal gammopathy of undetermined significance; MIR210HG, MIR210 host gene; MM, multiple myeloma; MSC, mesenchymal stem cells; MM-MS, MSC from MM patients; NRN1, neuritin 1; OPC, osteogenic precursor cells; PPAR γ , proliferator-activated receptor γ ; PBMCs, peripheral blood mononuclear cells; PGF, placental growth factor; RANKL, receptor activator of NF- κ B ligand; RT, reverse transcriptase; SPAG4, sperm associated antigen 4; T_A, annealing temperature; VEGFA, vascular endothelial growth factor A.

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1. Introduction

Multiple myeloma (MM) is a plasma cell neoplasia that is still largely incurable, although the median 10-year survival has increased to 40% in patients < 50 years of age due to the development of new drugs and bone marrow transplantation strategies during the last two decades [1]. MM is preceded by the non-cancerous plasma cell neoplasm monoclonal gammopathy of undetermined significance (MGUS) [2,3]. Progression to malignancy is driven, among other factors, by changes that occur in the tumor microenvironment leading to enhanced angiogenesis, immunosuppression, and bone resorption [4]. Myeloma bone disease is the result of intensive interaction between myeloma cells and the bone that often precipitates in osteolytic lesions, severe osteoporosis with pathological fractures, and bone pain. Together with other complications such as hypercalcemia and paralysis, myeloma bone disease has a very strong impact on the course of the disease, patient's quality of life, and overall survival. Over long periods it may even be the dominant determining factor for the individual burden and the clinical outcome [5,6].

Myeloma bone disease is the result of disturbed bone remodeling with enhanced osteoclastogenesis and decreased bone formation. The molecular mechanisms have been partly unraveled, in analogy to bone metastases from other solid tumors like breast and prostate cancer. Tumor-derived signaling substances, such as receptor activator of NF- κ B ligand (RANKL), directly or indirectly stimulate osteoclast activity causing bone loss and pathological fractures. More recently, it has been demonstrated that myeloma and other tumor cells also produce factors inhibitory for osteogenic differentiation and bone formation, such as inhibitors of the Wnt signaling pathway dickkopf (DKK)-1 and sclerostin, which results in completely abolished anabolic bone metabolism and regeneration [6,7,8]. Besides soluble factors, physical interaction between myeloma and mesenchymal stem cells (MSC) or osteogenic precursor cells (OPC) leads to a hampered osteoblast differentiation of the skeletal precursor cells [9] which can be partially restored by the anti-cancer agent and proteasome inhibitor bortezomib [10].

MSC have been reported to inhibit myeloma progression *via* pro-apoptotic mechanisms [11], but generally, their role in myeloma development and survival is described as a supportive one [12,13,14,15,16]. Comparison of gene expression profiles between MSC from MGUS patients/healthy donors with those from MM patients demonstrate tumor-promoting abnormalities in the latter [17,18]. The support of MM development by MSC may be genuine and intrinsic, *e.g.* as part of genetic predisposition. However, several studies revealed that signature changes of MSC are also acquirable by physical contact with myeloma cells [19] (unpublished results from Herman and colleagues: cited in [18]). Nonetheless, genome-wide analyses of MM cell contact-induced changes in the MSC transcriptome are scarcely published [18,19]. Moreover, to our knowledge, no study has been performed with MSC-derived osteogenic precursors, although it is known that their osteogenic potential is also suppressed by direct interaction with MM cells [9].

Therefore, we performed a thorough transcriptome analysis of both MSC and OPC after direct contact with MM cells. These results show that OPC are also capable of supporting MM progression, albeit to a lesser extent than MSC, which seem to have a greater tumor-promoting potential according to the genetic signature. Besides a general overview of altered signaling pathways that support the importance of bone-forming cells in myeloma bone disease the data indicate that osteogenesis and angiogenesis may be uncoupled at the bone-tumor interface. Furthermore we identified *Angiopoietin-Like 4 (ANGPTL4)* as a novel interaction-specific target of skeletal precursors that mediates myeloma cell attachment. These data support the potent role of stroma in pathophysiological mechanisms favoring MM bone disease and provide insights into molecular mechanisms and signaling pathways that are associated with the development of bone metastases and niche hijacking in general.

2. Materials and methods

2.1. Ethics statement

Bone material was used in accordance with the local Ethics Committee of the Medical Faculty of the University of Würzburg with written, informed consent of each patient.

2.2. Cells and cell culture

2.2.1. Primary cells and cell lines

Primary human MSC were isolated from the cancellous bone from the acetabulum received from donors after total hip arthroplasty (gender: 11 females, 13 male; average donor age in years: mean \pm SD: 63 \pm 9; range: 48–77 years). MSC were isolated by surface adherence and characterized as previously described [20,21,22]. Expansion was performed in DMEM/Ham's F-12 (1:1) medium (Life Technologies GmbH, Darmstadt, Germany), supplemented with 10% (v/v) fetal calf serum (FCS) (Biochrom, Berlin, Germany), 100 U/ml penicillin, 100 μ g/ml streptomycin (Life Technologies GmbH) and 50 μ g/ml L-Ascorbic acid 2-phosphate (Sigma-Aldrich Chemie GmbH (Sigma), Schnellendorf, Germany). MSC were passaged at least once before they were used for experiments.

The plasmacytoma cell line INA-6 [23] was authenticated by DNA profiling using 8 different highly polymorphic short tandem repeat loci (performed at the Leibniz Institute, Braunschweig, Germany). For cultivation, cells were incubated in RPMI 1640 medium (Life Technologies GmbH) supplemented with 20% (v/v) FCS, 100 μ g/ml gentamicin, 2 mmol/l L-glutamine (Life Technologies GmbH), 1 mmol/l sodium pyruvate (Sigma), and 2 ng/ml recombinant human interleukin-6 (IL-6; R&D Systems, Wiesbaden, Germany). In case of experiments comprising MSC and OPC, INA-6 cells were washed with PBS and resuspended in the respective propagation medium without IL-6 before used for co-culture studies [24]. OPM-2 [25], MM.1S [26], AMO1 [27], and U266 cells [28] were propagated and cultivated in RPMI 1640 medium comprising 10% (v/v) FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mmol/l L-glutamine, and 1 mmol/l sodium pyruvate.

CD19⁺ B-cells were used as a control for myeloma cell lines as previously published [29]. Primary CD19⁺ B-cells were purified from peripheral blood mononuclear cells (PBMCs) by magnetic activated cell sorting (MACS) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). PBMCs were obtained by ficoll density centrifugation of leukocyte concentrate. Leukocyte concentrate from whole blood donation (purchased from the Blood Donation Service of the Bavarian Red Cross, Wiesentheid, Germany) was diluted 1:1 (v/v) by PBS, coated onto Ficol[®] Paque Plus (GE Healthcare Europe GmbH, Munich, Germany) in a ratio of 2:1, and centrifuged for 30 min (609g, brake off). The interphase was washed with PBS buffer comprising 0.9% (w/v) sodium chloride (AppliChem GmbH, Darmstadt, Germany), 1% (v/v) FCS, and 2 mM EDTA (AppliChem GmbH). Isolation of CD19⁺ B-cells was performed according to the manufacturer's instructions using CD19 MicroBeads and LS columns (Miltenyi Biotec GmbH). CD19⁺ B-cells were incubated in RPMI1640 medium, 10% (v/v) FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mmol/l L-glutamine, and 50 μ mol/l beta-mercaptoethanol (Life Technologies GmbH) for one day prior to the experiments.

2.2.2. Differentiation of MSC to OPC

For differentiation of MSC to OPC, MSC were incubated for two weeks with DMEM High Glucose medium (Life Technologies GmbH) including 10% (v/v) FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and additionally supplemented with 10 mmol/l beta-glycerophosphate (Sigma), 100 nmol/l dexamethasone (Sigma), and 50 μ g/ml L-Ascorbic acid-2-phosphate (Sigma). Osteogenic differentiation medium and DMEM High Glucose medium (control) were changed every 3 to 4 days. Osteogenic differentiation was demonstrated by staining of alkaline phosphatase (ALP) and mineralized extracellular matrix using

alizarin red S. Staining of active ALP was performed using the Alkaline Phosphatase, Leukocyte Kit 86-C (Sigma) according to the manufacturer's instructions. For alizarin red S staining, cell monolayers were fixed with ice-cold methanol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and incubated for 2 min in 1% (w/v) alizarin red S (Sigma)/0.25% (v/v) ammonia (Merck KGaA, Darmstadt, Germany) staining solution. Stained monolayers were washed with distilled H₂O and embedded in mounting media (Tissue-Tek, Sakura, purchased from A. Hartenstein GmbH, Würzburg, Germany), before being examined microscopically. Alizarin Red S and ALP stainings of OPC, which had also been used for preparation of the OPC microarray specimens, are displayed as supplementary data (Supplemental Fig. 1).

2.2.3. Differentiation of MSC to OPC under co-culture with INA-6 cells

To investigate the effect of INA-6 cell contact on osteogenic differentiation of MSC, MSC were co-cultured with INA-6 cells while differentiating to the osteogenic lineage for 14 days. The protocol was similar to the procedure described by Giuliani et al. [9]. Briefly, 5×10^5 MSC were seeded per well (6-well cell culture plate) and allowed to attach overnight. The following day (day 1), 2×10^5 INA-6 cells per well were added and osteogenic differentiation was initiated by incubating the co-culture with osteogenic differentiation medium. Every third day, 50% of medium was changed. On day 15, co-cultures were washed with PBS and monolayers were stained for active ALP.

2.2.4. Co-culture of myeloma cells or B-cells with MSC or OPC

MSC (passage 0) were detached with 0.05% trypsin/EDTA (Life Technologies GmbH) at 70–90% confluency and re-seeded with 5×10^4 cells/cm² in 175 cm² cell culture flasks (for microarray sample preparation) or in 6-well cell culture plates (for all other experiments). In the case of OPC, osteogenic differentiation started the following day (day 1) as described above. For co-culture studies with myeloma cells, MSC and OPC were adapted to cell culturing conditions by incubating them with a 1:1 (v/v) mixture of MSC/myeloma cell medium and OPC/myeloma cell medium for 24 h at day 1 or day 15, respectively. For co-culture studies with B-cells, MSC were incubated for 1 day with a 1:1 (v/v) mixture of MSC/B-cell medium. The day after, bone-forming cells were co-cultured with CellTracker[®] Green 5-chloromethylfluorescein diacetate (5 μmol/l) (CMFDA; Lonza Group, Basel, Switzerland)-stained myeloma cells (2×10^6 cells in total, volume: 5 ml, 6-well cell culture plate; 3.5×10^7 cells in total, volume: 90 ml, 175 cm² cell culture flask) or B-cells (1.2×10^7 cells in total, volume: 5 ml, 6-well cell culture plate), resuspended in the respective 1:1 (v/v) media mixture. The CMTDA staining and co-culture procedure have been performed as recently described [30]. After 24 h, co-cultures were trypsinized and separated by fluorescence activated cell sorting (FACS) (BD FACS Aria[™] III cell sorter, Becton Dickinson GmbH, Heidelberg, Germany; performed at the Cell sorting core facility, Institute of Virology and Immunobiology, University of Würzburg; for microarray sample preparation) or by MACS (for all remaining experiments) using LD columns, CD45 MicroBeads (for INA-6 cells), CD38 and CD138 MicroBeads (for all remaining myeloma cell lines), or CD19 MicroBeads (for B-cells) according to manufacturer's instructions. Purity of CMTDA-negative MSC and OPC fractions were estimated by post-sort analysis (for FACS-separated cells) or microscopically (for MACS-separated cells). The respective control cells underwent the same procedure.

The indirect co-culture studies were performed according to the direct co-culture protocol described above in 6-well cell culture plates using transwell inserts of 0.4 μm pore membrane (Corning Inc. purchased from VWR International GmbH Darmstadt Germany) and by seeding INA-6 cells on top of the membrane.

2.2.5. Adhesion assay

The assay was performed similarly to the protocol that has been described for INA-6 cells by Holt et al. [31]. Briefly, white, clear-bottom 96-well cell culture plates (Greiner Bio-One GmbH, Frickenhausen,

Germany) were washed once with 100 μl distilled H₂O per well and supplemented with the solution of recombinant ANGPTL4 protein (ANGPTL4 full-length, R&D systems) or 1% bovine serum albumin (BSA) (control; Sigma). After incubation overnight, the wells were blocked with 1% BSA for 1 h, and washed three times with 100 μl hank's buffered salt solution (HBSS). INA-6 cells, starved for 1 h by incubating in RPMI 1640 medium without FCS and IL-6, were washed with HBSS two times, seeded in HBSS (5×10^4 cells/well), and allowed to attach for an additional 1 h at 37 °C and 5% CO₂. Following one washing step, adherent cells were quantified with the CellTiter-Glo[®] assay (Promega GmbH, Mannheim, Germany) according to manufacturer's instructions.

2.3. Isolation of total RNA and synthesis of cDNA

Isolation of total RNA was performed with the NucleoSpin[®] RNA II kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Briefly, synthesis of cDNA was carried out with 1 μg of total RNA, 1 μg of random primers (Life Technologies GmbH) or 50 pmol of oligo(dT)₁₅ primers (Promega GmbH), and 200 U of moloney murine leukemia virus reverse transcriptase (RT) (Promega GmbH) according to the manufacturer's instructions.

2.4. Semi-quantitative RT PCR analysis

The primers (listed in Supplemental Table 1) were created with the online software Primer3Plus. The PCR master mix was composed as previously described [32]. PCR analyses were run in a peqSTAR 2× thermocycler (Peqlab Biotechnologie GmbH, Erlangen, Germany) according to the following protocol: 1) 95 °C for 2 min, 2) 95 °C for 30 s, 3) annealing temperature (T_A) (primer specific, see Supplemental Table 1 for T_A) for 30 s, 4) 72 °C for 1 min, 5) 72 °C for 5 min, 6) chilled to 4 °C. PCR products were separated by agarose gel electrophoresis (2% agarose/0.5× GelRed[®] (Genaxxon Bioscience GmbH, Ulm, Germany)). Quantitation of the respective PCR band was performed densitometrically with the softwares Bio 1D (LTF, Wasserburg, Germany) and ImageJ (ImageJ 1.47; National Institutes of Health, Bethesda, MD, USA). Data were normalized to the expression level of the house-keeping gene *EEF1A1*. Specificity of PCR products was validated by sequence analyses using the BigDye Terminator v3.1 cycle sequencing kit (Life Technologies GmbH) and ABI 3130xL Genetic Analyzer (Applied Biosystems, Darmstadt, Germany; sequencing was conducted by the Institute of Human Genetics, University of Würzburg). Sequence data alignment was performed using the software BioEdit (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA) and the NCBI/Primer BLAST database.

2.5. Microarray analysis

Total RNA of co-cultured MSC and OPC as well as of the respective controls (each n = 5) were used for whole genome array analysis (Affymetrix, High Wycombe, United Kingdom; performed at the Institute of Cell Biology (Tumor Research), University of Duisburg-Essen). RNA (approximately 200 ng) was labeled with the GeneChip 3' IVT Express Kit according to the manufacturer's instructions. Fragmentation of cRNA was followed by 16 h hybridization of 10 μg sample at 45 °C on Affymetrix HG-U133 Plus 2.0 GeneChips, washing as well as staining using the Affymetrix Hybridization, Wash and Stain Kit and the Affymetrix Fluidics Station 450 (Affymetrix). Hybridization was recorded at 570 nm and analyzed with the Gene Chip Scanner 3000 with G7 update and GeneChip Operating Software 1.2, respectively (Affymetrix). Raw data are accessible at NCBI's Gene Expression Omnibus database through GEO accession number GSE87073. Raw data were processed with the statistical software R and the respective packages as recently described by Schlegelmilch et al. [33]. Probe sets with a log-Fold change (Fc) ≥ 0.5 and a log-Fc ≤ −0.5 were determined to be differentially expressed. The ClueGo plug-in for the software Cytoscape [34] was

used for comparative microarray analyses including differentially expressed probe sets with a $\log\text{-Fc} > 1.0$ (upregulated functionally grouped terms) or $\log\text{-Fc} < -0.5$ (downregulated functionally grouped terms).

2.6. Statistical analysis

Statistical tests were calculated with the software GraphPad Prism 6 (version 6.03; GraphPad Software, Inc., La Jolla, CA, USA). Analyses were performed using two-tailed Mann-Whitney test (validation of microarray analysis, direct co-culture with B-cells) or Kruskal-Wallis test with Dunn's post-hoc test (direct vs. indirect co-culture, adhesion assay). Differences were considered significant for $p \leq 0.05$ (*), $p \leq 0.01$ (**), or $p \leq 0.001$ (***)

3. Results

3.1. Contact with INA-6 cells inhibits the osteogenic differentiation of MSC

To investigate the effect of myeloma cell contact on skeletal precursor cells, we used the representative, MSC-dependent plasmacytoma cell line INA-6 [23,24] for co-culture experiments.

Since myeloma cells are known to hamper osteogenic lineage commitment of MSC [9], we first investigated the effect of INA-6 cell contact on the osteogenic commitment of MSC by staining active ALP in 2D co-culture that had been incubated with osteogenic differentiation medium for two weeks (Fig. 1A). While the amount of cells with active ALP increased in the MSC monolayer that underwent osteogenic differentiation, this effect was abolished in the presence of INA-6 cells, confirming an inhibitory influence of INA-6 cells on the osteogenic potential of MSC and thus its suitability for co-culture studies.

3.2. Contact with INA-6 cells induces changes in the transcriptome of skeletal precursor cells

As there are age-dependent differences in the transcriptome of MSC [35] and as MM is a disease of the elderly [36], we used MSC from older donors for our analyses. In addition to the experiments with MSC, we performed co-culture studies with MSC-derived OPC. Although osteogenic differentiation of pre-osteoblasts has been shown to be blocked by myeloma cell contact [9], to our knowledge no whole genome studies about MM cell contact-induced changes in the OPC transcriptome has been published yet.

To investigate the influence of myeloma cell contact on MSC and OPC transcriptomes, we performed 1 d co-culture studies comprising CMFDA-positive INA-6 cells and unstained bone-forming cells (Fig. 1B). The following day, MSC and OPC were purified from adherent INA-6 cells by FACS, gating the CMFDA-negative cells (Fig. 1C). Purity of MSC and OPC fractions was determined afterwards by post-sort analysis of the CMFDA-negative cell fractions (purity of MSC cell fractions: $98 \pm 0.3\%$ (mean \pm SEM, $n = 5$); purity of OPC fractions: $98.9 \pm 0.7\%$ (mean \pm SEM, $n = 5$)). The obtained RNA specimens were used for whole genome analyses.

A comparison between the transcriptome of co-cultured bone forming cells with the transcriptome obtained for the respective controls revealed INA-6 cell contact-induced changes in the gene expression of skeletal precursor cells (Fig. 2). In the case of MSC, 991 probe sets (783 up- and 208 down-regulated probe sets) were detected to be differentially expressed whereas co-cultured OPC harbored a changed expression pattern of 552 probe sets (292 up- and 260 down-regulated probe sets). Both transcriptome analyses showed consistent regulation in 181 probe sets (161 up- and 20 down-regulated probe sets), while no probe set was regulated contrarily.

Comparative analysis of probe sets with $\log\text{-Fc} > 1.0$ and $\log\text{-Fc} < -0.5$ displayed pathways that are differentially regulated (Supplemental Fig. 2 and Supplemental Fig. 3).

Physical interaction with INA-6 cells changed the transcriptome of MSC and OPC with regard to genes mediating an enhanced epithelial differentiation, playing a role in lipid and cholesterol storage, as well as cell-cell adhesion. Genes involved in DNA replication seem to be preferentially downregulated in co-cultured OPC while contact of INA-6 cells induces expression of genes in MSC that are associated with the impairment of osteoblast differentiation. In addition, upregulated pathways of MSC transcriptome analysis comprised B-cell receptor signaling. Due to the fact that MSC and OPC fractions could not be completely purified from INA-6 cells, differentially expressed probe set lists were additionally reworked by comparing these data with literature from NCBI's database PubMed and another transcriptome analyses comprising INA-6 cells (data not shown) to eliminate potentially B-cell- and myeloma cell-specific genes from the lists.

3.3. INA-6 cell contact changes the molecular signatures of skeletal precursors to a more tumor-promoting phenotype

Eleven genes were selected from each probe set list for re-evaluation. Selected genes code for disease-relevant factors, such as vascular endothelial growth factor A (VEGFA) [37], or give rise to a potential, targetable molecule and/or factor that is of relevance in bone and/or tumor biology (genes and abbreviations are listed in Supplemental Table 1). Differential expression of the mRNA transcripts was confirmed for microarray specimens by semi-quantitative RT-PCR using sequence-specific primers and densitometric analysis (Fig. 3). To investigate whether the transcriptional dysregulation of selected genes is transferable to bone-forming cells that have been in contact with other myeloma cell lines, we performed additional co-culture studies with MSC as well as OPC and the myeloma cell lines OPM-2, MM.1S, AMO1, and U266 and investigated the gene expression (with the exception of the recently published KISS1R [30]) of the specimens by PCR. In the case of upregulated probe sets, we were able to confirm an enhanced level of mRNAs in the co-cultured MSC and co-cultured OPC compared to the respective control cells for almost all selected genes, whereas the intensity of mRNA upregulation was dependent on the respective myeloma cell line used for co-culturing (Supplemental Figs. 4 and 5). However, we were not able to verify a downregulation of *MEOX2* and *FIGN* in MSC and OPC that had been in contact with OPM-2, MM.1S, AMO1, or U266 cells.

Database search of differentially expressed probe sets revealed that INA-6 cell contact changed the gene expression profiles of skeletal precursor cells to a more tumor-promoting transcriptome since differentially expressed genes are associated with disease activity and are known to interfere with bone metabolism (see Table 1 for abbreviations and references). Several genes coding for factors (APLN, STC1) or receptors/ligands (OSMR, MCHR1, SEMA4D, EFN2) that exert anti-anabolic action were upregulated after INA-6 cell contact, exclusively in undifferentiated MSC.

Since bone-forming cells are important interaction partners of MM cells throughout the course of the disease, we screened the lists for genes that are associated with mechanisms of malignant progression (see Table 2 for abbreviations and references). We noted an upregulation of the potential homing factors *CCL28* and *CCL26* in co-cultured MSC as well as OPC and OPC, respectively. As direct cell-cell contact to MSC is implicated in chemoresistance of MM cells (reviewed in [38]), the lists were scanned for genes coding for adhesion molecules. It is known that leukocyte function-associated antigen-1 (LFA-1) is expressed on myeloma cells and mediates adhesion to MSC by interacting with the ligand ICAM1 [39]. In addition to *ICAM1*, the MSC microarray list revealed an INA-6 cell contact-dependent upregulation of LFA-1 ligand *ICAM5* [40] and *Col11A1*. For OPC, the transcriptome analysis revealed an enhanced expression of *JAM2*. Moreover, another group of differentially expressed genes included those that code for pro-angiogenic factors, such as VEGFA, PGF, and ANGPTL4. These genes were expressed in co-cultured MSC and OPC, whereas the

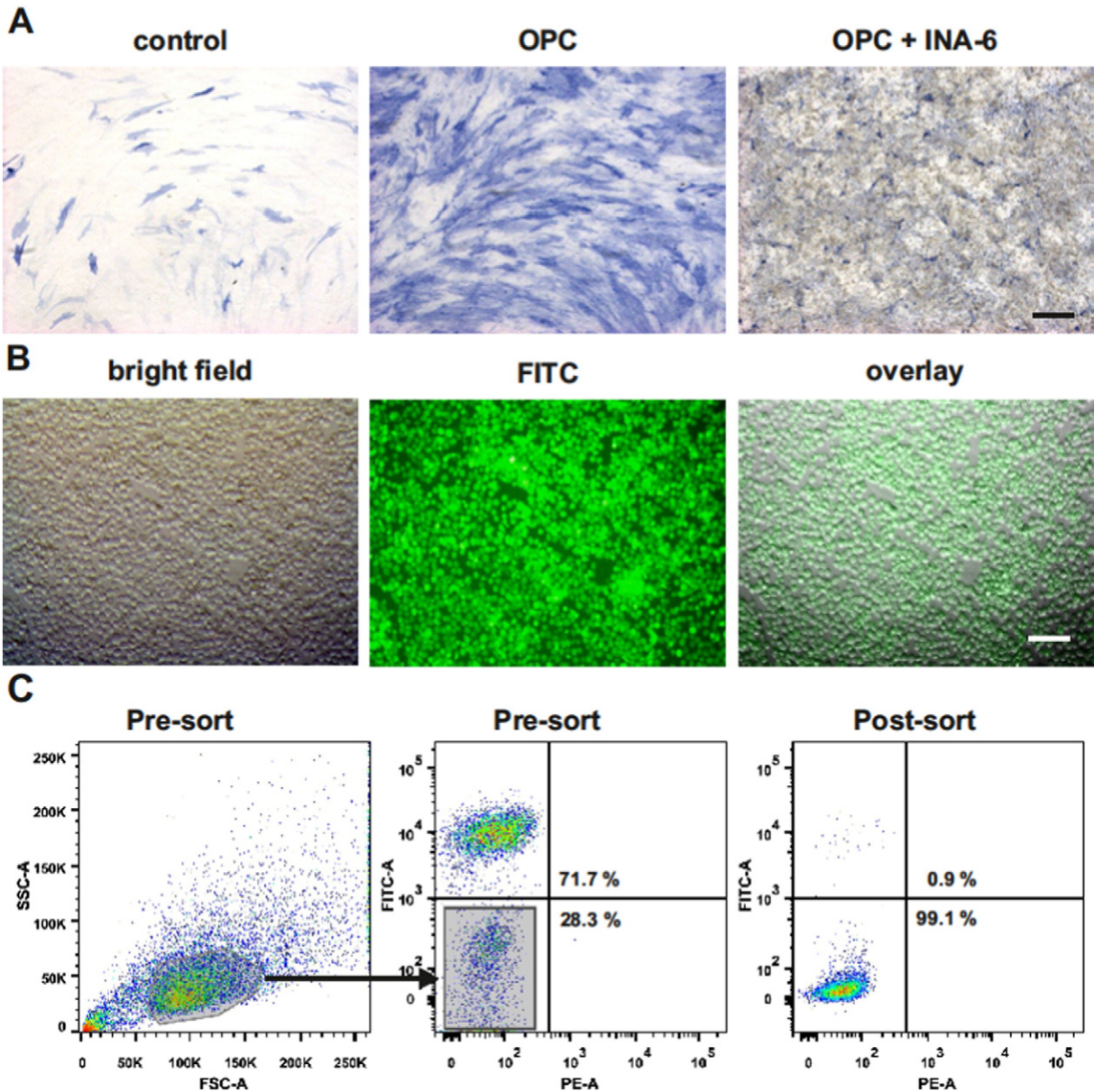


Fig. 1. MSC-INA-6 cell co-culture. (A) Representative ALP stainings of undifferentiated MSC (control), MSC cultured in osteogenic medium (OPC), and MSC that underwent osteogenic differentiation for two weeks while co-cultured with INA-6 cells (OPC + INA-6). Scale bar: 100 μ m (B) Displayed are 24 h co-cultures showing CMFDA-positive INA-6 cells attached to the MSC monolayer. Scale bar: 100 μ m (C) Representative dot plots of pre- and post-sort FACS analyses are shown.

undifferentiated stem cells have, according to the transcriptome analysis, a higher potential to stimulate endothelial cells. Additionally, we detected an upregulation of genes that code for adipokines (APLN, ANGPTL4), lipid storage proteins (PLIN2, HILPDA), and cholesterol transporters (ABCA1, VLDLR). In accordance with the MSC transcriptome, co-cultured OPC showed an induced expression of genes that are involved in lipid storage and fat metabolism. However, expression of several adipogenic transcription factors (*KLF8*, *KLF9*, *CEBPD*) and prostaglandin E2 synthesis relevant genes (*PTGS2*, *PTGES*, *PLCG2*) were exclusively upregulated in the undifferentiated MSC.

3.4. Upregulation of ANGPTL4 in MSC is mainly induced by direct cell-cell contact with INA-6 cells but not with CD19⁺ B-cells

In further studies, we concentrated on *ANGPTL4* since differential expression of this gene has not only been shown in our array results but

also in other independently performed transcriptome analyses comprising MM patient-derived MSC [17,41].

First, we were interested whether the differential expression of *ANGPTL4* is mediated by soluble factors or by direct cell-cell interaction. Therefore, indirect co-culture experiments with MSC and INA-6 cells using transwell inserts were performed in addition to direct co-culture studies. Semi-quantitative RT-PCR analysis revealed that differential expression of *ANGPTL4* in MSC is mainly mediated by physical interaction with INA-6 cells. The mRNA level of *ANGPTL4* was increased by 2-fold in MSC after indirect co-culture with INA-6 cells while gene expression was enhanced by 7-fold in directly co-cultured MSC (Fig. 4A).

Furthermore, we were interested whether differential expression of *ANGPTL4* is generally mediated by contact with B-cells. Therefore, expression analyses were performed with MSC specimens that were directly co-cultured with CD19⁺ B-cells, isolated from the blood of healthy donors. Semi-quantitative RT-PCR analysis revealed no differential expression of *ANGPTL4* mRNA in co-cultured MSC compared to the respective controls (Fig. 4B).

3.5. ANGPTL4 mediates adhesion of INA-6 cells

Since ANGPTL4 is an extracellular matrix protein and known to mediate cell-matrix interaction (reviewed in [42]), we tested the role of ANGPTL4 in MM cell adhesion. INA-6 cells were plated in wells that had been coated with different concentrations (1, 2.5, 5, and 10 $\mu\text{g/ml}$) of recombinant human ANGPTL4 protein (full-length). After 1 h of incubation, the number of adherent INA-6 cells was increased in a concentration-dependent manner. The amount of attached INA-6 cells was significantly enhanced by an average of 285% and 557% on 5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ ANGPTL4, respectively, compared to cells attached to control wells coated with BSA instead (Fig. 5).

4. Discussion

Results of the present study expand knowledge of signatures in skeletal precursor cells, coined by physical interaction with myeloma cells. Our data show for the first time, to the best of our knowledge, microarray analysis of osteogenically pre-differentiated MSC (OPC) that had been in contact with MM cells. These findings support the importance of the crosstalk of MM cells with skeletal precursor cells for myeloma bone disease development. Data reveal several novel mechanisms triggered in MSC and OPC that may lead to misbalanced bone remodeling, resulting in osteolytic bone loss with stunted bone regeneration.

Although the importance of direct cell-cell contact between skeletal precursor and myeloma cells for myeloma bone disease is known, whole genome analyses to evaluate myeloma cell contact-induced changes in skeletal precursor cells are limited. Unpublished results from Herman and colleagues, cited in the review of Reagan and Ghobrial [18], revealed the development of specific signatures in MSC and myeloma cells after 18 h of co-culture. Garcia-Gomez and co-workers confirmed that MSC are influenced by 24 h contact with MM.1S myeloma cells, resulting in a transcriptome with enhanced tumor-promoting features [19]. They performed direct co-culture studies in an elegant way by seeding the top and bottom of transwell inserts (pore size: 1 μm) with MM.1S and MSC, respectively [19]. In our hands, however, a direct cell culture system based on transwell inserts (pore size: 0.4 μm) was less efficient in terms of gene regulation (data not shown) when compared to physical contact without meshes between the cells. For this reason, we followed the latter strategy although the separation of the co-cultured cells is more challenging.

Using the plasmacytoma cell line INA-6, we were able to confirm that myeloma cell “barrier free” physical contact profoundly changes the transcriptome of MSC. In addition to MSC, the OPC transcriptome was also affected by physical interaction with INA-6 cells, although a smaller number of targets were differentially regulated. Co-culture with INA-6 cells changed the transcriptome of MSC and OPC with regard to 991 and 552 probe sets, respectively. The weaker response of differentiated cells is in line with a previous study showing that the transcriptome of osteoblasts from myeloma patients with and without osteolysis is less modulated compared to that of the MSC counterpart [43]. The number of differentially expressed probe sets in co-cultured OPC may be lower compared to co-cultured MSC, on the one hand, due to higher inter-experimental variability mediated by differences in the osteogenic potential and, on the other hand, by a lower adhesion of myeloma cells to OPC that were already protected by initial mineralization at time of co-culture. Nonetheless, INA-6 cells strongly adhere to

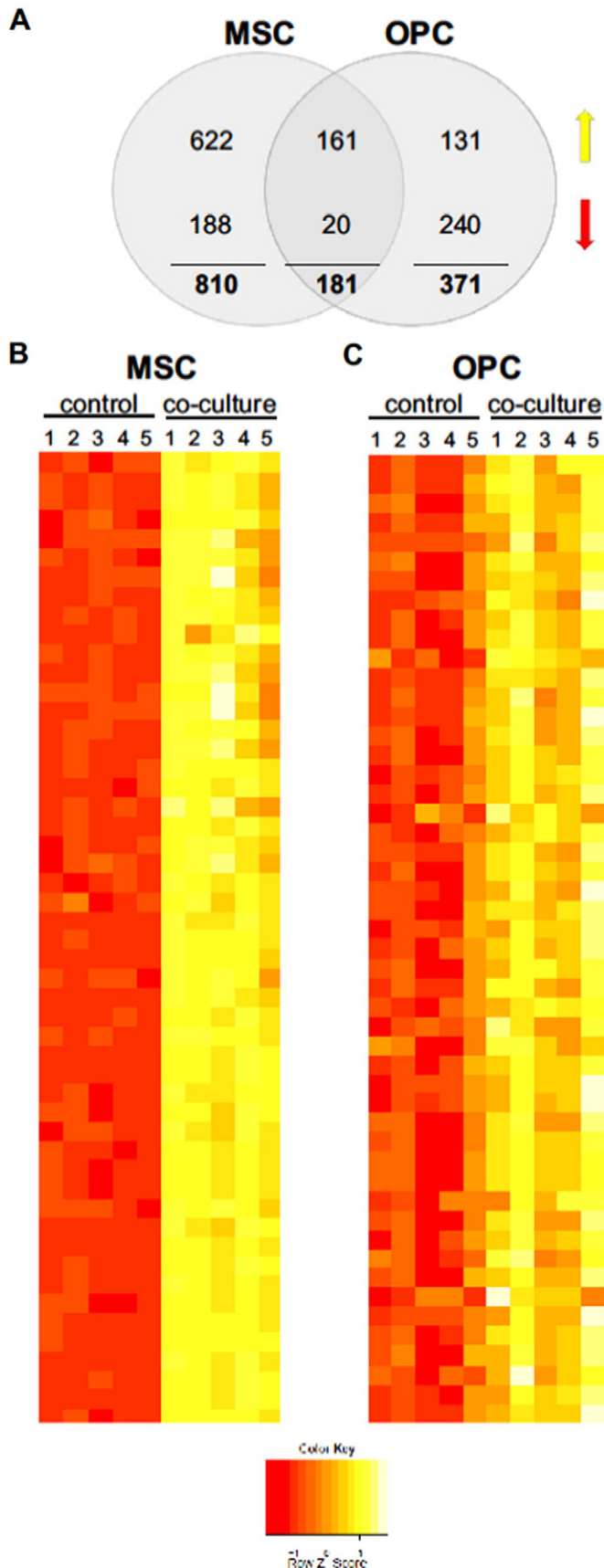


Fig. 2. Differential gene expression in MSC and OPC after INA-6 cell contact. (A) The Venn diagram shows the distribution of genes that are up- and downregulated according to the microarray analyses in MSC and OPC alone as well as in both cell types (depicted by the intersection). (B–C) Heatmap of the 50 most significantly up-regulated probe sets in (B) MSC or (C) OPC specimens (each $n = 5$) after 24 h contact to INA-6 cells (co-culture) in comparison to the respective control. Differential gene regulation is indicated by a color scheme: Yellow and red color represents up- and downregulation of the probe set, respectively.

Table 1

Differential expression of genes in skeletal precursor cells after 24 h INA-6 cell contact that are linked to MM or metastatic disease and dysregulated bone metabolism.

Symbol	Gene name	log-Fc MSC	log-Fc OPC		Reference
1.1 Genes coding factors that correlate with disease activity					
<i>VEGFA</i>	Vascular endothelial growth factor A	2.2	2.0	Level of VEGF correlates with disease activity markers. Bone marrow is suggested as the major source for VEGF.	[58]
<i>ANG</i>	Angiogenin, ribonuclease, RNase A family, 5	1.8		Serum level of ANG correlates negatively with survival rate of MM patients and positively with infiltration of plasma cells into the bone marrow.	[49]
<i>IL-16</i>	Interleukin 16	1.3		Bone marrow of myeloma patients shows overexpression of IL-16. IL-16 supports myeloma cell growth via an autocrine loop as both the cytokine and the receptors CD4 and/or CD9 were shown to be expressed by myeloma cells.	[48]
<i>BMP8B</i>	Bone morphogenetic protein 8b	1.6		Elevated expression of BMP8B in the bone marrow of gastric cancer patients correlates with metastatic disease.	[59]
<i>ANGPTL4</i>	Angiopoietin-like 4	3.0	2.0	Expression of ANGPTL4 correlates with esophageal squamous cell carcinoma as well as the metastatic potential of oral squamous cell carcinoma and early colorectal cancer to lymph nodes.	[60,61,62]
1.2 Genes coding factors that are associated with bone metabolism					
<i>APLN</i>	Apelin	1.1		Apelin knockout mice showed an increased bone mass by affecting osteoblast proliferation and differentiation.	[63]
<i>OSMR</i>	Oncostatin M receptor	1.0		Deficiency of osteoblasts for OSMR resulted in increased bone mass with limited bone resorption and formation in mice.	[64]
<i>MCHR1</i>	Melanin-concentrating hormone receptor 1	2.5		MCHR1 (−/−) mice develop osteoporosis.	[65]
<i>STC1</i>	Stanniocalcin-1	2.2		Expression of STC1 has a negative effect on bone mass and bone size.	[66,67]
<i>MEOX2</i>	Mesenchyme Homeobox 2	−1.6		<i>In vivo</i> studies in mice showed that both homeobox genes <i>MEOX1</i> and <i>MEOX2</i> are necessary for the proper development of axial skeleton and skeletal muscles.	[68]
<i>FIGN</i>	Fidgetin	−1.5	−1.1	Involved in mammalian development, including skeleton.	[69]
<i>ANGPTL4</i>	Angiopoietin-like 4	3.0	2.0	<i>ANGPTL4</i> is upregulated in high fracture bone of osteoporotic patients.	[70]
<i>SEMA4D</i>	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	1.1		Osteoclast-derived semaphorin 4D diminishes bone formation by acting via its receptor Plexin-B1, expressed on osteoblasts.	[71]
<i>EFNB2</i>	Ephrin-B2	−1.6		Bidirectional signaling between ligand ephrin-B2, expressed on osteoclasts, and EPH receptor B4, expressed on osteoblasts, regulates osteoclastogenesis negatively while osteoblastogenesis is enhanced; overexpression of EPH receptor B4 in osteoblasts resulted in a higher bone mass in transgenic mice.	[50]
<i>OMD</i>	Osteomodulin	−1.3		Overexpression of osteomodulin favors osteogenic differentiation of MC3T3E1 osteoblasts <i>in vitro</i> .	[72]

Fc = Fold change.

log-Fc MSC = log-Fc in array "MSC after INA-6 cell contact".

log-Fc OPC = log-Fc in array "OPC after INA-6 cell contact".

Indicated is the most differentially regulated probe set of the corresponding gene.

MSC and OPC, which requires certain efforts for separation of both cell types.

Except for a dysregulation of *ANGPTL4*, transcriptome analyses of MSC from MM patients (MM-MS), which have been published so far [17,19,41,43], largely lack consistency to our results. In addition, it has to be noted that comparisons of MM-MS transcriptome analyses only lead to minor overlaps, which may be the result of different experimental set-ups as discussed by Andre and colleagues [41]. Since harvesting of MM-MS is preceded by long-term cultivation and expansion, it is likely that the published analyses comprising MM-MS reflect a rather inherited and epigenetically modified phenotype [44], while our studies reproduce a physical contact-mediated profile. Thus, our data support the important influence of MM cell contact on MSC cell functions that are relevant for the progression of the disease. Nonetheless, our data show that mRNA expression of co-cultured bone-forming cells differs between the myeloma cell lines that were used. Therefore, we assume that the heterogeneity of myeloma [45] leads to diverse signatures in the bone marrow. Whether such differences can be assigned to myeloma subtypes and risk groups remains to be dissected. It seems reasonable to evaluate, in future research, whether specific myeloma cell-induced footprints in the bone marrow correlate with a high-risk myeloma phenotype and at the same time a high risk of developing myeloma bone disease.

According to our studies and the gene expression analyses, the bone-forming cells are susceptible to MM cell contact. The results of the microarray analyses indicate that homing of MM cells to bone and bone marrow induces new molecules and signaling pathways in the microenvironment. Transition of MGUS to MM is linked to changes in the bone marrow microenvironment, including an increased attraction of MM

cells to the bone marrow (reviewed in [46]) and an enhanced angiogenesis [47]. The transcriptome profiles presented here reflect these disease-relevant processes including several differentially expressed probe sets that code for (potential) homing factors (CCL26, CCL28), adhesion molecules (ICAM1, ICAM5, Col11A1, JAM2), and pro-angiogenic factors (among others *ANGPTL4*, *VEGFA*, *PGF*) (for references see Table 2). Supporting our *in vitro* data, previous *in vivo* data confirm that *ANG*, *VEGF*, and *IL-16* play a role in multiple myeloma and are detectable in plasma cell infiltrated bone marrow [48,49]. Our data show that the transcriptome of skeletal precursor cells changes with regard to an enhanced osteoclast-activating potential within hours of physical contact with myeloma cells. In addition to an enhanced activation of osteoclasts, myeloma bone disease is characterized by a hampered osteogenic differentiation potential of MSC and enhanced production of angiogenic factors that is, according to this study, also triggered in MSC early after INA-6 cell contact. The whole-genome analysis may provide novel targets and mechanisms, which result in blunted osteogenic potential. These include, for example, the expression of genes that code for osteoclastic proteins, such as semaphorin 4D and ephrin-B2 [50,51] and a lost expression of anabolic factors, indicated by downregulation of *OMD* in INA-6 cell co-cultured MSC. In addition, we detected a cluster of genes, possibly indicating a switch to a more lipogenic phenotype. Whether an adipogenic lineage commitment is favored in MSC and OPC at the expense of osteogenic regeneration remains to be proven, but has already been considered to play a role in the MM pathophysiology [52]. Although the whole genome analysis is limited to INA-6 cell co-cultured bone-forming cells, this lists may harbor a number of targets and mechanisms that are frequently or even generally involved in

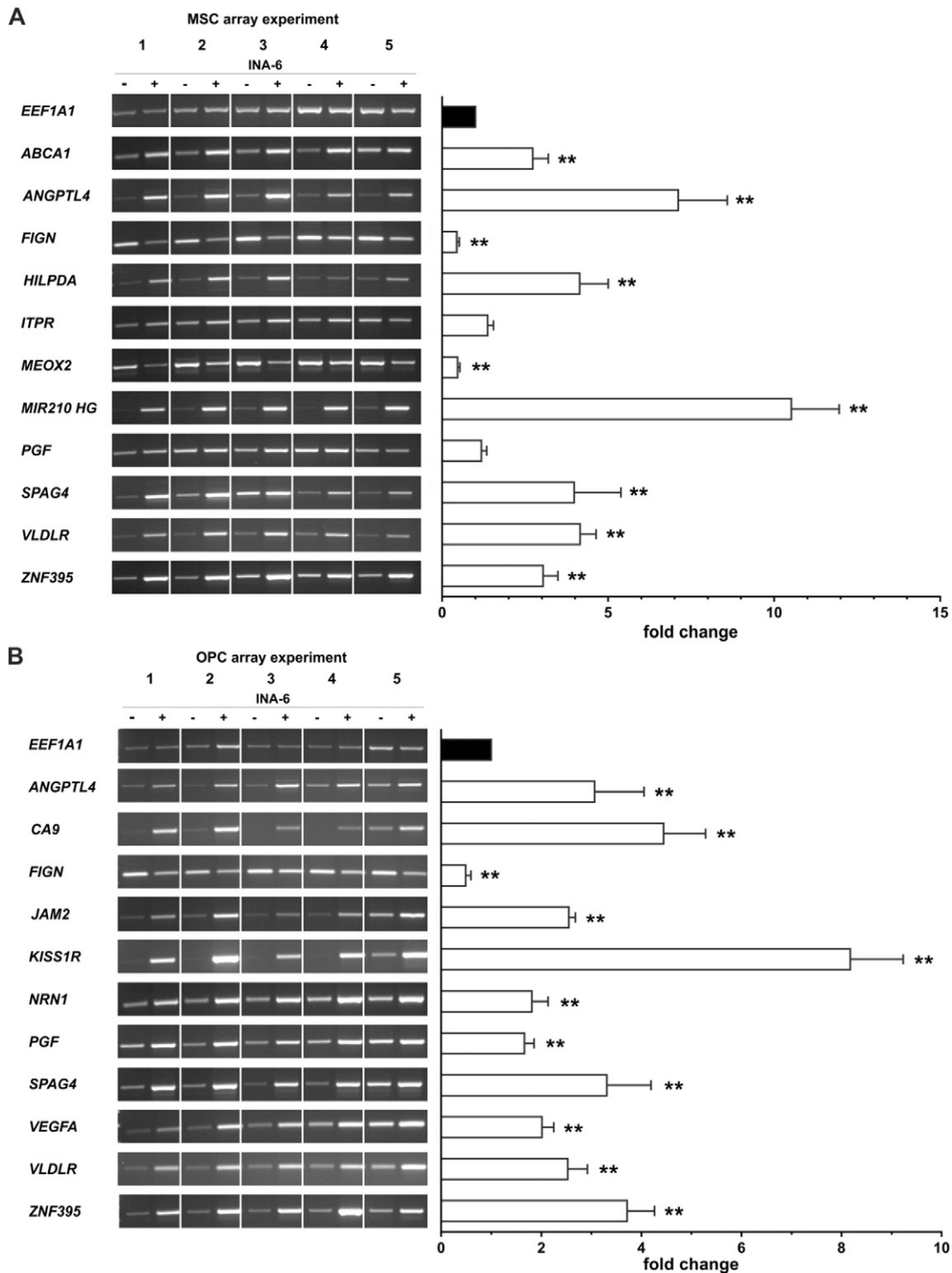


Fig. 3. Validation of differential gene expression in MSC and OPC after INA-6 cell contact. Microarray results were re-evaluated by examining the expression of 11 genes that were selected from the (A) MSC and (B) OPC microarray lists. Differential expression of the genes was validated using sequence-specific primer pairs, semi-quantitative RT-PCR, and cDNA from the same RNA specimens that had been used for gene chip hybridization prior to this. For quantitation, PCR products were analyzed densitometrically and normalized to the housekeeping gene *EEF1A1*. Representative agarose gel pictures and bar graphs, displaying the amount of amplified PCR products are shown. Bar graphs display mean transcript levels \pm SEM, $n = 5$. The basal expression level (black bar; control) was set to 1 while the respective changes of the mRNA levels in the co-cultured specimens are depicted as white bars. Statistic was performed with two-tailed Mann-Whitney test, analyzing the differences in expression levels between control cells (-) and cells after co-culture (+). Statistically significant differences are indicated by ** if the p -value was ≤ 0.01 . Gene symbols and descriptions are listed in Supplemental Table 1.

myeloma bone disease initiation and progression and are worth further investigation. The list of differentially regulated probe sets included a high number of genes with a putative impact on regulation of angiogenesis, for example the *KISS1R* (reviewed in [53,54]). Being the highest upregulated gene of the OPC array validation, the *KISS1R* was recently

published as an *in vivo* imaging biomarker of MM bone disease [30]. As a second significantly regulated gene involved in angiogenesis, we focused on *ANGPTL4* (reviewed in [55]) since this gene has been shown to be differentially expressed in MSC isolated from MM patients [17,41]. We were able to show a significant upregulation of *ANGPTL4*

Table 2

Differential expression of genes in skeletal precursor cells after 24 h INA-6 cell contact that may contribute to MM disease progression.

Symbol	Gene name	log-Fc MSC	log-Fc OPC		Reference
2.1. Homing					
<i>CCL26</i>	Chemokine (C–C motif) ligand 26		1.0	CCL26 activates chemokine (C–C motif) Receptor (CCR) 3, expressed on MM cells.	[73,74]
<i>CCL28</i>	Chemokine (C–C motif) ligand 28	1.5	1.5	Expressed by bone marrow stromal cells and possibly involved in the homing of plasma cells to bone marrow by binding to CCR3 and CCR10.	[74]
2.2 Adhesion					
<i>ICAM1</i>	Intercellular adhesion molecule 1	1.2		ICAM1 is a ligand for the leukocyte adhesion protein LFA-1, expressed on MM cells. Upregulated in osteoblasts after co-culture with myeloma cells. ICAM1/LFA-1 system may mediate apoptogenic signaling.	[39,75]
<i>ICAM5</i>	Intercellular adhesion molecule 5, telencephalin	1.2		ICAM5 is a ligand for LFA-1.	[40]
<i>COL11A1</i>	Collagen, type XI, alpha 1	1.3		Upregulated in osteoclasts co-cultured with myeloma cells.	[76]
<i>JAM2</i>	Junctional adhesion molecule 2		2.2	Jam2/3 interaction mediates adhesion between lung endothelial cells and melanoma cells, favoring metastasis of tumor cells to the lungs.	[77]
2.3 Angiogenesis					
<i>ANG</i>	Angiogenin, ribonuclease, RNase A family, 5	1.8		ANG shows activity in <i>in vitro</i> angiogenesis assay. It activates inositol-specific phospholipase C in endothelial cells.	[78]
<i>APLN</i>	Apelin	1.1		APLN shows angiogenic activity in an <i>in vitro</i> angiogenesis assay using retinal endothelial cells.	[79]
<i>STC1</i>	Stanniocalcin-1	2.2		STC1 exerts angiogenesis by inducing expression of VEGF in gastric cancer cells.	[80]
<i>VEGFA</i>	Vascular endothelial growth factor A	2.2	2.0	Ligand of Flk-1. Plays a role in blood vessel formation.	[81]
<i>ANGPTL4</i>	Angiopoietin-like 4	3.0	2.0	ANGPTL4 mediates VEGF-independent angiogenesis.	[82]
<i>PGF</i>	Placental growth factor	1.3	1.2	PGF is a ligand for VEGF receptors, like Flt-1, and enhance bioactivity of VEGF.	[83]
<i>NRN1</i>	Neuritin 1		1.7	NRN1 is a neurotrophic factor that exerts also angiogenic function <i>in vitro</i> and <i>in vivo</i> .	[84]
2.4 Adipogenesis and lipid metabolism					
<i>ZNF395</i>	Zinc Finger Protein 395	3.0	2.2	ZNF395 has a pro-adipogenic role. ZNF395 enhances adipogenic differentiation from dermal fibroblasts in concert with proliferator-activated receptor γ (PPAR γ) 2.	[86]
<i>KLF8</i>	Kruppel-like factor 8	1.2		Expression of transcription factor KLF8 is enhanced in 3T3-L1 cells that underwent adipogenic differentiation, upstream of PPAR γ and CCAAT/enhancer-binding protein α (C/EBP α).	[87]
<i>KLF9</i>	Kruppel-like factor 9	1.1		Expression of transcription factor KLF9 is upregulated in 3T3-L1 cells that underwent adipogenic differentiation. KLF9 is upstream of PPAR γ .	[88]
<i>CEBPD</i>	CCAAT/enhancer binding protein (C/EBP), delta	1.3		Induced in the early phase of adipogenic differentiation.	[89]
<i>ANGPTL4</i>	Angiopoietin-like 4	3.0	2.0	Inhibits lipoprotein lipase and regulates triglyceride metabolism. <i>ANGPTL4</i> expression is induced by PPAR and transforming growth factor- β ligands.	[90,91]
<i>APLN</i>	Apelin	1.1		APLN is an adipokine, which expression is regulated by insulin.	[92]
<i>PLIN2</i>	Perilipin 2	1.8	1.2	PLIN2 is a member of the Perilipin family, involved in lipid storage.	[93]
<i>HILPDA</i>	Hypoxia inducible lipid droplet-associated	3.1		Target gene of HIF-1. HILPDA overexpression potentiates intracellular lipid accumulation and expression of <i>VEGFA</i> , <i>migration inhibitory factor</i> , and <i>IL-6</i> .	[94]
<i>ABCA1</i>	ATP-binding cassette, sub-family A (ABC1), member 1	2.4		Cell-membrane transporter of lipophilic molecules, like cholesterol and phospholipids.	[85]
<i>VLDLR</i>	Very low density lipoprotein receptor	3.4	2.1	VLDLR is involved in cholesterol uptake.	[95]
<i>PTGS2</i>	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.0		PTGS2 is involved in prostaglandin E2 synthesis.	[96]
<i>PTGES</i>	Prostaglandin E synthase	1.8		PTGES is involved in prostaglandin E2 synthesis.	[96]
<i>PLCG2</i>	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	1.1		PLCG2 is involved in prostaglandin E2 synthesis.	[97]

Fc = Fold change.

log-Fc MSC = log-Fc in array "MSC after INA-6 cell contact".

log-Fc OPC = log-Fc in array "OPC after INA-6 cell contact".

Indicated is the most differentially regulated probe set of the corresponding gene.

mRNA in bone-forming cells after contact with INA-6, AMO1, and OPM-2 cells. According to our studies, the upregulation of *ANGPTL4* mRNA is not universal for B-cell contact, at least not for CD19⁺ B-cells. However, whether or not this induction in bone-forming cells is exclusively mediated by malignant B-cell contact remains to be proven by studies comprising other B-cell subpopulations, e.g. mature plasma cells.

Since *ANGPTL4* is an extracellular matrix protein [42], a potential role in adhesion of MM cells to MSC seemed likely. We were able to confirm an enhanced number of attached INA-6 cells to *ANGPTL4*-coated wells, implicating a potential role of *ANGPTL4* in mediating cell-cell interaction. However, this adhesion attitude is diminished in long-term cultures of INA-6 cells (data not shown).

Presence of key factors like *ANGPTL4* may mediate a disconnection between osteoblastogenesis and angiogenesis in myelomatous bones,

different to the healthy counterpart where angiogenesis is tightly coupled with osteogenesis [56]. A consistent, increased serum level of *ANGPTL4* and RANKL, a serum marker of bone resorption, has already been described for rheumatoid arthritis patients [57]. In addition, our group has shown that *ANGPTL4* is differentially expressed in the MSC transcriptome of osteoporotic patients [35] – reinforcing a potential role of this protein in pathological bone loss. Whether *ANGPTL4* serum levels correlate with osteolytic bone loss in myeloma and increase during the course of disease has to be evaluated. However, measurement of this protein in patient's serum may be an alternative to the measurement of the bone resorption biomarker RANKL, which is affected by treatment with anti-catabolic agents, like denosumab. The more in-depth evaluation of this gene product may represent another proof that mRNA changes are in fact followed by translational and functional consequences.

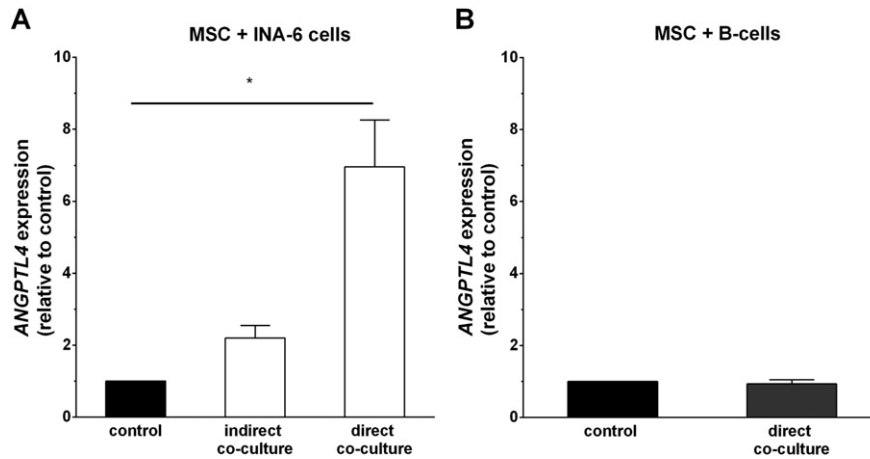


Fig. 4. *ANGPTL4* expression in MSC after co-culture with INA-6 cells and CD19⁺ B-cells. Levels of *ANGPTL4* mRNA in MSC after 24 h of (A) direct and indirect co-culture with INA-6 cells and (B) direct co-culture with CD19⁺ B-cells ($n = 3$). In case of direct co-culture, INA-6 and B-cells were depleted from MSC by MACS using CD45 and CD19 MicroBeads, respectively. Respective controls were treated accordingly. Expression of *ANGPTL4* was determined with semi-quantitative RT-PCR by using densitometric analysis and normalization to levels of *EEF1A1* mRNA. Bar graphs display the mean transcript levels \pm SEM. Statistic was performed with non-parametric Kruskal-Wallis and Dunn's post-hoc multiple comparison tests (Fig. 4A) as well as two-tailed Mann-Whitney test (Fig. 4B). Differences were considered to be significant if the p-value was ≤ 0.05 (*).

In summary, we identified a characteristic genomic signature in skeletal precursor cells after 24 h physical interaction with the myeloma cell line INA-6. The transcriptome analyses reflect changes in the gene expression profiles that have previously been linked to malignancy and are supporting a tumor-induced bone loss. According to the analysis, intrinsic pro-osteogenic pathways are downregulated in MSC that may be mediated by enhanced expression of osteoclast-derived coupling factors, an imbalanced ratio of anti-anabolic/anabolic factors, and a potential commitment to the adipocyte lineage. Furthermore, this hampered osteogenesis is associated with enhanced pro-angiogenic activity. This uncoupling between angiogenesis and osteogenesis may be triggered by an enhanced expression of key factors, like *ANGPTL4*, which further promote MM cell adhesion. These data reveal novel, potential trigger mechanisms and targets that trace the early processes of myeloma bone disease and offer new opportunities in the treatment scenario.

Conflict of interest

All authors declare no conflicts of interest except: Franz Jakob receives honoraria for lectures and consulting from Novartis, Procter & Gamble, Servier, Lilly, MSD, and Roche.

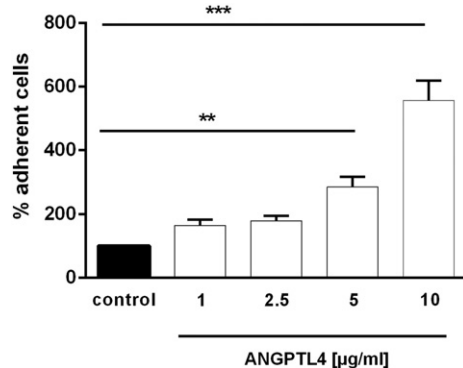


Fig. 5. Adhesion of INA-6 cells to *ANGPTL4* coating. INA-6 cells were pre-starved for 1 h and seeded on *ANGPTL4*-coated wells. After 1 h of incubation and plate washing, number of adherent INA-6 cells was determined using a luminescence-based assay. Percentage of attached cells was calculated by assessing cells attached at control wells (coated with 1% BSA) at 100%. Bar graphs display the means \pm SEM of nine values (three experiments, triple values).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bone.2016.08.006>.

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