Original article

Quantitative proteomics reveals novel functions of osteoclast-associated receptor in STAT signaling and cell adhesion in human endothelial cells

Claudia Goettsch a,1, Stefanie Kliemt b,1, Kathrin Sinningen a, Martin von Bergen b,c, Lorenz C. Hofbauer a,d, Stefan Kalkhof b,⁎

a Division of Endocrinology, Diabetes, and Bone Diseases, Dresden Technical University Medical Center, Dresden, Germany
b Department of Proteomics, Helmholtz-Center for Environmental Research-UFZ, Leipzig, Germany
c Department of Metabolomics, Helmholtz-Center for Environmental Research-UFZ, Leipzig, Germany
d DFG Research Center and Cluster of Excellence for Regenerative Therapies, Technical University, Dresden, Germany

A B S T R A C T

Previous studies indicate a novel role for the osteoclast-associated receptor (OSCAR) in oxidative stress-mediated atherogenesis. However, the functional role of OSCAR in endothelial cells is unknown. Here we characterized OSCAR signaling in human endothelial cells using a proteomic approach. OSCAR was either overexpressed or silenced, and the functional effects were assessed by an in-depth proteomic study using stable isotope labeling with amino acids in cell culture (SILAC). Reduction of complexity using subcellular protein fractions from the membrane, the cytosol, and the nucleus of human endothelial cells enabled the detection of 4975 unique proteins. Of these proteins, OSCAR overexpression regulated 145 and OSCAR silencing regulated 110. These proteins were mainly involved in cellular proliferation, inflammatory response and cell-to-cell signaling. Interestingly, OSCAR modulation reciprocally regulated signal transducer and activator of transcription 1 (STAT1) and 3 (STAT3). Thus, STAT1 and several interferon-induced proteins showed a clear inverse correlation to OSCAR expression, which was further verified by Western blot analysis. In contrast, it was found that OSCAR overexpression activated STAT3. Furthermore, OSCAR overexpression increased proteins involved in cell adhesion, which correlated with an increased adhesion of monocytes to the endothelium after OSCAR overexpression. In conclusion, using a comprehensive proteomic approach, endothelial cell-derived OSCAR was found to be involved in the STAT signaling pathway and to affect monocyte adhesion. This indicates a novel role of OSCAR in the vascular-immune cross-talk.

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relative protein quantification. The regulated proteins were clustered according to related biological processes and molecular functions to reveal the involved cellular processes influenced by OSCAR in human endothelial cells.

2. Materials and methods

2.1. SILAC sample preparation

Four biological replicates were prepared for SILAC experiments of OSCAR silencing and of OSCAR overexpression. Endothelial cells were labeled by cultivation in a SILAC-DMEM-flex-kit-medium (Invitrogen, Karlsruhe, Germany) containing stable isotope labeled ([U-13C6, 15N4]-l-arginine, [U-13C6]-l-lysine; heavy) or unlabeled arginine/lysine (light) for at least seven days before transfection. Beforehand, it was determined to allow for at least 7 million cell deviations to ensure at least 95% incorporation of heavy amino acids. The cells of the biological replicates were washed and lysed with 6 M urea, 2 M thiourea and protease inhibitor (Roche, Mannheim, Germany) to get whole cell extracts as described elsewhere [12]. For two of the four replicates, fractions of nuclei, membrane and cytoplasm were prepared using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany). Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL, USA).

2.2. Construction of plasmids encoding fusion proteins

All vector plasmids were obtained from Invitrogen. The coding cDNA sequence of human OSCAR (NM_206818) was amplified by PCR and cloned into the pcR-ii-TOPO vector (Invitrogen, Darmstadt, Germany). Stop codon was deleted by PCR and cDNA was cloned in the same vector. This vector subsequently cloned into the pcDNA4/TO/myc-His<sup>TM</sup> vector (Invitrogen, Darmstadt, Germany), resulting in pcDNA4/TO/OSCAR-myc-His plasmid. The integrity of each construct was confirmed by DNA sequencing.

2.3. Transfection and overexpression of fusion proteins

Human microvascular endothelial cells (HMEC)-1 (21) were kindly provided by Francisco J. Candal [13] and cultured in endothelial cell growth medium 2 (PromoCell, Heidelberg, Germany). Since primary cultures of human endothelial cells can only be transfected at very low efficiency levels, HMEC-1 cells already labeled with either heavy or light amino acids (described in ‘SILAC sample preparation’) were transfected with the pcDNA4/TO/OSCAR-myc-His plasmid or pcDNA4/TO/LacZ-myc-His plasmid as control using Fugene HD transfection Reagent (Roche, Mannheim, Germany). After 6 h, the supernatant was replaced by fresh medium. After 48 h proteins were lysed and processed for SILAC measurements.

2.4. Transfection of siRNA

HMEC-1 cells were treated with either siRNA against OSCAR (ON-TARGETplus SMART-pool, L-012922-00-0010) or non-target negative control (Dharmacon RNAi Technologies, Chicago, IL, USA), respectively. Transfection of 50 nM siRNAs was performed using DharmaFECT-1 transfection reagent (Dharmacon RNAi Technologies, Chicago, IL, USA). After 48 h proteins were lysed and processed for SILAC measurements.

2.5. Western blot analysis

Western blots were performed as described previously using the parameters given in the Supplementary Table 1 [10]. Quantification of protein expression was performed using ImageJ software.

2.6. ELISA

Cells were lysed in 20 mM Tris–HCl (pH 7.4), 1% SDS and protease inhibitor (Roche, Mannheim, Germany). OSCAR concentration was measured in 1 μg of whole-cell lysate using the ELISA kit for Osteoclast-associated Receptor from Uscln Life Science Inc. (Wuhan, China).

2.7. RNA preparation and real-time PCR

Total RNA from cell culture was isolated using the High Pure RNA Isolation kit (Roche, Mannheim, Germany). The mRNA expression was determined by SYBR green-based real-time PCR reactions as described previously [10]. Expression was normalized to β-actin. The results were calculated using the delta CT method and are presented as an x-fold increase relative to control.

2.8. Monocyte adhesion

HMEC-1 cells were transfected with pcDNA4/TO/OSCAR-myc-His or pcDNA4/TO/LacZ-myc-His as a control. THP-1 cells were labeled with CellTracker-green (Invitrogen, Darmstadt, Germany). Forty-eight hours after transfection 300,000 labeled THP-1 cells were added to the endothelial cells and incubated for 15 min. HMEC-1 cells were stimulated with 100 μg/ml oxLDL for 3 h prior to THP-1 addition. After two washing steps, attached monocytes were determined by fluorescence measurement using the FluoStar-Optima (BMG, Offenburg, Germany).

2.9. Protein separation, liquid chromatography/tandem mass spectrometry and data analysis

Equismol amounts of each of the protein lysates (heavy-labeled: light-labeled) were mixed prior to separation by 1D-SDS-PAGE and prepared for LC-MS analyses [14]. Protein mixtures (in total 30 μg) were precipitated with acetone and dissolved in 0.5 M Tris–HCl buffer pH 6.8 containing 40% SDS (v/v), 20% (v/v) glycerol, 2% (v/v) bromo phenol-blue, and 10% (v/v) 2-mercaptoethanol. After heating for 5 min at 60 °C, a 1D-SDS-PAGE (12% resolving gel, 4% stacking gel) was carried out according to Laemmli [15]. Gel lanes were cut into five pieces; proteins were digested with trypsin and extracted peptides were measured by nano-HPLC/nano-ESI-MS/MS by an online reversed-phase nanoscale liquid chromatography tandem mass spectrometry on a NanoAcuity UPLC system (Waters Corporation, Milford, MA, USA). This UPLC system was connected to an LTQ-Orbitrap XL ETD (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a nano-ESI source (TriVersaNanoMate, Advion, Ithaca, NY, USA) as described earlier [16] using a 4 h acetonitrile gradient (2–85% phase B) with a flow rate of 300 nl/min. MS analysis was performed in positive ion mode using a continuous scanning of eluted peptide ions in a mass range m/z 350–1600. The system automatically switched to CID-MS/MS mode on the six most intense ions with an intensity of more than 3000 counts and a charge state higher than 1. For protein identification and relative quantification MaxQuant software (version 1.1.1.25, www.maxquant.org) with default parameter settings was used [17]. Database search was carried out by the integrated search engine Andromeda against the IPI human database version 3.68. A peptide FDR specification of 0.01 was applied, whereas the precursor mass tolerance was set to 10 ppm, and refined during MaxQuant processing as described [18]. Oxidation (methionine), acetylation (protein N-termi) and deamination (glutamine) were used as variable modifications. Carbamidomethylation (cysteine) was set as fixed modification and a maximum of two tryptic missed cleavages were allowed. MaxQuant combines proteins into one group whenever the set of identified peptides for one protein is equal to a set of peptides from another protein. Those shared peptides are then associated with the group with...
the highest number of identified peptides (so called razor peptides). Nevertheless they remain in all protein groups where they occur [18]. Due to this fact we used a minimum of two unique peptides for a distinct peptide assignment as well as for an accurate protein identification and quantification. Of each remaining protein group which were indistinguishable by the identified peptides representative entries one protein was selected for further pathway analysis. Raw files of the LC-MS/MS analyses are available on Tranche file sharing server (https://proteomecommons.org/tranche/).

2.10. Clustering of proteins according to biological function and involved transcription factors

Proteins, which were differentially expressed with a fold change ±0.5 in the replicates were considered to be regulated and were used for a cluster analysis with the web-delivered application ‘Ingenuity Pathway Analysis’ (IPA; ‘Winter Release (2011)’, www.ingenuity.com, Ingenuity Systems, Redwood City, CA, USA). Regulated proteins of either the silencing or the overexpression experiments were mapped to their corresponding gene object in the Ingenuity Pathways Knowledge Base and further used for generating biological networks, biological functions and involved transcription factor were inferred. Thereby, the overexpression and silencing experiments were treated independently. The results of the tool ‘functional analysis’ were used to extract biological functions which were significantly altered in the data sets. Biological networks with a size of no more than 35 genes are built upon generating interactions between the so called ‘focus genes’ and all other genes stored in the knowledge base. The score for each network is calculated according to the set of the user’s significant genes. It indicates the likelihood of the genes in a network being found together due to random chance and is derived from a p-value calculated by Fisher’s exact test which is then further used for ranking the functions according their significance within this network.

Additionally ‘transcription factor analysis’ was used to identify transcription factors that are activated or inhibited within the dataset with a p-value < 0.05. This analysis uses literature-based effects between transcription factors and target genes compiled in the Ingenuity Knowledge Base. Targets in the dataset are examined and compared according to the targets’ direction of change to the literature findings. IPA uses the z-score algorithm to make predictions. If the direction of change is consistent IPA predicts that the transcription factor is more active (z-score is ≥2) and if it is more inconsistent the transcription factor is less active in the experimental dataset (z-score ≤ −2). No prediction is made when there is no clear pattern related to the literature.

2.11. Statistical analysis

Data are given as means ± s.d., and n indicates the number of independent experiments. Statistical analyses were performed using a one-way ANOVA with Bonferroni’s post hoc test, single group comparisons using a Student’s t test or as described above. A value of p < 0.05 was considered statistically significant.

3. Results

3.1. Modulation of OSCAR expression

To determine cellular functions of OSCAR in human endothelial cells, we used either silencing or overexpression in combination with comparative proteomics and functional cluster analysis of regulated proteins (Fig. 1). Overexpression caused a 1.5-fold OSCAR induction (155.0% ± 0.1%), whereas the transfection of the LacZ control vector did not affect OSCAR expression as assessed by ELISA (Fig. 2A). The use of siRNA technology led to a 44.0% ± 0.2% decrease in OSCAR expression compared to endothelial cells treated with scrambled siRNA (Fig. 2A). These data were confirmed by Western blot (Fig. 2B; fold increase to control (100%): OSCAR overexpression 207% ± 47%; OSCAR silencing 19% ± 5%) and real-time PCR (Fig. 2C). None of the tested conditions altered cell morphology and cell growth.

3.2. Determination of differentially expressed and affected proteins by quantitative proteomics

In order to elucidate the function of endothelial-derived OSCAR we applied a three-dimensional separation procedure, in which proteins were first separated based on cellular localization to reduce...
The mean±s.d. *P<0.05. Three independent experiments are shown. Numbers indicate the fold increase.

ments, respectively (Supplementary Tables 2 and 3).

pairs of SILAC peptides in the overexpression or silencing experi-

cient was used as loading control. 

expression (n=3). Data present the mean±s.d., *P<0.05.

complexity, secondly by molecular weight (SDS-PAGE), and finally

proteolytic peptides were separated according to their hydrophobici-
ty (reverse-phase HPLC). 

To provide a comprehensive and reproducible analysis, we used

four biological replicates, where replicates 1 and 2 were measured

by a standard gel LC-MS analyses and replicates 3 and 4 were

subjected to a subcellular fractionation prior gel LC-MS analyses.

Using this data in combination with the three-dimensional separation

procedure we were able to identify a total of 4975 unique proteins

subjected to a subcellular fractionation prior gel LC-MS analyses.

found to be normally distributed (Figs. 3B, D). Furthermore the log2-ratios (OSCAR overexpression vs. control and OSCAR silencing vs. control) of protein expressions were found to be normally distributed (Figs. 3B, D).

3.3. Differential expression of proteins caused by OSCAR overexpression

In response to OSCAR overexpression the expression of 145 pro-
teins (5.1% of the quantified proteins) was determined to be signifi-
cantly up or down-regulated by more than 50% (Supplementary Table 4a, Fig. 3B). Using the IPA-software, ten networks with highly significant scores between 24 and 46 were identified (Supplementary Table 5a). According to the enrichment analysis, the six most signifi-
cant biological functions for each network were: cellular growth and proliferation (score 45, 24 proteins), inflammatory response (score 42, 22 proteins), cellular development (score 38, 21 proteins), con-
nective development and function (score 36, 20 proteins), metabolic disease (score 31, 18 proteins) and cell-to-cell signaling and interac-
tion (score 28, 18 proteins) (Supplementary Table 5c).

3.4. Differentially expressed proteins in response to OSCAR silencing

Of the 2105 proteins which have been quantified in the silencing

experiment, 110 proteins (5.2%) were determined to be differentially

expressed (Supplementary Table 4b, Fig. 3D). Following OSCAR sili-

cening, the most affected functions issued by IPA were: cellular
growth and proliferation (score 45, 24 proteins), inflammatory re-

response (score 31, 16 proteins), cell death (score 27, 15 proteins), en-

ergy production (score 26, 14 proteins) and cell-to-cell signaling and interaction (score 22, 13 proteins) (Supplementary Table 5b and d).

3.5. Transcription factor analysis

Next, IPA ‘transcription factor analysis’ was used to identify trans-

cription factors that were responsible for altered gene expression

causd by OSCAR overexpression or silencing. N-Myc was predicted
to be the most relevant transcription factor with p-values of

4.19×10−13 and 3.31×10−10, respectively. In OSCAR silenced cells

N-Myc was predicted based on 15 altered genes, whereas in OSCAR

overexpression, the expression of 16 differentially expressed proteins

was controlled by N-Myc (Supplementary Table 5e, f). These findings

were supported by Western blot analysis demonstrating slightly re-

duced N-Myc expression by OSCAR overexpression, whereas OSCAR

silencing tremendously induced N-Myc expression (Fig. 4C). In the

transcriptional networks, most of the affected genes are involved in

cell adhesion (e.g. integrin β1, fibronectin, tissue growth factor) or

cellular component organization (e.g. elongation factor 2, caveolin 1).

3.6. OSCAR is involved in STAT signaling pathway

Expression of STAT1 (log2 ratio (OSCAR regulation/control) −0.70±0.23) as well as eight IFN-inducible proteins was suppressed by

OSCAR overexpression (Fig. 4A, Table 1). To confirm this finding, we performed Western blot analysis of interferon-induced GTP-

binding protein Mx1 and total or phosphorylated STAT1 and STAT3. In agreement with the SILAC data, OSCAR overexpression caused signi-

ficant reduction of Mx1 and STAT1 expression, whereas OSCAR silencing induced Mx1 and STAT1 protein expression as well as STAT1 activation, as evident from its markedly increased phosphorylation at Tyr701 (Figs. 4B, C). In line with these findings, MHC class II transactivator CIITA, a known downstream target of STAT1, was repressed by OSCAR overexpression and induced by OSCAR silencing (Fig. 4C). On the other hand, overexpression of OSCAR promoted STAT3 expression as

Fig. 2. Validation of OSCAR modulation. (A) OSCAR overexpression and silencing was measured by ELISA (n=4). (B) Western blot shows the induction of OSCAR by overexpression (OSCAR) and suppression by siRNA (si OSCAR). LacZ and a scramble control (scramble con) served as controls, respectively. β-actin was used as loading control. Three independent experiments are shown. Numbers indicate the fold increase. *P<0.05 vs. corresponding control. (C) OSCAR mRNA expression. n=3. Data present the mean±s.d., *P<0.05.
well as activation (phosphorylation at Tyr705) and its repressor protein inhibitor of activated STAT3 (PIAS3), whereas OSCAR silencing reduced STAT3 and PIAS3 levels (Figs. 4B, C). Thus, our results suggest that STAT1 and STAT3 are reciprocally activated by OSCAR.

3.7. OSCAR regulates cell-adhesion mediated signaling as well as monocyte adhesion

OSCAR overexpression increased several proteins which are involved in the cell-adhesion-mediated signaling up to 2.1-fold (Fig. 5A, log2 ratio (OSCAR regulation/control): vinculin 2.1±0.1, filamin A 1.6±1.2, catenin α1 1.0±0.3), whereas OSCAR silencing caused a decrease of cell-adhesion proteins (log2 ratio: ICAM-1 −1.2±0.4, PECAM-1 −1.0±0.1, integrin β1 −0.8±0.3, Fig. 5A). Representative, the expression of integrin β1 (overexpression 0.8±1.2, silencing −0.8±0.3) and ICAM-1 was confirmed by Western blot (Figs. 5B–D). ICAM-1 protein expression increased by OSCAR overexpression (Fig. 5C), whereas OSCAR silencing caused ICAM-1 repression (Fig. 5D). The functional relevance of these findings was confirmed by assessing monocyte adhesion. While monocyte adhesion to OSCAR-silenced endothelial cell did not alter (data not shown); OSCAR overexpression significantly increased monocyte adhesion to
the endothelium (206%±26%, n=4, p<0.05), which was comparable with the effect of oxidized low-density lipoprotein (oxLDL) (Fig. 5D). The potential functions of OSCAR in human endothelial cells are summarized in Fig. 6.

4. Discussion

OSCAR, a receptor initially characterized as co-stimulatory regulator of osteoclast and dendritic cell function, was recently identified as a novel receptor on vascular endothelial cells that is regulated by oxLDL in a calcium/NFAT-dependent manner [10]. In order to explore the signaling pathways influenced by OSCAR in endothelial cells, we analyzed the effects of OSCAR overexpression and silencing on global protein expression by quantitative proteomics.

In vascular endothelial cells, we found an inverse regulation of STAT1 and STAT3, with activation of STAT3 occurring with OSCAR overexpression, and activation of STAT1 occurring with OSCAR silencing. Among others such as paired Ig-like receptor A, platelet glycoprotein VI or leucocyte Ig-like receptor, OSCAR is one of the known receptors that do interact with the immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecule Fc receptor γ-chain [19]. Thus, regulation of STAT signaling by cross-talks to ITAM-dependent pathways [20] might be directly linked to and controlled by OSCAR. It has been shown that strong activation of ITAM signaling suppresses JAK/STAT-signaling. Conversely, low-level basal activation of ITAM-signaling can result in an increased tyrosine or serine phosphorylation of STAT1 and thus enhanced transcriptional activity [21]. In this context, it is interesting to note that in agreement with STAT1 activation in endothelial cells, caused by silencing of OSCAR, we observed a reduction of several IFN-induced proteins known to lie downstream of STAT1.

In osteoclasts, OSCAR itself is controlled by both, the STAT3 and the STAT1 pathways, either by the protein inhibitor of activated STAT3 (PIAS3) [22] or by IFN-γ induction of the MHC class II transactivator CIITA, which diminishes OSCAR expression in osteoclasts [23,24]. Thus, we postulate the potential existence of a feedback loop in endothelial cells in which STAT1 and OSCAR regulate each other by a strong ITAM activation by OSCAR, which could result in a reduced activation of STAT1 and thus finally in a reduced activity of the OSCAR suppressing CIITA.

STAT1 and STAT3 are well-known antagonistic transcription factors which are activated by the opposing cytokines IFN-γ and interleukin (IL)-10, respectively [25]. A balanced expression/activation of STAT1 and STAT3 is important to modulate the responses to cytokines and growth factors such as macrophage activation (activation by STAT1 vs. inhibition by STAT3), cell proliferation (suppression by STAT1 vs. promotion by STAT3), and T cell differentiation (Th1 responses promoted by STAT1, Th17 responses supported by STAT3) [26].

Additionally, in endothelial cells, the balance of STAT1 and STAT3 is critical, since coordinated STAT1 activation and STAT3 inactivation are
required to control STAT1-dependent gene transcription such as that of cytokine-activated GTP cyclohydrolase [27]. Based on the reciprocal responses of STAT1 and STAT3 observed in our experiments, we suggest that in human endothelial cells, the cytokine-dependent balanced activation of these two STAT proteins may be mediated by OSCAR. STAT proteins have also been implicated in the development and progression of cardiovascular diseases. STATs are activated in atheroma-tous plaques and may be involved in the stimulation of cells by vascular endothelial growth factor, hepatocyte growth factor and angiotensin II. STATs cause thrombogenesis due to the regulation of either major histocompatibility complex class II molecules or phospholipase A2 [28]. Both, STAT1 and especially STAT3, play important roles in vascular remodeling [28] and wound re-epithelialization [29]. Additionally, it has been shown that STAT3 increased resistance to apoptosis in pulmonary artery endothelial cells [30] and prevented endothelial cell dysfunction and inflammation induced by chronic alcohol intake [31]. This is consistent with our previous findings of reduced apoptosis and increased cell viability upon OSCAR activation in human endothelial cells [10], which in turn might be mediated by the STAT3 activation observed in the current experiments.

Interestingly, N-Myc, which is induced by IFN-γ via STAT1 [32], was determined to be the most affected transcription factor by OSCAR silencing and overexpression. N-Myc limits cell adhesion to the extracellular matrix and promotes cell migration by down-regulating integrin [32–35], which strengthens our observation of integrin β1 repression by OSCAR silencing. A cluster of proteins related to cell adhesion and cell adhesion-mediated signaling was altered by OSCAR overexpression or silencing. We observed an induction of vascular adhesion molecules such as integrins, ICAMs and PECAM by OSCAR overexpression, which are established markers in the development and progression of atherosclerosis [36]. In accordance with these findings, we found an increased monocyte adhesion to the endothelium in response to OSCAR overexpression. Interestingly, activation of OSCAR on dendritic cells increases the production of monocyte chemo-attractant protein 1, which is involved in the recruitment of leukocytes to sites of inflammation [37]. In addition, aggregation of OSCAR on human monocytes triggers intracellular calcium release and is responsible for the sustained secretion of high levels of IL-8, which is an established factor for the induction of monocyte adhesion to the endothelium [38,39]. Based on this data, OSCAR may modulate monocyte cell
Fig. 6. Potential role of OSCAR in STAT signaling and cell adhesion processes—a working model. The STAT1 pathway is activated by OSCAR silencing, whereas the STAT3 pathway activation is induced by OSCAR overexpression. Activation of STAT1 induces, CIITA, IFN-inducible genes and activates the transcription factor N-myc, which could inhibit integrin. OSCAR overexpression induces cell adhesion molecules (like ICAM-1) and thereby may promote monocyte adhesion to the endothelium. ICAM, intracellular adhesion molecule; ITAM1, immunoreceptor tyrosine-based activation motif containing adaptor molecules; oxLDL, oxidized low density lipoprotein; PIAS3, protein inhibitor of activated STAT3; CIITA, class II, major histocompatibility complex, transactivator. This model reflects the data from this and other studies.

In conclusion, we provide evidence for an active role of OSCAR in vascular-immune cell cross-talks by affecting STAT signaling as well as cell adhesion processes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2012.09.003.

Disclosures

None declared.

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