Int J Clin Exp Pathol 2015;8(5):4953-4962
www.ijcep.com /ISSN:1936-2625/IJCEP0008352

Original Article

Expression and function of microRNA-188-5p in activated rheumatoid arthritis synovial fibroblasts

Anke Ruedel[2*], Peter Dietrich[1*], Thomas Schubert[3], Simone Hofmeister[2], Claus Hellerbrand[4], Anja-Katrin Bosserhoff[1]

[1]Institute for Biochemistry, Biochemistry and Molecular Medicine, Emil-Fischer Zentrum, Friedrich-Alexander-University of Erlangen-Nürnberg, Fahrstraße 17, D-91054 Erlangen, Germany; [2]Institute of Pathology, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany; [3]Institute of Pathology, University Hospital of Erlangen, Krankenhausstr.12, D-91054 Erlangen, Germany; [4]Department of Internal Medicine I, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany. [*]Equal contributors.

Received March 23, 2015; Accepted April 15, 2015; Epub May 1, 2015; Published May 15, 2015

Abstract: Activated synovial fibroblasts in rheumatoid arthritis (RASF) play a critical role in the pathology of rheumatoid arthritis (RA). Recent studies suggested that deregulation of microRNAs (miRs) affects the development and progression of RA. Therefore, we aimed to identify de-regulated miRs in RASF and to identify target genes that may contribute to the aggressive phenotype of RASF. Quantitative real-time PCR revealed a marked downregulation of miR-188-5p in synovial tissue samples of RA patients as well as in RASF. Exposure to the cytokine interleukine-1β lead to a further downregulation of miR-188-5p expression levels compared to control cells. Re-expression of miR-188-5p in RASF by transient transfection significantly inhibited cell migration. However, miR-188-5p re-expression had no effects on glycosaminoglycan degradation or expression of repellent factors, which have been previously shown to affect the invasive behavior of RASF. In search for target genes of miR-188-5p in RASF we performed gene expression profiling in RASF and found a strong regulatory effect of miR-188-5p on the hyaluronan binding protein KIAA1199 as well as collagens COL1A1 and COL12A1, which was confirmed by qRT-PCR. *In silico* analysis revealed that KIAA1199 carries a 3'UTR binding site for miR-188-5p. COL1A1and COL12A1 showed no binding site in the mRNA region, suggesting an indirect regulation of these two genes by miR-188-5p. In summary, our study showed that miR-188-5p is down-regulated in RA *in vitro* and *in vivo*, most likely triggered by an inflammatory environment. MiR-188-5p expression is correlated to the activation state of RASF and inhibits migration of these cells. Furthermore, miR-188-5p is directly and indirectly regulating the expression of genes, which may play a role in extracellular matrix formation and destruction in RA. Herewith, this study identified potential novel therapeutic targets to inhibit the development and progression of RA.

Keywords: Rheumatoid arthritis, synovial fibroblasts, miR-188-5p, KIAA1199

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease affecting synovial joints eventually leading to cartilage degradation and loss of flexibility of the joints. However, the origin of RA still remains unknown [1]. Synovial fibroblasts (SF) are key players in the development of RA. Activated SF in RA (RASF) are localized in the hyperplastic synovium of patients with RA. Supported by adhesion molecules, RASF attach to cartilage and release matrix-degrading enzymes like MMP3 [2], thereby destructing the joint [3]. Compared to normal synovial fibroblasts (nSF), RASF display 'tumor-like' features and show increased migration and invasiveness into cartilage [4]. Over the past 2 years, major advances have continued to emerge in understanding of the relationship between synovial fibroblasts and the regulation of inflammatory pathways in the rheumatoid arthritis synovium [5], but the exact mechanisms still remain elusive.

In recent years, microRNAs (miRs) have been shown to play an important role for the development and progression of RA. MiRs are 18-22 nucleotides long RNA molecules, regulating stability and translation of messenger RNA (mRNA). Together with argonaute proteins, miRs can be loaded into a RNA-induced silencing complex (RISC), and the miRNA/RISC complex then can

bind to the target mRNAs. Depending on perfect or imperfect complementarity to their target 3'UTR binding sites, miRNA/RISC can either lead to degradation or inhibition of translation of target mRNAs. Several studies suggested that miRs, regulated *via* inflammatory signals, may provide new targets for RA therapy [6, 7]. For example, reduced expression of miRs results in up-regulation of their target genes due to less microRNA induced mRNA degradation. In the context of inflammatory lung diseases, decreased expression of miR-25, miR-140, miR-188, and miR-320 induced by pro-inflammatory stimuli was found [8].

As pro-inflammatory cytokines play a crucial role in RA, the regulation of miRs and their target genes *via* these cytokines could contribute to the activation of SF. However, for the up-regulation of activation-related genes in RASF, responsible miRs would have to be down-regulated in these cells. Therefore, the aim of this study was to find microRNAs which are down-regulated in early passage RASF and to identify target genes that may contribute to the aggressive phenotype of RASF. Furthermore, the role of cytokines like IL-1β in the context of RASF associated microRNA dysregulation was addressed.

Material and methods

Cell culture

Synovial tissue samples from patients with RA were obtained immediately after opening the knee joint capsule as previously described [9]. RASF were cultured in DMEM (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% fetal calf serum (FCS, PAN Biotech, Aidenbach, Germany) and penicillin and streptomycin (P/S, PAA). The cell line HSE (immortalized RASF) and K4Im (immortalized nSF) were also cultured in DMEM (plus 10% FCS and P/S). All cells were incubated under humid conditions in a 5% CO_2 incubator at 37°C. Synovial tissue harvesting and processing was in accord with the guidelines of Office for Human Research Protections (OHRP) Human Subject Protections.

Transfection experiments

Cells were transfected using Lipofectamine® RNAiMAX Transfection Reagent (Life technologies, Carlsbad, California, USA) according to the manufacturer's protocol. 100,000 to 200,000 cells were transfected with 90 pmol hsa-miR-188-5p mimic (Qiagen, Hilden, Germany) and the respective control (Qiagen) for 3 times over 7 d or mirVana™ hsa-miR-188-5p inhibitor (Life technologies) or the control (Life technologies) for 72 h.

Isolation of total RNA, reverse transcription and quantitative real-time PCR

Total cellular RNA was isolated from cultured cells using the Total RNA Kit (VWR, Darmstadt, Germany) and cDNAs were generated by reverse transcriptase reaction as described previously [10]. Real-time quantitative PCR (qRT-PCR) was performed using SYBR Green master mix (QIAGEN) with the following primer pairs: Collagen1A1 (5'-CGG CTC CTG CTC CTC TT-3' and 5'-GGG GCA GTT CTT GGT CTC-3'), Collagen12A1 (5'-TGA GGC AGA AGT TGA CCC AC-3' and 5'-GTA GGC CCA TCC GTT GTA GG-3'), ROBO3 (5'-ACA TCC CTC AGG AGA TCT GG-3' and 5'-TCA CTT TGC CTC CCT TGG-3'), UNC5B (5'-GAC GAA TTC GCC GCG GGA GCA TGG-3' and 5'-GAC GAA TTC TAA TCC TCC AGC CCA AAG AGC-3'), UNC5C (5'-GAC GAA TTC ACG ATG AGG AAA GGT CTG CG-3' and 5'-GAC GAA TTC GTC TCA TCT GGA TTT CCT CCT C-3'), Gremlin2 (5'-CGC TTC TCT TAT GGG CGT CT-3' and 5'-CCA GAA CAT CCT GCA ATG ACG-3'), Integrin-α7 (5'-GCT GTG GCT GCC CTC CAT CCC TTC-3' and 5'-CCC TCT AGG TTA AGG CAC TTC CGG G-3') and KIAA1199 (5'-TCT CTG CCA GAT ACA GCC CT-3' and 5'-AGG GCC CCA GAT CCT ATT GT-3'). Relative gene expression was normalized to β-actin (5'-CTA CGT GGC CCT GGA CTT CGA GC-3' and 5'-GAT GGA GCC GCC GAT CCA CAC GG-3') mRNA levels using the comparative cycle threshold (Ct) method.

Isolation of miRNA, reverse transcription and quantitative real-time PCR

MicroRNA was isolated using mirVana™ miRNA Isolation Kit (Life technologies). Reverse transcription was performed using mirVanaTM qRT-PCR miRNA Detection Kit (Life technologies) and specific primers for hsa-miR-188-5p (5'-CGC GCC TGC AGG TCG ACA ATT AAC CCT CAC TAA AGG GCC CCT CCU CCU TG-3'). Quantitative RT-PCR was performed on a LightCycler (Roche, Mannheim, Germany) using 10 µl N', N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-yli-

dene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine (SYBR) MIX (TaKaRa, Shiga, Japan) [4] and a specific Primer for miR-188-5p (5'-GTA ATA CGA CTC ACT ATA GGG AGA AGA GCA UCC CUU GCA-3').

Protein isolation and western blot analysis

Protein extracts from primary cells and cell lines were homogenized in 100 µl or 200 µl RIPA-buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% (w/v) Nonidet® P40; 0.5% (w/v) sodium deoxycholate; 0.1% (w/v) SDS; protease inhibitors), respectively. Insoluble fragments were removed by centrifugation at 13000 rpm for 10 min. The supernatant was stored at -20°C. For western blot, protein lysates were separated on 10% SDS-PAGE gels and blotted onto a PVDF membrane. After blocking with 5% milk powder/TBS-T (1%), primary antibodies were applied (anti-KIAA1199 (Sigma-Aldrich), 1:3,000; anti-ß-Actin, 1:4,000). Alkaline phosphatase-conjugated anti-rabbit antibody (1:4,000, Cell Signaling Technology, Boston, USA) or anti-mouse antibody (1:3,000, Chemicon/Millipore, Darmstadt, Germany) served as secondary antibodies. Protein bands in western blots were quantified using computer based colorimetric quantification analysis (ImageJ, by Wayne Rasband).

Migration assays using the xCELLigence system

Migratory potential was determined using CIM plates in the xCELLigence System of Roche Diagnostics (Penzberg, Germany) as described before [11]. The experiments were performed with 20,000 cells per 100 µl per well. Cell migration was analyzed for up to 8 h in the xCELLigence instrument.

Cartilage destruction assay

Cartilage was obtained from donors after informed consent and approval of the local ethics committee. Normal cartilage with no significant softening or surface fibrillation was obtained during autopsy within 48 h after death. Chondrocytes in the cartilage were destroyed by storage at -80°C before use. SF in early passages, 50,000 cells per attempt, were incubated with 8 pieces of cartilage of 2 mm^3 in 400 µl of medium for 10 days as described previously [4]. After 5 and 10 days, the supernatant was collected and amounts of glycosaminoglycans (GAG) were determined using the sGAG quantitative kit (Euro-Diagnostica, Malmo, Sweden). Each attempt was performed three times with different donors of SF and measured as duplicates.

Cytokine stimulation assay

Cytokine stimulation with IL-1β was performed as described previously [12]. Briefly, $6.5x10^4$ cells were seeded in 6-well plates until a confluency of 80% was reached. Afterwards, cells were stimulated with 10 U/ml recombinant human (rh) IL-1β· for 24-48 h. The DMSO concentration within each sample including negative controls was adjusted equally. RhIL-1β· was purchased from PAN Biotech.

Expression profiling

Gene expression profile of early or late passage RASF, transfected with either hsa-miR-188-5p or anti-miR-188-5p, respectively, was determined using GeneChip® PrimeView™ Human Gene Expression Array (Affymetrix, Santa Clara, CA, USA).

Statistical analysis

Results are expressed as mean ± standard deviation or mean ± standard error of the mean, respectively. Comparison between groups was made using the Student's unpaired t-test or one-way or two-way ANOVA, if appropriate. *P*-values below 0.05 was considered as statistically significant (ns: non-significant, *p<0.05). All calculations were performed using the GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Decreased expression of microRNA-188-5p in RA

Because RASF show de-regulated mRNA expression patterns as compared to normal synovial fibroblasts (nSF), we searched for a general mechanism responsible for this phenomenon. MicroRNAs (miRs) provide a ubiquitous and conserved machinery to simultaneously regulate the expression of several proteins in a cell. Therefore, the expression levels of different miRs were analyzed. As mentioned above, decreased expression of miR-25, miR-140, mir-188, and miR-320 induced by pro-

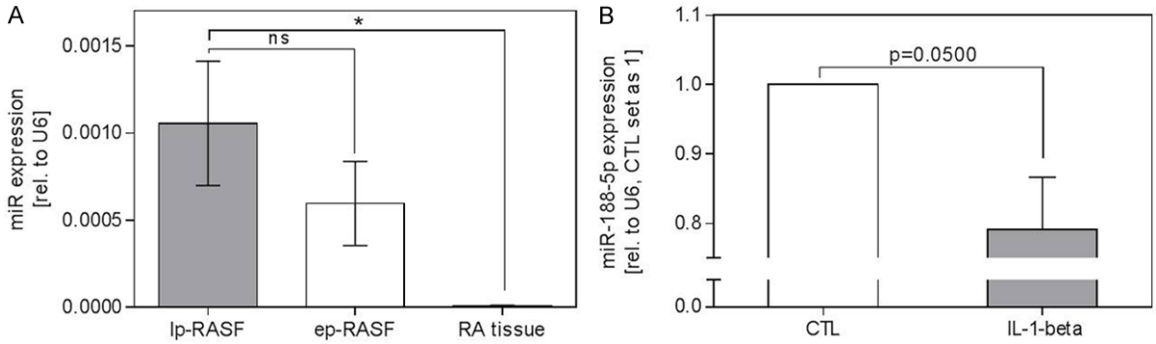


Figure 1. Expression of microRNA-188-5p in RA cells and tissues and influence of interleukin-1β on mirRNA-188 expression in RA cells. A: Hsa-miR-188-5p expression was determined in isolated activated synovial fibroblasts in rheumatoid arthritis (RASF) in early passages (ep) and late passages (lp) as well as in RA synovial tissue by qRT-PCR. B: miR-188-5p expression in RASF after exposure to interleukin-1β (IL-1β) and control cells (CTL) analyzed by qRT-PCR. *: $p<0.05$; ns: non-significant.

inflammatory stimuli was found in the context of inflammatory lung disease [8]. In RA, the synovia of joints also represents an inflammatory environment and could therefore lead to de-regulation of miRs in RASF. Quantitative RT-PCR revealed a decreased expression of hsa-miR-188-5p in aggressive RASF as compared to long-time cultivated (and less aggressive) RASF in late passages (**Figure 1A**). The lowest expression of hsa-miR-188-5p was found in samples isolated directly from synovial tissue of RA patients, supporting the hypothesis that the inflammatory environment in joints of these patients is involved in the down-regulation of hsa-miR-188-5p (**Figure 1A**).

Influence of interleukin-1-beta (IL-1β) on microRNA-188-5p expression

Because microRNAs can be regulated by inflammatory signals [6-8], we examined miR-188-5p expression after stimulation with IL-1β. After exposure to IL-1β, RASF showed approximately 20% decreased miR-188-5p expression levels (**Figure 1B**) with a strong trend to statistical significance ($p=0.05000$). Treatment with tumor necrosis factor alpha (TNF-α) showed similar results (data not shown).

MicroRNA-188-5p regulates migration of RASF

A reduced expression of miRs can result in up-regulation of target genes due to less microRNA induced mRNA degradation. Therefore, we hypothesized that diminished miR-188-5p expression might contribute to up-regulation of genes that are involved in RASF associated cartilage destructive mechanisms such as migration and glycosaminoglycan degradation. We once re-expressed miR-188-5p in early passage (ep) aggressive RASF (showing low levels of miR-188-5p). Furthermore, we down-regulated miR-188-5p in late passage (lp) less aggressive RASF (showing high levels of miR-188-5p as compared to ep-RASF, **Figure 1A**). Interestingly, re-expression *via* transient transfection of a miR-188-5p-mimic (premiR-188-5p) in ep-RASF markedly reduced migration analyzed with the xCELLigence system (**Figure 2A**). Here, we used a common method by analyzing the slope caused by the increasing cell index (induced by migration of cells). Migration of aggressive ep-RASF was significantly reduced after miR-188-5p re-expression (**Figure 2C**). Correspondingly, inhibition of enhanced miR-188-5p expression in less aggressive lp-RASF with transient transfection of a miR-188-5p inhibitor (antimiR-188-5p) resulted in elevated migration of lp-RASF as compared to control-treated lp-RASF, indicating a conversion into a more aggressive phenotype (**Figure 2B**). Again, the slope depicting the increasing cell index was significantly enhanced in antimiR-188-5p treated lp-RASF (**Figure 2C**).

MicroRNA-188-5p does not affect glycosaminoglycan degradation

As cartilage destruction *via* glycosaminoglycan (GAG) degradation depicts a crucial pathomechanism in RA, we performed a cartilage destruction assay to examine a possible regulation *via* miR-188-5. However, in contrast to migration, transient transfection of miR-188-5p in ep-

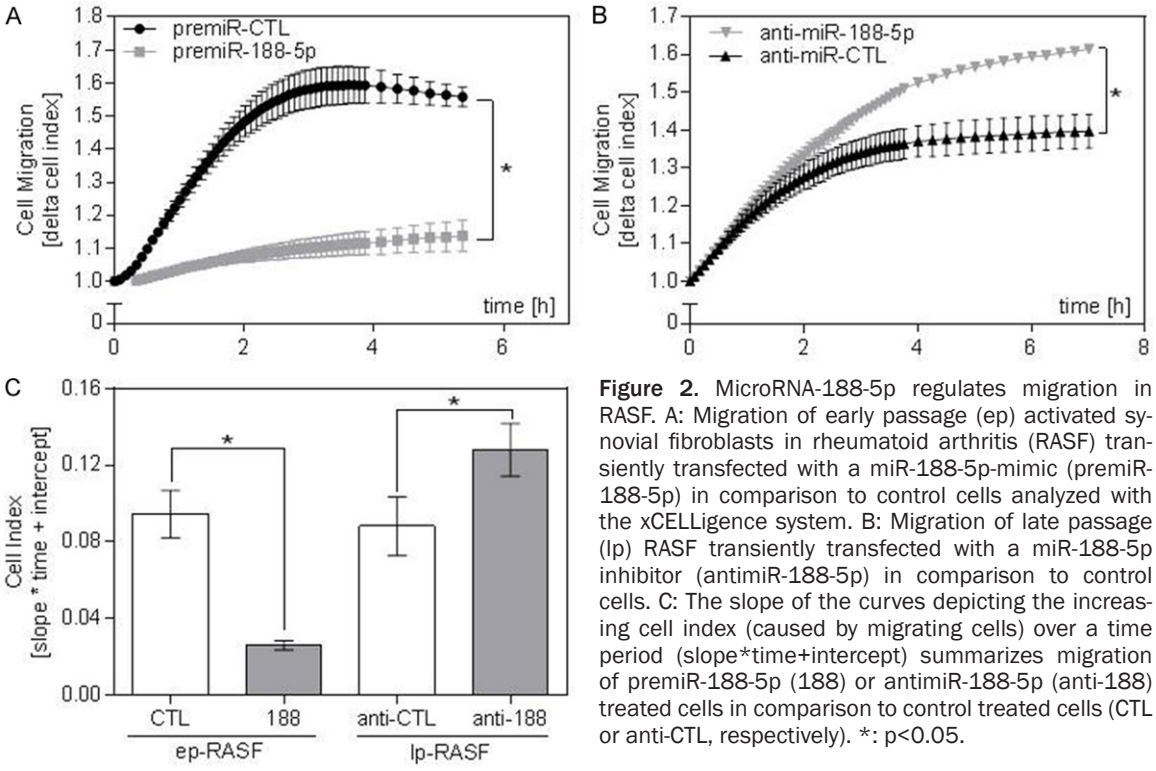


Figure 2. MicroRNA-188-5p regulates migration in RASF. A: Migration of early passage (ep) activated synovial fibroblasts in rheumatoid arthritis (RASF) transiently transfected with a miR-188-5p-mimic (premiR-188-5p) in comparison to control cells analyzed with the xCELLigence system. B: Migration of late passage (lp) RASF transiently transfected with a miR-188-5p inhibitor (antimiR-188-5p) in comparison to control cells. C: The slope of the curves depicting the increasing cell index (caused by migrating cells) over a time period (slope*time+intercept) summarizes migration of premiR-188-5p (188) or antimiR-188-5p (anti-188) treated cells in comparison to control treated cells (CTL or anti-CTL, respectively). *: $p<0.05$.

RASF or antimiR-188-5p in lp-RASF did not significantly affect GAG degradation (Supplementary Figure 1A). This leads to the assumption that miR-188-5p regulates migration but not GAG degradation in RASF *in vitro*.

MicroRNA-188-5p does not influence repellent factors

Changes of the expression of repellent factors, i.e., Netrins and their receptors, may be responsible for the invasive behavior of the synovial tissue cells in patients with RA [4, 13, 14]. RASF are known to have an aggressive phenotype *in vivo* and in early passages also *in vitro* and several genes have been shown to be differentially regulated in early passage RASF compared to RASF in late passages. Examples are the repellent receptors roundabout (ROBO3), axon guidance receptor, homolog 3 (*Drosophila*) or unc-5 homolog B or C (*C. elegans*) (UNC5B/C)]. Interestingly, *in silico* analysis showed that the repellent factors of the Netrin family, ROBO3, UNC5B and UNC5C have a predicted 3'UTR binding site for the miR-188-5p in common. However, transfection of miR-188-5p or antimiR-188-5p in ep-RASF or lp-RASF, respectively, revealed no regulation of these repellent factors via miR-188-5p on the mRNA level (Supplementary Figure 1B).

Gene expression array indicates targets of microRNA-188-5p in RASF

To identify target genes of the hsa-miR-188-5p, late passage RASF (lp-RASF) or aggressive early passage RASF (ep-RASF) were transfected with anti-miR-188-5p or miR-188-5p, respectively. Subsequently, a GeneChip® PrimeView™ Human Gene Expression Array was performed. Out of the numerous genes found to be regulated by miR-188-5p (data not shown), we focused our further analysis on those genes, which were down-regulated by hsa-miR-188-5p. *In silico* analysis of the 3'UTRs of the most strongly regulated genes predicted binding sites for the hsa-miR-188-5p in the 3'UTR of KIAA1199, GREM2 and ITGA7 (**Figure 3A**). COL1A1and COL12A1 showed also strong regulation by miR-188-5p but had no binding site in the mRNA region, suggesting indirect regulation of these two genes.

MicroRNA-188-5p regulates KIAA1199, COL1A1and COL12A1 in RASF

Next, we wanted to confirm the data from the gene expression array and *in silico* analysis by qRT-PCR analysis. We focused on genes which showed the strongest regulation and genes that are known to be involved in RA or other inflammatory disease (see discussion). RASF in

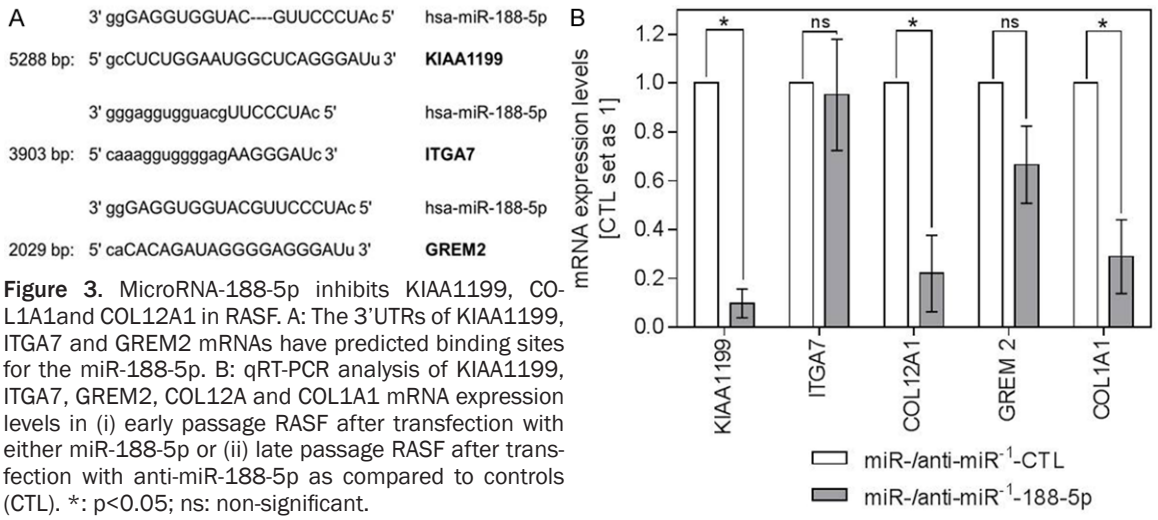


Figure 3. MicroRNA-188-5p inhibits KIAA1199, COL1A1and COL12A1 in RASF. A: The 3'UTRs of KIAA1199, ITGA7 and GREM2 mRNAs have predicted binding sites for the miR-188-5p. B: qRT-PCR analysis of KIAA1199, ITGA7, GREM2, COL12A and COL1A1 mRNA expression levels in (i) early passage RASF after transfection with either miR-188-5p or (ii) late passage RASF after transfection with anti-miR-188-5p as compared to controls (CTL). *: $p<0.05$; ns: non-significant.

early passage (ep-RASF) were transfected with hsa-miR-188-5p and RASF in late passages (lp-RASF) were transfected with anti-miR-188-5p, respectively. To compare the qRT-PCR results of both ep-RASF and lp-RASF, we used the quotient of the PCR expression values for lp-RASF (1/antimiR). A significant, approximately 90% down-regulation was observed for KIAA1199, which shows a predicted binding site for miR-188-5p in its 3'UTR (**Figure 3A**). In contrast, ITGA7 and GREM2 expression was not significantly altered by hsa-miR-188-5p (**Figure 3B**). Moreover, COL1A1 and COL12A1 mRNA levels were markedly down-regulated after miR-188-5p transfection (**Figure 3B**). In summary, qRT-PCR analysis supports that KIAA1199 is a direct target of miR-188-5p. Also ITGA7 and GREM2 exhibit binding sites for miR-188-5p in their 3'UTR and the gene expression array showed their regulation. However, we could not confirm their regulation on the mRNA level *via* miR-188-5p in qRT-PCR analysis. On the contrary, COL1A1 and COL12A1 were also confirmed to be regulated by miR-188-5p in qRT-PCR (approximately 80% and 70% down-regulation, respectively). However, these genes do not reveal 3'UTR binding sites for the miR-188-5p *in silico*, which suggests an indirect mechanism of regulation.

KIAA1199 is a target of microRNA-188-5p in RA

We further focused on KIAA1199 because of its strong regulation after hsa-miR-188-5p transfection on the mRNA level detected by qRT-PCR, its potential direct regulation *via* the 3'UTR binding site, and its possible role in RA and hyaluronic acid degradation [15]. We analyzed KIAA1199 expression in early passage RASF after transfection with miR-188-5p control or miR and found a strong regulation of the expression of KIAA1199 on protein levels in RASF from two different donors (**Figure 4**) confirming that KIAA1199 is directly regulated by miR-188-5p in RA.

Discussion

Synovial fibroblasts in rheumatoid arthritis (RASF) are known to have an aggressive phenotype *in vivo*, which is also seen *in vitro* in early cell passages. Several genes have been shown to be differentially regulated in early passages of RASF compared to RASF in late passages, e.g. the repellent receptors roundabout (ROBO3), axon guidance receptor, homolog 3 (Drosophila) or unc-5 homolog C (C. elegans) [3, 9]. However, the exact mechanisms for de-regulation of gene expression in RASF still remain elusive. Recently, several studies indicated that microRNAs (miRs) are involved in disease development and progression and to play a crucial role as small molecular regulators in RA. Several authors suggest that miRs, regulated *via* inflammatory signals, may provide new targets for RA therapy [6, 7]. Therefore, the purpose of this study was to find microRNAs which are down-regulated in early passage RASF and to identify target genes that may contribute to the aggressive phenotype of RASF. Furthermore, we addressed the role of cytokines like IL-1β in the context of RASF associated microRNA dysregulation.

We founs that mirR-188-5p is down-regulated in RA *in vitro* (RASF) and *in vivo* (RA synovia tissue), most likely triggered *via* an inflammatory

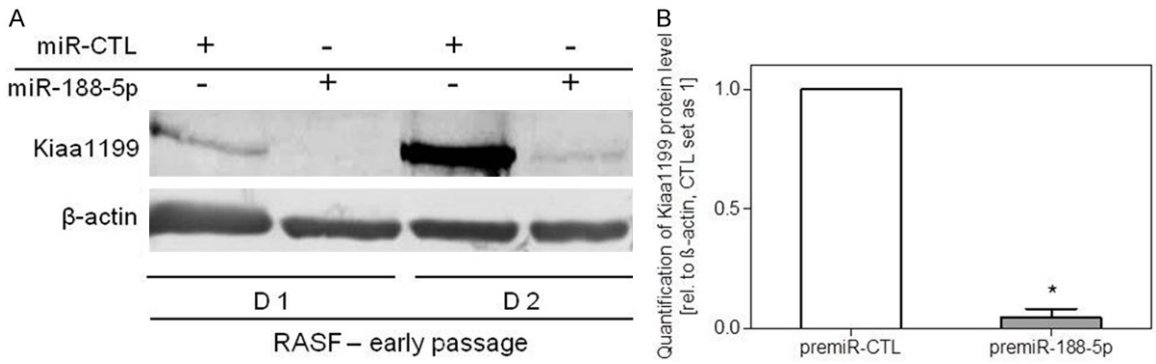


Figure 4. KIAA1199 is a target of microRNA-188-5p in RA. A: Western blot analysis of Kiaa1199 protein expression in early passage (ep) activated synovial fibroblasts in rheumatoid arthritis (RASF) of two donors after transfection with miR-188-5p. B: Colorimetric based quantification analysis (*ImageJ*) of western blot analysis of Kiaa1199 protein expression in premiR-CTL vs. premiR-188-5p treated ep-RASF. D1/D2: RASF Donor 1/Donor 2. *: $p<0.05$.

environment. Moreover, miR-188-5p expression is correlated to the activation state of RASF and inhibits migration but not GAG degradation in RASF. Finally, miR-188-5p directly targets the hyaluronan binding protein KIAA1199 and indirectly regulates COL12A1 and COL1A1.

Since decreased expression of several miRs induced by pro-inflammatory stimuli was found in the context of inflammatory lung disease [8], we examined the expression levels of different miRs in RA synovial tissue and in early passage (ep) RASF, which are known to have a more aggressive phenotype as compared to late passage (lp) RASF. Here, we could identify miR-188-5p to be significantly down-regulated in RA synovial tissue samples and most interestingly, ep-RASF also showed about 50% decreased miR-188-5p expression levels as compared to lp-RASF. The 50% decreased miR-188-5p expression in ep-RASF as compared to lp-RASF did not reach statistical significance due to large standard deviation. We think that this might be explained by the fact that we have fluent passages between an early passage RASF and a late passage RASF state *in vitro*. However, the tendency seems to be apparent, and corresponds well with the very low and significantly decreased miR-188-5p expression in RA synovia tissue (**Figure 1**).

MicroRNAs can be regulated by inflammatory signals [6-8]. In line with this, RASF exposed to IL-1β showed an approximately 20% decrease miR-188-5p expression levels although this down-regulation did not reach statistical significance ($p=0.05000$). However, *in vivo*, RASF are exposed to a strongly pronounced inflammatory environment with a mixture of hundreds of pro-inflammatory signals as compared to our *in vitro* setting. Thus, the cytokine induced down-regulation of miR-188-5p may be much more prominent *in vivo*.

As the aggressive phenotype of RASF is associated with their passage in cell culture and diminishes during the course of *in vitro* cultivation [4], the inverse correlation between aggressive phenotype and miR-188-5p expression levels suggests a regulatory role of miR-188-5p in RASF biology. Indeed, re-expression experiments showed that transfection of miR-188-5p significantly reduced *in vitro* migration of early passage RASF, whereas inhibition of miR-188-5p led to increased aggressiveness regarding migratory activity in late passage RASF. The effects of enhancing migration in lp-RASF were significant but less pronounced than the effect of migration in ep-RASF. This might be due to the non-inflammatory environment in cell culture, which normally contributes to the aggressive phenotype of RASF *in vivo*. We think that this might also explain why the knock-down of miR-188-5p seems to play a central role in the activation of RASF *in vivo* by up-regulation of several genes, e.g. KIAA1199, but it did not prove to reduce cartilage destruction of RASF *in vitro*.

Interestingly, miR-188 was recently shown to play a role in regulation in osteoarthritis in a bioinformatics approach [16], a disease also

exposing an inflammatory environment in joints. Another study revealed an up-regulation of miR-188 under mechanical stress in renal podocytes, hinting to a potential protective role in response to kidney injury [17]. MiR-188 was also found to be involved in dendritic cell plasticity by targeting neuropilin-2 [18] and to play a role in the regulation of bone regeneration [19] as well as peripheral nerve regeneration after injury [20]. A genome wide miRNA screen also showed involvement of miR-188 in controlling human ovarian cell proliferation and apoptosis [21]. Strong down-regulation of miR-188 was also found to be associated with cardiac remodeling in chronic heart failure [22]. Recently, miR-188 was even shown to be de-regulated in nasopharyngeal carcinoma and exerts anticancer effects *via* down-regulation of multiple G1/S related cyclin/CDKs and Rb/E2F signaling pathway in nasopharyngeal carcinoma [23]. Finally, as mentioned, decreased expression of miR-188 induced by pro-inflammatory stimuli was found in the context of inflammatory lung disease [8], strongly resembling our results of down-regulation in inflammatory joint disease. Together, all of these studies indicate that miR-188 is a potent regulator of cellular functions such as regeneration, proliferation, migration, apoptosis and inflammation, making this microRNA very interesting in the context of RA and RASF.

Searching for possible target genes of miR-188-5p *via* gene expression arrays and subsequent qRT-PCR confirmation, we also identified COL12A1 and COL1A1 to be down-regulated after miR-188-5p transfection, most likely due to an indirect mechanism, because these two genes do not exhibit predicted binding sites for miR-188-5p in their 3'UTRs. Both COL12A1 [24] and COL1A1 [25] are considered as profibrotic genes associated with TGF-β and IL-6 in inflammatory diseases and eventually leading to enhanced fibrosis and tissue stiffening as seen in synovia from RA patients. Thus, the indirect down-regulation of these two genes seems to be of high relevance concerning miR-188-5p induced regulation in RA.

In addition, gene expression arrays revealed three strongly regulated genes with predicted 3'UTR binding sites (KIAA1199, ITGA7 and GREM2). Of these, KIAA1199 mRNA levels were down-regulated significantly as measured by qRT-PCR with subsequent confirmation of decreased Kiaa1199 protein levels. This strongly suggests its involvement in the miR-188-5p associated regulation of RASF. Since we indeed could show marked inhibition of migration of aggressive early passage RASF and enhanced migration of antimiR-188-5p treated late passage RASF, it appears of high relevance that KIAA1199 has recently been identified as hyaluronan binding protein involved in hyaluronan depolymerization [15, 26, 27]. Therefore, KIAA1199 is one of the factors mediating aggressiveness in terms of migration potential and cartilage destruction of RASF. However, the regulation of KIAA1199 has not been analyzed in RA so far, whereas the role of KIAA1199 and its regulation in different kinds of cancer were published in the last years. Here, most interestingly, KIAA1199 was highly expressed in gastric cancer, and was associated with prognosis and lymph node metastasis [28]. Furthermore, KIAA1199 knockdown attenuates the effects of the Wnt/β-catenin signalling and decreased the proliferation of colon cancer cells [29]. Recently, KIAA1199 was described as an oncogenic protein induced by HPV infection and constitutive NF-κB activity that transmits pro-survival and invasive signals through EGFR signaling [30] and to play an important role in breast tumor growth and invasiveness [31]. Most interestingly, strongly supporting our data, KIAA1199-enhanced cell migration and silencing of KIAA1199 in breast cancer cells resulted in a mesenchymal-to-epithelial transition that reduced cell migratory ability *in vitro* and decreased metastasis *in vivo* [32]. Therefore, reduced levels of miR-188-5p in aggressive (early passage) RASF could lead to reduced inhibition KIAA1199, thereby promoting migration and invasiveness of RASF, contributing to progression of RA. In contrast, re-expression of miR-188-5p significantly down-regulated KIAA1199 mRNA and protein levels and inhibited *in vitro* migration markedly.

In summary, we report miR-188-5p to be down-regulated in RA tissue and in early passage RASF as compared to late passage (less aggressive) RASF, contributing to the regulation of RASF. Furthermore, we identify COL12A1, COL1A1 and, most interestingly, the hyaluronan depolimerization associated protein KIAA1199 as indirect or direct targets of miR-188-5p, respectively. Moreover, we found that miR-188-5p exerts strong migration inhibiting

effects on early passage RASF *in vitro* and thereby contributes to the aggressiveness of these cells. Together, these findings indicate the miR-188-5p-KIAA1199-axis as a promising novel therapeutic target in RA.

Acknowledgement

This study was supported by a grant from German Research Association DFG to AKB and to TS.

Disclosure of conflict of interest

Nothing to declare.

Address correspondence to: Dr. Anja-Katrin Bosserhoff, Institute for Biochemistry, Biochemistry and Molecular Medicine, Emil-Fischer Zentrum, Friedrich-Alexander-University of Erlangen-Nürnberg, Fahrstraße 17, D-91054 Erlangen, Germany. Tel: +49 9131 85 24191; Fax: +49 9131 85 22485; E-mail: anja.bosserhoff@fau.de

References

[1] Mertsch S, Schmitz N, Jeibmann A, Geng JG, Paulus W, Senner V. Slit2 involvement in glioma cell migration is mediated by Robo1 receptor. J Neurooncol 2008; 87: 1-7.

[2] Zeisel MB, Druet VA, Wachsmann D, Sibilia J. MMP-3 expression and release by rheumatoid arthritis fibroblast-like synoviocytes induced with a bacterial ligand of integrin alpha5beta1. Arthritis Res Ther 2005; 7: R118-26.

[3] Huber LC, Distler O, Tarner I, Gay RE, Gay S, Pap T. Synovial fibroblasts: key players in rheumatoid arthritis. Rheumatology (Oxford) 2006; 45: 669-75.

[4] Denk AE, Kaufmann S, Stark K, Schedel J, Lowin T, Schubert T, Bosserhoff AK. Slit3 inhibits its Robo3-induced invasion of synovial fibroblasts in rheumatoid arthritis. Arthritis Res Ther 2010; 12: R45.

[5] Turner JD, Filer A. The role of the synovial fibroblast in rheumatoid arthritis pathogenesis. Curr Opin Rheumatol 2015; 27: 175-82.

[6] Ammari M, Jorgensen C, Apparailly F. Impact of microRNAs on the understanding and treatment of rheumatoid arthritis. Curr Opin Rheumatol 2013; 25: 225-33.

[7] Olivieri F, Rippo MR, Procopio AD, Fazioli F. Circulating inflamma-miRs in aging and age-related diseases. Front Genet 2013; 4: 121.

[8] Kuhn AR, Schlauch K, Lao R, Halayko AJ, Gerthoffer WT, Singer CA. MicroRNA expression in human airway smooth muscle cells: role of miR-25 in regulation of airway smooth muscle phenotype. Am J Respir Cell Mol Biol 2010; 42: 506-13.

[9] Miller LE, Justen HP, Scholmerich J, Straub RH. The loss of sympathetic nerve fibers in the synovial tissue of patients with rheumatoid arthritis is accompanied by increased norepinephrine release from synovial macrophages. FASEB J 2000; 14: 2097-107.

[10] Wenke AK, Bosserhoff AK. Roles of AP-2 transcription factors in the regulation of cartilage and skeletal development. FEBS J 2010; 277: 894-902.

[11] Braig S, Bosserhoff AK. Death inducer-obliterator 1 (Dido1) is a BMP target gene and promotes BMP-induced melanoma progression. Oncogene 2013; 32: 837-48.

[12] Radons J, Bosserhoff AK, Grassel S, Falk W, Schubert TE. p38MAPK mediates IL-1-induced down-regulation of aggrecan gene expression in human chondrocytes. Int J Mol Med 2006; 17: 661-8.

[13] Schubert T, Denk A, Mägdefrau U, Kaufmann S, Bastone P, Lowin T, Schedel J, Bosserhoff AK. Role of the netrin system of repellent factors on synovial fibroblasts in rheumatoid arthritis and osteoarthritis. Int J Immunopathol Pharmacol 2009; 22: 715-22.

[14] Kaufmann S, Kuphal S, Schubert T, Bosserhoff AK. Functional implication of Netrin expression in malignant melanoma. Cell Oncol 2009; 31: 415-22.

[15] Yoshida H, Nagaoka A, Kusaka-Kikushima A, Tobiishi M, Kawabata K, Sayo T, Sakai S, Sugiyama Y, Enomoto H, Okada Y, Inoue S. KIAA1199, a deafness gene of unknown function, is a new hyaluronan binding protein involved in hyaluronan depolymerization. Proc Natl Acad Sci U S A 2013; 110: 5612-7.

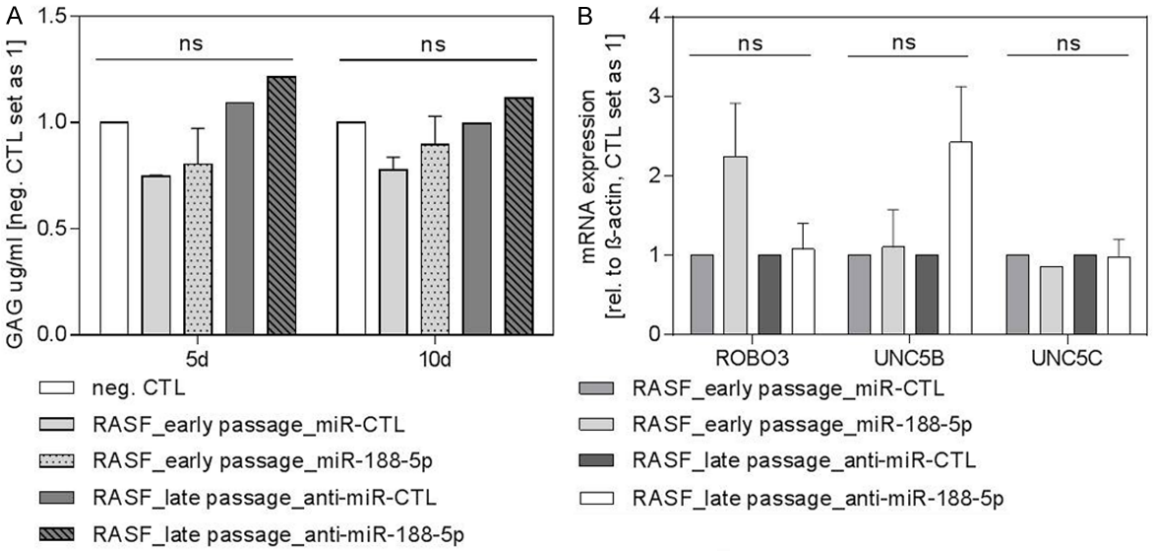
[16] Clement T, Salone V, Charpentier B, Jouzeau JY, Bianchi A. Identification of new microRNAs targeting genes regulating the Pi/PPi balance in chondrocytes. Biomed Mater Eng 2014; 24: 3-16.

[17] Li D, Lu Z, Jia J, Zheng Z, Lin S. Changes in microRNAs associated with podocytic adhesion damage under mechanical stress. J Renin Angiotensin Aldosterone Syst 2013; 14: 97-102.

[18] Lee K, Kim JH, Kwon OB, An K, Ryu J, Cho K, Suh YH, Kim HS. An activity-regulated microRNA, miR-188, controls dendritic plasticity and synaptic transmission by downregulating neuropilin-2. J Neurosci 2012; 32: 5678-87.

[19] Palmieri A, Pezzetti F, Brunelli G, Martinelli M, Lo Muzio L, Scarano A, Scapoli L, Arlotti M, Guerzoni L, Carinci F. Anorganic bovine bone (Bio-Oss) regulates miRNA of osteoblast-like cells. Int J Periodontics Restorative Dent 2010; 30: 83-7.

[20] Zhou S, Yu B, Qian T, Yao D, Wang Y, Ding F, Gu X. Early changes of microRNAs expression in the dorsal root ganglia following rat sciatic nerve transection. Neurosci Lett 2011; 494: 89-93.
[21] Sirotkin AV, Laukova M, Ovcharenko D, Brenaut P, Mlyncek M. Identification of microRNAs controlling human ovarian cell proliferation and apoptosis. J Cell Physiol 2010; 223: 49-56.
[22] Mishra PK, Tyagi N, Kundu S, Tyagi SC. MicroRNAs are involved in homocysteine-induced cardiac remodeling. Cell Biochem Biophys 2009; 55: 153-62.
[23] Wu J, Lv Q, He J, Zhang H, Mei X, Cui K, Huang N, Xie W, Xu N, Zhang Y. MicroRNA-188 suppresses G 1/S transition by targeting multiple cyclin/CDK complexes. Cell Commun Signal 2014; 12: 66.
[24] Castro NE, Kato M, Park JT, Natarajan R. Transforming growth factor beta1 (TGF-beta1) enhances expression of profibrotic genes through a novel signaling cascade and microRNAs in renal mesangial cells. J Biol Chem 2014; 289: 29001-13.
[25] Li C, Iness A, Yoon J, Grider JR, Murthy KS, Kellum JM, Kuemmerle JF. Noncanonical STAT3 Activation Regulates Excess TGF-beta1 and Collagen I Expression in Muscle of Stricturing Crohn's Disease. J Immunol 2015; 194: 3422-31.
[26] Yoshida H, Nagaoka A, Nakamura S, Tobiishi M, Sugiyama Y, Inoue S. N-Terminal signal sequence is required for cellular trafficking and hyaluronan-depolymerization of KIAA1199. FEBS Lett 2014; 588: 111-6.
[27] Yoshida H, Nagaoka A, Nakamura S, Sugiyama Y, Okada Y, Inoue S. Murine homologue of the human KIAA1199 is implicated in hyaluronan binding and depolymerization. FEBS Open Bio 2013; 3: 352-6.
[28] Matsuzaki S, Tanaka F, Mimori K, Tahara K, Inoue H, Mori M. Clinicopathologic significance of KIAA1199 overexpression in human gastric cancer. Ann Surg Oncol 2009; 16: 2042-51.
[29] Birkenkamp-Demtroder K, Maghnouj A, Mansilla F, Thorsen K, Andersen CL, Øster B, Hahn S, Ørntoft TF. Repression of KIAA1199 attenuates Wnt-signalling and decreases the proliferation of colon cancer cells. Br J Cancer 2011; 105: 552-61.
[30] Shostak K, Zhang X, Hubert P, Göktuna SI, Jiang Z, Klevernic I, Hildebrand J, Roncarati P, Hennuy B, Ladang A, Somja J, Gothot A, Close P, Delvenne P, Chariot A. NF-kappaB-induced KIAA1199 promotes survival through EGFR signalling. Nat Commun 2014; 5: 5232.
[31] Jami MS, Hou J, Liu M, Varney ML, Hassan H, Dong J, Geng L, Wang J, Yu F, Huang X, Peng H, Fu K, Li Y, Singh RK, Ding SJ. Functional proteomic analysis reveals the involvement of KIAA1199 in breast cancer growth, motility and invasiveness. BMC Cancer 2014; 14: 194.
[32] Evensen NA, Kuscu C, Nguyen HL, Zarrabi K, Dufour A, Kadam P, Hu YJ, Pulkoski-Gross A, Bahou WF, Zucker S, Cao J. Unraveling the role of KIAA1199, a novel endoplasmic reticulum protein, in cancer cell migration. J Natl Cancer Inst 2013; 105: 1402-16.



Supplementary Figure 1. MiR-188-5p influence on glycosaminoglycan degradation and on repellent factors. A: Amounts of glycosaminoglycans (GAG) in the supernatant of early passage activated synovial fibroblasts in rheumatoid arthritis (RASF) or late passage RASF transiently transfected with miR-188-5p or antimiR-188-5p or controls (CTL), respectively, after 5 and 10 days (d). B: ROBO3, UNC5B and UNC5C mRNA levels as measured by qRT-PCR in early passage RASF or late passage RASF transiently transfected with miR-188-5p or antimiR-188-5p or controls (CTL), respectively. neg: negative; ns: non-significant.