1 **Identification of miRNAs and associated pathways regulated by Leukemia**

2 **Inhibitory Factor in trophoblastic cell lines**

3

4

5 **Diana M. Morales-Prieto¹**

6 **Emanuel Barth^{2,3}**

7 **Jose Martín Murrieta-Coxca^{1,4}**

8 **Rodolfo R. Favaro¹**

9 **Ruby N. Gutiérrez-Samudio¹**

10 **Wittaya Chaiwangyen¹**

11 **Stephanie Ospina-Prieto¹**

12 **Bernd Gruhn⁵**

13 **Ekkehard Schleußner¹**

14 **Manja Marz^{2,3,6*}**

15 **Udo R. Markert^{1*}**

16

17 1. Placenta-Labor, Jena University Hospital, Am Klinikum 1, 07747 Jena, Germany

18 2. Friedrich-Schiller-University Jena, Germany. Faculty of Mathematics and

19 Computer Science. RNA Bioinformatics and High Throughput Analysis

20 3. Leibniz Institute for Age Research – Fritz Lipman Institute (FLI),

21 Beutenbergstrasse 11, 07745 Jena, Germany

22 4. Departamento de Inmunología, Instituto Politécnico Nacional, Escuela Nacional

23 de Ciencias Biológicas, Mexico City, Mexico

24 5. Children's Hospital, Friedrich-Schiller University Jena, Kochstraße 2, 07745 Jena,

25 Germany

1 6. European Virus Bioinformatics Center, Leutragraben 1, 07743 Jena,
2 Germany

3 * These authors contributed equally

4

5

6 Corresponding author:

7 Prof. Dr. Udo R. Markert

8 Phone: +49-3641-9390850

9 Fax: +49-3641-9390851

10 markert@med.uni-jena.de

11 www.placenta-labor.de

12

13 **Short title: LIF-induced miRNAs in trophoblastic cells**

14 **Keywords:** Pregnancy, trophoblast, LIF, microRNA, miR-21, placenta, cell lines.

15

1 **ABSTRACT**

2 Introduction: Leukemia Inhibitory Factor (LIF) regulates behavior of trophoblast cells
3 and their interaction with immune and endothelial cells. *In vitro*, trophoblast cell
4 response to LIF may vary depending on the cell model. Reported differences in the
5 miRNA profile of trophoblastic cells may be responsible for these observations.
6 Therefore, miRNA expression was investigated in four trophoblastic cell lines under
7 LIF stimulation followed by *in silico* analysis of altered miRNAs and their associated
8 pathways.

9 Methods: Low density TaqMan miRNA assays were used to quantify levels of 762
10 mature miRNAs under LIF stimulation in three choriocarcinoma-derived (JEG-3,
11 ACH-3P and AC1-M59) and a trophoblast immortalized (HTR-8/SVneo) cell lines.
12 Expression of selected miRNAs was confirmed in primary trophoblast cells and cell
13 lines by qPCR. Targets and associated pathways of the differentially expressed
14 miRNAs were inferred from the miRTarBase followed by a KEGG Pathway
15 Enrichment Analysis. HTR-8/SVneo and JEG-3 cells were transfected with miR-21-
16 mimics and expression of miR-21 targets was assessed by qPCR.

17 Results: A similar number of miRNAs changed in each tested cell line upon LIF
18 stimulation, however, low coincidence of individual miRNA species was observed
19 and occurred more often among choriocarcinoma-derived cells (complete data set at
20 <http://www.ncbi.nlm.nih.gov/geo/> under GEO accession number GSE130489).
21 Altered miRNAs were categorized into pathways involved in human diseases, cellular
22 processes and signal transduction. Six cascades were identified as significantly
23 enriched, including JAK/STAT and TGFB-SMAD. Upregulation of miR-21-3p was
24 validated in all cell lines and primary cells and STAT3 was confirmed as its target.

25 Discussion: Dissimilar miRNA responses may be involved in differences of LIF
26 effects on trophoblastic cell lines.

1 INTRODUCTION

2 Leukemia inhibitory factor (LIF) is a pleiotropic cytokine known to be
3 indispensable for human reproduction. LIF controls uterine receptivity and influences
4 trophoblast behavior by promoting proliferation, invasion and differentiation, and its
5 dysregulation is associated with infertility and poor pregnancy outcome (reviewed in
6 [1, 2]).

7 To investigate the molecular mechanisms involved in trophoblast response,
8 several cell lines have been employed as models for primary cells [3-7]. Unlike
9 primary cells, these models have the advantage to proliferate easily in culture while
10 maintaining characteristics of desired trophoblast phenotypes. These models have
11 been employed for decades, but it is still discussed how their limitations may
12 influence translation of results to the *in vivo* situation and which markers are required
13 to consider them as suitable trophoblast models [8]. Secreted proteins (e.g. IGF2,
14 CSH1), trophoblast lineage markers (e.g. KRT7, VE-Cadherin, HLA-G) and
15 expression of homeobox and imprinting genes (e.g. H19, IGF2, CDKN1C) are among
16 the markers used to characterize trophoblast phenotypes [8, 9]. Recently, specific
17 mRNA and microRNA (miRNA) expression profiles have been added to that list [10-
18 12].

19 Cumulating evidence demonstrates that physiological characteristics of
20 trophoblast biology (immunoregulation, invasion, proliferation among others) are
21 tightly regulated by miRNAs [13-15]. These short non-coding RNA sequences (~22
22 nt) regulate gene expression by sequence-specific binding to 3'UTR of mRNA targets
23 resulting in their degradation or translational repression. We have previously reported
24 differences in the miRNA expression profiles of four cell lines widely used as
25 trophoblast models [10]: JEG-3, a human choriocarcinoma cell line preserving
26 several trophoblast-like capacities; two hybrids of a JEG-3 mutant with human first

1 and third trimester trophoblast cells, ACH-3P and AC1-M59 cells respectively [3-5];
 2 and the immortalized human first-trimester trophoblast cell line HTR-8/SV40 [6].
 3 Unlike primary trophoblast cells and choriocarcinoma cell lines, HTR-8/SV neo cells
 4 do not express the placenta-related Chromosome 19 MicroRNA Cluster (C19MC),
 5 but retain expression of C14MC [10]. In contrast, the mentioned choriocarcinoma-
 6 derived cell lines express C19MC, but only low levels of C14MC miRNA [10].
 7 Likewise, these cell lines have differences in proliferation and invasion rates and
 8 respond dissimilarly under stimulation with LIF and other IL-6 family cytokines [16,
 9 17]. Altogether, these data demonstrate that no cell line resembles all characteristics
 10 of primary trophoblast cells. Therefore, to understand the *in vivo* relevance of findings
 11 obtained by using these cell lines, comparison of several models is advised.

12 In order to gain more insights into the trophoblast response to LIF, four cell
 13 models were investigated to identify in common altered miRNAs, their associated
 14 pathways and targets. The LIF/STAT3/miR-21-3p axis was additionally validated in
 15 primary trophoblast cells.

16

1 MATERIALS AND METHODS

2 *The Placenta Lab strictly applies quality management and is certified after DIN*
3 *EN ISO 9001.*

5 Primary trophoblast cells and cell lines

6 Healthy term placentae were obtained from the Department of Obstetrics,
7 Jena University Hospital, Jena, Germany. Trophoblast isolation was performed using
8 a modified Kliman method as described in detail elsewhere [10, 18]. Isolated cells
9 have been checked for purity by flow cytometry using anti-EGF-receptor, anti-
10 cytokeratin-7, and anti-HLA-G antibodies. The levels of impurity (cells negative for
11 mentioned markers) did not exceed 10%.

12 JEG-3 cell line was purchased from DSMZ (Braunschweig, Germany). ACH-
13 3P and AC1-M59 cells were a kind gift from Prof. G. Desoye (Graz, Austria). The
14 immortalized cell line HTR-8/SV40 was kindly provided by Dr. Charles H. Graham,
15 (Queen' University, Kingston, Canada).

17 Cell culture

18 Cell cultures were maintained under standard conditions (37°C, 5% CO₂,
19 humid atmosphere) in Ham's F-12 Nutrient Mixture with L-glutamine (GIBCO) or
20 RPMI Medium (GIBCO; HTR-8 cells) supplemented with 10 % heat-inactivated fetal
21 calf serum (FCS; GIBCO) and 1 % penicillin/streptomycin antibiotic solution (GIBCO).
22 Cells were checked regularly for mycoplasma contamination and their identity was
23 confirmed by Short Tandem Repeat (STR) genotyping compared to the databanks of
24 the American Type Culture Collection (ATCC) and the Leibniz Institute DSMZ -
25 German Collection of Microorganisms and Cell Cultures (DSMZ).

RNA isolation and miRNA TaqMan low density arrays

Cells were cultivated in 12-well plates and allowed to attach overnight. Subsequently, they were serum deprived for at least 2 h and then challenged 4 h with 10 ng/ml LIF (Millipore) [19, 20]. Total RNA was isolated using the mirVana isolation kit (Life Technologies), according to the manufacturer's protocol. RNA purity was assessed by the ratio of spectrophotometric absorbance at 260 and 280 nm (A260/280nm) using NanoDrop ND-1000 (NanoDrop Inc). Total RNA (100 ng) was reverse transcribed using the specific Megaplex RT kit (Life Technologies) followed by a pre-amplification step. Expression of 754 miRNAs was profiled by using the TaqMan® Array Human MicroRNA A+B Cards Set v3.0 (Life Technologies) on a 7900HT Sequence detection system. Experimental data were analyzed using the DataAssist v3.0 Software (Life Technologies) using the formula $2^{-\Delta\Delta Ct}$ with RNU48 and RNU44 as endogenous controls.

14

Validation of miRNA expression by qPCR

The expression levels of miR-885-5p, miR-21-3p and miR-34b-3p were verified by individual TaqMan miRNA Assays (Applied Biosystems, Foster City, CA, USA) according to the protocol provided by the supplier. Reverse transcription was performed with miRNA specific stem-loop RT primer (miR-21-3p Assay-ID 002438, miR-885-5p 002296, miR-34b 000427) using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was done using the specific TaqMan Assay and TaqMan Universal PCR Master Mix no UNG. All reactions were run in duplicates including no-template controls in 96-well plates on a 7300 Real Time PCR System (Applied Biosystems). Fold changes were calculated using the formula $2^{-\Delta\Delta Ct}$ relative to non-stimulated cells with RNU48 as endogenous control.

26

1 Prediction of targets and pathway analyses

2 Potential target genes of miRNA affected upon LIF stimulation ($|FC| \geq 1.5$)
3 were obtained from the miRTarBase [21] (release 7.0) to get non-redundant lists of
4 target proteins for each of the different conditions. In the next step, all 299 available
5 regulatory human pathways of the Kyoto encyclopedia of genes and genomes
6 (KEGG) database [22] were ranked individually for the investigated conditions,
7 according to the amount of targeted proteins within each pathway.

8 To evaluate whether a given pathway was overrepresented in the list of
9 differentially affected miRNA targets, the hypergeometric test [23] was used to
10 calculate corresponding p-values for each pathway:

$$11 \quad p - Value = \frac{R!n!(N-R)!(N-n)!}{N!} \sum_{i=r}^{\min(n,R)} \frac{1}{i!(R-i)!(n-i)!(N-R-n+i)!},$$

12 where N is the total number of miRNAs that have targets in any of the given
13 pathways, R the number of miRNAs of interest (here miRNAs with an absolute fold
14 change ≥ 1.5 upon LIF stimulation), n the total number of miRNAs with targets in the
15 currently observed pathway and r the number of miRNAs of interest that have targets
16 in the currently observed pathway.

17 After Benjamini-Hochberg false discovery rate (FDR) adjustment [24], only
18 pathways with an enrichment p-value of ≤ 0.05 were considered statistically
19 significant.

20 Gene Ontology analysis (GO) was used for identifying biological functions of
21 the enriched KEGG pathways. Significantly altered pathways were organized within
22 the seven networks of the KEGG pathway database [22]. Analysis of sub-pathways
23 was performed by manual identification of altered key regions within the significantly
24 enriched pathways.

25

Validation of miR-21-3p targets

HTR-8/SVneo and JEG-3 cells were seeded in 6-well plates and allowed to attach overnight to reach 50% confluence at the time of transfection. Transfection was performed for 24 h using Oligofectamine (ThermoFisher) according to the manufacturer's protocol as described before [25]. Sequences mimicking miR-21-3p (ID: MC12979) or a non-genomic scrambled sequence SCR (ID: MH12979) were transfected at a final concentration of 20 nM.

Expression level of STAT3 and SMAD7 was determined 24h after transfection by qPCR. RNA was isolated as described above. 100 ng were reverse transcribed using High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Darmstadt, Germany). Quantitative real-time PCR was performed using TaqMan assays (STAT3, Assay ID: Hs00374280_m1; SMAD7, Assay ID: Hs00998193_m1 and GAPDH, Assay ID: Hs02758991_g1) and TaqMan Universal PCR Master Mix reagents (Applied Biosystems). qPCR was run on a Mx3005P qPCR System (Applied Biosystems). Expression of STAT3 and SMAD7 was normalized using the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method relative to GAPDH and the respective experimental control.

1 RESULTS

2 miRNA profile after LIF stimulation changes in a cell-specific manner

3 After 4 h of LIF treatment (10 ng/ml), 754 miRNAs were profiled and miRNAs
4 with $Ct \leq 35$ displaying more than 1.5-fold change were considered altered. In HTR-
5 8/SVneo cells, expression of 110 miRNAs was influenced (39 up- and 71 down-
6 regulated). In the choriocarcinoma-derived JEG-3 cell line, 91 affected miRNAs were
7 identified: 45 down- and 36 up-regulated. In the hybrid cell lines AC1-M59 and ACH-
8 3P cells, a total of 88 (45 down- and 43 up-regulated) and 130 (45 down- and 85 up-
9 regulated) were identified, respectively (Figure 1). The microarray dataset is available
10 at the Gene Expression Omnibus database <http://www.ncbi.nlm.nih.gov/geo/> under
11 GEO accession number GSE130489.

12 Under the selected conditions for the data analysis of the arrays, no miRNA
13 species was identified simultaneously altered in all four tested cell lines. ACH-3P and
14 AC1-M59 cells had the most similar response with 25 altered miRNAs in common (14
15 up- and 11- down regulated), followed by pairs of JEG-3:ACH-3P and JEG-3:AC1-
16 M59 cells, each with 17 shared altered miRNAs (Figure 1).

17 To confirm the results obtained from the arrays and to validate the observed
18 effects on primary cells, three miRNAs were selected for further analysis. Both miR-
19 885-5p and miR-21-3p were identified altered in three cell lines and their basal
20 expression in primary trophoblast cells resembles that of HTR-8/SVneo and
21 choriocarcinoma cell lines [10]. Additionally, miR-34b-3p was included for verification
22 by qPCR as it was found altered only in two cell lines. Although having a tendency to
23 decrease upon LIF stimulation in all tested models, reduction of miR-885-5p was only
24 significant in JEG-3 and ACH-3P cells. miR-21-3p was significantly up-regulated in all
25 tested cell lines and in primary trophoblast cells. No significant changes were
26 detected in miR-34b-3p (Figure 2).

1

2 **Gene oncology and KEGG pathway analysis of the affected miRNAs**

3 Targets of the differentially expressed miRNAs were inferred from the
4 miRTarBase release 7.0. All 299 available regulatory human pathways of the KEGG
5 database were ranked individually according to the amount of targeted proteins after
6 LIF stimulation within each pathway.

7 Only two pathways were identified in JEG-3 as most likely to be affected after
8 LIF treatment: Epithelial cell signaling in Helicobacter pylori infection hsa05120 and
9 Adherens junction hsa04520. In ACH-3P, AC1-M59 and HTR-8/SVneo cells, 17, 41
10 and 31 statistically significantly enriched pathways were identified, respectively
11 (Suppl. Figure 1). Among them, a group of 13 KEGG pathways were found
12 significantly enriched in at least two cell lines (Suppl. Figure 1).

13

14 **Analysis of subpathway regions and associated functions**

15 To further establish conserved pathways activated by LIF in trophoblastic cell
16 lines, local areas of high concentration of target genes were identified within high
17 ranked pathways and designated as sub-pathways. Six sub-pathways were identified
18 as influenced by LIF affected miRNAs: a) NFkB, b) TGFB-Smad, c) RAS-RAF-MEK-
19 ERK, d) PI3K-Akt, e) NWASP-ARP2/3-(F-)actin and f) JAK/STAT. Among them,
20 NWASP-ARP2/3-(F-)actin was identified altered in all tested cell lines. Alterations of
21 a, c, d and f were identified in ACH-3P, HTR-8/SVneo and AC1-M59 cells, and of
22 TGFB-Smad in JEG-3 and HTR-8/SVneo cells (Figure 3A and 3B). Additionally, a
23 cross-talk between components of subpathways (a, c, d and f) was also described
24 (Figure 3A).

25 Occurrence analysis revealed that some subpathways were more frequently
26 altered across enriched pathways resulting in different profiles among cell lines

1 (Figure 3C). Enrichment of PI3K/AKT was observed in HTR-8/SVneo, ACH-3P and
2 AC1-M59 cells, while RAS/RAF enrichment was also frequent in HTR-8/SVneo cells
3 (Figure 3C).

4 Finally, to identify biological functions of LIF in trophoblast cells, significantly altered
5 pathways were organized within the KEGG categories. Those which were identified
6 in all cell lines were “human diseases” and “cellular processes” (Figure 3D).

7

8 **Validation of putative targets of miR-21-3p**

9 miRTarBase (release 7.0) [21] predicts STAT3 and SMAD7 expression as
10 affected by the LIF-induced increase of miR-21-3p (Figure 4A). After challenging with
11 LIF, qPCR showed that STAT3 mRNA was significantly increased in all tested cell
12 lines and in primary trophoblast cells (Figure 4B). SMAD7 was altered in JEG-3 but
13 not in HTR-8/SVneo cells and was therefore not considered for further analysis (data
14 not shown). Overexpression of miR-21-3p in HTR-8/SVneo and JEG-3 cells resulted
15 in a significant decrease of STAT3 mRNA confirming it as target (Figure 4C).

1 DISCUSSION

2 Leukemia Inhibitory Factor (LIF) is one of the most important factors within the
 3 uterine environment. It is involved in fundamental processes for human reproduction
 4 including endometrium receptivity and blastocyst implantation [2]. LIF and its receptor
 5 LIFR are localized in the chorionic villi, decidua and extravillous trophoblast cells
 6 (EVTs) of first trimester and term placenta [26]. There, LIF exerts an important role in
 7 the regulation of EVT invasion and the communication of trophoblast with decidua
 8 and immune cells, which is required for an adequate spiral artery remodeling [27].
 9 Activation of several signaling pathways by LIF and the resulting biological functions
 10 in trophoblastic cells have been described previously by our group and others [17,
 11 28-33]. However, miRNAs involved in these processes and the similarities across
 12 different trophoblastic models are not known.

13 To circumvent the disadvantages of working with primary cells and still
 14 allowing the study of intracellular signals, several cell lines have been established to
 15 investigate functions on trophoblastic cells. Some of the most widely used models
 16 have been derived from choriocarcinoma (e.g. JEG-3, ACH-3P and AC1-M59) or
 17 from primary cells immortalized with the gene encoding for simian virus 40 large T
 18 antigen (HTR-8/SVneo) [3-6]. Due to the differences in their origin, these models
 19 exhibit unique expression patterns of genes, proteins [12, 34] and miRNAs [10].
 20 These differences are reflected in their ability to proliferate, migrate and invade, thus,
 21 constituting an important aspect when selected as suitable models for trophoblast
 22 research.

23 Rather than to authenticate the “most suitable” trophoblast cell line for human
 24 research, this study aimed to determine the most affected miRNAs and their
 25 associated pathways involved in the response of trophoblast cells to LIF. Expression
 26 of 754 miRNAs was investigated by miRNA arrays revealing a similar number of

1 altered miRNAs in all four analyzed cell lines upon stimulation with LIF. Remarkably,
2 overlapping of specific miRNAs was low and occurred mostly between
3 choriocarcinoma cell lines. This may rely on the shared origin of JEG-3, AC1-M59
4 and ACH-3P cells, but it also may be the result of miRNA redundancy in HTR-
5 8/SVneo cells to compensate lack of some placenta-related miRNAs (e.g. C19MC
6 miRNAs) [10]. However, further investigation should confirm this assumption.

7 Identification of affected miRNAs was performed under a strict threshold of
8 $|\text{fold change}| \geq 1.5$ and an expression of $Ct \leq 35$. We consider that these strict
9 selection criteria may also contribute to the low coincidence of results among cell
10 lines, but this strategy allows the identification of the most relevant changes while
11 avoiding false positive results. Likewise, a single time point (4h) and a unique
12 concentration (10 ng/ml) were selected for this study based on our previous reports
13 on LIF functions and LIF-mediated alterations of miRNAs, mRNAs and proteins in
14 trophoblastic cells [17, 28-30]. Due to this selection, differences in the kinetic and
15 sensitivity of the cell lines to LIF may be disregarded resulting in oversight of
16 additional shared miRNAs.

17 Further, the miRNA arrays used in this study allow the simultaneous
18 measurement of hundreds of miRNA species, but this technique may neglect the
19 variation in melting temperatures of miRNA probes and the results depend greatly on
20 the RNA input and normalization [35]. Thus, qPCR is still considered the “gold
21 standard” for the corroboration of results obtained from miRNA arrays. We selected
22 three miRNAs for verification based on their similarities in the basal expression to
23 primary trophoblast cells. E.g. in primary cells expression of miR-885-5p was similar
24 to that of HTR-8/SVneo cells whilst expression of miR-21-3p and miR-34b-3p was
25 closer to that of choriocarcinoma-derived cell lines [10]. Approximately, 67% of the
26 results in the arrays were confirmed by qPCR which is consistent with other studies

[35]. Furthermore, miR-21-3p, which was identified upregulated in three cell lines by using the miRNA arrays, was confirmed upregulated in all cell lines and in primary trophoblast cells by qPCR. Previously, we reported upregulation of the miR-21 guide strand (miR-21-5p) in trophoblastic cells stimulated with LIF [28]. miR-21-5p correlates positively with the ability of trophoblastic cells to invade and proliferate, but its targets and associated pathways differ between JEG-3 and HTR-8/SVneo cells [13]. The function of miR-21-3p on trophoblast cells remains to be determined but it is to hypothesize that it also plays a role in controlling cell proliferation and invasion.

To further examine the biological relevance of the array results, an *in silico* analysis was carried out. This strategy has been employed in other studies to assign functional meaning to regulation at mRNA level in trophoblast cells [11, 36]. In our study, upon LIF stimulation, targets of the differentially expressed miRNAs were firstly predicted by miRTarBase [21] and then mapped to KEGG pathways. To avoid a biased selection of the most relevant pathways associated to LIF-regulated miRNAs, all annotated KEGG pathways were included in the study without filtering for those directly related to trophoblast cells. These pathways were then ranked based on the hit numbers and using the hypergeometric test to identify significantly altered pathways. Once more, the resulting pathways diverge among cell lines. Despite the similarities observed in single miRNA species, analysis of ACH-3P and AC1-M59 cell lines resulted in dissimilar enriched pathways. Likewise, while in JEG-3 only two pathways were significantly altered upon LIF stimulation, in AC1-M59 41 affected pathways and HTR-8/SVneo cells 31 pathways were identified.

Some of the enriched pathways belong to the KEGG DISEASE and KEGG DRUG databases [22] and thus, they may not necessarily have been described in the context of trophoblast behavior or reproduction. For instance, “Estrogen signaling pathway has04915” and “JAK-STAT signaling hsa04630” pathways significantly

1 enriched in HTR-8/SVneo cells have been respectively identified in endometrium
2 during the implantation period [37] and already recognized as a pivotal cascade for
3 signaling of IL-6 cytokines in trophoblast cells [38]. Nevertheless, other apparently
4 unrelated pathways were also identified such as “Alcoholism hsa05034” and “Non-
5 alcoholic fatty liver disease (NAFLD) hsa04932” with low p-values in HTR-8/SVneo
6 and AC1-M59, respectively.

7 To improve the interpretation of biological phenomena associated to enriched
8 KEGG pathways, analysis of local regions or sub-pathways has been recently
9 proposed [39]. By applying this method, mapped regions of high enrichment can be
10 manually identified within the pathways of the previous step highlighting the core
11 components targeted by specific miRNAs. For instance, LIF-altered miRNAs target in
12 the pathway “Alcoholism” the “RAS-RAF-MEK-ERK1/2” subpathway, whilst in
13 “NAFLD” the “IKK-NFkB” and “PI3K-Akt” subpathways. Using the same method for all
14 enriched pathways, six sub-pathways were consistently mapped across cell lines:
15 NFkB, TGFB-SMAD, RAS-RAF-MEK-ERK1/2, PI3K-Akt, NWASP-ARP2/3-(f)-actin
16 and JAK-STAT. Because most of the enriched pathways can be organized within
17 these sub-pathways, it can be suggested that they may be major cascades involved
18 in the LIF response of trophoblast cells. Indeed, activation of JAK/STAT, MAPK and
19 PI3K/Akt in trophoblast cells upon LIF stimulation has been described previously [17,
20 29, 30, 33].

21 Most of the enriched pathways identified by the *in silico* analysis were
22 categorized into the KEGG PATHWAY networks of “Cellular processes” and “Human
23 diseases” [22]. LIF is known as one of the most important factors mediating
24 trophoblast biology [1, 2, 17, 32] and its dysregulation has been reported in
25 pregnancy disorders such as preeclampsia [33] and hydatidiform mole [40].

1 Altogether, our results agree with those reports and contribute to identify the miRNAs
2 and their associated pathways involved in those processes.

3 Finally, STAT3, an inductor of cell growth and invasion [41], and also pivotal
4 regulator of trophoblast behavior [17, 41] was identified as putative target of miR-21-
5 3p. We demonstrate increased expression of STAT3 mediated by LIF in all cell lines
6 and in primary cells and confirmed it as target of miR-21-3p using two different cell
7 lines. A recent report has shown based on bioinformatic analysis followed by ChIP-
8 PCR that STAT3 acts as a transcription factor initiating miR-21 expression [42]. Our
9 results indicate that by reducing STAT3 expression miR-21-3p may be part of the
10 complex negative feedback system of STAT3.

11 Although the specific function of the miR-21-3p strand remains to be
12 determined, the common response in the models suggests the LIF/STAT3/miR-21-3p
13 axis to contribute in controlling major trophoblast functions.

14 In previous reports, we demonstrated that cellular behavior upon LIF treatment
15 differs among trophoblastic cell lines. For instance, invasion is induced by LIF in
16 JEG-3 and ACH-3P but not in AC1-M59 or HTR-8/SVneo cells [17, 29]. Now, we
17 have demonstrated that these dissimilar LIF responses of trophoblastic cells may be
18 influenced by the cell-specific set of altered miRNAs. However, these alterations
19 converged in six major conserved pathways demonstrating the redundancy of miRNA
20 species and highlighting the importance of further studying these pathways. Because
21 the investigated cell lines have particular features and responses, they may reflect
22 aspects of discrete populations of trophoblast cells present *in vivo*. Thus, their
23 applicability as trophoblast models should be considered depending on the question
24 to be addressed.

1 Altogether, this study demonstrates that LIF induces responses in miRNA
2 expression in trophoblastic cells and reveals further differences in the behavior of the
3 most frequently used trophoblast model cell lines.

4

5 **ACKNOWLEDGEMENT**

6

7 The project has been supported by the German Research Foundation (DFG,
8 Ma1550/12-1 to URM and RRF; Mo2017/3-2 to DMMP; MA 5082/7-1 to MM). It has
9 been partially financed by the ProExzellenz program of the Thuringian Ministry for
10 Research (EB: TMWWDG; RegenerAging – FSU-I-03/14).

11

12

13

1 LEGENDS TO TABLE AND FIGURES

2

3 Figure 1. miRNAs in trophoblastic cells regulated upon LIF treatment. Venn
4 diagrams show altered miRNAs (mean fold-change ≥ 1.5 ; n=3) in cells treated with
5 LIF (10 ng/ml; 4h) compared to controls. miRNAs in boxes were altered in at least
6 two cell lines. miRNA species are reported as hsa-miR-Number-AssayID.

7

8 Figure 2. Verification of miRNA array expression data in primary cells. Expression of
9 miRNAs identified as altered in several cell lines upon LIF treatment was confirmed
10 by qPCR in cell lines and primary trophoblast cells (PTC). Fold change expressed as
11 $2^{-\Delta CT}$ to RNU48. Values are presented as mean $\pm$ SEM (n = 4), * p < 0.05, ** p< 0.01
12 (vs.CTR)

13

14 Figure 3. Subpathways and biological functions of miRNAs modulated by LIF. A)
15 Venn diagram depicts the identified modulated subpathways and how
16 they are shared between cell lines. B) Identified subpathways a - NFkB activation by
17 members of the IKK complex; b - TGF-beta/SMAD
18 pathway; c - Ras-Raf-MEK-ERK pathway; d - PI3K/AKT/mTOR pathway;
19 e - N-WASP and Arp2/3 regulation of actin; f - JAK/STAT signaling pathway. Known
20 interactions between four of those subpathways are also depicted. C) Overview of
21 the relative number of occurrences of the identified subpathways within the
22 significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG)
23 pathways. D) Putative biological processes affected by LIF based on the KEGG
24 orthology reference hierarchy.

25

1 Figure 4. miR-21-3p targets STAT3 in trophoblast cells. A) miRNA/mRNA alignment
 2 of miR-21-3p/STAT3. B) Expression of STAT3 mRNA upon LIF stimulation (10 ng/ml;
 3 4h) in cell lines and primary trophoblast cells (PTC). C) Expression of STAT3 mRNA
 4 in HTR-8/SVneo and JEG-3 cells after transfection with SCR- and miR-21-3p-mimics.
 5 Data expressed as $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$ to GAPDH. One-way ANOVA test *p < 0.05
 6 **p<0.01.

7

8 Suppl. Figure 1. Signal pathway analysis of the target genes of LIF-altered miRNAs.
 9 Gene targets of miRNAs changing after LIF were inferred by TargetScan and
 10 classified into Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.
 11 Pathways were considered as significantly altered when p<0.05. Letters represent
 12 key subpathway regions a) NK-kB, b) TGFβ-Smad, c) Ras-Raf-MEK-ERK, d) PI3K-
 13 Akt, e) NWASP-ARP/2/3-(F)-actin, f) JAK-STAT

14

15

1 Fig 1.

DOWN

UP

2

3

4

1 **Fig 2**

2

3

4

5

Fig 3

Fig 4

Suppl. Figure 1

a) NK-κB; b) TGFβ-SMAD; c) RAS-RAF-MEK-ERK; d) PI3K-AKT; e) NWSAP-ARP2/3(f)-ad(in); f) JAK-STAT

References

1. Markert, U.R., D.M. Morales-Prieto, and J.S. Fitzgerald, *Understanding the link between the IL-6 cytokine family and pregnancy: implications for future therapeutics*. Expert Rev Clin Immunol, 2011. **7**(5): p. 603-9.
2. Fitzgerald, J.S., et al., *Cytokines regulating trophoblast invasion*. Advances in neuroimmune biology, 2011. **2**: p. 61-97.
3. Hiden, U., et al., *The first trimester human trophoblast cell line ACH-3P: a novel tool to study autocrine/paracrine regulatory loops of human trophoblast subpopulations--TNF-alpha stimulates MMP15 expression*. BMC Dev Biol, 2007. **7**: p. 137.
4. Gaus, G., et al., *Parent cells for trophoblast hybridization I: Isolation of extravillous trophoblast cells from human term chorion laeve Placenta*, 1997. **18**(Supplement 2): p. 181-190.
5. Funayama, H., et al., *Parent cells for trophoblast hybridization II: AC1 and related trophoblast cell lines, a family of HGPRT-negative mutants of the choriocarcinoma cell line JEG-3*. Trophoblast Research, 1997. **10**: p. 191-201.
6. Graham, C.H., et al., *Establishment and characterization of first trimester human trophoblast cells with extended lifespan*. Exp Cell Res, 1993. **206**(2): p. 204-11.
7. Straszewski-Chavez, S.L., et al., *The isolation and characterization of a novel telomerase immortalized first trimester trophoblast cell line, Swan 71*. Placenta, 2009. **30**(11): p. 939-48.
8. Adibi, J., et al., *IFPA meeting 2016 workshop report II: Placental imaging, placenta and development of other organs, sexual dimorphism in placental function and trophoblast cell lines*. Placenta, 2017.
9. Ji, L., et al., *Placental trophoblast cell differentiation: physiological regulation and pathological relevance to preeclampsia*. Mol Aspects Med, 2013. **34**(5): p. 981-1023.
10. Morales-Prieto, D.M., et al., *MicroRNA expression profiles of trophoblastic cells*. Placenta, 2012. **33**(9): p. 725-34.
11. Apps, R., et al., *Genome-wide expression profile of first trimester villous and extravillous human trophoblast cells*. Placenta, 2011. **32**(1): p. 33-43.
12. Bilban, M., et al., *Trophoblast invasion: assessment of cellular models using gene expression signatures*. Placenta, 2010. **31**(11): p. 989-96.
13. Chaiwangyen, W., et al., *Dissimilar microRNA-21 functions and targets in trophoblastic cell lines of different origin*. Int J Biochem Cell Biol, 2015. **68**: p. 187-96.
14. Morales-Prieto, D.M., et al., *Elsevier Trophoblast Research Award Lecture: origin, evolution and future of placenta miRNAs*. Placenta, 2014. **35** Suppl: p. S39-45.
15. Dumont, T.M.F., et al., *The expression level of C19MC miRNAs in early pregnancy and in response to viral infection*. Placenta, 2017. **53**: p. 23-29.
16. Suman, P., et al., *AP-1 transcription factors, mucin-type molecules and MMPs regulate the IL-11 mediated invasiveness of JEG-3 and HTR-8/SVneo trophoblastic cells*. PLoS One, 2012. **7**(1): p. e29745.
17. Chaiwangyen, W., et al., *Oncostatin M and leukaemia inhibitory factor trigger signal transducer and activator of transcription 3 and extracellular signal-regulated kinase 1/2 pathways but result in heterogeneous cellular responses in trophoblast cells*. Reprod Fertil Dev, 2016. **28**(5): p. 608-17.
18. Stenqvist, A.C., et al., *An efficient optimized method for isolation of villous trophoblast cells from human early pregnancy placenta suitable for functional and molecular studies*. Am J Reprod Immunol, 2008. **60**(1): p. 33-42.

- 1 19. Fitzgerald, J.S., et al., *Leukemia inhibitory factor triggers activation of signal*
2 *transducer and activator of transcription 3, proliferation, invasiveness, and altered*
3 *protease expression in choriocarcinoma cells*. *Int J Biochem Cell Biol*, 2005. **37**(11):
4 p. 2284-96.
- 5 20. Poehlmann, T.G., et al., *Trophoblast invasion: tuning through LIF, signalling via*
6 *Stat3*. *Placenta*, 2005. **26 Suppl A**: p. S37-41.
- 7 21. Chou, C.H., et al., *miRTarBase update 2018: a resource for experimentally validated*
8 *microRNA-target interactions*. *Nucleic Acids Res*, 2018. **46**(D1): p. D296-D302.
- 9 22. Kanehisa, M., et al., *KEGG: new perspectives on genomes, pathways, diseases and*
10 *drugs*. *Nucleic Acids Res*, 2017. **45**(D1): p. D353-D361.
- 11 23. Godard, P. and J. van Eyll, *Pathway analysis from lists of microRNAs: common*
12 *pitfalls and alternative strategy*. *Nucleic Acids Research*, 2015. **43**(7): p. 3490-3497.
- 13 24. Benjamini, Y. and Y. Hochberg, *Controlling the False Discovery Rate: A Practical*
14 *and Powerful Approach to Multiple Testing*. *Journal of the Royal Statistical Society.*
15 *Series B (Methodological)*, 1995. **57**(1): p. 289-300.
- 16 25. Ospina-Prieto, S., et al., *MicroRNA-141 is upregulated in preeclamptic placentae and*
17 *regulates trophoblast invasion and intercellular communication*. *Transl Res*, 2016.
18 **172**: p. 61-72.
- 19 26. Kojima, K., et al., *Expression of leukemia inhibitory factor in human endometrium*
20 *and placenta*. *Biol Reprod*, 1994. **50**(4): p. 882-7.
- 21 27. Winship, A., et al., *Leukemia Inhibitory Factor (LIF) Inhibition during Mid-Gestation*
22 *Impairs Trophoblast Invasion and Spiral Artery Remodelling during Pregnancy in*
23 *Mice*. *PLoS One*, 2015. **10**(10): p. e0129110.
- 24 28. Morales-Prieto, D.M., E. Schleussner, and U.R. Markert, *Reduction in miR-141 is*
25 *induced by leukemia inhibitory factor and inhibits proliferation in choriocarcinoma*
26 *cell line JEG-3*. *Am J Reprod Immunol*, 2011. **66 Suppl 1**: p. 57-62.
- 27 29. Morales-Prieto, D.M., et al., *Intranuclear crosstalk between extracellular regulated*
28 *kinase1/2 and signal transducer and activator of transcription 3 regulates JEG-3*
29 *choriocarcinoma cell invasion and proliferation*. *ScientificWorldJournal*, 2013. **2013**:
30 p. 259845.
- 31 30. Pereira de Sousa, F.L., et al., *Involvement of STAT1 in proliferation and invasiveness*
32 *of trophoblastic cells*. *Reprod Biol*, 2017. **17**(3): p. 218-224.
- 33 31. Mary Photini, S., et al., *PIM kinases 1, 2 and 3 in intracellular LIF signaling,*
34 *proliferation and apoptosis in trophoblastic cells*. *Exp Cell Res*, 2017. **359**(1): p. 275-
35 283.
- 36 32. Suman, P., S.S. Malhotra, and S.K. Gupta, *LIF-STAT signaling and trophoblast*
37 *biology*. *JAKSTAT*, 2013. **2**(4): p. e25155.
- 38 33. Zheng, Q., et al., *Leukemia inhibitory factor promote trophoblast invasion via*
39 *urokinase-type plasminogen activator receptor in preeclampsia*. *Biomed*
40 *Pharmacother*, 2016. **80**: p. 102-108.
- 41 34. Novakovic, B., et al., *Wide-ranging DNA methylation differences of primary*
42 *trophoblast cell populations and derived cell lines: implications and opportunities for*
43 *understanding trophoblast function*. *Mol Hum Reprod*, 2011. **17**(6): p. 344-53.
- 44 35. Git, A., et al., *Systematic comparison of microarray profiling, real-time PCR, and*
45 *next-generation sequencing technologies for measuring differential microRNA*
46 *expression*. *RNA*, 2010. **16**(5): p. 991-1006.
- 47 36. Bilban, M., et al., *Identification of novel trophoblast invasion-related genes: heme*
48 *oxygenase-1 controls motility via peroxisome proliferator-activated receptor gamma*.
49 *Endocrinology*, 2009. **150**(2): p. 1000-13.

- 1 37. Wang, Y., et al., *Analyses of Long Non-Coding RNA and mRNA profiling using RNA*
2 *sequencing during the pre-implantation phases in pig endometrium*. Sci Rep, 2016. **6**:
3 p. 20238.
- 4 38. Fitzgerald, J.S., et al., *Signal transduction in trophoblast invasion*. Chem Immunol
5 Allergy, 2005. **88**: p. 181-99.
- 6 39. Li, X., et al., *Subpathway Analysis based on Signaling-Pathway Impact Analysis of*
7 *Signaling Pathway*. PLoS One, 2015. **10**(7): p. e0132813.
- 8 40. Yucel Cicek, O.S., et al., *Differential expression of leukemia inhibitory factor and*
9 *insulin like growth factor-1 between normal pregnancies, partial hydatidiform moles*
10 *and complete hydatidiform moles*. Placenta, 2018. **69**: p. 64-70.
- 11 41. Fitzgerald, J.S., et al., *Trophoblast invasion: the role of intracellular cytokine*
12 *signalling via signal transducer and activator of transcription 3 (STAT3)*. Hum
13 Reprod Update, 2008. **14**(4): p. 335-44.
- 14 42. Chen, L.Y., et al., *Activation of the STAT3/microRNA-21 pathway participates in*
15 *angiotensin II-induced angiogenesis*. J Cell Physiol, 2019. **234**(11): p. 19640-19654.
- 16
- 17
- 18