

RESEARCH ARTICLE

STAT3 signaling induced by the IL-6 family of cytokines modulates angiogenesis

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ABSTRACT

Aberrant angiogenesis is a hallmark of cardiovascular and retinal neovascular disease. The STAT3 signaling pathway represents a potential pharmacological target for these diseases due to its impact on angiogenesis. Surprisingly, some STAT3 activators, such as the IL-6 cytokine family member oncostatin M (OSM), enhance angiogenesis, whereas others, such as ciliary neurotrophic factor (CNTF), reduce it. This study aimed to clarify these conflicting effects. In contrast to the anti-angiogenic cytokine CNTF, the pro-angiogenic cytokine OSM was able to activate intracellular signaling pathways beyond the STAT3 pathway, including the ERK and AKT pathways. These differences translated into transcriptomic and metabolic shifts. siRNA-mediated STAT3 knockdown experiments showed a decrease in VEGF-induced endothelial migration and sprouting, enhancing the pro-angiogenic drive of OSM and switching the CNTF response from anti-angiogenic to pro-angiogenic. These effects correlated with a transcriptomic shift representing enhanced STAT1 and ERK activity following STAT3 knockdown, including a compensatory prolonged phosphorylated STAT1 activity. In conclusion, the angiogenic effect of STAT3 appears to be determined by cytokine-induced STAT3 specificity and simultaneous activity of other intracellular signaling pathways, whereas the STAT3 pathway, predominantly recognized for its pro-angiogenic phenotypes, reveals novel anti-angiogenic potential.

KEY WORDS: Angiogenesis, Endothelial cell, STAT3, IL-6 family cytokines, Oncostatin M, Ciliary neurotrophic factor

INTRODUCTION

Angiogenesis is a complex process that is critical to physiological development as well as disease progression. It plays a key role in macrovascular as well as microvascular diseases including arteriosclerosis (Camaré et al., 2017), stroke (Navaratna et al., 2009; Deveza et al., 2012) and diabetic retinopathy (Crawford et al., 2009). Moreover, tumors tend to stimulate angiogenesis to fuel their need of metabolites for uninhibited growth (Viallard and Larrivé, 2017).

Targeting angiogenesis hence represents an important therapeutic approach. Understanding the underlying mechanisms of angiogenesis on a molecular level is a crucial prerequisite for the basis of identifying novel therapeutic targets.

Vascular endothelial growth factor (VEGF or VEGFA) is to date the most prominent cytokine that regulates angiogenic processes in disease (Melincovici et al., 2018). However, a deeper understanding of the pathomechanisms behind specific diseases such as arteriosclerosis and retinal neovascular disease has revealed that inflammatory processes also modulate angiogenesis (Dan-Brezis et al., 2019; Wolf and Ley, 2019). Among others, the interleukin 6 (IL-6) cytokine family plays a key role in mediating inflammatory responses and shows potential to modify inflammation-induced angiogenesis (Zhang et al., 2015; Jones and Jenkins, 2018).

The IL-6 cytokine family consists of multiple cytokines, including IL-6, ciliary neurotrophic factor (CNTF) and oncostatin M (OSM). They all bind to a similar receptor complex, share intracellular signaling pathways and ultimately share downstream targets as well (Heinrich et al., 2003). All IL-6 cytokines signal through the gp130 (IL6ST)-mediated recruitment of receptor-associated Janus kinases (JAKs), which leads to phosphorylation and therefore activation of signal transducer and activator of transcription (STAT) proteins, especially STAT3 (Heinrich et al., 2003; Jones and Jenkins, 2018). STAT3 was often reported to promote tumor growth (Johnson et al., 2018; Hu et al., 2019) and angiogenesis (Jung et al., 2005). However, the effects of IL-6 family cytokines on angiogenesis are inconsistent, despite their shared activation of the STAT3 signaling pathway (Vasse et al., 1999; Bucher et al., 2016, 2020).

Recent studies on CNTF suggest an anti-angiogenic effect of CNTF-induced STAT3 signaling on vascular endothelial cells and retinal angiogenesis (Bucher et al., 2016, 2020). CNTF activates STAT3 by binding to a heterotrimeric receptor complex consisting of gp130, LIF-receptor β (LIFR β) and the CNTF receptor α (CNTFR α). Interestingly, only a few cell types, such as neurons and skeletal muscle cells, express CNTFR α (Davis et al., 1991), but a soluble CNTFR α enables other cell types, including vascular endothelial cells, to respond to CNTF signaling (Davis et al., 1993).

In contrast to CNTF, OSM elicits a pro-angiogenic response in vascular endothelial cells (Vasse et al., 1999), enhances tumor progression (Zhu et al., 2015) and is associated with cardiovascular disease (Modur et al., 1997; Abe et al., 2019). OSM-induced intracellular signaling hence represents a potential target for new therapeutic approaches in neovascular disease (Kucia-Tran et al., 2018). Receptors necessary for OSM signaling, gp130 and OSMR β , are widely distributed among different cell types (Richards, 2013), causing OSM to not only influence diseases related to angiogenic processes but also other diseases, including diseases associated with bone remodeling, lung fibrosis or inflammatory skin conditions (Richards, 2013).

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The reported inverse angiomodulatory effect of CNTF and OSM on vascular endothelial cells, despite them sharing STAT3 signaling as the predominant intracellular signaling pathway, underlines that more research is needed to fully understand all aspects of cytokine-induced STAT3 signaling. Using *in vitro* angiogenesis models, we confirmed that OSM enhances vascular endothelial cell migration and sprouting, whereas CNTF limits VEGF-induced endothelial sprouting. The degree of STAT3 specificity correlated with the angiogenic phenotype, whereas STAT3 knockdown experiments suggested that the effect of STAT3 signaling on angiogenesis is highly context dependent and can result in pro- and anti-angiogenic reactions. A detailed understanding of STAT3 activity in disease is therefore necessary to successfully use STAT3 as therapeutic target.

RESULTS

OSM and CNTF have opposite angiomodulatory effects on vascular endothelial cells

To evaluate the effect of OSM and of CNTF co-stimulated with its soluble receptor CNTFR α (CNTF+R) on angiogenesis *in vitro*, both cytokines were tested in the spheroid-sprouting assay, the scratch-wound assay and proliferation assay. OSM treatment provided a strong pro-angiogenic stimulus on vascular endothelial sprouting (25% increase) compared to the basal sprouting rate [endothelial basal medium (EBM) control group]. An additional pro-angiogenic impulse of 31% on top of VEGF-induced sprouting (VEGF control group) was induced after OSM+VEGF stimulation (Fig. 1A). In contrast, CNTF+R significantly reduced VEGF-initiated sprouting by 21.9% (Fig. 1A).

In the scratch-wound assay, OSM treatment significantly enhanced endothelial migration after 10 h by 28.4% compared to that of the EBM control (Fig. 1B). Interestingly, the OSM-induced pro-migratory stimulus surpassed the VEGF-induced effect within the first 10 h but stagnated afterwards, whereas VEGF showed a consistent wound-closing kinetic. In line with the observation in the spheroid-sprouting assay, after 10 h, OSM+VEGF significantly enhanced endothelial cell migration by 29.3% in comparison to VEGF alone (Fig. 1B). In contrast, CNTF+R did not affect migration significantly with or without VEGF (Fig. 1B). Similar effects were seen with regards to cell proliferation: OSM significantly increased cell proliferation by 63.7% compared to that seen in the EBM control and by 55.3% on top of VEGF-induced proliferation (Fig. 1C). As before, CNTF+R with or without VEGF did not achieve a significant effect on cell proliferation.

OSM, in contrast to CNTF+R, activates intracellular signaling pathways beyond STAT3

We hypothesized that the observed divergent effects of OSM and CNTF+R on endothelial cells correlated with distinct intracellular signaling patterns. Western blot analysis showed that OSM and CNTF+R both activated the STAT3 signaling pathway in human umbilical vein endothelial cells (HUVECs) by phosphorylating the Tyr705 site of STAT3 (pSTAT3, Tyr705), but only OSM was able to activate phosphorylation of the Ser727 site (pSTAT3, Ser727) (Fig. 2A). STAT1 activation, as indicated by levels of phosphorylated (p) STAT1 (pSTAT1) upon exposure to cytokines, was the most prominent after OSM treatment, whereas CNTF+R led to a very weak pSTAT1 response (Fig. 2A). STAT5 (STAT5A) activation was not evident for either cytokine (Fig. 2A). Co-stimulation with VEGF led to the same results (Fig. 2A). In contrast to CNTF+R, OSM also led to activation of other pathways

including ERK and AKT, represented by the increase in pERK and pAKT levels in response to OSM treatment (Fig. 2B), which was especially stable in the presence of VEGF (Fig. 2B).

To screen for an explanation of these different signaling patterns between OSM and CNTF+R, we further analyzed more upstream-located proteins in the signaling cascade by measuring phosphorylation of JAKs. Interestingly, OSM and CNTF+R with or without VEGF co-stimulation did not generate a pJAK1 or pJAK2 signal in protein lysates after 5 min of stimulation, but only OSM was able to introduce a statically significant pTYK2 activation (Fig. S1A).

The time course of the intracellular signaling pathways revealed that both OSM as well as CNTF+R rapidly increased pSTAT3 levels, peaking at approximately 15 min, with a more prolonged activation observed for OSM (Fig. S1B). ERK signaling induced by OSM appeared more rapid and stronger, but of shorter duration than ERK activation induced by VEGF (Fig. S1B). Interestingly, pSTAT3 Ser727 activation showed a slower activation pattern in contrast to the Tyr705 kinetics by peaking later at approximately 15 min (Fig. S1B). This signaling kinetics aligned with the observed wound-closure dynamic in OSM- versus VEGF-stimulated HUVECs. In summary, OSM activated multiple signaling pathways, whereas CNTF+R showed STAT3 specificity, with minor activation of STAT1 (Fig. 2C).

OSM+VEGF and CNTF+R+VEGF induce a transcriptomic shift with many similarities but also distinct differences

Based on the observed differences in the angiomodulatory potential as well as intracellular signaling patterns between OSM and CNTF+R in the presence of VEGF, we next examined whether these changes were associated with distinct gene expression profiles using RNA-sequencing (RNAseq) analysis. Principal component analysis (PCA) of whole-transcriptome-sequencing data revealed that OSM+VEGF and CNTF+R+VEGF led to transcriptomic profiles that were clearly separated from those of the VEGF control group by the first principal component (PC1, Fig. 3A) while the second principal component separated biological replicates within the same group (PC2, Fig. 3A). In comparison to VEGF, only a small shift in PC1 was observed between OSM+VEGF and CNTF+R+VEGF, suggesting that the transcriptomes of OSM+VEGF and CNTF+R+VEGF share many similarities and are distinct from those of VEGF, but are also different from each other. Comparing OSM+VEGF to the VEGF control group, 1392 differentially expressed genes (DEGs) were upregulated and 706 were downregulated. Between CNTF+R+VEGF and the VEGF control group, 1083 DEGs were upregulated and 431 downregulated. In contrast, only 92 genes were differentially upregulated and 35 differentially downregulated for OSM+VEGF in comparison to CNTF+R+VEGF (Fig. 3B). To identify genes responsible for the OSM-associated pro-angiogenic effect, we compared the transcriptomes of OSM+VEGF and CNTF+R+VEGF as the angiogenic differences would be reflected in them. A Gene Ontology (GO) term enrichment analysis of OSM+VEGF compared to CNTF+R+VEGF for biological processes identified 'inflammatory response', 'response to cytokine' and 'cell migration' as part of the ten most enriched terms for OSM+VEGF (Fig. 3C). A GO term enrichment analysis for molecular functions identified 'cytokine receptor binding', 'growth factor binding' and 'growth factor receptor binding' as some of the top enriched terms. Besides these, GO term clusters associated with immunological processes were enriched, and this enrichment was also measured in an enrichment analysis using the Reactome database, which resulted in

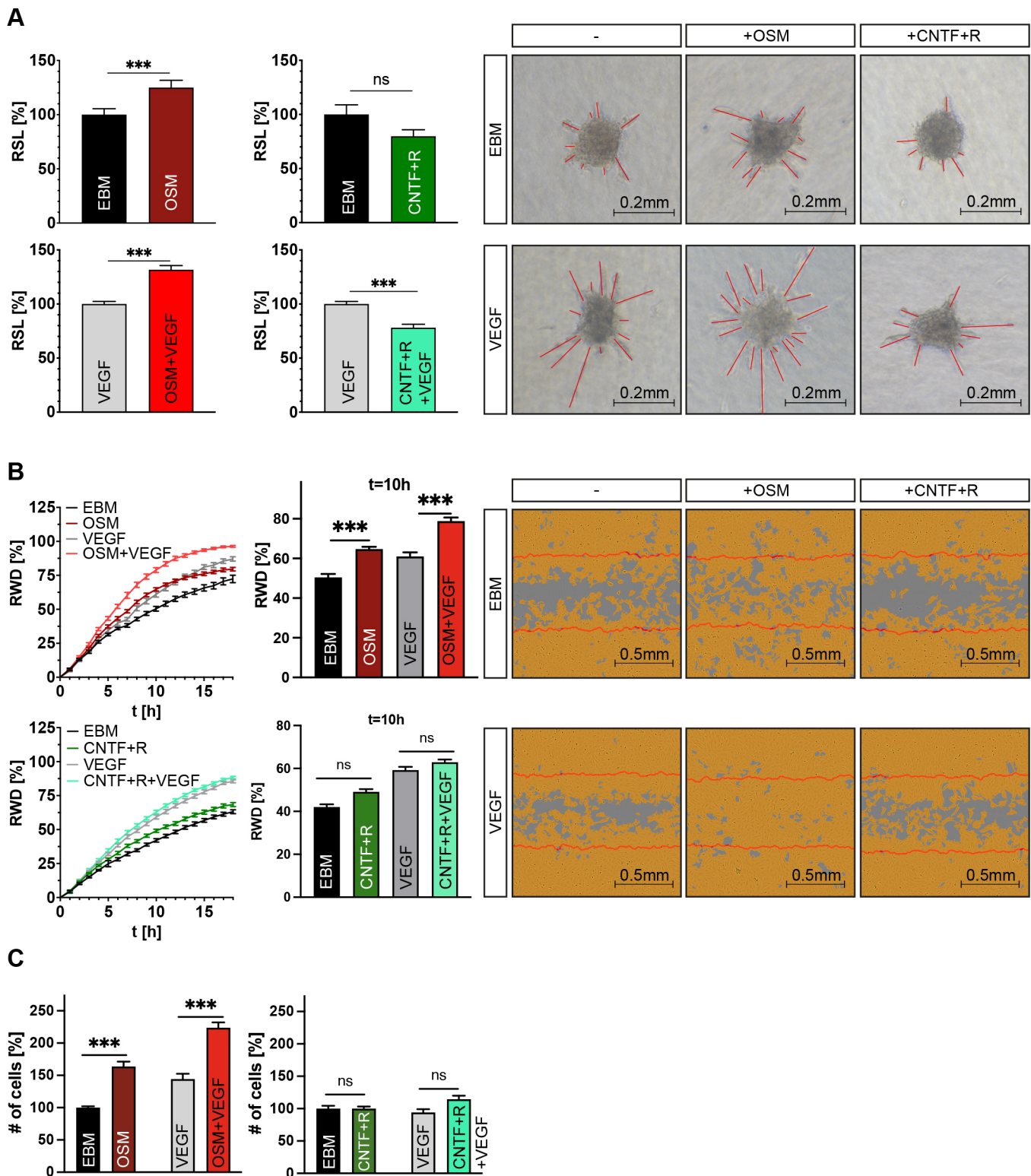


Fig. 1. OSM shows pro-angiogenic potential by activation of a broader range of signaling pathways in contrast to the anti-angiogenic CNTF+R.

(A) Relative sprouting length (RSL, left) of HUVECs stimulated by endothelial basal medium (EBM) as a negative control, OSM and CNTF+R, with or without VEGF measured in the spheroid sprouting assay (right, red lines indicated sprouts). $n=3-5$ independent experiments with 12–20 spheroids per group and experiment. A Mann–Whitney U -test was used for statistical analysis. (B) Relative wound density (RWD, left and middle) was measured over 18 h to determine the migratory effect of EBM, OSM, CNTF+R or VEGF on HUVECs in the scratch-wound assay (right, red lines indicate original wound edge at $t=0$ h). $n=3$ independent experiments each including six to eight technical replicates. A Kruskal–Wallis Test adjusted for multiple testing was used to determine significance 10 h after the start of the experiment. (C) Proliferation assay of HUVECs stimulated by specific cytokine treatment for 3 days. $n=3$ independent experiments with eight technical replicates each. A Kruskal–Wallis Test adjusted for multiple testing was used to determine significance. Data show the mean \pm s.e.m. ns, not significant; *** $P<0.001$.

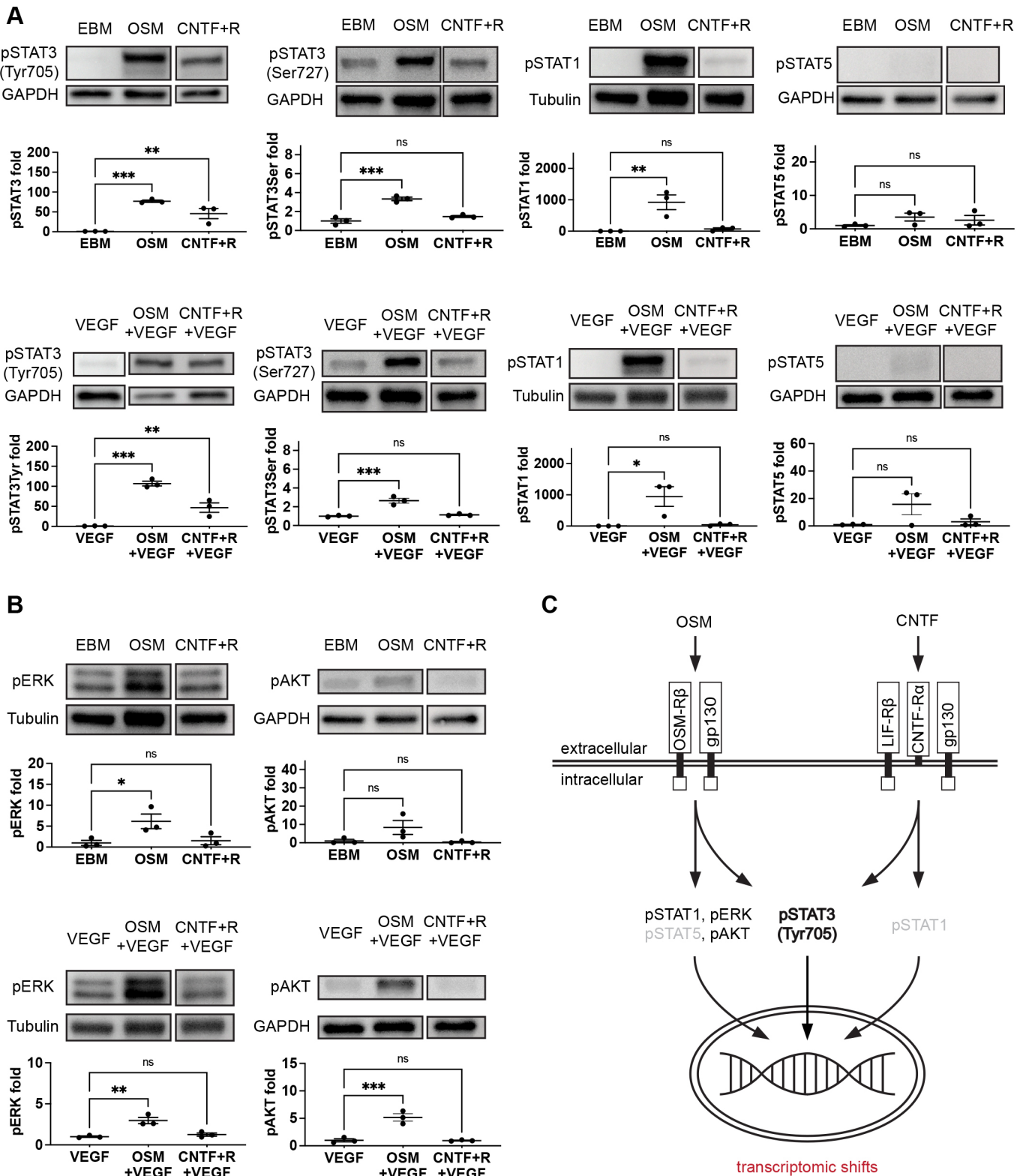


Fig. 2. OSM activates multiple other pathways beyond STAT3 signaling in contrast to CNTF+R. (A) Western blot analysis of HUVECs stimulated by EBM or cytokines for activation of STAT-related pathways. Protein lysates were collected after 15 min of stimulation, except for the pSTAT3 Ser727 blots, for which cells were incubated for 30 min. Statistical significance was calculated by one-way ANOVA adjusted for multiple testing with the two-stage step-up method of Benjamini, Krieger and Yekutieli. (B) Western blot analysis of HUVECs stimulated by EBM or cytokines for activation of the ERK and AKT pathways. Protein lysates were collected after 15 min of stimulation. Statistical significance was calculated by an ordinary one-way ANOVA adjusted for multiple testing with the two-stage step-up method of Benjamini, Krieger and Yekutieli. Data show the mean \pm s.e.m. ns, not significant; * P <0.05; ** P <0.01; *** P <0.001. (C) Graphical summary of the activated pathways by OSM or CNTF+R as measured in the signaling analysis.

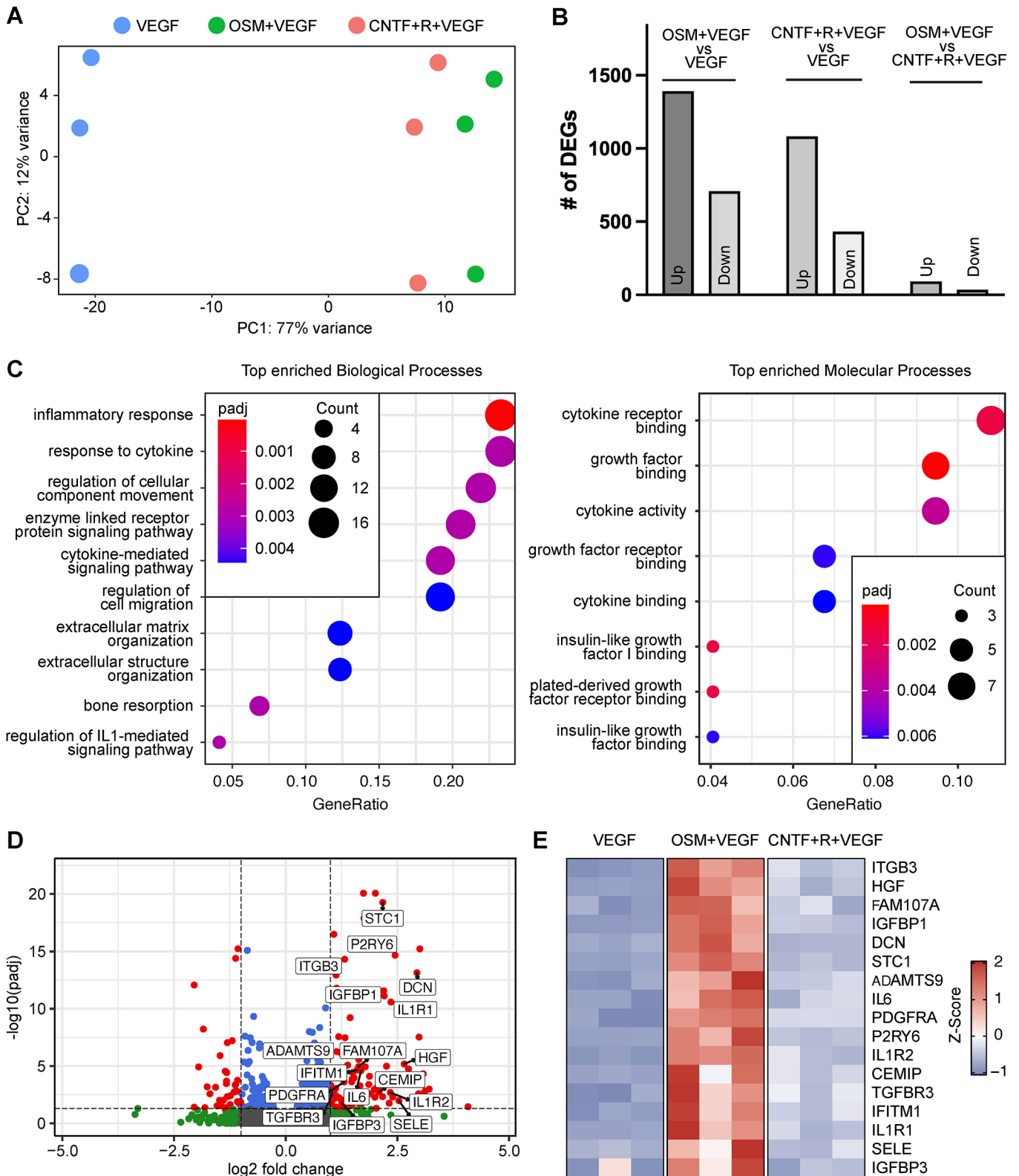


Fig. 3. See next page for legend.

enriched interleukin signaling clusters for OSM+VEGF-treated samples (Fig. S2). Most of the DEGs annotated to the GO terms ‘cell migration’, ‘cytokine receptor binding’, ‘growth factor binding’ and ‘growth factor receptor binding’ showed strong fold changes and were highly significant for OSM+VEGF (Fig. 3D).

Gene-expression changes were consistent among biological replicates within the groups (Fig. 3E). Thus, the sequencing analysis suggests a conceivable role of the transcriptomic shifts inducing the observed angiogenic phenotype between the OSM+VEGF- and CNTF+R+VEGF-treated samples.

Fig. 3. The diverse angiomodulatory effect of OSM and CNTF+R on HUVECs corresponds to a distinct transcriptomic shift. (A) PCA of the HUVEC transcriptome following treatment with VEGF, VEGF+OSM or CNTF+R+VEGF for 24 h. (B) Bar graph visualizing the amount of DEGs measured for each condition. Up, upregulated DEGs; Down, downregulated DEGs. (C) Gene Ontology (GO) enrichment analysis of DEGs enriched for HUVECs treated with OSM+VEGF in contrast to CNTF+R+VEGF. The ten most significant GO terms according to the adjusted P -value (P_{adj}) are shown and ranked based on their share of the total number of DEGs (GeneRatio). Diagrams show terms enriched for OSM+VEGF and therefore depleted for CNTF+R+VEGF. The number of DEGs and the total number of genes for each GO term can be found in Tables S3 and S4. (D) Volcano plot of all genes with at least one count in both samples of HUVECs treated with OSM+VEGF or CNTF+R+VEGF. Genes defined as upregulated DEGs after OSM+VEGF treatment are marked in red. Genes which were not considered differentially expressed due to an absolute $\log_2(\text{fold change}) < 1$ even though $P_{adj} < 0.05$ are colored blue. Features with a $\log_2(\text{fold change}) > 1$ but $P_{adj} > 0.05$ are marked green. Genes annotated with the GO terms 'regulation of cell migration' (GO: 0030334), 'growth factor binding' (GO:0019838) or 'growth factor receptor binding' (GO:0070851) are labeled. (E) Heatmap of the Z-score of all labeled genes in D, visualizing the three replicates of each condition.

STAT3 knockdown enhances the pro-angiogenic potential of OSM and inverts the anti-angiogenic potential of CNTF+R on HUVECs

Having established significant differences in angiogenic cell behavior in response to OSM or to CNTF, we next assessed how the shared STAT3 pathway contributes to these phenotypes. We established a STAT3 knockdown protocol using siRNA, which resulted in a potent knockdown persisting for up to 96 h after transfection (Fig. S3A). In the spheroid-sprouting assay, STAT3 knockdown abrogated the basal sprouting rate in the EBM group and decreased VEGF-induced sprouting by 39% compared to cells transfected with control siRNA (Fig. 4A). In contrast, STAT3 knockdown led to the opposite effect in OSM+VEGF-stimulated HUVECs, characterized by a 2.06-fold increase in sprouting compared to the respective control siRNA-transfected cells and a 4.7-fold increase compared to STAT3 knockdown cells treated with VEGF alone.

STAT3 knockdown in HUVECs not only abolished the ability of CNTF+R to reduce VEGF-induced sprouting significantly, it also increased endothelial sprouting in the CNTF+R+VEGF group compared to the VEGF group by 1.78-fold. HUVECs transfected with control siRNA and CNTF+R+VEGF treatment still reduced sprouting by 40.8% (Fig. 4A). Comparing CNTF+R+VEGF-treated HUVECs with or without STAT3 knockdown, only a small difference was measurable, despite the massive decrease in sprouting caused by the STAT3 knockdown in samples treated only with VEGF (Fig. 4A).

Similar changes in response to STAT3 knockdown could be observed in the scratch-wound assay. STAT3 knockdown reduced the wound-closing kinetics of HUVECs in the EBM group by 27.6% and in the VEGF-treated group by 32.3% compared to the respective control siRNA-treated cells after 14 h (Fig. 4B). Stimulation with OSM in STAT3 knockdown cells led to a strong increase by a 3.258-fold change compared to EBM-stimulated STAT3 knockdown cells (Fig. 4B). Comparable effects were observed in the presence of VEGF: co-stimulation with OSM+VEGF following STAT3 knockdown increased migration by 2.05-fold compared to VEGF after 14 h (Fig. 4B). OSM treatment in control siRNA-transfected cells still led to a 1.51-fold increase in wound-closing kinetics and, upon co-stimulation with VEGF, it led to a 1.16-fold increase in wound-closing kinetics. Surprisingly, STAT3 knockdown significantly decreased

OSM+VEGF-induced cell proliferation (Fig. S4), whereas VEGF-induced proliferation was also significantly reduced, suggesting that the observed phenotype in the spheroid-sprouting and migration assays are not based on enhanced proliferation but rather on migration and hypertrophy.

To ensure that the observed knockdown results were not skewed by siRNA off-target effects, we repeated selected knockdown experiments with a second STAT3 siRNA of a different sequence. STAT3 siRNA #2 resulted in a long-lasting, efficient knockdown (Fig. S3B). The spheroid-sprouting assay showed comparable changes in endothelial cell sprouting following STAT3 knockdown and OSM stimulation compared to STAT3 siRNA #1 (Fig. S3C), thus validating our STAT3 knockdown data. The same tendencies for the second STAT3 siRNA were also measurable in a screening experiment for CNTF+R using the second STAT3 siRNA (Fig. S3D).

To further validate the specificity of the observed STAT3-dependent phenotype, we established a STAT1 knockdown using siRNA (Fig. S3E) and repeated the sprouting assay. Similar to the STAT3 knockdown, the STAT1 knockdown decreased the basal sprouting rate in every group (average decrease of 46.3%, Fig. S3F). The increase in sprouting in the OSM treatment group was abolished following STAT1 knockdown. In contrast to the STAT3 knockdown that led to a significant increase in sprouting, OSM+VEGF only induced a weak increase in sprouting by 18.1% compared to VEGF following STAT1 knockdown (Fig. S3F). These data further validate the specificity of the observed STAT3 knockdown data.

The increase in the pro-angiogenic effect of OSM following STAT3 knockdown correlates with a shift in the balance of intracellular signaling pathways

To better understand the molecular mechanisms behind the enhanced pro-angiogenic potential of OSM following STAT3 knockdown, we next performed RNAseq analysis of HUVECs co-stimulated with OSM and VEGF following STAT3 knockdown (OSM+VEGF STAT3 siRNA) compared to control siRNA-transfected cells (OSM+VEGF control siRNA). In the OSM+VEGF STAT3 siRNA group, 2410 DEGs were detected. GO enrichment analysis of all DEGs revealed many immunological terms, in line with the well-characterized role of STAT3 in inflammation. Interestingly, multiple GO terms associated with the regulation of the MAPK pathways and interferon- γ signaling pathway, which is closely connected to the STAT1 signaling pathway and known for its role in angiogenesis, were enriched as well (Fig. 5A). A pro-migrative transcriptome was also triggered by STAT3 knockdown in OSM+VEGF-treated HUVECs, indicated by the enrichment of the GO term 'positive regulation of cell migration'. Although STAT3 knockdown cells showed a depletion of genes associated with 'negative regulation of growth' (Fig. 5A), as indicated by an enrichment in GO terms in the RNAseq data from the control siRNA samples treated with OSM+VEGF, this did not translate in a functional alteration as described earlier (Fig. S4). Most of the DEGs annotated to GO terms associated with MAPK, cell migration and interferon- γ activity presented good clustering on a sample-to-sample level (Fig. 5B). Three out of the five most strongly expressed upregulated DEGs (Fig. 5C, marked in red) in OSM+VEGF-treated STAT3 knockdown cells were part of the GO 'angiogenesis' cluster (GO:0001525), which was not among the top 15 regulated GO terms shown in Fig. 5A, but was significantly enriched in knockdown cells. Gene set enrichment analysis (GSEA) confirmed that overall, genes associated with the GO term angiogenesis were significantly enriched in the

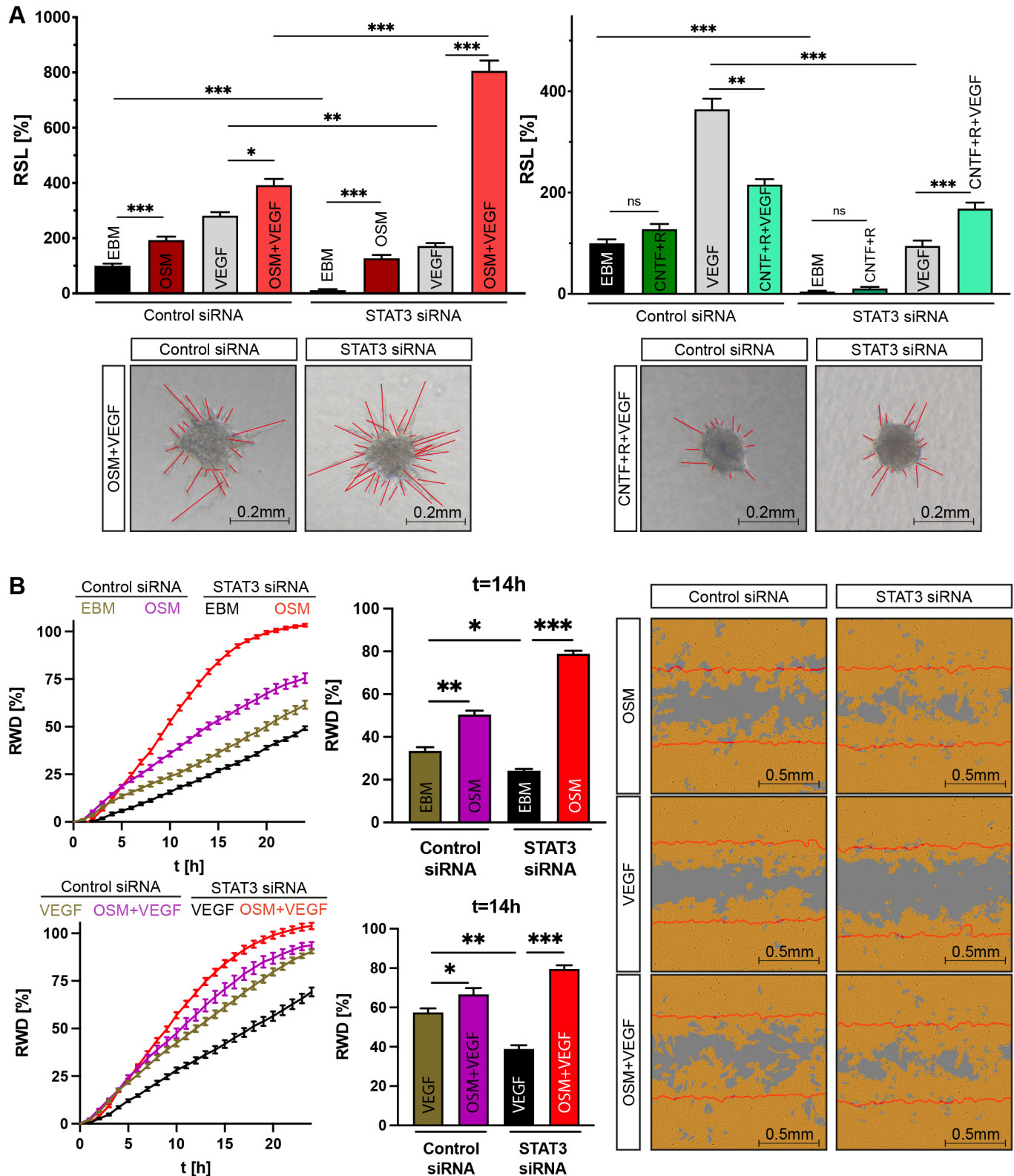


Fig. 4. STAT3 knockdown enhances the pro-angiogenic effect of OSM and abolishes the anti-angiogenic influence of CNTF on HUVECs. (A) Endothelial sprouting of HUVECs in response to the EBM control, OSM, CNTF+R, VEGF or a combination of the above was measured by the relative sprouting length (RSL, top) in the spheroid sprouting assay (bottom) following STAT3 knockdown and compared to controls treated with control siRNA. Data are representative of three independent experiments each consisting of 12–20 spheroids per group and experiment. Statistical testing was conducted by a Kruskal–Wallis test adjusted for multiple testing. (B) Relative wound density (RWD, left and middle) of HUVECs in response to stimulation with EBM, OSM, VEGF or a combination was determined in the scratch-wound assay of HUVECs transfected with STAT3 siRNA or control siRNA. $n=3$ individual experiments which include six to eight technical replicates each. A Kruskal–Wallis test adjusted for multiple testing was used to determine significance 14 h after stimulation. Data show the mean \pm s.e.m. ns, not significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

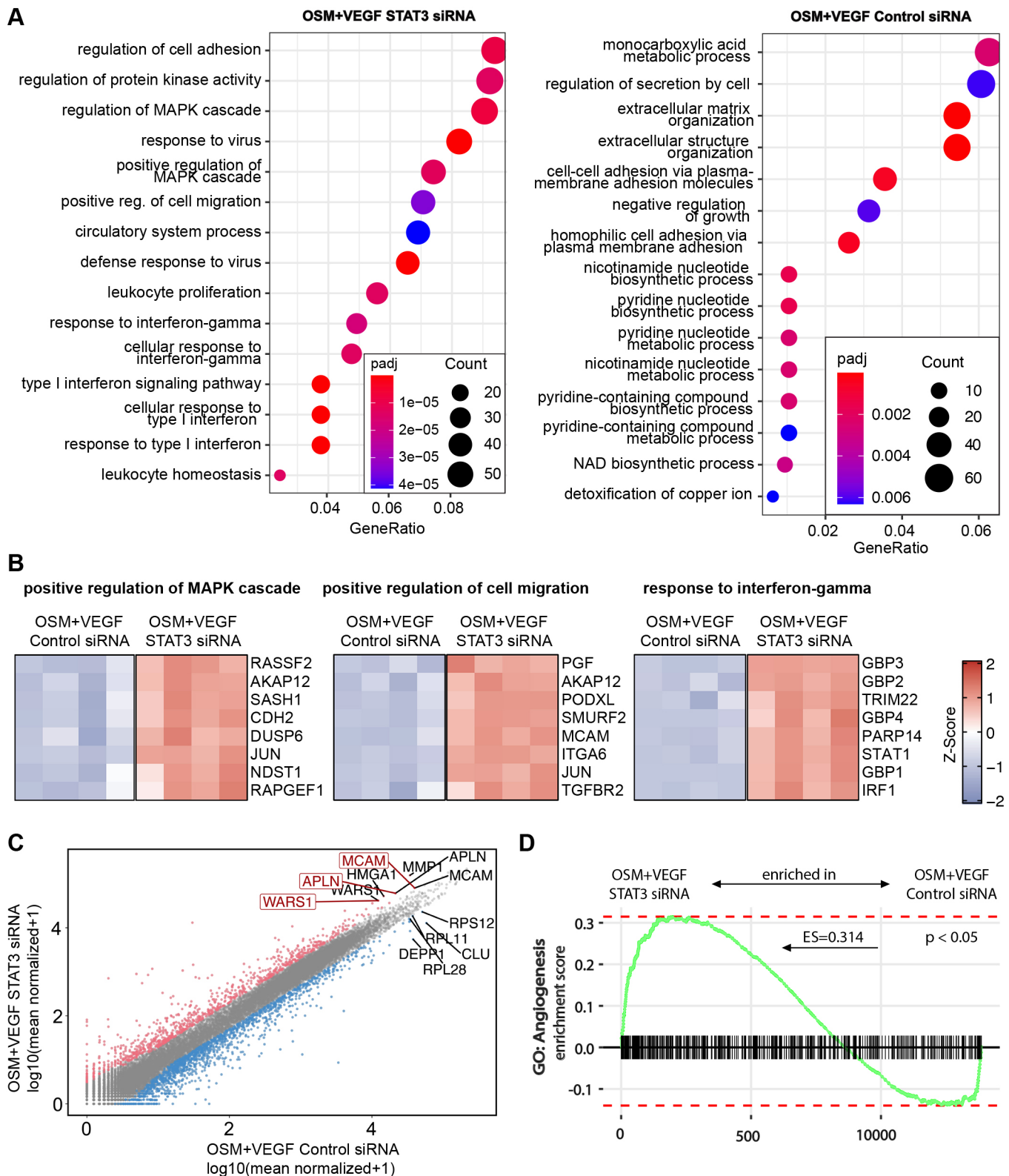


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OSM+VEGF STAT3 siRNA group (Fig. 5D). Taken together, these results strongly suggest that the enhanced pro-angiogenic effect of OSM in the absence of STAT3 is related to the upregulation of STAT1- and ERK-dependent genes associated with cell proliferation and angiogenesis.

To understand whether a suspected compensatory upregulation of STAT1 and ERK signaling in response to STAT3 knockdown and OSM+VEGF treatment was specific to OSM, we also analyzed the transcriptomic profile of HUVECs treated with CNTF+R+VEGF following STAT3 knockdown (CNTF+R+VEGF STAT3 siRNA)

Fig. 5. STAT3 knockdown in HUVECs induces a transcriptomic shift towards a dysregulated state of proliferation, migration and vascular development in response to OSM treatment, including upregulation of ERK- and STAT1-associated signaling pathways on the RNA level.

(A) GO term enrichment analysis of DEGs enriched for HUVECs treated with OSM+VEGF following STAT3 knockdown (STAT3 siRNA) or treatment with control siRNA. The 15 statistically most significant GO terms according to the adjusted P -value (P_{adj}) are shown and ranked by their share of the total number of DEGs (GeneRatio). The diagrams show the enriched terms for the labeled conditions. The number of DEGs and the total number of genes for the GO terms can be found in in Tables S5 and S6. (B) Heatmaps of the eight most expressed DEGs tagged with GO terms associated with positive regulation of MAPK signaling (GO:0043410), response to the interferon- γ pathway (GO:0034341) and positive regulation of cell migration (GO:0030335) visualized by the Z-score in each replicate. (C) Scatterplot of all genes with at least one count in HUVECs treated with OSM+VEGF following STAT3 knockdown compared to the control siRNA group. Genes defined as upregulated DEGs in response to OSM+VEGF treatment following STAT3 knockdown are marked in red and differentially downregulated genes are marked in blue. The top five upregulated and downregulated DEGs according to their absolute expression are labeled. Genes which are related to the GO term 'angiogenesis' (GO:0001525) are highlighted. (D) GSEA for the GO term 'angiogenesis' in HUVECs treated with OSM+VEGF following STAT3 knockdown.

as well as with VEGF following STAT3 knockdown (VEGF STAT3 siRNA). Comparing CNTF+R+VEGF-treated knockdown and control cells, multiple enriched GO terms associated with interferon- γ signaling, but not MAPK signaling, were identified (Fig. S5A; Tables S7 and S8). These data align with initial analyses indicating that CNTF+R, in contrast to OSM, activated STAT3 and STAT1 signaling but not ERK signaling (Fig. 2B). A scatter plot of the data again illustrated many highly expressed DEGs annotated for GO angiogenesis including *WARS1* and *APLN* (Fig. S5B). GSEA was also significantly enriched for the GO term 'angiogenesis' (Fig. S5C). In contrast, VEGF-stimulated HUVECs with or without STAT3 knockdown retrieved only six significantly enriched biological processes in the GO enrichment analysis (Fig. S6A,B; Tables S9 and S10). Again, this aligns with the observation that VEGF does not induce STAT3 signaling in HUVECs.

The observed transcriptomic shifts in the OSM+VEGF- as well as the CNTF+R+VEGF-treated groups following STAT3 knockdown point towards compensatory upregulation of STAT1 signaling and, in the case of OSM+VEGF, ERK signaling. We therefore re-evaluated the activity of intracellular signaling pathways following STAT3 knockdown with cytokine stimulation on protein level using western blotting. Following STAT3 knockdown and 5 min of OSM+VEGF stimulation, there was no enhanced activation of pSTAT1, pSTAT5, pAKT and pERK, whereas the pSTAT3 signal was clearly diminished (Fig. 6A). However, time-course experiments that screened for differences in pSTAT1 activity over 24 h revealed that STAT3 knockdown cells showed a clearly prolonged pSTAT1 activity after 6 h and 24 h in response to OSM+VEGF treatment (Fig. 6B).

In summary, these data suggest that the extent of the pro-angiogenic effect of OSM is tightly regulated by the balance of intracellular signaling activity. Blocking STAT3 enhances cell migration and sprouting, which is reflected by significantly upregulated genes associated with cell migration and compensatory signaling pathways, including ERK and AKT, which in turn have been associated with a pro-angiogenic effect

in previous studies (Fig. 6C) (Zhang et al., 2019; Jiang and Liu, 2008).

OSM shifts HUVECs to an active metabolic state by increasing mitochondrial performance

Examining alternative phosphorylation sites of STAT3 in response to OSM and CNTF, we observed that only OSM promoted phosphorylation of STAT3 not only at Tyr705, but also at Ser727 (pSTAT3 Ser727) (Fig. 2A). pSTAT3 Ser727 has been described to enhance the transcriptomic impact of STAT3 and it modulates mitochondrial function to consequently change the metabolic rate of cells (Gough et al., 2009, 2014; Wegrzyn et al., 2009; Balic et al., 2020). We hypothesized that OSM increases the mitochondrial function of HUVECs by phosphorylation of STAT3 at the Ser727 site and thus provide the energy and metabolites necessary for the enhanced angiogenic process. Using OSM-stimulated bovine aorta endothelial cells (BAECs) transfected with plasmids expressing STAT3 tagged with a fluorescent protein, co-localization of STAT3 and stained mitochondria was evident (Fig. 7A). Unstimulated cells did not yield this result (Fig. 7A). Extracellular flux analysis of HUVECs pretreated with cytokines overnight for 15 h provided a precise readout for further functional metabolic switches. Pretreatment with OSM or OSM+VEGF led to an increase in basal respiration, ATP production and maximal respiration in comparison to cells treated only with EBM (Fig. 7B,C). VEGF and CNTF+R did not significantly alter any of these functions.

To screen for potential molecular reasons that might explain the enhanced metabolic activity of HUVECs in response to OSM, we next studied the levels of mitochondrial respiratory chain complexes. Western blot analysis of all important complexes in the respiratory chain did not show any changes at the protein level (Fig. 8A) or at the RNA level by RNASeq (Fig. S7). The major production of superoxide was also not changed compared to control groups (Fig. 8B). Although OSM induced mitochondrial activity consistently, the underlying molecular mechanisms remain to be elucidated.

OSM treatment enhances glycolysis

While measuring mitochondrial activity by extracellular flux analysis, OSM was also found to enhance glycolytic activity in vascular endothelial cells (Fig. 8C,D). This observation might be of particular relevance in the context of angiogenesis as glycolysis is believed to represent the main energy provider in hypoxia-driven angiogenic processes (Potente and Carmeliet, 2017). Quantification of the extracellular acidification rate (ECAR) results revealed that baseline glycolytic activity was only significantly enhanced in the VEGF+OSM group compared to the VEGF group, whereas maximal glycolytic capacity was significantly increased in cells treated with OSM or OSM+VEGF compared to the respective controls (Fig. 8C,D). In contrast, CNTF+R stimulation did not result in similar changes (Fig. 8C,D).

Taken together, the pro-angiogenic potential of OSM appears to be rooted in the activation of strong proliferative stimuli on a transcriptomic as well as metabolic level, affecting mitochondrial respiration as well as glycolysis.

DISCUSSION

In this study, we examined the angiomodulatory effects and underlying molecular changes of the IL-6 family members and STAT3 activators OSM and CNTF to better characterize the role of STAT3 signaling in angiogenesis. Using *in vitro* angiogenesis assays, we confirmed the previously described pro-angiogenic

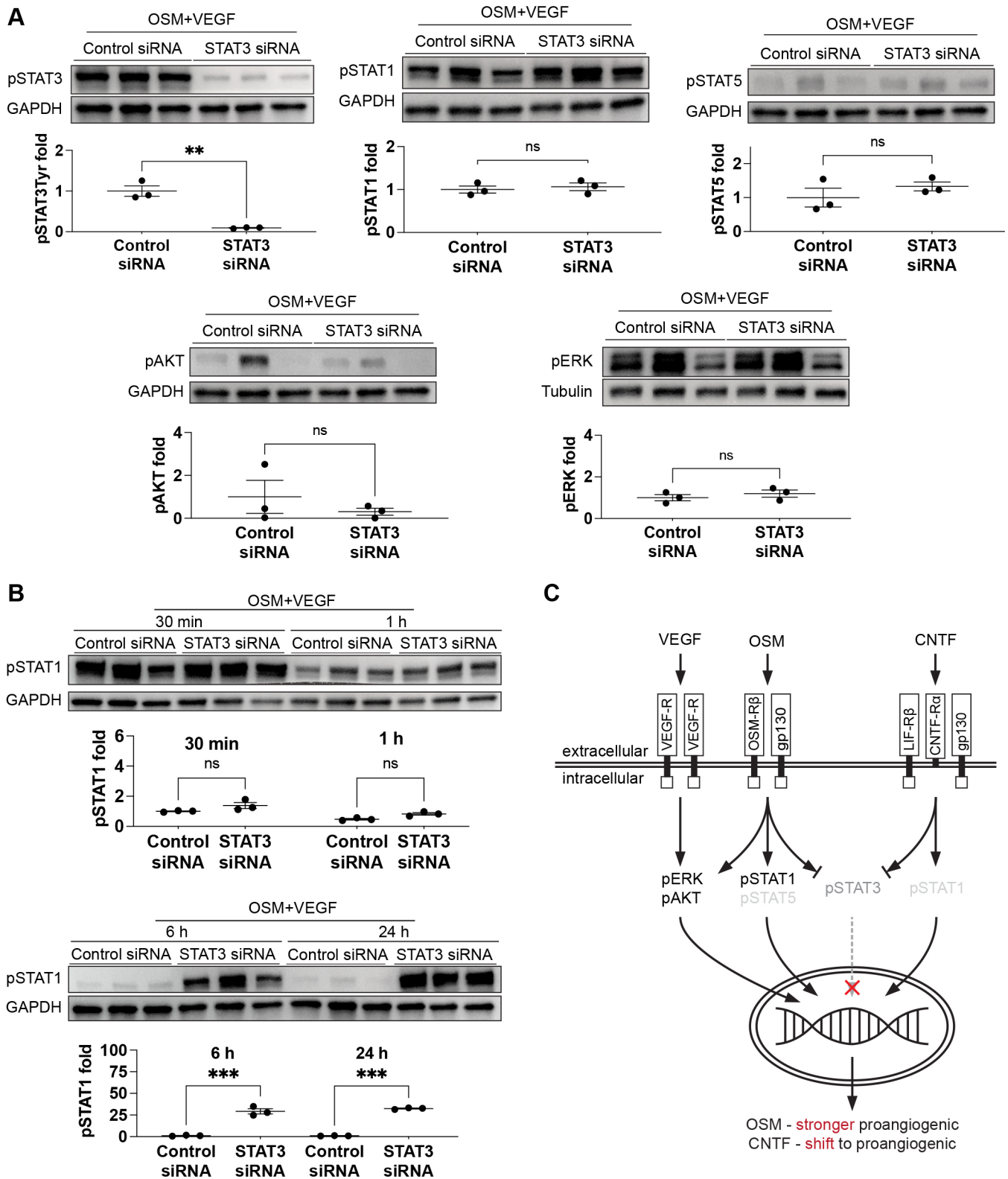


Fig. 6. Compensatory changes of intracellular signaling pathways in response to STAT3 knockdown and OSM+VEGF treatment. (A) Comparison of intracellular signaling patterns in HUVECs after successful STAT3 knockdown and treatment with different cytokines for 5 min using western blotting. $n=3$ independent experiments; two-tailed unpaired Student's t -test. (B) Western blot analysis for pSTAT1 at 30 min, 1 h, 6 h and 24 h in OSM+VEGF-treated HUVECs with or without STAT3 knockdown. $n=3$ independent experiments; two-tailed unpaired Student's t -test. Data show the mean \pm s.e.m. ns, not significant; $**P<0.01$; $***P<0.001$. (C) Graphical summary of the altered signaling patterns by OSM and CNTF+R and how the angiogenic phenotype changed due to knockdown.

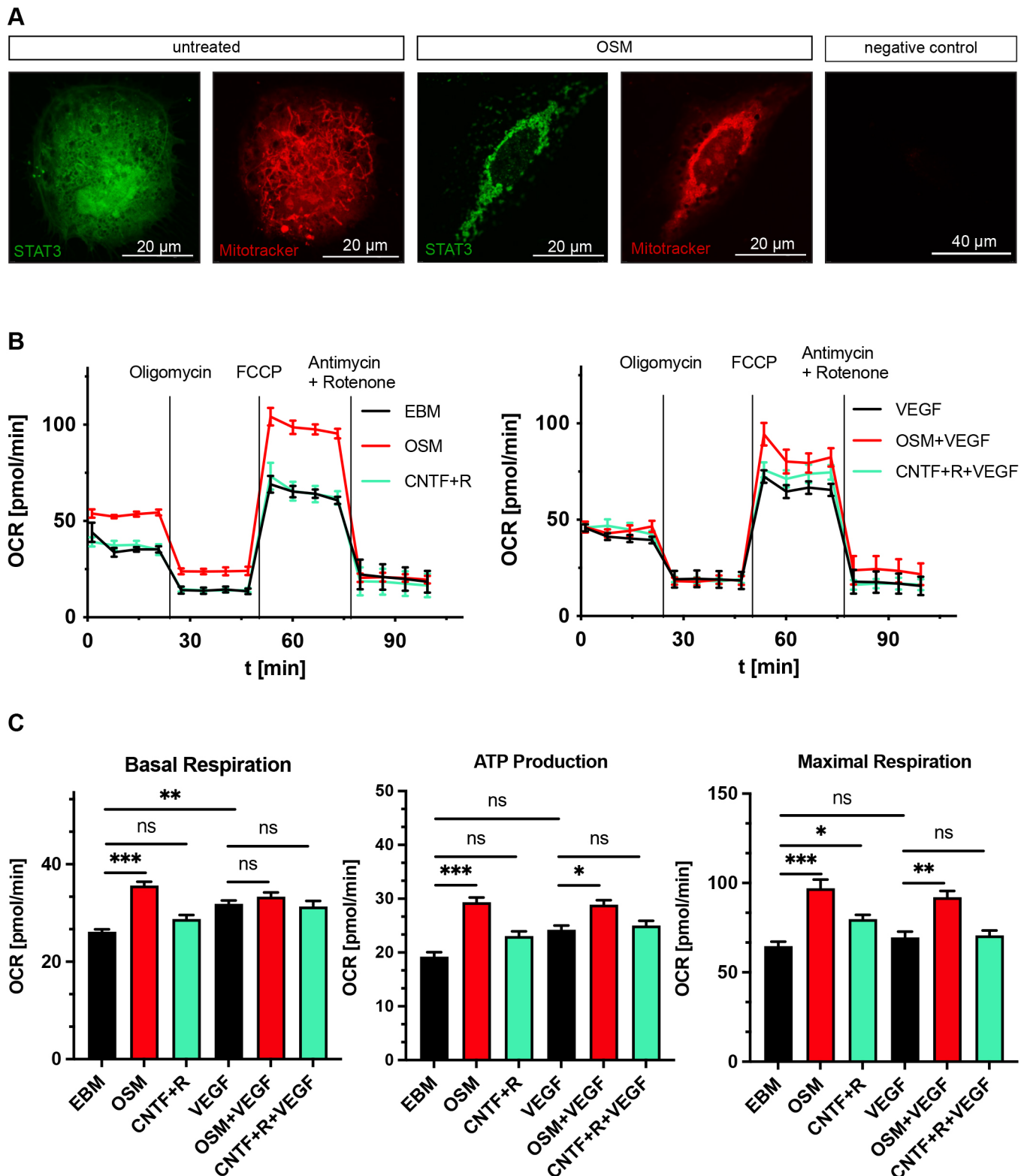


Fig. 7. STAT3 accumulates in mitochondria after OSM treatment and increases mitochondrial activity. (A) Immunocytochemical analysis of BAECs transfected with Venus-WTSTAT3 plasmids. BAECs were stimulated with OSM or negative control for 30 min and stained with MitoTracker. The negative controls were BAECs transfected with no plasmid and no incubation with MitoTracker. (B) Representative graphs of the oxygen consumption rate (OCR) of HUVECs pretreated overnight with different cytokines or EBM using Seahorse XF Cell Mito Stress Test. Each graph includes six to eight technical replicates. Data were normalized to the measured cell count in each well. (C) Normalized quantification of the Seahorse XF Cell Mito Stress Test results from HUVECs pretreated with cytokines or EBM overnight. Data are representative of three independent experiments including six to eight technical replicates each. A Kruskal–Wallis adjusted for multiple testing was used for statistical analysis. Data show the mean±s.e.m. ns, not significant; * P <0.05; ** P <0.01; *** P <0.001.

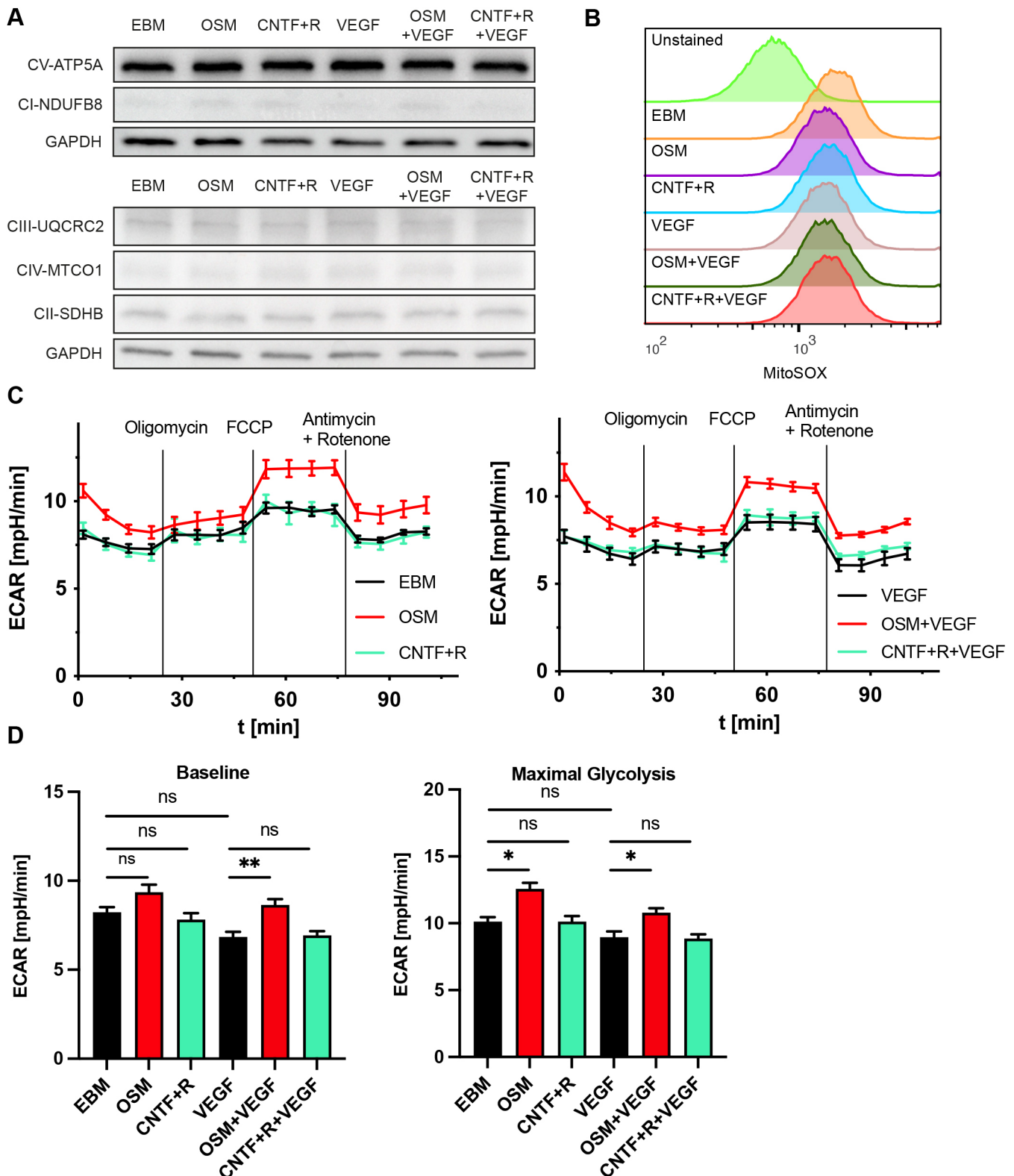


Fig. 8. Analysis of metabolic changes induced by OSM or CNTF+R treatment in HUVECs. (A) Western blot analysis of the expression of mitochondrial complex I (CI-NDUFB8), complex II (CII-SDHB), complex III (CIII-UQCRC2), complex IV (CIV-MTCO1) and complex V (CV-ATP5A) after overnight treatment with cytokines or EBM. (B) Exemplary flow cytometry measurements for the production of ROS after pretreatment of HUVECS with cytokines or EBM overnight using the MitoSOX assay. (C) Representative graphs of the extracellular acidification rate (ECAR) of HUVECs pretreated overnight with EBM or different cytokines using the Seahorse XF Cell Mito Stress Test. Each graph includes six to eight technical replicates. Data are normalized to the measured cell count in each well. (D) Normalized quantification of the glycolytic activity of HUVECs pretreated with EBM or cytokines overnight. $n=3$ independent experiments including six to eight technical replicates each. A Kruskal–Wallis test was used for statistical analysis and adjusted for multiple testing. Data show the mean \pm s.e.m. ns, not significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

effect of OSM (Vasse et al., 1999) and anti-angiogenic effect of CNTF (Bucher et al., 2016, 2020) on vascular endothelial cells in standardized experimental settings (Fig. 1). Next, we elucidated the underlying mechanisms responsible for the inverse phenotype by characterizing intracellular signaling activity beyond STAT3 signaling (Fig. 2). STAT3 knockdown experiments and RNAseq analyses were used to further address the impact of STAT3 in the context of other pro-angiogenic signaling pathways (Figs 4–6).

At first, our results about the role of STAT3 signaling in vascular endothelial cells appear to be contradictory: STAT3 knockdown decreased endothelial cell migration and sprouting in untreated or VEGF-stimulated cells, whereas STAT3 knockdown in IL-6 family cytokine-induced angiogenesis led to increased migration and sprouting (Fig. 4). Earlier studies on the role of STAT3 signaling in cell biology reveal that STAT3 modulates cellular functions on different levels depending on its subcellular localization and phosphorylation status (Avalle and Poli, 2018).

Our observation that STAT3 knockdown in unstimulated and VEGF-stimulated HUVECs significantly decreased endothelial cell migration and sprouting is in line with previously published data, which led to the common perception of STAT3 being a driver of angiogenesis (Jung et al., 2005; Johnson et al., 2018; Hu et al., 2019). Interestingly, both conditions (EBM as well as VEGF treatment) did not induce any baseline activation of the STAT3 signaling pathway, represented by phosphorylation of STAT3 (Fig. 2). The measured decrease of angiogenesis might be due to a cytoplasmic function of STAT3 independent of its phosphorylation status. Teng et al. (2009) proposed an interaction of STAT3 with the cytoskeleton that negatively affects cell migration after STAT3 loss. Ng et al. (2006) provided evidence for the interaction of STAT3 and the microtubule network, showing that STAT3 knockdown led to microtubule disassembly, resulting in decreased endothelial capacity to migrate into a cell-free area *in vitro*. Our own immunohistochemical staining of the actin cytoskeleton by phalloidin suggested that STAT3 knockdown significantly reduced lamellipodium formation (data not shown), which is reported to be dependent on proper microtubule formation (Mikhailov and Gundersen, 1998). Furthermore, a role of unphosphorylated STAT3 as a transcription factor is also discussed in the literature (Timofeeva et al., 2012). This suggests that changes in expression levels of the downstream targets of unphosphorylated STAT3 could also be sufficient to induce shifts in phenotype owing to STAT3 knockdown. Although the idea of a protein–protein interaction of unphosphorylated STAT3 in the cytosol is still controversial, our own and previously published data suggest a more diverse role of STAT3 in cell biology beyond its role as a transcription factor in its phosphorylated form.

Starting from the observed decrease in endothelial cell migration and sprouting in STAT3 knockdown cells following control and VEGF treatment, it was intriguing to observe an increase in endothelial cell migration and sprouting in response to OSM+VEGF or CNTF+R+VEGF stimulation following STAT3 knockdown (Fig. 4). Treatment with OSM+VEGF or CNTF+R+VEGF led to phosphorylation of STAT3 as well as other signaling molecules, including ERK (Fig. 2), which was associated with distinct shifts in the transcriptomic profile (Fig. 3). Under the circumstance of STAT3 loss due to knockdown, other pathways in response to OSM+VEGF and CNTF+R+VEGF, such as pERK, remained active (Fig. 6). We therefore assume that pSTAT3 balances the influence of pro-angiogenic drivers like ERK or AKT on a transcriptomic level. Besides clustering of ERK-associated GO terms, RNAseq analysis of OSM+VEGF STAT3 siRNA cells showed enriched clusters of type I interferon and interferon- γ associated signaling (Fig. 5A). We

assume that this is an indication for the increased impact of pSTAT1 on the transcriptome because type I interferon and interferon- γ use the STAT1 pathway as a second messenger (Jung et al., 2021). Owing to increased pSTAT1 impact on the transcriptome induced by STAT3 knockdown, both of those GO terms were enriched without any actual stimulation by type I interferons or interferon- γ . Western blot analysis revealing prolonged pSTAT1 activity over 24 h after STAT3 knockdown support this hypothesis (Fig. 6B). Normally, pSTAT1 activity is associated with anti-angiogenic properties (Huang et al., 2002), which questions the role of the prolonged pSTAT1 activity in the increased pro-angiogenic drive of OSM+VEGF after STAT3 knockdown. We therefore assume that our data underline the important regulatory function of STAT3 on the activity of other signaling pathways that modulates their ability to change their capability of shifting the transcriptome, which in total dictates the behavior of the endothelial cell.

The complexity of STAT3 signaling is further increased due to the existence of multiple phosphorylation sites that modulate STAT3 activity in different subcellular compartments. Besides the Tyr705 phosphorylation site, STAT3 signaling can further be altered by phosphorylation at Ser727, which is reported to increase mitochondrial performance and oxidative phosphorylation activity (Gough et al., 2009, 2014; Wegrzyn et al., 2009; Balic et al., 2020). In contrast to CNTF, only OSM led to STAT3 Ser727 phosphorylation (Fig. 2A). Although vascular endothelial cells are known to mainly rely on glycolysis to fuel their need for ATP, they can also use mitochondrial oxidation under stress and mitochondria as a biosynthetic hub to produce metabolites necessary for angiogenesis (Potente and Carmeliet, 2017). The molecular mechanism behind the impact of pSTAT3 Ser727 on cell metabolism is still not fully understood. Current work suggests an interaction between STAT3 and respiratory complexes I and II to increase their activity, leading to membrane potential enhancement (Wegrzyn et al., 2009), and a regulatory function of STAT3 in the production of reactive oxygen species (Gough et al., 2009). Mitochondrial STAT3 is also recognized to change mitochondrial gene expression (Carbognin et al., 2016). The ability of OSM to increase mitochondrial performance and biogenesis while reducing apoptotic behavior has been reported in cardiomyocytes (Sun et al., 2015). Hanlon et al. (2019) recently described increased glycolysis in endothelial cells treated with OSM, but no improvement in mitochondrial performance was observed, whereas our data suggest an increase in mitochondrial respiration as well as glycolysis (Fig. 7B,C, Fig. 8C,D). In contrast to our setup, Hanlon et al. (2019) pretreated HUVECs for 3 h in comparison to our 15 h overnight incubation. The increase in oxidative phosphorylation might need more time to unfold, whereas the boost in glycolysis measured by Hanlon et al. (2019) represents a potentially immediate reaction. An instantaneously increased ECAR by an OSM injection to untreated cells in a Seahorse extracellular flux assay, which we also measured (data not shown), supports this hypothesis. However, we did not observe OSM-associated differences by screening for major changes in the expression levels of mitochondrial complexes or the production of reactive oxygen species induced by severe oxidative stress (Fig. 8A,B). The increased metabolic rate could be triggered by an unknown protein–protein interaction, which warrants further understanding of the link between OSM and metabolism, especially because pSTAT3 Ser727-induced metabolic switches were linked to disease progression (Zhang et al., 2013; Gough et al., 2014).

In summary, our study outlines the complex role of STAT3 signaling in vascular endothelial cells in response to cytokine treatment, which consequently results in pleiotropic angiogenic

effects. The angiomodulatory effect of STAT3 appears to be dependent on the activity of other intracellular signaling pathways as well as its subcellular localization in the cytosol, mitochondria or nucleus. OSM and CNTF differ in their intracellular signaling patterns and their STAT3 specificity, which translates into unique transcriptomic profiles and metabolic activity. A profound knowledge of these fine differences is necessary to understand the contribution of STAT3 signaling to disease progression and to subsequently use STAT3 or STAT3-activating cytokines as therapeutic targets.

MATERIALS AND METHODS

HUVEC culture

HUVECs (pooled in EGM-2, C2519A, Lonza, Basel, Switzerland) were grown in endothelial cell growth medium (EGM, CC-3162, Lonza) and used for all *in vitro* experiments.

For protein and RNA analyses, cells were stimulated by cytokines (summarized in Table S1), which were diluted in endothelial cell growth basal medium 2 (EBM-2, CC-3156, Lonza) supplemented with 6% fetal bovine serum (FBS, S0615, Biochrom, Berlin, Germany). Cytokines and their concentrations can be found in Table S1. For protein analysis, cells were lysed after 5 min of cytokine stimulation using T-PER tissue protein extraction reagent (78510, Thermo Fisher Scientific) supplemented with phosphatase and proteinase-inhibitor (87786 und 78420, Thermo Fisher Scientific). For RNA sample generation, cells were lysed in QIAzol lysis reagent (79306, QIAGEN, Hilden, Germany) 24 h after stimulation.

Transfection

HUVECs were transfected using a loading solution of Opti-MEM (31985062, Thermo Fisher Scientific) containing 0.4% Lipofectamine RNAiMAX (137780309, Thermo Fisher Scientific) and 15 nM STAT3 Stealth siRNA (1299001, siRNA ID: VHS40491, Thermo Fisher Scientific), 120 nM STAT1 Stealth siRNA (1299001, siRNA ID: VHS40871, Thermo Fisher Scientific) or control siRNA (Stealth RNAi siRNA negative control, Med GC, 12935300, Thermo Fisher Scientific) for 6 h. After 6 h, wells were filled up to 2 ml using EGM. Cells were used for assays 48 h after transfection. On-target accuracy of the knockdown was evaluated by comparing effects of a second STAT3 Stealth siRNA (1299001, siRNA ID: VHS40497, Thermo Fisher Scientific) with a completely different sequence.

SDS PAGE and western blotting

Protein samples were prepared for electrophoresis by denaturation using 75% sample, 22.5% Laemmli Buffer (4× Laemmli Sample Buffer, 1610747, Bio-Rad) and 2.5% 2-mercaptoethanol (M3148, Sigma-Aldrich). Samples were separated by SDS-PAGE and then transferred to a Immobilon-P PVDF Membrane (IPVH00010, Millipore). Following a blocking step with 3% bovine serum albumin (BSA, Albumin, Rind, Fraction V, Protease-free, 11926.03, Serva, Heidelberg, Germany) for 30 min, membranes were stained with primary antibodies overnight at 4°C. Staining for GAPDH and secondary antibodies was conducted on the following day for 1 h. All antibodies and their respective dilutions can be found in Table S2. A Fusion FX system (Vilber, Collégien, France) detected the signal using the ECL Prime Western Blotting System (RPN2232, GE Healthcare). Figures show representative western blot results of three independent experiments. Where applicable, western blots were semiquantitatively analyzed by using ImageJ Fiji and its gel analyzer platform. Data were normalized by using GAPDH expression levels for each lane, which served as protein loading control. The relative fold change of protein expression was finally calculated as a percentage by normalizing data to the specific control group. Uncut blots can be found in Figs S8–S12.

Spheroid sprouting assay

Spheroids formed in hanging drops of EGM containing 0.25% methylcellulose (M0512, Sigma-Aldrich) overnight were seeded in 0.5 ml of a collagen matrix consisting of 50% rat tail collagen (354236, Corning)

and 48% EBM containing 0.25% methylcellulose and 2% FBS. Collagen was titrated to a physiological pH by using sodium hydroxide (P031.2, Roth, Karlsruhe, Germany) and buffered with 1 µl of a 1 M HEPES buffer (P05-01100, PAN Biotech, Aidenbach, Germany) After solidifying the gel for 30 min, the wells were layered with 100 µl EBM containing cytokines to match the desired final concentration. After incubation for 1 day, images of spheroids were taken using an inverse microscope (Zeiss Axio Vert A1, Jena, Germany) and the imaging software 'ProgPres CapturePro 2.10.0.1' (Jenoptik Optical Systems, Jena, Germany). Quantification of all sprouts in each image took place using the measuring tool of ImageJ Fiji. The relative sprouting length (RSL) per spheroid was used as a final readout by calculating the average cumulative sprouting length per spheroid normalized to the respective control from the same experiment.

Scratch-wound assay

The migratory potential of HUVECs was measured by a standard scratch assay. Approximately 20,000 HUVECs per well were seeded in EGM in IncuCyte ImageLock 96-well plates (4379, Sartorius, Göttingen, Germany) and starved overnight using EBM supplemented with 2% FBS and antibiotics. On the following day, the scratch was created using the IncuCyte WoundMaker (IncuCyte Cell Migration Kit, 4493, Sartorius) and wells were imaged every hour for 18–24 h by the IncuCyte S3 Live-Cell Analysis System (4647, Sartorius). For analysis, the integrated IncuCyte software (Integrated Cell Migration analysis module, 9600-00-12, Sartorius) primed for HUVECs automatically detected cells and cell migration. The relative wound density (RWD) was calculated automatically by the software and used as a final readout by dividing the confluence inside the originally scratched area by the confluence outside this area.

Proliferation assay

Approximately 3000 HUVECs were seeded into 96-well plates in EBM with 6% FBS. After 3 h, cells were stimulated by cytokines diluted in EBM supplemented with 6% FBS for a total of 72 h, with a medium change containing fresh cytokines every 24 h. Cell number was determined by following the CyQUANT Cell Proliferation Assay protocol (C7026, Thermo Fisher Scientific). The readout was normalized to the specific control group during data analysis.

Extracellular flux analysis

Metabolic shifts were assessed using extracellular flux analysis by a Seahorse XFe96 Analyzer (Agilent Technologies), measuring the oxygen consumption rate (OCR) and ECAR. Approximately 20,000 cells were seeded in 96-well Seahorse cell culture plates (Seahorse XF96 V3 PS Cell Culture Microplates, 101085-004, Agilent Technologies) and pretreated for 15 h overnight with cytokines. The medium of cells was changed after one washing step 1 h before the assay to plain Dulbecco's modified Eagle medium (DMEM, D5030, Sigma-Aldrich) titrated to pH 7.4 and supplemented with 10 mM glucose (Seahorse XF 1.0 M glucose solution, 103577-100, Agilent Technologies), 2 mM glutamine (Seahorse XF 200 mM glutamine solution, 103579-100, Agilent Technologies) and 10 mM HEPES. A typical injection strategy for the mitochondrial stress test was performed by injecting oligomycin (495455, Sigma-Aldrich) first, followed by carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, C2920, Sigma-Aldrich), and finally antimycin A (A8674, Sigma-Aldrich) and rotenone (R8875, Sigma-Aldrich). The final concentration for oligomycin was 20 µM, 1.25 µM for FCCP and 2 µM for antimycin A and rotenone. The gathered data were normalized by the cell count in each well measured by CyQUANT Cell Proliferation Assay and the average of cells in the EBM control group wells as reference. The smallest measurement of OCR after the last injection defined the non-mitochondrial oxygen consumption. The baseline was determined by the lowest measurement of OCR before oligomycin injection, subtracted from the non-mitochondrial oxygen consumption. ATP production-related OCR was calculated by the difference between the baseline and the lowest measurement following oligomycin injection. Finally, the highest OCR after FCCP injection subtracted from non-mitochondrial oxygen consumption defined the maximum respiration.

Co-staining of STAT3 and mitochondria

Plasmids coding for Venus1-STAT3 [Venus1-WTSTAT3 (Letra-Vilela et al., 2020), 123164, Addgene] and Venus2-STAT3 [Venus2-WTSTAT3 (Letra-Vilela et al., 2020), 123165, Addgene] were purchased and transformed in bacteria following the One Shot Stbl3 (C737303, Thermo Fisher Scientific) protocol. Then, 1 ml of the generated stock solution was plated on agar plates and grown overnight in the incubator. Plasmids were isolated using the Plasmid DNA Purification Mini Prep Kit (S5369.0050, Genaxxon BioScience, Ulm, Germany). For quality control, plasmids were digested using the enzymes BsrGI (R0575S, New England Biolabs) and NotI (R0189S, New England Biolabs) according to the protocol generated for these enzymes by NEBcloner (<https://nebcloner.neb.com/#!/protocol/re/double/BsrGI,NotI>). The plasmid fragments were amplified by standard PCR and DNA was detected on a 1% agarose gel.

For transfection experiments, BAECs (GM-7373, Leibniz Institut – German Collection of Microorganisms and Cell Cultures, Leipzig, Germany) cultured on fibronectin-coated flasks (Human Plasma Fibronectin Purified Protein, FC010, Merck, Darmstadt, Germany) were used. BAECs were transfected with the plasmids encoding Venus1-STAT3 (1.04 ng/μl) and Venus2-STAT3 (1.04 ng/μl) in 8.3% Lipofectamine 2000 (11668030, Thermo Fisher Scientific)-containing medium. After 24 h, the cells were stimulated with cytokines for 30 min, then fixed with 2% paraformaldehyde for 20 min and blocked with 10% goat serum (005-000-121, Jackson ImmunoResearch Laboratories) for 1 h. Cells were stained by MitoTracker Orange CMTMROS (M7510, Thermo Fisher Scientific) following the recommended protocol using a 1:3000 dilution. A Leica TCS SP8 confocal microscope and the LAS X 3.5.7 software generated high-quality representative images.

Mitochondrial assays

Reactive oxygen species (ROS) production following cytokine pretreatment overnight was measured using MitoSOX Red Mitochondrial Superoxide Indicator (M36008, Thermo Fisher Scientific). The signal was analyzed by a LSRFortessa flow cytometer (BD Bioscience). Debris and duplets were excluded from the analysis by gating using the FlowJo v10 software (FlowJo, Becton Dickinson, Franklin Lakes, NJ).

RNA sequencing

RNA isolation and all steps necessary for total RNA sequencing were conducted by Center of Excellence for Fluorescent Bioanalytics (KFB, University of Regensburg, Germany; www.kfb-regensburg.de). Samples were lysed in QIAzol and shipped on dry ice. Whole RNA was isolated using the RNeasy Micro Kit (74004, QIAGEN), including on-column DNase digestion and homogenization by Precellys CK14 ceramic beads (432-3751, VWR) before extraction. A combination of the Illumina TruSeq Stranded mRNA Sample Preparation Guide (TruSeq Stranded mRNA, 20020594, Illumina) and the Illumina NextSeq 2000 Sequencing System was used for library preparation and processing samples. Equimolar amounts of each library quantified by the KAPA Library Quantification (ABI Prism, KK4835, Roche Sequencing Solutions, Basel, Switzerland) were sequenced on a NextSeq 2000 instrument controlled by the NextSeq 2000 Control Software v1.1.0.27334, using two 100-cycle P2 Flow Cells with the single-index, single-read run parameters. Base calling and imaging analysis were done by the Real Time Analysis software v3.6.14. Resulting CBCL files were converted into FASTQ files with the bcl2fastq conversion software v2.20.

Bioinformatics

Raw data were uploaded to the European Galaxy server (<https://usegalaxy.eu>) for further analysis (Afgan et al., 2018). FastQC (Galaxy Version 0.72) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) provided quality assessment of all raw files and STAR aligner (Galaxy Version 2.7.5b) (Dobin et al., 2012) mapped all reads to a human reference genome provided by GENCODE (GRCh38, release date 03.2020). The aligned reads were assigned to specific genes by featureCounts (Galaxy Version 1.6.4) (Liao et al., 2013) using the annotation file provided by GENCODE for the same reference genome. Standard settings were applied for all tools. Downstream analysis was performed in R 4.0.2 (<https://www.r-project.org>) by normalizing counts using the DESeq2 package (Love et al., 2014).

Ensembl IDs (v101) were matched to HGNC gene abbreviations by BioMart (Durinck et al., 2009). Genes were considered to be differentially expressed at strict thresholds of adjusted P -value ($P_{\text{adj}} < 0.05$) using the Benjamini–Hochberg method and an absolute $\log_2(\text{fold change}) > 1$. GO enrichment analysis was conducted by the clusterProfiler R package (Yu et al., 2012), pathway enrichment analysis using the Reactome database by ReactomePA (Yu and He, 2016) and GSEA by ranking for shranked $\log_2(\text{fold change})$ by fgsea (Sergushichev, 2016 preprint) and MSigDB gene sets for GO terms (Liberzon et al., 2011; downloaded 8 April 2021).

Statistics

Error bars show mean \pm s.e.m. Statistical testing was performed by a Mann–Whitney test or, when multiple samples were compared, a Kruskal–Wallis test adjusted for multiple testing using the methods described by Benjamini, Krieger and Yekutieli (Benjamini et al., 2006). $P < 0.05$ was considered significant and marked with an asterisk. $P < 0.01$ was visualized by two asterisks and $P < 0.001$ by three asterisks.

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Competing interests

F.B. has the following potential conflicts of interest to declare: funding for scientific research projects from Roche and Neurotech pharmaceuticals; and speaker activity for Novartis and Bayer.

Author contributions

Conceptualization: J.R., O. Gorka, C.L., G.S., F.B.; Methodology: J.R., R.F.U.K.; Validation: J.R., M.J., F.B.; Formal analysis: J.R., M.J., J.W., J.A., O. Gorka, F.B.; Investigation: J.R., J.A.; Resources: J.R.; Data curation: J.R., J.W., F.B.; Writing - original draft: J.R., F.B.; Writing - review & editing: J.R., J.W., O. Gorka, C.L., H.A., G.S., F.B.; Visualization: J.R.; Supervision: O. Groß, C.L., H.A., G.S., F.B.; Project administration: G.S., F.B.; Funding acquisition: J.R., O. Gorka, O. Groß, F.B.

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Data availability

RNA sequencing data have been deposited to the Gene Expression Omnibus with the accession numbers GSE196776 and GSE198484.

Peer review history

The peer review history is available online at <https://journals.biologists.com/jcs/article-lookup/doi/10.1242/jcs.260182.reviewer-comments.pdf>.

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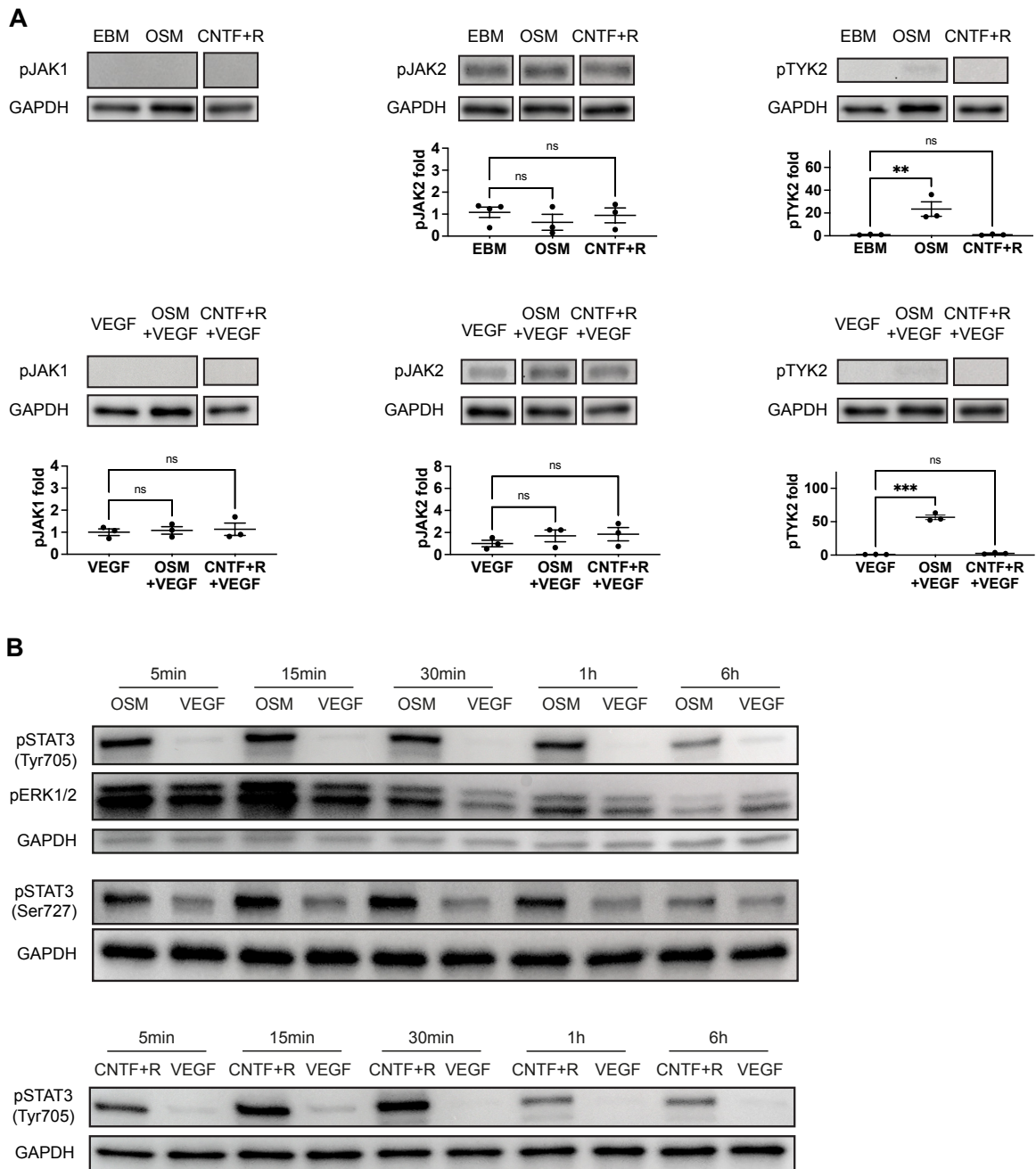


Fig. S1. JAK activation and time kinetic of pSTAT3 Tyr 705 activation in HUVECs following cytokine treatment. (A) Western blot analysis of HUVEC lysates stimulated by cytokines or EBM/VEGF as control for 5 min. Selected images show representative results. N = 3 biological replicates. * $p < 0.05$ one-way ANOVA adjusted for multiple testing. Uncut blots can be found in the supplementary material. (B) HUVECs were treated with VEGF, OSM or CNTF+R for up to 6 h and their potential to activate their respective pathways investigated by western blot.

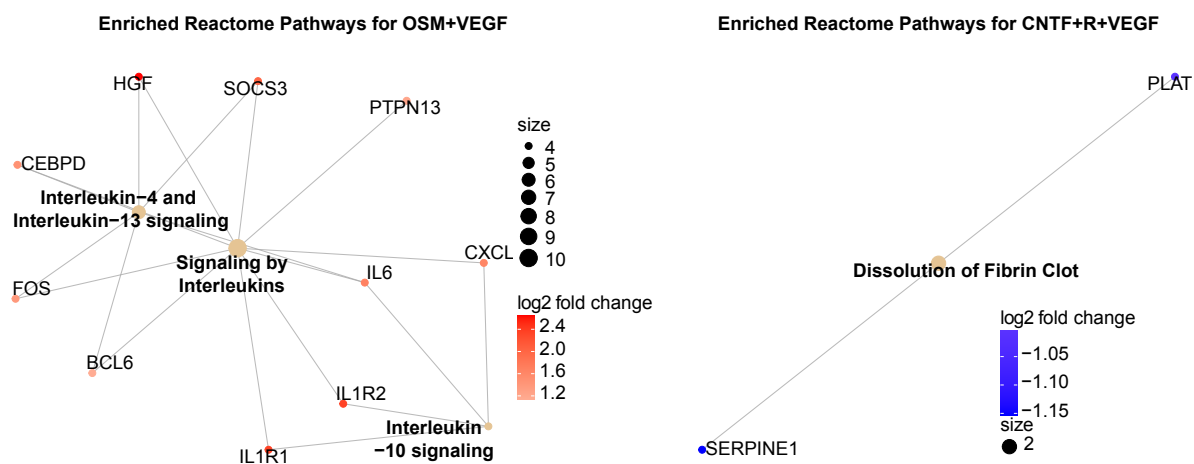


Fig. S2. Pathway enrichment analysis for DEGs of the RNA sequencing data of OSM+VEGF stimulated HUVECs in comparison to CNTF+R+VEGF treatment using the Reactome database. Size refers to the number of DEGs in the enriched pathway.

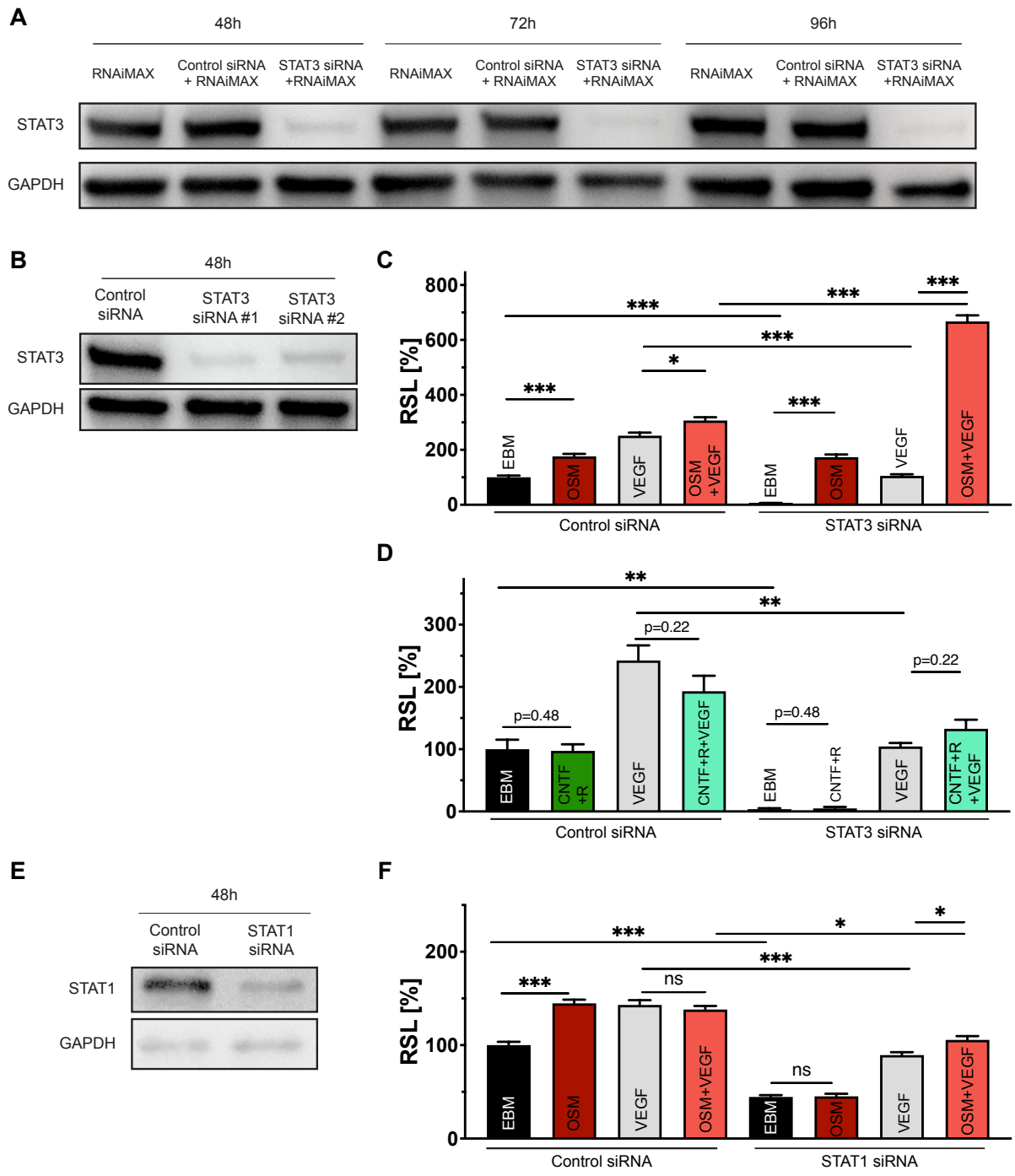


Fig. S3. Validation of STAT3 knock-down and on-target accuracy. (A) Western blot analysis of HUVECs transfected with plain RNAiMAX or in combination with Control siRNA or STAT3 siRNA. Time points refer to time passed after transfection. (B) Validation of a second STAT3 siRNA with a different target sequence using western blot. (C) Spheroid-Sprouting Assay of HUVECs transfected with the second STAT3 siRNA or Control siRNA treated with Endothelial Basel Medium (EBM), OSM, OSM+VEGF or VEGF. N = 3 independent experiments consisting of 12-20 spheroids

per group and experiment. Statistical testing: Kruskal–Wallis Test adjusted for multiple testing. **(D)** Spheroid-Sprouting Assay of HUVECs transfected with the second STAT3 siRNA or Control siRNA treated with Endothelial Basal Medium (EBM), CNTF+R, CNTF+VEGF or VEGF. N = 1 independent experiment consisting of 12-20 spheroids per group. A Kruskal–Wallis Test adjusted for multiple testing was used to determine statistical significance. **(E)** Western blot analysis to determine sufficient STAT1 knock-down in HUVECs 48 after transfection. **(F)** Spheroid-Sprouting Assay of HUVECs transfected with STAT1 siRNA or Control siRNA treated with Endothelial Basal Medium (EBM), OSM, OSM+VEGF or VEGF. N = 3 independent experiments consisting of 12-20 spheroids per group and experiment. Statistical testing: Kruskal–Wallis Test adjusted for multiple testing.

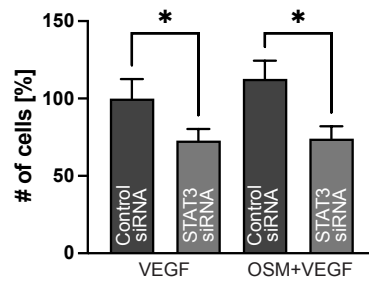


Fig. S4. Influence of STAT3 Knock-Down on OSM+VEGF induced proliferation.

Proliferation assay of HUVECs with or without STAT3 knock-down stimulated with OSM+VEGF for 3 days. Data is representative for three independent experiments with 8 technical replicates each. A Kruskal-Wallis test adjusted for multiple testing was performed for statistical analysis.

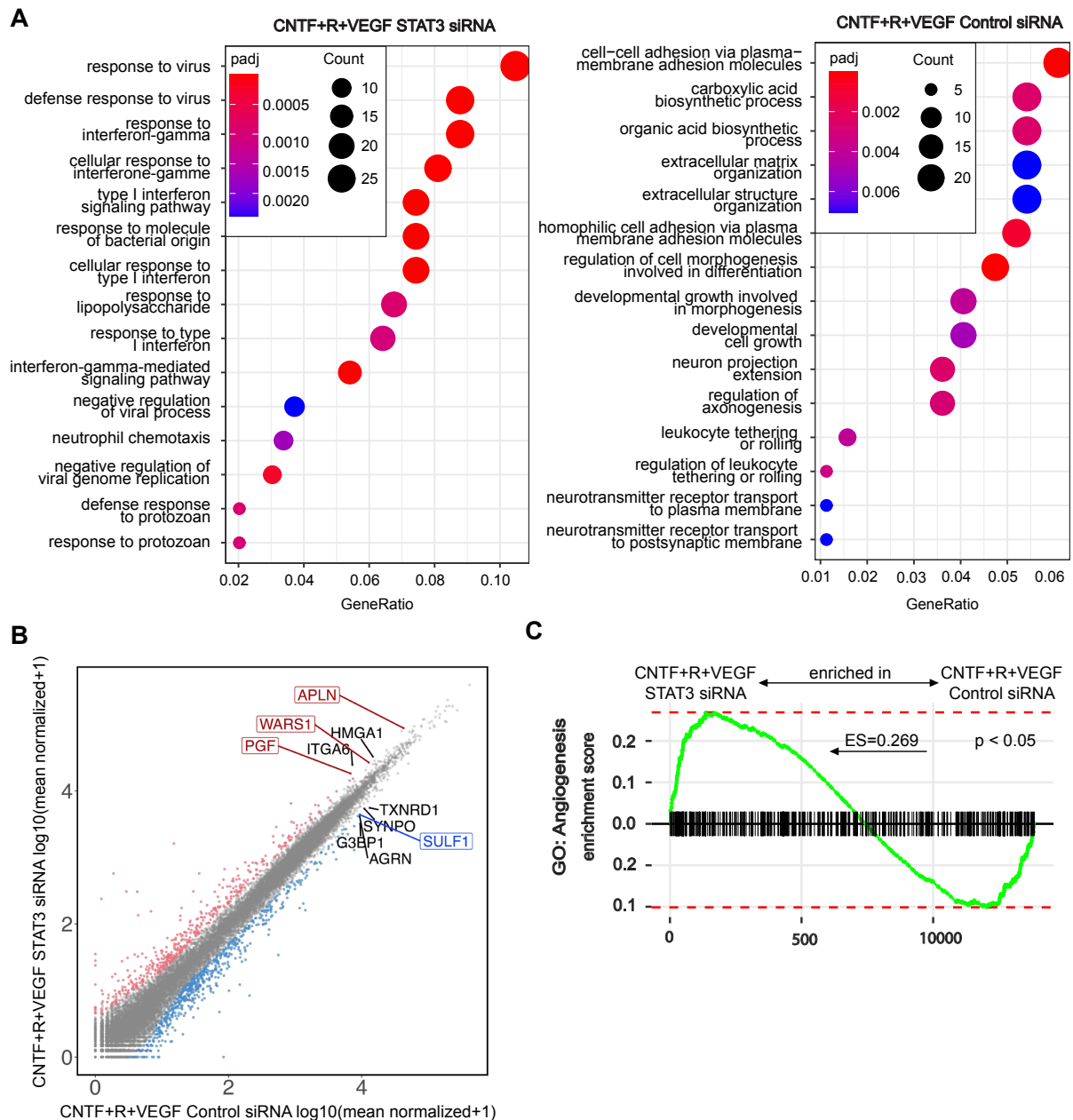


Fig. S5. The transcriptome of CNTF+R+VEGF stimulated STAT3 knock-down cells shows similarly tendencies as for OSM+VEGF stimulated STAT3 knock-down cells. (A) GO enrichment analysis for enriched biological processes between CNTF+R+VEGF stimulated HUVECs with or without STAT3 knock-down for each condition. The ten most significant enriched GO terms according to their adjusted p-value are shown and sorted based on their share at the total number of DEGs (GeneRatio). The diagrams show the enriched terms for the labeled condition. The number of DEGs and the total number of genes for one of the GO-terms can be found in Supplementary Table S7-8 **(B)** Scatterplot of all DEGs with at least one count in both groups comparing CNTF+R+VEGF stimulated HUVECs with or without

STAT3 knock-down. Genes defined as upregulated DEGs are labeled red and downregulated DEGs blue. The five most expressed DEGs from each category are highlighted by name and DEGs linked to the GO term Angiogenesis (GO:0001525) additionally highlighted. **(C)** GSEA between CNTF+R+VEGF HUVECs with or without STAT3 knock-down for the GO term Angiogenesis (GO:0001525). A positive enrichment score refers to enrichment for STAT3 knock-down cells stimulated with CNTF+R+VEGF.

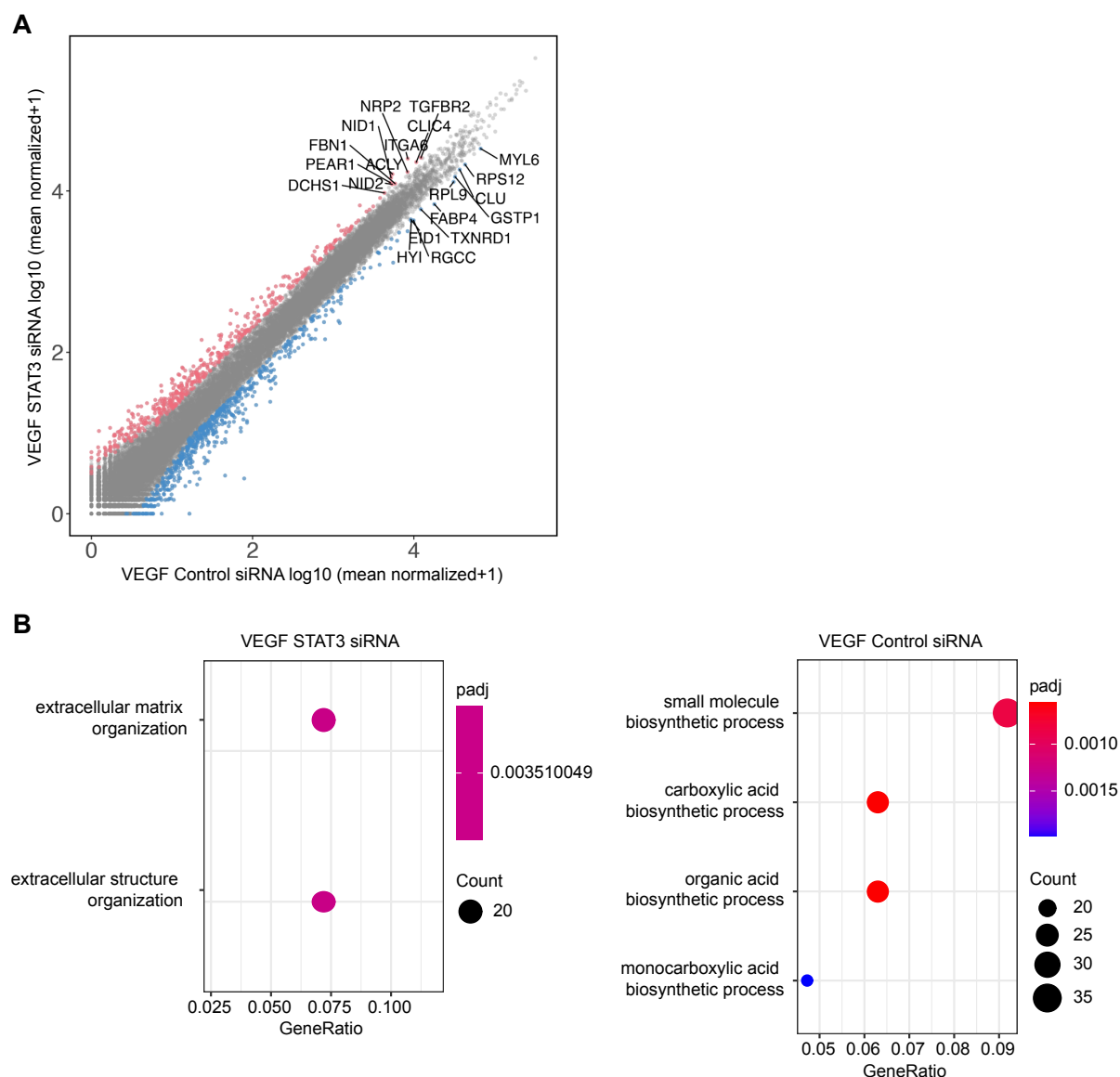


Fig. S6. STAT3 knock-down in VEGF stimulated HUVECs does not shift the transcriptome in a clear direction. (A) Scatterplot of all DEGs with at least one count in both groups comparing VEGF stimulated HUVECs with or without STAT3 knock-down. Genes defined as upregulated DEGs are labeled red and downregulated DEGs blue. The ten most expressed DEGs from each category are highlighted by name. **(B)** GO enrichment analysis for enriched biological processes between VEGF stimulated HUVECs with or without STAT3 knock-down for each condition. GO terms are ranked based on their share at the total number of DEGs (GeneRatio). The diagrams show the enriched terms for the labeled condition. The number of DEGs and the total number of genes for one of the GO-terms can be found in Supplementary Table S9-10.

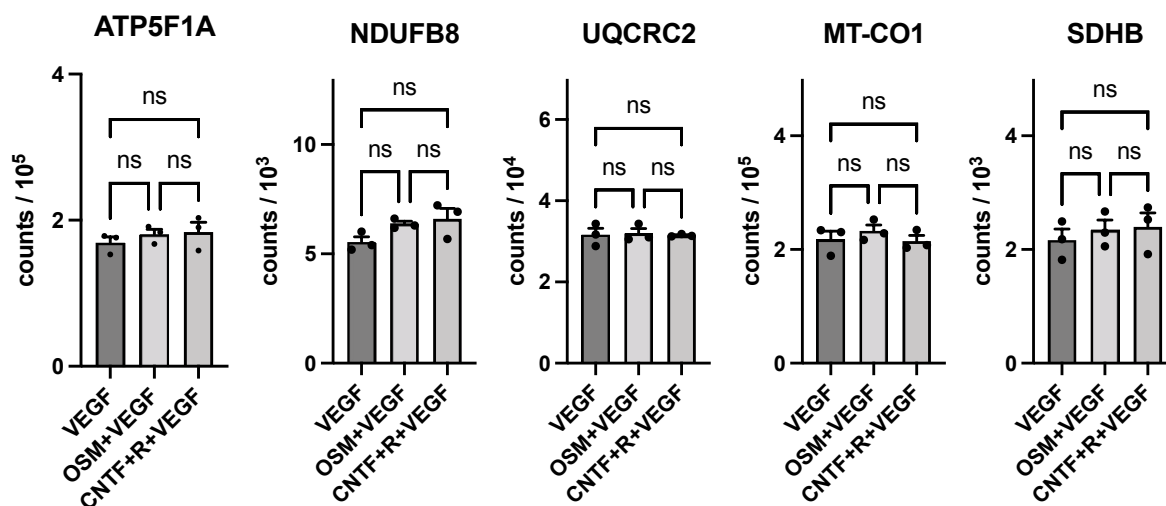


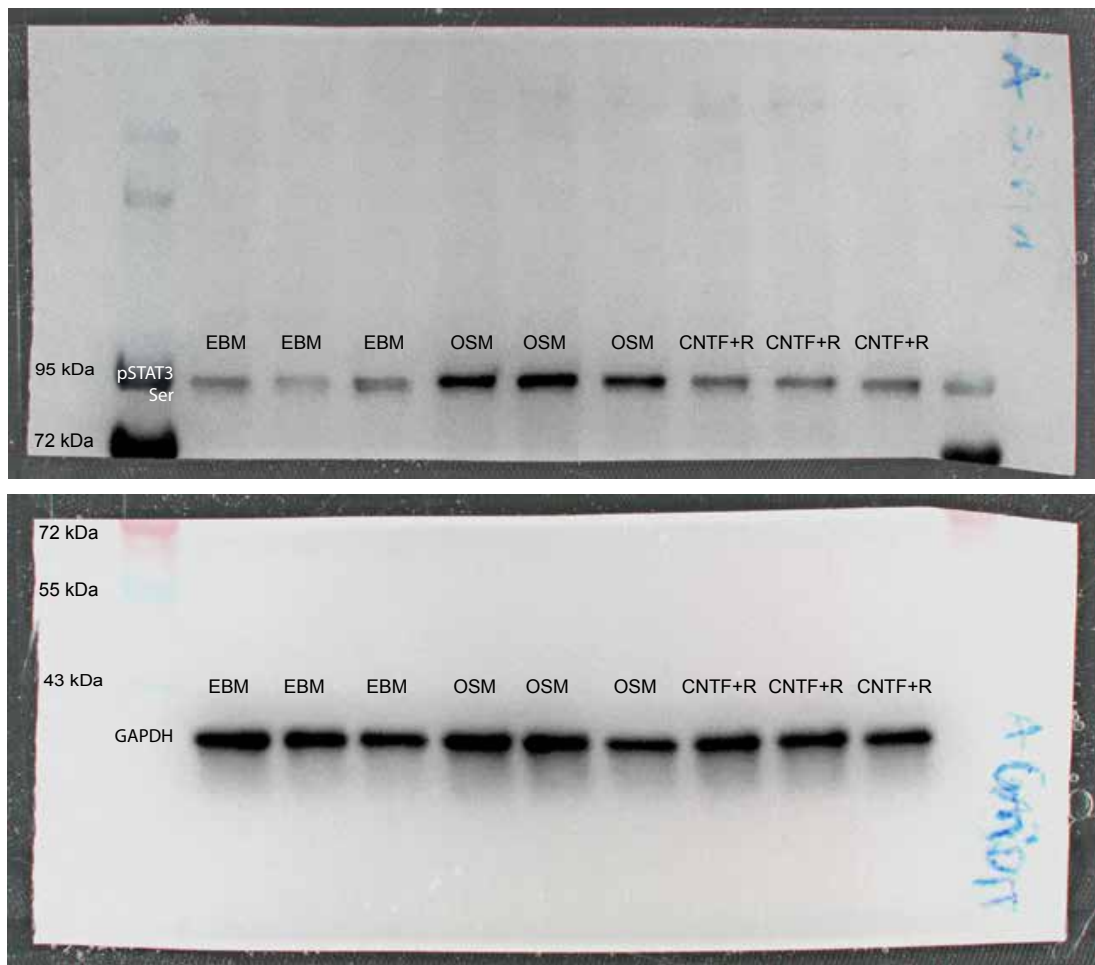
Fig. S7. RNA sequencing further validates the western blot analysis of mitochondrial complexes. Expression of different components of mitochondrial complexes in HUVECs stimulated with different cytokines for 24h according to RNASeq.

Fig. S8. Blot transparency

(I) pSTAT3Tyr, no co-stimulation with VEGF



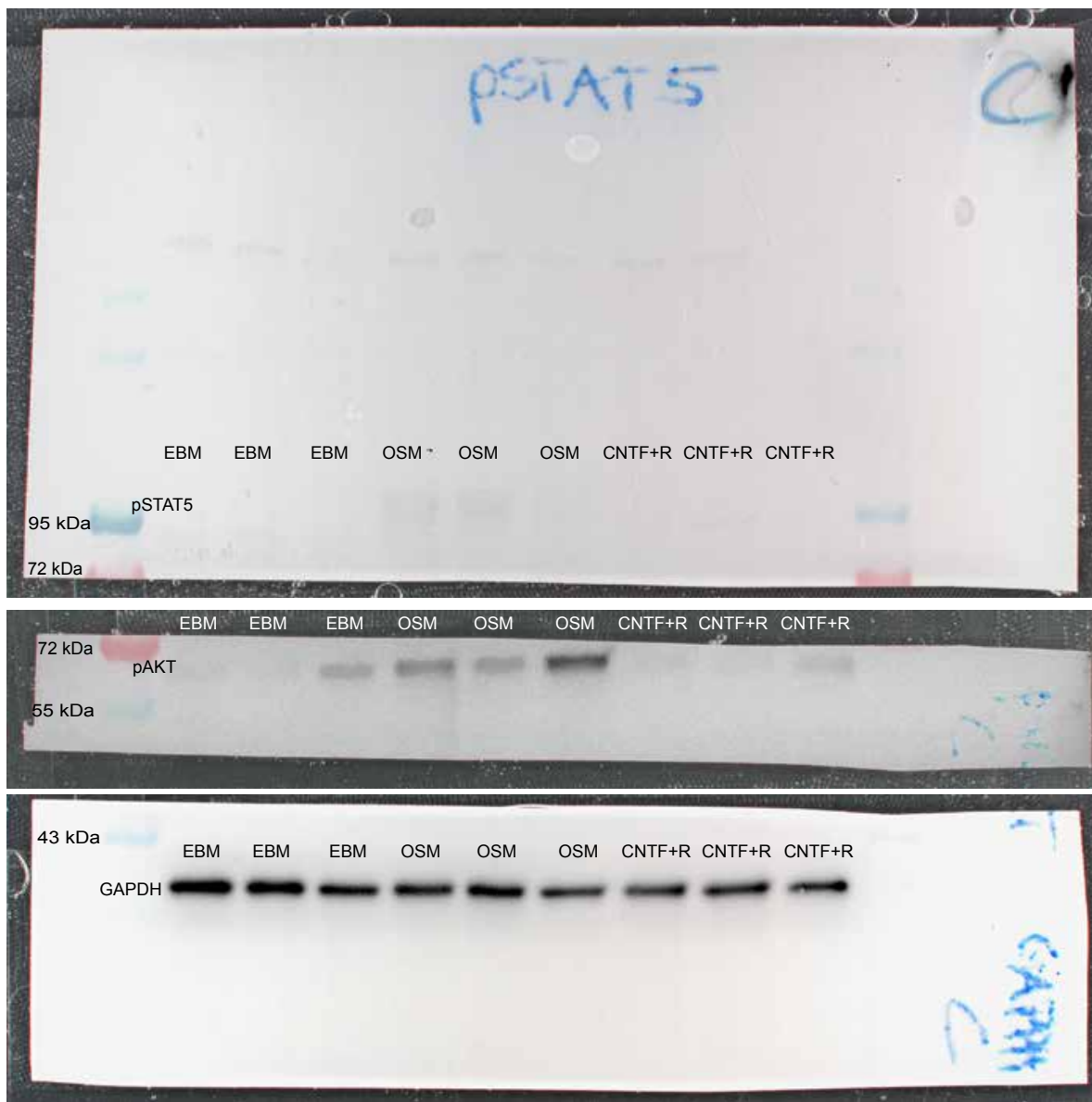
(II) pSTAT3^{Ser}, no co-stimulation with VEGF



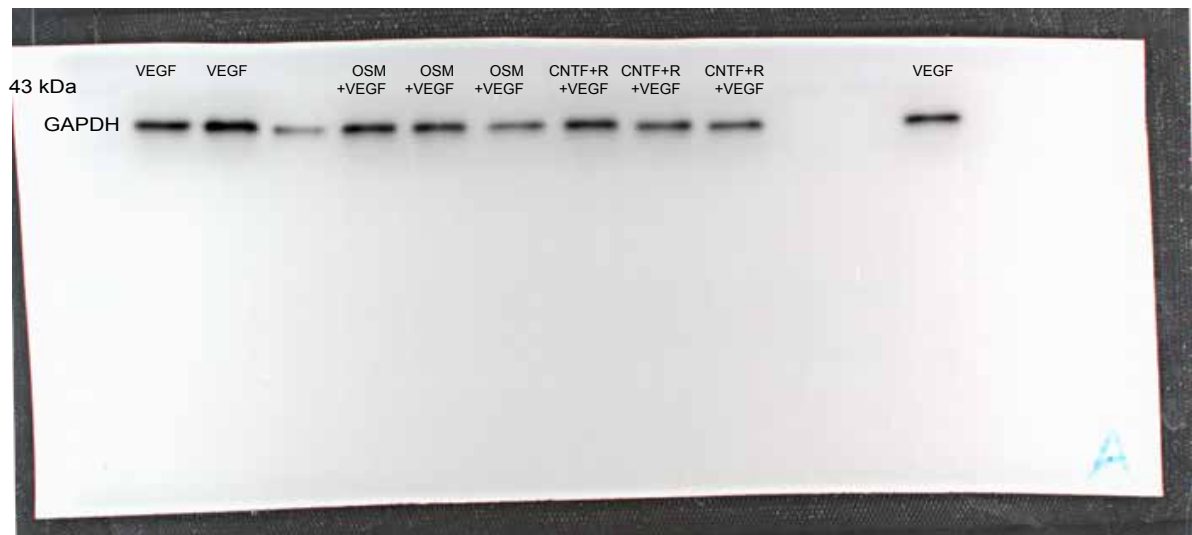
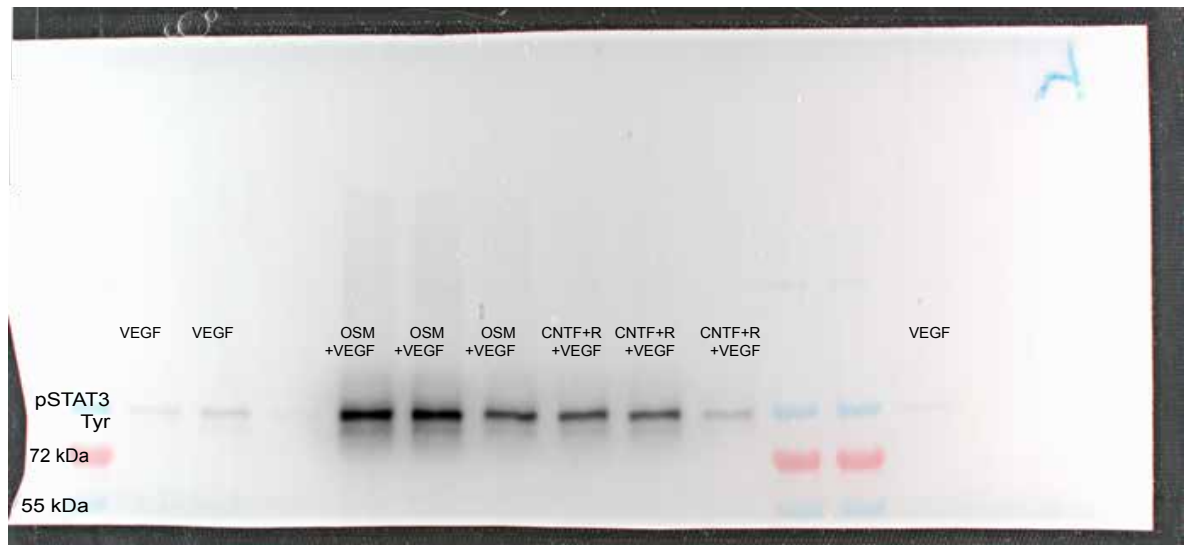
(II) pSTAT1, pERK, no co-stimulation with VEGF



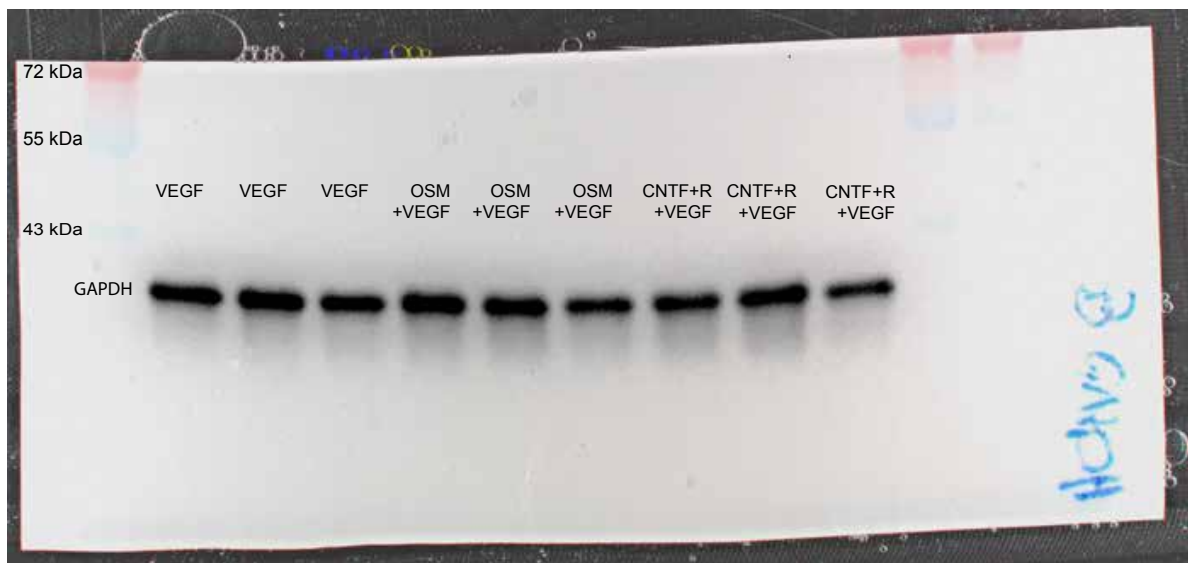
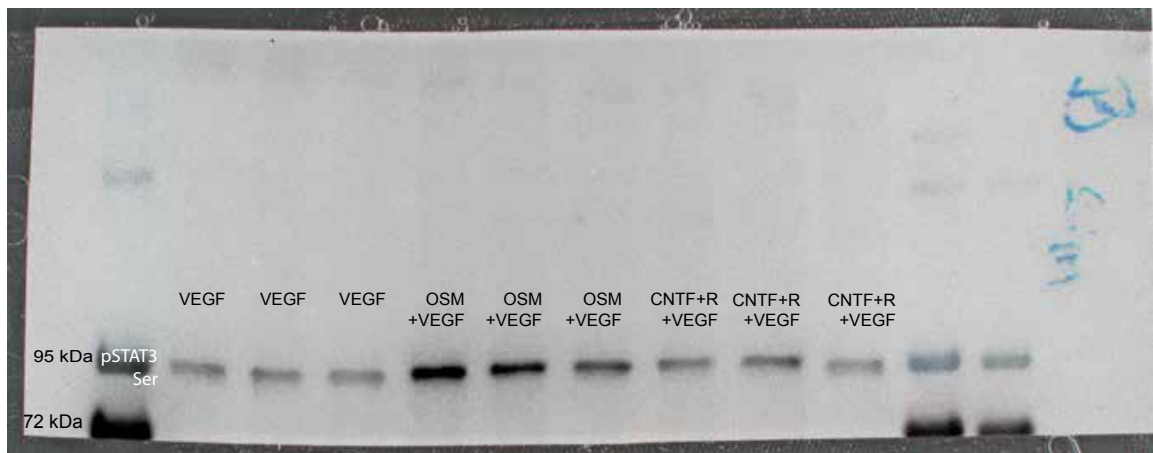
(III) pSTAT5, pAKT, no co-stimulation with VEGF



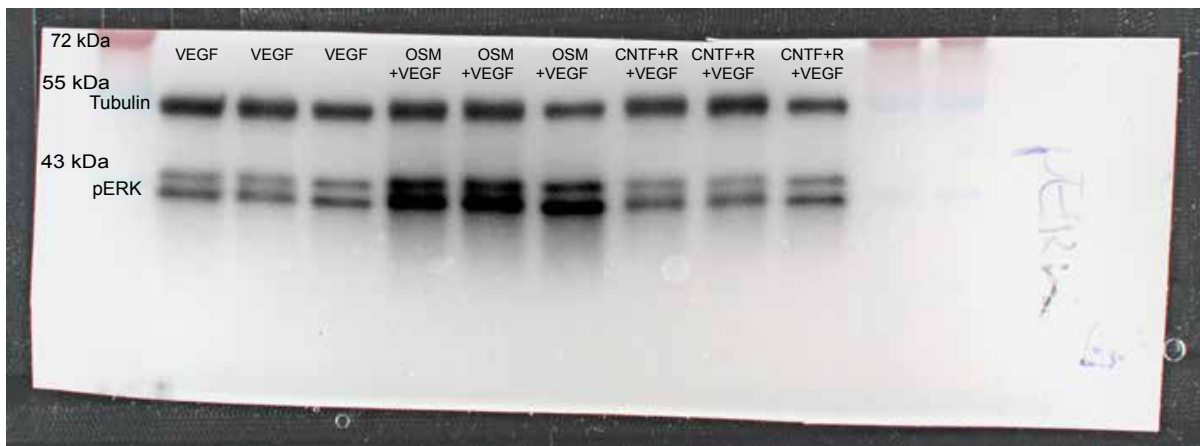
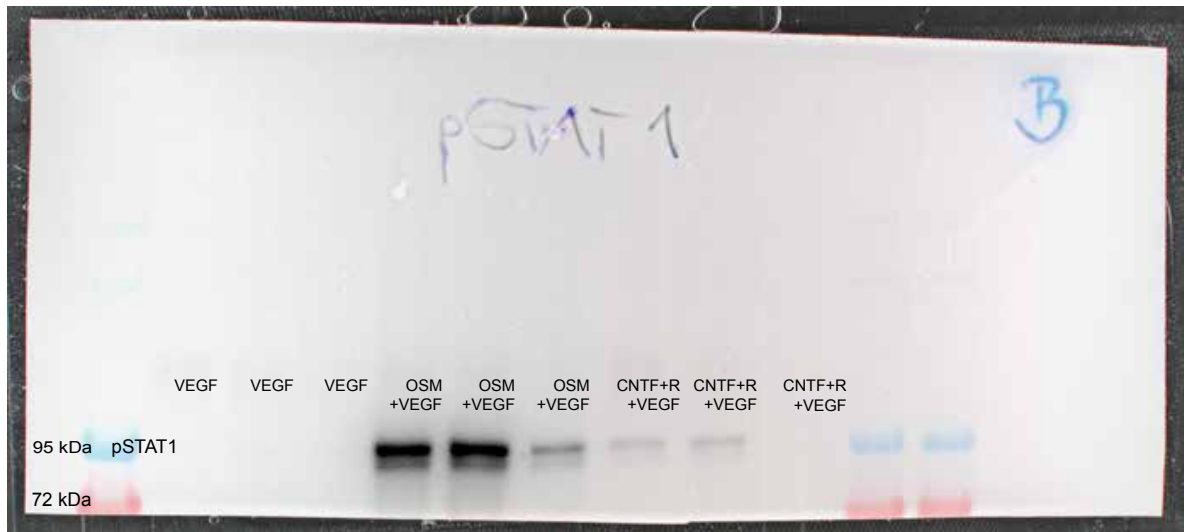
(IV) pSTAT3Tyr, co-stimulation with VEGF



(V) pSTAT3Ser, co-stimulation with VEGF



(VI) pSTAT1, co-stimulation with VEGF



(VII) pSTAT5, pAKT, co-stimulation with VEGF

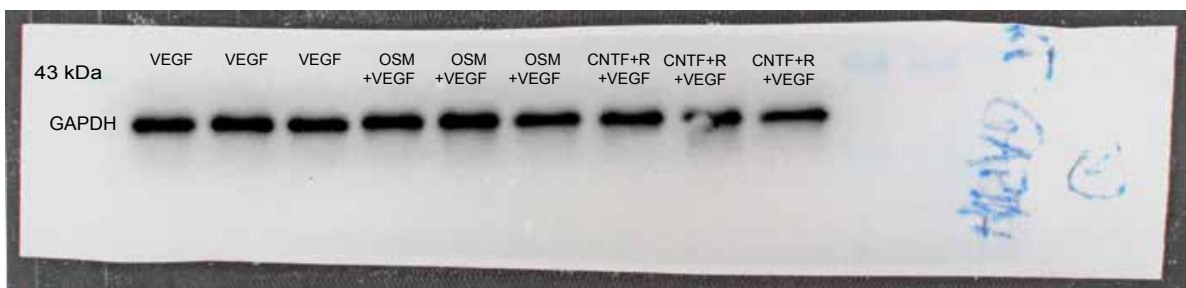
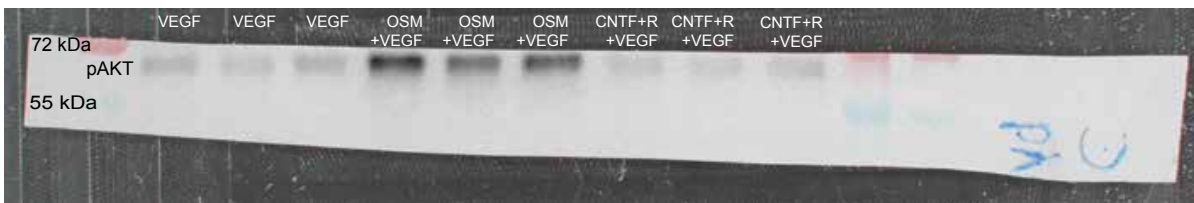
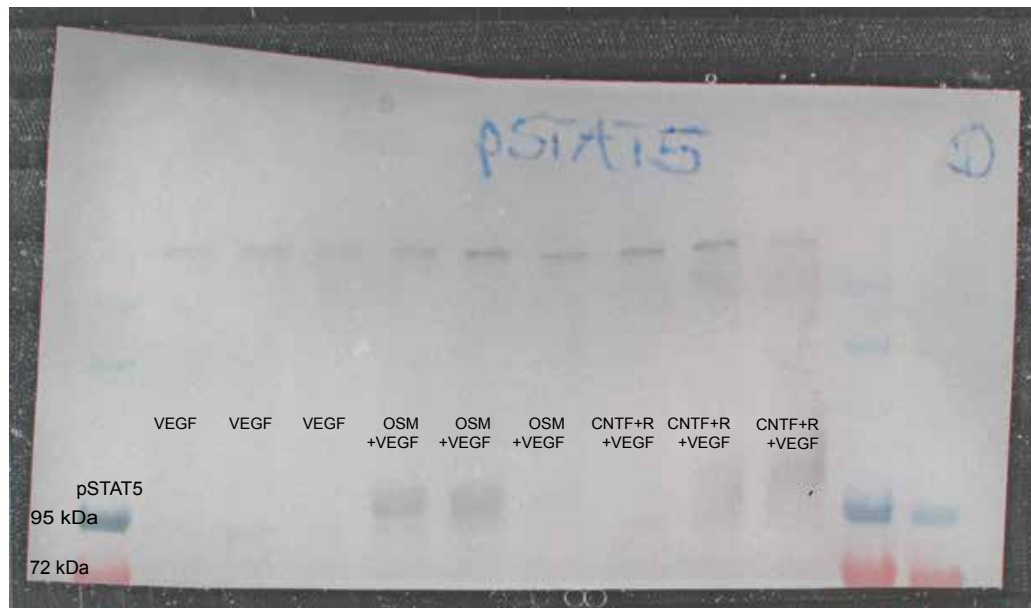
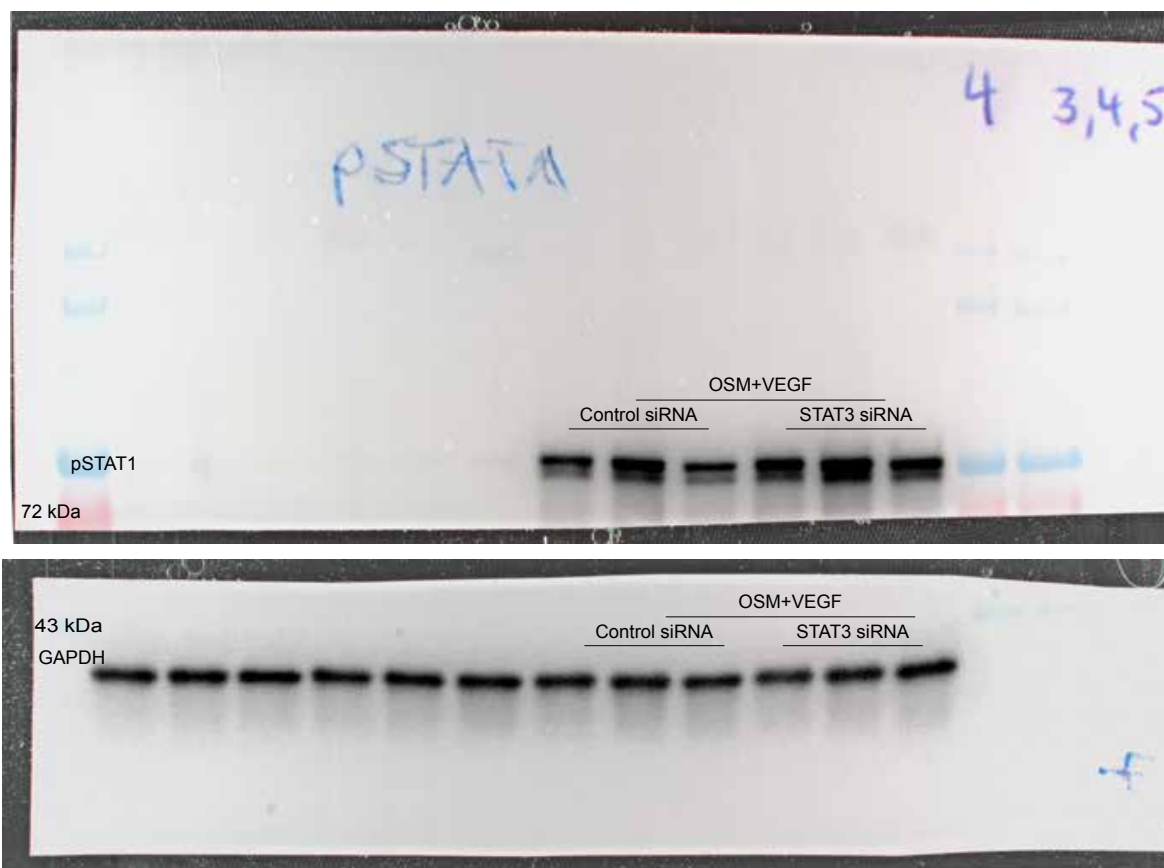


Fig. S9. Blot transparency

(I) STAT3 knock-down, OSM+VEGF (5 min), pSTAT3, pAKT



(II) STAT3 knock-down, OSM+VEGF (5 min), pSTAT1



(III) STAT3 knock-down, OSM+VEGF (5 min), pSTAT5



(IV) STAT3 knock-down, OSM+VEGF (5 min), pERK



(V) STAT3 knock-down, OSM+VEGF (30 min, 1 h), pSTAT1



(VI) STAT3 knock-down, OSM+VEGF (6 h, 24 h), pSTAT1

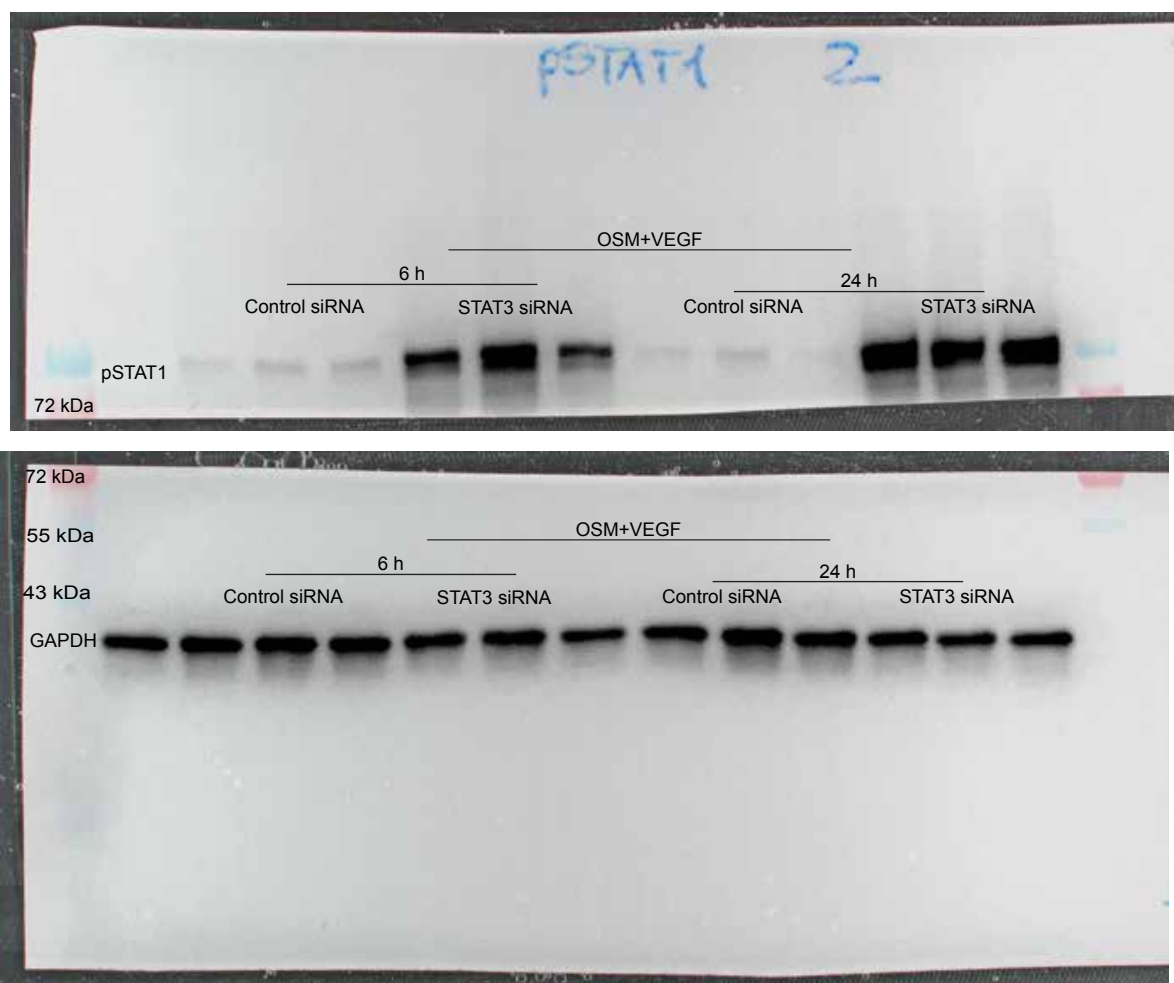
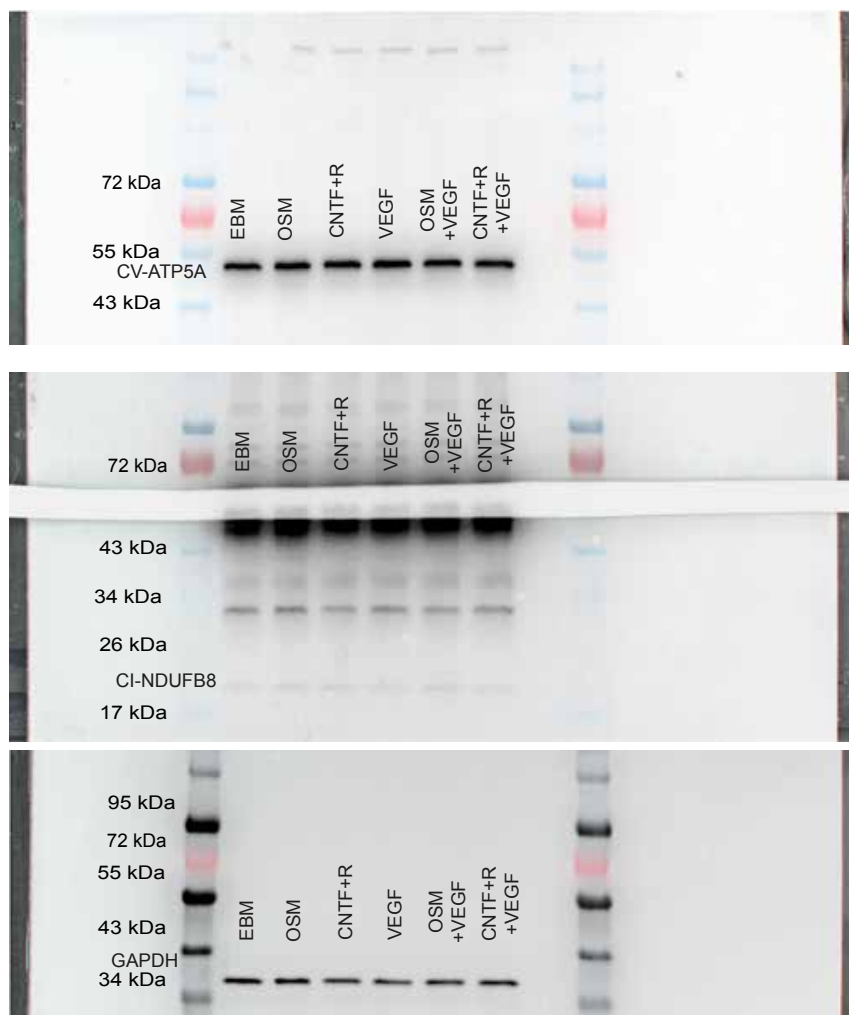


Fig. S10. Blot transparency

(I) Mitochondrial complexes 1



(II) Mitochondrial complexes 2

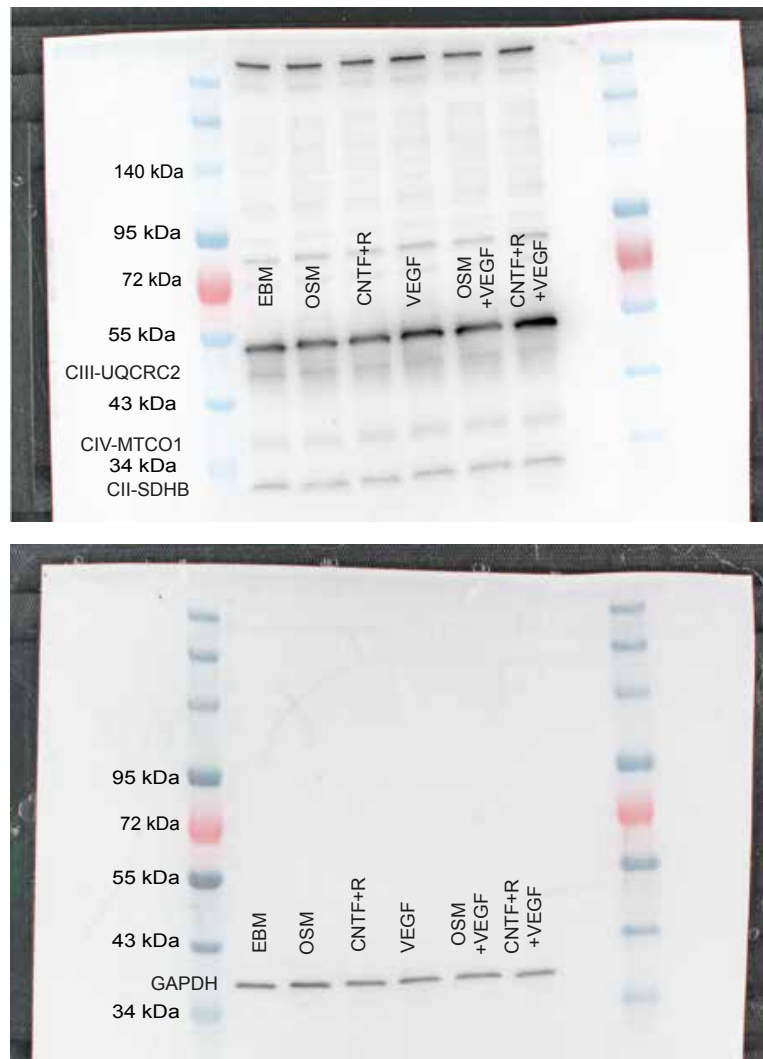
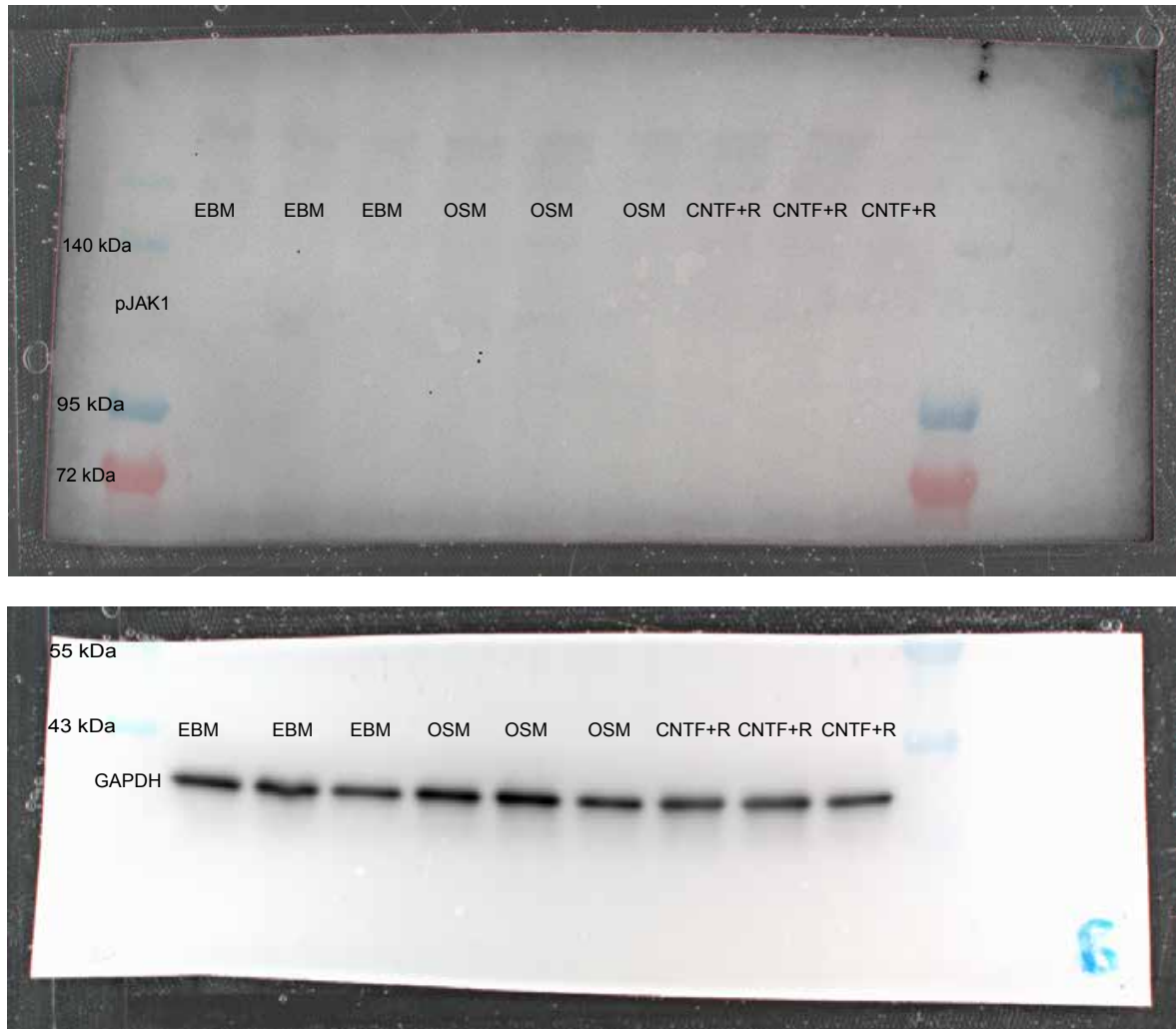
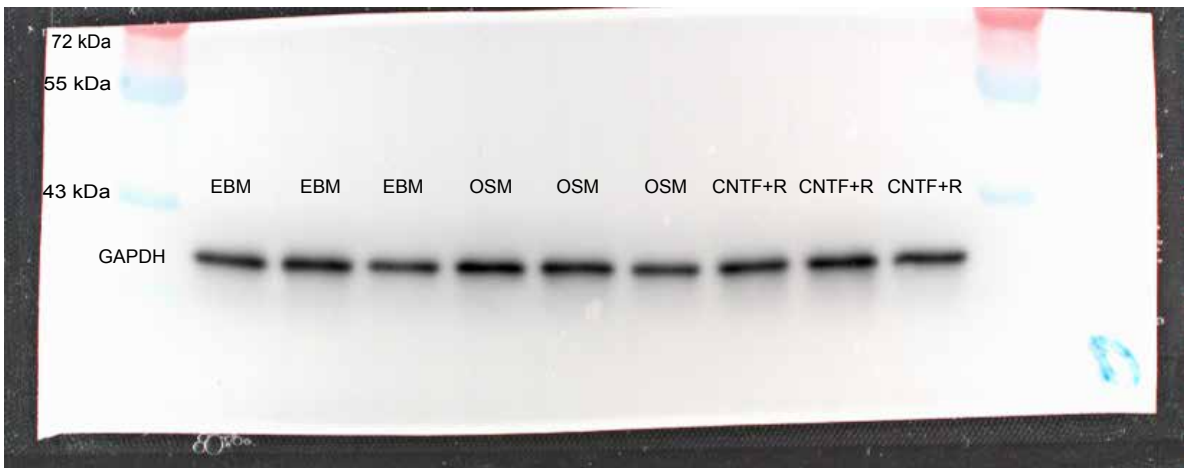
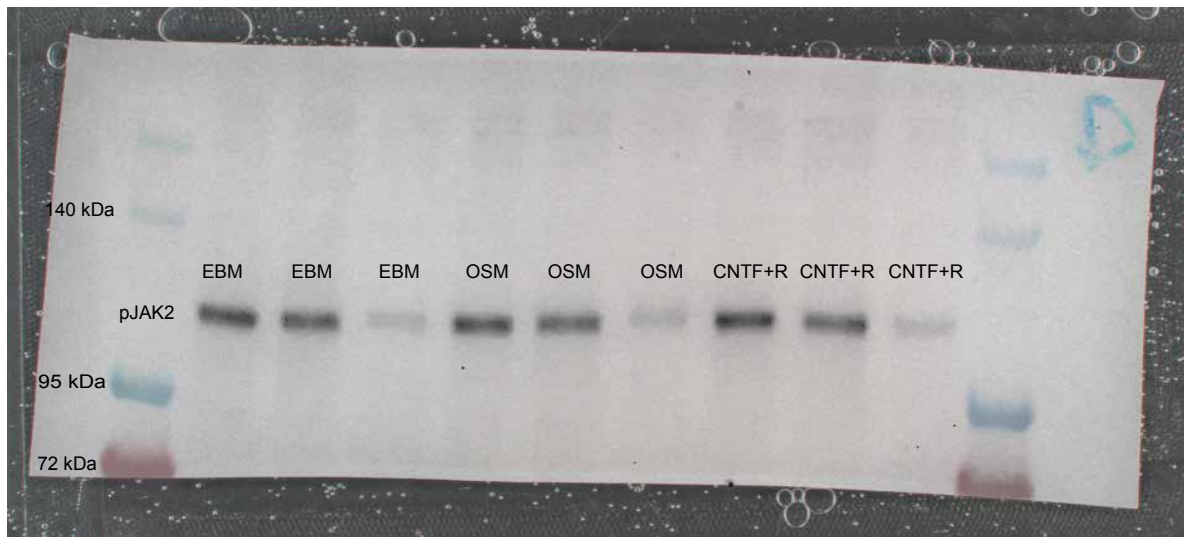


Fig. S11. Blot transparency

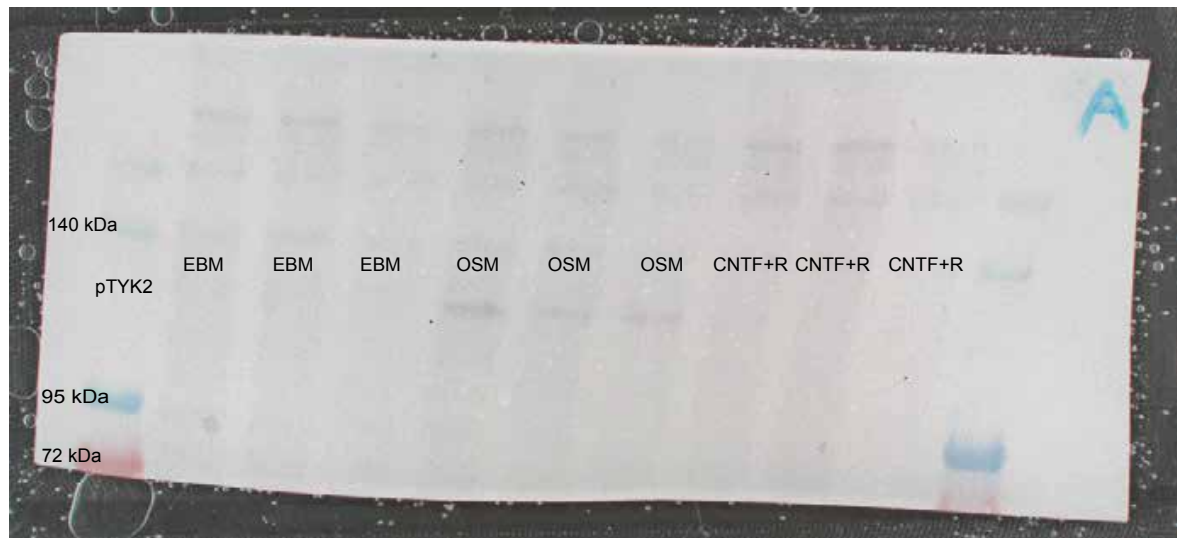
(I) pJAK1, no co-stimulation with VEGF



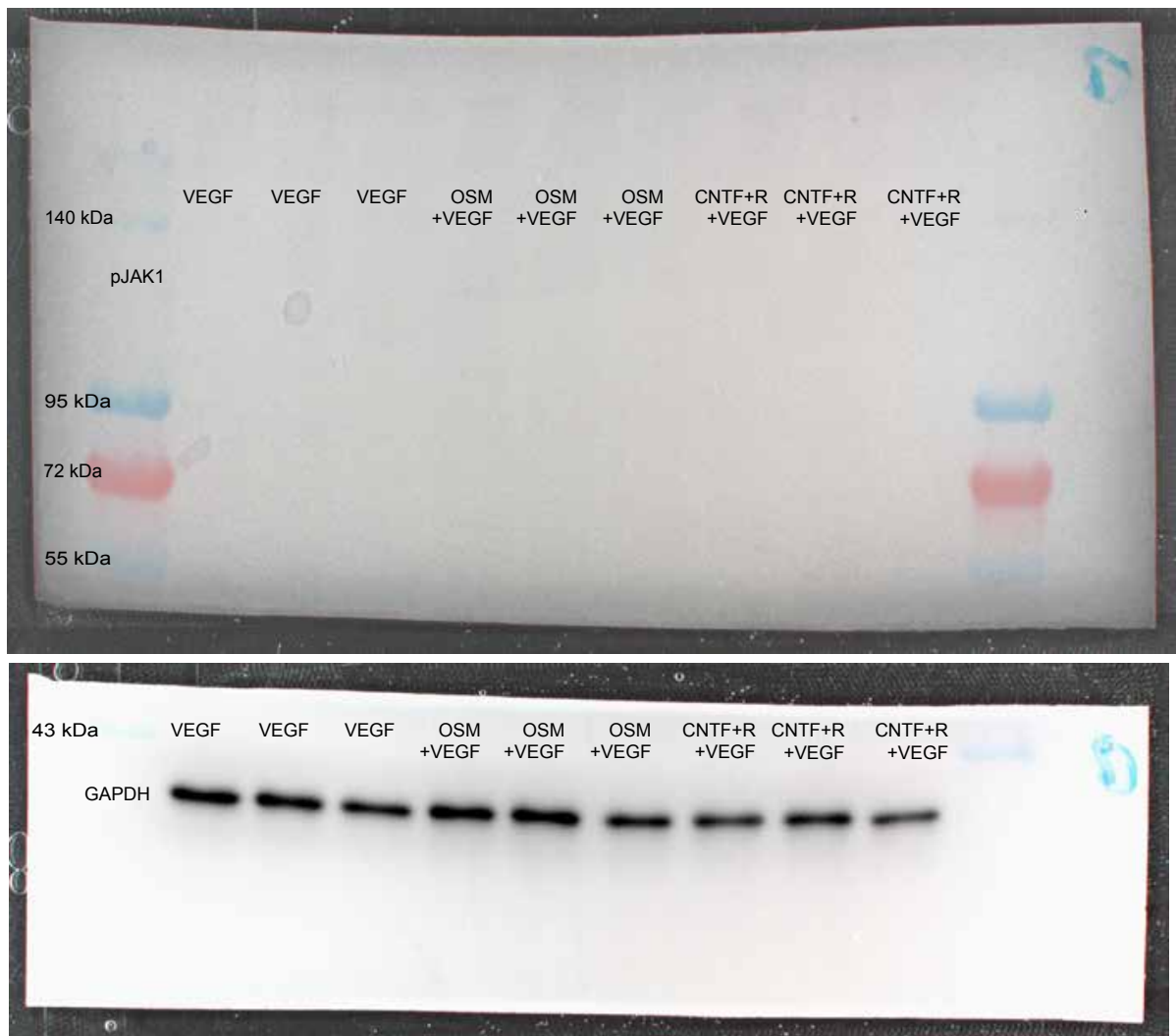
(II) pJAK2, no co-stimulation with VEGF



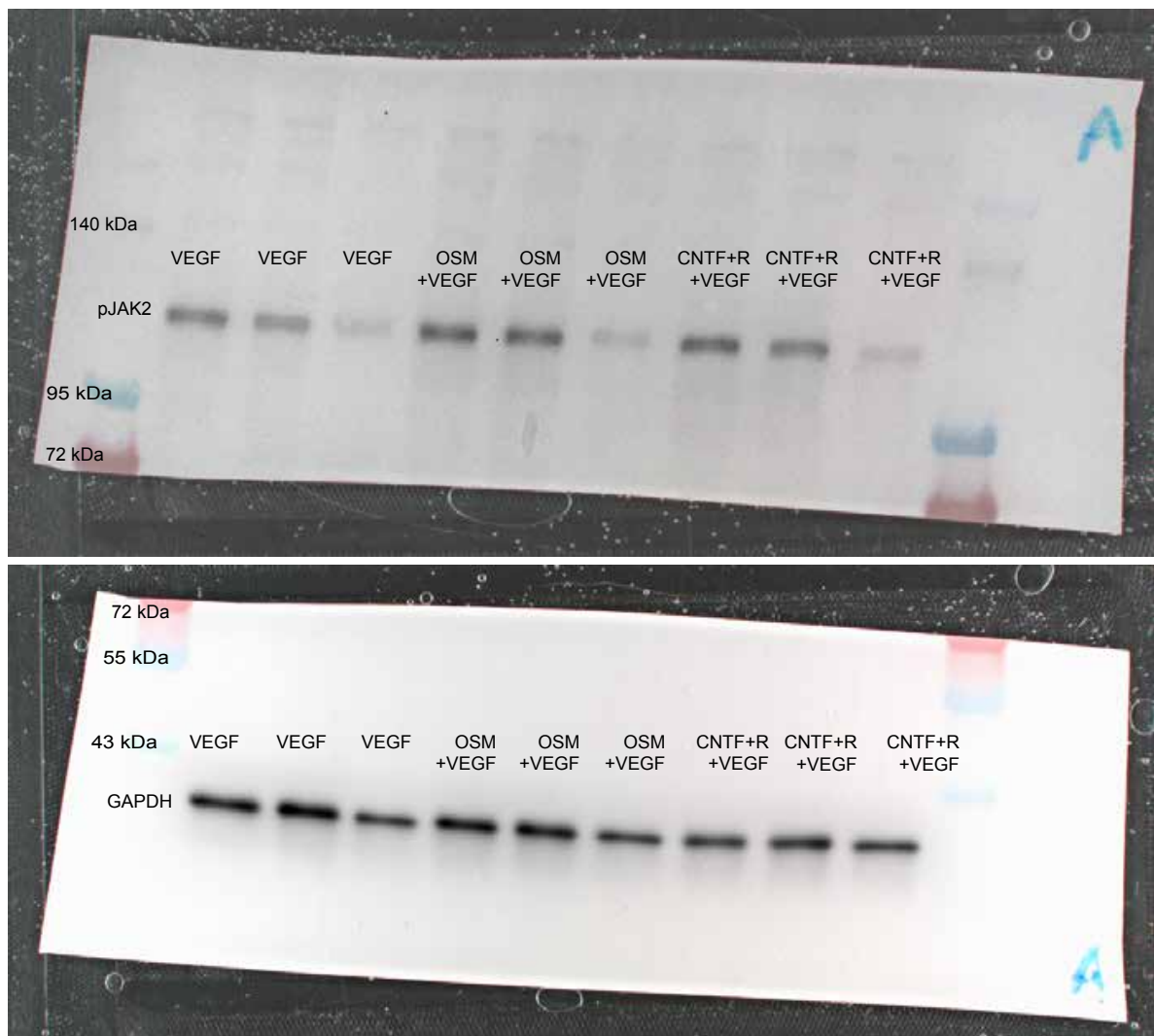
(III) pTYK2, no co-stimulation with VEGF



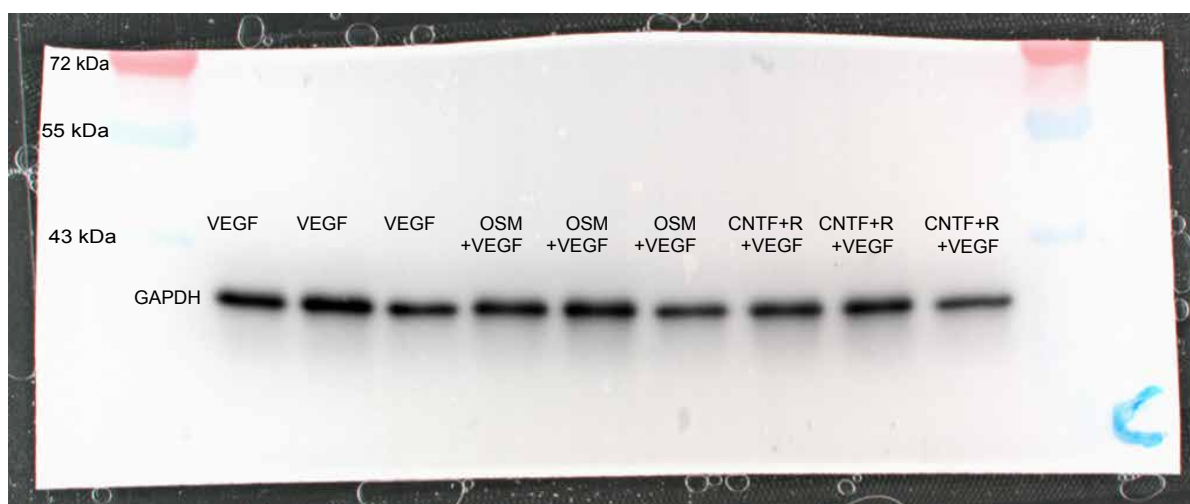
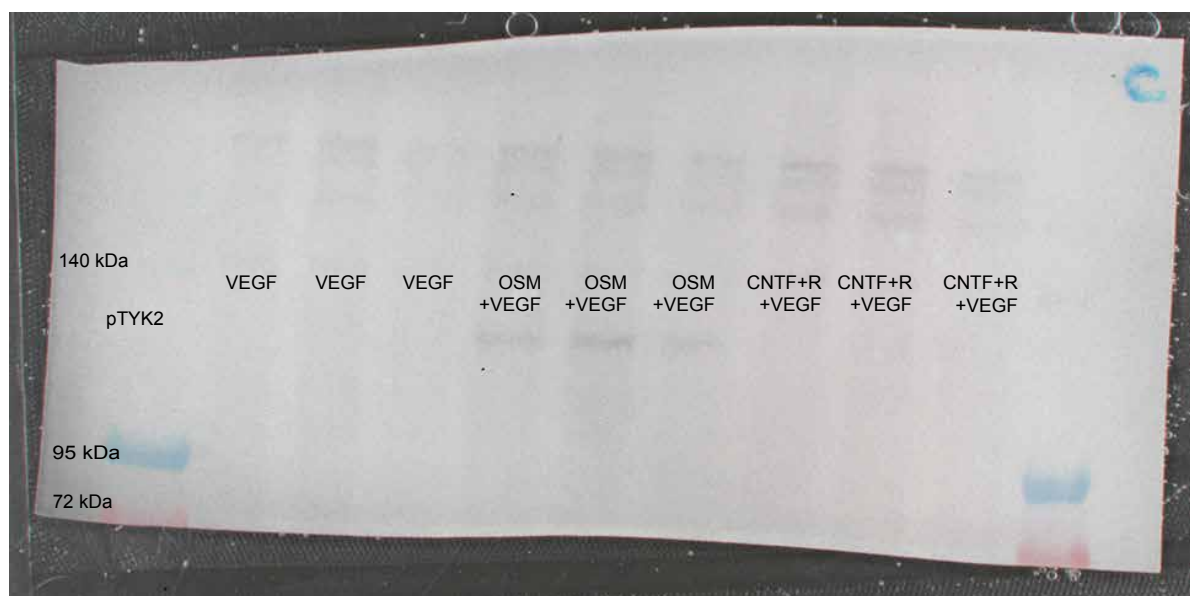
(IV) pJAK1, co-stimulation with VEGF



(V) pJAK2, co-stimulation with VEGF



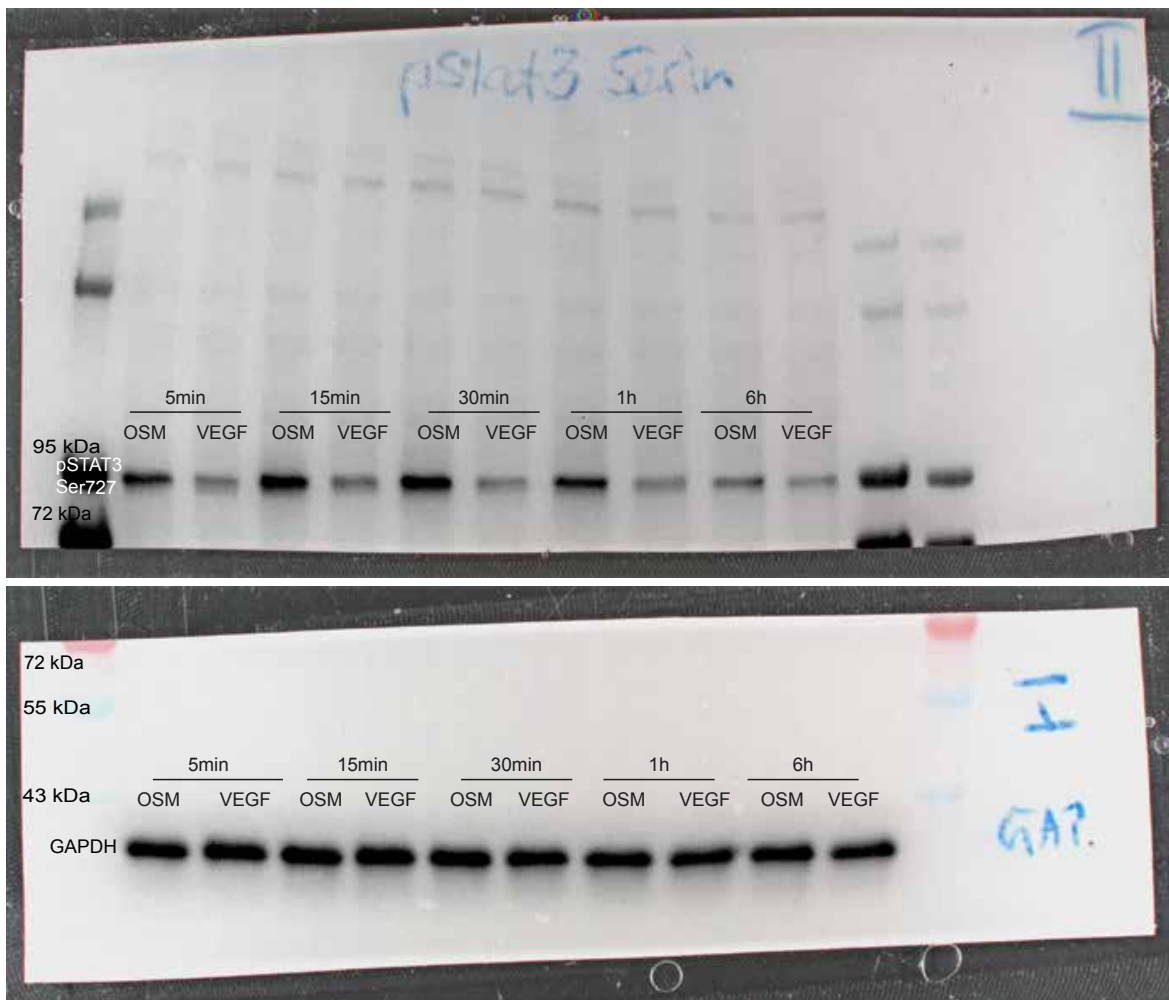
(VI) pTYK2, co-stimulation with VEGF



(VII) OSM kinetics (1)



(VIII) OSM kinetics (2)

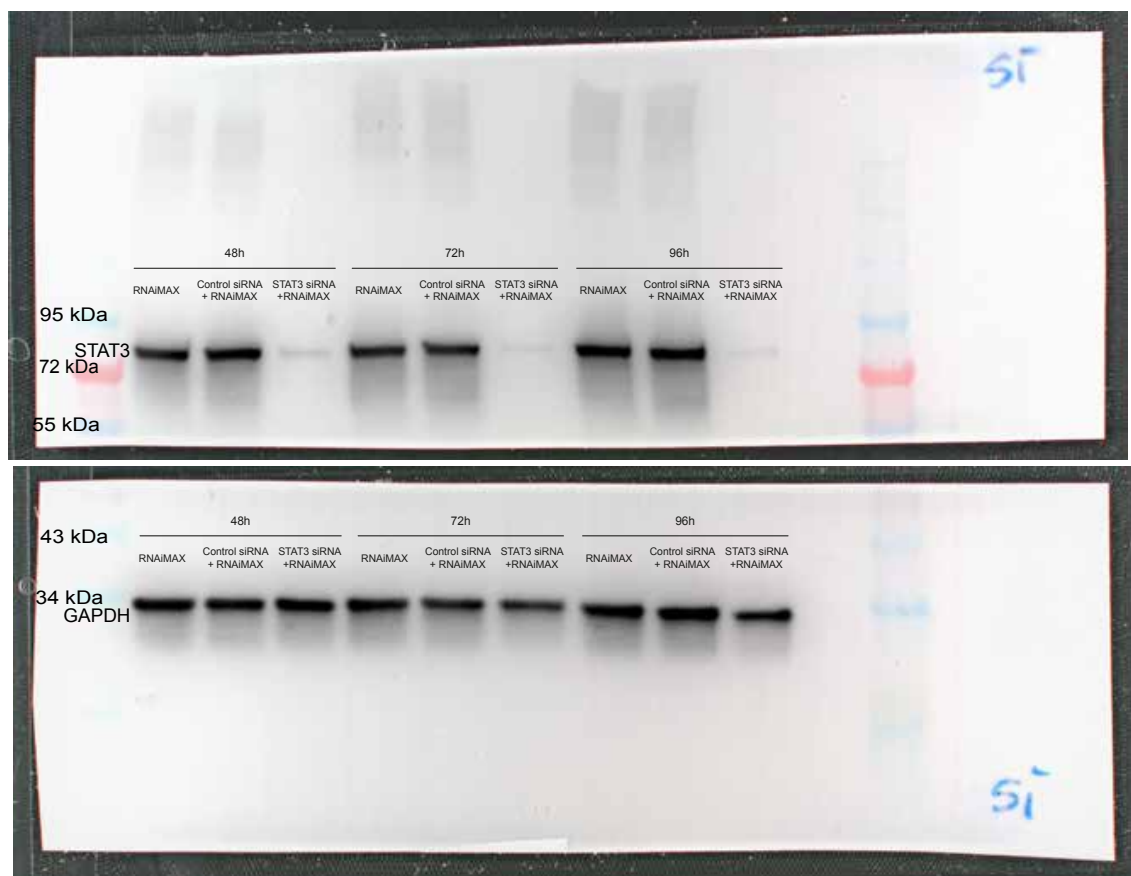


(IX) CNTF+R kinetics

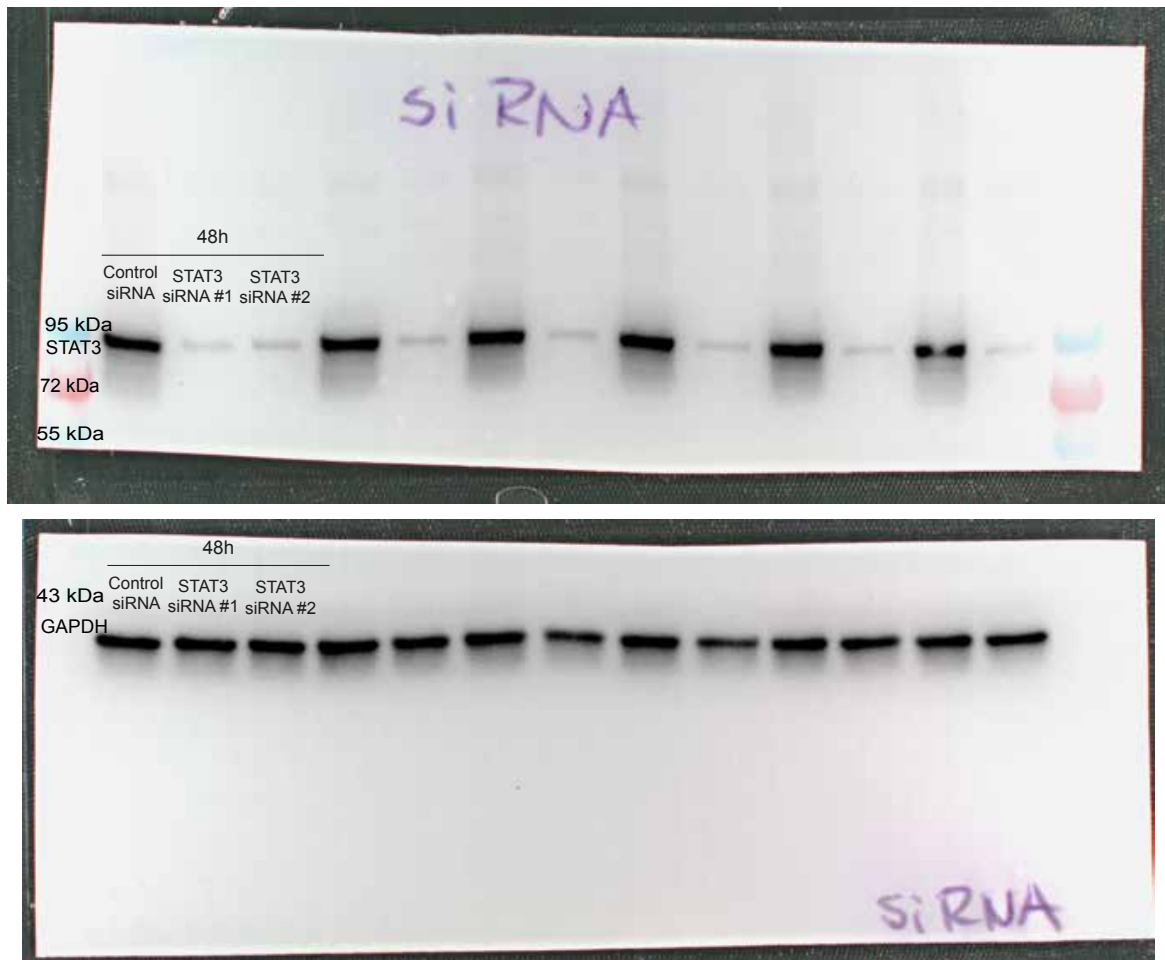


Fig. S12. Blot transparency

(I) STAT3 knock-down, siRNA 1



(II) STAT3 knock-down, siRNA 2



(III) STAT1 knock-down

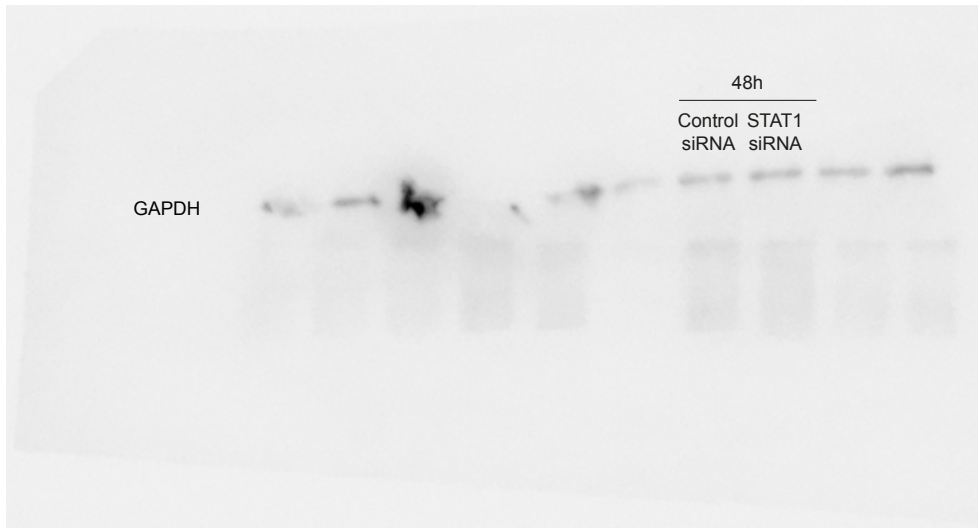
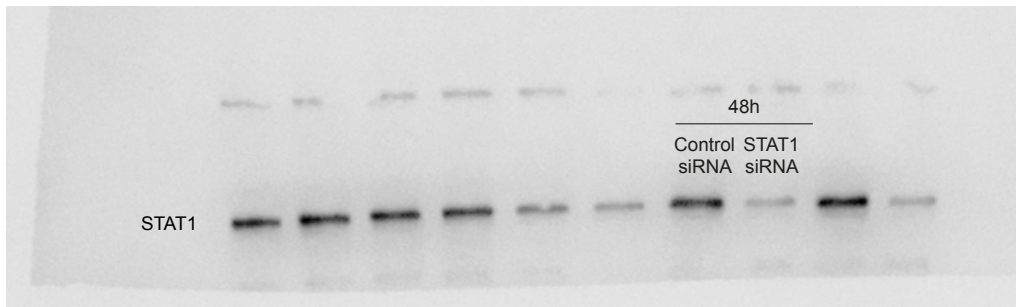


Table S1. Cytokines used.

Cytokine	Source	Cat#:	Final Concentration
OSM	Peprotech	300-10	100 ng/mL
CNTF	Peprotech	450-13	100 ng/mL
CNTFR	R&D Systems	303-CR	200 ng/mL
VEGF	Peprotech	100-20	25 ng/mL

Table S2. Antibodies used.

Antigen	Source	Cat#:	Final Concentration
pSTAT3 (Tyr705)	Cell Signaling	9145	1:2000
pSTAT3 Ser(727)	Cell Signaling	9134	1:1000
pSTAT1	Cell Signaling	9167	1:1000
pSTAT5	Cell Signaling	9359	1:1000
pAKT	Cell Signaling	4060	1:2000
pERK	Cell Signaling	4370	1:2000
pJAK1	Cell Signaling	74129	1:1000
pJAK2	Cell Signaling	8082	1: 1000
pTYK2	Cell Signaling	68790	1:1000
STAT3	Cell Signaling	4904	1:2000
OxPhos Rodent WB Antibody Cocktail	Thermo Fisher	45-8099	1:250
GAPDH	Merck	MAB374	1:10000
Goat Anti Rabbid	Jackson	111-035-003	1:10000

Table S3. Top 10 GO Terms (Biological processes) with their specific ID and the corresponding number of DEGs annotated to those terms in the RNA sequencing analysis between OSM+VEGF vs CNTF+R+VEGF stimulated HUVECs which enriched for OSM+VEGF.

GO Term	ID	# of DEGs	Total Number
inflammatory response	GO:0006954	17	788
response to cytokine	GO:0034097	17	1113
regulation of cellular component movement	GO:0051270	15	12006
enzyme linked receptor protein signaling pathway	GO:0007167	15	1002
cytokine-mediated signaling pathway	GO:0019221	14	438
regulation of cell migration	GO:0030334	14	1010
extracellular matrix organization	GO:0030198	9	323
extracellular structure organization	GO:0043062	9	324
bone resorption	GO:0045453	5	80
regulation of IL1-mediated signaling pathway	GO:2000659	3	7

Table S4. GO Terms (Molecular function) with their specific ID and the corresponding number of DEGs annotated to those terms in the RNA sequencing analysis between OSM+VEGF vs CNTF+R+VEGF stimulated HUVECs which enriched for OSM+VEGF.

GO Term	ID	# of DEGs	Total Number
cytokine receptor binding	GO:0005126	8	321
growth factor binding	GO:0019838	7	146
cytokine activity	GO:0005125	7	232
growth factor receptor binding	GO:0070851	5	151
cytokine binding	GO:0019955	5	147
insulin-like growth factor I binding	GO:0005520	3	18
plated-derived growth factor receptor binding	GO:0005161	3	14
insulin-like growth factor binding	GO:0005520	3	18

Table S5. Top 15 GO Terms (Biological processes) with their specific ID and the corresponding number of DEGs annotated to those terms in the RNA sequencing analysis between OSM+VEGF stimulated HUVECs with or without STAT3 knock-down which enriched for the knock-down group.

GO Term	ID	# of DEGs	Total Number
regulation of cell adhesion	GO:0030155	57	826
regulation of protein kinase activity	GO:0045859	56	614
regulation of MAPK cascade	GO:0043408	55	752
response to virus	GO:0009615	50	367
positive regulation of MAPK cascade	GO:0043410	45	540
positive reg. of cell migration	GO:0030335	43	596
circulatory system process	GO:0003013	42	579
defense response to virus	GO:0051607	40	305
leukocyte proliferation	GO:0070661	34	395
response to interferon-gamma	GO:0034341	30	148
cellular response to interferon-gamma	GO:0071346	29	119
type I interferone signaling pathway	GO:0060337	23	58
cellular response to type I interferon	GO:0071357	23	58
response to type I interferon	GO:0034340	23	64
leukocyte homeostasis	GO:0001776	15	137

Table S6. Top 15 GO Terms (Biological processes) with their specific ID and the corresponding number of DEGs annotated to those terms in the RNA sequencing analysis between OSM+VEGF stimulated HUVECs with or without STAT3 knock-down which enriched for the control siRNA group.

GO Term	ID	# of DEGs	Total Number
monocarboxylic acid metabolic process	GO:0032787	60	674
regulation of secretion by cell	GO:1903530	58	703
extracellular matrix organization	GO:0030198	52	323
extracellular structure organization	GO:0043062	52	324
cell-cell adhesion via plasmamembrane adhesion molecules	GO:0098742	34	218
negative regulation of growth	GO:0045926	30	267
homophilic cell adhesion via plasma membrane adhesion	GO:0007156	25	114
nicotinamide nucleotide biosynthetic process	GO:0019359	10	16

pyridine nucleotide biosynthetic process	GO:0019363	10	19
pyridine nucleotide metabolic process	GO:0019362	10	71
nicotinamide nucleotide metabolic process	GO:0046496	10	69
pyridine-containing compound biosynthetic process	GO:0072525	10	23
pyridine-containing compound metabolic process	GO:0072524	10	80
NAD biosynthetic process	GO:0009435	9	12
detoxification of copper ion	GO:0010273	6	6

Table S7. Top 15 GO Terms (Biological processes) with their specific ID and the corresponding number of DEGs annotated to those terms in the RNA sequencing analysis between CNTF+R+VEGF stimulated HUVECs with or without STAT3 knock-down which enriched for the knock-down group.

GO Term	ID	# of DEGs	Total Number
response to virus	GO:0009615	31	367
defense response to virus	GO:0051607	26	305
response to interferon-gamma	GO:0034341	26	148
cellular response to interferone-gamme	GO:0071346	24	119
type I interferon signaling pathway	GO:0060337	22	58
response to molecule of bacterial origin	GO:0002237	20	411
cellular response to type I interferon	GO:0071357	22	58
response to lipopolysaccharide	GO:0032496	19	390
response to type I interferon	GO:0034340	22	64

interferon-gamma-mediated signaling pathway	GO:0060333	16	25
negative regulation of viral process	GO:0048525	11	112
neutrophil chemotaxis	GO:0030593	10	104
negative regulation of viral genome replication	GO:0045071	9	67
defense response to protozoan	GO:0042832	6	39
response to protozoan	GO:0001562	6	44

Table S8. Top 15 GO Terms (Biological processes) with their specific ID and the corresponding number of DEGs annotated to those terms in the RNA sequencing analysis between CNTF+R+VEGF stimulated HUVECs with or without STAT3 knock-down which enriched for the control siRNA group

GO Term	ID	# of DEGs	Total Number
cell-cell adhesion via plasmamembrane adhesion molecules	GO:0098742	27	218
carboxylic acid biosynthetic process	GO:0046394	24	307
organic acid biosynthetic process	GO:0016053	24	310

extracellular matrix organization	GO:0030198	24	323
extracellular structure organization	GO:0043062	24	324
homophilic cell adhesion via plasma membrane adhesion molecules	GO:0007156	21	114
regulation of cell morphogenesis involved in differentiation	GO:0010769	23	130
developmental growth involved in morphogenesis	GO:0060560	18	288
developmental cell growth	GO:0048588	18	284
neuron projection extension	GO:1990138	16	208
regulation of axonogenesis	GO:0050770	16	187
leukocyte tethering or rolling	GO:0050901	7	38
regulation of leukocyte tethering or rolling	GO:1903236	5	22
neurotransmitter receptor transport to plasma membrane	GO:0098877	5	23
neurotransmitter receptor transport to postsynaptic membrane	GO:0098969	5	21

Table S9. Top 15 GO Terms (Biological processes) with their specific ID and the corresponding number of DEGs annotated to those terms in the RNA sequencing analysis between VEGF stimulated HUVECs with or without STAT3 knock-down which enriched for the control siRNA group

GO Term	ID	# of DEGs	Total Number
extracellular matrix organization	GO:0030198	20	323
extracellular structure organization	GO:0043062	20	324

Table S10. GO Terms (Biological processes) with their specific ID and the corresponding number of DEGs annotated to those terms in the RNA sequencing analysis between VEGF stimulated HUVECs with or without STAT3 knock-down which enriched for the control siRNA group

GO Term	ID	# of DEGs	Total Number
small molecule biosynthetic process	GO:0044283	35	564
carboxylic acid biosynthetic process	GO:0046394	24	307
organic acid biosynthetic process	GO:0016053	24	310
monocarboxylic acid biosynthetic process	GO:0072330	18	215