Molecular mechanisms in trophoblastic cells after LIF-stimulation with special regard to microRNAs

MicroRNAs in trophoblast cells

Dissertation
zur Erlangung des akademischen Grades *doctor rerum naturalium (Dr. rer. nat.)*

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät
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Verteidigungsdatum: 23.03.2012

To my family

"El ser humano además de materia,
es pensamiento, energía y tomadera de pelo"

Carmenza Prieto CAPRI.

Contents

List of abbreviations

C19MC	Chromosome 19 microRNA cluster
C14MC	Chromosome 14 microRNA cluster
CAM	Cell adhesion molecule
CL	*Corpus Luteum*
CNTF	Ciliary Neurotrophic Factor
ECM	Endometrial extracellular matrix
EGF	Epidermal-Growth-Factor
ERK	Extracellular-signal-Regulated Kinases
GAPs	GTP-ase activating proteins
GTD	Gestational trophoblastic disease
hES	human embryonic stem cells
hGC	Human chorion gonadotropine
IL-11	Interleukin- 11
IL-6	Interleukin- 6
IUGR	Intrauterine Growth Restriction
IVF	*In vitro* fertilization
JAK/STAT	Janus kinase/Signal Transducer and Activator of Transcription
JNK	Jun N-terminal kinase
LH	Luteinizing Hormone
LIF	Leukemia Inhibitory Factor
MEK	Mitogen-activated kinase
MiRNA	MicroRNA
MiRNome	MicroRNA expression signature
MMPs	Matrix metalloproteinases
mTOR	Mammalian Target Of Rapamycin
ncRNAs	Non-coding RNAs
OSM	Oncostatin M
PIAS	Protein Inhibitors of Activated Stats
PKC	Protein kinase C
PlGF	Placental Growth Factor
PTPs	Protein tyrosine phosphatases
RAS/MAPK	Ras/Mitogen Activated Protein Kinase
RNAi	RNA interference
RISC	RNAi-induced silencing complex
SOCS	Suppressors Of Cytokines Signalling
TFR	Total fertility rate
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene

In most women, cyclical ovulation at 25- to 35-day intervals continues during almost 40 years between menarche and menopause, which represents approximately 400 opportunities for pregnancy, if no contraception is used (Cunningham und Williams 2010). Nevertheless, the total fertility rate (TFR), understood as the average number of children that would be born to a woman over her lifetime, arises only 2.56 in the world and is even less than 2.0 in developed countries (Agency 2010). Besides the cultural and social implications, this low birth rate may also be attributed to poor pregnancy outcome.

Despite belonging to mammals, human beings do not exhibit the characteristic high fertility of this genealogical class. Whereas fertility rates of baboons and rabbits can reach 80%, in humans it only arises ca. 20% (Evers 2002). This difference is caused, among others, by the high embryo wastage and pregnancy loss in humans, estimated to be 30% prior to implantation (preimplantation loss), 30% before 6 weeks gestation and 10% miscarries, mostly prior to 12 weeks gestation (Teklenburg et al. 2010, Macklon et al. 2002). Therefore, the study of the embryo implantation and the molecular mechanisms underlying this process is essential in the understanding of the natural limits of human fertility and their implications in the success of *in vitro* fertilization (IVF) techniques.

1.1. Where do babies come from? The first stages: From fertilization to implantation

Union of egg and sperm at fertilization represents one of the most important and fascinating processes in biology. Two haploid nuclei fusion to form the genome of a diploid organism by a very complex process which includes binding of the head of the sperm with the surrounding glycoprotein layer of the unfertilized egg, following by digestion of this zona pellucida finally allowing sperm and egg to fuse (Cunningham und Williams 2010, Alberts 2002). Several regulatory mechanisms like depolarization of the egg plasma membrane and egg cortical reaction occur to ensure that only one sperm fertilizes the egg (Alberts 2002). Fertilization, however, does not seem to be the main problem for pregnancy establishment. As described before, fertilized eggs are often lost during implantation, a process in which the blastocyst embeds itself into the lining of the uterus and which requires a receptive endometrium, a normal and functional embryo at

the blastocyst stage and coordinated embryo-maternal dialogue (Achache und Revel 2006).

1.1.1. Menstrual cycle and functional windows

The endometrium is the inner layer of the uterus and changes with the menstrual cycle to provide the optimal environment for the blastocyst implantation. A sequence of hormonal events defines the follicular, ovulation and lutheal phases within the menstrual cycle. During the follicular phase (1-14 days), gradual increasing amounts of estrogen stop the menses and stimulate thickening of the endometrium. Simultaneously within the ovary, selection of the dominant "ovulatory" follicle occurs. When the egg is almost mature, levels of estradiol reach a threshold above which the Luteinizing Hormone (LH) can be expressed, thus the dominant follicle releases an egg, an event called ovulation (Nussey und Whitehead 2001, Gilbert 2000). After ovulation, the follicular phase starts, and the vestige of the dominant follicle remains in the ovary and becomes a corpus luteum (CL). This temporary structure has the function of producing estrogen and progesterone which prepare the endometrium for implantation. If implantation occurs, the blastocyst produces human chorion gonadotropine (hGC) and rescues the CL, thus maintaining progesterone production. On the other hand, if implantation does not occur, the corpus luteum decreases in size causing reduction in progesterone and estrogen levels which leads back to menses (Nussey und Whitehead 2001, Cunningham und Williams 2010).

Two main periods of time within the menstrual cycle are recognized for their relevance in conception and pregnancy, and are known as "fertile window" and "implantation window", respectively (Figure 1) (Teklenburg et al. 2010, Wilcox et al. 2000). Since most of the human pregnancies result from intercourse during a 6-day interval ending on the day of the ovulation, this period has been termed "fertile window" and is characterized by increasing pre-ovulatory estradiol levels on vaginal mucus, cervical opening and subendometrial contraction waves that allow sperm transport trough the female reproductive tract (reviewed by (Teklenburg et al. 2010)). Between days 5 and 10 following the luteinizing hormone (LH) surge, a second interval of time occurs, in which the blastocyst is allowed to implant in the lining of the uterus, this interval is called "implantation window". During this time, decidualization starts around the spiral arteries and expands to the endometrium. As the endometrial extracellular matrix

(ECM) attracts water, it becomes distended allowing the blastocyst to implant (Bischof und Campana 1996).

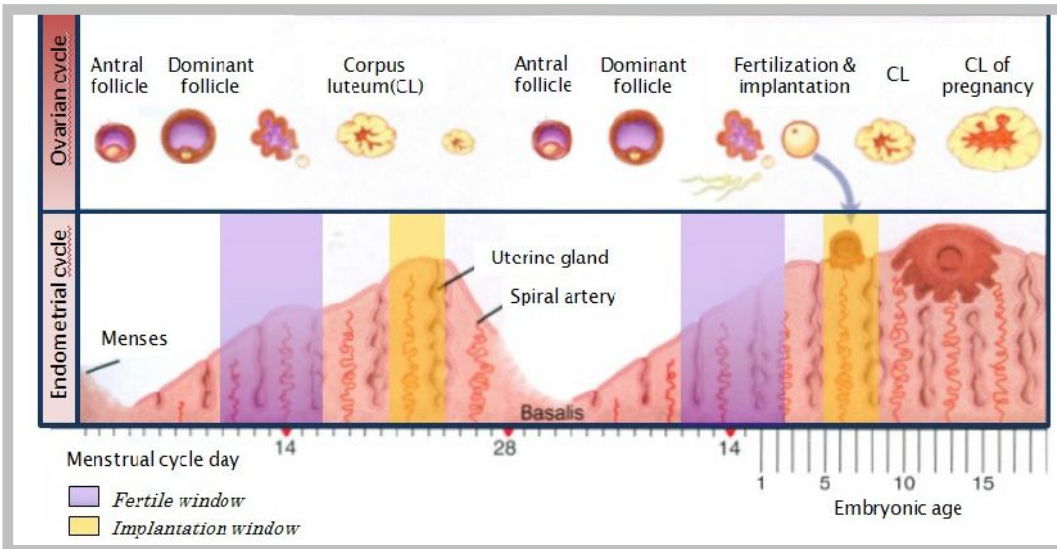

Figure 1. Menstruation cycle including alterations of the endometrium. The "implantation window" that corresponds to the period of maximum uterine receptivity is depicted in yellow, "fertile window" that constitutes the maximum period of conception, in purple. (Modified after (Cunningham und Williams 2010)).

1.1.2. Blastocyst implantation

As early as 4 to 5 days after fertilization, the blastula differentiates into the embryo-producing cells (inner cell mass) and the outer cells destined to form trophoblasts. Once the blastocyst arrives in the uterus, the embryonic pole is oriented to the potential implantation sites (Fitzgerald et al. 2008). As soon as the zona pellucida dissolves, the blastocyst can interact with the endometrium and adhere it in a process called *apposition*, but the connections between blastocyst and endometrium are not strong enough at this point and can be disrupted by washing. An increase in the physical contact between blastocyst and the uterine epithelium occurs during the second big process termed *adhesion*, after which the embryo cannot be dislodged. Finally, the embryo embeds itself in the uterus by a process called *invasion*, by which trophoblast cells coming from the embryo intrude between the endometrium, inner third of the myometrium, and uterine vasculature (Figure 2) (Dimitriadis et al. 2010b, Bischof und Campana 2000, Bischof und Campana 1996).

Invasion of trophoblasts into maternal tissues is an outstanding process that aims to connect maternal bloodstream with the embryonal tissue. Maternal spiral arteries should be transformed into large vessels of low resistance to ensure an effective

uteroplacental circulation, which constitutes a prerequisite for normal fetal growth. An inappropriate blood supply to the fetus results in pregnancies complicated by pre-eclampsia or intrauterine growth retardation (IUGR) (Moffett-King 2002, Ashton et al. 2005, Parham 2004). Conversely, hyperactive trophoblast invasion can lead to placenta accreta or percreta (Dimitriadis et al. 2010a), or results also in malignancies mostly related to gestational trophoblastic disease (GTD). Among other pathologies of the GTD, molar pregnancies are distinguished by hyperplasia of trophoblast cells and grapelike vesicles; as a result, pregnancy ends almost always as a spontaneous abortion. In some cases, molar pregnancies may lead to choriocarcinoma, a very aggressive cancer which may be fatal if metastasis to brain or lungs occurs (Fu et al. 2009, Seckl et al. 2010).

Interestingly, during blastocyst implantation trophoblasts cells resemble cancer cells as both cell types exhibit high proliferation, lack of cell-contact inhibition and the ability to protect themselves from the maternal immune system ("host" in the case of tumor cells) (Fitzgerald et al. 2008). In contrast, trophoblast cells are distinct from tumor cells in a very important feature uniquely happening in pregnancy, which is the tightly regulated proliferation and invasion depending on surrounding tissues and progress of gestation (Chakraborty et al. 2002, Fitzgerald et al. 2005a, Knofler 2010). The molecular mechanisms that control trophoblast invasiveness are therefore of great interest because they may be useful in the development of treatments for pregnancy diseases and cancer (Cheng et al. 2009).

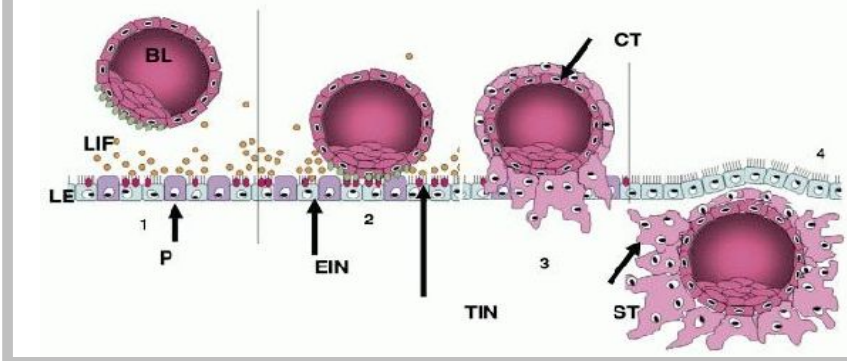

Figure 2. Blastocyst implantation to endometrium. Novel biomarkers: integrins (red), pinopodes (violet) and LIF (orange) and trophectodermal integrins (green) are illustrated. (1) Blastocyst is floating in uterus and then oriented to the implantation site. (2) Blastocyst hatching occurs when LIF is secreted by the endometrium and the blastocyst exhibit LIF receptors. (3) Trophoblast differentiate into cytotrophoblast and syncytiotrophoblast, the last ones invade the luminal epithelium (4) Blastocyst ist completely embedded in the myometrim and the implantation is complete (Taken from (Fitzgerald et al. 2007))

1.2. Regulating implantation: A plethora of small molecules

Several substances are recognized to play a role in the establishment of a receptive endometrium and in the regulation of trophoblast invasion, either in an autocrine way (trophoblastic factors) or in a paracrine way (uterine factors) (Bischof et al. 2000). The group of regulatory molecules includes hormones (e.g. Progesterone)(Szekeres-Bartho et al. 2009), cell adhesion molecules (CAMs) (Achache und Revel 2006), growth factors (e.g. EGF, PlGF)(Guzeloglu-Kayisli et al. 2009), enzymes (e.g. MMPs) (Cohen und Bischof 2007) and cytokines.

Of the hormones involved in the female menstrual cycle, progesterone is well-known for playing a critical role in the establishment and maintenance of pregnancy. This steroid hormone mediates interaction between the endocrine and immune systems creating a favorable immunological environment for the fetus. Besides, progesterone triggers genes that contribute to the regulation of blastocyst implantation including cell cycle regulatory genes like *p53* and *p27*, both recognized for their role in the establishment of a receptive endometrium and in the control of trophoblast invasion (Szekeres-Bartho et al. 2009, Chen et al. 2011).

The family of cell adhesion molecules (CAM) is composed by integrins, cadherins, selectins and immunoglobulins. Mostly, these proteins mediate cell-to-matrix and cell-to-cell adhesion in many physiologically processes including embryological development, haemostasis, thrombosis, wound healing, immune and non-immune defense mechanisms, and oncogenic transformation. Some members of the CAM family like L-selectine, ICAM-1 and some integrins are expressed by trophoblasts cell and/or endometrium during the time of implantation and their deregulation is associated with unexplained infertility and endometriosis, which suggests a regulatory role in the implantation process. Cadherins like E-cadherin are expressed at the cell surface during the preliminary phases, but should be down-regulated to enable epithelial cells dissociation and blastocyst invasion. Lastly, some mucins like MUC-1, which is found in the human endometrium, serve as negative factors for embryo implantation and are vanished in the area where implantation takes place (Reviewed in (Achache und Revel 2006)).

Finally, several cytokines and growth factors are found in the site of implantation or expressed by trophoblasts. For several years, research in Placenta-lab group has been

focused mostly on the interleukin-6 family of proinflammatory cytokines, which is known to be critical in the establishment and maintenance of a pregnancy and whose deregulation results in endometriosis, infertility or recurrent miscarriage (Paiva et al. 2009, Fitzgerald et al. 2005b). Six cytokines belong to the IL-6 family: Interleukin- 6 (IL-6) and 11 (IL-11), oncostatin M (OSM), the ciliary neurotrophic factor (CNTF), the leukemia inhibitory factor (LIF) and the recently identified cardiotrophin-1 (Cullinan et al. 1996).

The study of cytokines and growth factors with biological relevance in the control of trophoblast behavior is, however, more extensive. In order to summarize the vast amount of information about these mediators and their signal transduction pathways, we decided to write a review in cooperation with scientists from different continents. The central goal was to describe the main characteristics of these mediators, including their distribution within the reproductive tract, cellular origin, signaling transduction pathways and their implication with human pregnancy pathologies. Likewise, in a second paper, we reviewed the information of IL-6, IL-11 and LIF covering the current knowledge and the possible future applications of these cytokines in the field of human reproduction.

1.2.1. Classical signaling pathways

Depending on the cellular context, cytokines and growth factors mediate their effects trough activation of different intracellular cascades. Mechanistically, transmembrane cell receptors recognize these cytokines and activate signaling pathways that translate extracellular stimuli into cellular responses like increase of proliferation or invasiveness. Two main signaling pathways are essential in the response of trophoblast to stimulus and thus, relevant in the control of their proliferative and invasiveness properties: The Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) and the Mitogen Activated Protein Kinase (RAS/MAPK) (Cooper 2000, Rawlings et al. 2004, Dhillon et al. 2007, Plaza-Menacho et al. 2007).

The JAK/STAT pathway comprises three main steps: 1) Juxtaposition and trans-phosphorylation of two JAK molecules in the extracellular membrane. 2) Phosphorylation of STATs, a familiy of transcriptional factor located in the cytoplasm and 3) Hetero- or homo-dimerization of STATs which allow them to be translocated into

the nucleus and control gene expression (Rawlings et al. 2004, Maj und Chelmonska-Soyta 2007, Decker und Kovarik 2000). Since JAK/STAT cascade is involved in the regulation of implantation and maternal immune response in early pregnancy, and its deregulation is associated of malignancy, several molecules are responsible for modulate the signal or turning it off. Three major regulator families have been identified: Suppressors Of Cytokines Signalling (SOCS), Protein Inhibitors of Activated Stats (PIAS) and protein tyrosine phosphatases (PTPs), but the inhibition mechanism differs between them. In the cytoplasm, PTPs lead to dephosphorylation of JAKs or the cytokine receptor, SOCS inhibits activation of STATs, while PIAS bind to STAT dimers preventing them from binding DNA (Rawlings et al. 2004, Fitzgerald et al. 2005a) (Figure 3).

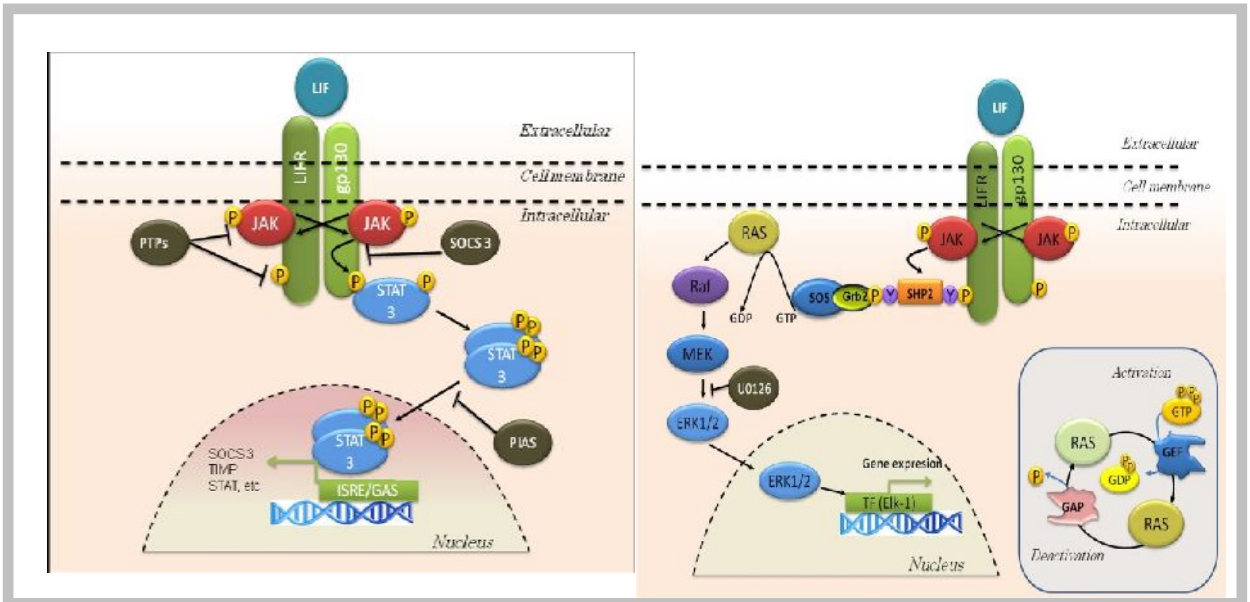

Figure 3. Schematic diagram of pathways activated by LIF. Left panel: JAK/STAT cascade, right panel: RAS/MAPK pathway. Negative regulators are displayed in black. (Design by Morales-Prieto 2011)

Likewise, JAKs are also able to trigger Ras activation. Ras is a GTP-binding protein kinase that alternates between an active and an inactive state when bound to GTP or GDP, respectively. By doing so, ras proteins activate RAF kinases. Consecutively, Raf activate Mitogen-activated protein kinases 1/2 MEK1/2), which in turn phosphorylate Extracellular signal-Regulated Kinases 1 and 2 (ERK1/2). ERKs are translocated into the nucleus where they phosphorylate some transcription factors including Elk-1, resulting in the control of gene expression (Landes Bioscience., Dhillon et al. 2007). Activation of MAPK pathway is terminated mostly by GTP-ase activating proteins

(GAPs), which inactive Ras causing the hydrolyzation of active Ras-GTP into inactive Ras-GDP (Dhillon et al. 2007). For the study of MAPK pathway, however, some compounds chemically synthesized have been found to interfere in the signaling trough this cascade. U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) inhibits MEK1/2 in a highly specific manner by suppressing their kinase activity and is one of the most widely used inhibitors in the study of ERK1/2 effects (Figure 3).

Although the mechanisms of signaling in JAK/STAT and MAPK pathways may appear to be simple, the biological effects are complicated by cross-talks between them. These interactions permit to enhance the effect of a specific stimulus or conversely, inhibit its signal. For instance, STAT3 activation and translocation results in the expression of SOCS3, a terminating molecule in the JAK/STAT cascade. SOCS3, however, has the ability to bind RasGAP, a negative regulator of Ras signaling, thus promotes activation of the MAPK pathway. Likewise, MAPKs specifically phosphorylate a serine near the C-terminus of most STATs enhancing their transcriptional activation, and thereby increasing the effects mediated by STATs (Rawlings et al. 2004, Plaza-Menacho et al. 2007). There is still conflicting evidence about the kinase responsible for this phosphorylation. Specifically, ERK1/2, p38, the Protein kinase C (PKC), Jun N-terminal kinase (JNK), and the mammalian target of rapamycin (mTOR), may have the ability to activate STAT3 Ser727 phosphorylation, but this interaction seems to be dependent on the cellular context and the stimuli and therefore, needs to be particularly investigated (Schuringa et al. 2000b, Schuringa et al. 2000a, Liu et al. 2008) .
The possible cross-talk between ERK1/2 and STAT3 will be analyzed in this work, using the JEG-3 choriocarcinoma cell line as model. The activation of both cascades mediated by LIF, and the implication of ERK1/2 inhibition on the cell proliferation and invasion will be investigated. Finally, it is aimed to find changes on STAT3 phosphorylation and transcriptional activity after abrogation of ERK1/2 activation and thus, to establish the molecular “dialogue” between these cascades.

1.2.2 Novel regulatory molecules: MicroRNAs

Numerous scientists seek regulatory molecules with the potential to control JAK/STAT and MAPK cascades simultaneously, mainly because of their implications on the regulation of trophoblast behavior, but also because this information may also be extrapolated to cancer research.

A novel group of regulatory molecules are the micro-RNAs (mi-RNAs), endogenous small RNA sequences that do not code for proteins, but instead exercise control over those that do. Non-coding RNAs (ncRNAs) were characterized for the first time in 1965, but their physiological role was not investigated until 1993, when Lee and colleagues demonstrated for the first time the involvement of lin-4, a so called "small temporal RNA" (stRNA), in the developmental timing in *C. elegans* (Lee et al. 1993). Seven years after, a genetic analysis of the *C. elegans* heterochronic gene pathway revealed that let-7, also a 21-nucleotide stRNA, was able to regulate expression of several genes involved in the control of developmental events (Reinhart et al. 2000). Over the years, numerous 21-25nt RNAs were cloned from different organisms confirming the existence of a new class of RNAs. This family was initially known as "tiny RNAs" and the term microRNA (miRNA) was introduced when the intracellular mechanisms started to be described (Ruvkun 2001).

By 1998, the study of posttranscriptional gene silencing (PTGS) had described the phenomenon of RNA interference (RNAi) that refers to gene silencing caused by introducing double-stranded RNA into the cell (Fire et al. 1998). RNAi is a natural cell process found in almost all eukaryotes and represents an antiviral defense mechanism against viruses and transposable elements (Dillin 2003). Nowadays, it is used for numerous biological applications and even some RNAi-based approaches are being studied in preclinical and clinical trials as new strategies for the treatment of skin diseases, respiratory diseases and cancer (Davidson und McCray 2011). Two types of RNA molecules trigger their effects through the RNAi pathway: small interfering RNAs (siRNAs) and miRNAs. Although they share some similarities (e.g. small length 20-25nt), they differ in a main feature that is their origin: siRNA are synthetic sequences whilst miRNAs are endogenous (Qavi et al. 2010, Prieto und Markert 2011).

Mechanistically, miRNAs are transcribed from DNA as longer sequences known as pri-miRNAs, which are then cleaved by the nuclear enzyme Drosha to form ~70 nucleotide precursors named pre-miRNAs. Pre-miRNAs associate with Exportin-5 and are exported to the cytoplasm. Once in the cytoplasm, pre-miRNAs and external siRNAs are processed by a Dicer-containing complex and then associated with the RNAi-induced silencing complex (RISC). The guide strand (if siRNA was used) or the mature miRNA directs the complex to the target mRNA thus, it represses protein translation (Bueno et al. 2008, Qavi et al. 2010, Cheng et al. 2005, Davidson und McCray 2011, Prieto und Markert 2011). The grade of complementarity between miRNA and its target mRNA

defines the mechanism used for gene repression. If alignment is perfect, the cascade ends in mRNA degradation, while partial complementarity and alignment lead to translational repression of the target mRNA (Cheng et al. 2005, Navarro und Monzo 2010, Hamilton und Baulcombe 1999) (Figure 4).

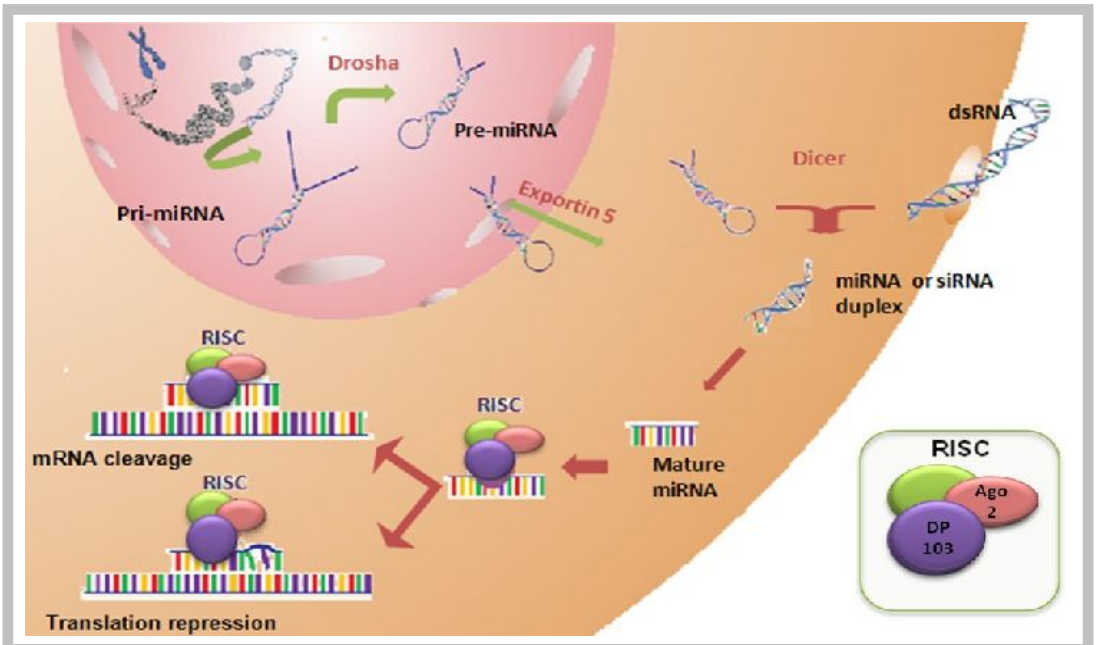

Figure 4. Principle mechanism of RNA interference. Inside the nucleus, pri-miRNA are cleaved by Drosha to pre-miRNA and transported into the cytoplasm by Exportin 5 (green arrows). The subsequent cascade is shared with exogenous siRNA (blue arrows). Processing by Dicer results in mature miRNA or functional siRNA which bind to RISC and to complementary RNA sequences. Perfect complementarity induces degradation whilst partial annealing leads to translational repression (Taken from (Morales Prieto und Markert 2011))

Since a perfect sequence match between miRNA and its mRNA target is not necessary, a miRNAs can regulate simultaneously more than one gene, but also different miRNAs target the same mRNA. This characteristic provides different grades of regulation and explains the current estimation that about 30% of the human genome may be regulated by miRNAs (Bueno et al. 2008). Since the introduction of the term microRNAs, numerous groups focused their investigation on this topic, mostly aimed to identify the location, regulation and function of these RNAs. Up to date, ~12000 reports have been published (Pubmed) and the number of miRNAs described arises approximately 1000 (MiRBase V16), this rapid growth demonstrates the interest caused but also the importance of their study in numerous research fields including human reproduction.

The signature of miRNAs expression, also known as miRNome, is regulated in a tissue- and developmental stage-specific manner and, thereby, their regulation is associated

with cancer (Navarro und Monzo 2010, Bueno et al. 2008, Zhang et al. 2007). This characteristic allows them to be used as a biomarker for the identification of certain physiological or pathological events including malignancies. Additionally, since miRNAs are known to participate in the control of several cellular processes, new therapies based on miRNAs are expected to be the future of cancer treatment. Their study in physiological processes like pregnancy is still incipient and their role in the control of pregnancy establishment remain unclear. In order to establish the "state-of-art" of miRNAs in pregnancy, we will summarize the current knowledge on miRNA biogenesis, targets and functions with relevance for pregnancy and placenta development.

Furthermore, human placenta, mainly trophoblast cells, produces miRNA-containing exosomes which transport regulating signals into the maternal organism and may play a role in the establishment of maternal immune tolerance (Frangsmyr et al. 2005). It can be expected that these circulating miRNAs will be useful for the diagnosis of pregnancy disorders, such as preeclampsia. Altogether, these observations suggest the role of miRNAs as regulators of inflammation and immune responses induced by mechanisms that include control of transcriptional factors and relevant for embryo implantation and placentation.

Additionally, the effect of LIF on the microRNA signature of trophoblast has not been studied and may provide crucial information about the molecular mechanisms involved in the regulation of LIF effects. Currently, the work of RNA signatures in primary cells represents a great challenge due to the limitations in obtaining these cells. Therefore, most of the work should be performed in trophoblastic cell lines before and after LIF-treatment and only afterwards, they may be compared with the expression in primary cells.

1.3. Objectives and design of this work

The objective of this work is to investigate the molecular mechanisms underlying the effects of LIF-stimulation o proliferation and invasion of trophoblastic cells with special regard on two main intracellular processes: a possible cross talk between LIF-induced JAK/STAT and RAS/MAPK cascades, and the identification of novel miRNAs involved in the LIF-response of trophoblastic cell lines.

Due to the extension of the topic, this study will be divided into three parts in order to answer the following questions:

1. What is known about LIF in pregnancy?
2. Is there any cross-talk between JAK/STAT and RAS/MAPK cascades in trophoblastic cells and how does it affect cellular proliferation and invasion?
3. Which miRNAs are associated with pregnancy or LIF responses in trophoblastic?

Ten papers will be included in this work. Initially, the role of LIF and other related cytokines in pregnancy will be analyzed and summarized in two reviews. Afterwards, the LIF-induced cross-talk between ERK1/2 and STAT3 in JEG-3 and HTR-8/svneo cells will be examined, as well as its implication in the cell proliferation and invasion. Subsequently, the state of art of miRNAs in pregnancy will be reviewed followed by an analysis of some miRNAs in LIF-induced JEG-3 cells. Finally, the microRNA expression signature (miRNome) of four cell lines will be analyzed and compared with that of isolated trophoblast cells before and after LIF stimulation (Figure 5), with the aim to find novel miRNAs involved in the control of trophoblast behavior.

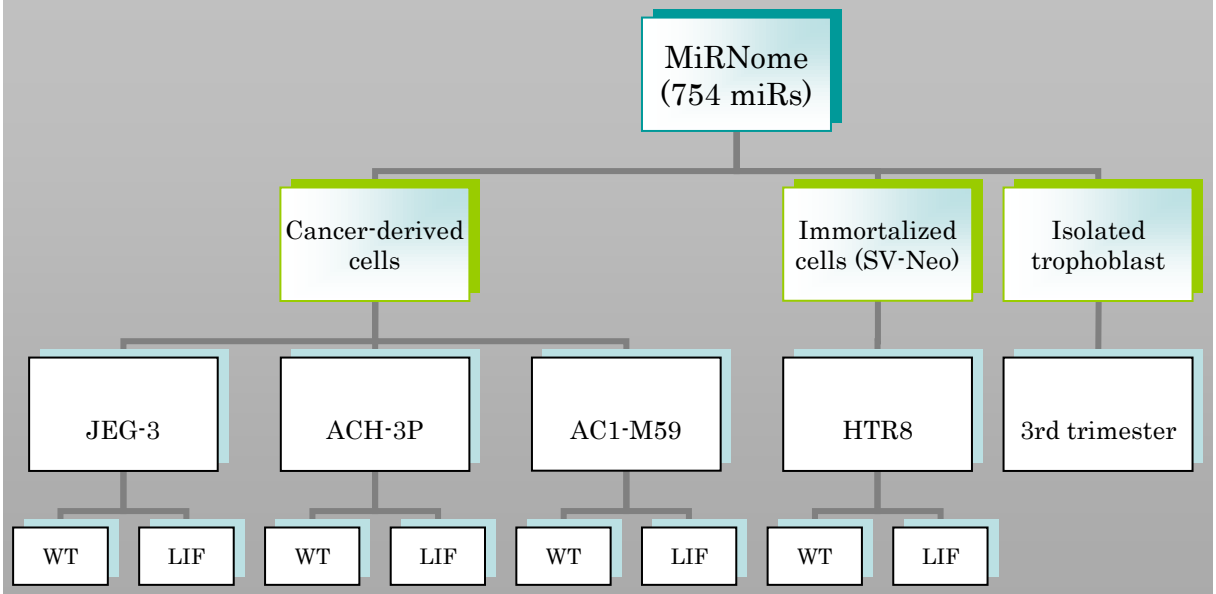

Figure 5. Experimental design of miRNome profiling in trophoblastic cells. Analysis of 754 miRNAs will be performed for three choriocarcinoma-derived cell lines, an immortalized trophoblastic cell line (HTR8/svneo) and isolated trophoblasts of third trimester placentas. MiRNA profiles of cell lines will be repeated after LIF treatment.

The following techniques should be established or optimized for this study:

- Western blot
- DNA-binding capability assay
- Matrigel Invasion Assay
- RNA isolation and Array assays
- qRT-PCR for miRNAs
- Over expression and knock-down of miRNAs
- Small-interference RNA
- Primary trophoblast isolation protocol

2.1. LIF biological relevance in pregnancy

Several investigations have been carried out during the last years in order to elucidate the specific role of LIF and other cytokines in the establishment and maintenance of pregnancy. Two works are presented here, both of them summarizing the current knowledge of cytokines in human reproduction.

The first review was written in cooperation with young investigators belonging to eleven research groups from different continents. The main goal was to summarize cytokines that are vital for human reproduction, their distribution within the reproductive tract, source of expression and function. Since the number of studied factors was very high, we decided to organize them according to their receptor family aiming to elucidate the characteristic signal transducing pathways. I have contributed in the chapter 2 "Type I cytokine receptor" with special focus on the subchapter on the role and functions of LIF in reproduction. The entire manuscript has approximately 68 pages and more than 430 cites. Therefore, in this thesis only the section on "Type I Cytokine Receptor" was included.

Similar to the previous one, the second article summarizes current knowledge on IL-6-like cytokines and their role in reproductive medicine. Additionally, their potential for future diagnostic and therapeutic applications in regard of new strategies in the treatment of reproductive pathologies was discussed. I contributed with the LIF subchapter and the revision of the manuscript.

2.1.1. Publication 1. "Cytokines regulating trophoblast invasion"

Authors: Fitzgerald JS, Abad C, Alvarez AM, Bhai Mehta R, Chaiwangyen W, Dubinsky V, Gueuvoghlanian B, Gutierrez G, Hofmann S, Hölters S, Joukadar J, Junovich G, Kuhn C, **Morales-Prieto DM**, Nevers T, Ospina-Prieto S, Pastuschek J, Pereira de Sousa FL, San Martin S, Suman P, Weber M, Markert UR.
Journal: Advances in Neuroimmune biology (NIB)

Status: Accepted May 2011

Impact Factor: Not yet, new journal (Online Date: August 2011)

2.1.2. Publication 2. "Understanding the link between the interleukin-6 cytokine family and pregnancy: implications for future therapeutics"

Authors: Markert UR, Morales-Prieto DM, Fitzgerald JS

Journal: Expert Review of Clinical Immunology (Expet Rev Clin Immunol)

Status: Published. Expert Rev Clin Immunol. 2011 Sep;7(5):603-9.

Impact Factor: 0.593

2.2. Uncovering the crosstalk between JAK/STAT and RAS/MAPK cascades

STAT3 and ERK1/2 are intracellular molecules relevant in the trophoblast response to extracellular stimuli. Based on current investigations that have suggested a possible crosstalk between these molecules, it was decided to investigate the activation of ERK1/2 and STAT3 after stimulation with LIF, and the possible crosstalk between their pathways.

Two different cell models were used in these works: JEG-3 and HTR8/SVneo cells. The first study included exclusively JEG-3 cells and aimed to analyze the cross-talk at cytoplasmic and nuclear levels, as well as their implications in trophoblast proliferation and invasion. Some of the experiments were assisted by Maja Weber, Sebastian Hölters and Stephanie Ospina and the adjustments and revisions were done by Prof. Dr. Ekkehard Schleussner, Dr. Justine Fiztgerald and Prof. Dr. Udo R. Markert.

The second report was supported by an Indo-German exchange program between the Department of Science and Technology (DST), Government of India, and the German academic exchange service (DAAD), Germany. Aim of this study was to determine the significance of ERK1/2- and STAT3-dependent signaling pathways in LIF-mediated proliferation and survival of trophoblast cells using HTR-8/SVneo cells. The exchange program included a scholarship of three weeks in India. During this time, I presented the methodology and experimental design of our study and we performed the first experiments. After returning to Germany, my contribution was the peroxidase staining for ERK1/2 and STAT3 phosphorylation after stimulation with LIF, as well as the participation in the writing and revision of the manuscript.

2.2.1. Publication 3. "Intranuclear, but not intracytoplasmic, crosstalk between Extracellular Regulated Kinase1/2 and Signal Transducer and Activator of Transcription3 regulates JEG-3 choriocarcinoma cell invasion and proliferation"

Author: Morales-Prieto DM, Ospina-Prieto S, Weber M, Hoelters S, Fiztgerald JS, Schleussner E, Markert UR

Journal: Journal of Cellular Biochemistry

Impact Factor: 3.122

Status: Submitted (July 2011)

Re-submitted after reviewers modifications (March 2012)

2.2.2. Publication 4. "Leukemia Inhibitory Factor mediated proliferation of HTR8/SVneo trophoblastic cells is dependent on Extracellular Regulated Kinase 1/2 activation"

Authors: Golla JP, Suman P, **Morales Prieto DM**, Markert UR, Gupta SK.

Journal: Reproductive Fertility and Development (Reprod Fert Develop)

Impact Factor: 2.553

Status: Published. Reprod Fert Develop 23(5) 714-724

2.3. MicroRNAs regulating throphoblast behavior

2.3.1. Publication 5. "MicroRNAs in pregnancy".

Authors: Morales Prieto DM, Markert UR.
Journal: Journal of Reproductive Immunology
Impact Factor: 2.204
Status: Published. J Reprod Immunol. 2011 Mar;88(2):106-11

This review provides a general overview of the current knowledge on miRNAs in pregnancy. The available information concerning profiles and functions of microRNAs in the peri-implantation period, embryonic stem cells, placentation and pregnancy, as well as in several pregnancy-related pathologies are summarized in this work. The main achievement is the description of a miRNA cluster (C19MC) that is highly expressed in placenta tissues and has been described in several independent studies. As first author, I carried out most of the investigation and wrote the first draft. Prof. Dr. Markert contributed with important ideas and a critical and academic review of the manuscript.

2.3.2. Publication 6. Reduction of miR-141 is induced by Leukemia Inhibitory Factor and inhibits proliferation in choriocarcinoma cell line JEG-3

Authors: Morales-Prieto DM, Schleussner E, Markert UR.
Journal: American Journal of Reproductive Immunology
Impact Factor: 2.451
Status: Published. Am J Reprod Immunol. 2011; 66S1:57–62

Since the study of microRNAs and their functions in pregnancy is very incipient, we decided to analyze the function of some miRNAs in the proliferation of choriocarcinoma cells and their expression after LIF stimulation. We selected 5 miRNAs, which have been previously described to participate in the control of cancer development, to be implicated in pregnancy, or to be related with members of the signaling intracellular cascade of LIF, especially STAT3.
Prof. Dr. Schleussner was involved in the project design; Prof. Dr. Markert is responsible for the design of the study and the revision and finalization of the manuscript.

2.3.3. Publication 7 . MiRNA expression profiles of trophoblastic cells

Authors: **Morales-Prieto DM**, Chaiwangyen W, Gruhn B, Markert UR.
Journal: Placenta
Impact Factor: 2.985
Status: Submitted (September 2011)

The study of the miRNA signature (miRNome) in normal human tissues has revealed some universally expressed miRNAs and also several groups of miRNAs exclusively or preferentially expressed in a tissue-specific manner (Liang et al. 2007). MiRNA signatures are frequently altered in cancer (Selcuklu et al. 2009, Zhang et al. 2007), and they can be successfully used to distinguish between cancer and normal tissues (Murakami et al. 2006, Cohn et al.) or even to clarify poorly differentiated tumors (Lu et al. 2005). This part of the work was designed to analyze the miRNA expression profiles of different cell lines before and after LIF stimulation. Due to the vast amount of data, two manuscripts were written. The first one contains the information about the signatures of trophoblastic cells and the comparison with those of isolated third trimester trophoblast cells. The full set of data is published and accessible at NCBI Gene Expression Omnibus: GSE32346. The expression of some miRNAs, previously described to be involved in cancer development, was also analyzed. This work provides the first comprehensive miRNA encyclopedia of trophoblastic cells and may also be useful for the design of further experiments. Wittaya Chaiwangyen assisted in the isolation of primary trophoblast cells and contributed with some of the single assays. PD. Dr. Gruhn supported the qPCR studies, Prof. Dr. Markert is responsible for the design of the study and the revision and finalization of the manuscript.

2.3.4. Publication 8. Leukemia Inhibitory factor alters miRNome of trophoblastic cells

Authors: **Morales-Prieto DM**, Ospina-Prieto S, Chaiwangyen W, Gruhn B., Markert UR.

Journal: Placenta
Impact Factor: 2.985
Status: In preparation

As mentioned above, two manuscripts were prepared to publish the miRNA signatures of trophoblastic cells and the alteration after LIF treatment. In this second manuscript, the miRNome of trophoblastic cells before and after LIF stimulation is compared. Here, some miRNAs were identified as possible mediators of LIF effects. It was also demonstrated that HTR8/svneo and JEG-3 cells differ in the expression of miR-141 and one of its putative targets (PIAS3). Since the relation between miR-141 and PIAS3 should be further confirmed, this manuscript has not yet been submitted. Some additional experiments carried out by Wittaya Chaiwangyen and Stephanie Ospina will be also included. PD. Dr. Gruhn supported the qPCR studies, Prof. Dr. Markert is responsible for the design of the study and the revision and finalization of the manuscript.

2.4. Additional Publications

In addition to the manuscripts described above, several minor results of this thesis are included in two manuscripts that have been submitted recently. They are presented below but will be shorter discussed, because their focus is distinct from that of the others.

2.4.1. Publication 9. AP-1 transcription factos, mucin-type molecules and MMPs regulate the IL-11 mediated invasiveness of JEG-3 and HTR-8/SVneo cells

Authors: Suman P, Godbole G, Thakur R, **Morales Prieto DM**, Modi D, Markert UR, Gupta SK.
Journal: PLOS one
Impact Factor:4.411
Status: Published . PLoS ONE 2012; 7(1): e29745.

The cooperation with the group in India continued during this year in an Indo- The cooperation with the National Institute of Immunology in India continued during this year in an Indo-German exchange program between the Department of Science and Technology (DST), Government of India, and the German academic exchange service (DAAD), Germany. The focus of this program was the delineation of molecular mechanisms of HTR8/svneo cells, especially with regard to the IL-6 family of cytokines. Based on the previous results with LIF, the cooperation program has been focused on the regulation of trophoblastic cells mediated by IL-11, another cytokine of the IL-6 family. The aim of this publication was the analysis of the expression of matrix metalloproteinases and changes in the invasion capability of HTR8/svneo cells.

2.4.2. Publication 10. It's a woman thing: Part II - The placenta under the influence of tobacco

Authors: Fitzgerald JS, **Morales-Prieto DM**, Suman P, San Martin S, Poehlmann T, Gupta SK, Markert UR.
Journal: Human Reproduction Update (Hum.reprod.update)
Impact Factor: 8.755
Status: Under revison

This work is the second section of a two-part review concerning the clinical and pathophysiological effects of maternal tobacco during pregnancy. The first part was focused on the clinical effects including the histological and physiological modification of the placenta during pregnancy. In this manuscript, the literature on experimental data on smoke effects has been summarized in an attempt to correlate with the clinical effects reviewed in the first part.
My contribution to the above mentioned manuscript is the overview on trophoblast behavior under the influence of toxic insults from the cigarette. The effects of some molecules, e.g. nicotine, cadmium and some antioxidants are summarized in this section. The entire manuscript has approximately 47 pages and more than 160 cites. Therefore, in this thesis only the section on "Maternal smoking and trophoblast cells" is included.

3.1. LIF biological relevance in pregnancy (Publications 1-2)

Trophoblast and cancer cells share several features including high proliferation, lack of cell-contact inhibition and the ability to escape from the host immune system (Fitzgerald et al. 2008). Trophoblast cells, however, exhibit a tightly time-regulated proliferation and invasion (Chakraborty et al. 2002, Fitzgerald et al. 2005a, Knofler 2010), which turns them into an excellent model for understanding the molecular mechanisms involved in this regulation. Numerous cytokines are expressed within the female reproductive track and regulate the trophoblast response to external stimuli. These cytokine patters are also responsible for the communication between fetus and mother during blastocyst implantation and therefore, their deregulation causes a variety of pregnancy disorders.

Several studies have been performed in order to establish the intracellular mechanisms and the specific function of some cytokines. Since numerous models and experiments have been carried out, a large amount of information is available. By summarizing this information (Markert et al. 2011), it was found that dysregulation of some cytokines like IL-6, IL-10 and IL-11 is closely associated with infertility and recurrent miscarried (Lim et al. 2000, von Wolff et al. 2000, Gutierrez et al. 2004, Koumantaki et al. 2001, Murphy et al. 2005), while aberrant expression of G-CSF and IFN-γ is relevant in preeclampsia and preterm birth (Matsubara et al. 1999, Whitcomb et al. 2009, Szarka et al. 2011). Among the variety of cytokines considered for this work, Leukemia Inhibitory Factor (LIF) apperared to be one of the most extensively studied due to its implication in almost all processes associated with pregnancy.

Even when LIF is associated with inflammatory cell responses and cell differentiation, during pregnancy, LIF expression is up-regulated by progesterone, one of the major hormones responsible for pregnancy establishment and maintenance (Markert et al. 2011). Moreover, the concentration of LIF in follicular fluids correlates with embryo quality, its concentration in flushing is a measure of uterine receptivity prior to blastocyst implantation (Arici et al. 1997, Laird et al. 1997) and finally LIF can also influence trophoblast behavior (proliferation, invasion and differentiation) (Fitzgerald et

al. 2005b, Fitzgerald et al. 2008). Altogether, these investigations demonstrate the vital role of LIF during ovulation, implantation and pregnancy outcome and therefore, emphasize the need to understand the molecular mechanisms associated to its function and regulation.

It is expectable that LIF-based treatments will improve the outcome of IVF treatments in women with recurrent implantation failure or recurrent miscarriege. The first multicenter study failed in showing LIF as a therapeutic agent (Brinsden et al. 2009). However, the lack of prior assessment of LIF expression and its administration during the trial are discussed. LIF signaling is regulated through a negative feedback mechanism, meaning that both too much, as well as too little LIF will induce similar functional effects (Fitzgerald et al. 2009). Therefore, cytokine supplementation in IVF treatments may still be optimized. Currently, LIF is used as a supplement to culture media in embryo cultures previous to implantation, because the percentage of embryos that reach the implantation stage increases in presence of LIF (United States Patent 5962321; Inventors: Gough, Nicholas Martin; Willson, Tracey Ann, Seamark, Robert Frederick (Beulah Park, AU), http://www.freepatentsonline.com/5962321.html).

The possible applications of LIF in human reproduction are not only focused on the improvement of pregnancy achieving and maintenance but also on the contraception methodologies. Oral steroid contraceptives (OC) are the most common method of reversible contraception but their use is associated with several hormone withdrawal symptoms including bleeding, pelvic pain, breast tenderness, bloating/swelling, and increased use of pain medications (Sulak et al. 2006). Recent reports have even indicated that combined oral contraceptive containing drospirenone carries a higher risk of venous thromboembolism than do formulations containing levonorgestrel (Parkin et al. 2011). Despite the recent attempts to reduce the hormonal concentration, side effects are still high and numerous studies are carried out to reduce the frequency of menstruation and the acceptance of OC regimes (Coffee et al. 2007). The appearance of non-hormonal contraceptives, thus, represents an alternative to improve the quality of life for millions of women. Recently a new non-hormonal contraceptive has emerged. Known as PEGylated (conjugated to polyethylene glycol) LIF antagonist (PEGLA), this formulation has become a promising contraceptive which, by intra-vaginal application, may guarantee implantation block and simultaneously eliminate the systemic effect on bone (Menkhorst et al. 2011). The main concern about this new medication is the severe side effects of targeting IL-6-like cytokines, which include alteration in muscles,

cardiovascular development, immune system and nervous system development (Bauer et al. 2007).

Summarizing, the potential to use LIF as biological marker for embryo selection, as medicatione to achieve and maintain pregnancy (Aghajanova 2010), or as a treatment in the control of pregnancy-associated diseases (Koehn et al. 2011) is enormous. However, it is crucial to understand the signaling mediators responsible for its regulation. For instance, the analysis of STAT3 and MAPK pathways may contribute to clarify the effects of LIF on trophoblastic cells and the future implications of a LIF-based therapy.

3.2. Uncovering the cross talk between JAK/STAT and RAS/MAPK cascades (Publications 3-4)

STAT3 is a well studied intracellular molecule which plays a crucial role in the regulation of trophoblast invasion mediated by LIF (Poehlmann et al. 2005, Fitzgerald et al. 2008). STAT3 becomes fully activated after phosphorylation at its tyr705 and ser727 residues, which allows it to dimerize and translocate into the nucleus (Schuringa et al. 2000b, Liu et al. 2008, Schuringa et al. 2001). A previous report of our group on JEG-3 cells had demonstrated that LIF triggers STAT3 Tyr705 phosphorylation, and this activation correlates with an increase of cell proliferation and invasion (Fitzgerald et al. 2005b). STAT3 Ser727 phosphorylation has been less studied but it is known that its inhibition decreases DNA binding activity of STAT3 after stimulation with IL-6 (Decker und Kovarik 2000, Boulton et al. 1995). The mechanisms involved in the activation and regulation of p-STAT3 Ser727 remain unclear and several studies are carried out to establish the kinase responsible for this phosphorylation, principally due to the potential to control STAT3-mediated cell responses. Since STAT3 contains a characteristic ERK-MAPK phosphorylation site (-pro-X-ser/thr-pro-) (Chung et al. 1997), a possible cross-talk between STAT3 and ERK activated by LIF may be expected.

By using JEG-3 cells as model, we have demonstrated that LIF triggers phosphorylation of both STAT3 Ser727 and Tyr705 residues. In addition, activation of MAPK pathway, measured as phosphorylation of ERK1/2 was also observable within 5 min of stimulation. This rapid activation of both pathways provided the first evidence of an independent activation after LIF stimulation, which was lately confirmed by Western blotting and immunocytochemistry. The methodology included the pre-treatment of JEG-3 cells with U0126, a specific p-ERK1/2 inhibitor, followed by LIF-stimulation and the

determination of STAT3 phosphorylation. Results demonstrated a successful inhibition of ERK1/2 activation in all experiments including a reduction of basal levels. Conversely, phosphorylation of STAT3 Ser727 and Tyr705 was not altered by application of U0126 and also no changes in the localization of the protein were observed by immunocytochemistry.

As mentioned before, activation of STAT3 depends on the cell-type and the stimuli, and therefore, responses may vary among different trophoblastic subtypes or cell lines. In order to confirm the role of ERK1/2 in the LIF-mediated STAT3 activation, a parallel study was carried out in cooperation with the Reproductive Cell Biology Laboratory in New Dehli, India. In this study, HTR-8/svneo cells were used as a model. HTR-8/svneo cells were established through transfection of isolated first trimester trophoblast cells with a simian virus 40 (SV40), and represent a model for trophoblast study, as they share several characteristics with first trimester trophoblast cells (Graham et al. 1993). Nevertheless, the results were almost identical, with a fully abrogation of ERK1/2 activation that does not change the LIF-mediated activation of STAT3. Interestingly, HTR-8 cells exhibit higher basal levels of p-STAT3 Ser727 and p-ERK1/2 in comparison with JEG-3 cells. This can be explained by the cellular transformation by SV40, which in other cell lines has been associated with an increase of ERK1/2 and STAT3 activation by a mechanism including inhibition of protein phosphatase 2A (Cheng et al. 2009, Sablina und Hahn 2008). Incubation with a low concentration of U0126 (10ng/ml) was sufficient to abrogate ERK1/2 phosphorylation, independently of the basal levels. There results demonstrate that the methodology was optimal and also confirm the efficiency and specificity of U0126.

Altogether, our studies demonstrated that STAT3 Ser727 phosphorylation in trophoblastic cells is independent of ERK1/2 activation, and therefore, further experiments are needed to clarify the signaling mediator. Based on previous studies in our laboratories, in which mammalian target of rapamycin (mTOR) was found to be required for the constitutive, LIF-independent phosphorylation of STAT3 Ser727 in HTR8/svneo cells (Busch et al. 2009), and also in a recent publication of mTOR as likely responsible for the phosphorylation of STAT3 Ser727 upon IL-6 stimulation in the human hepatocarcinoma cell line HepG2 (Kim et al. 2008), one can hypothesize that this may be the major signaling pathway responsible for the activation of p-STAT3 Ser727 in trophoblast and choriocarcinoma cells.

A major finding of the present work is that after stimulation with LIF, ERK1/2 inhibition does not influence STAT3 phosphorylation, but it does augment STAT3 nuclear translocation in JEG-3 cells. Besides the numerous reports describing a positive regulation of cytokine-mediated STAT3 phosphorylation by ERK1/2 (Tian und An 2004), there is also cumulating evidence describing the negative regulation of STAT3 by ERK1/2 (Krasilnikov et al. 2003). In CHO (Chinese hamster ovary) cells, constitutive expression of MEK1 cells inhibited the activation of STAT3 and hampered the binding of phosphorylated STAT3 to DNA (Sengupta et al. 1998). In addition, a recent report demonstrated that hepatic stimulator substance (HSS)-induced ERK1/2 activation in human hepatoma HepG2 cells exerted negative modulation on STAT3 accumulation into the nucleus (Tian und An 2004).

IL-6 family members induce STAT3 activation and translocation into the nucleus, which is essential for mediating invasion in trophoblast and choriocarcinoma cells (Poehlmann et al. 2005, Suman et al. 2009, Dubinsky et al.). In the current study, inhibition of ERK1/2 induces accumulation of STAT3 in the nucleus and thus, increases its transcriptional activity, resulting in an augmention of JEG-3 invasion. This cross-talk might be useful for the development of new therapies based on the regulation of trophoblast invasion. However, studies *in vivo* are required to clarify this potential therapy.

On the other hand, proliferation of trophoblastic cells is a process mostly mediated by MAPK activation, rather than by JAK/STAT. LIF-treatment triggers activation of ERK1/2 and STAT3, and results in an increase of proliferation in both HTR-8/svneo (Prakash et al. 2011) and JEG-3 cells. As previously demonstrated, ERK1/2 has no intracytoplasmic crosstalk with STAT3, but it antagonizes STAT3 DNA-binding capacities in the nucleus. Hence, decrease in proliferation caused by U0126 addition can be attributed to the loss of ERK1/2 activation, independent of activation of STAT3. In JEG-3 cells treated with U0126, further addition of LIF rescues slightly cell proliferation, showing that STAT3 is also be involved. The proliferation of P19 embryonal carcinoma cells following LIF stimulation is also independent of the activation of STAT3 (Schuringa et al. 2002), which supports our findings that ERK1/2 is the major mediator of trophoblast proliferation, even in absence of cytokine stimulation.

It may be concluded that LIF is a major inducer of invasion and proliferation in trophoblastic cells, and triggers its effects through activation of JAK/STAT and MAPK

pathways. These cascades are connected by an intracellular cross-talk, in which ERK1/2 is a negative regulator of STAT3 nuclear activity (Figure 4). This connection may explain the disorders observed when dysfunctions of the pathways occur, but also provides information for understanding the role of individual factors which may lead to the development of new therapeutic strategies.

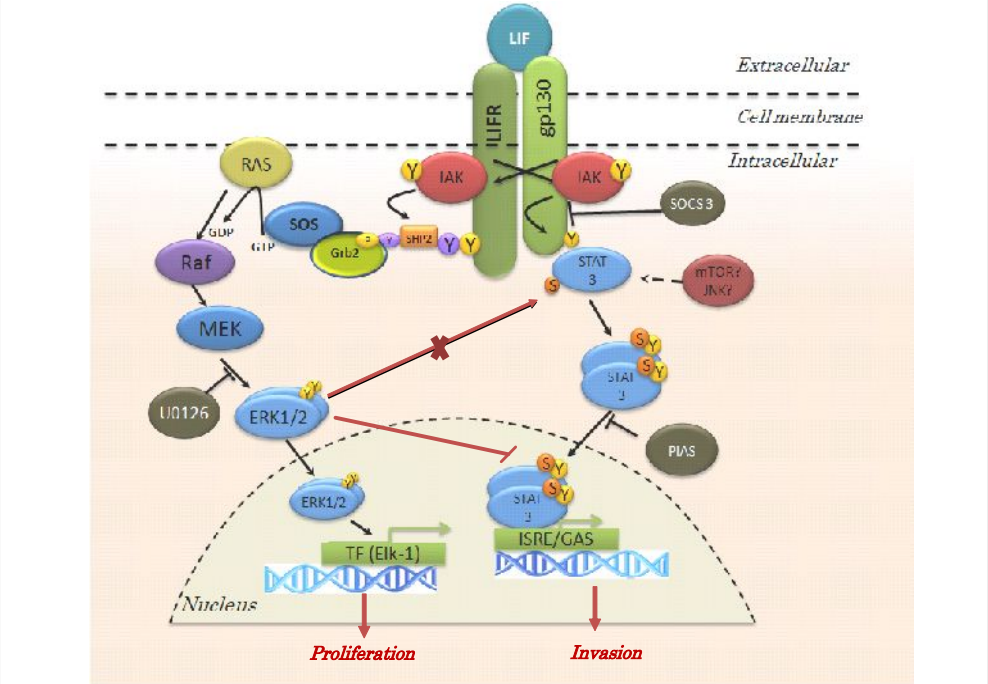

Figure 6. Diagram of the proposed LIF signaling pathway in trophoblast cells. LIF trigger activation of JAK/STAT and MAPK independently. ERK1/2 does not regulate STAT3 Ser727 phosphorylation but antagonize to STAT3 translocation into the nucleus. JAK/STAT and MAPK activation result in different cell responses increasing proliferation and invasion, respectively. Taken from (Morales-Prieto et al. 2011).

3.3. MicroRNAs regulating throphoblast behavior (Publications 5-8)

MiRNAs constitute a novel group of regulatory molecules which play a pivotal role in the control of gene expression at post-transcriptional level, and it is thought that 30% of the human genome is regulated by these molecules (Bueno et al. 2008). The study of miRNAs in pregnancy is still incipient, albeit some pioneer studies in pregnancy-associated diseases (e.g. preeclampsia) have been published (Noack et al. 2011). Therefore, it was important to investigate the state-of-art of miRNAs in pregnancy reviewing the current data of microRNAs in pregnancy and highlighting some perspectives of their study in human reproduction (Prieto und Markert 2011).

This review article summarizes current reports that demonstrate participation of miRNAs in several processes associated with pregnancy achievement and maintenance. For instance, during the menstrual cycle, inflammation-like processes must occur to prepare the endometrium for implantation (Pan und Chegini 2008). However, altered endometrial gene expression is responsible for inappropriate tissue regeneration, resulting in dysfunctional uterine bleeding, failure in embryo implantation, as well as many other endometrial disorders (Kuokkanen et al. 2009). MiRNAs participate in regulating dynamic changes in uterine gene expression patterns by controlling genes associated with inflammatory responses (Pan und Chegini 2008, Chakrabarty et al. 2007), or by repressing expression of immune tolerance-associated genes, such as HLA-G (Veit und Chies 2009). Altogether, these observations support the role of miRNAs as regulators of inflammation and immune responses by mechanisms that include control of transcriptional factors. Therefore, they appear to be highly relevant for tuning of embryo implantation and placentation.

The main goal of our miRNA review (Prieto und Markert) was to summarize the information relevant for the miRNAs exclusively expressed by placenta. Three recent reports have independently identified a cluster of miRNAs located in the chromosome 19 and which constitutes the largest miRNA cluster ever reported (Bentwich et al. 2005, Bortolin-Cavaille et al. 2009, Liang et al. 2007). The chromosome 19 microRNA cluster (C19MC) comprises 54 predicted miRNAs, 43 of them already cloned and sequenced. Two main characteristics of C19MC demonstrate its importance in human embryonic development: the fact that it is conserved among eutherian species, and its imprinting expression exclusively from the paternally inherited chromosome (Bortolin-Cavaille et al. 2009). Imprinting genes play important roles in the regulation of cellular differentiation and fate, and they are frequently expressed only in embryonic stages or placenta tissues, which revealed C19MC as a miRNA cluster involved in human embryonic development (Tsai et al. 2009). Located close to C19MC, a second cluster has been identified. It maps to chromosome C19q13.42 and comprises only three miRs (miR-371, miR-372 and miR-373). These miRNAs are found exclusively expressed by human embryonic stem cells hES (Laurent et al. 2008) and their study may provide information about the regulatory mechanisms involved in the embryonic development.

The next step was to investigate the miRNome of isolated trophoblast cells and compare them with the miRNA signatures of several trophoblastic cell lines, which share characteristics with isolated trophoblast cells but differ in the proliferation and invasion

rates. In this study we demonstrated that the miRNome signature of the choriocarcinoma and choriocarcinoma-like cells (JEG-3, ACH-3P and AC1-M59) was very similar, but it differs significantly from that of HTR8/svneo cells. Surprisingly, it was also demonstrated that the miRNA signature of isolated trophoblast cells from term placentas is more similar to that of choriocarcinoma-derived cell lines than of the immortalized cell line HTR-8/svneo. A recent report focused on the mRNA signature of several cell lines has also described more similarities of the mRNA expression of isolated trophoblast cells with choriocarcinoma-derived cell lines than with HTR-8/SVneo cells (Bilban et al. 2010). In conclusion, these results suggest that cell lines derived from choriocarcinoma preserve large parts of the mRNA and miRNAs expression of trophoblast cells, while the immortalization process of HTR-8/svneo generates changes in the gene expression that result in a less appropiate model for trophoblast gene expression analyses.

Furthermore, our study emphasized the importance of the C19MC because of its high expression in primary trophoblast cells and also in JEG-3 and their hybrids, but more significantly, because here it was demonstrated that these miRNAs confer the identity to the trophoblastic cells. It is to expect thus, that dysregulation of their expression may be associated with pregnancy disorders. A recent report in serum of pre-eclampsia versus normal pregnant women has confirmed partially this hypothesis, as an aberrant expression of some members of the C19MC was observed in the pre-eclamptic women (Yang et al. 2011).

Recent studies have also reported alterations in the expression of some miRNAs in choriocarcinoma cells when compared to normal trophoblast (Chao et al. 2010). Similarly, some miRNAs were reported to be altered in placentas injured or exposed to toxic agents versus normal tissues (Maccani et al. 2010). Here, the complete miRNAs signature of the most studied trophoblastic cell lines is provided and is compared with the expression of normal isolated trophoblasts. When used as a data bank, this information will be of value to design experiments related to gene expression and functional analyses. As an example, over-expression experiments on miR-519e, which is located within C19MC, can be carried out in HTR-8/svneo cells, while downregulation experiments can be performed in JEG-3 cells, as no basal expression in HTR-8 is observable.

3.3.1. MiRNome after LIF

By reviewing the investigations carried out during the last decade, it was established that there were no studies published on LIF-induced miRNA in any cell type, albeit several miRNAs have been described as regulators of some members of JAK/STAT or MAPK pathways (Meng et al. 2007, Taganov et al. 2006, Bazzoni et al. 2009). As LIF plays an important role in the achievement, maintenance and regulation of pregnancy, the study of miRNAs expression in response to LIF is imperative for understanding cellular processes associated with pregnancy.

The number of miRNAs already described arises 1000, but initially only five miRNAs were selected for the study (miR-9, miR-21, miR-93, miR-141 and let-7g). They were previously published to correlate with tumor-grade, to be implicated in pregnancy or to be related with members of the intracellular signaling cascade of LIF. Three miRNAs were identified to be significantly altered after LIF-treatment: miR-21, miR-93 (upregulated) and miR-141 (downregulated). Interestingly, the strongest effect was observable in the expression of miR-141, which was downregulated by far more than 50%(Morales-Prieto et al. 2011). MiR-141 was found significantly elevated in plasma from pregnant women in comparison with non-pregnant women (Gilad et al. 2008), and therefore, may be expected to display a specific or even crucial role during pregnancy. On the other hand, our finding of increased miR-21 expression in trophoblastic cells after LIF stimulation coincides with previous reports in head and neck carcinoma, osteosarcoma, ovarian carcinomas and others, and in which miR-21 promotes proliferation, migration and invasion (Zheng et al., Lou et al., Ziyan et al.).

As previously mentioned, LIF increases proliferation of trophoblastic cells. Therefore, an effect of miR-141 over-expression or silencing on proliferation was expectable. Due to the small sequences used for transfection and the low cell viability after transfection, this methodology should be initially optimized. Two different small chemically altered RNA molecules were used for transfection: dsRNAs that mimic endogenous miRNA (overexpression) or single-stranded RNAs that inhibit specific miRNA (down-regulation). By using these methods, we were able to establish that silencing of miR-141 results in a reduction of JEG-3 proliferation. This finding goes in line with a report in nasopharyngeal carcinoma, where miR-141 positively correlates with proliferation, migration and invasion (Zhang et al.), but differs from the observed in gastric cancer cells (Du et al. 2009), reinforcing the idea of a cell-type specific response of miRNAs.

Finally, the effect of LIF on the miRNome of four trophoblastic cell lines was investigated. We identified three miRNAs dysregulated in all cell lines after four hours of LIF-treatment and therefore, which may contribute tor the LIF-response in trophoblast cells: miR-511, miR-550 and miR-885-5p. Among those, miR-511 has been more intensively studied because of its significantly lower expression in adenocarcinomas compared with normal tissues (Tombol et al. 2009) and its potential role as modulator of human immune responses (Tserel et al.). MiR-885-5p was also found down-regulated in primary neuroblastoma and seems to have a tumor suppressive role interfering with cell cycle progression and cell survival (Afanasyeva et al.). These associations allow us to hypothesize that these miRNAs may be involved in the trophoblast response to LIF stimulation. In future, research on their target genes may be of great importance to understand the LIF-mediated invasion and proliferation of trophoblast cells and thus, to generate novel therapeutical strategies.

Summarized, this thesis describes the molecular mechanisms involved in the LIF-response in trophoblastic cells. Starting with the intracellular processes occurring within the cytoplasm, when the cytokine receptors allow the activation of MAPK and JAK/STAT cascades, through the cross-talk between STAT3 and ERK1/2 and their association with proliferation and invasion, and finally, reporting for the fist time miRNAs specifically expressed by some trophoblastic cells and their implication in the proliferation of trophoblast cells.

3.4. Final Comments and future prospects

Working with trophoblast primary cells represents a challenge due to some problems including the relatively low yield of isolation and the small life expectative of these cells. Several models have been established with the aim to avoid these disadvantages allowing the study of intracellular regulatory mechanisms including proliferation, migration and invasion. However, in this thesis we could demonstrate that these models differ significantly in their behavior and responses on stimuli, such as LIF, as in our focus. Therefore, we recommend to use generally more than one cell line in order to distingiush molecular mechanisms which are cell-type dependent and which are not.

Among the cell models analyzed in this study JEG-3 and HTR-8 are the most different cell lines, as previously demonstrated in studies on their mRNA and protein expression. Our work describes an intracellular cascade shared by these cell lines, which includes activation of STAT3. Some additional works in our group have found further dissimilarities in the LIF-response between these cells lines including large differences in the expression of protein inhibitors of activated STAT3 (PIAS3), a negative regulator of the STAT3 cascade. Therefore, a deeper study of the expression of PIAS3, its possible control through miRNAs and the implications in the proliferation and invasion of trophoblast cells should be further carried on.

Furthermore, since trophoblast cells release miRNA into the maternal circulation (Frangsmyr et al. 2005), placenta-specific miRNA expression in serum changes during the course of pregnancy and thereby, reflects the physiological state (Pinzani et al. 2010, Gilad et al. 2008). This association revealed miRNA profiling in serum as a future tool for diagnosis of pathological conditions, including pre-eclampsia or intrauterine growth restriction (IGR). MiR-141 has been already reported to be higher in serum from pregnant women and we found that it is involved in the regulation of trophoblast proliferation and LIF-responses. It may be hypothesized that miR-141 may be useful as biomarker for pregnancy disorders associated with trophoblast dysfunction.

Likewise, this thesis reveals miR-511, miR-550 and miR-885 as possible mediators of LIF-responses in trophoblast cells and therefore, we propose to further investigate their functions and targets. In this study, a miRNA encyclopedia is provided, which contains key information about the expression and regulation of miRNAs in primary trophoblast

cells and different trophoblstic cell lines. This information may be useful for designing new strategies in order to establish the full functionality of miRNAs in pregnancy and their application as biomarkers or for new therapeutical strategies.

Chapter 4 | Summary

The present Ph.D. thesis is a cumulation of ten mostly published or accepted scientific papers on Leukemia Inhibitory Factor (LIF) and trophoblastic cells.

In contrast to what may be thought, human beings are not very fertile. About 70% of the fertilized eggs are lost within the first 12 weeks of pregnancy and the main reason seems to be dysregulation during the blastocyst implantation. In this process, the trophoblast cells of the outer layer of the blastocyst invade the decidua connecting maternal and fetal bloodstreams. In a "dialogue" between maternal and fetal cells, several molecules are released in order to control trophoblast proliferation and invasion. The group of secreted molecules includes hormones, enzymes, cytokines, chemokines and growth factors and their dysregulation can result in miscarries or pregnancy associated diseases like pre-eclampsia or choriocarcinoma. One of those cytokines is LIF.

LIF is a pleiotropic cytokine which belongs to the IL-6 family of cytokines. It is known for mediating cellular responses including proliferation and invasion and therefore, it plays a critical role in pregnancy establishment and maintenance. In this thesis, two review articles are included which summarize LIF production, and LIF-induced effects and molecular processes in trophoblastic cells. Likewise, the information about the potential clinical applications of LIF, its role in pregnancy and its association with pregnancy disorders was reviewed.

Despite the fact that LIF has been studied for several years, the molecular mechanisms controlling LIF-induced cell-responses have not been analyzed in detail. On the cell membrane, transmembranal receptors recognize LIF and activate several intracellular pathways. One part of this thesis was focused on the JAK/STAT and MAPK cascades, due to their implications in the control of trophoblast cell behavior. STAT3 is a molecule downstream LIF receptor (LIFR) that plays a pivotal role in the signaling of extracellular stimuli to the nuclei. STAT3 is activated by phosphorylation at its ser727 and tyr705 residues, which allows it to dimerize and cross from the cytoplasm to the nucleus. STAT3 tyr705 has been more extensively studied previously, while the relevance of ser727 was not yet known. Recent reports highlighted the importance of STAT3 ser727 in the cell response and new investigations are carried out to identify the

kinase responsible for this phosphorylation. ERK1/2, a molecule of the MAPK cascade, was predicted to be involved in the control of STAT3 ser727 phosphorylation and a cross-talk between these molecules was hypothesized. In this thesis, it was demonstrated that ERK1/2 plays an important role in the proliferation of trophoblast cells, which is not dependent on STAT3 activation. We have also defined a cross-talk between ERK1/2 and STAT3, which, conversely to the expected, does not occur in the cytoplasm, but in the nucleus: ERK1/2 is not responsible for the STAT3 ser727 phosphorylation, but it has a negative effect on the translocation of STAT3 into the nucleus, which results in a decrease of trophoblast invasiveness.

Recently discovered, microRNAs constitute a group of regulatory molecules that can control gene expression at post-transcriptional level. About 30% of the human genome is regulated by these molecules and their dysregulation is associated with cancer and malignancy. This thesis summarizes in a published review article the studies on miRNAs and placenta with special emphasis on those miRNAs specifically expressed by trophoblast cells. Additionally in this work, the miRNA expression profiles, also known as miRNome, of four different trophoblastic cell lines were analyzed and compared with that of isolated term trimester trophoblast cells. Some miRNAs were identified as potential markers responsible for the differentiation of trophoblast cells. Finally, the effect of LIF treatment on the miRNome of the same cell lines was investigated. Four miRNAs were found to be altered in all cell lines: miR-511, miR-550 and miR-885-5p (down-regulated), and miR-641 (up-regulated), suggesting an association between their expression and the LIF-induced cell response. The analysis of the putative targets suggested an association with the control of cell proliferation.

Altogether, this work analyzes intracellular signalling mechanisms involved in the regulation of LIF-responses in trophoblastic cells and highlights some novel miRNAs which may be responsible for the control of trophoblast proliferation and invasion and, therefore, may contribute to new strategies for future treatments and clinical approaches

Chapter 5 | Zusammenfassung

Die vorliegende Doktorarbeit ist eine Zusammenstellung („kumulative Arbeit") von zehn Manuskripten, die in den meisten Fällen bereits veröffentlicht oder angenommen sind, und das Thema „Leukemia Inhibitory Factor und trophoblastäre Zellen" bearbeiten.

Im Gegensatz zur allgemeinen Meinung, sind die Menschen nicht sehr fruchtbar. Ungefähr 70% der befruchteten Eier werden innerhalb der ersten 12 Wochen der Schwangerschaft verloren. Der Hauptgrund scheint die Fehlregulation während der Implantation der Blastozyste zu sein. In diesem Prozess invadieren die Trophoblastzellen von der äußeren Zellschicht der Blastozyste in die Dezidua und fügen den mütterlichen und fetalen Blutkreislauf zusammen. In einem "Dialog" zwischen mütterlichen und fetalen Zellen werden zahlreiche Faktoren freigesetzt, welche die Trophoblastenproliferation und -invasion kontrollieren. Die Gruppe der sezernierten Moleküle enthält Hormone, Enzyme, Zytokine, Chemokine und Wachstumsfaktoren, deren Fehlregulation im Verlauf der gesamten Schwangerschaft zu Erkrankungen wie Wachstumsretardierungen, Präeklampsie, vorzeitigen Wehentätigkeiten bis hin zu Aborten führen kann. Eines der entscheidenden Zytokine ist Leukemia Inhibitory Factor (LIF).

LIF ist ein pleiotropes Zytokin, das zur IL-6-Familie der Zytokine gehört. Es induziert zelluläre Antworten wie Proliferation und Invasion. Außerdem spielt es eine entscheidende Rolle zu Beginn und im Verlauf der Schwangerschaft. In diese Arbeit werden zwei Übersichtsartikel einbezogen, welche die LIF Produktion, die LIF-induzierten Effekte und die molekularen Prozesse in den Trophoblasten zusammenfassen. Auch Informationen in Bezug auf die mögliche klinische Anwendungen von LIF, seine Rolle in der Schwangerschaft und seine Verbindung zu Schwangerschaftsstörungen wurden zusammengestellt.

Trotz der Tatsache, dass LIF seit mehreren Jahren untersucht worden ist, wurden die molekularen Mechanismen, welche die LIF-induzierten Zell-Antworten kontrollieren noch nicht in allen Details analysiert. Transmembrane Rezeptoren auf der Zellmembran erkennen LIF und aktivieren daraufhin mehrere intrazelluläre Signalwege. Ein Teil

dieser Dissertation hat sich auf die JAK / STAT-und MAPK-Kaskaden konzentriert, welche aufgrund ihrer Auswirkungen auf die Kontrolle des Trophoblastverhaltens von Bedeutung sind. STAT3 ist ein „downstream“ Molekül des LIF-Rezeptors (LIFR), das eine zentrale Rolle in der Signalübertragung von extrazellulären Stimuli auf den Kern spielt. STAT3 wird durch Phosphorylierung an seinen Ser727- und Tyr705-Resten aktiviert, wodurch es die Fähigkeit erlangt Dimere zu bilden und in den Zellkern zu wandern. Die aktivierten Dimere regulieren die Expression spezifischer Zielgene.

Während STAT3 Tyr705 schon ausführlich untersucht wurde, ist über die Phosphorylierung und Funktion von Ser727 noch relativ wenig bekannt. Jüngste Berichte hoben die Bedeutung von STAT3 Ser727 in der Zellantwort hervor und neue Untersuchungen wurden durchgeführt, um die Kinase zu identizifieren, welche für diese Phosphorylierung verantwortlich ist. Es wurde erwartet, dass ERK1/2, ein Molekül der MAPK-Kaskade, an der Kontrolle der STAT3-Ser727-Phosphorylierung beteiligt sein sollte. Daher wurde die Möglichkeit eines „cross-talk“ zwischen diesen beiden Molekülen als Hypothese aufgestellt. In dieser Dissertation wurde gezeigt, dass ERK1/2 eine wichtige Rolle bei der Proliferation von Trophoblastzellen spielt, die nicht abhängig von STAT3-Aktivierung ist. Wir haben auch einen „cross-talk“ zwischen ERK 1/2 und STAT3 beschrieben, welcher nicht im Zytoplasma, sondern im Zellkern auftrat: ERK1/2 ist nicht für die STAT3-ser727-Phosphorylierung verantwortlich, hat aber einen negativen Effekt auf die Translokation von STAT3 innerhalb des Zellkerns, was eine Verminderung der Trophoblast-Invasivität zur Folge hat.

Vor einigen Jahren wurde entdeckt, dass microRNAs eine Gruppe von regulatorischen Molekülen darstellen, welche Genexpressionen auf post-transkriptioneller Ebene steuern können. Über 30% des menschlichen Genoms wird durch diese Moleküle reguliert und deren Fehlregulation sind unter anderem mit malignen Erkrankungen verbunden. Diese Dissertation fasst in einer veröffentlichten Übersichtsarbeit die Studien über miRNAs und Plazenta zusammen, mit besonderer Betonung auf die speziell durch Trophoblastzellen exprimierten miRNAs. Zudem wurden in dieser Dissertation die miRNA Expressions-Profile, auch als miRNom bekannt, in vier verschiedenen trophoblastären Zelllinien analysiert und mit denen von isolierten primären Trophoblastzellen des dritten Trimenon verglichen. Einige miRNAs wurden als potenzielle Marker für die Differenzierung von Trophoblastzellen identifiziert. Schließlich wurden die Effekte von LIF auf die miRNA-Profile der selben Zelllinien

untersucht. Dabei fanden wir vier miRNAs, welche in allen Zelllinien signifikant verändert wurden: miR-511, miR-550 und miR-885-5p (herunterreguliert) und miR-641 (hochreguliert), was auf ihre besondere Bedeutung hindeutet. Die Datenbank-Analyse der möglichen Zielgene legt einen Zusammenhang mit der Regulation der Zellteilung nahe.

Zusammengefasst wurden in dieser Arbeit intrazelluläre Signalmechanismen untersucht, die an der Regulation der LIF-Reaktionen in Trophoblasten beteiligt sind. Außerdem wurden miRNAs identifiziert, die zur Regulation von Trophoblastzellproliferation beitragen. Diese miRNAs bieten daher das Potenzial zur Entwicklung neuer Strategien für die Erkennung oder Behandlung von Schwangerschaftsstörungen.

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Ziyan W, Shuhua Y, Xiufang W, Xiaoyun L. MicroRNA-21 is involved in osteosarcoma cell invasion and migration. Med Oncol.

Personal Information:

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E-mail	diana.morales@med.uni-jena.de
Date of Birth	September 16th, 1983
Place of Birth	Bogotá
Nationality	Colombian Resident in Germany
Martial Status	Single

EDUCATION

04.2007- 03.2012	Friedrich - Schiller University Jena, Germany. Faculty of Biology and Pharmacy. PhD thesis: "Molecular mechanisms in trophoblastic cells after LIF-stimulation with special regard to microRNAs. MicroRNAs in trophoblast cells". Magna Cum Laude Place of work: University Hospital Jena, Department of Obstetrics, Placenta-Labor, under supervision of Prof. Udo Markert
02.2005-12.2005	Universidad Nacional de Colombia, Bogotá, Colombia Department of Chemistry. Laboratory of Hormones. Diploma Thesis: Determination of the expression of Insulin-like Growth Factor IGF-II type-2 Receptor (IGF-IIR) in

	Gestational Trophoblastic Disease (GTD). Final mark: 5.0 highest possible 5.0
01.2000-12.2005	Universidad Nacional de Colombia. Bogotá, Colombia. Department of Chemistry. Obtained degree: Chemist Final Examination: Final mark: 118.27 Highest possible 128.7 Final Average: 3.8 Highest possible: 5.0
01.1989 -11.1999	Colegio de la presentación Sans Façon. Bogotá, Colombia. High School and Elementary School. Final Test ICFES: 361/400

LANGUAGES

Spanish: First language
English: Advance
German: Advance

AWARDS

May 2011	NIH Travel Award. 31st Annual Meeting of the American Society for Reproductive Immunology. Salt Lake City, Utah, USA
August 2010	Travel Award and Nomination for "New Investigator Award". International Society for Immunology of Reproduction. XI International Congress of Reproductive Immunology. Cairns, Australia
May 2010	NIH Travel Award. 30th Annual Meeting. American Society for Reproductive Immunology. Pittsburgh
June 2009	NIH Travel Award. 29th Annual Meeting. American Society for Reproductive Immunology. Orlando, USA.
February 2009	Best Oral Presentation. International Congress on Bio-Immunoregulatory Mechanisms associated with Reproductive Organs: Relevance in Fertility and in sexually transmitted infections. National Institute of Immunology, New Dehli, India.

SCHOLARSHIPS

September 2011	DAAD Travel grant for attending the IFPA-14th European Placenta Group meeting. Geilo, Norway
April 2007- April 2011	PhD Scholarship. Graduate Academy at the Friedrich Schiller University. Jena, Germany
May 2011	"Pro-Chance" grant 2011. Friedrich Schiller University. Jena, Germany. Travel allowance for attending the 31st Annual Meeting of the American Society of Reproductive Immunology. Salt Lake City, USA

November 2010	Merck Serono. Sponsorship for attending the "10. Arbeitskreis Molekularbiologie der Deutschen Gesellschaft für Gynäkologische Endokrinologie und Fortpflanzungsmedizin DGGEF". Düsseldorf, Germany.
August 2010	Travel Allowance of The International Society for Immunology of Reproduction to attend the XI ICRI 2010 in Palm Cove, Australia
May 2010	"Pro-Chance" grant 2010. Friedrich Schiller University. Jena, Germany. Travel allowance for attending the 30th Annual Meeting of the American Society of Reproductive Immunology. Pittsburgh, USA.
June 2009	"Pro-Chance" grant 2009. Friedrich Schiller University. Jena, Germany. Travel allowance for attending the 29th Annual Meeting. American Society for Reproductive Immunology. Orlando, USA.
February 2009	National Institute of Immunology. New Dehli, India. German Academic Exchange Program. DAAD, Jena, Germany. Internship
April 2008	Institute "Humanitas". Immunology Department. Milan-Italia. EMBIC, European Network of Excellence. Internship
September 2008	"Pro-Chance" grant 2008. Friedrich Schiller University. Jena, Germany. Travel allowance for attending the IFPA meeting 2008- 12th EPG Conference. Seggau Castle, Austria.
May 2008	Deutsche Forschungsgemeinschaft. DFG. Travel allowance for an invited lecture, Medellín, Colombia.
November 2007- March 2008	PhD Scholarship. German Academic Exchange Program. DAAD, Jena, Germany
July 2006	EMBIC. European Network of Excellence. Travel allowance for the 2nd EMBIC Summer School, Pecs, Hungary
June –September 2006	Boehringer Ingelheim Fonds. Travel allowance, Jena, Germany

INVITED LECTURES

May 2008	Visit to the "Reproduction Group" at the University of Antioquia. Lecture entitled: "Ras in trophoblastic cells and the possible regulative role of microRNAs". Medellin, Colombia

INTERNSHIP

February 2009	National Institute of Immunology, New Dehli, India. PCR techniques training in the scope of the DFG cooperation program.
March –April 2008	Institute "Humanitas". Immunology Department. Milan-Italia. Micro-RNA techniques training.
September 2007	First Embic summer training. Friedrich Schiller Universität. Placentalabor. Jena- Germany
June –September 2006	Friedrich Schiller Universität. Placentalabor. Jena, Germany
September 2005	Universidad Nacional de Colombia. Molecular Biology Training. AEXMUN. Bogotá, Colombia

CONGRESSES AND MEETINGS

September 2011	IFPA-14th European Placenta Group meeting. Geilo, Norway. Poster presentation
August 2011	ESRI/ESHRE Early Pregnancy Congress. Copenhagen, Denmark. Oral presentation and Award finalist.
May 2011	31st Annual Meeting of the American Society of Reproductive Immunology. Salt Lake City, USA. Poster presentation
November 2010	2nd Jena InTReST-DGRM. International Training in Reproductive Sciences and Technologies. Jena, Germany. Organization Committee
November 2010	8th European Congress on Reproductive Immunology ESRI. Munich, Germany. Poster Presentation
November 2010	10. Arbeitskreis Molekularbiologie der Deutschen Gesellschaft für Gynäkologische Endokrinologie und Fortpflanzungsmedizin DGGEF. Düsseldorf, Germany. Oral Presentation
September 2010	Treffen der Arbeitskreises Reproduktionsimmunologie. Leipzig, Germany. Assistant
August 2010	XI International Congress of Reproductive Immunology ICRI. Cairns, Australia. Oral presentation and Award finalist.
May 2010	30th Annual Meeting of the American Society of Reproductive Immunology. Pittsburgh, USA. Poster presentation

August 2009	9. Treffen des Arbeitskreises Molekularbiologie der Deutschen Gesellschaft für Gynekologische, Endokrinologie und Fortpflanzungsmedizin (DGGEF). Düsseldorf, Germany. Oral presentation.
June 2009	29th Annual Meeting. American Society for Reproductive Immunology. Orlando, USA. Poster Presentation.
February 2009	International Congress on Bio-immunoregulatory Mechanisms associated with Reproductive Organs: Relevance in Fertility and in sexually transmitted infections. National Institute of Immunology, New Dehli, India. Oral Presentation.
September 2008	IFPA meeting 2008-12th EPG Conference. Seggau Castle, Austria. Poster presentation
June 2008	4th EMBIC Summer School. Barcelona, Spain. Poster presentation
September 2007	3rd EMBIC Summer School. Jena. Germany. Poster presentation
September 2007	5th European Congress of Reproductive Immunology. Berlin. Germany. Poster Presentation
July 2006	4th European Congress of Reproductive Immunology. Graz. Austria. Assistant
July 2006	2nd EMBIC Summer School. Pecs. Hungary. Oral Exposition, poster presentation
November 2005	II Latin – American Symposium of Materno-Fetal Interaction and Placenta. Santiago de Chile. Chile. Poster Presentation
October 2005	XL Congreso Nacional de Ciencias Biológicas. Cali. Colombia. Oral Presentation
September 2005	II Simposio de Química Aplicada. VII Congreso de Estudiantes de Química. Armenia. Colombia. Oral Presentation
October 2004	X Encuentro Nacional de Estudiantes de Química. Bogotá. Colombia. Organizing Committee. Congress Chair.

PROFESSIONAL EXPERIENCE

February – March 2007	Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ). Agencia de la GTZ en Bogotá. Logistic Assistant

May 2005 – March 2007	Colombian Chemistry Asociation. Asociación Química Colombiana ASQUIMCO. Substitute Fiscal. Duties: Organization of the Chemical annual meeting, project management
October 2003 – October 2005	Universidad Nacional de Colombia. Asociación de Estudiantes de Química UNESQUI. President Duties: Manage activities within the association. Organization of the meeting "X Encuentro Nacional de Estudiantes de química".
Januar 2004 – October 2005	Chymeia Magazine ISSN: 0121-6074 Revista de la Asociación de Estudiantes de Química de la Universidad Nacional de Colombia UNesqui Director Duties: Magazine edition and management.

Chapter 8| List of Publications

8.1. Scientific papers

Published

- **Morales Prieto DM**, Markert UR. MicroRNAs in pregnancy. Journal of Reproductive Immunology. J Reprod Immunol. 2011 Mar;88(2):106-11

- **Morales-Prieto DM**, Schleussner E, Markert UR. Reduction of miR-141 is induced by Leukemia Inhibitory Factor and inhibits proliferation in choriocarcinoma cell line JEG-3. Am J Reprod Immunol. 2011 Jul;66 Suppl 1:57-62.

- Markert UR, **Morales-Prieto DM**, Fitzgerald JS. Understanding the link between the Interleukin-6 cytokine family and pregnancy: implications for future therapeutics. Expert Rev Clin Immunol. 2011 Sep;7(5):603-9

- Golla JP, Suman P, **Morales Prieto DM**, Markert UR, Gupta SK. Leukemia Inhibitory Factor mediated proliferation of HTR8/SVneo trophoblastic cells is dependent on Extracellular Regulated Kinase 1/2 activation. Reprod Fert Develop 23(5) 714-724

- Fitzgerald JS, Abad C, Alvarez AM, Bhai Mehta R, Chaiwangyen W, Dubinsky V, Gueuvoghlanian B, Gutierrez G, Hofmann S, Hölters S, Joukadar J, Junovich G, Kuhn C, **Morales-Prieto DM**, Nevers T, Ospina-Prieto S, Pastuschek J, Pereira de Sousa FL, San Martin S, Suman P, Weber M, Markert UR. Cytokines regulating trophoblast invasion. Advances in Neuroimmune biology (NIB).2012 Jan;2(1):61-97.

- Suman P, Godbole G, Thakur R, **Morales Prieto DM,** Modi D, Markert UR, Gupta SK. IL-11 Reduces the Invasion of Trophoblastic HTR-8/SVneo Cells through Decrease in the Expression of Matrix Metalloproteinases and Mucin-1. PLoS ONE 2012; 7(1): e29745.

Submitted

- **Morales-Prieto DM**, Ospina-Prieto S, Weber M, Hoelters S, Schleussner E, Markert UR. Intranuclear, but not intracytoplasmic crosstalk between Extracellular Regulated Kinase1/2 and Signal Transducer and Activator of Transcription3 in JEG-3 choriocarcinoma cells. Journal of cellular biochemistry (Under revisions September 2011 ID JCB-11-0464)

- **Morales-Prieto DM,** Chaiwangyen W, Gruhn B, Markert UR. MicroRNA expression profiles in trophoblastic cells. Placenta (Submitted September 2011)

- Fitzgerald JS, **Morales Prieto DM**, Suman P, San Martin S, Poehlmann T, Gupta SK, Markert UR. It's a woman thing: Part II - The placenta under the influence of tobacco. Hum. Reprod.update (Preparation).

8.2. Thesis

- **Morales Prieto DM**. Molecular mechanisms in trophoblastic cells after LIF-stimulation with special regard to microRNAs. 2012. Friedrich Schiller Universität. Jena, Germany.

- **Morales DM.** Determination of the expression of Insulin-like Growth Factor IGF-II type-2 Receptor (IGF-IIR) in Gestational Trophoblastic Disease (GTD). 2005. Universidad Nacional de Colombia.

8.3. Published Abstracts

- Pereira de Sousa FL, **Morales Prieto DM**, Ospina Prieto S, Chaiwangyen W, Sass N, Daher S, Markert UR. Effects of STAT1 suppression on ERK1/2 in trophoblastic cells. Placenta 32 (2011) A1-A149. Poster presentation.

- Weber M, Weise A, Mrasek K, Párraga San Roman M, Khachaturyan L, **Morales DM**, Liehr T, Markert UR, Fitzgerald JS. Cytogenetic and STAT3 expression analysis of HTR8/SVNEO. Placenta 32 (2011) A1-A149. Poster presentation.

- Knöfler I, Röhler C, Hölters S, Fitzgerald JS, **Morales Prieto DM**, Schleussner E, Markert UR. Trophoblast migration is activated via chemokine receptor 1 and 3. Placenta 32 (2011) A1-A149. Poster presentation.

- **Morales Prieto DM**, Weber M, Ospina Prieto S, Fitzgerald JS, Schleussner E, Gruhn B, Markert UR. MicroRNA expression profiles in trophoblastic cells. Placenta 32 (2011) A1-A149. Oral presentation.

- Chaiwangyen W, Pereira de Sousa FL, **Morales Prieto DM**, Ospina Prieto S, Markert UR. Comparison of Leukemia Inhibitory Factor-Induced intracellular signalling in different trophoblastic cell lines. Placenta 32 (2011) A1-A149. Poster presentation.

- **Morales DM,** Weber M, Ospina S, Fitzgerald JS, Schleussner E, Gruhn B, Markert UR. MicroRNA expression profiles in trophoblastic cells. J. Reprod Immunol 90 (2011) 164-183. Oral presentation. Award finalist.

- Chaiwangyen W, Pereira de Sousa FL, **Morales-Prieto DM**, Ospina S, Markert UR. Comparison of leukemia inhibitory factor-induced intracellular signaling in different trophoblastic cell lines. J. Reprod Immunol 90 (2011) 164-183. Poster presentation.

- Knöfler I, Röhler C, Hölters S, Fitzgerald JS, **Morales-Prieto DM**, Wartenberg M, Schleussner E, Markert UR. Trophoblast migration is activated via chemokine receptors 1 and 3. J. Reprod Immunol 90 (2011) 164-183. Poster presentation.

- Pereira de Sousa FL, **Morales Prieto DM**, Ospina S, Chaiwangyen W, Daher S, Sass N, Markert UR. Effects of STAT1 suppression on ERK1/2 in trophoblastic cells. J. Reprod Immunol 90 (2011) 164-183. Poster presentation.

- Ospina S, **Morales DM**, Markert UR. EGF induces proliferation of trophoblastic cells trough STAT5 activation. J. Reprod Immunol 90 (2011) 164-183. Poster presentation.

- Pereira de Sousa FL, **Morales Prieto DM**, Ospina S, Chaiwangyen W, Markert UR. Cytokine induced crosstalk between STAT1 and ERK1/2. 31st Annual Meeting of the American Society of Reproductive Immunology, May 2011, Salt Lake City, USA. Am J Reprod Immunol 2011; 65(Suppl 1):9. Poster Presentation

- Chaiwangyen W, **Morales Prieto DM**, Ospina S, Pereira do Sousa FL, Markert UD. Characterization of cellular signalling pathways involved in the regulation of trophoblast cell functions. 31st Annual Meeting of the American Society of Reproductive Immunology, May 2011, Salt Lake City, USA. Am J Reprod Immunol 2011; 65(Suppl 1):14. Poster Presentation

- Weber M, Weise A, Mrasek K, Khachaturyan L, **Morales Prieto DM**, Liehr T, Markert UR, Fitzgerald JS. Cytogenetic and STAT3 expression analysis of HTR8/SVneo. 31st Annual Meeting of the American Society of Reproductive Immunology, May 2011, Salt Lake City, USA. Am J Reprod Immunol 2011; 65(Suppl 1):15. Poster Presentation

- **Morales Prieto DM**, Weber W, Ospina S, Fitzgerald JS, Markert UR. MicroRNA expression profiles in trophoblastic cells. 31st Annual Meeting of the American Society of Reproductive Immunology, May 2011, Salt Lake City, USA. Am J Reprod Immunol 2011; 65(Suppl 1):18. Poster Presentation

- Ospina S, Pereira de Sousa FL, **Morales Prieto DM**, Markert UR. EGF induces proliferation of trophoblastic cells through STAT5 activation. 31st Annual Meeting of the American Society of Reproductive Immunology, May 2011, Salt Lake City, USA. Am J Reprod Immunol 2011; 65(Suppl 1):19. Poster Presentation

- Knöfler I, Röhler C, Hölters S, Fitzgerald JS, **Morales Prieto DM**, Wartenberg M, Schleussner E, Markert UR. Chemokine Receptor 1 and 3 fundamental for trophoblast migration. 31st Annual Meeting of the American Society of Reproductive Immunology, May 2011, Salt Lake City, USA. Am J Reprod Immunol 2011; 65(Suppl 1):28. Poster Presentation

- **Morales DM**, Ospina S, Markert UR. Micro-RNA profiles and functions in response to LIF in trophoblastic cells. J Reprod Immunol 2010; 86:79-111

- Ospina S, **Morales DM**, Markert UR. Induction of signal transducer and activator of transcription 5 (STAT5) signaling in trophoblastic cells by epidermal growth factor (EGF). J Reprod Immunol 2010; 86:79-111

- **Morales DM**, Markert UR. ERK1/2 Aktivierung ist an der LIF-induzierten STAT3 ser727 Phosphorylierung in Trophoblast-Zellen nicht beteiligt. 10. Arbeitskreis Molekularbiologie der DGGEF, Düsseldorf, Germany. J Reproduktionsmed Endokrinol 2011;8:35. Oral presentation

- Khachaturyan L, Poehlmann TG, Weber M, Forti ALL, **Morales DM**, Fitzgerald JS, Schleussner E, Markert UR. Protein inhibitors of activated STATs (PIAS) control major trophoblastic functions. 9. Arbeitskreis Molekularbiologie der

DGGEF, Düsseldorf, Germany. J Reproduktionsmed Endokrinol 2010;7:120-121. Oral presentation

- **Morales DM**, Ospina S, Markert UR. Micro-RNA-Profiles in Response to LIF induction in Trophoblastic cells. International Federation of Placenta Associations Meeting. October 2010, Santiago de Chile. Placenta 2010; 31: A126. Poster Presentation

- Ospina S, **Morales DM**, Markert UR. Signal Tranducer and Activator of Transcription 5 (STAT5) Signaling in Trophoblastic cells is Induced by Epidermal Growth Factor (EGF). International Federation of Placenta Associations Meeting. October 2010, Santiago de Chile. Placenta 2010; 31: A134. Poster Presentation

- Markert UR, **Morales DM**, Ospina S. JAK/STAT signalling in trophoblast differentiation. XI International Congress of Reproductive Immunology, August 2010, Cairns, Australia. J Reprod Immunol 2010; 86:18. Invited lecture.

- **Morales DM**, Ospina S, Markert UR,. Micro-RNA-profiles in response to LIF in trophoblast cells. XI International Congress of Reproductive Immunology, August 2010, Cairns, Australia. J Reprod Immunol 2010; 86:32.

- Ospina S, **Morales DM**, Markert UR. STAT5 signaling in trophoblastic cells is induced by Epidermal Growth Factor. XI International Congress of Reproductive Immunology, August 2010, Cairns, Australia. J Reprod Immunol 2010; 86:62. Poster Presentation

- Ospina S, **Morales DM**, Markert UR. Epidermal Growth Factor (EGF) induces p-STAT5 signaling in trophoblastic cells. 30th Annual Meeting of the American Society of Reproductive Immunology, May 2010, Farmington, USA. Am J Reprod Immunol 2010; 63(Suppl 1):36. Poster Presentation

- **Morales DM**, Ospina S, Markert UR. Micro-RNA-response to LIF induction in trophoblastic cells. 30th Annual Meeting of the American Society of Reproductive Immunology, May 2010, Farmington, USA. Am J Reprod Immunol 2010; 63(Suppl 1):35. Poster Presentation

- Markert UR, **Morales DM**, Fitzgerald JS, Weber, Ospina S. Regulation of trophoblast invasion: from signalling molecules to micro-RNAs. 30th Annual Meeting of the American Society of Reproductive Immunology, May 2010, Farmington, USA. Am J Reprod Immunol 2010; 63(Suppl 1):16. Oral presentation

- Markert UR, Weber M, Khachaturyan L, **Morales DM**, Poehlmann TG, Fitzgerald JS. Trophoblast invasion: the role of intracellular cytokine signalling via signal transducer and activator of transcription 3 (STAT3) and its potential negative intracellular regulators. 2nd Symposium on Reproductive Immunology, October 2009, Rio de Janeiro, Brazil. Am J Reprod Immunol 2009; 62:219. Poster Presentation

- **Morales DM**, Markert UR. Inhibition of ERK1/2 does not affect LIF-induced STAT3 ser727 phosphorylation in trophoblastic cells. 7th European Congress on Reproductive Immunology, September 2009, Marathon, Greece. J Reprod Immunol 2009; 81:174-75. Poster presentation. Poster Presentation

- Khachaturyan L, Poehlmann TG, Weber M, Forti ALL, **Morales DM**, Fitzgerald JS, Schleussner E, Markert UR. The pivotal role of protein inhibitors of activated STATs (PIAS) in regulating trophoblastic functions. 7th European Congress on Reproductive Immunology, September 2009, Marathon, Greece. J Reprod Immunol 2009; 81:174. Poster presentation.

- **Morales DM**, Markert UR. STAT3 ser727 phosphorylation in trophoblastic cells is induced by LIF, but not via ERK1/2 activation. 29th Annual Meeting of the American Society of Reproductive Immunology, June 2009, Orlando, USA. Am J Reprod Immunol 2009; 61:416. Poster presentation.

- Khachaturyan L, Poehlmann TG, Weber M, Forti ALL, **Morales DM**, Fitzgerald JS, Schleussner E, Markert UR. Protein inhibitors of activated STATs (PIAS) control major trophoblastic functions. 29th Annual Meeting of the American Society of Reproductive Immunology, June 2009, Orlando, USA. Am J Reprod Immunol 2009; 61:417. Poster presentation.

- **Morales DM**, Poehlmann TG, Forti ALL, Schleussner E, Rubio I, Markert UR. Ras-activation by IL-6 in trophoblastic cells. 14th Annual Congress of the International Federation of Placenta Associations, September 2008, Seggau, Austria. Placenta 2008; 29:A89. Poster presentation.

- Forti ALL, Poehlmann TG, **Morales DM**, Schleussner E, Markert UR. IL-6 and LIF activated intracellular signalling pathways in trophoblastic cells. 14th Annual Congress of the International Federation of Placenta Associations, September 2008, Seggau, Austria. Placenta 2008; 29:A22. Poster presentation

- Khachaturyan L, Poehlmann TG, **Morales DM**, Forti ALL, Ermisch C, Weber M, Trück M, de la Motte T, Fitzgerald JS, Schleussner E, Markert UR Das System von Janus Kinasen (JAKs), Signal Transducers and Activators of Transcription (STATs) und deren Inhibitoren reguliert die Invasivität von trophoblastären Zellen. 8. Arbeitskreis Molekularbiologie der DGGEF, Essen, Germany. J Reproduktionsmed Endokrinol 2008;5:368. Poster Presentation

- Forti ALL, Poehlmann TG, **Morales DM**, Schleussner E, Markert UR. Activated intracellular signaling pathways by IL-6 and LIF in trophoblastic cells. 28th Annual Meeting of the American Society of Reproductive Immunology, June 2008, Chicago, USA. Am J Reprod Immunol 2008; 59:492. Poster presentation

- **Morales DM**, Poehlmann TG, Forti ALL, Rubio I, Markert UR. IL-6-mediated Ras-activation in Trophoblastic cells. 28th Annual Meeting of the American Society of Reproductive Immunology, June 2008, Chicago, USA. Am J Reprod Immunol 2008; 59:490. Poster presentation.

- Khachaturian L, **Morales Prieto DM**, Bozic M, Poehlmann TG, Schleussner E, Markert UR. Protein Inhibitors of Activated STATs (PIAS) in Trophoblast Cells. 5th European Congress of Reproductive Immunology, August 2007; Berlin, Germany. Am J Reprod Immunol 2007; 58: 231. Poster presentation

- Poehlmann TG, Bachmann S, Rudloff I, Neblung C, **Morales Prieto DM**, Schleussner E, N Rodde, Sandra O, Markert UR. Invasive or not? The STAT3 - SOCS3 balance regulates trophoblast and choriocarcinoma behavior. 10th

International Congress of Reproductive Immunology, June 2007, Opatija, Croatia. Am J Reprod Immunol 2007; 57:459. Oral presentation

8.4. Other conference publications

- **Morales DM**, Markert UR LIF-mediated ERK1/2 activation is not related with STAT3 Ser727 phosphorylation. Bio-immunoregulatory Mechanisms Associated with Reproductive Organs: Relevance in Fertility and in Sexually Transmitted Infections, February 2009, New Delhi, India

- **Morales DM**, Poehlmann TG, Forti ALL, Schleussner E, Markert UR. Functional analysis of IL-6 induced Ras Activation in Trophoblastic Cells. 4th Embic Summer School, June 2008, Barcelona, Spain.

- Khachaturian L, **Morales Prieto DM**, Bozic M, Poehlmann TG, Schleussner E, Markert U. Protein Inhibitors of Activated STATs (PIAS) in Trophoblast Cells. 3rd EMBIC Summer School. 2007.

- **DM Morales**, M.S. Carrasco-Rodriguez, M. Sanchez – Gomez. Insulin-like growth factor –II and IGF-IIR mRNA expression in woman with hydatidiform mole and spontaneous abortions. 2nd EMBIC Summer School Memories. 2006. P1.

- Pinzon ML, **Morales DM**, Ortiz BL, Sánchez-Gómez M. Increased IGF-II expression and MMP-2 activity in hydatidiform mole in comparison with first trimestre pregnancy. Placenta 2006; 27: A55

8.5. Additional publications

- **Morales DM.** ¿Culpable o inocente? La aplicación de la química en las ciencias Forenses". *Chymeia*. Revista de la Asociación de Estudiantes de Química de la Universidad Nacional de Colombia UNesqui. 2005; 5: 14-17.

Acknowledments

I would like to acknowledge and extend my heartfelt gratitude to the following persons who have made the completion of this thesis possible:

First and foremost, I would like to thank my family for their vital encouragement and support. My mother for been my inspiration during all these years and for being the best mother of the world. To my father, who taught me how to go through difficult situations and continue laughing. To my brother, who always makes me laugh with all his suggestions and comments. To my dear sister, who is my best friend and a scential part of my life. To Stephanie and Ingrid for their help and support during this time. To all my aunts for their love and encouragement.

I would also like to express my sincere gratitute to my advisor Prof. Dr. Udo Markert for the continuos support of my PhD, but also for making possible to work in a nice place with an amazing atmosphere. To the placenta-team, all people currently working and those who have been there during this period. I am sorry I can not mention them all in name but I really appreciated working with all of you. Thanks specially to Justine, Maja, Sebastian, and Wittaya for their friendship and for their help in the realization of these manuscripts.

Thank to the group of Dr. Massimo Locatti in the "Istituto Humanitas" in Milan. This was a very enjoyable experience and I am grateful for the team-group for their help with the experiments but also for the great experiences after work.

To my friends in Germany and in Colombia for being always by my side and for all the good memories. Thanks specially to Jonathan, Julian, Caro, Jorge, Ricardo and Pili for all the laughts and support. To Micha, Agustina and Angela for cheered me up and shared this experience.

Last but not least, I gratefully acknowledge the funding sources that made my Ph.D. work possible. I was funden by the Graduate Academy at the Friedrich Schiller University for the 3 last years of my PhD. My work was also funded by the German Academic Exchange Service (DAAD) and the Boehringer Ingelheim Fonds (Bifonds).

- Die vorliegende Arbeit wurde im Labor von Prof. *Dr. med.* Udo R. Markert im Klinikum der Friedrich-Schiller-Universitat Jena, Abteilung fur Gynakologie und Geburtshilfe angefertigt.

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Hiermit erklare ich, dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät bekannt ist und ich die vorliegende Dissertation selbst verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe und, dass alle Stellen, die dem Wortlaut oder Sinn nach, anderen Werken entnommen sind, durch Angaben deren Quellen kenntlich gemacht wurden. Folgende Personen haben mich bei der Auswertung von Ergebnissen und der Erstellung des Manuskriptes unterstutzt: Prof. Dr. med. Udo R. Markert, Dipl. biol. Stephanie Ospina, MsC. Wittaya Chaiwangyen, Dipl. Ing. Sebastian Hölters, Dipl. biol. Maja Weber. Die Hilfe eines Promotionsberaters wurde nicht Anspruch genommen. Dritte haben keine geldwerte Leistungen im Zusammenhang mit der vorgelegten Arbeit erhalten. Weiterhin wurde die vorliegende Dissertation oder Teile daraus keiner weiteren Institution/Universitat als Prufungsarbeit vorgelegt.

Jena, 13.04.2012 Diplom-Chemikerin. Diana Maria Morales Prieto

Advances in Neuroimmune Biology 2 (2011) 61–97
DOI 10.3233/NIB-2011-023
IOS Press

Cytokines Regulating Trophoblast Invasion

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Abstract. Pregnancy is personally special to every woman expecting a child, but is also interesting from the perspective of an immunologist. During a physiological pregnancy, the mother's immune system decides to tolerate and foster an incorporated, non-self, non-dangerous organism. Whether the maternal reaction stems from deciphering the foreigness or safeness of this new individual, it is the general consensus that there is a foeto-maternal, bidirectional "dialogue" occurring and that the "messages" that are "spoken" are relayed through signaling mediators, which are capable of transmitting a functional command to a target cell. Much information dedicated to this theme has been gleaned in the past decade; however, the complex nature of cytokine networks jeopardizes clarity.

In this review, we touch upon a list of mediators that are vital for reproduction. These factors are divided according to their receptor family, because this elucidates the characteristic signal transducing pathway, which is expected to mediate their signal within the target cell. The target cells of interest are the trophoblast, upon which we focus for several reasons: 1. the trophoblast represent the foetal compartment while participating in foeto-maternal interplay (e.g. while invading the decidua, trophoblasts are in constant communication with uterine, maternal immunocytes, which check and contain this function), 2. trophoblasts are responsible for foetal well-being (e.g. nutrition, protection from the environment) and 3. dysfunctional trophoblast function results in several pregnancy complications (e.g. preeclampsia, intrauterine growth retardation, miscarriage, preterm delivery).

We summarize what is described in the literature on how these mediators are distributed within the reproductive tract, which cells are expressing their respective receptors (especially which trophoblast subsets) and how they modify trophoblast function

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ISSN 1878-948X/11/$27.50

(namely invasion, proliferation, differentiation and apoptosis). Furthermore, we unearth for which mediator the signal transducing pathway is verifiably used in trophoblast (ic) cells. Finally, we correlate actual biological importance of the mediator for reproduction by comparing murine knockout phenotypes and known positive and negative associations of these mediators with human pregnancy pathologies (as listed above). We expect this concise review to be useful to both basic researchers and clinicians who wish to obtain an overview of the reproductive cytokine network in respect to the trophoblast.

Keywords: Placenta, pregnancy, trophoblast, cytokines, cytokine receptors, chemokine receptors, immunoregulation

INTRODUCTION

The immunological situation found during pregnancy is of special importance. Most often, pregnancy, although generally accepted as a miraculous milestone in the lifeline of a woman, is not perceived as a critical condition in which the mother is incorporating a complex organism of foreign origin. Indeed, normal and physiological pregnancies are usually concluded in an uncomplicated fashion without the maternal organism reacting in any adverse way toward the foetus or placenta inside the gravid uterus, so that the accomplishments of this non-reaction are quite ignored [1].

During pregnancy, it is of utmost consequence that the so-called foreign object, the foetus, is not recognized as such, but instead accepted as a "friend". There are two most prominent theories committed to explaining this physiology. One maintains that an active *induction* of tolerance of the foetal allograft is initiated through *bidirectional* dialogue between the foetus (or placenta) and the mother during physiological pregnancies [2]. Another, newer hypothesis, which proposes that the immune system is more concerned with damage than with foreignness, describes that without a so-called "danger signal" stemming either directly from the pregnancy or from a precarious setting during pregnancy, the foetus will not be recognized as anything that requires an aggressive immunological response [3, 4]. The discussion between both of these fields is quite controversial and does not promise to be resolved completely in the near future (reviewed in [5, 6]). However, both sides realize that a major contributor to any immunological reaction that might be seen during pregnancy would be identified, amongst others, per cytokines [5, 7].

Cytokines, being the main mode of communication between immunological cells and their targets, would be instigators of tolerance, rejection or any other immunological reaction toward a pregnancy. When these signals are intercepted, blocked or amplified, dire consequences can be expected. During pregnancy, communication between the foetus (placenta) and the mother (decidua) is an intricate network intercalated with that of the hormonal network. Cytokines are produced by immunocytes that are in dialogue with their environment, and these immunocytes are in turn responsive to other cytokines. Many of these cytokines are produced in a spaciotemporal fashion, indicating that they are in cinque with maternal pregnancy homeostasis, and are responsible for the fine tuning of specific functions within the placenta.

In this review, we focus on a spectrum of cytokines which are known to be important for reproduction. We are mainly interested in their effects on the trophoblasts, a main subset of cells that constitute the placenta and which are of foetal origin. Maternal, uterine immunocytes come into direct contact with trophoblast cells, thus initiating a bi-directional transfer of information.

In short, there are three main trophoblast subsets in the placenta: the villous cytotrophoblast (CTB), the syncytiotrophoblast (STB) and an intermediate trophoblast subset that is also termed extravillous trophoblast (EVT). The first two subsets are found coating the villous tree of the placenta. The STB layer is found on the outside coating, and comes into direct contact with maternal blood of the intervillous space (and with maternal immunocompetent cells), and thus is also responsible for such jobs as transportation of nutrition and oxygen to the foetus. It is also important in metabolic changes including detoxification and protection from microbes. The layer just underneath the STB consists of CTB, which are often considered a sort of trophoblast stem cell that replenishes the STB layer when areas of the villous tree thin out and are exhausted [8, 9]. STB consist namely of fused CTBs, which after fusion, have become aproliferative, meaning that the only manner in which the STB layer can grow, is through a constant replenishment from the below CTB layer [9]. The CTB however have several functions: they either differentiate to replenish STB or they differentiate along the invasive pathway. This situation occurs in areas where the floating villi are attached to the basement membrane. Upon doing so, some CTB differentiate first to anchor the villi to the basement membrane, but some further differentiate into an

invasive trophoblast phenotype, coined EVT due to its location or intermediate trophoblast due to its size. These EVT proceed to invade the maternal decidua and, mainly during first trimester, the myometrium [10]. A part of these EVT has the main goal of reaching maternal uterine spiral arteries [11, 12]. These arteries are important to supply the intervillous space with maternal blood (containing nutrients and oxygen), which is then rerouted into the intervillous space. When EVT reach these arteries, the endothelium is eroded by the EVT and replaced with EVT which then differentiate into the so-called endovascular EVT (endEVT), which are not capable of producing vasotension in reponse to vasoreactive substances. In this manner, the spiral arteries are transformed into low-resistance vessels that supposedly allow for optimal blood flow into the intervillous space [12]. EVT invasion, although very similar to cancerous invasion, is also spaciotemporally controlled. Currently, it is deemed that EVT come into contact with decidual/ uterine immunocytes, which communicate with each other, and thus, EVT invasion is controlled in terms of intensity and direction [13].

Insufficient EVT invasion is hypothesized to lead to faulty spiral artery transformation and thus to a situation within the placenta that promotes placental insufficiency, and secondary to that intrauterine growth retardation (IUGR) and/or preeclampsia [14]. Faulty trophoblast invasion into the decidua at earlier points of pregnancy could lead either to infertility (due to implantation failure) or to miscarriage [15]. In some situations, trophoblast invasion is not under adequate control. This could result in benign, but life-threatening, diseases such as placenta accreta, increta and percreta. Some trophoblast cells dedifferentiate. This situation can lead either to benign molar disease, but also malign trophoblastic disease, choriocarcinoma. All of these settings are dire for reproductive success in the least, and in the most for the mother and the foetus.

We focus now on an assortment of mediators that are known to play a role during reproduction. In this review, we mainly summarize the available literature on the interactions of these cytokines with trophoblast cells (proliferation, invasion, differentiation and others). Furthermore, we list:

- what is known about cytokine distribution within the reproductive organs,
- which trophoblast subsets are known to express their receptors,
- which signal transduction pathways are utilized in trophoblast(ic) cells,
- the impact of murine cytokine deficiency on viability and reproduction and finally
- how the cytokine is associated with human pregnancy pathologies.

The mediators are categorized here according to their classical receptor family, although we stress that this scheme might not necessarily hold true for the trophoblast. We refrain from describing the exact mode of signal transduction via these receptors since this would far overreach the scope of this topic. Further information on this cytokine categorization principle in the immune system in general can be found in Coico and Sunshine [16] as well as http://en.wikipedia.org/wiki/Cytokine_receptor and http://de.wikipedia.org/wiki/Zytokin.

TYPE I CYTOKINE RECEPTOR

Interleukin-6 (IL-6)

IL-6 is a 26-kD pleiotropic protein that belongs to the family of glycoprotein 130 (gp130) cytokines along with leukemia inhibitory factor (LIF) and IL-11. It is produced by a large numbers of cells, such as fibroblasts, macrophages, dendritic cells, T and B lymphocytes, endothelial cells, glial cells and keratinocytes. In addition, IL-6 has been shown to be expressed by STBs and EVTs (2).

Although most biological functions of IL-6 occur by activation of its membrane receptor gp130, there is a specific receptor (IL-6R) which forms a complex agonist. Both IL-6 membrane receptor gp130 and the specific receptor IL-6R are present in the maternal and foetal tissues (endometrium, decidua and trophoblast) during implantation and placentation. Within the human endometrium, IL-6 expression follows a regulated temporal pattern (3–5), indicating a role in endometrial function and in implantation. Both increase during secretory phase of menstrual cycle, however, during the early first trimester pregnancy the soluble form predominates over the membrane-bound form until pregnancy week 10. At pregnancy week 11, the longer membrane-bound form increases. This increase proceeds during the second trimester [17].

It is also implicated in the prevention of recurrent abortion in mice and humans [18–20], and it has been demonstrated that deficient IL-6 mice show a reduction in fertility and a decrease in viable implantation

sites [21]. Abortion in mice can be prevented by rIL-6 treatment [20].

IL-6 activates the Janus kinase/ signal transducer and activator of transcription pathway (JAK/STAT) [11, 12]. IL-6 has been shown to stimulate system A (but not system L) amino acid transporter activity in primary trophoblast cells through STAT3 and increased expression of Na(+)-coupled neutral amino acid transporter (SNAT)2 isoform. STAT3 was phosphorylated at Tyr705 in these experiments. The importance of the JAK/STAT signal-transduction pathway in embryo implantation has been demonstrated by the embryonic lethality of STAT3 deficient mice [22]. The role of STAT3 activity in trophoblast invasion suggests a potential participation of IL-6 in this process [12, 13].

On the other hand, the invasive CTB cells express high levels of IL-6 [23] which increases the activity of matrix metalloproteinase-9 (MMP-9) and MMP-2 [24]. Moreover, transwell migration and Matrigel invasion of JEG-3 cells have been significantly reduced by transfection with IL-6 siRNA (small inhibitory RNA), while silencing of both IL-6 receptors was able to significantly decrease trophoblastic cell proliferation [25]. In addition, IL-6 enhanced the invasiveness of different tumor cells in an extracellular matrix membrane system [17, 18]. Furthermore, recent researches has shown that IL-6 increased the invasion ability of human pancreatic cancer cells [26] and that serum levels of this cytokine correlate with the extent of tumor invasion, lymph node metastasis, distant metastasis and all aspects of breast cancer [27]. These findings indicate that tumor cells tend to invade and metastasize in an environment rich in IL-6. In the context of reproduction, an increased ratio of soluble gp130/soluble IL-6R (sIL-6R) and/or reduced sIL-6R production combined with down-regulation of IL-6R occur in placentas from pre-eclampsia women [28].

Taken together these data suggest a contributing role for IL-6 in stimulation of trophoblast invasion, regulation of endometrial function and in implantation.

Interleukin-11 (IL-11)

IL-11, an IL-6 group cytokine, is present at the site of implantation and has been observed to be indispensable for the murine embryonic development [29]. It transduces its signal through the IL-11 receptor α (IL-11Rα; IL-11 specific receptor) and gp130 (common co-receptor for IL-6 family of cytokines) through activation of JAK1/2 and STAT3 mediated signaling pathway. IL-11 has pleiotropic effects on cells depending upon the cellular microenvironment. It is involved in regulation of biological functions, such as cellular proliferation and differentiation as well as in progression of several carcinomas [30–32]. The IL-11Rα knockout female mice, though physiologically normal are infertile because of defective decidualization of the endometrial stromal cells [33, 34]. Defective decidualization leads to lack of critical endometrial signals essential for normal proliferation and differentiation of trophoblastic cells of the developing embryo. In humans, IL-11Rα has been found to be expressed consistently in the endometrium from proliferative and secretory phase to 7–9 weeks of gestation [35]. In contrast to this, IL-11 expression is barely detectable in the proliferative and secretory phase of endometrium, but was found to be significantly higher in the chorionic villi as well as in decidua [35]. The endometrium of rhesus monkey shows maximum immunoreactivity for both IL-11 and IL-11Rα during the secretary phase of the menstrual cycle and their co-localization at the site of implantation [36]. From the foetal side, immunoreactive IL-11Rα is expressed by subpopulations of interstitial and endEVT cells of first trimester human placenta as well as by JEG-3 choriocarcinoma cells [37, 38]. Furthermore, a defective production of IL-11 correlates with a reduced fertility rate in humans [35]. The plasma level of IL-11 was found to be low in women suffering from spontaneous abortion [39]. Another study on human endometrial cells confirmed that IL-11, in either an autocrine or paracrine manner, works in conjunction with progesterone to bring forth their differentiation into a functional decidua [40]. Though IL-11 plays a defined role in endometrial decidualization, its role in trophoblastic cell invasion has been held in controversy. Exogenous treatment with IL-11 of JEG-3 choriocarcinoma cells and a cell line derived from the hybridoma of human EVT and JEG-3 cells led to an increase in the invasion and migration respectively through activation of STAT3 Tyr705 phosphorylation [37]. In contrast to these, treatment of HTR-8/SVneo trophoblastic cells with IL-11 decreases their invasion through activation of STAT3 Tyr705 [41].

Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

Among the colony-stimulating factor family members, GM-CSF is an hematopoietic cytokine secreted by macrophages, mast cells, endothelial cells, T cells, fibroblasts and bone marrow stromal cells [42]. GM-CSF affects the proliferation, differentiation and

survival of myeloid leukocytes and their precursors [43]. The receptor of GM-CSF consists of α (GM-Rα) and β subunits. The β subunit is shared with receptors for GM-CSF, IL-3 and IL-5 [44]. On the cell membrane of choriocarcinoma cell lines such as BeWo, JEG-3 and JAR and of primary trophoblast cells (CTB and EVT; only weak expression on STB), GM-Rα protein can be detected [45]. Researches demonstrate, also, a role in the modulation of Th1 and Th2 immune responses for this cytokine [46, 47]. Studies of *in vitro* angiogenesis assays and *in vivo* Matrigel plug assays with endothelial cell of mice indicated that GM-CSF and monocytes play a key role in angiogenesis through the regulation of vascular endothelial growth factor (VEGF) [48]. Acting as an immunoregulatory agent, GM-CSF, which is regarded as an important determinant of pregnancy outcome, contributes to regulation of placental morphogenesis and maternal immune tolerance [49]. Furthermore, as an embryotrophic factor, it is indispensable for providing ideal foetal evolution after implantation, such as foetal and post-natal growth, and the likelihood of obesity in adult descendants and it regulates the morphological and functional development of the placenta [50]. It contributes as a driving force in a tightly regulated sequence of events involving CTB cell proliferation and terminal differentiation to generate STB cells [51]. By accommodating these trophoblastic functions, it is believed to be involved in invasion of maternal decidual tissues and blood vessels, although actual *in vitro* data is yet to be generated.

GM-CSF seems relevant to human reproductive medicine, since its deficiency is associated with placental insufficiency, as well as immunological disorders, and it is shown to be involved in miscarriage, low birth weight, pre-term delivery and preeclampsia [49]. According to other studies concerning these complications, GM-CSF expression is increased in the decidua of preeclamptic women and mice [52]. During the post-conceptional period, the GM-CSF which is secreted into the uterus and the salpinges is implicated as a regulator of the growth and development of the pre-implantation embryo [53]. One study of women suffering from recurrent miscarriage showed that an increase in serum GM-CSF content, which is seen in normal pregnancy, did not occur in the miscarriage group [54]. Researchers have demonstrated that the outcome of gestation is radically modified by the administration of exogenous GM–CSF to mice [55]. Other studies in mice with a null mutation in the GM-CSF gene show that fertility and the number of surviving pups are impaired in the absence of GM-CSF [56–58]. These studies reveal that a GM-CSF deficiency leads to growth retardation and small litter sizes probably secondary to placental anomalies, including a diminished proportion of glycogen cells in the spongiotrophoblast layer [56].

Uterine and placental tissues are recognised as potent sources of hematopoietic colony stimulating activity [59, 60]. GM-CSF is produced by uterine epithelial cells, and GM-CSF is found in the luminal and glandular epithelium. GM-CSF synthesis by uterine epithelial cells is predominantly stimulated by estrogen; its expression stays high for the first few days after conception, but then declines around the time of embryo implantation, which occurs under the inhibitory influence of progesterone [61]. Once implantation begins, cell lineages in the chorionic villi of the early developing placenta contribute to GM-CSF production including the invading CTB cells [62], villous fibroblasts [63], and placental macrophages [64]. Other analyses also demonstrated that in women and in mice, GM-CSF synthesis by reproductive tract epithelial cells is responsive to ovarian steroid hormones and to male seminal fluid factors (59, 60).

Several identified polymorphisms in the genes that encode GM-CSF are identified conferring endogenous variability in GM-CSF bioavailability and signaling networks [65–67]. At least three signaling pathways have been described for this cytokine: the mitogen-activated protein kinase (MAPK) pathways, the JAK/STAT pathway and the phosphatidylinositol 3-kinase pathway (PI3K) [68–72]. Furthermore, there is one study that supports the idea that STAT5 is recruited to the membrane from the cytosol upon GM-CSF stimulation and is tyrosine-phosphorylated by JAK2 [44], but it is yet to be discovered which pathway is used for signal transduction of GM-CSF in the trophoblast. Our own unpublished results indicate that at least STAT3 is not involved in mediating its signal. At least in the inner cell mass it has been demonstrated that GM-CSF signaling occurs independent of its ß common subunit [53]. Considering the relevance of GM-CSF in the early stages of pregnancy, a complete understanding of its role represents an opportunity for developing interventions for achieving favorable obstetrical outcomes.

Leukemia Inhibitory Factor (LIF)

LIF, a member of the IL-6 family, is a widely known pleiotropic cytokine which posseses a pivotal role in human reproduction [73, 74]. LIF was first identified in 1987 by Metcalf and colleagues as a factor that induced

the differentiation of mouse myeloid leukemic M1 cells into macrophages [75]. Currently, LIF is known to be expressed in numerous cell types including neurons, hepatocytes, and kidney and breast epithelial cells [76, 77] and its main role is the mediation of inflammatory cell responses [78, 79]. Nevertheless, LIF is also recognized to control uterine receptivity for blastocyst implantation, or to influence trophoblast behavior by promoting proliferation, invasion and differentiation [80, 81].

LIF appears to be an important modulator of pregnancy in humans. Both granulosa-lutein cells and ovarian stromal cells expressed LIF mRNA and protein. Furthermore, LIF concentration in follicular fluids correlates with the embryo quality suggesting an important role of LIF in the physiology of ovulation and early embryo development [82]. On the other hand, LIF is expressed by the endometrium, predominantly in the glandular and luminal epithelium, and its concentrations reach maximal levels during the secretory/postovulatory phase of the menstrual cycle, when the implantation is expected to commence [80, 83]. During the implantation window, trophoblast cells also express mRNA for the LIF receptor which maximizes interaction with the endometrium. After adhesion, the blastocyst is able to produce LIF mRNA by itself, which leads to an increase in cell proliferation and triggers differentiation into CTBs and STBs, and enhances invasive behavior of trophoblast cells. [81, 84–86]. The LIF receptor is expressed by both villous as well as EVT cells throughout pregnancy. EVT express the LIF receptor as they pass decidual leukocytes which secrete LIF, and thus they come into dialogue [87].

LIF's crucial role during embryo implantation is evident in LIF deficient female mice, which albeit being infertile by the inability of the blastocyst to attach, could recover fertility by LIF infusion into the uterus [88]. Conversely, LIF receptor knockout mice implant, but exhibit impaired placenta function, which results in death within 24 h of birth [89]. In humans, LIF expression levels are diminished in endometrial cell cultures from infertile women with repeated abortions or unexplained infertility [90, 91]. In fact, women wearing a copper T380A intrauterine device (IUD), one of the most effective anticonceptive devices, showed also lower expression of LIF compare with control [73]. But it is not only LIF protein expression deregulation which may have a negative impact on the pregnancy outcome, functionally relevant mutation of the LIF gene are found higher in infertile women in comparison with fertile controls resulting in poor outcome in IVF treatment [92].

LIF triggers its effects by induction of a signaling heterodimer consisting of the specific LIF receptor (LIFR) and the subunit gp130 [74]. This causes the activation of the RAS/MAPK (RAt Sarcoma/ MAPK) and JAK/STAT cascades [93–95]. STATs are a family of transcription factors located in the cytoplasm, which after activation can form hetero- or homo-dimers and be translocated into the nucleus to control gene expression [96, 97]. STATs are associated with regulation of implantation and maternal immune response in early pregnancy [98]. Furthermore, we have demonstrated that STAT3, a member of the STAT family, plays a crucial role in the regulation of trophoblast invasion mediated by LIF. LIF induces alteration of proteases such as tissue inhibitor of metalloproteinase 1 (TIMP1) and Caspase4 via STAT3, which elevates trophoblast(ic) proliferation and invasion, and STAT3 knockdown annuls these functions even in the presence of LIF [81, 86, 99]. LIF has been patented as a supplement to culture media to promote the development of mammalian embryos to the implantation stage, since growth in the presence of LIF increases the percentage of embryos that reach the implantation stage than growth without LIF (United States Patent 5962321; Inventors: Gough, Nicholas Martin; Willson, Tracey Ann, Seamark, Robert Frederick (Beulah Park, AU), http://www.freepatentsonline.com/5962321.html).

Granulocyte-Colony Stimulating Factor (G-CSF)

G-CSF is a macrophage- and granulocyte-inducing (MGI) protein, mainly produced by macrophages, which induces the proliferation and differentiation of macrophage and granulocyte precursor cells. Furthermore, G-CSF is able to induce terminal differentiation in murine leukemic cells and thereby suppress leukemic cell populations. The murine and human G-CSF protein show almost complete cross-reactivity regarding biological effects and receptor-binding in human or murine normal and leukaemic cells [93–97]. The molecular weight of G-CSF amounts 19.6 kDa, consist of 174 amino acids and is o-glycosylated at Thr-133 [100–102]. The encoding gene of G-CSF is located at chromosome 17, 17q11.2–21 [103]. The G-CSF receptor is a 150 kD single subunit protein [104].

G-CSF is produced by those decidual cells that are in contact with anchoring villi but not by trophoblast cells of the chorionic villi [105]. G-CSF-receptor (G-CSFR) is expressed in human placental membranes as well as CTBs and STBs and decidual stromal and endometrial gland cells [104, 105]. G-CSFR is intensely expressed in first and third trimester, but not

expressed in second trimester placental tissue. The strongest expression was found in invasive, extravillous CTBs, and differentiated STBs but expression was weak in undifferentiated CTBs. Furthermore, G-CSFR positive interstitial trophoblasts were found in decidual tissue, distal and proximal to the materno-foetal interface and in dense maternal connective tissue. Thereby, the G-GSFR expression was weak in first, strongest in second, and weak or absent in third trimester. These data suggest a possible role of G-CSFR in decidual invasion of CTBs [106, 107].

The immortalised first trimester trophoblast cell line HTR-8/SV neo increase G-CSF expression after lipopolysaccharide (LPS) incubation, suggesting a participation of trophoblast cells in cytokine based immune signaling [108]. G-CSF is able to increase proliferation of CTB cells derived from human chorionic villous tissue [105], but inhibits the proliferation of two choriocarcinoma cell lines [109], which the authors cannot presently specify due to inavailability of the full publication.

G-CSF activates the JAK/ STAT and MAPK signaling pathway in chorioncarcinoma cell line JEG-3. G-CSF had no positive effect on JEG-3 proliferation, but protects JEG-3 cells from serum starvation [110]. All these data suggest a potential role of G-CSF, secreted by decidual cells, in controlling trophoblast invasion, but *in vitro* experiments substantiating this notion are yet to be published.

Follicular fluid G-CSF has recently been described as a new biomarker identifying the competent oocyte, and this concentration correlated in positive prediction of live birth in assisted reproduction techniques (ART) [110, 111]. In a recently published pilot study, administration of G-CSF during ART in patients who suffered from repetitive implantation failure (and were also lacking killer-cell immunoglobulin-like receptors) elevated the pregnancy rate to a stunning ca 73%, albeit the abortion rate was also high (ca 39%) [111]. Women suffering from primary recurrent miscarriage also profited from G-CSF treatment (ca 83% versus 39% in control group for live birth rate) [112]. G-CSF is also associated with other immune-related pregnancy complications such as preeclampsia [113] and spontaneous preterm birth [114]. Taking the above into consideration, it is no surprise that G-CSF has been patented as an intervention to prevent abortion, implantation failure during ART or to treat or prevent preeclampsia or preterm labor (United States Patent 7615531, inventor: Carter, Darryl (Owings Mills, MD, US) Nora Therapeutics; http://www.freepatentsonline.com/7615531.html).

Leptin

Leptin is a hormone that was originally thought to be produced only by adipocytes to aid in modulating satiety and energy homeostasis [115, 116]. However, this cytokine is now known to be produced by placental tissues [117] and secreted to both maternal and foetal circulations during the pregnancy [118, 119].

Expression of the leptin gene was found abundantly in the human first trimester chorionic villi, and slightly in the third trimester chorion laeve, and amnion. Immunohistochemical experiments demonstrated that both STBs and CTBs were stained positively for leptin [120].

Leptin receptors have been described in trophoblast cells of several species [121–125] as well as in the JAR and BeWo derived trophoblast cells lines [126]. Bodner et al. [127], showed that theleptin receptor mRNA was expressed in the cytoplasm of the STB. Moreover, throughout immunohistochemistry technique, the leptin receptors produced a strong reaction in the STB of placental villi at term. The apical membranes were continuously stained, whereas basal membranes and cytoplasm lacked reactivity with both antibodies. CTB cells, stroma cells and endothelial cells were not labeled. EVT display high expression of the leptin receptor [128].

The role of this cytokine during pregnancy was confirmed in ob/ob mice, which lack a functional leptin protein. These animals are infertile, however, leptin treatment promoted embryo implantation and initial placental development in these mice [129]. Translated to human pregnancy pathologies, it can be stated that leptin is associated with gestational hypertension, IUGR and gestational diabetes. The polymorphism of the leptin receptor is related with severe preeclampsia [130]. Leptin has been found to be associated with maternal hypertension that may or may not proceed to preeclampsia [131, 132]. In IUGR decrease of placental leptin and mRNA leptin in umbilical cord was observed [133]. Pregnancy associated with diabetes is linked with an increase in the placental and circulatory leptin [134, 135].

During embryo implantation and the development of the placenta, trophoblast invasion is currently considered as the most limiting factor for the establishment of normal pregnancy. There is evidence suggesting that leptin produced by the maternal endometrium plays an important role in the signaling necessary to these processes [136]. In particular, leptin has been proposed to play a relevant role in implantation and trophoblast invasion by virtue of its stimulatory effect

on MMP expression in CTB [137]. Leptin increases, in a dose-dependent manner, the secretion of MMP-2 and enhanced the activity of MMP-9 in cytotrophoblastic cells [137]. Moreover, Magarinos et al, illustrated the antiapoptotic and proliferative effects of leptin in trophoblastic cells lines [138].

Leptin seems to promote trophoblast invasiveness in primary cultures of mouse trophoblasts. This cytokine stimulated the phosphorylation of MAP or ERK kinase (MEK, also termed MAP2K1), but not that of STAT3 in the cultures, while it increased the concentration of the suppressor of cytokine signaling 3 (SOCS3) protein, and up-regulated metalloproteinase activity [139].

Leptin is now known to play a wide range of important roles, which extend from pregnant physiology as well as implantation and from paracrine effects in the placenta to regulation of trophoblast invasiveness.

TYPE II CYTOKINE RECEPTORS

Interleukin-10 (IL-10)

IL-10 is an immunosuppressive cytokine that has been shown to be produced by a wide variety of cell types, including macrophages, dendritic cells, natural killer (NK) cells, T cells, B cells, as well as cells found at the maternal-foetal interface, namely endothelial, placental trophoblast and decidual stromal cells [140–144]. Functionally, IL-10 binds to its cognate receptor IL-10R and in turn activates the JAK and STAT signaling pathways [145], but this is yet to be demonstrated in the trophoblast.

In the context of pregnancy, IL-10 has been shown to play a prominent role. The kinetics of IL-10 expression in both mice and human placental trophoblast exhibit a temporal pattern. IL-10 is expressed early in gestation and remains elevated throughout the second trimester [143].

As mentioned above, different cellular populations are involved in its production at the maternal-foetal interface. Particularly, CTBs produce IL-10. Studies indicate that IL-10 can inhibit the activity of MMP-9, an enzyme which increases CTB invasiveness [144, 146]. Furthermore, one publication illustrates how infection of differentiating and invasive CTB with cytomegalovirus (CMV) leads to production of both cmv- and human IL-10. Both of these cytokines apparently inhibited CTB migration (into an endothelial cell wounding assay) and invasion (into a Matrigel) [147]. On the other hand, extrawillous trophoblast (EVT) cells are poor producers of IL-10 (unpublished data), thus possibly allowing MMP expression and invasion capability. Additionally, in preeclampsia, a pregnancy disorder associated with decreased trophoblast invasion and remodeling of uterine spiral arteries, IL-10 production is significantly reduced [148].

In an *in vitro* model that recapitulates the interaction between first trimester EVT and endothelial cells, exogenous IL-10 could rescue the polychlorinated biphenyls (PCBs)-induced disruption of endovascular activity and restored impaired spiral artery remodeling *in vivo* [149] implying its role in vascular activity. Additional evidence, from the lab of Surrendra Sharma suggests that IL-10 may play a protective role in preeclampsia. In this context, unlike the IL-10 proficient wild-type mouse, pregnant IL-10−/− counterparts were sensitive to human preeclampsia serum treatment that impaired spiral artery remodeling and precipitated the full spectrum of clinical features of the disease [150]. Importantly, recombinant IL-10 reversed the hypoxia induced preeclampsia-like features in pregnant IL-10−/− mice providing further evidence to the pleiotropic vascular role of IL-10 [151, 152]. Likewise, IL-10 can promote trophoblast invasion indirectly by disrupting macrophages that inhibit trophoblast invasion [153] or protect against LPS and angiotensin II-induced vascular dysfunction [154, 155].

IL-10 is not essential for normal pregnancy outcome in mice [156] and reviewed in [157]. When IL-10−/− females are mated with IL-10 −/− males, implantation sites are increased with more viable foetuses than pregnant wild-type IL-10+/+mice [158]. However, IL10 is vital in protecting pregnancy during inflammatory alterations as seen during LPS-confrontation. In these instances (IL-10 deficiency), LPS mediates an elevated incidence of miscarriage [159] and preterm labor [160] and predisposes to growth retardation [157], while administration of IL-10 on E9.5 of gestation to these mice reduced foetal loss and growth restriction [157].

Single nucleotide polymorphisms of IL-10 are associated with the development of preeclampsia [161]. Some IL-10 gene promoter polymorphisms associated with cytokine down-regulation seem to be constitutional risk factors for early embryonic pregnancy failure [162]. An increase in the production of IL-10 early after implantation is related to the success of pregnancy [163].

Taken together, IL-10 plays an important part in the regulation of trophoblast invasion and vascular activity at the maternal-foetal interface.

Interferon-gamma (IFN-γ)

IFN -γ is a type II proinflammatory cytokine involved in the activation of innate and adaptive

immune response via receptor (IFN -γ Rs)-mediated JAK/STAT1 signaling pathway [164]. It has also potent anti viral activity, as all other IFNs.

IFN -γ is expressed in the reproductive tract in implantation and in pregnancy [165]. Histochemical analyses in human trophoblast cells have shown a stronger expression of IFN -γ during the first trimester as compared to term [166]. In mice IFN -γ has been detected in giant trophoblast cells [167]. However, the majority of the human [168] and mice [169] endometrial IFN -γ stems from CD56bright CD16- uterine natural killer (uNK) cells homing in from the systemic circulation. IFN -γ Rs are known to be expressed throughout the pregnancy by trophoblast cells (namely villous CTBs) and in the CTB cell columns [168]) and in uterine epithelium and stroma [170], and is in particular localized to those areas adjacent to attaching trophoblast [171].

IFN -γ and IFN-γ R null mice have a large number of undifferentiated uNK cells causing wide spread necrosis in the decidua suggesting the significance of the IFN -γ pathway during early pregnancy [169].

IFN -γ was shown to decrease excessive trophoblast invasion in a Matrigel assay using first trimester extravillious trophoblast cells and JEG-3 choriocarcinoma cells. The effect was mediated via down regulation of MMP-2 and MMP-9 mediated by STAT1 and IFN-γ -inducible class II transactivator (CIITA) [172–174]. Also, IFN-induced and regulated genes were found to be upregulated in decidualized endometrial cells cultured in the presence of the human trophoblast conditioned medium, suggesting a role for IFN -γ in regulating the maternal side of the foetal maternal interface [175]. However, harmful effects of IFN -γ can also be anticipated since the inhibition of IFN-γ signaling in human trophoblast cells, exerted by protein tyrosine phosphatase, prevented transplant rejection directed against the foetus [176].

A shift towards a Th1-type immunity, as expressed either through an increased IFN-gamma/IL-4 ratio in maternal serum or elevated placental concentrations of IFN -γ levels, is observed during preeclampsia [170, 171]. Although IFN-γ polymorphisms do not seem to be associated with preeclampsia, a higher frequency of a specific IFN -γ polymorphism was observed in a Brazilian population of eclamptics [177, 178]. In a China-based study, some IFN-γ receptor 1 polymorphisms are associated with the development of PE [179]. Umbilical cord serum levels of IFN -γ was associated with a decreased risk of small for gestational age (SGA) birth, especially amongst preterm babies [180]. In terms of miscarriage, systemic levels of IFN-γ were not associated with implantation rate or miscarriage rate in women undergoing IVF treatment. However, high levels of IFN -γ correlated with elevated levels of activated NK cells and this may subsequently exert a negative impact on reproduction [181]. Two South American based studies, including a meta-analysis, reported an association between IFN-γ gene polymorphisms and (unexplained) recurrent, spontaneous pregnancy loss [182, 183]. However among an Iranian population, the studies of IFN -γ gene polymorphisms did not show any association with the pathology [184].

IMMUNOGLOBULIN SUPERFAMILY

Interleukin-1beta (IL-1ß)

IL-1ß is a potent inflammatory mediator produced by monocytes, dendritic cells and a variety of other cells. An experimental study suggests that IL-1ß effects depend on inflammatory conditions.

IL-1ß−/− mice had reduced acute-phase response in a model of local, sterile inflammation (without microbial stimulus) but presented a normal reaction when LPS was added [185]. It was viable to generate homozygous IL-1b KO mice, they developed normally and they were healthy and fertile [186].

Besides its role in autoimmune diseases and inflammatory disorders [187, 188], IL-1ß seems to play a relevant role during human embryo implantation [189]. IL-1ß was weakly expressed in epithelium and stroma of human endometrium, but highly expressed in first trimester decidua and in term placental membranes. It seems that progesterone can regulate the IL-1ß expression, since IL-1ß mRNA was detected in the late secretory endometrium, when progesterone is high, but not in proliferative endometrium, when the progesterone level is low [190–192]. Despite IL-1ß seeming to be a potent inhibitor of decidualization, IL-1ß mRNA increases in cultures of endometrial stroma cells during decidualization [193]. The expression of IL-1 receptor type I (IL1RI) had three phases trough the menstrual cycle, low expression in the proliferative phase, moderate during ovulation and the implantation phase, and intense at the end of the cycle [190, 194]. Furthermore, IL-1 receptor type II (IL1RII) mRNA expression was low in the early-to mid-proliferative phase, increased in the late proliferative/ early secretory phase, decreased in mid-secretory phase and increased again in late secretory phase [195]. IL-1ß and IL-1R tI are detected in human placentas and IL-1ß is expressed both in CTB and STB of chorionic villi

[196]. IL-1ß was also detected in foetal and maternal blood cells from placenta samples [197], while IL-1R tI was present in STB of chorionic villi and in the endometrial glands of maternal decidua [196]. It seems that IL-1ß controls human placental trophoblast growth. Recombinant human IL-1ß inhibited JAR and BeWo (choriocarcinoma cell lines) proliferation and this effect can occur by induction of apoptosis [198, 199]. An experiment to investigate the molecular interactions on EVT differentiation showed that IL-1ß had no effect in TCL1 cell differentiation into an invasive phenotype (human EVT cell line) [200].

It has been suggested that IL-1ß plays a relevant role in trophoblast invasion. First trimester CTB and EVT stimulated by IL-1ß increased invasiveness by approximately 50% on a Matrigel system [201, 202]. Moreover, stimulation with IL-1ß increased the invasion of human placental choriocarcinoma cells (JEG-3) and immortalized trophoblast cells (HTR-8/SVneo) [202, 203], but did not affect the invasiveness of trophoblastic SGHPL-4 cells in the same system [204]. Different studies suggest that IL-1ß's tissue invasiveness effect is due to its regulatory role on the production of MMPs, including MMP-2, MMP-3, MMP-9 and monocyte chemoattractant protein-1 (MCP-1) [204–209]. This inductor process apparently occurs via MAPK and AKT (RAC-alpha serine/threonine-protein kinase) signalling, given that inhibitors of theses kinases decrease MMP-3 expression in SGHPL-4 cells [204]. Moreover, low molecular mass polypeptide-2 (LPM2) may be necessary for IL-1ß-induced trophoblast invasion, because it seems to regulate the expression and activity of MMP-2 and MMP-9 in HTR-8/SVneo. Additionally, LPM2 knockdown in HTR-8/SVneo inhibited IL-1ß cell invasion in Matrigel system [203, 210]. Therefore, these data suggest that IL-1ß seems to have an important role in trophoblast invasion, through the activation of MMPs.

There seems to be no apparent difference in the maternal serum levels of IL-1ß in preeclamptics versus control [170, 211]. However, the placental expression of this cytokine seems to be increased in preeclampsia patients [212]. Furthermore, placental levels of IL-1ß were not altered in between pregnancies with or without foetal growth retardation [213]. Increased amniotic fluid levels of IL-1ß (as measured after amniocentesis) was correlated with an increased risk for delivery <34 weeks gestation [214, 215]. In women threatening to abort, elevated serum levels IL-1ß were correlated to an adverse outcome [216]. Reduced intra-amniotic IL-1ß concentrations and an increased occurrence of spontaneous abortions in previous pregnancies are associated with the foetal carriage of polymorphic IL-1 receptor antagonist allele 1 [217]. Furthermore, in contrast to older study results, newer studies indicate that combinations of polymorphisms for the promoter region of the IL-1ß gene with other polymorphisms or homozygotous polymorphisms of this promoter region seems to confer a risk for recurrent pregnancy loss through TH1 immunitiy to trophoblast [218–220].

Colony Stimulating Factor-1 (CSF-1)

CSF-1, also known as M-CSF (macrophage- colony stimulating factor) [221] or MGF (macrophage growth factor [222]) is one of a group of at least 18 glycoproteins, collectively known as hematopoietic growth factors [223], which implies membership to the type I cytokine receptor group, however, CSF-1 classically belongs to the immunoglobulin superfamily (in terms of receptors), which shares structural homology with immunoglobulins. The CSF-1 homodimer is produced in a variety of adult tissues and influences the proliferation and differentiation of numerous of cell types [224]. Ninety-five percent of circulating CSF-1 is cleared by sinusoidally-located macrophages, primarily Kupffer's cell in liver [225]. CSF-1 and its receptor are an important receptor/ligand pair in the biology of breast cancer and tumors of the female reproductive tract [226]. They are initially implicated as essential to normal monocyte development and trophoblastic implantation [227] and it is one of the important cytokines for the function of monocytes and macrophages [228].

CSF-1 bioactivity is high in the uterus, placenta and amniotic fluid [229]. CSF-1 is secreted by human trophoblast as well as endometrial cells [228]. CSF-1 and endothelin-1 are co-localized in same cells in the amniotic membrane [230]. Female sex steroids, progesterone and estradiol, regulate CSF-1 synthesis by luminal and glandular secretory epithelial cells of the uterus [225]. CSF-1 mRNA and protein factors of its receptor c-fms are identified in the human placenta and decidua; both are expressed by normal human 1st trimester invasive EVT (217). The expression of CSF-1 and c-fms also possess intrinsic tyrosine kinase activity which suggests that this is another factor playing a potentially important role in regulating trophoblast function (218). CSF-1 is present in uterine glandular epithelium (as mentioned above), vascular endothelium and villous as well as in EVT, and mRNA expression of CSF-1 is found in the placenta and decidua but not in the non-pregnant endometrium

[224]. The presence of CSF-1 and c-fms mRNA and protein at human foetomaternal interface suggest that CSF-1 may be involved in autocrine and/or paracrine interactions which may regulate trophoblast and/or decidual cell function [224]. Quantitative mRNA analysis showed that c-fms mRNA expression in placental tissues was lowest in first trimester samples and highest at the end of pregnancy [231]. It has been demonstrated that the level of CSF-1 in the amniotic fluid of term gravidas is significantly increased compared to mid-gestational controls [232]. These results suggest that elevated levels of M-CSF in amniotic fluid have an important immunological function in the maintenance of pregnancy and foetal growth.

There exists some unclearness in the exact functional effects of CSF-1 on trophoblast proliferation, differentiation and invasion (commented in [233]). While the two studies described here are congruent in the aspects that EVT produce both CSF-1 and express c-fms, the effects were disparate especially on EVT proliferation and invasion. It seems that primary (invasive) EVT react to CSF-1 supplementation with an increase in growth (proliferation), but no difference in Matrigel invasion. Furthermore, in these cells, both MMP-9 (type IV collagenase) and TIMP-1 expression were up-regulated in response to this cytokine, which could explain why no alteration in invasion was detected [234]. In the study accomplished by Lewis et al. [235], immortalized EVT cells, termed TCL-1 cell line, did not respond in a proliferative manner to CSF-1, while blockage of CSF-1 promoted trophoblast growth. The activity of MMP was unaltered in this experiment. Thus, this group suggested that CSF-1 may be a differentiation or maturation factor that suppresses the proliferation of trophoblasts. The differences seen here are probably explainable by the cell types used in both experiments. Lala comments that in their HTR8/SVneo cell line, another immortalized EVT cell line, proliferation was not altered through CSF-1 treatment (unpublished data mentioned in reply to comment [236]). However, in a recent study, HLX (H_20-like homeobox protein) was found to be a regulator of CSF-1-dependent trophoblast proliferation. The homeobox gene HLX is expressed in proliferating and migrating human trophoblast cells and HLX expression is significantly decreased in human IUGR. CSF-1 apparently stimulated HLX expression, and silencing of HLX in the presence of the cytokine resulted in a significant decrease of trophoblastic proliferation. These experiments were accomplished on SGHPL-4 and HTR-8/SVneo trophoblast cells [237, 238].

Studies of osteoporotric (op/op) mice show the crucial role of CSF-1 in pregnancy. These mice experience severely reduced fertility, largely due to a male deficiency to mate, but also related to low implantation rates and greater embryonic wastage compared with the wild-type female [221]. However, CSF-1 deficient embryos (op/op) can develop normally. Infertility of op/op×op/op matings, and lower implantation rates and higher resorption rates in op/op females mated to +/op or +/+ males, clearly indicate a role for CSF-1 in both pre- and post-implantation embryos [239]. Many effects of op/op mutation could be rescued by injection of neonatal mice with human recombinant CSF-1 [225].

Data suggest that the success of human pregnancy is associated with the production of the Th2-type cytokines LIF and CSF-1 (M-CSF) by T cells at maternal-foetal interface [240]. There are also significant increases in M-CSF and GM-CSF levels in the placenta as well as in the serum of preeclampsia patients which is bound to have various physiological effects on tissues at the maternal-foetal interface [230]. The serum level of CSF-1 is significantly elevated not only in preeclampsia, but also in normotensive pregnancies with IUGR compared with those in normal pregnancy [230]. Paradoxically, excess decidual macrophage infiltration has been linked to preeclampsia and IUGR in the human, and studies also showed that TNF and IL-1ß induced CSF-1 output by cultured first trimester and term decidua cells [221]. CSF-1, or M-CSF, is a potent inducer of Th2-type cytokines, as well as LIF production, by T cells [240]. Defective IL-10, LIF and M-CSF production by decidual CD4+ T cells were detectable in women with unexplained recurrent abortion at the time of miscarriage [240]. Thus, it seems that CSF-1 is important in the pathogenesis of human pregnancy pathologies as well.

TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY

Tumor Necrosis Factor-alpha (TNF-α)

The tumor necrosis factor (TNF) superfamily is involved in several physiological functions, such as immune response and morphogenesis. It has also been implicated in tumorigenesis, transplant rejection, septic shock and viral replication [241]. TNF-α is a non-glycosilated protein with potent pro-inflammatory activity which is required for a normal immune response, but its overexpression has severe

pathological consequences. TNF-α is associated with many autoimmune diseases [242] and anti-TNF-α drugs have been used in arthritis rheumatoid treatment. Actually, anti-TNF-α active immunization with a vaccine started the clinical phase of development [243].

In the reproductive field, TNF production is involved in the balance of trophoblast turnover and renewal [242]. TNF-α reduces proliferation in EVT [244] and primary trophoblast cultures [245]. During pregnancy, several decidual cells types, EVT and villous CTB produce TNF-α. Recently, two receptors have been detected in the placental bed: TNF-αRI and TNF-αRII. Immunostaining for TNF-αRI was greatest in intramural EVT and for TNF-αRII in endEVT [244].

To investigate the individual role of TNF-α in the regulation of immune response, a TNF-α −/− mouse strain was generated. This strain was viable and fertile, indicating that TNF-α is not required for normal embryo development [246]. However, another model demonstrated that the association TNF-α/INF-γ was related with miscarriage in mice, demonstrating how Th1-type cytokines are involved in abortion [247].

The TNF-α effect on cell invasion seems to be cell type-dependent. The cytokine did not have any effect on human CTB invasion [248] but had an inhibitory effect on HTR8/SVneo cells in Matrigel assays [249, 250]. Recently, its inhibitory effect on EVT was shown through an explant/matrigel invasion assay [244, 251]. The mechanism for exerting this effect remains unclear, but the Knöfler group concluded that TNF-α restricts trophoblast invasion through the induction of plasminogen activator inhibitor-1 (PAI-1) [249] and the study published by Otun speculates that TNF-α alters EVT invasion by regulation of trophoblast apoptosis [244]. On the other hand, it has been demonstrated that extracellular matrix remodeling, a key process in invasion, is variable between several cell types: TNF-α exerts inhibition of matrix metaloproteinase MMP-2 and integrins in JEG-3 cells, [252] but does not in EVT [251]. TNF-α increases proMMP-9 secretion in human chorionic trophoblast cells [253] and EVT [244], but not over the MMP-9 in JEG-3 cells. The last findings suggest an inhibitory effect of TNF-α in trophoblast(ic) invasion, but it will be necessary to develop new approaches to understand the involved mechanisms better. The general consensus is that the the MAPK pathway is involved in mediating the TNF signal in trophoblast cells: Erk1/2 (which then initiates NF-KappaB), stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK, which activates activator protein-1 or AP-1) [254, 255].

Circulating levels of the TNF-α, amongst others, were raised in preeclampsia compared with healthy pregnancy [170], and these levels were significantly higher in patients with severe preeclampsia than in mild preeclampsia [256]. In this respect, TNF-α up-regulating gene variants are associated with preeclampsia [257]. Furthermore, elevated maternal serum levels of TNF-α, in preeclamptic women correlate with (low) foetal birth weight in the early third trimester [256, 258]. Finally, an elevated umbilical cord blood concentration of TNF-α was associated with an increased risk of preterm birth, especially among babies who were appropriate for gestational age [180]. Polymorphisms in the cytokine genes TNF-α (and IL1ß) is associated with an increased risk of preterm birth, which possibly reflects a dysregulation of the immune system in pregnancy [259]. Maternal TNF-α levels appear to be dependent on maternal body mass index, since, although elevated TNF-α levels were associated with an elevated risk for preterm delivery (34–36 weeks' gestation) in general, these elevated levels had disparate associations for the under- and over weight. Elevated TNF-α concentrations were associated amongst obese women with a *reduced*, and in underweight women with an *elevated,* risk for spontaneous preterm delivery <34 weeks' gestation [260]. In terms of miscarriage (recurrent spontaneous abortion, recurrent pregnancy loss, reproductive failure), it has been demonstrated that serum levels of TNF-α are higher in women suffering from reproductive failure than in fertile controls [261]. Several studies have identified an association between TNF-α gene polymorphisms and this pregnancy pathology [181, 261, 262].

CHEMOKINE RECEPTORS

Interleukin-8 (IL-8)

IL-8 is an 8 kD protein member of the neutrophil-specific subfamily of chemokines (CXC) with ELR (Glu-Leu-Arg) motif. It is produced by multiple cell types, including leukocytes, fibroblasts, trophoblast and some tumor cells [263]. The 77-amino-acid predominant form of IL-8 is produced by endothelial and anchorage-dependent cells. During early pregnancy, uNK cells constitute an important source of decidual IL-8 [264]. IL-8RA (CXCR1) and IL-8RB (CXCR2) receptors, which coupled to G-Protein, are expressed in EVT, STB, CTB, Hofbauër cells and human endometrium among other multiple cell types [265–268].

IL-8 has been reported to be present in the human reproductive tract, and is detected in the cervix, the fallopian tubes [269], placenta [264], and endometrium [270]. It has been associated with endometrial proliferation, angiogenesis and apoptosis [271]. Moreover, IL-8 is abundantly secreted by villous and extravillous CTB during first trimester pregnancy, while 12–14 gestational weeks' EVT cells produce higher levels than 8–10 gestational weeks' villous cells. IL-8R is expressed by isolated first trimester trophoblast cells, CTB and EVT. This cytokine seems to stimulate trophoblast invasion and plays a role in spiral artery remodeling, although the mechanism of action has not been investigated yet [272]. IL-8 stimulates EVT Matrigel invasion [267, 273, 274] by increasing MMP-2 and MMP-9 gelatinase activity in EVT cells [267, 274]. Moreover, it has been shown that IL-8 stimulates these gelatinases together with urokinase plasminogen activator (uPA) in various different cancer cell types [275–277], and in such cases this situation is associated with an increased tumor growth and metastases. Enhanced tumoral growth is completely blocked by IL-8 or FosB gene silencing which also reduced microvessel density [278]. Moreover, Matrigel tumor invasion is decreased by down-regulating IL-8 protein production with the use of IL-8 targeted siRNA or by blocking CXCR1 receptor [279]. In EVT, IL-8 neutralising antibody partially abrogated uNK cell stimulated invasion of these cells [267]. In terms of proliferation, exogenous IL8 increases the proliferation of the EVT cell line HTR-8/SVneo, but did not affect proliferation of BeWo cells [280].

Currently, no data is available about IL-8KO mice, so that it is difficult to state how indelible IL-8 is for mammalian reproduction. However, IL-8 aberrations are correlated to some pregnancy pathologies. Elevated mRNA of IL-8 was found in chorionic villous samples derived from women at ca. 11 gestational weeks' and later developed preeclampsia, although patient size in these experiments indicate the preliminary nature of the study [281]. Furthermore significantly higher levels of this cytokine were found in preeclampsia patient and in umbilical cord serum as compared to healthy controls [256]. There are also indications that an elevated production of IL-8 is associated with intrauterine growth reduction (IUGR), since IL-8 mRNA was elevated in the placentae of term-born IUGR cases in comparison to their normally developed counterparts [213]. The cervical mucous of patients (with a history of recurrent miscarriage), who subsequently miscarried contained significantly higher of IL-8, so that IL-8 has been put forward as a possible predictor of miscarriage [282]. Therefore, IL-8 appears to play a role at the foeto-maternal interface and dysregulation of this chemokine may contribute to some complications of pregnancy.

TRANSFORMING GROWTH FACTOR-β (TGF-β) (OR SERIN/ THREONINKINASE)RECEPTORS

Transforming Growth Factor-beta (TGF-ß)

Cytokine mediators of tissue growth and remodeling, such as TGF-β, are important regulators of implantation and placentation. Members of the TGF-β superfamily involved in these processes are TGF-β1-3 as well as activins [283]. TGF-β1, -2, and -3 each consist of a homodimeric structure of 25 kD and are stored in the extracellular matrix until activation, when a covalently-bound propeptide is cleaved from the molecule [284–286]. TGF-β was first shown to induce anchorage-independent growth in rat kidney cells [287] and mouse fibroblast cells [285] in the presence of epidermal growth factor (EGF) [287], but it can either induce or inhibit cell growth depending on the cell type and its environment [284].

TGF-β1 and -2 are expressed in human CTB [288] and active TGF-β has been detected on epithelial cells at the maternal-foetal interface in the pig [289], suggesting a role in maternal-foetal cross-talk. TGF-β receptors in trophoblast cell lines suggest a possible autocrine signaling as well [290]. TGF-β receptors have been detected on murine trophoblast giant cells, spongiotrophoblast and maternal decidua [291, 292] and on human microvillus membrane, STB and CTB [293, 294].

On binding TGF-β, the TGF-β receptor induces a hetero-tetrameric complex of serine/threonine kinase transmembrane receptors, which then phosphorylate Smad, a signaling molecule which then translocates to the nucleus[295]; this pathway is known to be utilized in trophoblast cells [296]. TGF-β can also utilize the MAPK and Rho-A/Rho-associated kinase (ROCK) signaling pathways [297]; the latter two have been suggested to be involved in trophoblast migration and are detected in CTB and syncytia [296]. Smad ubiquitination regulatory factor 2 (Smurf2) is an E3 ubiquitin ligase which can target Smad for degradation; its overexpression in the human trophoblast cell line HTR8 enhances migration and invasion [298]. While TGF-β and macrophage inhibitory cytokine (MIC-1), a TGF-β superfamily member, inhibits MMP-2 and MMP-9 production and trophoblast migration *in vitro*

[288, 299, 300], activin A promotes CTB invasion by stimulating MMP production [301]. Inhibition of TGF-β3 or the ROCK pathway increases invasion and MMP production in human trophoblasts [172, 302, 303], and exogenous TGF-β decreases invasion of trophoblasts *in vitro* [172, 303–305]. However, it has been shown to increase invasion in a rat placental cell line [306]; controversial findings in this area have been reviewed by Karen Forbes and Evangelia Pardali [297, 307]. In general, however, TGF-ß is understood to contain physiological trophoblast invasion, while choriocarcinoma (JAR, JEG-3) is impervious to this cytokine [308]. TGF-ß has been described to maintain trophoblast stem cell proliferation, which is unusual, since this cytokine is often considered an inhibitor of epithelial cell proliferation [309]. Indeed, TGF-ß has also been described as inhibiting EVT proliferation [310].

Knockout models are not commonly utilized because most TGF-β or TGF-βR knockouts are embryonic lethal on gestational day 10.5 in mice due to a defective yolk sac vasculogenesis [297, 311, 312]; in humans, TGF-β gene defects are associated with the Camurati-Engelmann disease [297]. One Korean based study indicated that a TGF-beta1 polymorphism may be a genetic risk factor for PE and IUGR-complicated PE [313], and maternal plasma concentrations of TGF-ß1 were significantly higher in PE patients than in healthy, pregnant controls [314]. In terms of recurrent spontaneous abortion, no association with TGF-ß1 gene polymorphism could not be traced [315].

TYROSINE KINASE RECEPTORS

Insulin-like Growth Factor (IGF)

IGF is a hormone and key growth factor and is one part of a huge family of proteins, which all can be summarized as the IGF family or the "IGF axis". In general the IGF axis includes the receptor ligands for IGF1 and IGF2, six different high-affinity binding proteins (IGFBP1-6), several more low-affinity binding proteins, known as IGFBP-related proteins (IGFBP-rPs) and finally receptors type-1 IGF receptor (IGF1R) and type-2 IGF/mannose-6-phosphate receptor (IGF2R/M6PR) (reviewed in [307, 316–321]). All of these members, expressed ubiquitously in tissue-specific ratios and amounts, regulate important cellular functions like proliferation, survival, differentation, cellular metabolism and others [316]. For this reason, more or less all IGF axis proteins play crucial roles in a wide variety of cellular processes in normal physiology and pathophysiology including growth [322], tumorgenesis [316] and many reproductive events such as follicular development [323, 324], oocyte maturation [325], preimplantation- and embryo development [326] and finally placental function/placentation and, associated with that, foetal growth [307, 317].

Within human placental regions IGF1 and IGF2 mRNAs are expressed with a similar tissue distribution and primarily by foetal tissues including CTB, EVT for IGF1- and 2 and the chorionic plate, mesoderm and leave (IGF2). The IGF2 mRNA was abundant at all gestational ages. In contrast to this, IGFBP mRNAs are expressed almost exclusively by maternal tissues (decidual cells) and were identified in variable abundance [327, 328]. Generally, IGFBPs are modulatory regulators of IGF proteins, which means they have controversial functions and they can inhibit or stimulate IGFs [329].

The classical and preferential mode of action of the two IGF ligands is through the two IGF receptors, but they can also bind to the insulin receptor (IR). Conversely, insulin can also bind to IGF receptors, which results finally in a complex interacting receptor network [316]. IGF1 and IGF2 mostly prefer the IGF1R and IGF2 the type-2 IGF receptor and the insulin receptor IR-A [316, 317, 330]. The IGF1R mRNA is expressed in all cell types of the placenta and [327], for this, IGF1Rs are localized in villous endothelium and stroma, trophoblast and decidua [331, 332]. The activation of the tyrosine kinase receptor, IGF1R, by ligand binding leads to the activation of a complex signaling network across the two major signaling pathways PI3K-AKT and RAS-RAF-MAPK [318, 333–335]. The IGF2R/M6PR like IGF1R are expressed primarily on the maternal-facing microvillous membrane of the STB [331]. The type-2 IGF receptor has no intrinsic kinase activity and is consequently regarded as non-signaling [336] and it was suggested that the primary function of this receptor is to clear IGF2 from circulation and to prevent excessive IGF2 effects on the placenta (293). However, McKinnon et al. [337], showed in transwell migration assays with HTR8/SVneo cells that IGF2 stimulates EVT cell migration by signaling through IGF type-2 receptors independently of IGF1R and IGFBPs and further involves signaling via inhibitory G proteins and the MAPK pathway.

It is well known that placental development correlates with foetal growth and that alterations in the placental structure and function leads to infants with intrauterine growth restriction (IUGR) [338]. The involvement of IGF axis proteins, especially the ligands and the receptors, in placentation can be supported

by the fact that null-mutations and knockout of IGFs and/or IGF receptors in mice mostly lead to IUGR [330]. However, IGF2R/M6PR knockout mice present foetal overgrowth and higher perinatal lethality, which leads to the conclusion that IGF axis proteins, especially the receptors, are crucial for the regulation of normal growth [339]. For a good overview of mice with many different null mutations of the IGF family, please refer to the review of Nakae et al. [330].

Besides many functions including proliferation, migration, nutrient exchange and amino acid uptake, as well as inhibition of apoptosis of IGF axis proteins during placentation [317, 340], many authors showed that IGF proteins, especially IGF2 via the type-2 receptor and IGFBP1, stimulate EVT cell migration and enhance the invasion of EVT cells to the maternal decidua [337, 341–344]. To underline and detail this statement, Hamilton et al. [342] conclude that after transwell migration and additional Matrigel invasion assays with human first trimester EVT cells, that trophoblast-derived IGF2 and decidua-derived IGFBP1 provide autocrine/paracrine enhancement of trophoblast invasiveness largely by stimulation of migration. Furthermore Hamilton and his colleagues showed that EVT cell proliferation was unaffected by a wide range of IGF1, IGF2 and IGFBP1 concentrations and they also showed that blocking of the type-1 receptors by antibodies did not affect the IGF2-mediated invasion, indicating that IGF1R was not responsible for the IGF2 effect. The migration stimulatory action of IGFBP1 occurs by binding of its RGD (Arg-Gly-Asp) domain to the α5ß1 integrin (fibronectin receptor) on the EVT cell surface, leading to activation of focal adhesion kinase (FAK) and stimulation of the MAPK pathway [337, 344].

While IGF2 and the IGF2R play an important role for placenta development, this is also the case IGF1, which promotes CTB differentiation and IGF1R are crucial for normal placenta function [292]. For example decreased maternal serum IGF1 levels were found in women who developed preeclampsia [345] and also mutations in the human IGF1R gene are associated with intrauterine and postnatal growth retardation [346]. A G1125A mutation was identified which resulted in a kinase-deficient IGF1R, which likely caused the phenotype of intrauterine and postnatal growth retardation in a girl and members of her family who had a history of this phenotype [347]. Maternal diabetes resulted in inverse changes of circulating foetal IGF-1 and IGFBP-1 at birth. A decrease in circulating IGFBP-1 and to a lesser extent an increase in circulating IGF-1 may present an important mechanism that contributes to increased birth weight in diabetic pregnancies [348].

Epidermal Growth Factor (EGF)

The CTBs, as mentioned in the introduction section, are a progenitor stem cell population of trophoblast which continuously proliferate and differentiate into EVTs or STBs. EVT invasion play an important role for placental growth and successful pregnancy, especially during the first trimester [349, 350]. Previous investigations have revealed that the trophoblast cell is strongly influenced by cytokines and growth factors, of which one of the most relevant is EGF. Fourteen different ligands comprise the EGF family [351], including heparin-binding EGF-like growth factor (HBEGF) [352] and Transforming growth factor alpha (TGF-α) [350]. Binding of EGF to its receptor (EGFR) stimulates intrinsic tyrosine phosphorylation activity and transautophosphorylation [353], which results in the activation of a variety of intracellular pathways, comprising of the Ras/MAPK, PI3K/Akt, phospholipase C-γ/protein kinase C (PLCγ/PKC), transcription factor AP-2α and STAT [354–357]. In the human placenta, it has been established that MAPK11/14 and AP-2α are important for EGF-mediated functions in the trophoblast.

The importance of EGF has been demonstrated using *in vitro* or *in vivo* studies, in which human placental cell lines, isolated primary trophoblasts, and explant tissues are the most widely used [350]. EGF is able to influence positively or negatively a variety of fundamental (trophoblast) cell properties, such as proliferation [358–360], differentiation [358], apoptosis [361–364], motility [355, 356, 365], secretion [355, 366] and invasion/migration [367–370]. CTB proliferation and differentiation into syncytial units can be stimulated in culture by EGF [358]. Interestingly, EGF-stimulated differentiation requires MAPK11/14 activation, while EGF-driven trophoblast proliferation is enhanced when MAPK11/14 is inhibited [358]. AP-2α has been found to promote EGF-dependent EVT invasion, probably through alteration of MMP-2, uPA and PAI-1 activity [371]. EGF also induces MMP-9 and TIMP-1 secretion in the trophoblast through activation of both PI3K and MAPK signalling pathways [355]. It should be noted here though, that differences in EGF effects have been seen between primary trophoblasts and cell lines (SGHPL-4) – gene silencing of AP-2α had no effect on SGHPL cells, while the invasive capacity of primary EVT was reduced [371].

EGF and its receptors are expressed both in decidual and trophoblastic cells. CTB, STB, and foetal connective tissue cells in first-trimester tissues stained positive for both the EGF binding domain of the human EGF receptor or to the activated (tyrosine-phosphorylated) human EGF receptor. In contrast, staining of third-trimester placentae for both targets yielded only a sparse staining of either trophoblast cell layers but an intense staining of the foetal connective tissue cells [372]. EGF was localized immunohistochemically in the human endometrium throughout the menstrual cycle, in gestational decidua, and in first, second, and third trimester placenta. Stromal cells, but not glandular epithelium localisation suggested that EGF-production occurs in the proliferative endometrium and this secretion seemed more intense during the secretory phase especially in those stromal cells surrounding the spiral arterioles. Immunostaining for EGF in stromal cells is again moderate in gestational decidua, but intense in the surface epithelium. EGF immunostaining was intense in STB of first trimester placenta, with a moderate staining of CTB, but decreased in both layers of trophoblast as pregnancy progressed [373].

Despite the above, the role of this growth factor in foetal growth regulation is still only partially understood. Animal models have been able to confirm that EGFR is required for numerous developmental and physiological processes Conditional knock-out of EGFR (sometimes termed HER-1 or erbB-1) cause embryonic or perinatal lethality of the affected mice due to growth retardation, smaller placentas and impaired epithelial development of several organs [374, 375]. However, EGF −/− mice show no effect of genotype or background strain on litter size, gender ratio, pup body weight, and survival of foetuses in utero [376].

Investigations in humans have found that a polymorphism in the 59 untranslated region of EGF that results in increased EGF expression is associated with lower birth weight and foetal growth restriction in pregnant women from Western Europe [377, 378]. In the human placenta, an altered expression of the EGF receptor is associated with preeclampsia, intrauterine growth restriction (IUGR), persistent trophoblastic disease and pathological trophoblast invasion [379].

Vascular Endothelial Growth Factor (VEGF)

VEGF is a heparin-binding homodimeric glycoprotein of 30–40 kD which is involved in many processes related to reproductive physiology [380, 381]. It is a potent mitogen of endothelial cells which promotes the remodeling of the early vasculature and stimulates the formation of a capillary network of the placenta [382]. This cytokine also positively regulates the proliferation of trophoblast [383–385] (probably through better vascularisation) and its expression is altered in recurrent abortion [386, 387].

The VEGF family is an important mediator of angiogenesis and consists of six members: VEGF-A-E and placenta growth factor (PlGF) [388-393] have been identified up to date. The angiogenesis function is regulated by the receptors VEGFR-1 (flt-1 or FMS-like tyrosine kinase), VEGFR-2 (KDR or kinase domain-containing receptor), VEGFR-3 which have tyrosine kinase activity [394]. VEGF and its receptors have been detected in giant trophoblast cells, early yolk sac [395], human endometrium [396], human and animal placentae [397, 398], fallopian tube and ovary [399] suggesting a role for this factor in the induction of vascular growth in decidua, placenta and vascular membranes [400]. In the ovary, VEGF is produced by both thecal and granulosa cells [401, 402]. In fact, in situ hybridization studies in rat ovary provided the first evidence that VEGF may be a regulator of physiological angiogenesis [403]. Post-implantation embryos in VEGF± mice have shown several malformations in the vascular system resulting in lethality on days 11 and 12 of pregnancy, strongly suggesting a regulation of foetal vascular development by VEGF [404]. Furthermore, results of targeted gene disruption in the mouse have revealed that the two VEGF receptors, Flt-1 and KDR/fetal liver kinase-1 (Flk-1), are functionally important for embryonic vasculogenesis. Heterozygous VEGF-deficient mouse embryos show impaired or abnormal blood vessel formation, leading to embryonic death by mid-gestation [404, 405]. Homozygous Flk-1/KDR deficient mice died in utero as a result of early defects in the development of hematopoietic and endothelial cells, which are necessary for yolk-sac blood island formation and vasculogenesis [406]. Homozygous Flt-1 gene-deficient mice also died in utero, most likely because of deficient cell-cell or cell-matrix interactions, causing endothelial cells to form abnormal vascular channels [407].

VEGF mRNA has been detected in human preimplantation embryos at the blastocyst stage supporting a possible role in others reproductive events [405]. The receptor for VEGF, Flt-1, is produced in the decidua by chorionic vascular endothelium and especially by invasive EVT and, *in vitro*, by BeWo choriocarcinoma cells [408]. However, VEGF seems to mediate proliferation rather than invasion/migration in EVT [408]. In the trophoblastic cell line JEG-3, cultures

containing VEGF showed that *in vitro* migration of JEG-3 cells through a transwell membrane was significantly reduced by silencing of VEGF. Therefore, a role of VEGF was postulated in the regulation of trophoblast migration [25]. It is also known that VEGF acts via autocrine stimulation loop in trophoblastic cells [409]: in normal human CTB, TGF-beta1 induced an hypoxia inducible factor-1α (HIF-1α)-mediated VEGF secretion (and a TGF-ß1-stimulated-ERK1/2 activation may be involved in this process) [410]. Moreover, both the PI3K–AKT–mTOR (mammalian Target of Rapamycin)–HIF-1α and ERK–HIF-1α signaling pathways are crucial for increasing VEGF and endoglin expression in response to hypoxia in BeWo cells [411].

It has been shown that VEGF is predominantly expressed in tumour cell lines derived from female reproductive organs [412]. In tumors, VEGF is known to significantly contribute to pathological angiogenesis, tortuosity of tumor vasculatures and vasculogenesis, which all together lead to accelerated growth rates of tumors, invasion and metastasis [413]. VEGF family members regulate CTB survival and expression of a subset of its family members is dysregulated in severe forms of preeclampsia [414]. Currently, assays have been implemented clinically to detect the concentration of soluble Flt-1 (sFlt-1), which binds and inactivates VEGF, and PlGF in maternal serum at mid-gestation. Unfavorable ratios of sFlt-1/PlGF can predict the development of PE [415].

Considering all these data, VEGF is most likely involved in many processes related to reproductive physiology, and which are essential for correct implantation and placentation.

Hepatocyte Growth Factor (HGF)

HGF is a pleiotropic cytokine whiuch was first defined as a potent mitogen for hepatocytes *in vitro* [416, 417]. HGF acts through binding to the receptor, c-Met, causing tyrosine kinase activation and autophosphorylation at tyrosine residues [418]. Following auto- phosphorylation, the PI3K and MAPK pathways are two main signaling cascades involved in mediating the HGF signal [419]. HGF is associated with cell proliferation, differentiation, invasion and angiogenesis in other cell systems [420].

The placenta has been shown to express a significant amount of HGF [416]. Serum of HGF levels increase throughout the first, second and third trimesters of pregnancy, respectively [417]. Furthermore, the second trimester period displays the highest HGF level in amniotic fluid, but the rate of HGF production from placental tissue is not significantly different between the three periods [417]. Mesenchymal cells of the villi express HGF mRNA in situ and CTB express HGF receptor, c-Met [421, 422]. In addition, c-Met protein is expressed in choriocarcinoma cell lines [423, 424].

In vivo "gene knockout" studies in mice lacking the HGF gene show placental defects and embryonic lethality which were identified as a complete lack of development of labyrinthine trophoblast at 13.5–14.5 ([425] and reviewed in [426]). A single injection of HGF/SF at embryonic day 9.5 (E9.5) into the amniotic cavity of HGF/SF −/− embryos rescued the placental defect [427]. In c-Met mutant mouse embryos, identical placental and additional liver abnormalities were found [428]. Transgenic mice that overexpress HGF, seem to have lower tumor yields or a lower incidence of hepatocellular cancers [429].

HGF stimulates nitric oxide (NO) synthesis involved in the human EVT cell line SGHPL-4 invasion of fibrin gel [430]. HGF regulates trophoblast invasion through the activation of c-Met and consequent secretion of 92-kDa collagenase as determined by the Boyden chamber invasion assay [431]. Trophoblast motility is an important step for the invasive process. Cartwright et al found that HGF stimulates SGHPL-4 motility and invasion by activation of the PI3K pathway [432]. Inhibition of the MAPK pathway also inhibited HGF-induced motility of primary human EVT, whereas not effect on basal motility [432]. The homeobox gene HLX is expressed in proliferating and migrating (but not invading) human trophoblast cells and HLX expression is significantly decreased in human IUGR. In SGHPL-4 and HTR-8/SVneo cells, HGF stimulated the production of HLX mRNA and protein expression, which resulted in increased trophoblast cell migration. Reciprocally, HLX inactivation significantly decreased trophoblast migration, thus the HLX gene is also a key of trophoblast cell migration via HGF/c-Met signaling pathway [238]. Finally, although much attention has been dedicated to HGF and its effects on trophoblast invasion, only little to no attention is paid to its effects on trophoblast proliferation.

As to be expected from the above studies, HGF seems to be associated with IUGR. HGF and c-met expression are reduced in IUGR placentae [433]. Recently, plasmatic HGF measurements at around mid-gestation (14–20 gestational weeks) were successfully implemented as a predictive marker for small-for-gestational age foetuses, but not for preeclampsia [434]. In this context, no direct information can

Table 1
Selected Type I receptor cytokines in reproduction

Cytokine	Distribution in reproduction	Trophoblast effects					Murine KO model	Positive/or negative association with human pregnancy pathology	Signal transduction • trophoblast(ic) cells ○ other cells	Effector
		Receptor expression	Invasion/ migration	Proliferation	Differenttiation	other effects				
IL6	E: EM, decidua, EVT, STB, CTB, human and animal placenta	STB, EVT	JEG-3: + (indirect proof, since siRNA of IL6 and its rec resulted in ↓ invasion)	JEG-3: + (indirect proof, since siRNA of IL6 and its rec resulted in ↓ proliferation)	+ into thee invasive phenotyp		Reduced fertility and viable implantation sites	Human and mice recurrent abortion, infertility, PE	• STAT3	↑ MMP-9, -2
IL-11	E: chorionic villi, decidua R: EM, implantation site	IT, endEVT, JEG-3	JEG-3: + HTR-8/SVneo: -	No effect	Not known		Infertility due to defective deciualization	Reduced fertility, spontaneous abortions	○ JAK1/2 ○ STAT1 ○ ERK1/2 • STAT3	Not known
GM-CSF	E: chorionic villi, placental macrophage, villous fibroblast, epithelial cells of reproductive tract, EM-glands	JEG-3 JAR Invading CTB, EVT, (weak on STB)	Not known	CTB: +	+ terminally into STB		Impaired fertility and offspring survival, IUGR, small litter sizes, diminished glycogen cells in placenta	PE, recurrent miscarriage, placental insufficiency, IUGR, preterm delivery	○ MAPK ○ JAK2/ ○ STAT5 ○ PI3K	Not known
LIF	E: endometrial glands, blastocyst, granulose-lutein cells, ovarian stromal cells,	Trophectoderm, villous trophoblast, EVT	JEG-3: + (Matrigel)	+	+ into CTB, STB, EVT		LIF−/−: fertile, but no implantation; LIFR −/−: impaired placental function	↓ endm. LIF expression in recurrent abortion, unexplained infertility; ↑ LIF gene mutation in infertile women	○ RAS/ ○ MAPK • STAT3	↑TIMP ↓ Casp4
G-CSF	E: decidual cells in contact with anchoring villi (not chorionic trophoblast) R: decidual stromal + EM gland cells	EVT, ST, CT	Not known	CTB: + Chorio-Ca cell lines:-JEG-3: 0	Not known		Not known	PE, preterm birth, biomarker for competent oocyte	• JAK/STAT • MAPK	Not known
Leptin	E: 1st trim chorionic villi, 3rd trim. chorion leave, amnion, STB, CTB	EVT, STB, IT, JAR, BeWo, (weak in CTB)	Mouse trophoblast: + CTB: +	Cell line: + JEG-3 JAR(in presence of 25 mM glucose)	Not known	Anti-apoptotic	Infertility due to implantation problems and placental anomalies	PE, IUGR, gestational diabetes	• MEK/ • SOCS3 (mouse trophoblast)	↑ MMP-9, 2

Table 2
Selected Type II cytokine receptors in reproduction

8, 5	Distribution in reproduction	Trophoblast effects					Murine KO model	Positive/or negative association with human pregnancy pathology	Signal transduction • trophoblast(ic) cells ○ other cells	Effector
		Receptor expression	Invasion/ migration	Proliferation	Differentiation	Other effects				
IL10	E: CTB, decidual stroma cells	CT EVT (little)	CTB:↓ Also indirectly by disrupting macrophage	Not known	↓ to invasive phenotype		- implantation sites are increased + more viable foetuses, - higher rates of LPS-mediated miscarriage, preterm birth, IUGR, - human PE-serum mediated impaired spiral artery remodelling and PE-like symptoms	production in PE, ↑ single nucleotide polymorphism in PE, early pregnancy failure	○ JAK/STAT	↓ MMP-9
IFN-γ	E: uNK R: uterine epithelium and stroma (especially adjacent to attaching trophoblasts)	Villous CTB, CTB cell columns	1st trim EVT: - JEG-3: - (both Matrigel)	Not known	Not known		Large number of undifferentiated uNK cells causing necrosis	↑ production in PE, ↑ levels in umbilical cord serum associated with decreased risk for IUGR; gene polymorphisms associated with recurrent pregnancy loss	• STAT1 • CIITA	↓ MMP2/9

Table 3
Immunglobulin receptor. Tumor necrosis factor receptor, Chemokine receptor and TGF-ß receptor cytokine families

Cytokine	Distribution in reproduction	Trophoblast effects: Receptor expression	Invasion/ migration	Proliferation	Differentiation	Other effects	Murine KO model	Positive/or negative association with human pregnancy pathology	Signal transduction • trophoblast(ic) cells ○ other cells	Effector
A: Immonoglobulin receptors										
IL-1β	E: CTB, STB, 1st trimester decidua, secretory EM R: decidual EM- glands, STB	STB, CTB, EVT	CTB, EVT: + by 50% (Matrigel) JEG-3: + HTR8: + SGHPL-4: 0 EVT cell line: -	JAR:- Bewo:-	No effect toward invasive phenotype	Induction of apoptosis	Normal, healthy, fertile	↑ in placenta of PE (not IUGR), ↑ level in amniotic fluid of preterm birth pregnancies, ↑ maternal level in miscarriage	• MAPK • LPM2	↑ MMP-2, -3, - and -9, MCP-1
CSF1	E: EM-glands, placenta, decidua into uterus +amniotic fluid, vascular endothelium. R: EVT, decidua	1st trim EVT	EVT: 0 (Matrigel)	EVT: + TCL-1: 0 HTR8/ SVneo: 0/ + BeWo: - SGHPL-4: +	Not known	Not described	Reduced fertility due to low implantation rates; normal phenotype	PE, IUGR, recurrent abortion	• TK • HLX	EVT: ↑ MMP-9, ↑ TIMP-1 TCL-1: 0 for MMP
B: Tumor necrosis factor receptor										
TNF-α	E: deciduas,: EVT, villous CTB R	Intramural EVT, endEVT	CTB: 0 EVT: - HTR8/ SVneo: - (all Matrigel)	EVT: - Other primary trophoblast: -		Apoptotic (EVT)	Viable, fertile; however, TNF-α and IFN- needed for Vα14 NKT-cell mediated abortion	PE, IUGR, preterm birth (however risk alterations according to maternal BMI), reproductive failure	• ERK $\frac{1}{2}$ • SAPK/JNK	PAI-1 ↑ pro-MMP-9, but not active MMP-9 (EVT) ↓ MMP-2 (for JEG-3, not EVT)
C: Chemokine receptors										
IL-8	E: villi, EVT, uNK cells, Hofbaur cells, EM into cervix, Fallopian tubes, placenta	1st trimester CTB, EVT	EVT: + (Matrigel)	HTR-8/SVneo: + Bewo: 0	Not known		Not known	PE, IUGR, miscarriage	○ FosB	↑ MMP-2, -9 ↑ uPA (in other tumors)
D: TGF-β receptors (serine-threonine recptors)										
TGFβ	E: CTB, pig placental epithelial cells R: murine giant- and spongio- trophoblast, human decidua	CTB, STB,	CTB: + HTR-8: + EVT: - JAR: 0 JEG-3: 0	Trophoblast stem cell: + EVT: -	Not known		Embryonic lethal E 10.5 due to defective yolk sac vasculogenesis	Increased TGF-β1 gene polymorphisms in PE, elevated plasma levels of TGF-β1 in PE	• Smad, • Rho-A/ ROCK • Smurf2	↓ MMP-9, -2 production

Table 4
Tyrosine kinase receptors

Cytokine	Distribution in reproduction	Trophoblast effects:					Murine KO model	Positive/or negative association with human pregnancy pathology	Signal transduction • trophoblast(ic) cells ○ other cells	Effector
		Receptor expression	Invasion/ migration	Proliferation	Differenttiation	Other effects				
IGF-1/2	E: CTB, EVT, chorion R: villous endothelium, stroma, decidua	STB	EVT: + (Matrigel) HTR8: +	EVT: 0	IGF-1: CTB +	Antiapoptotic	IUGR or foetal macrosomia	PE, IUGR, SGA	IGF-1: • MAPK IGF-2: • non signalling • G-protein, • MAPK	α5β1 activation
EGF	E: EM (stroma surrounding spiral aterioles), decidua, placenta (STB, later CTB + STB) R: foetal connective tissue	STB, CTB	EVT: + JEG-3: + JAR: + (MAtrigel)	CTB: +	CTB: + (into syncytial units)		Midgestational embryonic lethality, IUGR and placental abnormalities (small)	PE, IUGR, persistent trophoblastic disease, Gene polymorphism for ↑ EGF production: ↑ rate IUGR	○ Ras ○ MAPK ○ PI3K/Akt ○ PLCγ/PKC ○ RhoA/C ○ JAK/STAT • MAPK11/-14 • AP-2α	↑MMP-2/9 uPA PAI-1 ↑TIMP-1
VEGF	E:, placenta (+ animal), Fallopian tube, ovary (thecal + granulosa cells) R: giant trophoblast, yolk sac, human EM	JEG-3, CTB,	JEG-3: + (indirectly, since silencing of VEGF resulted in ↓ invasion)	EVT: + (indirectly through better vascularization)	Not known	Antiapoptotic (CTB)	Post-implantation vascular malformations with lethality at E11 + E12	Recurrent abortion, PE	• HIF-1 α • ERK • PI3K • Akt • mTOR	
HGF	E: placenta, mesenchymal cells of villi	CTB, chorio-ca cell lines	EVT: + (Boyden chamber) SGHPL-4: +	Not known	Not known	Antiapoptotic	Placenta defect caused by decrease of labyrinthine trophoblast with homozygous embryo lethality between E13.5–E15.5	PE, IUGR	• PI3K • MAPK • Homeobox gene HLX	↑collagenase

Table 5
ACRONYMS, ABBREVIATIONS & SYMBOLS/GLOSSARY

-: negative effect
+: positive effect
↑: up-regulation
↓: down-regulation
Akt: RAC-alpha serine/threonine protein kinase
AP: activator protein
BeWo: a choriocarcinoma cell line

Ca: carcinoma

Casp.: caspase

CIITA: IFN-inducible class II transactivator

CMV: cytomegaly virus

CSF-1: Colony stimulating factor-1 (synonomous with M-CSF and MGF)
CTB: cytotrophoblast
CXC: chemokine super family with four conserved cystine (C) residues and X is any amino acid
HIF: hypoxia inducible factor
E: cytokine expression

EGF: epidermal growth factor
ELR: Glu-Leu-Arg or Glutamic acid-Leucine- Arginine
EM: endometrium
endEVT: endovascular EVT
EVT: extravillous trophoblast
FAK: focal adhesion kinase
Flk: fetal liver kinase
Flt: FMS-like tyrosine kinase (a VEGF receptor)

FosB: proteins encoded by the c-fos genes

G-CSF: granulocyte-colony stimulating factor

GM-CSF: granulocyte macrophage-colony stimulating factor
gp130: glycoprotein 130
HBEGF: heparin-binding EGF

HGF: hepatocyte growth factor

HLX: H2.0-like homeobox protein- a human protein encoded by the HLX gene
HTR8/SVneo: a trophoblast cell line (HTR8) derived through transfection of EVT with simian virus neo
IFN: interferon

IGF: insulin-like growth factor

IGFBP: IGF binding protein
IL: interleukin

IT: intermediate trophoblast)
IUD: intrauterine device (for contraception

IUGR: intrauterine growth retardation
JAK: Janus Kinase

JAR: a choriocarcinoma cell line
JEG-3: a choriocarcinoma cell line
JNK: Jun N-terminal kinase
KDR: kinase domain- containing receptor
LIF: leukemia inhibitory factor
MGI: macrophage granulocyte inducing (protein)

LPM-2: low molecular mass polypeptide

M6PR: mannose-6-phosphate receptor

MAPK: mitogen activated protein kinase, pathway includes : Ras/ Raf/ / ERK/ MEK/ JNK
MCP-1: monocyte chemoattractant protein-1
M-CSF: macrophage-colony stimulating factor (synonomous with CSF-1 and MGF)
MEK: MAP or ERK Kinase

MGF: macrophage growth factor (synonomous with M-CSF and CSF-1)
MIC: macrophage ihibitory cytokine

MMP: matrixmetalloproteinase

LPS: lipopolysaccharide

mTOR: mammalian Target of Rapamycin
NK cells: natural killer cells

NO: nitric oxide
PAI: plasminogen activator inhibitor

PCB: polychlorinated biphenyl
PI3K: phosphatidylinositol-3-kinase

PKC: protein kinase C
PLC: phospholipase C
PLCγ: Phospholipase C gamma
PlGF: placental growth factor
R: receptor expression
Ras: RAt Sarcoma; a protein subfamily of small GTPases

RGD: Arg-Gly-Asp or Arginine- Glycine- Aspartat

ROCK: Rho-A/Rho-associated kinase

s: soluble, example: sIL-6R (soluble IL-6 receptor)
SAPK: stress-activated protein kinase
SGA: small for gestational age

SGHPL-4: trophoblast cell line (primary EVT transfected with SV40)
siRNA: short inhibitory RNA

Smad: a signal transducing protein; homologs of " mothers against decapentaplegic" (MAD) and the C. elegans protein SMA
Smurf: Smad ubiquitination regularory factor
SNAT: Na(+)-coupled neutral amino acid transporter
SOCS: suppressor of cytokine signaling
STAT: Signal Transducer and Activator of Transcription
STB: yncytiotrophoblast
TCL1: choriodecidua,immortalized through transfection with a retrovirus gene coding for the SV40 large-T antigencell line
TGF: transforming growth factor
TIMP: tissue inhibitor of metalloproteinase

TNF: tumor necrosis factor
Trim.: trimester
uNK cells: uterine NK cells
uPA: Urokinase-Type Plasminogen Activator
VEGF: vascular endothelial growth factor

be gleaned thus far from the literature on an association between preeclampsia and HGF although HGF is reported to have an effect on trophoblast invasion. In this aspect, it is interesting that the hypothesis of trophoblast invasion being causative for preeclampsia has been challenged [435]. It has proposed instead that trophoblast invasion is causative of IUGR and related alterations of foetal growth, which is in line with the information available thus far on HGF and its association to foetal growth retardation. Also in line with the above findings, HGF seems to be associated with trophoblastic disease [436]. Miscarriage, spontaneous abortion and preterm delivery have also not been associated with HGF yet.

CONCLUSION

It may be concluded that cytokines play a vital role in mammalian reproduction, including during human pregnancy. Much information has been unearthed in terms of where and when these cytokines are produced, and which cells possess receptors, indicating that specific cells within the reproductive tract are potentially capable of reacting to its corresponding cytokine. In the past decade, a tremendous array of data has been generated concerning the functional actions of these cytokines on trophoblast populations. This denotes the vast interest and potential that many international scientists believe this field of research occupies. This is in part due to the impact that is supposed to emanate from the results found in these studies in terms of preeclampsia and IUGR, but also of cancer.

It must be stressed however, that we are not close yet from reaching a therapeutic goal. This review exposes several research gaps, especially in terms of factual knowledge on signal transduction in the trophoblast. It may not be assumed that the mode of signal transduction for a specific mediator in a specific cell will be the same in the trophoblast, or even between the trophoblast(ic) subsets. It is vital to comprehend the exact regulating mechanisms of signaling mediators if these mediators are proposed for therapeutic interventions. In the example of LIF, for instance, the negative feedback mechanism driven through the STAT signaling system warrants caution in using LIF as a therapeutic agent, since both low as well as high LIF concentrations would results in an under-utilization of STAT3. This would probably negatively regulate trophoblast invasion. Therefore, understanding trophoblast(ic) signaling functions well should help to enforce innovation towards novel therapeutic approaches that will assist in enhancing reproduction on the one side, and on the other, combating cancer.

ACKNOWLEDGMENTS

Fitzgerald JS was supported by grants from the Deutsche Akademische Auslands Dienst (DAAD grant number: Po-50735372) and by the Interdisziplinäres Zentrum für Klinishe Forschung Jena (IZKF Jena).

The following co-authors were also supported by various exchange scientist programs of the DAAD: Abad C, Chaiwangyen W, Dubinsky V, Gueuvoghlanian Silva B, Gutierrez G, Junovich G, Markert UR, Morales-Prieto DM, Ospina-Prieto S, Pereira de Sousa FL, Suman P, Weber M.

Alvarez AM has received a grant from the Boehringer Ingelheim Fonds.

San Martin S. is supported by grants from DIPUV (Universidad de Valparaiso, Valparaiso, Chile; grant no. CI 05/2006), Programa de Investigación Interdisciplinario (PIA) from Comisión Nacional de Investigación en Ciencia y Tecnología (CONICYT) (Anillos ACT-73, Chile) and Programa de Cooperación Científica Internacional CONICYT/DAAD (190-2009).

Furthermore, we would like to thank the German Society of Reproductive Medicine (DGRM) for their support of the 2nd Jena InTReST (International Training in Reproductive Science and Technologies) School in the year 2010, without which this cooperational project publication would not have been possible.

The Placenta-Lab of the University Hospital Jena kindly received major funding from the German Research Foundation (DFG), Bundesministerium für Bildung und Forschung (BMBF) and the Thüringer Ministerium für Bildung, Wissenschaft und Kunst (TMBWK) during preparation of this manuscript.

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EXPERT REVIEWS

Understanding the link between the IL-6 cytokine family and pregnancy: implications for future therapeutics

Expert Rev. Clin. Immunol. 7(5), 603–609 (2011)

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Cytokines are involved in almost all processes during the menstrual cycle, the fertilization period and pregnancy. They are expressed in numerous reproduction-related body fluids and tissues. Disorders of cytokine expression patterns may cause pregnancy pathologies. Therefore, cytokines have the potential as new biomarkers in different body compartments for a variety of such pathologies. Furthermore, cytokines may also serve to treat fertility and pregnancy disorders. The IL-6-like family of cytokines is an intensively investigated group of cytokines with well-accepted functions in fertility and pregnancy. This article summarizes current knowledge on IL-6-like cytokines in regard of their role in reproduction and their potential for new strategies in the treatment of reproductive pathologies.

KEYWORDS: abortion • IL-6 • IL-11 • implantation • infertility • leukemia inhibitory factor • LIF • placenta • pregnancy • STAT • trophoblast

The IL-6-like cytokine family

Cytokines are present at all stages and levels of reproduction and pregnancy. They are, in different compositions and concentrations, expressed in all body fluids related with reproduction including seminal plasma, follicular fluid, mucosal exudations and amnion fluid, but they are also expressed in all related tissues. A variety of fertility and pregnancy disorders may derive from dysregulation of cytokine patterns. Therefore, these patterns may provide potential for development of new biomarkers or even new therapeutic strategies. The IL-6-like cytokine family has been intensively investigated during recent years. This article provides an overview of current knowledge and possible future applications of these cytokines.

The IL-6-like cytokine family is known by different synonyms, such as the gp130 family (all members use the gp130 receptor chain for initiation of intracellular signals) and the neuropoietic family (because of its functions in hematopoiesis and nerves). The members are IL-6, IL-11, IL-27, leukemia inhibitory factor (LIF), ciliary neurotrophic factor, cardiotrophin 1, neuropoietin and cardiotrophin-like cytokine (CLC) and oncostatin M. They all share structural similarities (reviewed in [1]). This article will focus on the role of IL-6, IL-11 and LIF because, within this family, they are the most investigated in reproductive sciences.

IL-6

A wide spectrum of cells produce the pleiotropic 26-kDa protein IL-6, including T and B lymphocytes, macrophages, dendritic cells, fibroblasts, endothelial cells, glial cells and keratinocytes. Furthermore, syncytio- and extravillous trophoblasts, main constituents of the placenta, also express IL-6 [2].

The kinetics of endometrial IL-6 expression reveal how well synchronized this process is during the human menstrual cycle and in pregnancy [2–4], indicating a role in endometrial function and in implantation. IL-6 induces intracellular signals via activation of the transmembrane receptor chain gp130, which is shared with other members of the IL-6 family, and the

10.1586/ECI.11.60

ISSN 1744-666X

specific IL-6 receptor (IL-6R) chain [1]. Both IL-6R chains are present in maternal and fetal tissues (endometrium, decidua and trophoblast) during implantation and placentation. Furthermore, in these tissues, a soluble form of the receptor (sIL-6R) exists, and both increase during the secretory phase of the menstrual cycle. During the first 10 weeks of pregnancy, the soluble receptor outweighs the membrane-bound form in the decidua, while during the second trimester, the longer membrane-bound form progressively increases [5]. In reproductive medicine, increased ratios of soluble gp130:sIL-6R and/or reduced sIL-6R expression associated with downregulation of IL-6R have been observed in placentas from patients with preeclampsia [6].

A number of studies indicate that dysregulation of IL-6 may disturb implantation and be harmful for pregnancy. These include reports that describe that no or very low expression of IL-6 or its receptors may be involved in the pathomechanism of recurrent abortion in mice and humans [7–9] as well as that high expression of IL-6 in the reproductive tract or blood may induce or accompany abortions [10,11]. A study on endometrial tissues from the mid-luteal phase of women with recurrent miscarriage demonstrated, by using quantitative PCR, a 53% reduction of mRNA encoding IL-6 in comparison with the control group [12]. In mice, IL-6 deficiency reduces fertility and the number of viable implantation sites [13]. In the CBA/J × DBA/2 abortion-prone mouse model, the application of a well-defined concentration (2500 units intraperitoneally at days 6.5, 9.5 and 12.5 of pregnancy) of recombinant IL-6 or vitamin E, induced a slight increase of IL-6 and reduced fetal resorption (equivalent to human abortion), while in the same study the application of 5000 units of IL-6 completely abrogated murine pregnancy [9,14]. This is in line with an observation study in humans that describes significantly increased plasma levels of soluble IL-6R and IL-6, but low levels of soluble gp130, in women with recurrent spontaneous abortions [15]. The responsible mechanism may be an IL-6-induced reduction of FOXP3$^+$ Treg cells in combination with an increase of Th17 cells [11]. Finally, it has also been reported that IL-6 levels in serum are not changed in women with threatened abortion or embryo loss [16]. Summarized, the aforementioned studies are partially contradictive and it remains unclear how far observations made in mice will be translatable to humans. Nonetheless, they indicate an involvement of IL-6 dysregulation (be it up or down) in abortion processes, which certainly needs further detailed investigation, and also insinuates the future possibility of correction by external modulation of the system through medical intervention.

For intracellular signaling, IL-6 uses the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway [17,18]. STATs are a family of transcription factors located in the cytoplasm, which after activation can form hetero- or homo-dimers and be translocated into the nucleus to control gene expression [19,20]. Several STAT isoforms are associated with regulation of implantation and maternal immune response in early pregnancy [21]. IL-6 stimulates system A amino acid transporter activity in primary trophoblast cells through phosphorylation of STAT3 at its tyr705 residue and increases expression of a Na(+)-coupled neutral amino acid transporter 2 isoform [22]. The JAK/STAT signal-transduction pathway is crucial in embryo implantation as shown by the embryonic lethality of STAT3-deficient mice [17]. This pivotal function of STAT3 activity in implantation and trophoblast invasion implies a potential participation of IL-6 in this process [18,23].

High levels of IL-6 are detectable in invasive cytotrophoblast cells [24]. They seem to increase the activity of matrix metalloproteinase (MMP)-9 and MMP-2 [25]. When IL-6 is silenced by siRNA in the trophoblastic cell line JEG-3 (choriocarcinoma), migration and invasion are significantly reduced, while silencing of both IL-6 receptors significantly decreases proliferation [26]. Also, in the immortalized human trophoblast cell line HTR8/SVneo, IL-6 induces proliferation, migration and invasion [27]. In a number of tumor cells of different origin, IL-6 enhances invasiveness of cells in different extracellular matrix-based assays [28–30]. In breast cancer, serum levels of IL-6 correlate with the extent of tumor invasion, lymph node metastasis and distant metastasis [31]. These observations suggests that many tumor cells use an IL-6 rich environment to invade and metastasize.

Taken together, these data suggest a contributing role for IL-6 in stimulation of trophoblast invasion, regulation of endometrial function and in implantation.

IL-11

IL-11 has pleiotropic effects on cells depending upon the cellular microenvironment. A variety of biological functions, such as cell proliferation and differentiation, but also progression of several carcinomas, are regulated by IL-11 [32–34]. At the site of implantation in mice, IL-11 is indispensable for embryonic development [35]. It transduces its signal through IL-11Rα (IL-11 specific receptor) and gp130 (a common coreceptor for the IL-6 family of cytokines) chains.

IL-11 is one of a small number of factors that are indispensible for implantation in mice [36–38]. IL-11 also plays a fundamental role in the physiological development of the placenta [35]. IL-11-induced signals are crucial for the decidualization of endometrial stroma cells [37]. Female IL-11Rα knockout mice are infertile due to defective decidualization of their endometrial stroma cells, although they appear physiologically normal [36,39]. In such a defective environment, trophoblast cells do not receive the necessary signals to proliferate and differentiate normally. The endometrium of rhesus monkeys displays highest expression of both IL-11 and IL-11Rα during the secretory phase of the menstrual cycle. They are colocalized at the site of implantation [40]. IL-11Rα is permanently expressed in the human endometrium from proliferative and secretory phase until 7–9 weeks of gestation [39]. It is also expressed by subpopulations of interstitial and endovascular extravillous trophoblast cells of first trimester human placenta [41]. In contrast to its receptor, IL-11 is barely detectable in the proliferative and secretory phase of the endometrium but it is highly expressed in the chorionic villi and in the decidua [39]. An inadequate production of IL-11 is related to a decreased fertility rate in humans [39]. A further study described that women with spontaneous abortions have decreased plasma levels of IL-11 [42].

The role of IL-11 in terms of the endometrial function regulation is well characterized but its significance in the regulation of the invasion of the trophoblast and choriocarcinoma cells is still controversial. JEG-3 choriocarcinoma express IL-11R and IL-11 increases their invasiveness through activation of STAT3 as well as of STAT1 and extracellular signal regulated kinases 1/2 without any significant change in the proliferation of these cells. In the extravillous trophoblast cells and in AC1.88 (a JEG-3-primary trophoblast hybrid cell line), IL-11 also increases their migration through activation of the STAT3-dependent signaling pathway [43]. In contrast to these, treatment of HTR-8/SVneo trophoblastic cells with IL-11 decreases their invasion through activation of STAT3 tyr705 [44].

Leukemia inhibitory factor

Leukemia inhibitory factor is a prominent cytokine with characteristics pointing to its fundamental utility in the field of reproduction (including humans) [45,46]. LIF was initially discovered by Metcalf and colleagues as a protein that induced murine myeloid leukemic M1 cell differentiation into macrophages [47]. LIF is a recognized expression product of numerous cell types such as neurons, hepatocytes and kidney and breast epithelial cells [48,49]. Its key function is especially understood in the mediation of inflammatory cell responses [50,51]. LIF is a Th2-type cytokine whose production is upregulated by IL-4 and progesterone, downregulated by various inflammatory mediators, such as IFN-γ, and which encourages the production of the pregnancy-protecting human chorionic gonadotrophin [52]. Furthermore, LIF also seems to control uterine receptivity prior to blastocyst implantation, or to influence trophoblast behavior (proliferation, invasion and differentiation) in various species [23,53–55]. Although its manifold effects on trophoblast cells have been widely described, studies on humans that confirm a role in abortion are still rare and preliminary [56].

Leukemia inhibitory factor is often perceived as an important modulator of human pregnancy for a number of reasons. Granulosa-lutein cells and ovarian stromal cells express both *LIF* mRNA and the protein itself. Indeed, the concentration of LIF in follicular fluids correlates with embryo quality, which hints at its vital role during physiological ovulation and early embryo development [57]. Conversely, LIF is expressed by the endometrium. This is most observable in the glandular and luminal epithelium, where its concentrations peak during the secretory and postovulatory phase of the menstrual cycle concurrent to a time when implantation is liable to occur [53,58,59]. Interestingly, trophoblast cells also express mRNA for the *LIF* receptor during the implantation window, suggesting intense communication between the endometrium and the blastocyst. Subsequent to adhesion, the blastocyst, now capable of independent LIF mRNA expression, increases trophoblast cell proliferation while initiating differentiation processes (into cytotrophoblast and syncytiotrophoblast cells) which ultimately result in an augmentation of trophoblast invasion [23,60–62]. Both villous as well as extravillous trophoblast subsets express the LIF receptor throughout gestation. While extravillous trophoblasts pass LIF-secreting decidual leukocytes, they express the LIF receptor and thus these cells communicate with each other [63].

LIF expression levels have been found to be both diminished or increased in uterine flushings, endometrial cell cultures or decidual cells from infertile (human) women with repeated abortions or unexplained infertility [64–67]. Women with higher LIF levels during the implantation window may have a better chance of establishing pregnancy than women with low LIF expression [68]. A matter of interest is that women bearing a copper T380A intrauterine device as an effective contraceptive, also maintain a lower uterine expression of LIF in comparison to control [45]. Nonetheless, faulty LIF protein expression is not responsible for negative (LIF-related) pregnancy outcome alone. Mutations of the *LIF* gene that potentially result in the functional deviance of LIF are associated with infertile women in comparison to fertile controls, and this is thought to be expressed in poor outcome after *in vitro* fertilization (IVF) treatment [69]. However, in a large randomized, double-blinded, placebo-controlled multicenter study on 149 patients, recombinant human LIF failed to improve the outcome of IVF treatment in women with recurrent implantation failure. In this study, the embryos were first transferred and then the patients received subcutaneous shots of 150 mg recombinant human LIF (r-hLIF) twice-daily for a period of 7 days [70]. This trial appears to demonstrate the failed potential of LIF as a therapeutic agent; however, this should not completely discourage physicians and scientists who favor LIF as an implantation marker, because prior assessment of LIF expression was not performed in the intervention groups. This makes it likely that many women who had physiologic uterine LIF concentrations received LIF supplementation. In this context, it is important to keep in mind the fact that LIF signaling is regulated through a negative feedback mechanism, meaning that both too much, as well as too little LIF will induce similar functional effects [71]. The ideal treatment regimen would include the controlled administration of LIF subsequent to evaluation of endometrial LIF expression, allowing for a balanced and appropriate cytokine supplementation. This being said, exogenous LIF administration throughout the early pregnancy (very much like supplementation with estradiol and progesterone) may have a potential to improve chances of not only achieving, but also maintaining pregnancy [72].

Embryos of LIF-knockout mice fail to implant, but these embryos can be rescued through LIF supplementation [38,73,74]. By contrast, LIF receptor knockout mice can implant, but die within 24 h prior to birth due to placenta-mediated hypoxia [75]. In primates, injection of a monoclonal antibody against r-hLIF into the uterine cavity during the peri-implantation period resulted in a significant reduction in fertility [76].

LIF triggers its effects by induction of a signaling heterodimer consisting of the specific LIF receptor and the subunit gp130 [46,77]. This activates the RAS/MAPK and JAK/STAT cascades [78–80]. STAT3, a member of the STAT family, is a key regulator of LIF-mediated trophoblast invasion as made visible through STAT3 knockdown experiments (discussed later). LIF alters the expression of proteases such as tissue inhibitor of metalloproteinase 1 and caspase-4 via STAT3, which elevates trophoblast(ic) proliferation and invasion, and STAT3 knockdown annuls these functions even in the presence of LIF [18,23,62].

Expert commentary & five-year view

Implications for future therapeutics

Members of the IL-6 cytokine family have been considered seriously for therapeutic approaches in reproductive medicine and obstetrics. The most advanced studies are available for LIF, but potential is also seen for IL-6 and IL-11. The current status registers that LIF has been patented as a supplement to culture media to promote the development of mammalian embryos to the implantation stage, since growth in the presence of LIF increases the percentage of embryos that reach the implantation stage than growth without LIF (US Patent 5962321; Inventors: Gough: [invented by] Nicholas Martin; Willson: Tracey Ann; Seamark: Robert Frederick [Beulah Park, Australia] [101].

Another advanced application is the nonsteroidal contraception: vaginally-applied PEGylated (conjugated to polyethylene glycol) LIF antagonist (PEGLA) blocks implantation. PEGLA administered by intraperitoneal injection inhibits bone remodeling whereas vaginally-applied PEGLA has no effect on bone. Furthermore, PEGLA has no effect in an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis, suggesting PEGLA cannot target the CNS. Vaginally-administered PEGLA is a promising nonhormonal contraceptive, one which could be delivered alone, or in tandem with a microbicide. Vaginal application reduces the total dose of PEGLA required to block implantation and eliminated the systemic effect on bone, showing the vagina is a promising site of administration for larger drugs which target organs within the reproductive tract [81].

However, severe side effects of targeting IL-6-like cytokines may be secondary to alterations on muscles, cardiovascular development, the immune system and the development of the nervous system [1].

These two examples also stand for the potential of the other IL-6 family cytokines. Principally, the intravaginal application of IL-6-like cytokines pose a theoretical mode to improve implantation results or to employ specific inhibitors for contraception. Current state-of-the-art on nonsteroidal contraception by IL-11 and LIF is elegantly summarized by Dimitriadis and Menkhorst [82]. As described previously, STAT3 is a signaling molecule used by all members of the IL-6 family. Therefore, it is not surprising, that blocking the phosphorylation of STAT3 by injection of a specific peptide inhibitor into murine uteri reduces implantation [83]. Since STAT3 is present in almost all cells, this method is not specific and possess a high risk of nondesirable side effects.

For improvement of implantation, it may be considered to supplement embryo cultures with IL-6-like cytokines or to apply them intravaginally in preparation of the embryo transfer. This, however, constitutes an enormous challenge to define the optimal concentrations for these types of applications, and we are far from reaching trial concepts suitable for therapy in humans.

In established pregnancy, IL-6-like cytokines or also several of their mutants, which induce functional abnormalities, may have the potential as new biomarkers, mainly in body fluids or cells obtained from the reproductive tract. As their reduction is reported for several pregnancy disorders, applications to prevent (recurrent) abortion or other severe pregnancy disorders could be considered. Blocking IL-6-like cytokines might be contemplated as a new strategy in cases of excessive trophoblast cell invasiveness, mola or choriocarcinoma [84]. Such new strategies will need to compete with existing chemotherapeuticals in regard to efficacy, side effects and costs.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Key issues

- The IL-6-like family of cytokines is an intensively investigated group of cytokines with well-accepted functions in fertility and pregnancy.
- Trophoblast cells of all pregnancy stages express receptors for IL-6-like cytokines that activate intracellular signaling cascades, such as the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which controls cell behavior.
- A number of studies indicate that dysregulation (up or down) of IL-6 may disturb implantation and be harmful for pregnancy.
- Members of the IL-6 cytokine family including leukemia inhibitory factor (LIF), IL-6 and IL-11 have been considered seriously for therapeutical approaches in reproductive medicine and obstetrics.
- LIF inhibition has the potential for novel nonsteroidal contraception strategies.

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- •• of considerable interest

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Journal of Cellular Biochemistry

Intranuclear crosstalk between Extracellular Regulated Kinase1/2 and Signal Transducer and Activator of Transcription3 regulates JEG-3 choriocarcinoma cell invasion and proliferation

Journal:	*Journal of Cellular Biochemistry*
Manuscript ID:	JCB-11-0464.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	03-Apr-2012
Complete List of Authors:	Morales-Prieto, Diana; University Hospital Jena,, Department of Obstetrics, Placenta Laboratories Ospina-Prieto, Stephanie; University Hospital Jena,, Department of Obstetrics, Placenta Laboratories Chaiwangyen, Wittaya; University Hospital Jena, Department of Obstetrics, Placenta Laboratories Weber, Maja; University Hospital Jena,, Department of Obstetrics, Placenta Laboratories Hölters, Sebastian; University Hospital Jena,, Department of Obstetrics, Placenta Laboratories Schleussner, Ekkehard; University Hospital Jena,, Department of Obstetrics, Placenta Laboratories Fitzgerald, Justine; University Hospital Jena,, Department of Obstetrics, Placenta Laboratories Markert, Udo; University Hospital Jena, Department of Obstetrics, Placenta Laboratories
Keywords:	trophoblast cells, JAK/STAT, LIF, MAP kinase, ERK, placenta, pregnancy

Intranuclear crosstalk between Extracellular Regulated Kinase1/2 and Signal Transducer and Activator of Transcription3 regulates JEG-3 choriocarcinoma cell invasion and proliferation

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Short title: ERK1/2 and STAT3 in JEG-3 choriocarcinoma cells

Keywords: Pregnancy, trophoblast, STAT3, LIF, ERK, MAPK, placenta, invasion

Number of figures: 7

Abstract (197 words)

Background and aim: Invasiveness of trophoblast and choriocarcinoma cells is in part mediated via Leukemia Inhibitory Factor (LIF)-induced activation of Signal Transducer and Activator of Transcription 3 (STAT3). The regulation of STAT3 phosphorylation at its ser727 binding site, possible crosstalk with intracellular MAPK signaling and their functional implications are the object of the present investigation.

Methods: JEG-3 choriocarcinoma cells were cultured in the presence/ absence of LIF and the ERK1/2 inhibitor, U0126. Alternatively, ERK1/2 was silenced by small interfering RNA (siRNA). Phosphorylation of signaling molecules (p-STAT3(ser727), p-STAT3(tyr705), p-ERK1/2(thr 202/tyr 204) was assessed per Western blot. Immunocytochemistry confirmed results, but also pinpointed the location of phosphorylated signaling molecules. To illustrate the activated STAT3 pathway further, its DNA-binding capacity was studied with a colorimetric ELISA-based assay. Colorimetric proliferation and Matrigel invasion assays portrayed analyses of STAT3 influence on cellular functions.

Results: LIF-induced phosphorylation of STAT3(tyr705) and STAT3(ser727), is significantly increased after blocking ERK1/2. STAT3 DNA-binding capacity and invasiveness are enhanced after LIF stimulation and ERK1/2 blockage. In contrast, proliferation is enhanced by LIF, but reduced after ERK1/2 inhibition.

Conclusion: Blocking ERK1/2 in JEG-3 choriocarcinoma cells does slightly induce LIF-induced STAT3 phosphorylation and increases STAT3 DNA-binding capacity by an intranuclear crosstalk, which leads to enhanced invasiveness and reduced proliferation.

Introduction

Embryo implantation is a decisive stage in the establishment of human and murine pregnancy and is accomplished when trophoblast cells invade into uterine tissue [Cheng et al., 2001; Knofler, 2010]. An intricate interplay of cytokines, growth factors and hormones secreted into the fetomaternal interface tightly controls this process [Dimitriadis et al., 2010]. Leukemia Inhibitory Factor (LIF), a member of the interleukin-6 (IL-6) family, is a cytokine which seems to play a pivotal role in human and murine reproduction [Aghajanova, 2010; Cheng et al., 2001; Dimitriadis et al., 2010; Guney et al., 2007]. Although LIF is mainly recognized for its regulatory functions of inflammatory cell responses in several cell types [Knight et al., 1999; Okahisa et al.], it also controls uterine receptivity for blastocyst implantation and influences trophoblast behavior by promoting proliferation, invasion and differentiation in mice and humans [Cullinan et al., 1996; Fitzgerald et al., 2008].

LIF triggers its effects by induction of a signaling heterodimer receptor consisting of the specific LIF receptor and its subunit GP130 [Cheng et al., 2001]. This activates the RAS/ Mitogen Activated Protein Kinase (RAS/MAPK) and Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) cascades [Heinrich et al., 2003; Knight, 2001; Schuringa et al., 2002]. In short, STATs are a family of cytoplasmic transcription factors which form hetero- or homo-dimers upon activation and translocate into the nucleus to influence target gene expression, such as Suppressor of Cytokine Signaling 3 (SOCS3), a negative feedback molecule [Decker and Kovarik, 2000; Rawlings et al., 2004]. STATs are associated with regulation of implantation, placentation and maternal immune

response in early pregnancy in humans and mice [Corvinus et al., 2003; Fitzgerald et al., 2010; Maj and Chelmonska-Soyta, 2007]. We have demonstrated in the past that STAT3, a member of the STAT family, plays a crucial role in regulating LIF-mediated trophoblast invasion [Fitzgerald et al., 2008; Fitzgerald et al., 2005b; Poehlmann et al., 2005].

On the other hand, MAPKs are a group of protein kinases that play an essential role in signal transduction pathways modulating gene transcription in the nucleus in response to changes in the cellular environment [Whitmarsh and Davis, 1999]. Numerous mitogens, growth factors and cytokines trigger their effects through ERK1/2, thus contributing to normal cell growth, but also to malignant transformation [Roberts and Der, 2007]. A recent study has demonstrated that LIF induces proliferation in the extravillous trophoblastic cell line, HTR8/svneo, via phosphorylation of ERK1/2 [Prakash et al., 2011]. Similarly, decreased Akt and ERK1/2 are associated to developmental restriction of dexamethasone-induced rat placenta [Ozmen et al.]. Altogether these studies highlight the importance of ERK1/2 in pregnancy.

Crosstalks between the JAK/STAT and MAPK pathways have been described as occuring regularly: SOCS3 binds and inactivates RasGAP, a negative regulator of Ras signaling, leading to increased Ras/MAPK pathway activity [Rawlings et al., 2004]. Conversely, in other cell systems, such as in thyroid carcinoma, activated MAPKs enhance transcriptional activity of STATs by specifically phosphorylating a serine residue near its C-terminus [Plaza-Menacho et al., 2007]. Full activation of STAT3 requires phosphorylation at its tyr705 and ser727 residues, which allows it to dimerize and translocate into the nucleus [Liu et al., 2008; Schuringa et al., 2000a]. Ser727

phosphorylation is stimulus-regulated and its presence is necessary for complete STAT3 activation during oncogenesis [Schuringa et al., 2001]. Additionally, its inhibition decreases DNA-binding activity of STAT3 after stimulation with IL-6 [Boulton et al., 1995; Decker and Kovarik, 2000]. To date, conflicting evidence exists concerning the kinase responsible for STAT3(ser727) phosphorylation. Some members of the MAPK family, such as Protein kinase C, Jun N-terminal kinase, extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and mammalian target of rapamycin (mTOR) seem to be involved, but their implications remain unclear [Busch et al., 2009a; Liu et al., 2008; Schuringa et al., 2000a; Schuringa et al., 2000b]. The apparent divergence of results may be due to the variation of cell systems and stimuli employed in the different studies.

Taken together, a better understanding of functional trophoblast regulation seems to require further investigation of the intracellular mechanisms which govern STAT3. This study was performed to assess the phosphorylation of ERK1/2 and STAT3, especially in regard to serine727 phosphorylation in JEG-3 choriocarcinoma cells after stimulation with LIF and the possible crosstalk between these molecules at cytoplasmic and nuclear levels. We further aimed to detect influences that these pathways have on JEG-3 invasion and proliferation by inhibiting ERK1/2 with U0126, a specific blocker of mitogen-activated protein kinase kinase (MEK) that phosphorylates ERK1/2.

Materials and Methods

Cell culture

JEG-3 choriocarcinoma cells (DSMZ, Braunschweig, Germany) were cultured in Dulbecco's modified Eagle's medium- F12 (GIBCO), supplemented with 10% heat-inactivated fetal bovine serum (FBS; SIGMA, St. Louis, USA) and 1X Penicillin/Streptomycin (PAA Laboratories; Pasching, Austria), and maintained under standard conditions (37°C, 5% CO_2, humidified atmosphere).

Protein isolation

For protein analysis, cells were seeded in 6-well plates to reach 60-70% confluence. The succeeding morning, cells were starved for 2 h in serum-free medium and subsequently, incubated with or without 10 mM of the chemical MEK inhibitor U0126 (Cell Signaling, Boston, USA) for another 2 h. Following this treatment, cells were challenged with 10 ng/ml LIF (Millipore, Schwalbach, Germany), washed in PBS, harvested and lysed in cell lysis buffer supplemented with protease inhibitors (Cell Signaling). Three freeze-thaw cycles in liquid nitrogen were performed to ensure the complete lysis of cells. After centrifugation (18,000 rpm, 30 min, 4°C), supernatants were collected and protein concentrations were determined by using a Bradford-based Bio-Rad Protein Assay (BIO-RAD, Munich, Germany).

Small interfering RNA treatment

Alternatively to the MEK inhibitor, JEG-3 cells were treated with pre-designed small interfering RNA (siRNA) for ERK1/2 (Ambion). The following are the 5'-3' oligonucleotide

sequences: Sense: GCAGCUGAGCAAUGACCAUtt and Antisense: AUGGUCAUUGCUCAGCUGCtg The STAT-3 DNA binding capacity was measured after 24 hours of transfection. Briefly, cells were seed in 6-well plates to reach 40-60% confluence. The next morning cells were washed with OPTIMEM (GIBCO) and 800 µl fresh OPTIMEM was added. Transfections were performed with Oligofectamine (Invitrogen) as suggested by the manufacturer. Concentration of oligonucleotides and oligofectamine dilution were 66nM and 1:2.75, respectively. After 4 hours of treatment, transfections were stopped by addition of F-12 medium (GIBCO) containing 30% fetal bovine serum without antibiotics.

SDS-PAGE and Western blotting

20 µg of protein lysates were suspended in gel-loading buffer (62.5 mM Tris-HCl; pH 6.8; 2% SDS; 25% glycerol; 1% phenol blue; 5% ß-mercaptoethanol), boiled for 7 min and resolved on 7.5% acrylamide SDS gels. Proteins were then transferred to a hydrophobic polyvinylidene difluoride membrane (Hybond-P; GE Healthcare, Freiburg, Germany). After protein transfer, membranes were blocked in milk-containing buffer for 1 h (1X TBS containing 0.1% Tween-20 with 5% w/v nonfat dry milk). Antibodies against p-STAT3(ser727), p-STAT3(tyr705), p-ERK1/2(thr 202/tyr 204), STAT3, ERK1/2 and ß-actin (Cell Signaling) were applied in a 1:1000-dilution over night at 4°C. Membranes were then washed (1X TBS containing 0.1% Tween-20) and incubated with peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling) used in a 1:10000 dilution for 1 h at room temperature. For detection, a luminol-based system (LumiGlo; Cell Signaling) was used as described in the instructions of the manufacturer.

Immunocytochemistry

Cells were trypsinized, centrifugated and resuspended in 500 µl medium. Slides were washed and sterilized with ethanol, coated with cells and incubated over night at 37°C. Subsequently, fresh medium supplemented with or without 10 mM U0126 was applied for 2 h, followed by stimulation with 10 ng/ml LIF. Staining of cells was performed by using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, USA), as follows: Cells were fixed in ethanol/methanol 1:1 for 5 min, washed in 0.1 M PBS, and nonspecific antigens were blocked with normal goat serum for 20 min at room temperature. After blocking, slides were incubated 1 h with the primary antibody diluted 1:100 (p-ERK) or 1:200 (p-STAT3(tyr705 or ser727) in Antibody Diluent (DAKO, Hamburg, Germany), washed again and incubated 30 min with biotinylated affinity-purified anti-rabbit-IgG (Cell Signaling). Thereafter, slides were treated with a solution of Avidin/Biotinylated enzyme Complex (ABC; Thermo Fisher Scientific, Bonn, Germany) for 30 min, followed by 2 min staining with 3,3 diaminobenzidin (DAB; Dako), and cell nuclei were stained with hematoxylin for 2 min. Finally, slides were dehydrated by an ethanol-to-xylene treatment, covered with Histofluid (Paul Marienfeld, Lauda-Königshofen, Germany), and stored at 4°C. Analysis was performed at a microscope Axioplan 2 (Carl Zeiss, Jena, Germany).

DNA binding capability assay

JEG-3 cells were grown to subconfluence, serum-starved for 2 h, and then treated or not with 10 nM U0126 and 10 ng/ml LIF as for the above described experiments. From these cells, nuclear extracts were prepared by using the Nuclear Extract Kit (Active

Motif, Carlsbad, USA). Briefly, cells were collected in ice-cold PBS in the presence of phosphatase inhibitors, resuspended in hypotonic buffer and treated with detergent to separate the cytoplasmic fraction from nuclei by centrifugation. The nuclei were then lysed and nuclear proteins were solubilized in lysis buffer.

STAT3 DNA-binding capability was measured by using the TransAM STAT3 Kit (Active Motif). In brief, 10 ng nuclear extracts were incubated with immobilized oligonucleotides specific for STATs. STAT3 bound to DNA was then detected through use of an anti-STAT3 antibody and a secondary antibody conjugated to horseradish peroxidase (HRP), followed by a colorimetric reaction. STAT3-DNA binding was spectrophotometrically quantified in a SPECTROstar Omega (BMG Labtech, Offenburg, Germany) [Schust and Berg, 2004].

Proliferation Assay

The effect of LIF and U0126 on JEG-3 cell proliferation was analyzed by using a Cell Titer AQeous MTS assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. Assays were commenced with $1x10^4$ cells/well in 96-well plates. Cells were cultured in serum containing F12 medium in presence or absence of 10 ng/ml LIF and 10 mM U0126. Cell proliferation was measured in triplicates after 0, 24 and 48 h incubation by adding 20 µl/well methyl tetrazolium salt (MTS) solution and measuring the absorbance at 490 nm on the above-mentioned spectrometer.

Cell invasion assay

Cell invasion assays were conducted by using BD Matrigel™ Growth Factor Reduced Matrix (BD Biosciences, Heidelberg, Germany) according to the manufacturer's

instructions. Hanging Cell Culture Inserts (Millicell; Millipore) were coated with Matrigel matrix (1:3 dilution in F12 serum-free medium) and incubated 30 min at 37°C to form a semi-solid gel matrix. 5 x 10^4 JEG-3 cells were suspended in 500 µl of serum-free medium (containing or not LIF and U0126) and seeded into the upper chamber of inserts on the gel matrix. 500 µl of the respectively identical medium was also filled into the bottom of the well. The chambers were incubated 24 h at 37°C. After incubation, cells on the upper side of the filter were removed by using cotton swabs. Cells that had invaded to the underside of the filter were first fixed with pre-cooled 80% ethanol (20 min at 4°C), then stained with 0.1% crystal violet (5 min) and rinsed with water. The dried inserts were de-stained with acetic acid 10% and the absorbance was measured at 630 nm.

Statistical analyses

All Western blots and immunocytochemical analyses have been repeated 3 times with qualitatively similar results. For kinetics of phosphorlation intensity of ERK and STAT proteins as well as for analyses of dose-dependency of LIF and U0126 on STAT3-DNA-binding, a two-tailed Pearson test was performed and the correlation coefficient (r) was calculated. $p<0.05$ indicates a significant correlation between stimulation time and band intensity or positive dose-dependency, respectively. For comparison between band intensities of a concrete time point and the control, a Student's t test has been done. For the other assays, statistical evaluation was performed by a Student's t test (for invasion assays: n=7; proliferation assays: n=5) and using the software packages SPSS version 17.0 (WPSS Ltd., Surrey, United Kingdom). Differences were considered significant when $p<0.05$.

Results

LIF activates JAK/STAT and RAS/MAPK pathways

Western blots demonstrated that stimulation of JEG-3 with 10 ng/ml LIF induces rapid phosphorylation (visible after 2 min) of both STAT3 phosphorylation sites (ser727 and tyr705) and ERK1/2(thr202/ tyr204). Phosphorylation remains increased during the entire analyzed period of 30 min. The positive correlation between the stimulation time and band intensity is significant for all analyzed factors (Pearson' correlation). A slight constitutive phosphorylation of all factors is detectable before cells were stimulated (fig. 1).

LIF-induced p-STAT3(ser727)and its translocation capacity is ERK1/2 independent

JEG-3 cells were pre-treated for 2 h with or without 10 nM U0126 and then stimulated with 10 ng/ml LIF for 10 and 30 min. As assessed by Western blotting, application of the MEK inhibitor U0126 almost completely blocks constitutive and LIF-induced ERK1/2 phosphorylation. The inhibition of MEK led to a slight, but significant increase of the phosphorylation of STAT3(ser727) and STAT3(tyr705), when band density values from all experiments with U0126 application were compared with alll experiments without U0126 independently from the LIF stimulations time (fig. 2).

To further confirm these observations with an additional method, the phosphorylation of STAT3 and ERK1/2 in JEG-3 cells has been analyzed by immunocytochemistry before and after LIF stimulation and after the respective pre-treatment with U0126. This method also allows for localization of phosphorylated factors within the cells.

In control cells, p-ERK1/2 is slightly detectable in the cytoplasm as well as the nucleus. After stimulation with LIF, ERK1/2 activation increases dramatically and is located mostly within the nuclei. In cells pre-treated with U0126, p-ERK is slightly visible in the nuclei of a few cells but no further activation occurs after LIF stimulation. In analogy to the Western blot observations, low levels of p-STAT3(tyr705 and ser727) are detectable and located in the cytoplasm in control cells. Stimulation with LIF induces an increase of phosphorylation and translocation of p-STAT3(tyr705 and ser727) into the nucleus. The slight increase of STAT3(tyr705 and ser727) phosporylation observed in Western blots after pre-treatment with U0126 is hardly visible with this method. The translocation of pSTAT3 into the nucleus seems to be unaffected (fig. 3).

ERK1/2 activation suppresses intranuclear STAT3 DNA-binding capability (independent of LIF)

Stimulation of JEG-3 cells with LIF for 4 hours significantly increases STAT3 transcriptional activity dose dependently between 0 and 200 ng/ml in presence of 10 nm U0126 ($p<0.05$, Pearson's correlation). A significant increase was already detectable after 30 min LIF stimulation (not shown). Increase of STAT3 DNA-binding activity induced by 10 ng/ml LIF was significantly dependent on previously applied doses of U0126 ($p<0.05$, Pearson's correlation).

Inhibtion of ERK1/2 either by siRNA or by U0126 induced a slight, but significant increase of STAT3-DNA-binding also without subsequent LIF stimulation (fig. 4). These results demonstrate that the STAT3 transcriptional activity can be augmented upon inhibition of ERK1/2 without any additional external stimulation, but also after stimulation with LIF.

For a rough confirmation of these results, we have performed conventional reverse-transcriptase PCR for *Stat3* mRNA. Basically, we could demonstrate, that *Stat3* gene expression can be induced by stimulation of JEG-3 with LIF (n=2) and that pharmacological blocking of ERK1/2 by U0126 can enhance this effect (n=2; data not shown).

ERK1/2 activation is a major regulator of JEG-3 proliferation

JEG-3 cells were cultured in presence or absence of LIF and U0126 to assess proliferation rates. For this approach, the metabolic activity was measured after 24 h and 48 h in a MTS proliferation assay. Proliferation of JEG-3 cells is obvious after 48 h and LIF slightly increases this proliferation. The application of U0126 completely inhibits the proliferation, which is significant when compared with the respective control cells after 48 h of culture. This reduction is slightly, but significantly recovered by simultaneous treatment with LIF (fig. 5). Also in JEG-3 cells stimulated with LIF, ERK1/2 inhibition by application of U0126 leads to a significant reduction of proliferation. To exclude the possible effect of the U0126 vehicle DMSO, an independent assay was performed by adding the respective concentration of DMSO to the control cells and demonstrated that DMSO had no influence on proliferation (data not shown).

Blocking ERK1/2 increases JEG-3 cell invasion

LIF induces approximately 10-20% increase in invasiveness of JEG-3 cells through Matrigel, similar to previously published data. DMSO decreases the invasiveness of control cells slightly by 9%. Administration of U0126 (dissolved in DMSO) results in a significant 32% enhancement of the invasive activity. The combined application of LIF

and U0126 also induces a significant increase of invasiveness compared with the respective control cells, which is slightly higher than the application of both factors separately (fig. 6). These results correlate with the above described increased STAT3(tyr705 and ser727) phosphorylation and the STAT3-DNA-binding capacity after blocking ERK1/2.

Discussion

Activation of MAP kinases and JAK/STAT cascades is related with carcinogenesis and proliferation in numerous cell types including trophoblast cells and their malign derivates [Fitzgerald et al., 2005a; Knofler, 2010]. Previously, we demonstrated that LIF exerts a dose-dependent effect on STAT3(tyr705) activation, more intensively than other members of the IL-6 family of cytokines [Fitzgerald et al., 2005b]. It is also known that IL-6-like cytokines activate the MAPK pathway in several cell types [Auernhammer and Melmed, 2000; Rowley and Van Ness, 2002]. Here, we demonstrate that LIF triggers phosphorylation of both pathways simultaneously within 5 min of stimulation, which indicates that activation of both is independent of each other.

Complete STAT3 activation is dependent on the phosphorylation at the ser727 and tyr705 amino acid residues [Decker and Kovarik, 2000; Schuringa et al., 2000a]. Since STAT3 contains a characteristic ERK-MAPK phosphorylation site (-pro-X-ser/thr-pro-), ERK was expected to phosphorylate the ser727 residue of STAT3 [Chung et al., 1997]. Therefore, we focused on the effect of ERK1/2 inhibition on STAT3(ser727) phosphorylation. We have chosen to perform the current study on the JEG-3 choriocarcinoma cell line and not on the immortalized first trimester trophoblast cell line HTR8/SVneo because recently several reports remark major differences between HTR8/SVneo cells, primary trophoblast cells and choriocarcinoma cell lines [Bilban et al., 2010; Fu et al., 2010].

In JEG-3 cells, ERK1/2 phosphorylation was not necessary for phosphorylation of either STAT3(ser727) or STAT3(tyr705) induced by LIF as demonstrated by Western blotting and immunocytochemistry. These results correspond with a report on HepG2

hepatocellular carcinoma cells in which IL-6-induced STAT3(ser727) phosphorylation was also ERK1/2 independent [Schuringa et al., 2000a]. The variety of information from literature indicates that the kinase responsible for STAT3(ser727) phosphorylation depends on the individual cellular context and the respective stimulus. Several protein kinases such as Protein kinase C, Jun N-terminal kinase, p38 and mTOR may also be responsible for STAT3(ser727) phosphorylation [Busch et al., 2009a; Kim et al., 2008; Liu et al., 2008; Plaza-Menacho et al., 2007; Yokogami et al., 2000; Zhang et al., 2001]. Previous studies in our laboratories have shown that mTOR is required for the constitutive phosphorylation of STAT3(ser727) in the immortalized first-trimester trophoblast cell line HTR8/SVneo [Busch et al., 2009a].

In accordance with our previous investigations based on electrophoretic mobility shift assays [Fitzgerald et al., 2005b], we now observed an increase in the DNA-binding capacity of STAT3 after stimulation with LIF by using an alternative method (TransAM STAT3 kit, see methods section). In the current study, the spontaneous, LIF-independent DNA-binding activity of STAT3 increased, when the activation of ERK1/2 was abrogated by using the specific inhibitor U0126 or specific ERK1/2 siRNA. This result demonstrates that activated ERK1/2 functions as an inhibitor of the transcriptional activity of STAT3. This coincides with a report in LU1205 melanoma cells, in which STAT3 transcriptional activities can be activated upon inhibition of ERK and constitutively active ERK signaling resulted in down-regulation of STAT3 and STAT5 transcriptional activities [Krasilnikov et al., 2003].

To decipher functional correlates to the biochemical findings, we analyzed the effects of ERK1/2 blocking on proliferation and invasion of JEG-3 cells. LIF-induced activation of STAT3 has previously been found to enhance trophoblastic cell proliferation and invasion [Fitzgerald et al., 2005b; Prakash et al., 2011]. This elevated JEG-3 proliferation in the presence of LIF is employed as base for our current investigation. Blocking ERK1/2 significantly reduces proliferation, similar to the results recently published on HTR8/SVneo cells [Prakash et al., 2011], while elevating STAT3 DNA-binding ability as mentioned above. This indicates that ERK rather than STAT3 is responsible for proliferative effects in JEG-3. In a previous study, we have demonstrated that mTOR contributes not only to constitutive STAT3(ser727) phosphorylation, but also to trophoblastic (HTR8) proliferation [Busch et al., 2009b].

We have previously reported that several members of the IL-6 family of cytokines induce invasion of trophoblastic cells, and that IL-6 receptor-mediated STAT3 activation and translocation into the nucleus are essential for mediating the invasion promoting effects of LIF, IL-11 and IL-6 in trophoblast and choriocarcinoma cells [Dubinsky et al.; Poehlmann et al., 2005; Suman et al., 2009]. In the current study, we have shown that inhibition of ERK induces an increase in the transcriptional activity of STAT3. Functionally, this ERK inhibition also led to increased invasiveness of JEG-3 cells.

Conclusions

Summarized, the here presented findings demonstrate that in JEG-3 choriocarcinoma cells, LIF simultaneously employs two main intracellular signaling cascades, the JAK/STAT and MAPK pathways. ERK1/2 does not induce STAT3 phosphorylation, but instead, represses STAT3(tyr705 and ser727) phosphorylation and antagonizes STAT3 DNA-binding capacities in the nucleus (Figure 7).

Both pathways seem to have different functions. ERK1/2 is a major, but not sole, promoter of JEG-3 proliferation and is a negative regulator of STAT3, while STAT3 rather induces invasion (Figure 7). It may be concluded that dysfunctions of both pathways may be involved in placentation disorders and trophoblast malignancies. The better understanding of the role of individual factors may lead to the development of new therapeutic strategies.

Authors' roles

Diana M. Morales-Prieto has done major parts of experimental work and written the first draft of the manuscript. Stephanie Ospina Prieto and Wittaya Chaiwangyen have assisted in Western blotting and in DNA-binding assays, Maja Weber in immunocytochemistry, Sebastian Hoelters in invasion assays and. Ekkehard Schleussner has contributed in the design of the study and the revision of the manuscript. Justine S. Fiztgerald has contributed to the study design and critically revised the manuscript. Udo R. Markert is responsible for the design of the study and the revision and finalization of the manuscript.

Funding

The research group (Placenta Laboratories) has been supported by the German Research Foundation (DFG, project Ma1550/7-1), the German Ministry of Education and Research and the Thuringian Ministry of Education, Science and Culture. DMMP had a Ph.D. grant from the regional graduate academy of the Friedrich-Schiller-University Jena, Germany.

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Figure Legends

Figure 1. Kinetics of LIF-induced phosphorylation of STAT3 and ERK1/2 in JEG-3 choriocarcinoma cells. A) Representative Western blot of lysates from cells starved in serum-free medium and subsequently stimulated with 10 ng/ml LIF. The bands of all blots (n=3) have been scanned for density analysis. The density of bands from phosphorylated proteins (B: phospho-STAT3(ser727); C: phospho-ERK1/2; D: phospho-STAT3(tyr705)) has been normalized against the non-phosphorylated form. The so obtained relative density at 10 min LIF stimulation has been defined as "1" and the other values have been calculated respectively. Bars show means, error bars show standard error. * indicates $p<0.05$ when compared with value at 0 min LIF stimulation (Student's t test). Results of an analysis of correlation after Pearson of the respective kinetics are displayed in the left upper corner of each figure. $p<0.05$ indicates a significant correlation between stimulation time and band intensity.

Figure 2. Effects of ERK1/2 blocking on LIF-induced phosphorylation of STAT3 in JEG-3 choriocarcinoma cells. A) Representative Western blot of lysates from cells starved in serum-free medium, treated or not 2 h with the ERK1/2 blocker U0126 (10 mM) and subsequently stimulated with 10 ng/ml LIF for different time periods. The bands of all blots (n=3) have been scanned for density analysis. The density of bands from phosphorylated proteins (B: phospho-STAT3(ser727); C: phospho-ERK1/2; D: phospho-STAT3(tyr705)) has been normalized against the non-phosphorylated form. The so obtained relative density at 10 min LIF stimulation has been defined as "1" and the other values have been calculated respectively. Bars show means, error bars show standard

error. In B) and D) * indicates $p<0.05$ when all band densities of U0126 treated cells were compared with all without such treatment independent from LIF stimulation time (Student's t test). In C) * indicates a significant decrease when compared with the respective value without U0126 treatment.

Figure 3. Immunocytochemistry of JEG-3 cells after incubation with U0126 and LIF. Cells were settled and incubated overnight on microscope slides, where they attached, and then treated 2 h with or without 10 mM U0126, and subsequently stimulated or not with 10 ng/ml LIF. After 15 min, cells were fixed and stained (brown) for p-ERK, p-STAT3(tyr705) and p-STAT3(ser727). NC - negative control.

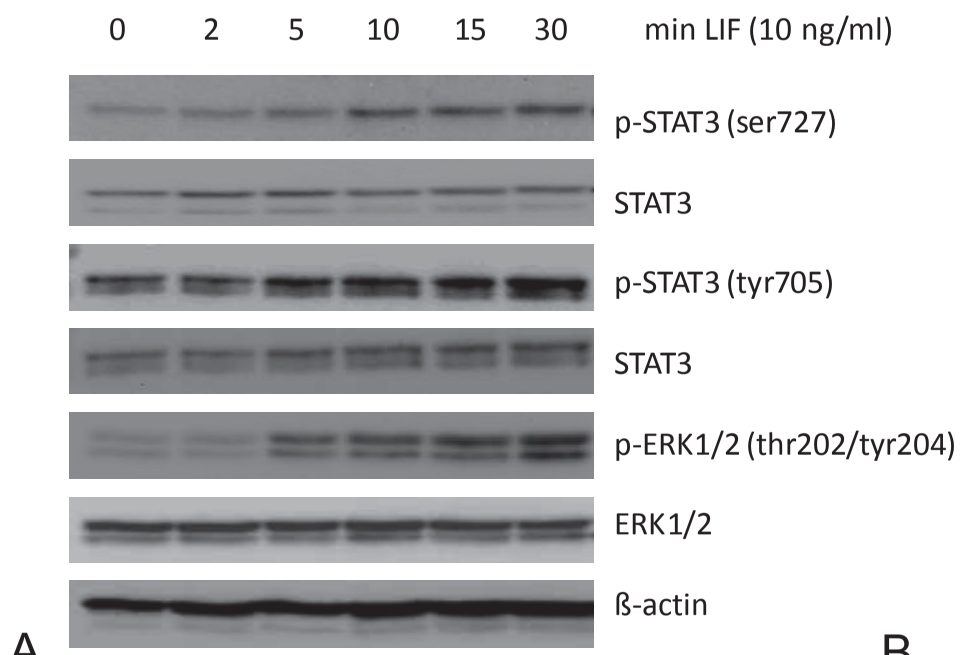
Figure 4. STAT3 DNA-binding capacity in JEG-3 cells after ERK1/2 inhibiton and stimulation with LIF. STAT3-DNA-binding capacities has been assessed by applying an ELISA-based colorimetric assay after a battery of different treatments. A) ERK1/2 was silenced by siRNA (silencing efficiency displayed by a representative Western blot, left upper corner) and stimulated or not with 10 ng/ml LIF. Non-transfected cells and cells treated with non-genomic siRNA served as controls. Error bars show standard error (n=4). * $p<0.05$ (Student's t test). B) Starved JEG-3 cells were treated or not with different concentrations of U0126 for 2 h and then, additionally, stimulated or not with different concentrations of LIF for another 4 h. The effect of both substances was significantly dose-dependent within the displayed range ($p<0.05$; two-tailed Pearson's correlation analysis). Bars represent the mean of n=3 independent assays. Error bars show standard error of the mean.

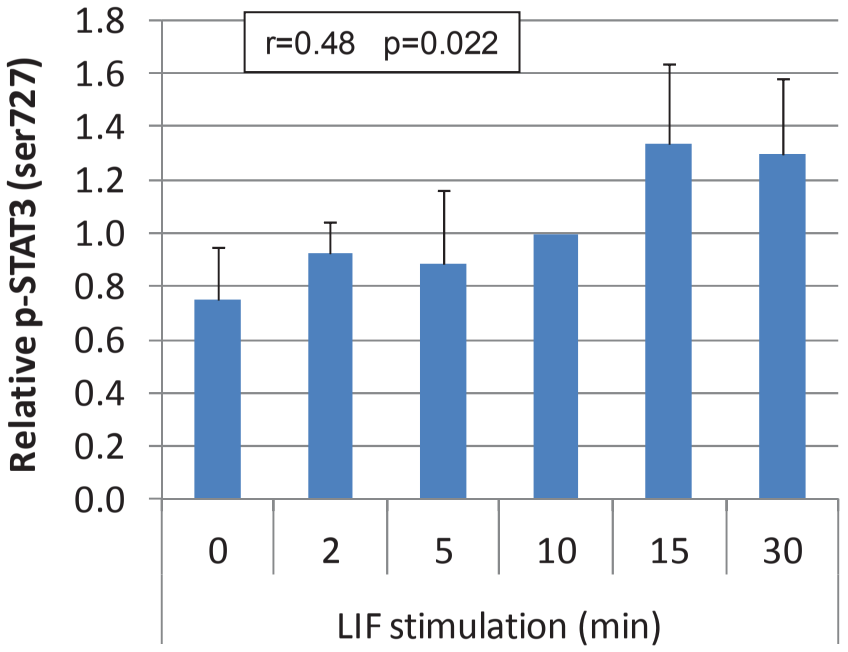
Figure 5. Effect of LIF and U0126 on the proliferation of JEG-3 cells. JEG-3 cells were incubated for up to 48 h in presence or absence of 10 mM U0126 and 10 ng/ml LIF. A MTS colorimetric assay was performed and optical density (OD) at 490nm was measured to assess cell proliferation. Bars show mean values of n=5 independent experiments, which been performed in triplicates (controls in 12 replicates). Error bars indicate standard error of the mean. * horizontal bars indicate $p<0.05$ for comparison of the respective means; Student's t-test.

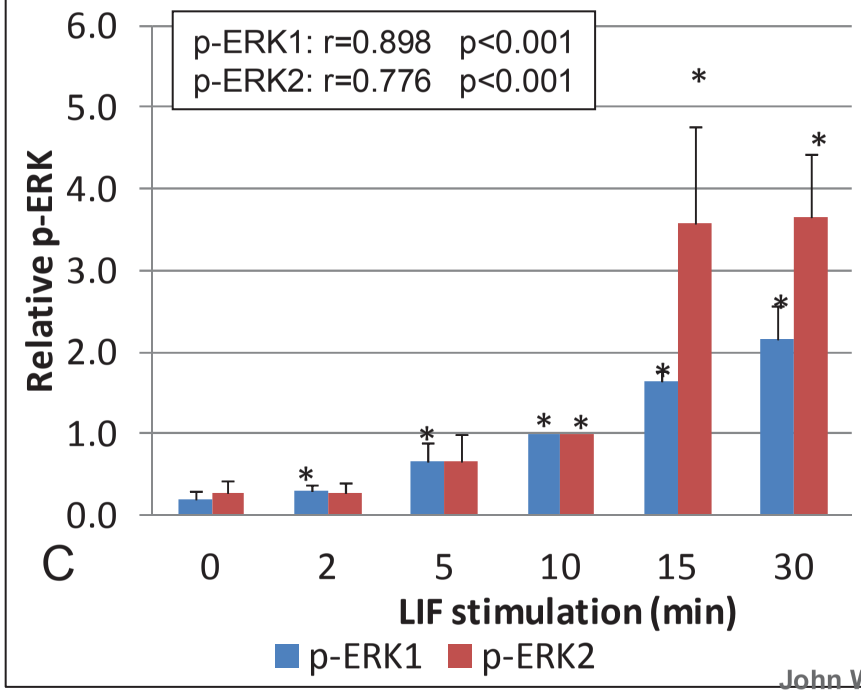
Figure 6. Relative invasiveness of JEG-3 cells upon ERK1/2 inhibiton and stimulation with LIF. JEG-3 cells were seeded on Matrigel-coated transwell chambers in presence or absence of 10 nM U0126 and10 ng/ml LIF as indicated. Relative invasion was assessed after 24 h as described in materials and methods, and measured as absorbance at 630nm. Results were normalized to non-stimulated cells and are expressed as mean ± standard error (n=7). * $p<0.05$; Student's t test (two-tailed).

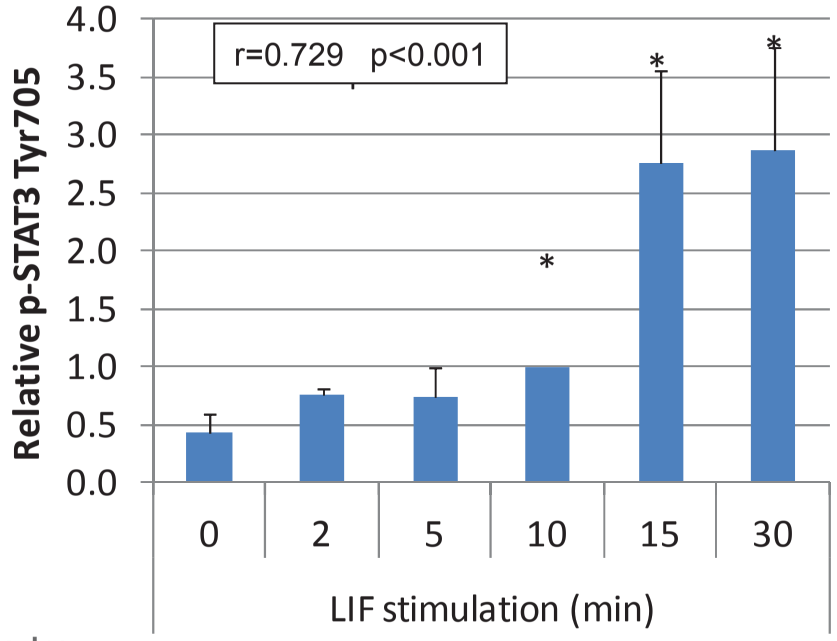
Figure 7. Scheme of the proposed LIF signaling pathway in trophoblasts. Red lines demonstrate the major findings of this paper: LIF triggers activation of JAK/STAT and MAPK pathways independently. ERK1/2 does not induce STAT3(ser727) phosphorylation but antagonizes STAT3 DNA-binding capacity in the nucleus. JAK/STAT and MAPK activation result in different cell-responses: increase of invasiveness and proliferation, respectively.

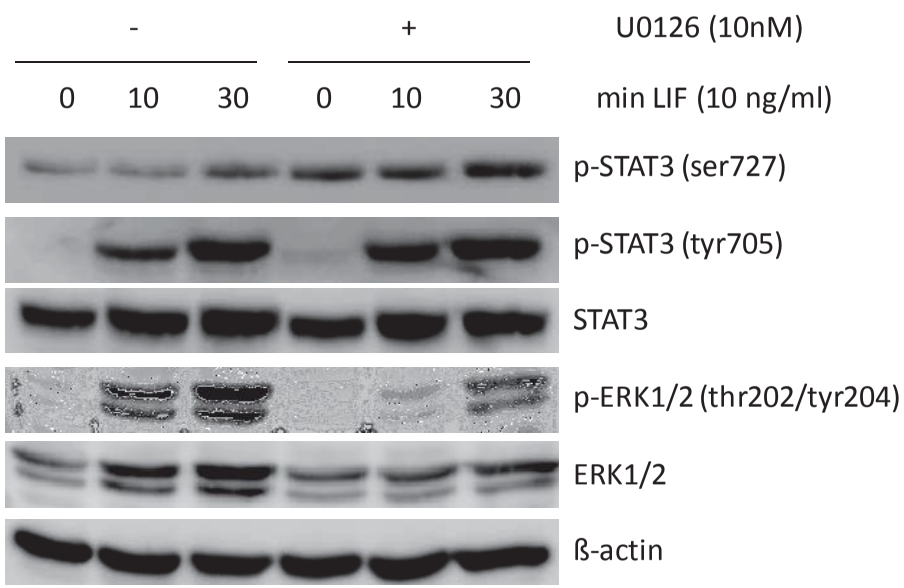
Figures










-
+
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0 10 30 0 10 30
min LIF (10 ng/ml)
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p-STAT3 (tyr705)
STAT3
p-ERK1/2 (thr202/tyr204)
ERK1/2
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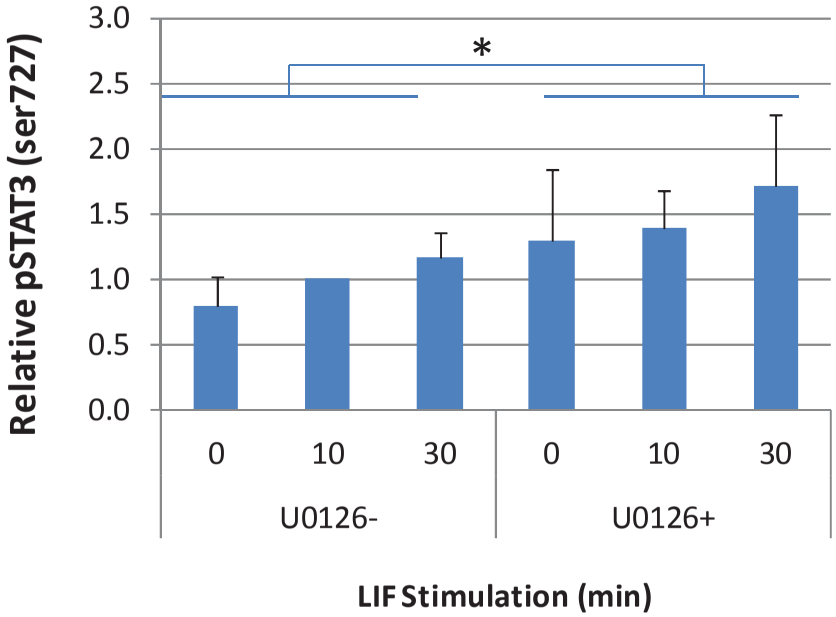
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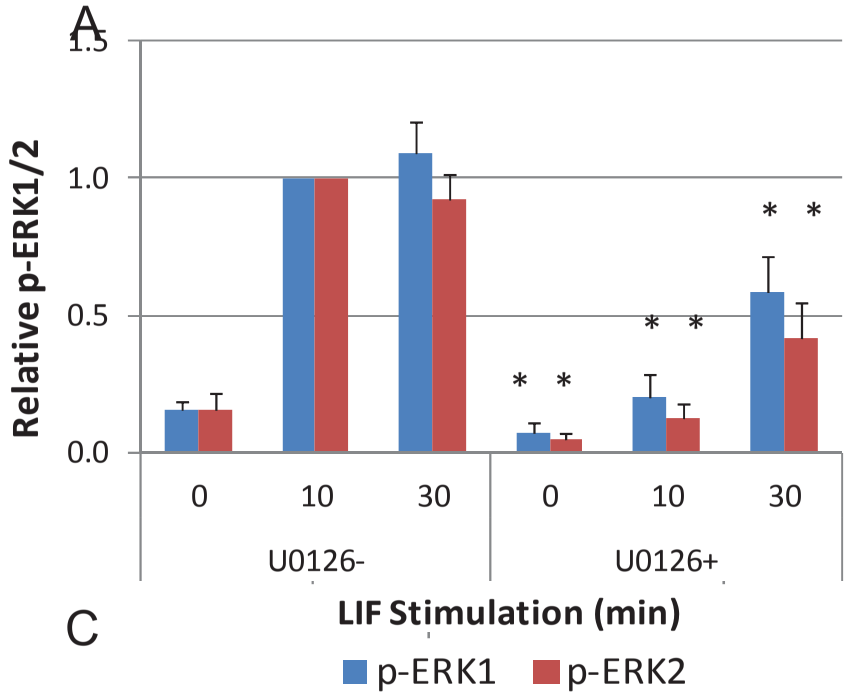
A
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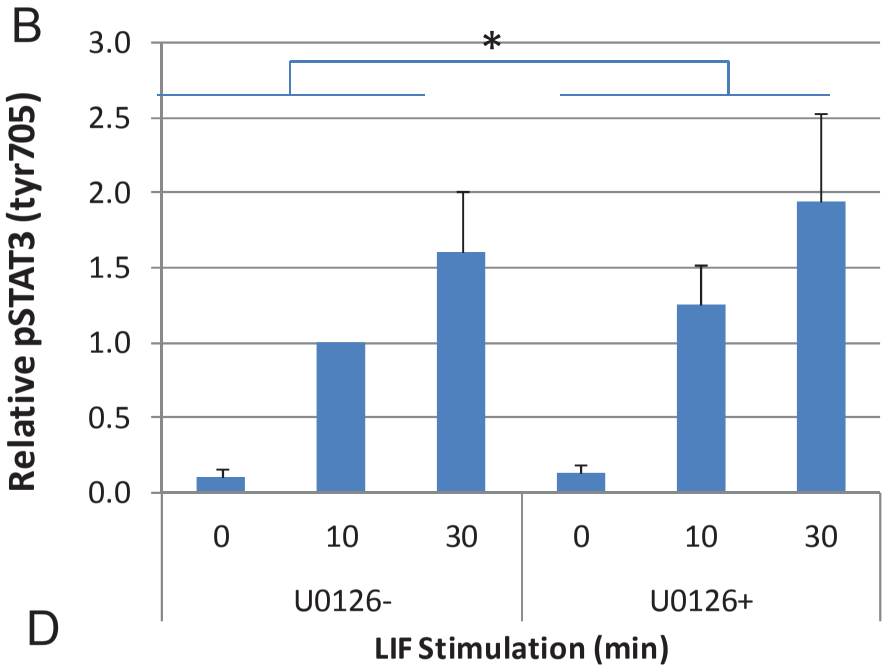
p-ERK1 p-ERK2


B
Relative pSTAT3 (tyr705)
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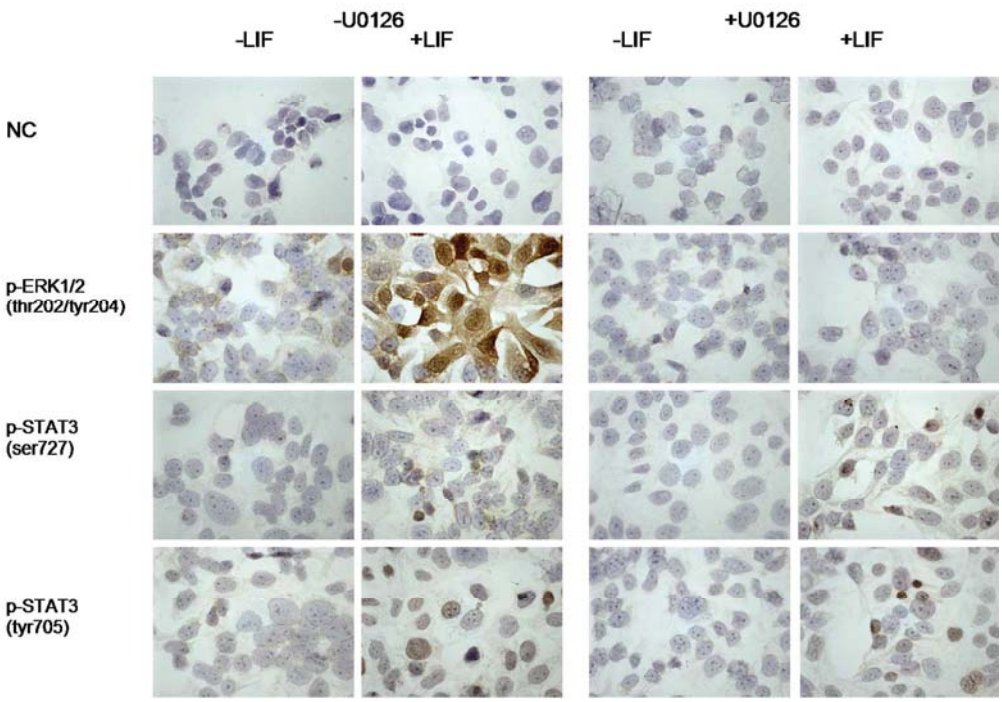

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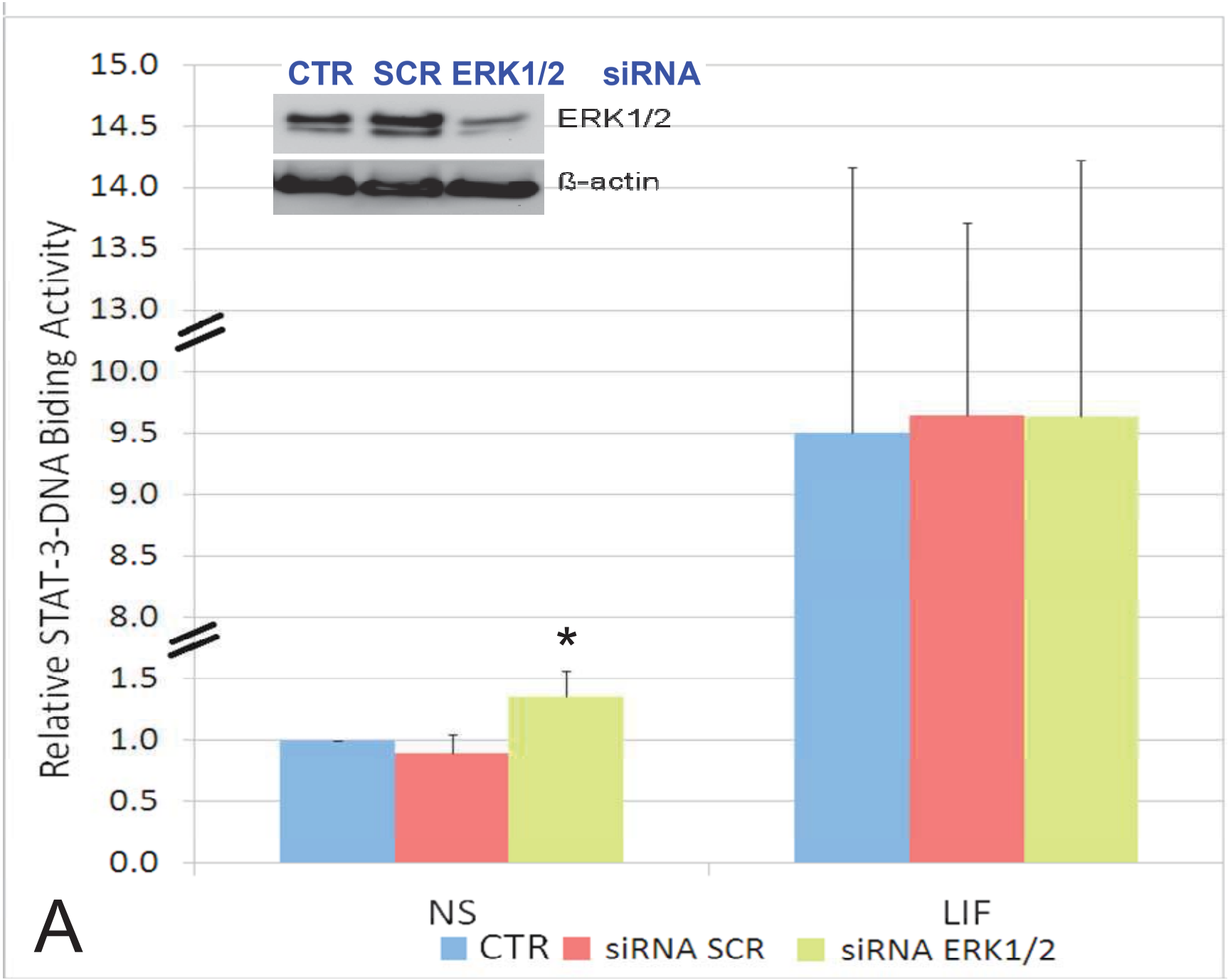

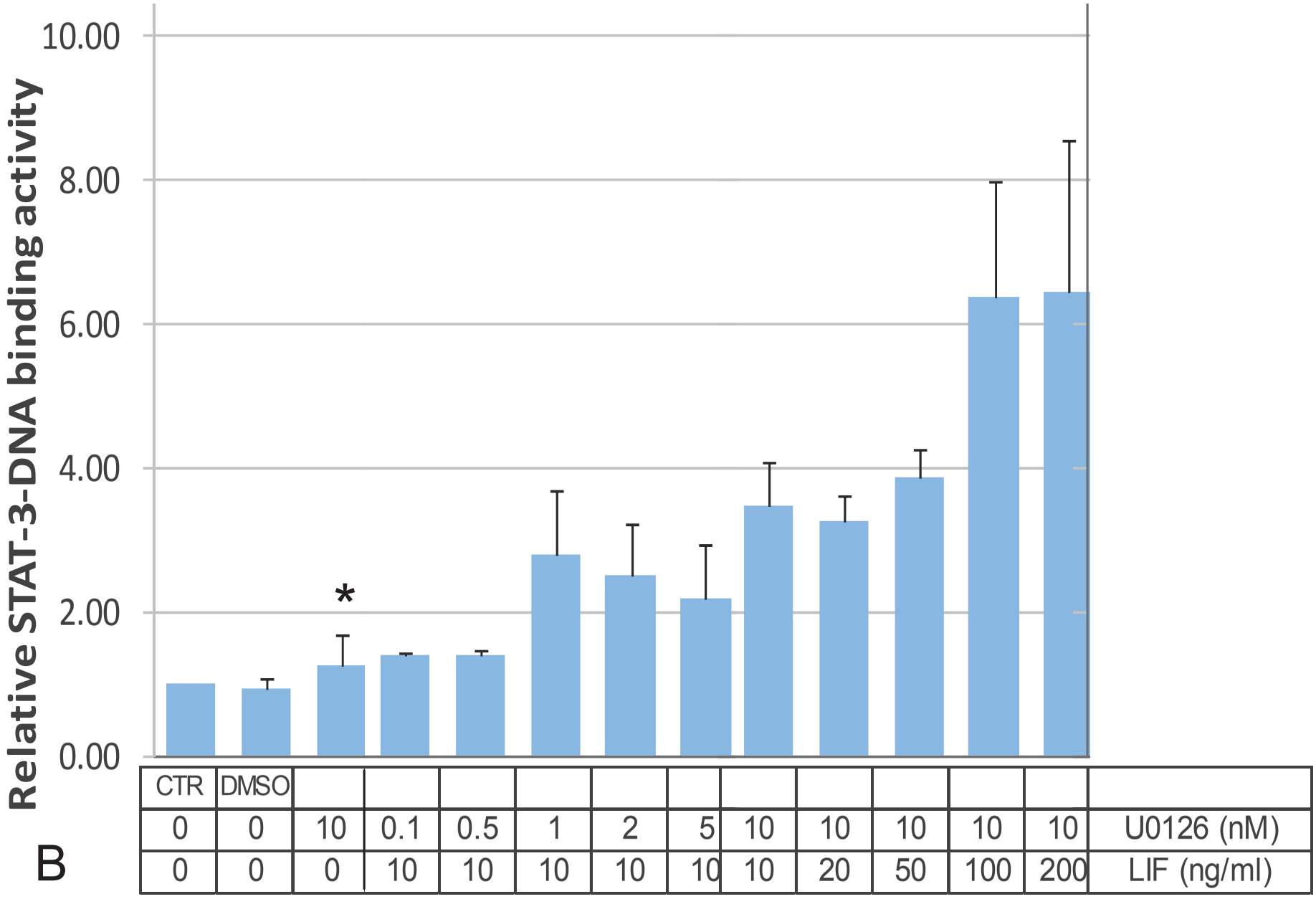

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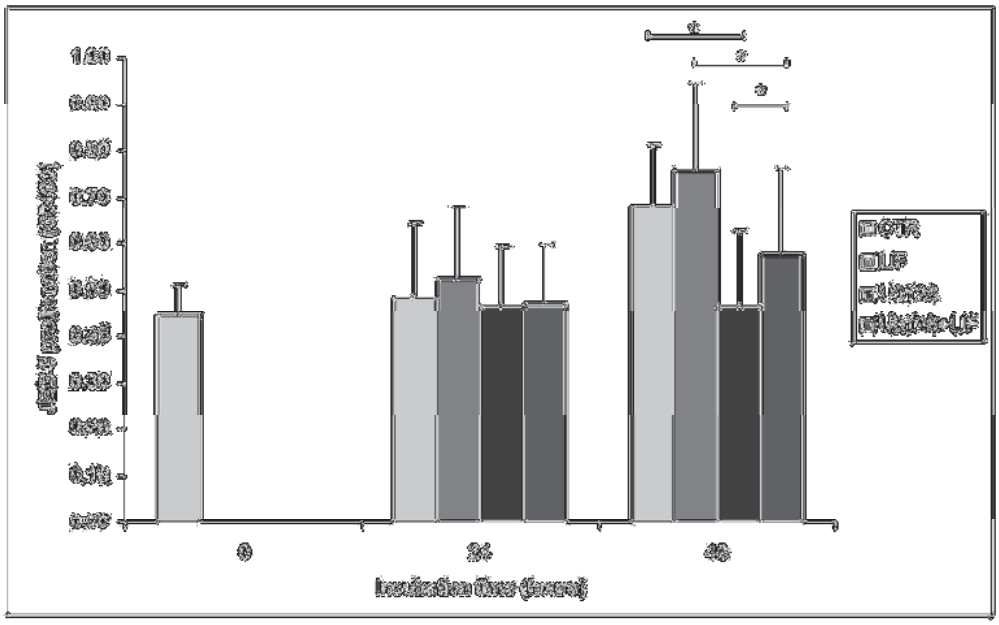

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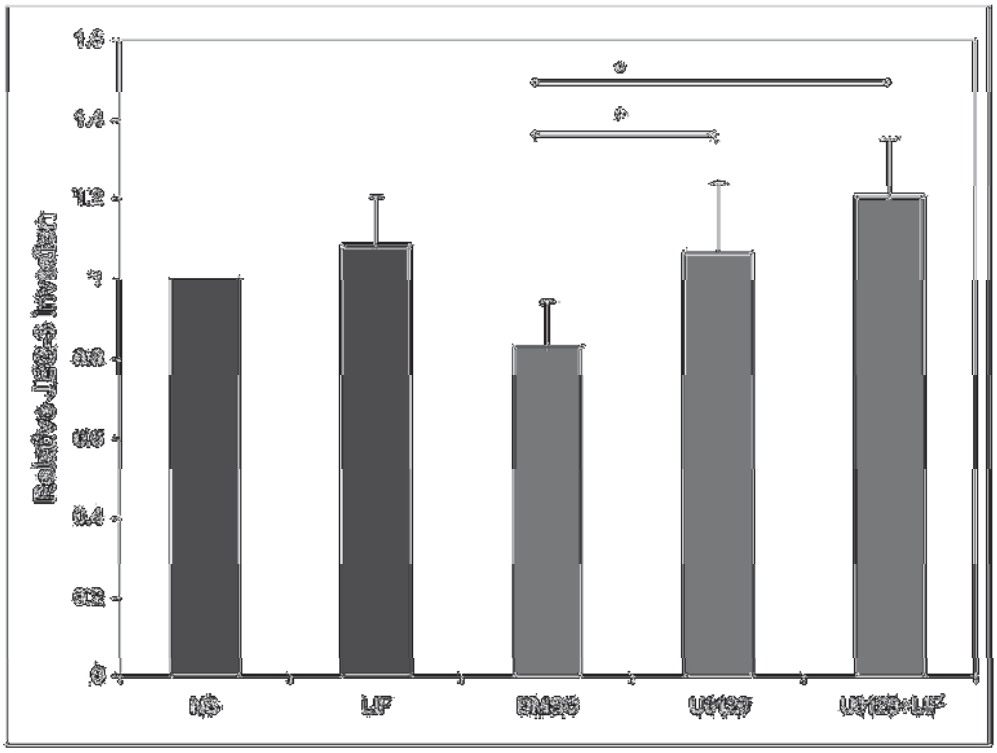

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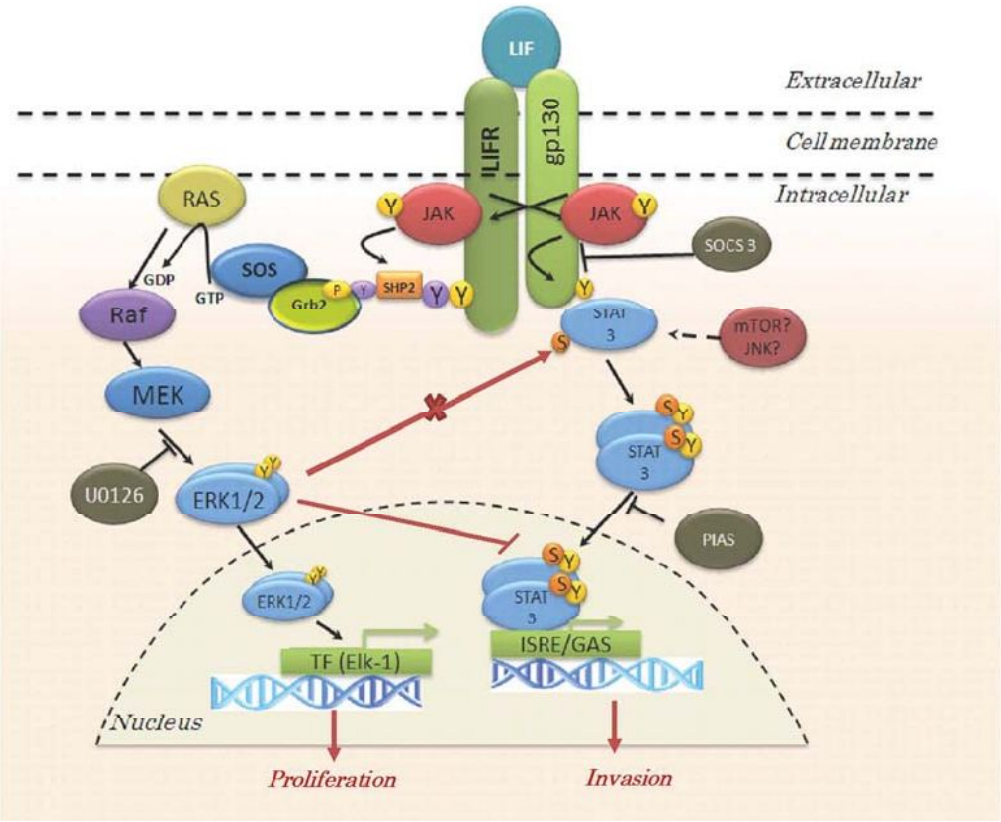

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Reproduction, Fertility and Development, 2011, **23**, 714–724 www.publish.csiro.au/journals/rfd

Leukaemia inhibitory factor mediated proliferation of HTR-8/SVneo trophoblast cells is dependent on activation of extracellular signal-regulated kinase 1/2

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Abstract. Leukaemia inhibitory factor (LIF) is one of the cytokines that is indispensable for embryo implantation. The aim of the present study was to investigate the role of activation of extracellular signal-regulated kinase (ERK) 1/2 in LIF-mediated proliferation of HTR-8/SVneo cells. Stimulation of HTR-8/SVneo cells with LIF (50 ng mL^{-1}) resulted in an increase in cell proliferation ($P < 0.05$) via increased transition of cells to the G_2/M phase of cell cycle. Stimulation with LIF resulted in the activation of both signal transducer and activator of transcription (STAT) 3 Tyr^{705} and ERK1/2, but inhibition of ERK1/2 signalling by pretreatment of cells with U0126 (10 μM) for 2 h resulted in abrogation of LIF-mediated increases in G_2/M transition, with a significant decrease ($P < 0.05$) in absolute cell numbers compared with control. Although STAT3 silencing had no effect on LIF-dependent proliferation of HTR-8/SVneo cells, it did result in an increase in cell apoptosis, which increased further upon inhibition of ERK1/2 activation irrespective of LIF stimulation. Stimulation of cells with LIF increased the Bcl-2/Bax ratio, whereas ERK1/2 inhibition decreased the Bcl-2/Bax ratio, even after LIF stimulation. Hence, it can be inferred that ERK1/2 activation is essential for LIF-mediated increases in proliferation and that both STAT3 and ERK1/2 activation are important for the survival of HTR-8/SVneo cells.

Additional keywords: apoptosis, trophoblast.

Introduction

After fertilisation, implantation of the blastocyst is one of the critical steps leading to establishment of pregnancy. This process involves extensive cross-talk between the trophoblast cells and the receptive endometrium through embryonic- as well as endometrial-derived factors at the site of implantation. Lack of any of the crucial embryonic or maternal signals at the site of implantation may result into shallow implantation or failure of implantation (Knöfler 2010).

During the first trimester of pregnancy, trophoblast cells proliferate, invade and differentiate to establish contact with the maternal circulation. Proliferation of trophoblast cells is positively regulated by factors such as leukaemia inhibitory factor (LIF), epidermal growth factor (EGF), insulin-like growth factor (IGF)-1, vascular endothelial growth factor (VEGF), granulocyte colony-stimulating factor, transforming growth factor (TGF)-α, but is negatively regulated by decorin and TGF-β1 (Li and Zhuang 1997; Athanassiades and Lala 1998; Athanassiades *et al*. 1998; Miyama *et al*. 1998; Fitzgerald *et al*. 2005; Iacob *et al*. 2008; Dubinsky *et al*. 2010; Hambruch *et al*. 2010). Depending upon the cell type and the predominance of cytokine/growth factors in the external milieu, cellular proliferation is regulated by activation of several signalling pathways, including phosphatidylinositol 3-kinase (PI3K), protein kinase C, mammalian target of rapamycin, wnt/β-catenin, Janus tyrosine kinase (JAK)–signal transducers and activators of transcription (STAT), p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) 1/2 (Davies *et al*. 2004; Meloche *et al*. 2004; Petersen *et al*. 2005; Wu *et al*. 2005; Masckauchan *et al*. 2006; Busch *et al*. 2009; Grivennikov *et al*. 2009; Fu *et al*. 2010; Keuling *et al*. 2010). This suggests that cellular proliferation is under the tight regulation of several external factors through the activation of various downstream signalling pathways (Fitzgerald *et al*. 2010).

As one of the cytokines predominantly present during the peri-implantation period, LIF plays a vital role in embryo implantation (Harvey *et al*. 1995). It has been reported that in LIF-knockout mice, embryos fail to implant, which can be rescued by injection of LIF (Stewart *et al*. 1992; Chen *et al*.

10.1071/RD10315 1031-3613/11/050714

2000; Makrigiannakis *et al*. 2006). In primates, injection of a monoclonal antibody against recombinant human LIF into the uterine cavity during the peri-implantation period resulted in a significant reduction in fertility (Sengupta *et al*. 2006). LIF activates the JAK-STAT pathway and increases the proliferation and invasion of JEG-3 choriocarcinoma cells (Fitzgerald *et al*. 2005; Poehlmann *et al*. 2005). However, LIF can transduce its signal through activation of either JAK-STAT or the MAPK/ERK pathways in different cell types like retinal cells, bronchial epithelial cells and cardiac myocytes to bring out their effective functions (Kodama *et al*. 2000; Hu *et al*. 2006; Rhee and Yang 2010).

Although both the JAK-STAT and MAPK/ERK pathways have been implicated in the regulation of the proliferation of trophoblast cells, the specific involvement of these pathways in LIF-mediated proliferation of trophoblast cells has not been deciphered. Hence, the aim of the present study was to determine the significance of ERK1/2- and STAT3-dependent signalling pathways in LIF-mediated proliferation and survival of trophoblast cells using HTR-8/SVneo cells (derived from human first-trimester placenta explant cultures immortalised by SV40 large T antigen) as a model of first trimester trophoblast cells (Graham *et al*. 1993; Busch *et al*. 2009).

Materials and methods

Culture of trophoblast cells

HTR-8/SVneo cells (kindly provided by Dr Charles Graham, Queen's University, Kingston, ON, Canada) were maintained in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with an antibiotic–antimycotic cocktail (100 U mL^{-1} penicillin; 100 μg mL^{-1} streptomycin; 0.25 μg mL^{-1} amphotericin B; Biological Industries, Kibbutz beit Haemek, Israel) and 10% heat-inactivated fetal bovine serum (FBS; Biological Industries). The cells were grown under a humidified atmosphere of 5% CO_2 in air at 37°C (Graham *et al*. 1993).

Cell proliferation assay

To investigate the role of LIF on cell proliferation, HTR-8/SVneo cells were seeded onto 12-well cell culture plates (1×10^4 cells per well; Greiner Bio-one, Frickenhausen, Germany). After 24 h, cells were washed with plain medium and separately treated with increasing concentrations of LIF (Sigma-Aldrich; 1, 5 and 50 ng mL^{-1}) in RPMI-1640 medium containing 1% FBS for 48 h under standard culture conditions of 5% CO_2 in air at 37°C. After 48 h, cells were harvested using a medium containing 0.5% trypsin and 0.2% EDTA and were then stained with 1 mg mL^{-1} propidium iodide (PI; Sigma-Aldrich) and counted on a flow cytometer (BD FACS Calibur; Becton Dickinson Biosciences, San Jose, CA, USA).

In another set of experiments, HTR-8/SVneo cells (1×10^4 cells per well), cultured as described above, were pretreated for 2 h with an optimised non-cytotoxic concentration (10 μM) of U0126 (Calbiochem, La Jolla, CA, USA), an inhibitor of mitogen-activated protein kinase kinase (MEK) 1/2 or with a vehicle control (dimethyl sulfoxide). After the 2-h incubation period, both control and U0126-treated cells were stimulated with an optimised concentration of LIF (50 ng mL^{-1}) for 48 h, followed by cell counting as described above.

DNA cell cycle analysis

The HTR-8/SVneo cells were seeded onto six-well cell culture plates (0.05×10^6 cells per well; Greiner Bio-one). After 24 h, cells were washed with plain medium and pretreated with 10 μM U0126 or vehicle control for 2 h. Then, 50 ng mL^{-1} LIF was added to both control and U0126-treated cells. Cells were incubated for 48 h and then harvested using a medium containing 0.5% trypsin and 0.2% EDTA. Cells were washed twice with 50 mM phosphate buffer, pH 7.4, with 0.9% NaCl (phosphate-buffered saline (PBS)) and fixed with 70% chilled ethanol. Subsequently, cells were kept for 20 min on a roller rotator at 4°C. Then, cells were washed twice with PBS, followed by the addition of RNase A (50 μg mL^{-1}) with 0.05% Triton-X to the cell suspension and a further 20 min on the roller rotator at 4°C. After this incubation period, PI was added to the RNase-treated cell suspension at a final concentration of 50 μg mL^{-1} and cells were kept in the dark for 15–20 min before DNA content was analysed using a BD FCAS Canto II (Becton Dickinson Biosciences). Cell cycle analysis was performed using FlowJo software (Tree Star, San Carlos, CA, USA) and the Dean–Jett–Fox model for fitting of cell cycle phases.

Western blot analysis of LIF-mediated activation of downstream signalling pathways

The HTR-8/SVneo cells (0.1×10^6 cells per well) were seeded into six-well cell culture plates (Greiner Bio-one) in RPMI-1640 medium supplemented with 10% FBS and cultured until 70–80% confluency. Cells were serum starved for 4–6 h and washed once with plain medium. Cells were further cultured in plain RPMI-1640 medium and treated with LIF (50 ng mL^{-1}) for 10, 30 or 60 min with or without U0126 pretreatment (10 μM, 2 h). After each time point, cells were lysed by the addition of 100 μL lysis buffer (20 mM TRIS-HCl, 10% glycerol, 0.2 mM EDTA, 137 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate) supplemented with complete protease and phosphatase inhibitor cocktails (Roche Diagnostics, Mannheim, Germany). The lysates were centrifuged at 12 000*g* for 15 min, the supernatant collected and the protein concentration estimated by the BCA colourimetric assay (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard.

For gel electrophoresis, 40 μg whole-cell extract was solubilised in gel-loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 25% glycerol, 1% bromophenol blue, 5% β-mercaptoethanol), boiled for 10 min and resolved by 0.1% SDS–10% polyacrylamide gel electrophoresis (SDS-PAGE) essentially as described previously (Laemmli 1970). The resolved proteins were transferred onto nitrocellulose membranes using the wet transfer method. After protein transfer, membranes were blocked with 50 mM Tris-buffered saline (TBS; 50 mM TRIS-HCl, 150 mM NaCl, pH 7.4) with 3% BSA for 1 h. Blots were incubated at 4°C overnight with a 1 : 1000 dilution of rabbit polyclonal antibodies against phosphorylated (p-) c-Raf (Ser^{338}), p-MEK1/2 ($Ser^{217/221}$),

p-ERK1/2 (Thr^{202}/Tyr^{204}), p-p90RSK (Thr^{359}/Ser^{363}) and p-STAT3 (Tyr^{705}; Cell Signalling Technology, Danvers, MA, USA) in TBS containing 0.1% BSA and 0.3% Tween-20 (TBST), followed by three washings with TBST and incubation with a 1 : 2000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Cell Signalling Technology) in TBST for 1 h at room temperature. Blots were developed using the chemiluminescent substrate Immobilon (Millipore, Billerica, MA, USA) and Hyperfilm-MP (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturers' instructions. All blots were then probed for β-actin, as described previously (Suman *et al.* 2009). The intensity of the bands on Western blots was quantified by LabWorks software version 4.5 (Ultra-Violet Products, Cambridge, UK).

Immunocytochemistry

The HTR-8/SVneo cells were seeded onto sterile microscopy slides and incubated in a drop of medium overnight at 37°C to allow them to adhere to the surface. Cells were starved in serum-free medium for 4 h. Subsequently, cells were treated or not treated with 10 μM U0126 for 2 h, followed by stimulation with 10 ng mL^{-1} LIF for 15 min (which was the time point with the strongest phosphorylation on Western blot analysis). Immunostaining was performed using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Briefly, cells were fixed in ethanol : methanol (1 : 1) for 5 min, washed in 0.1 M PBS and non-specific sites blocked with normal goat serum for 20 min at room temperature. After blocking, slides were incubated for 1 h with anti-p-ERK1/2 (Thr^{202}/Tyr^{204}) polyclonal rabbit antibody diluted 1 : 100 in Dako antibody diluent (Dako, Glostrup, Denmark) and then incubated for 30 min with biotinylated affinity-purified anti-rabbit IgG antibody (Vector Laboratories). Thereafter, slides were treated for 30 min with a solution of avidin/biotinylated enzyme complex (ABC; Vector Laboratories), followed by staining for 2 min with diaminobenzidine (DAB; Dako). Cell nuclei were counterstained with haematoxylin for 1 min. Finally, slides were dehydrated with an alcohol-to-xylol treatment, covered with Histofluid (Marienfeld, Lauda-Königshofen, Germany) and stored at 4°C until used for microscopy. Cells were analysed under a fluorescence microscope (Axioplan 2; Carl Zeiss, Jena, Germany). Density analysis of staining was performed using Scion Image software (Scion Image, Frederick, MD, USA).

Silencing STAT3 expression by short interference RNA

HTR-8/SVneo cells were transfected with STAT3 short interference (si) RNA (Thermo Scientific Dharmacon, Lafayette, CO, USA) as described previously with slight modification (Suman *et al.* 2009). Briefly, cells were transfected with 250 nM siRNA using Oligofectamine (Invitrogen, Carlsbad, CA, USA) for 4 h in OPTI-MEM I medium (Invitrogen) and, after 4 h, the medium was exchanged for RPMI-1640 supplemented with 10% FBS. Cells were retransfected after 48 h using half the amount of transfection mixture. The extent of silencing of STAT3 expression was ascertained by Western blot analysis of cell lysates collected 24 h after the second transfection with either non-genomic siRNA designed by scrambling the STAT3 siRNA (AAGCCACTTATAAATTCGTTC) or STAT3 siRNA (AATGTTCTCTATCAGCACAAT; Poehlmann *et al.* 2005). Blots were probed with a 1 : 1000 dilution of rabbit polyclonal antibody against STAT3 (Cell Signalling Technology) as the primary antibody and a 1 : 2000 dilution of HRP-conjugated anti-rabbit IgG antibody (Cell Signalling Technology) as the secondary antibody. Blots were reprobed for actin as an internal control, as described above. Silenced and non-silenced cells (24 h after the second transfection) were trypsinised and seeded as per the protocol described for DNA cell cycle analysis and assessment of apoptosis by annexin V staining. Briefly, cells were pretreated for 2 h with U0126 or with vehicle, followed by stimulation or not with LIF (50 ng mL^{-1}) for 12 h for annexin V staining or for 48 h for DNA cell cycle analysis.

Apoptosis and death scoring of HTR-8/Svneo cells following LIF and U0126 treatment

HTR-8/SVneo cells were treated or not with LIF (50 ng mL^{-1}) for 12, 24 and 48 h in the presence or absence of U0126 (10 μM). Cells were dislodged from the surface of six-well culture plate using medium containing 0.5% trypsin and 0.2% EDTA before being washed three times with PBS. Cells were then resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) and stained with PE-Annexin V (BD PharMingen, Franklin Lake, NJ, USA) for 15 min in the dark. To ascertain the specificity of cell death by apoptosis, cells were counterstained with SYTOX-green DNA binding dye (10 nM; Invitrogen). Stained cells were analysed using a BD FACSCanto II flow cytometer (Becton Dickinson Biosciences) and data were analysed using FlowJo software (Tree Star).

In addition, cells were stimulated for 12 and 24 h in the presence or absence of LIF (50 ng mL^{-1}) and U0126 (10 μM). After 12 and 24 h, cells were harvested for the preparation of whole-cell lysates as described above or to prepare a mitochondrial fraction. For the preparation of a mitochondrial fraction, cells were trypsinised and washed with cold PBS. Cells were then resuspended in isolation buffer (0.2 M sucrose, 0.1% BSA, 0.2 mM EDTA,10 mM HEPES, pH 7.4) and homogenised on ice using a Dounce homogenizer (Sigma-Aldrich). Homogenised fractions were centrifuged at 1000*g* at 4°C for 10 min and the supernatant collected. The supernatant was further centrifuged at 14 000*g* for 15 min at 4°C. The mitochondrial fraction, in the form of the pellet, was washed twice with cold isolation buffer and lysed in lysis buffer containing protease inhibitor cocktail (Roche Diagnostics). Whole-cell lysates and the mitochondrial fractions were used for Western blot analysis of the expression of Bcl-2 and Bax, respectively. For Western blotting, mouse monoclonal antibodies against Bcl-2 and Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 1000 dilution in TBST) were used as primary antibodies, whereas HRP-conjugated goat anti-mouse IgG, (H^+L) antibody (Pierce; 1 : 2000 dilution in TBST) was used as a secondary antibody. Blots were developed and reprobed for actin as described above.

Statistical analysis

All experiments were performed at least twice and the results are expressed as the mean ± s.e.m. For different sets of experiments,

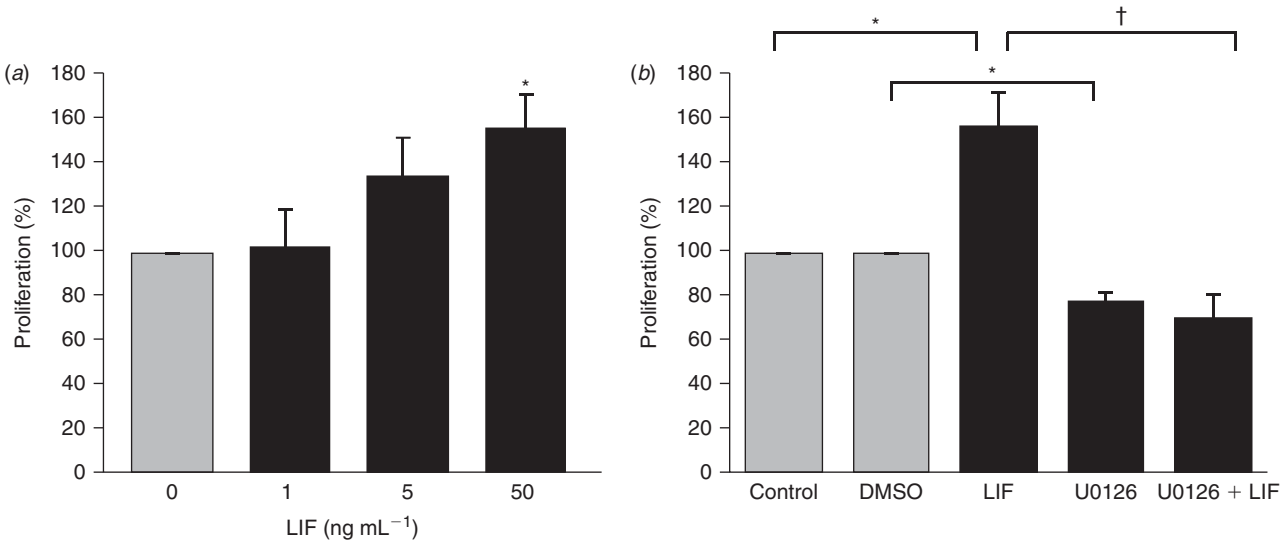

Fig. 1. Effect of leukaemia inhibitory factor (LIF) on the proliferation of HTR-8/SVneo cells in the presence or absence of U0126. Cells were treated with varying concentrations of LIF and processed for proliferation assay as described in the Methods. (*a*) Concentration-dependent effects of LIF on the proliferation of HTR-8/SVneo cells. (*b*) Effects of LIF on the proliferation of cells in the presence and absence of U0126. Data are the mean ± s.e.m. of cell proliferation as a percentage of control from three independent experiments performed in duplicate. *$P < 0.05$ compared with control, †$P < 0.01$ compared with cells stimulated with LIF.

such as proliferation assays and Western blot analysis (densitometric analysis), statistical analyses were performed by comparing mean values in control and experimental groups using paired Student's *t*-test and/or Wilcoxon's signed-rank test. $P < 0.05$ was considered significant.

Results

Effect of LIF on proliferation of HTR-8/SVneo cells

HTR-8/SVneo cells were treated with increasing concentrations of LIF and the extent of proliferation was assessed by counting the number of viable cells after 48 h treatment. The addition of 1 ng mL^{-1} LIF to the cells had no effect on proliferation, but a significant increase in proliferation ($P < 0.05$) was observed following treatment with 50 ng mL^{-1} LIF (Fig. 1*a*).

Relevance of the ERK1/2 signalling pathway in LIF-mediated proliferation of HTR-8/SVneo cells

U0126, a pharmacological inhibitor of MEK1/2 activation, was used to investigate the relevance of ERK1/2-associated signalling in LIF-mediated proliferation of HTR-8/SVneo cells. Treatment of cells with a non-cytotoxic concentration of U0126 (10 μM) resulted in a significant decrease ($P < 0.05$) in proliferation compared with vehicle control (Fig. 1*b*). Furthermore, when cell were costimulated with LIF and U0126, a significant decrease ($P < 0.01$) in proliferation was observed compared with cells treated with LIF alone (Fig. 1*b*). In addition, evaluation of the cell cycle phases by analysing the DNA content of HTR-8/SVneo cells after 48 h LIF stimulation revealed an approximate two-fold increase in the number of cells in the G_2/M phase of cell cycle compared with control, which is suggestive of a greater number of proliferating cells in LIF-treated samples (Table 1). Pretreatment of cells with U0126 in the presence or absence of LIF had no significant effect on the G_2/M cell population compared with control (Table 1).

Table 1. Effects of leukaemia inhibitory factor and U0126, an inhibitor of extracellular signal-regulated kinase 1/2 activation, on cell cycle progression of HTR-8/SVneo cells

DNA content was measured after propidium iodide staining by flow cytometry. The percentages of cells in different phases of the cell cycle are shown as the mean ± s.e.m. of at least two different experiments. *$P < 0.05$ compared with control; LIF, leukaemia inhibitory factor

Treatment	G_0/G_1 phase	S phase	G_2/M phase
Control	52.5 ± 2.5	35.0 ± 2.1	9.4 ± 1.4
LIF	40.5 ± 1.9	29.8 ± 3.4	18.6 ± 2.6*
U0126	44.8 ± 2.6	42.5 ± 3.1	10.5 ± 3.2
LIF + U0126	43.9 ± 2.1	40.6 ± 1.7	9.8 ± 2.5

Effect of MEK1/2 inhibition on LIF-mediated activation of the MAPK–ERK1/2 signalling pathway

HTR-8/SVneo cells were treated with 50 ng mL^{-1} LIF for 10, 30 and 60 min with or without 2 h pretreatment with 10 μM U0126. Irrespective of the addition of U0126, treatment of HTR-8/SVneo cells with LIF resulted in transient activation by phosphorylation of c-Raf and MEK1/2 as early as 10 min, which decreased by 60 min (Fig. 2*a–c*). Furthermore, treatment of cells with LIF resulted in the activation of ERK1/2 by 10 min, which was maintained at the same level until 60 min. However, pretreatment of cells with U0126 abrogated ERK1/2 activation following LIF stimulation. In addition, following the addition of U0126, there was an absence of the basal phosphorylation of ERK1/2 compared with untreated control (Fig. 2*a*, *d*). Immunocytochemistry for p-ERK1/2 also suggested that

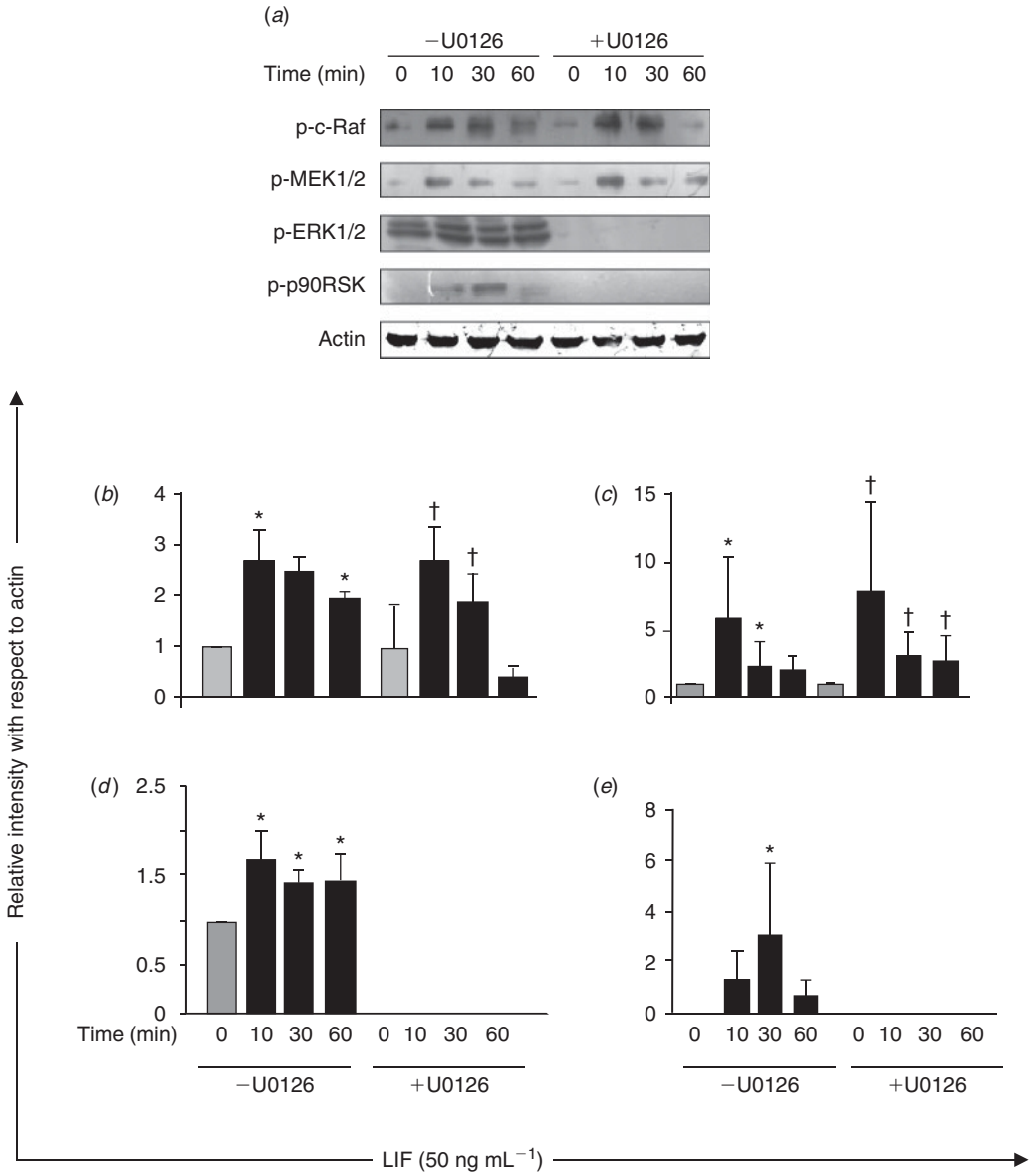

Fig. 2. Effect of U0126 on leukaemia inhibitory factor (LIF)-mediated activation of the extracellular signal-regulated kinase (ERK) 1/2 signalling pathway in HTR-8/SVneo cells. Cells were stimulated with LIF (50 ng mL^{-1}) for 10, 30 and 60 min in the presence or absence of U0126 (10 μM) and cell lysates were processed for Western blot analysis. (*a*) Representative blots for phosphorylated (p-) c-Raf, p-mitogen-activated protein kinase kinase (MEK) 1/2, p-ERK1/2 and p-p90RSK. (*b–e*) Band intensities for p-c-Raf, p-MEK1/2, p-ERK1/2 and p-p90RSK, respectively, normalised against actin. Data are given as the fold change compared with control (without LIF stimulation) and are expressed as the mean ± s.e.m. of three independent experiments. *$P < 0.05$ compared with control (without LIF stimulation); $^{\dagger}P < 0.05$ compared with U0126-pretreated with otherwise unstimulated cells.

stimulation of HTR-8/SVneo cells with 10 ng mL^{-1} LIF could increase the p-ERK1/2 levels, an effect that was abrogated following U0126 pretreatment (Fig. 3). One of the downstream molecular targets of activated ERK1/2 is p90 ribosomal S6 kinase (p90RSK), the phosphorylation of which was increased 10 min after stimulation with LIF, but returned to basal levels by 60 min (Fig. 2*a*, *e*). As observed for ERK1/2 activation, LIF-mediated activation of p90RSK was completely abrogated in cells pretreated with U0126 (Fig. 2*a*, *e*).

Effect of U0126 on LIF-mediated activation of STAT3 in HTR-8/SVneo cells

HTR-8/SVneo cells were stimulated with 50 ng mL^{-1} LIF for 10, 30 and 60 min in the presence or absence of U0126. An

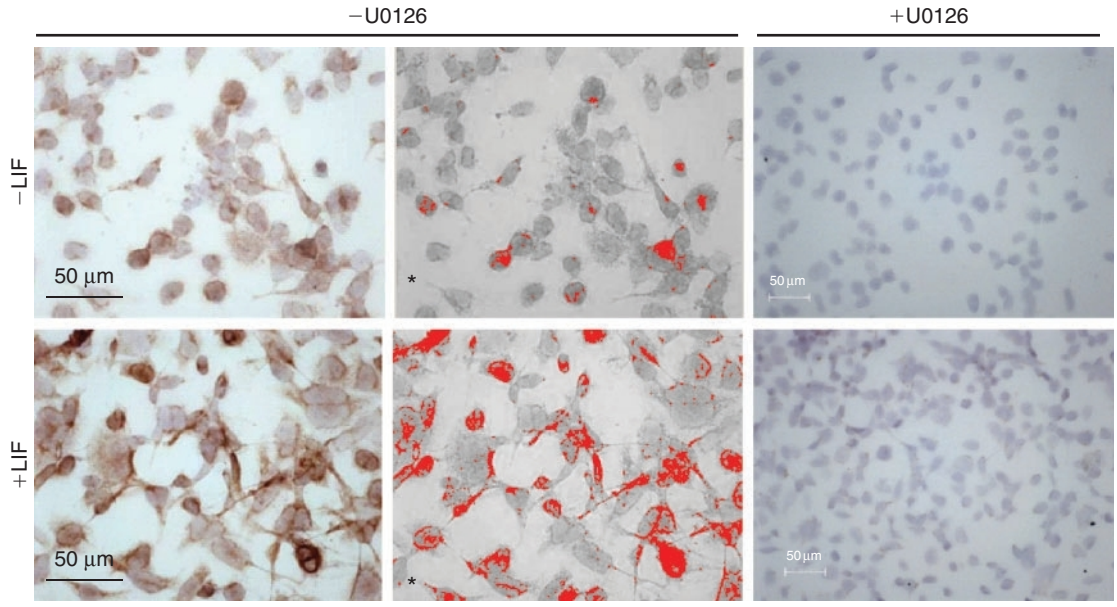

Fig. 3. Peroxide staining for extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in HTR-8/SVneo cells. HTR-8/SVneo cells were pretreated or not for 2 h with 10 µM U0126 in the absence of serum and then stimulated or not with 10 ng mL^{-1} leukaemia inhibitory factor (LIF) for 15 min. Slides marked with an asterisk are copies of the slides to their left. Strong staining has been highlighted in red using Scion Image software (Scion Image, Frederick, MD, USA).

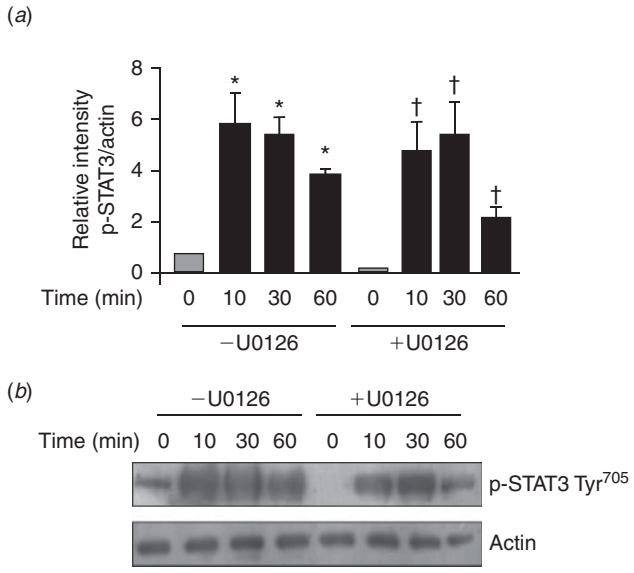

Fig. 4. Effect of extracellular signal-regulated kinase (ERK) 1/2 inhibition on leukaemia inhibitory factor (LIF)-mediated activation of signal transducer and activator of transcription (STAT) 3 phosphorylation. HTR-8/SVneo cells were stimulated with LIF (50 ng mL^{-1}) for 10, 30 and 60 min in the presence or absence of U0126 (10 µM) and cell lysates were processed for Western blot analysis of phosphorylated (p-)STAT3 Tyr^{705}. (*a*) Mean (± s.e.m.) band intensities compared with control (cells without LIF stimulation) following normalisation against actin from three independent experiments. (*b*) Representative blot for p-STAT3 Tyr^{705}. *$P < 0.05$, as compared with control (without LIF stimulation); $^{\dagger}P < 0.05$ compared with U0126-pretreated with otherwise unstimulated cells.

increase in the phosphorylation of STAT3 was observed 10 and 30 min after LIF treatment, which started to decrease at 60 min (Fig. 4). Despite treatment of HTR-8/SVneo cells with U0126, LIF stimulation resulted in a significant increase in STAT3 Tyr^{705} phosphorylation (Fig. 4).

Role of the ERK1/2 pathway in the regulation of apoptosis of HTR-8/SVneo cells

HTR-8/SVneo cells were stimulated with 50 ng mL^{-1} LIF for 12, 24 and 48 h, with or without 2 h pretreatment with U0126 (10 µM), and apoptotic cells were analysed by staining with PE-Annexin V. Following LIF stimulation, there was no change in the proportion of annexin V-positive cells compared with control at any time point, whereas pretreatment of cells with U0126 resulted in a progressive increase in the number of annexin V-positive cells compared with control (Fig. 5). Even LIF stimulation of U0126-pretreated cells resulted in an increase in annexin V-positive cells over time (Fig. 5).

In another experiment, HTR-8/SVneo cells were stained for death by PE-Annexin V (apoptosis) and SYTOX green DNA binding dye (total death), to differentiate between apoptotic and necrotic death, 12 and 48 h after LIF stimulation in the presence or absence of U0126. At 12 h, there was an increase in the number of annexin V-positive cells following U0126 treatment in the presence or absence of LIF compared with control cells. This was associated with an overall shift in the cell population in the U0126 treatment group (see Fig. S1 available as an Accessory Publication to this paper; Fig. 5). However, there was no change in the proportion of double-positive cells 12 h after LIF stimulation in the presence or absence of LIF. After 48 h

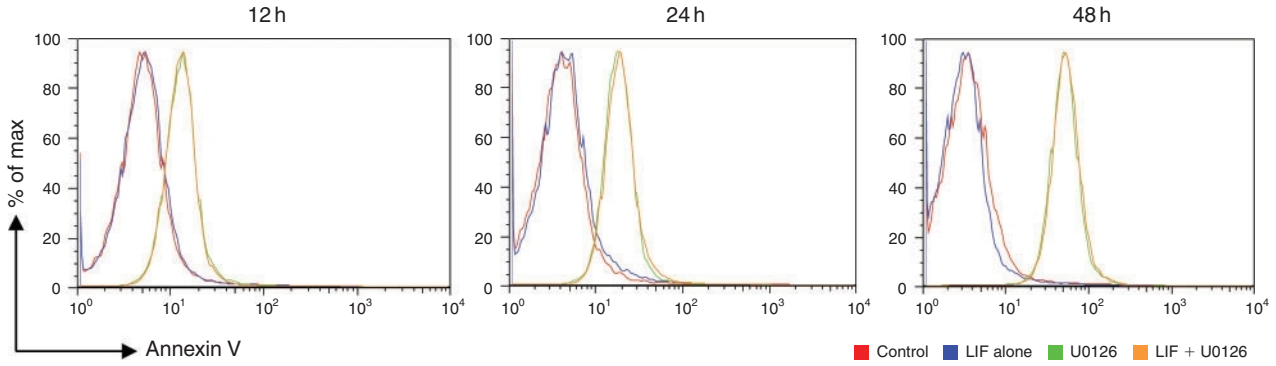

Fig. 5. Effect of extracellular signal-regulated kinase (ERK) 1/2 inhibition on survival of HTR-8/SVneo cells. HTR-8/SVneo cells were stimulated with 50 ng mL^{-1} leukaemia inhibitory factor (LIF) for 12, 24 and 48 h following 2 h pretreatment or not with U0126 (10 μM). Cells were stained with PE-Annexin V as described in the Methods. The diagram represents the histogram overlays of annexin V-stained cells following modulation of ERK1/2 activation by LIF and U0126.

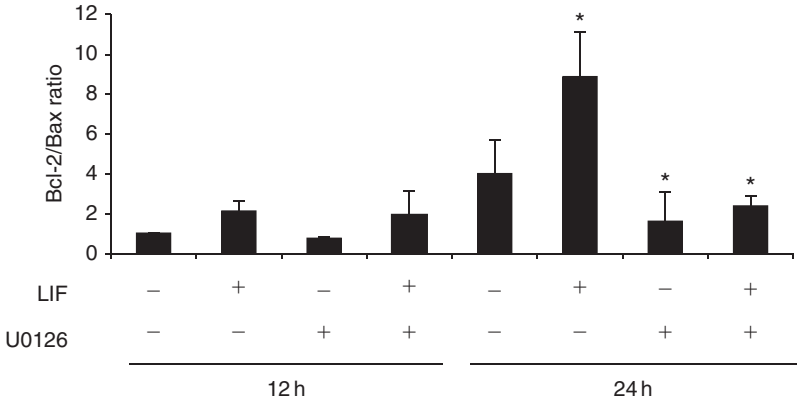

Fig. 6. Expression of pro- and anti-apoptotic molecules in HTR-8/SVneo cells following leukaemia inhibitory factor (LIF) stimulation and inhibition of extracellular signal-regulated kinase (ERK) 1/2 signalling pathways. HTR-8/SVneo cells were stimulated for 12 and 24 h with LIF (50 ng mL^{-1}) in the presence or absence of U0126 (10 μM). Whole-cell lysates were prepared after specified time points for Western blot analysis of Bcl-2 expression. In a parallel experiment, mitochondrial fractions were prepared and Western blot analysis was used to determine Bax expression at specified time points. Bands intensities were estimated by LabWorks software version 4.5 (Ultra-Violet Products, Cambridge, UK). Data are the mean ± s.e.m. of the Bcl-2 (after normalisation against actin) to Bax ratio from two different experiments. *$P < 0.05$ compared with cells without U0126 and LIF treatment.

incubation, there was an increase in SYTOX green-positive cells in all groups, but compared with the control and LIF-stimulated groups, there was significant increase in double-positive cells following ERK1/2 inhibition irrespective of the addition of LIF (Fig. S1).

Effect of LIF on the Bcl-2/Bax ratio in HTR-8/SVneo cells

HTR-8/SVneo cells were stimulated with LIF in the presence or absence of U0126 as described above and cells were lysed using lysis buffer after 12 and 24 h for Western blot analysis of Bcl-2 (anti-apoptotic molecule) expression. In another series of experiments, mitochondrial fractions were collected after 12 and 24 h for Western blot analysis of the expression of the pro-apoptotic molecule Bax. A significant increase ($P < 0.05$) was observed in the Bcl-2/Bax ratio 24 h after LIF stimulation compared with control; however, there was no significant difference at the 12 h time point (Fig. 6). In contrast, there was a significant decrease ($P < 0.05$) in Bcl-2/Bax ratio in cells pretreated with U0126 or both U0126 and LIF (Fig. 6).

Significance of STAT3 in the proliferation and apoptosis of HTR-8/SVneo cells

HTR-8/SVneo cells were transfected with STAT3 siRNA and, 72 h after transfection, Western blot analysis was used to evaluated the expression of STAT3 in siRNA-transfected cells. A significant decrease in STAT3 expression was observed in silenced cells compared with expression in cells transfected with scrambled siRNA (Fig. 7*a*).

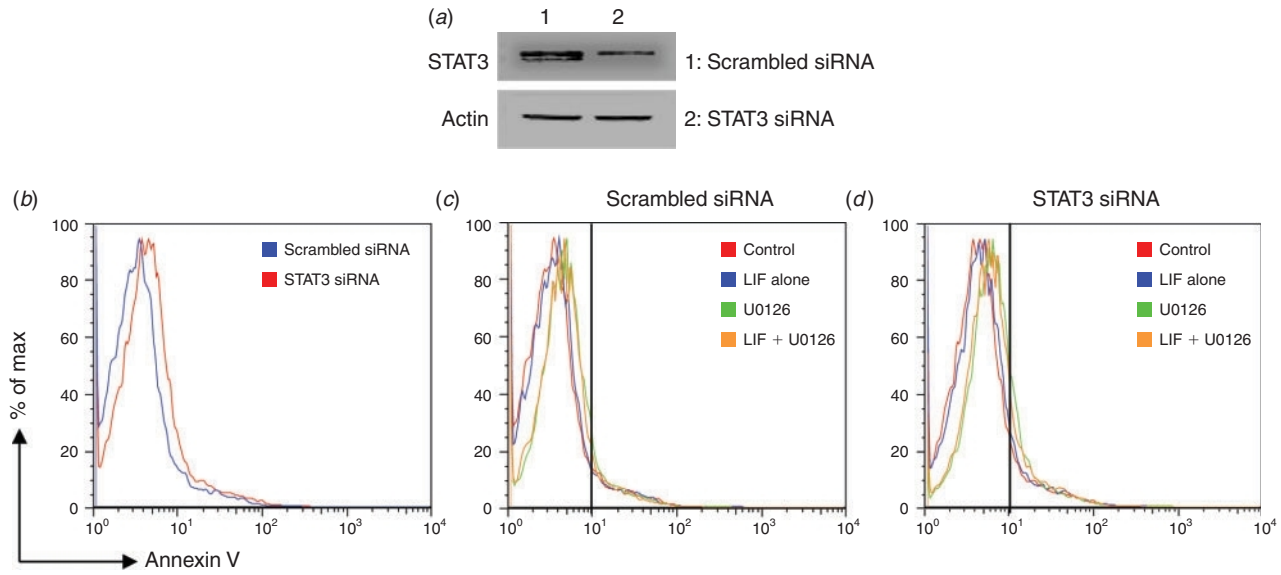

Fig. 7. Effect of signal transducer and activator of transcription (STAT) 3 silencing on the proliferation and apoptosis of HTR-8/SVneo cells in the presence or absence of leukaemia inhibitory factor (LIF) and the mitogen-activated protein kinase kinase (MEK) 1/2 inhibitor U0126. HTR-8/SVneo cells were transfected with STAT3 short interference (si) RNA for 72 h and the extent of silencing was assessed by Western blot analysis of STAT3 expression in silenced cells compared with that in cells transfected with scrambled siRNA. (*a*) Representative blots of STAT3 and actin (as internal control) after siRNA transfection. STAT3-silenced and scrambled siRNA-transfected cells were stained with annexin V after 12 h stimulation with or without LIF (50 ng mL^{-1}) and U0126 (10 μM). (*b*) Histogram overlays of scrambled siRNA-transfected cells and STAT3 siRNA-transfected cells following annexin V staining. (*c*, *d*) Histogram overlays of cells transfected with scrambled siRNA (*c*) and STAT3 siRNA (*d*) in the presence or absence of LIF and U0126. A line has been drawn to highlight the shift in the annexin V-positive population following STAT3 silencing compared with scrambled siRNA-transfected cells.

Table 2. Effect of signal transducer and activator of transcription 3 silencing and inhibition of extracellular signal-regulated kinase 1/2 activation on cell-cycle progression in the presence or absence of leukaemia inhibitory factor

Propidium iodide staining was used to measure DNA content by flow cytometry. The percentage of cells in different phases of the cell cycle are shown as the mean ± s.e.m. of two different experiments. *$P < 0.05$ compared with control. LIF, leukaemia inhibitory factor; siRNA, short interference RNA; STAT3, signal transducer and activator of transcription 3

Treatment	G_0/G_1 phase	S phase	G_2/M phase
Scrambled siRNA			
Control	51.6 ± 4.2	36.5 ± 3.1	8.9 ± 1.2
LIF	50.4 ± 2.6	33.1 ± 2.9	15.7 ± 2.5*
U0126	62.1 ± 3.1	25.4 ± 2.1	10.9 ± 2.1
LIF + U0126	64.6 ± 5.1	26.1 ± 4.5	10.8 ± 1.9
STAT3 siRNA			
Control	52.1 ± 2.8	39.2 ± 1.1	8.1 ± 1.9
LIF	49.8 ± 1.9	32.3 ± 3.6	17.4 ± 2.4*
U0126	54.9 ± 3.5	35.1 ± 2.3	9.1 ± 1.7
LIF + U0126	55.7 ± 4.6	34.5 ± 3.8	13.1 ± 2.0

Furthermore, the proliferation of STAT3-silenced cells was evaluated in the presence of LIF and after U0126 pretreatment. An increase in the proliferating cell population (G_2/M phase) was observed following LIF stimulation in both scrambled siRNA treated and STAT3-silenced cells, but no change was observed following inhibition of ERK1/2 activation by U0126 in both the above groups (Table 2). Compared with control cells, there was no change in the G_2/M phase cell population in scrambled siRNA-transfected cells treated with both U0126 and LIF, but there was an increase in the proportion of proliferating cells (G_2/M phase) in the STAT3-silenced group treated with both U0126 and LIF compared with untreated STAT3-silenced cells, which was not statistically significant (Table 2).

Apoptosis was also evaluated in STAT3-silenced cells using annexin V staining. An increase in apoptosis was observed in STAT3-silenced cells compared with scrambled siRNA-transfected cells (Fig. 7*b*). There was a further increase in apoptosis in STAT3-silenced cells treated with U0126 or with both U0126 and LIF for 12 h (Fig. 7*d*); however, this failed to reach statistical significance compared with scrambled siRNA-treated cells (Fig. 7*c*).

Discussion

During the peri-implantation phase of embryonic development, controlled proliferation and self-renewal of trophoblast cells play an important role in the successful establishment of pregnancy. This is facilitated by several cytokines and growth factors present in the uterine microenvironment. For example, EGF increases the survival of trophoblast cells by activating several pathways, such as ERK1/2, PI3K and p38 (Johnstone *et al*. 2005; Magarinos *et al*. 2007). Another growth factor present during the peri-implantation phase, namely IGF-1, acts in an autocrine and/or paracrine manner to regulate early placental growth and function. IGF-1 has been reported to increase the proliferation of primary trophoblast cells obtained from explant culture

(Maruo *et al*. 1995). Similarly, VEGF increases the proliferation of trophoblast cells by activating the MAPK–ERK1/2 pathway, which can be specifically inhibited by an MEK1/2 inhibitor (Cha *et al*. 2001). As observed in mice, LIF is secreted by the glandular epithelium of the human endometrium and is expressed at higher levels in women with proven fertility during the secretory and/or post-ovulatory phase of the menstrual cycle, suggesting its importance in human reproduction (Cullinan *et al*. 1996). In the human first trimester trophoblast cell line (HTR-8/SVneo), stimulation with LIF led to a concentration-dependent increase in proliferation through enhanced transition of cells from the resting to G_2/M phase of the cell cycle. This observation is consistent with previous observations that LIF increases the proliferation of JEG-3 choriocarcinoma cells, although the increase in the proliferation of those cells was observed at concentrations as low as 1 ng mL^{-1} (Fitzgerald *et al*. 2005). This may be due to differences in the cell lines used between the studies.

In the human placenta, villous trophoblast cells show immunolocalisation of p-ERK1/2 until Week 12 of gestation, which is indicative of their involvement in the early phase of trophoblast proliferation (Kita *et al*. 2003). It has been observed that ERK1/2 activation controls the interleukin (IL)-11-mediated proliferation of HT-29 cells (human colorectal adenocarcinoma cell line) as well as the LIF-mediated increase in the proliferation of P19 (human embryonal carcinoma) cells (Schuringa *et al*. 2002; Yoshizaki *et al*. 2006). Furthermore, the increase in the proliferation of P19 cells following LIF stimulation was found to be independent of the activation of STAT3. Activation of ERK1/2 also plays an important role in the leptin-mediated increase in trophoblast cell proliferation (Magarinos *et al*. 2007). These studies highlight the significance of ERK1/2 activation in the proliferation of trophoblasts and other cell types. Stimulation of HTR-8/SVneo cells with LIF resulted in the activation of both STAT3 Tyr^{705} and ERK1/2 (Figs 2, 4). ERK1/2 is a serine/threonine kinase that is activated by an upstream MEK1/2, which is activated through another kinase upstream to that (i.e. mitogen-activated protein kinase kinase kinase (MAPKKK) or c-Raf). To investigate the significance of the activation of the MAPK–ERK1/2 pathway, downstream signalling can be abrogated using pharmacological inhibitors such as U0126 or PD98059 for MEK1/2, which can block the activation of ERK1/2. In the present study, we used U0126, an inhibitor of both MEK1 and MEK2, at a concentration of 10 μM, which is non-toxic to the cells. Pretreatment of HTR-8/SVneo cells with U0126 for 2 h resulted in complete abrogation of ERK1/2 activation, at the basal level as well as after LIF stimulation. In contrast with observations that LIF was responsible for an increase in the G_2/M phase transition of HTR-8/SVneo cells, after inhibition of ERK1/2 activation, irrespective of LIF stimulation, there was no change in the proportion of cells undergoing G_2/M transition. This suggests that LIF-mediated activation of ERK1/2 signalling pathways plays an important role in trophoblast cell proliferation by promoting the G_2/M phase transition of cells. Activation of the ERK1/2 pathway has been documented during the increased proliferation of cells through an increase in the transition from the resting stage to the G2/M phase. For example, leptin increases vascular smooth muscle cell progression to the G_2/M phase via activation of ERK1/2 (Huang *et al*. 2010). In fibroblasts and epidermal cells, activation of ERK1/2 regulates cell cycle progression by increasing the expression of cyclins (Dumesic *et al*. 2009). So, it can be suggested that activation of ERK1/2 is key to the regulation of the proliferation of HTR-8/SVneo cells, which may itself be regulated by the activation and expression of several cell cycle-associated proteins.

Treatment of HTR-8/SVneo cells with LIF in the presence or absence of U0126 led to the inhibition of activation of signalling molecules such as ERK1/2 and p90RSK, although it had no effect on the activation of STAT3 Tyr^{705} (Figs 2, 4). These observations further suggest that the LIF-mediated increase in proliferation of HTR-8/SVneo cells was brought about by activation of the ERK1/2-mediated signalling pathway and not through activation of the STAT3 Tyr^{705} pathway. Furthermore, to verify the independent role of STAT3 on LIF-mediated proliferation of HTR-8/SVneo cells experimentally, we silenced its expression using siRNA. Silencing of STAT3 expression in HTR-8/SVneo cells did not affect the LIF-mediated increase in proliferation because there was a comparable increase in the level of the G_2/M phase cell population, but inhibition of ERK1/2 activation in STAT3-silenced cells inhibited the LIF-mediated proliferation of cells. These observations further suggest that although STAT3 has a role in increasing the invasion of trophoblast cells, it does not have a significant role in LIF-mediated proliferation (Fitzgerald *et al*. 2008).

Inhibition of the LIF-mediated increase in G_2/M progression by U0126 was associated with a decrease in the absolute cell count after 48 h of treatment (Fig. 1*b*). One reason for this could be the induction of apoptotic cell death following ERK1/2 inhibition because EGF and leptin enhance the survival of trophoblast cells by activating ERK1/2-dependent MAPK signalling (Garcia-Lloret *et al*. 1996; Magarinos *et al*. 2007; Perez-Perez *et al*. 2008). Furthermore, activation of p90RSK, downstream to ERK1/2, has been linked to the activation of several anti-apoptotic proteins, such as Bcl-xL/Bcl-2 associated death promoter, eukaryotic elongation factor-2 kinase and glycogen synthase kinase-3, so it may have a role in cell survival (Garcia-Lloret *et al*. 1996; Ticchioni *et al*. 2007; Quoyer *et al*. 2010). In addition, ERK1/2 activation has been reported to trigger a survival mechanism by increasing the Bcl-2/Bax ratio in TNF-related apoptosis-inducing ligand (TRAIL)-induced stress in HeLa cells (Lee *et al*. 2006). In HTR-8/SVneo cells, inhibition of ERK1/2 activation led to an increase in apoptosis, with a concomitant decrease in the Bcl-2/Bax ratio. In the present study, stimulation of HTR-8/SVneo cells with LIF after blockade of ERK1/2 activation could not rescue the apoptotic changes induced by U0126. However, LIF alone increased the Bcl-2/Bax ratio in HTR-8/SVneo cells but, after ERK1/2 inhibition, the ratio was reversed, favouring an increase in apoptosis. This change in the Bcl-2/Bax ratio was reflected by an increase in cell death at 48 h compared with 12 h after treatment with U0126 (Fig. S1).

Because STAT3 activation was not associated with the LIF-mediated increase in proliferation, we wanted to investigate whether this was associated with the regulation of apoptosis. Silencing the expression of STAT3 brought about an increase in

the apoptosis of HTR-8/Svneo cells, which increased further after inhibition of the ERK1/2-mediated signalling pathway.

In conclusion, the findings of the present study suggest that the LIF-mediated proliferation of HTR-8/SVneo cells is regulated by a ERK1/2-dependent signalling pathway by increasing G_2/M transition and is not associated with activation of STAT3 Tyr^{705}. In addition, LIF promotes survival of HTR-8/SVneo cells by upregulating the Bcl-2/Bax ratio, whereas ERK1/2 inhibition increases apoptosis by decreasing the Bcl-2/Bax ratio. Silencing STAT3 expression in HTR-8/SVneo cells had no effect on their proliferation.

Acknowledgements

This study was supported financially by the National Institute of Immunology, New Delhi, and the Indian Council of Medical Research, Government of India. G.J.P. is Postdoctoral Fellow of the Department of Biotechnology (DBT), Government of India; P.S. is a recipient of a Senior Research Fellowship from the Council of Scientific and Industrial Research (CSIR), Government of India; and D.M.M.P. is supported by a PhD grant from the Friedrich-Schiller University, Jena. The work reported in this paper was facilitated by an Indo-German exchange program between the Department of Science and Technology (DST), Government of India, and the Deutscher Akademischer Austausch Dienst (DAAD), Germany.

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Manuscript received 24 November 2010, accepted 24 December 2010

Journal of Reproductive Immunology 88 (2011) 106–111

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jreprimm

MicroRNAs in pregnancy

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ARTICLE INFO

Article history:
Received 30 September 2010
Received in revised form
24 December 2010
Accepted 12 January 2011

Dedicated to Professor Ursula Koldovsky, who died in October 2010 at the age of 73.

Keywords:
Micro-RNA
Post-transcriptional gene silencing
Pregnancy
Placenta
Trophoblast
Immune tolerance
Embryonic stem cells

ABSTRACT

Since the discovery of non-coding RNAs, several families of small regulatory molecules have been described including small nucleolar RNAs, piwi-interacting RNAs and microRNAs (miRNAs). MiRNAs are small single-stranded RNA molecules which play an important role in the regulation of gene expression at the transcriptional level. Recent studies demonstrated that about 30% of human genes are regulated by miRNAs and their deregulation has been associated with malignancies and poor outcome. Therefore, it is not surprising that profiling of miRNAs expression and studies on their regulation became a great field of interest in the last decade. However, miRNA-mediated regulation in pregnancy remains poorly investigated although several independent processes associated with placenta development have been shown to be miRNA-regulated. This review provides a general overview of the current data on profiles and functions of microRNAs in the peri-implantation period, embryonic stem cells, placentation and pregnancy, as well as in several pregnancy-related pathologies. We conclude that miRNAs present in the maternal circulation may provide a new promising diagnostic tool for pregnancy disorders.

1. Introduction

Small non-coding RNAs (ncRNAs) constitute a group of RNAs which do not code for proteins, but instead exercise control over those that do. The first ncRNA was characterized in 1965 in baker's yeast, but the physiological role of ncRNAs was not manifest until 1993 when Lee and colleagues described for the first time the involvement of *lin4*, a so called "small temporal RNA", in controlling developmental timing in *Caenorhabditis elegans* (Lee et al., 1993). It was only in the early 2000s that the term microRNA (miRNA) was introduced and the intracellular mechanisms of RNA interference (RNAi) started to be described. One of the first identified characteristics of the miRNAs was the highly conserved sequences throughout species and the fact that they are expressed in a tissue-specific manner. However, their importance in the control of genome expression became clear when the analysis of miRNA sequences revealed the vast amount of recognition sites on many mRNAs, which suggested a potential role of miRNAs in the control of transcription and translation of protein-coding-RNAs and provided information about the still unexplained 98% of genes which do not produce proteins (Zhang et al., 2007; Buckingham, 2003). It is hypothesised that miRNAs may be key factors in evolutionary processes and particularly in the evolution of the complexity of higher mammals (Bentwich et al., 2005).

During the last decade, about 800 miRNAs have been described in humans and their function in the regulation of cell proliferation and apoptosis in cancer has been demonstrated (Zhang et al., 2007). Currently, most of the miRNA-related studies compare cancer cells versus normal cells, but the analysis of miRNAs in the control of physiological processes including pregnancy is just incipient. Recent reports demonstrate that specific patterns of miRNAs are expressed only in embryonic stem cells and in early phases of embryonic development and some miRNAs are shown to

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doi:10.1016/j.jri.2011.01.004

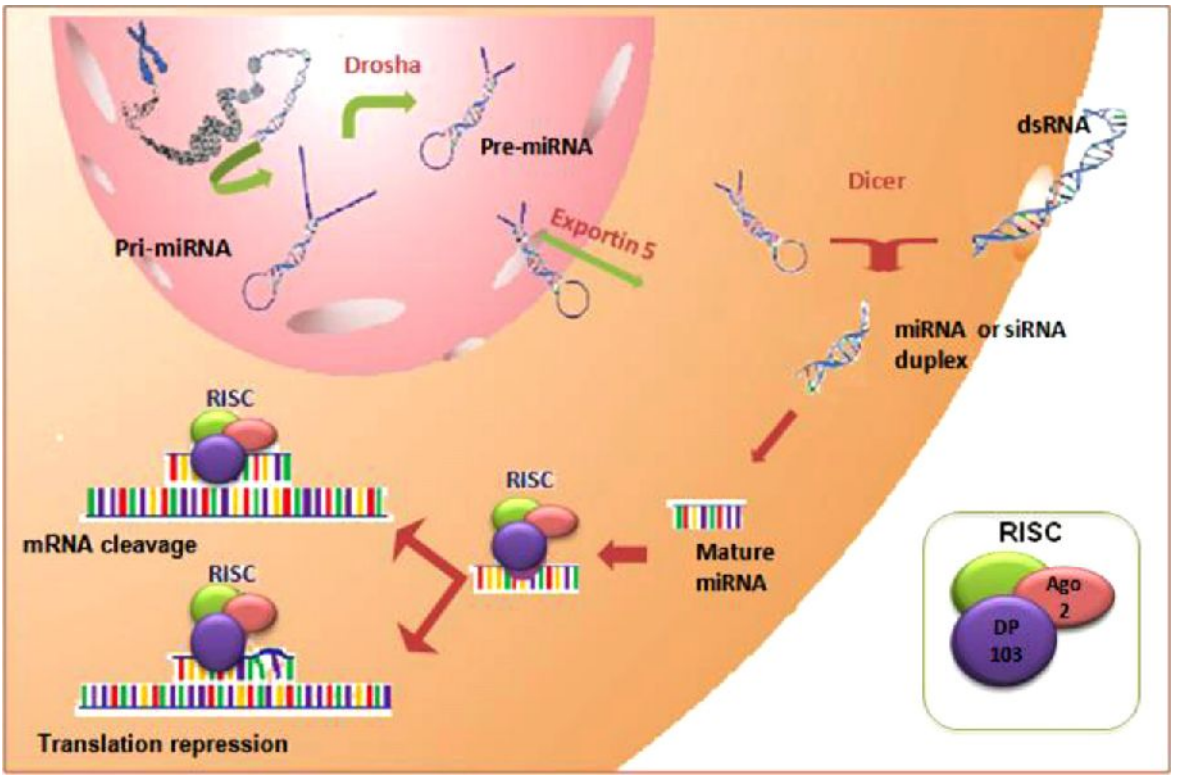

Fig. 1. The principal mechanism of RNA interference. Inside the nucleus, pri-miRNA are cleaved by Drosha to pre-miRNA and transported into the cytoplasm by Exportin 5 (green arrows). The subsequent cascade is shared with exogenous siRNA (red arrows). Processing by Dicer results in mature miRNA or functional siRNA which bind to RISC and to complementary RNA sequences. Two major enzymes involved in the RISC complex are Ago2 and DP130. Complete complementarity induces degradation and partial annealing leads to translational repression (modified after (Navarro and Monzo, 2010)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

be less strongly expressed in choriocarcinoma cells than in normal trophoblast (Chao et al., 2010; Navarro and Monzo, 2010). More surprisingly, placental miRNAs seem to be released into the maternal circulation and their concentration and patterns in plasma raise the potential for them to become markers for the detection of pregnancy disorders such as fetal growth restriction (FGR) (Mouillet et al., 2010a; Mincheva-Nilsson and Baranov, 2010; Frangsmyr et al., 2005).

In this review, we summarize the current knowledge on miRNA biogenesis, targets and functions with relevance for pregnancy and placental development.

2. MicroRNA biogenesis and RNA interference pathway

The phenomenon of RNA interference was first described in 1998 and refers to gene silencing caused by introducing double-stranded RNA into the cell (Fire et al., 1998). Two types of RNA molecules trigger their effects through the RNAi pathway: small interfering RNAs (siRNAs) and miRNAs. While siRNA are synthetic sequences, miRNAs are endogenous small sequences of RNA (~22 nt) which have been shown to be highly conserved throughout evolution (Qavi et al., 2010).

Most miRNA are not complementary to a specific mRNA and thereby regulate simultaneously more than one gene. Additionally, different miRNAs can target the same mRNA and have similar biological functions, which can intensify or amplify their effects, also in case of their deregulation. It is currently estimated that about 30% of the human genome may be regulated by miRNAs (Bueno et al., 2008), which explains the rapidly increasing number of studies and publications in the field.

MiRNAs are transcribed from DNA as longer sequences known as pri-miRNAs, which are then cleaved by the nuclear enzyme Drosha to form hairpin pre-miRNAs before being exported into the cytoplasm through Exportin-5. Once in the cytoplasm, pre-miRNAs are further cleaved by Dicer, an enzyme of the RNase III superfamily of bidentate nucleases, to form mature single-stranded miRNAs. Finally, mature miRNAs are associated with the RNA-induced silencing complex (RISC) and repress protein translation (Bueno et al., 2008; Qavi et al., 2010; Cheng et al., 2005). The mechanism used for miRNA-mediated translational inhibition depends on the complementarity grade between the miRNA and its target mRNA. If alignment is complete, the cascade ends in cleavage of the target mRNA, while partial complementarity and alignment leads to translational repression of the target mRNA (Cheng et al., 2005; Navarro and Monzo, 2010) (Fig. 1).

The relevance of miRNA in reproduction is underlined by the finding that the cascade of the pivotal miRNA biogenesis proteins Drosha, Exportin 5, Dicer, Argonaute 2 (Ago2)

Table 1
MiRNA cluster C19MC on chromosome 19. Most members have been detected in the placenta (Liang et al., 2007; Bentwich et al., 2005; Bortolin-Cavaille et al., 2009) or in maternal blood (Miura et al., 2010) as marked.

microRNA	Bortolin-Cavaillé	Bentwich	Liang	Miura	microRNA	Bortolin-Cavaillé	Bentwich	Liang	Miura
miR-498	√	√	√	√	hsa-mir-519a-2	√			
hsa-mir-512-1	√				miR-519b	√	√	√	
hsa-mir-512-2	√				miR-519c	√	√	√	
miR-512-3p			√		miR-519d	√	√	√	√
miR-512-5p			√		miR-519e	√	√	√	
hsa-mir-515-1	√		√		miR-520a	√	√	√	√
hsa-mir-515-2	√				miR-520b	√	√	√	
miR-515-3p		√	√		miR-520c	√	√	√	
miR-515-5p			√	√	miR-520d	√	√	√	
hsa-mir-516-1					miR-520e	√	√	√	
hsa-mir-516-2	√				miR-520f	√	√	√	
miR-516-3p		√	√		miR-520g		√	√	
hsa-mir-516-4	√				miR-520h		√		
miR-516-5p					miR-521-1		√	√	
hsa-mir-516b2-3	√				miR-521-2	√	√	√	
miR-517a	√	√	√	√	miR-522	√	√	√	
miR-517b	√		√		miR-523	√	√	√	
miR-517c	√	√		√	miR-524	√	√	√	
miR-518a		√	√	√	miR-525	√	√	√	
hsa-mir-518a-1	√				miR-525-3p				√
hsa-mir-518a-2			√	√	miR-525-5p				√
miR-518b	√	√	√	√	miR-526a		√	√	
miR-518c	√	√	√	√	miR-526a-1	√			
miR-518d	√	√	√		miR-526b		√	√	√
miR-518e	√	√	√	√	miR-526c		√	√	
miR-518f	√	√	√		miR-527		√	√	
miR-519a		√		√	hsa-mir-1283-1	√			
hsa-mir-519a-1	√				hsa-mir-1283-2	√			
					miR-1323	√			

and DP103 is fully present and functional in trophoblast cells (Donker et al., 2007). The expression of these proteins does not depend upon decidualization, normoxia/hypoxia or culture medium composition. This shows the nonspecific basis of production of active miRNAs, and in part also accounts for the efficiency of exogenous siRNA when applied to trophoblasts (Wengenmayer et al., 2004).

Knockout studies of these pivotal miRNA biogenesis proteins confirm the requirement of functional miRNA in reproduction. Loss of Dicer within ovarian granulosa cells, luteal tissue, oocyte, oviduct and, potentially, the uterus renders females infertile (Hong et al., 2008). Disruption of the gene for Ago2 leads to a phenotype similar to the disruption of Dicer1, but with embryonic lethality early after the implantation stage (Morita et al., 2007).

3. MiRNA in the peri-implantation period

During the menstrual cycle, inflammation-like processes occur aiming to prepare the immunological receptivity of the endometrium for implantation. These processes are controlled by several proteins, enzymes and angiogenic factors which are differentially expressed and tightly regulated. Altered endometrial expression of these molecules seems to be responsible for inappropriate tissue regeneration, resulting in dysfunctional uterine bleeding, failed embryo implantation, and other endometrial disorders. Current reports have demonstrated that miRNAs participate in regulating dynamic changes in uterine gene expression patterns by controlling genes associated with the inflammatory response (Pan and Chegini, 2008; Chakrabarty et al., 2007).

A number of miRNAs are specifically expressed during the peri-implantation and pre-implantation periods in mice. Among the 32 miRNAs identified as up-regulated during the receptive phase, miR-101 and miR-199a* were shown to target cyclooxygenase-2 (Cox2) gene, which is known for its critical role in implantation and also for its abilities to promote inflammation and tumorigenesis (Chakrabarty et al., 2007).

4. MiRNA expression in placenta

Current reports on miRNA expression patterns have exposed a group of miRNAs almost exclusively expressed by the placenta and fetal brain tissues (Miura et al., 2010). Located in chromosome 19, C19MC represents the largest miRNA cluster ever reported. It comprises 54 predicted miRNAs, 43 of which have been cloned and sequenced (Bentwich et al., 2005; Bortolin-Cavaille et al., 2009; Liang et al., 2007) (Table 1).

Interestingly, this cluster is only present in primates and seems to be the result of duplication and mutation events unique to this taxonomical order, reinforcing the hypothesis of miRNA development as a state of evolution. Besides the current increasing knowledge about miRNA clusters, it is still not clear whether all miRNAs located in a miRNA cluster are co-regulated by the same cis-elements and trans-factors (Tsai et al., 2009).

The study of C19MC hypermethylation demonstrated an association between their expression pattern and the methylation status of a distal CpG-rich region at 17.6 kb upstream. Imprinting genes play important roles in the regulation of cellular differentiation and fate, and they are frequently expressed only in embryonic stages or placental tissues, which revealed C19MC as a miRNA cluster involved in human embryonic development (Tsai et al., 2009).

Six miRNAs are up-regulated in hypoxic trophoblast (miR-93, miR-205, miR-224, miR-335, miR-451 and miR-491) and one is down-regulated in hypoxia (miR-424) (Mouillet et al., 2010b; Donker et al., 2007). Analysis of the potential mRNA targets of these miRNA revealed that miR-424 controls FGFR1 and MAP2K1 while miR-205 silences MED1 gene expression, which is essential for murine placental development (Mouillet et al., 2010b). MiR-205 is expressed in the hematopoietic and reproductive systems (Landgraf et al., 2007). It is highly expressed in primary trophoblast cells, marginally expressed in trophoblast-derived cells, and undetectable in cancer cell lines (Mouillet et al., 2010a,b) which suggests that miR-205 plays a role in the adaptation of placental epithelium to injury.

Several further miRNAs are altered in placental injuries or exposure to toxic agents. In cigarette smoke-exposed placentas miR-16, miR-21 and miR-146a are down-regulated after cigarette smoke challenge (Maccani et al., 2010). In the immortalized cell line TCL-1, derived from the choriodecidua, miR-146a was dose-dependently reduced by nicotine and benzo(a)pyrene treatment (Maccani et al., 2010). These observations insinuate a role of miR-146 in the reaction on cell stress. Interestingly, strong effects on miR-146 have also been found after bisphenole A exposure of immortalized trophoblastic cells (TCL-1, HTR8), which confirms and underlines its role in answering cell stress (Avissar-Whiting et al., 2010).

Recent studies have described changes of placental miRNA profiles in preeclampsia. In one study expression of miR-210, miR-1, a miRNA in the 14q32.31 cluster region, miR-584 and miR-34c-5p was affected (Enquobahrie et al., 2010), while in another study the differential expression of miR-15b, miR-181a, miR-200C, miR-210, miR-296-3p, miR-377, miR-483-5p, and miR-493 has been reported (Mayor-Lynn et al., 2010). Only miR-210 coincides in both papers, which may confirm its involvement in the pathomechanism of preeclampsia.

Expression of some miRNAs is elevated in choriocarcinoma cells compared with normal trophoblast cells (miR-9*, miR-96, miR-203, miR-372 and miR-200a), whereas others are down-regulated (miR-199a, miR-199s, miR-199s*, miR-154, miR-370, let-7b, miR-299, miR-134 and miR-199b) (Chao et al., 2010). Within this group, miR-199b may display a key role in the control of trophoblast proliferation as forced expression of miR-199b results in inhibition of choriocarcinoma cell proliferation.

Thus far, very little is known about miRNA expression in individual cell types in the decidua and placenta surrounding the trophoblast cells. Let-7a and miR-320 are induced by blastocysts and during decidualization of the uterus (Xia et al., 2010a,b). MiRNA-222 has been described as a major factor in differentiation of endometrial stromal cells during decidualization (Qian et al., 2009). To the best of our knowledge no reports have been published on miRNA in decidual or placental immune cells.

5. MiRNAs in embryonic stem cells

Similar to C19MC, study of the expression signature in human embryonic stem (hES) cells demonstrated a specific pattern of miRNA. Surprisingly, the majority of the characterized miRNAs are also located in chromosomes 19 and X (Navarro and Monzo, 2010; Suh et al., 2004). Among the 36 miRNAs identified, seven miRNAs were expressed exclusively in hES cells (miR-200c, miR-368, mir-154*, miR-371, miR-372, miR-373 and miR-373*) suggesting that these miRNAs control specific functions of hES. Further, this study also revealed a stage-specific group of miRNAs involved in the regulation of development and differentiation and comprising let-7a, miR-301, miR-374, miR-21, miR-29b, and miR-29. The authors speculate that these miRNAs may be the primary regulators of hES cell maintenance or differentiation. Therefore, identification of their target mRNAs will provide information about the complex network of regulation in hES cells (Suh et al., 2004).

Additional studies on the involvement of miRNAs in embryonic stem cell differentiation have been performed in mice. MiR-17 family members, miR-17-5p, miR-20a, miR-93, and miR-106a, are differentially expressed in developing mouse embryos and function to control differentiation of stem cells. MiR-93 and miR-17-5p are strongly expressed within the mesoderm of gastrulating embryos, and specifically, miR-93 localizes to differentiating primitive endoderm and trophectoderm of the blastocyst (Foshay and Gallicano, 2009). A more recent study has analyzed mouse pre-implantation embryos at each development stage and has found that only a small number of miRNAs are strongly induced or repressed at any given stage. They further observed that several miRNAs showed dramatic directional changes in expression between successive stages of development and suggest that they are strongly expressed only during narrow time windows. They found nine miRNAs which are potentially involved in trophectoderm development: miR-297, miR-96, miR-21, miR-29c, let-7, miR-214, miR-125a, and miR-424 are up-regulated while miR-376a is down-regulated upon blastocyst formation (Viswanathan et al., 2009). During blastocyst-derived stem cell differentiation, mainly members of the let-7 family and miR-24 were induced. MiR-24 targets sdx2 which is a stem cell marker that declines during this differentiation process (Viswanathan et al., 2009).

6. Involvement of miRNA in regulation of materno-fetal immunotolerance

Several miRNAs seem to repress expression of immune tolerance-associated genes, including HLA-G, but without altering trophoblast invasion. In different situations, HLA-G is involved in developing immune tolerance, such as in pregnancy, inflammatory and autoimmune diseases or cancer (Veit and Chies, 2009). Abnormal HLA-G expression occurs in almost 70% of breast cancer lesions and

is associated with poor outcome (Chen et al., 2010). A recent study demonstrated a relation between miRNAs and HLA-G. Among others, aberrant hypermethylation of miR-148 and miR-152 was observed in primary human breast cancer specimens, suggesting a regulatory role for these miRNAs in HLA-G expression (Lehmann et al., 2008). This correlation was confirmed by a study on JEG-3 choriocarcinoma cells. MiR-152 repressed HLA-G expression with no effect on JEG-3 invasion. In addition, over-expression of miR-152 increases NK cell-mediated cytolysis implying that miR-152 has a role as an immune response enhancer (Zhu et al., 2010).

7. Pregnancy related miRNAs in maternal peripheral blood

Placenta-derived miRNAs in the maternal circulation seem to play a pivotal role in adaptation of the organism to pregnancy, especially in regard to inducing immune tolerance. Numerous members of the above mentioned C19MC cluster of miRNA have been detected in maternal blood (Miura et al., 2010). Elevated plasma levels of placental DNA and RNA are associated with clinical conditions related to placenta dysfunction, such as preeclampsia and intrauterine growth restriction (Alberry et al., 2009; Zhong et al., 2001), but little is known about the (placental) miRNA patterns in maternal blood during pregnancy and its correlation with diseases.

Placenta-specific miRNA expression in serum changes during the course of pregnancy. Some miRNA change more than 600-fold which has allowed researchers to distinguish accurately pregnant from non pregnant women by analyzing three miRNA that are highly expressed during pregnancy (miR-526a, miR-527 and 520d-5p) (Gilad et al., 2008). Among the significantly elevated miRNAs in plasma from pregnant women, miRNA-424 and miR-141 have been previously described as useful for diagnosis of malignancies (Mouillet et al., 2010a). MiR-141 belongs to the miR-200 cluster, physiologically exclusively expressed in hES cells, and is involved in carcinogenesis in breast cancer (Neves et al., 2010). MiR-424 regulates differentiation in human leukemia cells (Kasashima et al., 2004).

Fetal growth restriction is the second leading cause of perinatal morbidity and mortality. Recently, an association between FGR and increased circulating miRNA levels has been described based on the hypoxic conditions which are frequently involved in the pathophysiology of fetal growth restriction.

8. Conclusions and perspectives

Pregnancy is a complex process which requires tightly regulated gene expression in the placenta. MiRNA tune and control gene expression post-transcriptionally, but manifold factors and situations tune and control miRNA. Therefore, their potential for becoming novel biomarkers and also drug targets is enormous. In pregnancy, miRNA may reflect disorders not yet detectable with other methods and contribute to understanding the underlying pathological mechanisms.

Acknowledgements

The group is currently working on miRNA expression in trophoblastic cells and supported by the Deutsche Forschungsgemeinschaft (Project Number MA 1550/7-1). DMM is receiving a Ph.D. grant from the Friedrich-Schiller-University Jena.

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Reduction in miR-141 is Induced by Leukemia Inhibitory Factor and Inhibits Proliferation in Choriocarcinoma Cell Line JEG-3

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Keywords
Micro-RNA, placenta, post-transcriptional gene silencing, pregnancy, proliferation, trophoblast

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Submitted April 28, 2011;
accepted May 10, 2011.

Citation
Morales-Prieto DM, Schleussner E, Markert UR. Reduction in miR-141 is Induced by Leukemia Inhibitory Factor and Inhibits Proliferation in Choriocarcinoma Cell line JEG-3. Am J Reprod Immunol 2011; 66 (Suppl. 1): 57–62

doi:10.1111/j.1600-0897.2011.01037.x

Problem
Starting from the peri-implantation period, leukemia inhibitory factor (LIF) is a major regulator of trophoblast functions. Micro-RNAs (miRNA) are short non-coding RNA sequences, which regulate expression of genes at post-transcriptional level. The influence of LIF on miRNA expression in trophoblastic cells has not yet been analyzed and was focus of this investigation.

Method of study
JEG-3 choriocarcinoma cells have been stimulated with LIF for 1, 2, 4, 6, and 24 hr. The expression of miR-9, miR-141, miR-21, miR-93, and let-7g has been analyzed by real-time PCR. Subsequently, miR-141 has been silenced and over-expressed to test its role in the proliferation of JEG-3 cells after 24 and 48 hr.

Results
MiR-141 has been significantly downregulated by more than 50% after LIF stimulation, while miR-21 and miR-93 expression has been significantly upregulated. Silencing of miR-141 completely inhibited the proliferation of JEG-3 cells, while over-expression had no effect.

Conclusion
LIF regulates expression of miRNA in trophoblastic cells, which may be responsible for several functional effects induced by LIF.

Introduction

Leukemia inhibitory factor (LIF) induces tyrosine phosphorylation of signal transducer and activator of transcription 3 (STAT3) in several trophoblast and choriocarcinoma cell types and lines (summarized in[1]). This event triggers several trophoblastic functions, such as migration, invasion or induction and suppression of expression of a variety of genes.[2,3] Because functional effects have been observed after several days, it cannot be excluded that parts thereof are secondary or indirectly induced. We argue that micro-RNA (miRNA) may be involved in the regulation of these previously observed LIF-induced functions. For this reason, we have selected a panel of five miRNAs which have been described to influence STAT3 expression or which are known to be expressed on full activation of STAT3.

MiRNAs constitute a novel group of regulatory molecules that play a pivotal role in the control of gene expression at post-transcriptional level. The number of miRNAs described thus far arises approximately 1000 (MiRBase V16), which may regulate up to 30% of the human genome.[4] The signature of miRNA expression is regulated in a tissue- and developmental stage-specific manner, and thereby, it

may be used as a biomarker for the identification of certain physiological or pathological events including malignancies. Current reports have demonstrated specific patterns of miRNAs regulating changes in uterine gene expression[5,6] or miRNAs that are specifically expressed in embryonic stem cells and whose expression is altered during embryonic development.[7] Likewise, some miRNAs are found less expressed in choriocarcinoma cells than in normal trophoblast, which suggests a role in carcinogenesis.[8]

We focused on five miRNAs previously published to correlate with tumor grade, to be implicated in pregnancy, or to be related with members of the signaling intracellular cascade of LIF. For instance, miR-141, belonging to the miR-200 cluster, is found upregulated in nasopharyngeal and ovarian carcinomas in comparison with normal tissues and correlates with poor prognosis.[9,10] As biological marker, levels of miR-141 are increased in plasma from pregnant women.[11] Also, expression of miR-9 may serve as a biomarker, which correlates with tumor grade and metastatic status in breast and cervical cancer.[12,13] Its inhibition results in increased levels of phospho-STAT3 in embryonic stem cells.[14] Among the miRNAs selected for the present investigation, to date, miR-21 is the most extensively studied. Because of its over-expression in at least six different solid cancers (lung, stomach, prostate, colon, pancreas, and breast), it has been considered an oncomir (reviewed in[15]). MiR-21 can be induced by STAT3.[7] Mir-93 seems to be related with the trophoblast response to hypoxia as it is upregulated in hypoxic trophoblast cells.[16] MiR-93 shares some features with miR-141 and miR-21 as they all are expressed in human embryonic stem cells, but their effects in cell maintenance or differentiation seem to be dissimilar. While miR-93 expression remains similar also in adult tissue, miR-141 attenuates differentiation and miR-21 expression intensifies it.[17–20] Finally, we selected let-7g, a member of one of the currently most important miRNA families (let-7), which is aberrantly expressed in human cancer.[21] Let-7g and also miR-21 were expressed *in vitro* as well as *in vivo* via STAT3 activation after IL-6 stimulation.[22]

Although the LIF-induced STAT3 activation in trophoblastic cells seems to be crucial for many cell functions, thus far, the LIF-induced miRNA expression in these cells has not yet been investigated. Therefore, in the present study, we aim to analyze the kinetics of the expression of miR-9, miR-21, miR-93, miR-141, and let-7g after LIF treatment in JEG-3 cells. Being the most affected, influence of miR-141 on proliferation has been analyzed by its experimental over-expression and silencing.

Materials and methods

Cell Cultures

JEG-3 (DSMZ, Braunschweig, Germany) is an adherent human choriocarcinoma cell line preserving several trophoblast-like capacities including production of pregnancy-related hormones and cytokines. JEG-3 cells cultures were performed at 10^6 cells/175 cm^2 flask and maintained under standard conditions (37°C, 5% CO_2, humid atmosphere) in Ham's F-12 Nutrient Mixture with L-glutamine (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco) and 1% penicillin/streptomycin antibiotic solution (Gibco).

Quantitative Real-time PCR

JEG-3 cells were seeded in 12-well plates, allowed to attach, and deprived of serum overnight. Thereafter, cells were challenged with 10 ng/mL LIF (Millipore, Schwalbach, Germany) up to 24 hr, and total RNA (containing miRNAs) was isolated with TRIzol (Invitrogen, Darmstadt, Germany). Mature miRNAs were reverse-transcribed, and real-time PCR was performed using TaqMan miRNA assays with specific primers for the selected miRNAs (Applied Biosystems, Darmstadt, Germany; see Table I). Each real-time PCR was performed in duplicates, including no-template controls. For normalization, several endogenous controls were tested, and RNU48 was selected after showing high stability and expression in our model. Fold changes were determined using

Table I MiRNA Assays Used for qRT-PCR (Applied Biosystems)

Assay ID	Assay name	Target sequence
002282	hsa-let-7g	UGAGGUAGUAGUUUGUACAGUU
000397	hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA
001090	mmu-miR-93	CAAAGUGCUGUUCGUGCAGGUAG
000463	hsa-miR-141	UAACACUGUCUGGUAAAGAUGG
000583	hsa-miR-9	UCUUUGGUUAUCUAGCUGUAUGA
001006	RNU48	GAUGACCCCAGGUAACUCUGA GUGUGUCGCUGAUGCCAUCAC CGCAGCGCUCUGACC

the 'delta-delta Ct' method relative to the expression at the beginning (0 hr) before LIF stimulation was initiated. The experiments were repeated independently five times for miR-9, miR-141, and let-7g and four times for miR-21 and miR-93. Differences in the quantified gene expression were statistically assessed using the non-parametric Wilcoxon test and considered significant when $P < 0.05$.

Over-expression and Silencing of miR-141

Anti-miR™ miRNA inhibitors are single-stranded nucleic acids specifically designed to bind and to inhibit endogenous miRNA molecules. Conversely, Pre-miR™ miRNA precursor molecules are double-stranded RNA molecules, which mimic endogenous mature miRNA. Owing to their small size, all these molecules can be easily delivered into the cells using transfection reagents similar to those used for small interfering RNA transfection.

To determine the effect of miR-141 on cell proliferation, JEG-3 cells were transfected with either anti-miR inhibitors or pre-miR precursors specifically designed for miR-141 or the respective non-genomic negative controls (assays IDs: AM10860, AM17010, PM10860, AM171010; Applied Biosystems). Transfection was performed by applying Nanofectin (PAA, Cölbe, Germany) as follows: 24 hr before transfection, cells were seeded in 12-well plates to obtain a 70–80% of confluence the day of transfection. The following day, two solutions were prepared: (1) Three microlitres of either anti- or pre-miR solution (5 μM each) was diluted in 32 μL serum-free medium. (2) Three microlitres of nanofectin was diluted in 30 μL of serum-free medium. Solutions 1 and 2 were mixed and incubated for 30 min at room temperature. Subsequently, the mix was added into the wells containing the cells in 500 μL serum-free medium and incubated at 37°C for 4 hr. Transfection was terminated by the addition of 250 μL of medium supplemented with 30% FCS. The next morning, cells were trypsinized and seeded into 96-well plates (1×10^4 cells/well).

Proliferation Assay

Cell proliferation was analyzed using a Cell Titer AQeous MTS assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. Assays were commenced with 1×10^4 cells in 96-well plates, and cells initiated spontaneous proliferation. After 0- (basal value), 24-, and 48-hr incubation, proliferation was measured by adding methyl tetrazolium salt (MTS) solution 20 μL/well and measuring the absorbance at 490 nm. Three independent cultures have been performed for each time point. Differences in the quantified proliferation rates of JEG-3 cells were statistically assessed by Student's *t*-test and considered significant when $P < 0.05$.

Results

MiRNA Expression Kinetics After LIF Stimulation

JEG-3 cells were stimulated up to 24 hr with 10 ng/mL LIF, and the expression of miRNAs was assessed at five different time points by real-time PCR. LIF stimulation significantly reduces the expression of miR-141 after 4 and 6 hr compared with the respective basal expression levels. MiR-93 increases at all time points (significantly after 2 and 24 hr of LIF stimulation up to 9.2-fold), and miR-21 increases significantly after 1, 6, and 24 hr with a maximum of 19.8-fold. After 4 hr of LIF stimulation, miR-21 expression is significantly reduced compared with that at the aforementioned time points. This strong reduction has been obvious in each individual experiment. All other changes, including the 2.3-fold increase in let-7g expression at 2 hr LIF stimulation, were not significant (Fig. 1).

Effects of miR-141 Silencing and Over-expression on Proliferation

Because we have observed the most stable LIF-induced changes in miR-141, we decided to analyze its impact on proliferation by silencing and over-expression in JEG-3 cells. Transfection of JEG-3 cells with control substances reduces proliferation at all analyzed time points. Only silencing of miR-141 leads to a block of proliferation, when compared with its respective control, and is, after 48 hr, approximately 50% lower than in cells transfected with a non-genomic control sequence. In all other settings, proliferation is time-dependent. Over-expression of miR-141 does not lead to a further increase in proliferation (Fig. 2).

Discussion

We have observed a significant influence of LIF on the expression of the miRNAs miR-21, miR-93

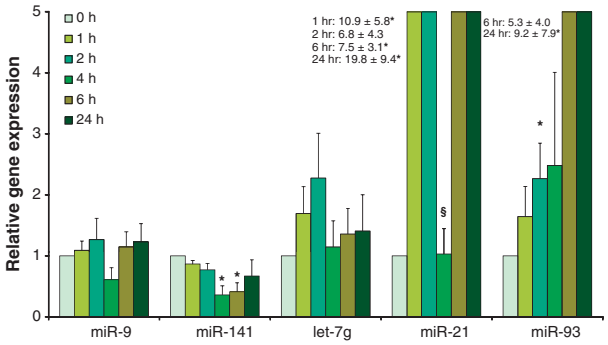

Fig. 1 Normalized expression kinetics of miRNAs after leukemia inhibitory factor (LIF) induction. JEG-3 cells were stimulated with LIF (10 ng/mL), and miRNA gene expression was measured by real-time PCR at several time points. Results were normalized to the basal (0 hr) expression levels of the respective miRNAs and are expressed as mean. Error bars show standard error of the mean. $n = 5$ independent experiments for miR-9, miR-141, and let-7g, $n = 4$ for miR-21 and miR-93. Values for the exceeding bars and SE are shown respectively on the left side. *$P < 0.05$ (Wilcoxon test) compared with initial values (0 hr); §$P < 0.05$ compared with values at 1, 6, and 24 hr.

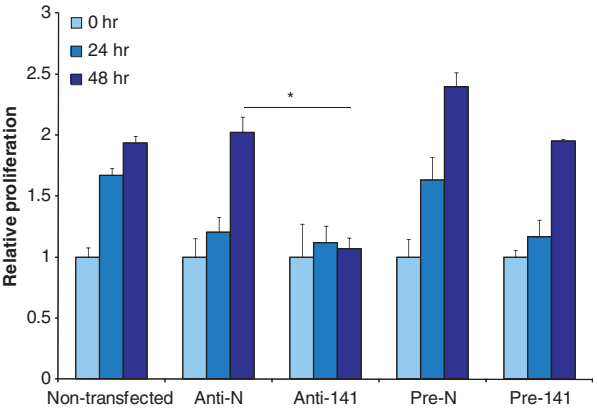

Fig. 2 Impact of miR-141 on JEG-3 proliferation. JEG-3 cells were transfected with either pre- or anti-miRNAs, and cell proliferation was measured by MTS at 0, 24, and 48 hr. Results are expressed as mean of three independent experiments. Error bars show standard error. *$P < 0.05$ (Student's *t*-test).

(upregulation), and miR-141 (downregulation). The strongest effects were observable 4 and 6 hr after stimulation with LIF when miR-141 was downregulated by far more than 50%. A surprising result was the downregulation of miR-21 after 4 hr of LIF stimulation compared with the earlier and later analyses. Silencing of miR-141 inhibits proliferation of JEG-3 cells, while over-expression does not further induce proliferation. To the best of our knowledge, thus far, no studies have been published on LIF-induced miRNA in any cell type, but several STAT3-induced miRNAs have been described. LIF is well known to phosphorylate and activate STAT3 in a variety of cells including trophoblastic cells, where it induces invasiveness.[3]

In our experiments, LIF stimulation of JEG-3 cells significantly increased miR-21 expression. This is compatible with previous reports that in head and neck carcinoma, osteosarcoma, ovarian carcinomas, and others, miR-21 promotes proliferation, migration, and invasion.[23–25] The significant downregulation of miR-21 after 4 hr of stimulation may be attributable to the initiation of negative intracellular feedback mechanisms, such as induction of suppressors of cytokine signaling 3, but which may then again be overcome by the permanence of LIF stimulation.[26] Let-7g was slightly, but not significantly, increased after LIF stimulation, which is in contrast to previous descriptions on let-7g in cancer. In hepatocellular carcinoma, ectopic expression of let-7g inhibits cell migration and growth.[27] In gastric cancer, low let-7g is associated with unfavorable outcome in overall survival independent of clinical covariates, including depth of invasion, lymph-node metastasis, and stage.[28] LIF-stimulated JEG-3 cells expressed significantly higher levels of miR-93, which is in line with previous observations on tumors. In human glioblastoma, miR-93 suppresses integrin-β8 expression, which promotes tumor growth and angiogenesis.[29] In human T-cell leukemia virus 1, miR-93 targets the mRNA for tumor protein 53–induced nuclear protein 1 (TP53INP1), which is a tumor suppressor protein.[30] In our experiments, miR-9 did not change considerably. In human embryonic stem cell-derived neural progenitors, loss of miRNA-9 reduces proliferation and increases migration.[31] On the other hand, miR-9 targets E-cadherin, which is a suppressor of metastasization and angiogenesis. Its high expression in breast cancer is correlated with the malign properties.[32]

In JEG-3 cells, LIF significantly downregulated miR-141. Repression of miR-141 induces invasiveness of breast cancer cells by targeting the endothelial mesenchymal transition activators ZEB1 and ZEB2, which downregulate E-cadherin expression.[18] Also in colorectal cancer, miR-141 negatively correlates with migration and invasion.[9] A different function has been observed for miR-141 in gastric cancer cells, where its over-expression by the application of

its precursors inhibited the proliferation.[33] In contrast, it is upregulated in nasopharyngeal carcinoma, where it positively correlates with proliferation, migration, and invasion.[34] In our hands, silencing of miR-141 inhibits proliferation of JEG-3 choriocarcinoma cells, which goes in line with these results. The observed strong impact of LIF on various miRNA in JEG-3 choriocarcinoma cells underlines the expected involvement of miRNAs in the regulation of essential functions in trophoblastic cells and thus in tuning placentation and other crucial processes in reproduction and pregnancy.

Acknowledgements

The project has been supported by the German Research Foundation (DFG, project Ma1550/7-1). DMMP has a Ph.D. grant from the regional graduate academy of the Friedrich-Schiller-University Jena, Germany.

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MiRNA expression profiles of trophoblastic cells

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Short title: microRNA expression in trophoblastic cells

Keywords: Pregnancy, trophoblast, microRNA, placenta,

Abstract

Background: MicroRNAs (miRNAs) are small single-stranded RNA molecules which are important post-transcriptional modulators of gene expression. Trophoblast cells are a heterogenous group a fetal cells in the placenta displaying a spectrum of functions. The regulation of their behavior may partly underly the control through miRNAs. Therefore, we aimed to compare the miRNA profile of primary third trimester trophoblast cells with that of different trophoblastic cell lines.

Material and methods: Total RNA was isolated from cytotrophoblast cells from 3 healthy term placentae and the cell lines HTR-8/SVneo (immortalized trophoblast cells), JEG-3 (choriocarcinoma), ACH-3P and AC1-M59, which are choriocarcinoma cells fused with first and third trimester trophoblast cells, respectively. The expression level of 762 different miRNAs was quantitatively analzed by using a TaqMan Human MicroRNA Array. The results for 10 important miRNA were confirmed by individual qPCR.

Results: The analyzed cell types share many similar patterns of miRNAs, but are significantly distinct in the expression of two major miRNA clusters: chromosome 19 miRNA cluster (C19MC; containing at least 54 different miRNAs) and C14MC (at least 34 miRNAs). Both clusters are expressed in primary term trophoblast cells, but C19MC is not expressed in HTR-8/SVneo and C14MC not in the choriocarcinoma-derived cell lines (complete array data at NCBI Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32346). Another minor cluster (miRNA-371 to miRNA-373 cluster), also located on chromosome 19 is also expressed in all cells except HTR-8/SVneo. Beside the miRNAs within the clusters, miR-24, miR-193b and miR-720 are the three highest expressed in all cell types,

while miR-200c expression is >50fold higher in primary trophoblast than in all cell lines.

Conclusion: Primary term trophoblast cells and trophoblastic cell lines display major differences in their miRNA fingerprints which may be involved in their different behavior and characteristics.

Introduction

Since the discovery of the first microRNA *lin-4* in 1993 [1], the study of microRNAs (miRNAs) has generated great interest due to their vast potential in the regulation of protein-coding genes. MiRNAs are highly conserved sequences of single-stranded RNA (~19-22nt) which repress gene expression by a mechanism involving the RNA interference pathway [2]. Depending on the complementary grade between the miRNA and its mRNA target, the pathway results in inhibition of translation, or cleavage of the target mRNA, when partially or fully complimentary, respectively [3]. This characteristic allows targeting of several genes simultaneously and therefore, it can be expected that 30% of the human genome may be regulated by miRNAs [4].

Remarkably, miRNA genes are frequently located at fragile sites and cancer-related genomic regions [5], and trend to be organized into clusters suggesting that miRNAs belonging to a same cluster are likely to have similar functions and be regulated by the same promoter and in the same transcriptional orientation [6, 7]. The analysis of the miRNA signature (miRNome) in normal human tissues revealed some universally expressed miRNAs but also several groups of miRNAs exclusively or preferentially expressed in a tissue-specific manner [8]. Likewise, the miRNA expression signature is frequently found altered in cancer [9, 10], and can be successfully used to distinguish between cancer and normal tissues [11, 12] or even to clarify poorly differentiated tumors [13].

Recent reports have described two large miRNA clusters expressed in placenta: The chromosome 19 miRNA cluster (C19MC), which maps to chromosome 19q13.41 and comprises 54 predictive miRNAs, 43 of which have been already cloned an

sequenced (reviewed in [3]); and the C14MC located in the 14q32 domain and which contains at least 34 miRNAs [14]. These clusters differ in some important features: C19MC is only found in primates while C14MC appears to be conserved among eutherian species [15]; and even when both of them are imprinted genes, C19MC is only expressed from the paternally inherited chromosome whilst C14MC is only expressed from the maternally inherited chromosome [15, 16]. Imprinting genes are known to be involved in human embryonic development and to play important roles in the regulation of cellular differentiation and fate [17]. Therefore, study of these clusters could provide information about the regulatory mechanisms involved in the embryonic development.

The study of the miRNome of trophoblast cells, however, is restricted by the limitations associated with the work on primary cells such as relatively low number of isolated cells, short lifespan or lack of proliferation *in vitro* [18]. Several trophoblastic cell lines have been established during the last three decades attempting to resemble primary trophoblasts and avoiding the limitations of isolation. Two main methodologies have been used: Introduction of the gene encoding simian virus 40 large T (sv40T) antigen [14] or establishment of human choriocarcinoma cell lines [19]. Therefore, the different genetic background and the methods used for immortalization should be taken into consideration for interpretation and discussion of results obtained from the respective cell line.

To our knowledge, there are no publications yet on the miRNA expression profiles in trophoblastic cells, or their comparison with primary isolated trophoblast cells. To overcome this lack of knowledge, we assessed the miRNA expression patterns of four cell lines and isolated trophoblast cells. We included the immortalized human

first trimester trophoblast cell line HTR-8/SVneo [20], the choriocarcinoma cell line JEG-3 and the two hybrids cell lines, ACH-3P and AC1-M59, which resulted of fusion of the AC-1 choriocarcinoma cell line with first and third trimester isolated trophoblast cells, respectively [14].

By fingerprinting miRNA gene expression we aimed to contribute to better understanding of differences and resemblances of these frequently used cell lines and primary trophoblast cells. Concluding from our observations, the above mentioned cluster C14MC and C19MC may play key roles in regulating their phenotypical and functional diversity.

Materials and Methods

Cell lines

Four cell lines were investigated in this study: the immortalized first-trimester trophoblast cell line HTR-8/SVneo (kind gift from CH Graham, Kingston Canada) [20], the choriocarcinoma cell line JEG-3 (DSMZ, Braunschweig, Germany), and two hybrids of JEG-3 with human first and third trimester trophoblast cells, ACH-3P and AC1-M59 cells, respectively (kind gift from G Desoye, Graz, Austria) [19, 21, 22].

Cell culture

Cell cultures were performed at 10^6 cells/175 cm^2 flask, and maintained under standard conditions (37ºC, 5% CO_2, humid atmosphere) in Ham's F-12 Nutrient Mixture with L-glutamine (GIBCO, Paisley, UK) or RPMI Medium (GIBCO) (HTR-8/SVneo cells) supplemented with 10 % heat-inactivated fetal calf serum (FCS; GIBCO) and 1 % penicillin/streptomycin antibiotic solution (GIBCO).

Primary Trophoblast Isolation Protocol

Trophoblast isolation was performed using a modified Kliman method as described in detail by Stenqvist et al [23]. Briefly, 20g tissue from healthy term placentae was physically disaggregated and enzymatically digested for 30 min. After washing, isolated cells were applied on a density gradient (Percoll, Pharmacia, Sweden) and the fraction retained within the layer of 25% Percoll was collected and washed. For depletion of non-trophoblastic cells, Dynabeads coated with CD45 and CD82 antibodies (Life Technologies, Darmstadt, Germany) were used.

RNA isolation and array analysis

Cells were seeded in 12-well plates, allowed to attach overnight and serum deprived for at least two hours. Total RNA was isolated by using a mirVana isolation kit (Life Technologies, Darmstadt, Germany), according to the manufacturer's protocol. Thereafter, 100 ng of total RNA containing miRNAs was reverse transcribed using the specific Megaplex RT primers (Life Technologies) followed by a pre-amplification of the obtained cDNAs. Finally, the expression level of 762 different miRNAs was performed using the TaqMan® Array Human MicroRNA A+B Cards Set v3.0 (Life Technologies). Card A includes historically “older” miRNAs, which have been described early than those of card B. This correlates with their generally higher expression and frequency in many tissues. Experimental data were analyzed by DataAssist v3.0 (Life Technologies) using RNU48 and RNU44 as endogenous controls. Due to software settings, results from card A and card B had to be analyzed separately and are displayed as heatmaps from unsupervised hierarchical clustering of all miRNAs and all individual samples. The arrays were repeated independently twice for ACH-3P, AC1-M59 cells and HTR8/SVneo, and three times for JEG-3 and trophoblast cells.

Real-time quantitative RT-PCR

The expression levels of five miRNAs (miR-518a-5p, miR-519e, miR-373, miR-411, miR-539) representing three different miRNA clusters (C19MC, cluster miR-371, C14MC) and with large differences between HTR-8/SVneo and the other cell lines were confirmed by applying individual TaqMan miRNA Assays (Applied Biosystems, Foster City, CA, USA) according to the protocol provided by the supplier. Additionally, the expression of another set of 5 miRNAs (miR-9, miR-21, miR-93,

miR-141, let-7g), which are known to correlate with tumor-grade, to be implicated in pregnancy or to be related with members of the signaling intracellular cascade of LIF was confirmed by use of the same method (analyzed and summarized in [24]). Total RNA was isolated by using a mirVana isolation kit (Life Technologies). RNA purity was assessed by the ratio of spectrophotometric absorbance at 260 and 280 nm (A260/280nm) on a NanoDrop ND-1000 (NanoDrop Inc, Wilmington, DE USA). Reverse transcription was performed with miRNA specific stem-loop RT primers and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), followed by qRT-PCR using specific TaqMan Assays and TaqMan Universal PCR Master Mix. 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 by the formula $2^{-\Delta\Delta Ct}$ relative to the expression in primary trophoblast cells. The experiments were repeated independently three times and differences in the quantified gene expression were statistically assessed by using a Student's t-test and considered statistically significant when $p<0.05$.

Results

Expression profiling of microRNAs in trophoblastic cells

We assessed the complete (miRBase v13.0) microRNA expression profile of the four trophoblastic cell lines HTR-8/SVneo, JEG-3, AC1-M59 and ACH-3P as well as that of trophoblast cells isolated from third trimester placentae (complete array data at NCBI Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32346). The detection of the total of 762 miRNAs was done on two different array cards A and B containing 381 miRNAs each. Around 65% of the miRNAs on card were notably expressed (ct < 35.0), but only approximately 35% on card B (Figure 1A, 1B and Supplement 1). Unsupervised cluster analysis of all ct values revealed that in both arrays (card A and B) choriocarcinoma-derived cell lines cluster together, whilst HTR-8/SVneo cells appear in a different branch of the dendrogram (Figure 1A and B).Isolated trophoblast cells clustered more closely to choriocarcinoma derived cell lines than to HTR-8/SVneo cells, except one sample of trophoblast cells on card B which had a generally very low miRNA expression.

Similarly, the analysis of the microRNA signature allowed us to identify two major groups of microRNAs, both present in primary trophoblast cells, but one of which almost exclusively expressed in HTR-8/SVneo cells and the other in the choriocarcinoma-derived contra-parts (Figure 1C and 1D). A deeper analysis revealed that among the 43 miRNAs highly expressed (Ct+ $\leq$ 28.0) in HTR-8/SVneo cells, 25 map to the chromosome 14, and 24 of them belong to the C14MC (Table 1). Likewise, we found 45 miRNAs almost exclusively expressed in JEG-3 cells and its hybrid cell lines, but not in HTR-8/SVneo cells (Ct > 35 in HTR-8 and Ct $\leq$ 25.0 in JEG-3 cells). All these miRNAs are located in chromosome 19: 42 of these miRNAs belong to the placenta-specific cluster C19MC, and 3 miRNAs to the miR-371 cluster,

which is also located on chromosome 19 (Table 1). Isolated trophoblast cells highly express miRNAs corresponding to C19MC but not those of the miR-371 cluster. They also express of some members of C14MC.

The mentioned clusters dominate the miRNA fingerprints and have high influence on the unsupervised clustering of analyzed samples. Due to the high expression levels of > more than 30 miRNAs within the C19MC cluster in all choriocarcinoma-derived cell lines and primary trophoblast cells, but their almost complete absence in HTR-8/SVneo cells, the dendogram is mainly organized depending on the statiscal power of these strong components. In order to investigate the relevance of further miRNAs, which do not belong to C14MC or C19MC, the unsupervised hierarchical clustering was repeated after depleting their respective results. When only C14MC miRNAs were excluded, the resulting dendogram was very similar to the original with a close association between choriocarcinoma cell lines and trophoblast cells (Figure 2A). When C19MC miRNAs were excluded, trophoblast cells appear in a separate branch of the dendogram, which demonstrates that miRNAs belonging to the C19MC are the mostly responsible for the observed similarities between choriocarcinoma derived cell lines and isolated trophoblast cells (Figure 2B). After elimination of C19MC miRNAs, JEG-3 cells clustered in a different branch than their hybrids, which indicates major systematic differences in other miRNAs, which do not belong to C19MC. The depletion of the combination of both, C19MC and C14MC miRNAs data, did not result in additional changes (Figure 2C). These results highlight on the one hand the leading relevance of C19MC in distinction of the analyzed cell types, but on the other hand, that the fingerprints and differences between the different analyzed cell types do not depend exclusively on C19MC and C14MC miRNAs.

Expression of miRNAs in isolated trophoblasts resembles choriocarcinoma cell lines more than immortalized first trimester trophoblast HTR-8/SVneo cells

For confirmation of array results, we analyzed individually by qPCR the expression of 2 miRNAs representing C14MC (miR-411 and miR-539), 2 miRNAs representing C19MC (miR-519e and miR-518a-5p) and miR-373, a member of the small cluster of miR-371. As observed in the arrays, HTR-8/SVneo cells differ significantly in the expression of the miRNAs located on the chromosome 19. The levels of miR-518a-5p, miR-519e and miR-373 were 89.9-, 5634.2-, and 286.0- fold, statistically significantly higher in trophoblast cells than in HTR-8/SVneo cells, respectively (Figure 3A-C). Conversely, only the expression of miR-539 was slightly, not significantly, higher in HTR-8/SVneo cells than in trophoblast cells (24.0-fold). Expression of miRNAs belonging to C14MC were between 1.3- and 7.2 higher in trophoblast cells than in JEG-3, ACH-3P and AC1-M59. (Figure 3 D-E). In comparison with the choriocarcinoma-derived cell lines, C14MC miRNAs expression in HTR-8/SVneo cells was higher but not always significantly. These results confirm the array data showing that microRNA expression of isolated trophoblast cells resembles more closely that of choriocarcinoma-derived cell lines than that of the immortalized trophoblast cell line HTR-8/SVneo.

Additionally, we have done qPCR for the analysis of expression of 5 further miRNAs which may be related with malignant properties. These analyses have been performed exclusively to compare the 4 above-mentioned cell lines subsequently to a previously published manuscript on their kinetics in JEG-3 cellss after LIF stimulation [24]: The expression of miR-9 and miR-141 is significantly lower in HTR-8/SVneo cells than in JEG-3 cells, while the expression of miR-21, miR-93 and let-7g is significantly higher.

Discussion

Recent studies indicate that miRNA expression signatures may be useful for the characterization and prediction of cancer [13], but investigations on their role in pregnancy are still incipient. Pioneer reports have revealed a group of miRNAs, the cluster C19MC, exclusively expressed by the placenta. Serum levels of some of its members are altered in preeclampsia [8, 25, 26]. However, the cellular origin of these miRNAs or their role in the control of trophoblast invasion and other functions is still unknown.

For the study of the molecular mechanisms involved in the regulation of trophoblast proliferation and invasion an increasing variety of cell lines are used as models due to the limitations of primary cultures. The here investigated cell lines include the most accepted models: HTR-8/SVneo, JEG-3,. AC1-M59 and ACH-3P). However, it is still controversially discussed to which extend they resemble trophoblast cells and how to extrapolate results from these models for generation of hypothesis for the different trophoblastic subtypes. On the one hand, HTR-8/SVneo cells have the advantage of being benign first trimester trophoblast cells, but vector transformation as used for their immortalization can be associated with uncontrolled amplification and splicing of viral DNA resulting in a heterogeneous genotype [21]. On the other hand, choriocarcinoma cells are not virus-treated, but have, due to their malign origin, different gene expression patterns when compared with normal trophoblasts [27].

A recent study of mRNA patterns performed on several trophoblastic cell lines and isolated trophoblast cells demonstrates that mRNAs signatures allow differentiation between choriocarcinoma-derived cell lines, immortalized trophoblast cell lines and primary trophoblast cells [18]. Also several functional differences, mainly in regard of

invasiveness and proliferation, in combination with different expression patterns of proteins have been described between HTR-8/SVneo cells and choriocarcinoma cells [18, 28]. Similar to these observations, in the current study, we demonstrate that miRNA profiles of the choriocarcinoma-derived cell lines JEG-3, ACH-3P and AC1-M59 share large congruences with each other, but not with HTR-8/SVneo. In comparison with primary third trimester trophoblast cells by performing unsupervised hierarchical clustering, miRNA profiles of choriocarcinoma-derived cell lines resemble more the primary trophoblast cells than profiles from HTR-8/SVneo do. We could also demonstrate that the placenta (and brain) specific miRNA cluster C19MC is highly expressed in trophoblast cells and choriocarcinoma-derived cells, but not in HTR-8/SVneo. Due to its placenta specifity, it can be expected that alterations of C19MC may be involved in pregnancy pathologies by being their cause or their conseuqence. In other cells than trophoblast and brain, a distal CpG-rich region on chromosome 19 is hypermethylated, but can be demethylated in human cancers, which leads to expression of the respective miRNAs [17]. In can be argued if C19MC miRNA expression in choriocarcinoma cells derives from their trophoblastic origin or their cancerous properties or from both, which may explain the mostly higher C19MC expression than in primary trophoblast cells. In contrast to C19MC, another placenta (embryonic tissue and brain) specific miRNA cluster, C14MC [26], is highly expressed in HTR-8/SVneo, little in primary third trimester trophoblast cells, but it is almost absent in the here analyzed choriocarcinoma-derived cell lines. In a previous study, several members of both clusters have been detected in plasma, where they are elevated during pregnancy [29].

Another major difference between choriocarcinoma and HTR-8/SVneo cells is, that JEG-3 cells and their hybrids express the human embryonic stem cell specific miRNA

cluster miR-371 (containing miR-371, miR-372 and miR-373), while HTR-8/SVneo do not. HTR-8/SVneo expresses high levels of miRNAs of the Let-7 family, which is generally related with malignancies, and miR-21, which is secreted strongly by human embryonic stem cells derived mesenchymal stem cells [30]. We conclude that these miRNAs regulate specific characteristics of the different trophoblastic cell lines.

Our study provides a comprehensive encyclopedia of the microRNA expression profile of four cell lines widely used as models of trophoblast cells, and their comparison with primary isolated term trophoblast cells. In regard of the current international discussion about the nature of HTR-8/SVneo cells, this study confirms their close relationship with primary trophoblast cells, but it also exhibits large inequalities. The obtained encyclopedia may be useful for comparison with other cell types and tissues, for interpretation of any experimental results from the analyzed cell lines, for future analysis of function of major trophoblast-related miRNA clusters, or for selection of new miRNA targets.

Acknowledgement

The project has been supported by the German Research Foundation (DFG, project Ma1550/7-1). DMMP had a Ph.D. grant from the regional graduate academy of the Friedrich-Schiller-University Jena, Germany. The Boerhinger Ingelheim Fonds provided her grants for starting in Jena and learning methods at the “Istituto Clinico Humanitas”, Milan, Italy. WC receives a Ph.D. grant from the German Academic Exchange Service (DAAD).

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Legends to Table and Figures

Table1. Selected miRNAs belonging to C14MC, C19MC, C19 and the let-7 family with relatively high expression (ct < 28) either in HTR-8/SVneo or choriocarcinoma-derived cell lines.

Table 2. Heatmapped list and chromosome localization of the 30 (out of 754) highest expressed miRNAs in isolated term trophoblast cells. Mean ct-values of these miRNAs are listed for all analyzed cell types. The mark indicates their belonging to the C19MC miRNA cluster. None of the listed miRNAs belongs to C14MC. Background colors: white: ct-value <25; light grey: ct-value 25-30; dark grey: ct-value 30-35; black: ct-value >35. EC: Endogenous control.

Figure 1. Unsupervised hierarchical clustering analysis of miRNAs expression profiles of all individually analyzed samples and miRNAs.The level (ct-value) of miRNA expression is color-coded. Red: higher miRNA expression; blue: lower miRNA expression. A) and B) represent the 377-containing miRNA Assays A and B, respectively. C) and D) zoom into the boxes marked in A, which display expression of miRNAs belonging to the clusters C19MC (purple) and C14MC (green).

Figure 2. Dendograms of the unsupervised hierarchical clustering as shown in figure 1 after exclusion of data from the leading clusters A) C14MC, B) C19MC or C) both, C14MC and C19MC.

Figure 3. Confirmation of array data by individual qRT-PCR. Mean relative expression of miRNAs belonging to either C19MC (miR-519e and miR-518a-5p), the

miR-371 cluster (miR-373) or C14MC (miR-539 and miR-411) were analyzed in four cell lines and isolated trophoblast. Data is presented as fold change (Log2RQ) compared to mean expression in isolated trophoblast cells ± SE. * $p<0.05$ when compared with isolated trophoblast cells; § $p<0.05$ when compared with any of the choriocarcinoma-derived cell lines (Student's t-test).

Figure 4. Confirmation of array data by individual qRT-PCR. Mean relative expression of miRNAs which have been analyzed in previous studies. Data is presented as fold change (Log2RQ) compared to mean expression in isolated trophoblast cells ± SE. * $p<0.05$ when compared with isolated trophoblast cells; § $p<0.05$ when compared with any of the choriocarcinoma-derived cell lines (Student's t-test).

Tables

Table1.

HIGH EXPRESSION IN HTR-8/SVneo cells			HIGH EXPRESSION IN JEG-3, AC1-M59 and ACH-3P cells		
miRNA	**Sequence (5' to 3')**	**Locus**	**miRNA**	**Sequence (5' to 3')**	**Locus**
miR-127-3p	UCGGAUCCGUCUGAGCUUGGCU	C14MC	miR-371-3p	AAGUGCCGCCAUCUUUUGAGUGU	C19
miR-134	UGUGACUGGUUGACCAGAGGGG	C14MC	miR-372	AAAGUGCUGCGACAUUUGAGCGU	C19
miR-136*	CAUCAUCGUCUCAAAUGAGUCU	C14MC	miR-373	GAAGUGCUUCGAUUUUGGGGUGU	C19
miR-154	UAGGUUAUCCGUGUUGCCUUCG	C14MC	miR-512-3p	AAGUGCUGUCAUAGCUGAGGUC	C19MC
miR-299-5p	UGGUUUACCGUCCCACAUACAU	C14MC	miR-512-5p	CACUCAGCCUUGAGGGCACUUUC	C19MC
miR-337-5p	GAACGGCUUCAUACAGGAGUU	C14MC	miR-515-3p	GAGUGCCUUCUUUUGGAGCGUU	C19MC
miR-369-5p	AGAUCGACCGUGUUAUAUUCGC	C14MC	miR-515-5p	UUCUCCAAAAGAAAGCACUUUCUG	C19MC
miR-370	GCCUGCUGGGGUGGAACCUGGU	C14MC	miR-516a-5p	UUCUCGAGGAAAGAAGCACUUUC	C19MC
miR-376a	AUCAUAGAGGAAAAUCCACGU	C14MC	miR-516b	AUCUGGAGGUAAGAAGCACUUU	C19MC
miR-379	UGGUAGACUAUGGAACGUAGG	C14MC	miR-517a	AUCGUGCAUCCCUUUAGAGUGU	C19MC
miR-380	UAUGUAAUAUGGUCCACAUCUU	C14MC	miR-517*	CCUCUAGAUGGAAGCACUGUCU	C19MC
miR-382	GAAGUUGUUCGUGGUGGAUUCG	C14MC	miR-517b	UCGUGCAUCCCUUUAGAGUGUU	C19MC
miR-409-3p	GAAUGUUGCUCGGUGAACCCCU	C14MC	miR-517c	AUCGUGCAUCCUUUUAGAGUGU	C19MC
miR-410	AAUAUAACACAGAUGGCCUGU	C14MC	miR-518b	CAAAGCGCUCCCCUUUAGAGGU	C19MC
miR-411	UAGUAGACCGUAUAGCGUACG	C14MC	miR-518c	CAAAGCGCUUCUCUUUAGAGUGU	C19MC
miR-431	UGUCUUGCAGGCCGUCAUGCA	C14MC	miR-518c*	UCUCUGGAGGGAAGCACUUUCUG	C19MC
miR-487b	AAUCGUACAGGGUCAUCCACUU	C14MC	miR-518d-5p	CUCUAGAGGGAAGCACUUUCUG	C19MC
miR-539	GGAGAAAUUAUCCUUGGUGUGU	C14MC	miR-518e	AAAGCGCUUCCCUUCAGAGUG	C19MC
miR-541	UGGUGGGCACAGAAUCUGGACU	C14MC	miR-518e*	CUCUAGAGGGAAGCGCUUUCUG	C19MC
miR-543	AAACAUUCGCGGUGCACUUCUU	C14MC	miR-518f	GAAAGCGCUUCUCUUUAGAGG	C19MC
miR-654-5p	UGGUGGGCCGCAGAACAUGUGC	C14MC	miR-518f*	CUCUAGAGGGAAGCACUUUCUC	C19MC
miR-758	UUUGUGACCUGGUCCACUAACC	C14MC	miR-519a	AAAGUGCAUCCUUUUAGAGUGU	C19MC
miR-889	UUAAUAUCGGACAACCAUUGU	C14MC	miR-519b-3p	AAAGUGCAUCCUUUUAGAGGUU	C19MC
miR-1247	ACCCGUCCCGUUCGUCCCCGGA	C14	miR-519c-3p	AAAGUGCAUCUUUUUAGAGGAU	C19MC
let-7b	UGAGGUAGUAGGUUGUGUGGUU	C22	miR-519d	CAAAGUGCCUCCCUUUAGAGUG	C19MC
let-7d	AGAGGUAGUAGGUUGCAUAGUU	C9	miR-519e	AAGUGCCUCCUUUUAGAGUGUU	C19MC
let-7e	UGAGGUAGGAGGUUGUAUAGUU	C19	miR-519e*	UUCUCCAAAAGGGAGCACUUUC	C19MC
let-7g	UGAGGUAGUAGUUUGUACAGUU	C3	miR-520a-3p	AAAGUGCUUCCCUUUGGACUGU	C19MC
let-7c	UGAGGUAGUAGGUUGUAUGGUU	C21	miR-520a-5p	CUCCAGAGGGAAGUACUUUCU	C19MC
let-7f	UGAGGUAGUAGAUUGUAUAGUU	C9	miR-520b	AAAGUGCUUCCUUUUAGAGGG	C19MC
let-7i*	CUGCGCAAGCUACUGCCUUGCU	C12	miR-520c-3p	AAAGUGCUUCCUUUUAGAGGGU	C19MC
			miR-520d-3p	AAAGUGCUUCUCUUUGGUGGGU	C19MC
			miR-520d-5p	CUACAAAGGGAAGCCCUUUC	C19MC
			miR-520e	AAAGUGCUUCCUUUUUGAGGG	C19MC
			miR-520f	AAGUGCUUCCUUUUAGAGGGUU	C19MC
			miR-520g	ACAAAGUGCUUCCCUUUAGAGUGU	C19MC
			miR-520h	ACAAAGUGCUUCCCUUUAGAGU	C19MC
			miR-521	AACGCACUUCCCUUUAGAGUGU	C19MC
			miR-522	AAAAUGGUUCCCUUUAGAGUGU	C19MC
			miR-523	GAACGCGCUUCCCUAUAGAGGGU	C19MC
			miR-524	GAAGGCGCUUCCCUUUGGAGU	C19MC
			miR-524-5p	CUACAAAGGGAAGCACUUUCUC	C19MC
			miR-525-3p	GAAGGCGCUUCCCUUUAGAGCG	C19MC
			miR-525-5p	CUCCAGAGGGAUGCACUUUCU	C19MC
			miR-526b	CUCUUGAGGGAAGCACUUUCUGU	C19MC

Table 2.

MiRNA	Troph	JEG3	HTR-8	ACH-3P	AC1-M59	C19 MC	Chr
hsa-miR-512-3p	19.59	17.39	40.00	16.81	16.48	√	
hsa-miR-24	20.23	21.11	19.66	20.87	20.31		9
hsa-miR-517c	20.46	18.58	36.64	17.86	17.74	√	
hsa-miR-517a	20.52	18.42	36.42	17.91	17.84	√	
hsa-miR-193b	21.24	19.71	23.77	19.96	19.29		16
hsa-miR-519a	21.63	19.05	35.72	18.04	17.98	√	
hsa-miR-200c	21.73	27.36	29.02	28.03	28.36		12
hsa-miR-720	21.80	18.13	21.92	26.23	20.58		3
hsa-miR-519d	22.10	19.52	29.94	18.06	17.49	√	
hsa-miR-1274B	22.24	17.79	19.95	27.33	21.42		19
hsa-miR-191	22.30	21.65	21.77	21.29	20.82		3
hsa-miR-525-3p	22.39	22.00	40.00	21.79	21.32	√	
hsa-miR-30b	22.56	20.91	21.04	20.67	20.41		8
hsa-miR-518e	22.63	19.25	29.61	17.94	17.72	√	
hsa-miR-484	22.68	20.99	23.05	21.12	20.91		16
hsa-miR-483-5p	22.89	22.64	31.72	24.68	24.40		11
hsa-miR-1274A	22.95	19.27	21.91	26.00	21.57		5
hsa-miR-30c	22.99	20.31	21.01	20.34	19.99		1
hsa-miR-342-3p	23.00	23.28	23.65	23.67	23.18		14
hsa-miR-518f	23.20	21.47	33.43	19.64	19.09	√	
hsa-miR-19b	23.22	17.88	20.09	17.96	18.00		X
hsa-let-7b	23.35	27.00	22.32	29.92	30.74		22
hsa-let-7e	23.44	24.99	22.15	25.89	27.06		19
hsa-miR-126	23.65	26.95	23.44	25.21	25.00		9
hsa-miR-106a	23.68	19.26	20.90	19.16	19.01		X
hsa-miR-574-3p	23.70	25.60	23.49	25.70	25.70		4
hsa-miR-200b	23.73	31.56	34.62	28.28	27.60		1
hsa-miR-518b	23.84	20.34	33.62	19.38	19.13	√	
hsa-miR-145	23.86	28.77	27.51	28.06	26.98		5
hsa-miR-20a	23.87	19.90	22.49	19.06	18.88		13
RNU48	18.98	17.71	20.09	19.50	17.57		EC
RNU44	22.61	18.54	21.93	20.26	18.43		EC
Definition of background color: Ct	<20.0	20-25	25-30	> 30			

Figures

Figure 1

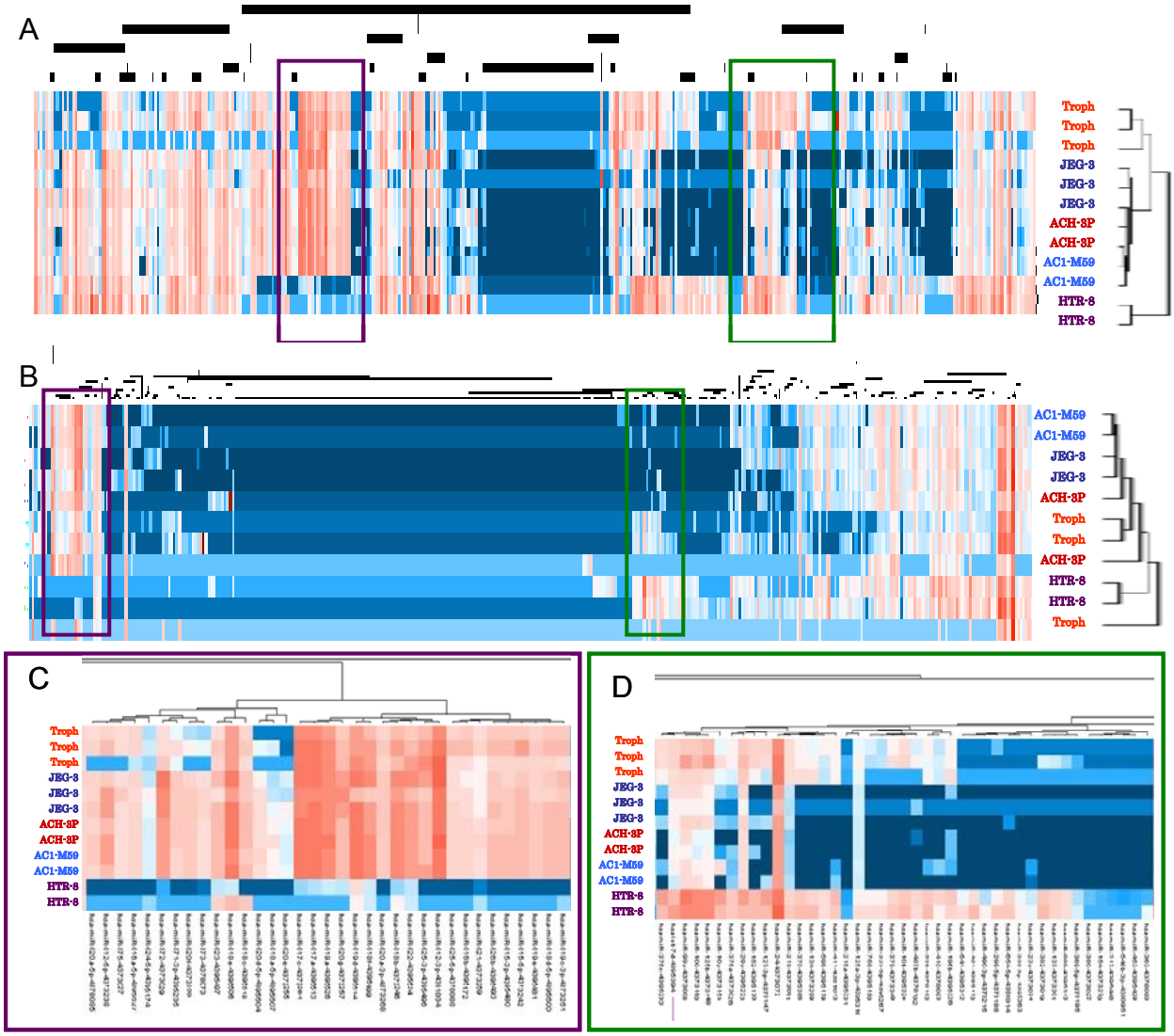


Figure 2

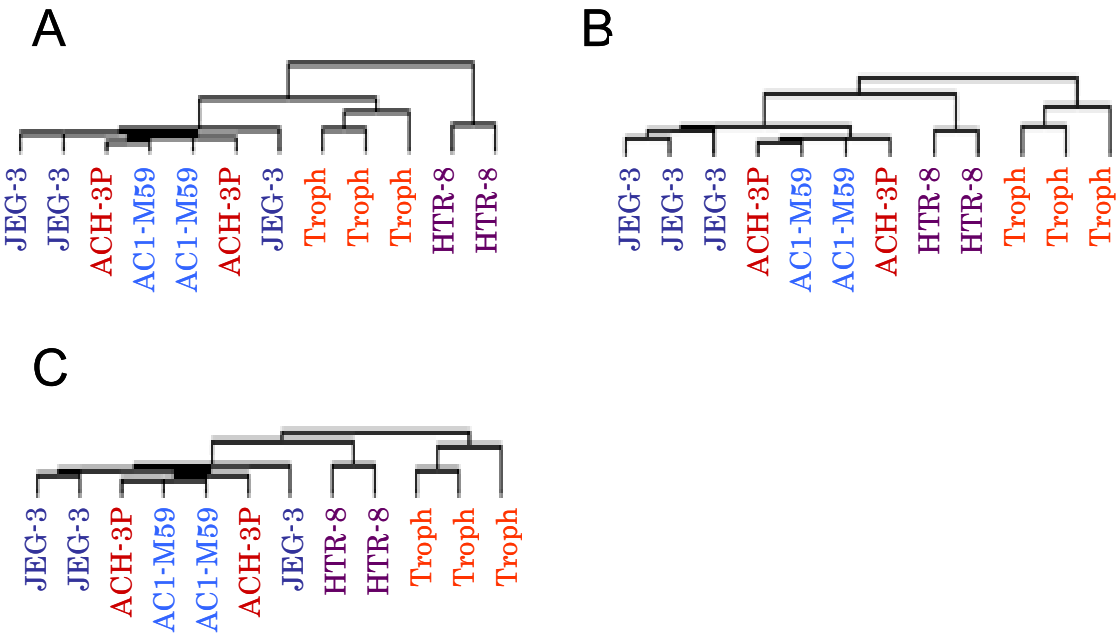

A
JEG-3
JEG-3
ACH-3P
AC1-M59
AC1-M59
ACH-3P
JEG-3
Troph
Troph
Troph
HTR-8
HTR-8
B
JEG-3
JEG-3
JEG-3
ACH-3P
AC1-M59
AC1-M59
ACH-3P
HTR-8
HTR-8
Troph
Troph
Troph
C
JEG-3
JEG-3
ACH-3P
AC1-M59
AC1-M59
ACH-3P
JEG-3
HTR-8
HTR-8
Troph
Troph
Troph

Figure 3

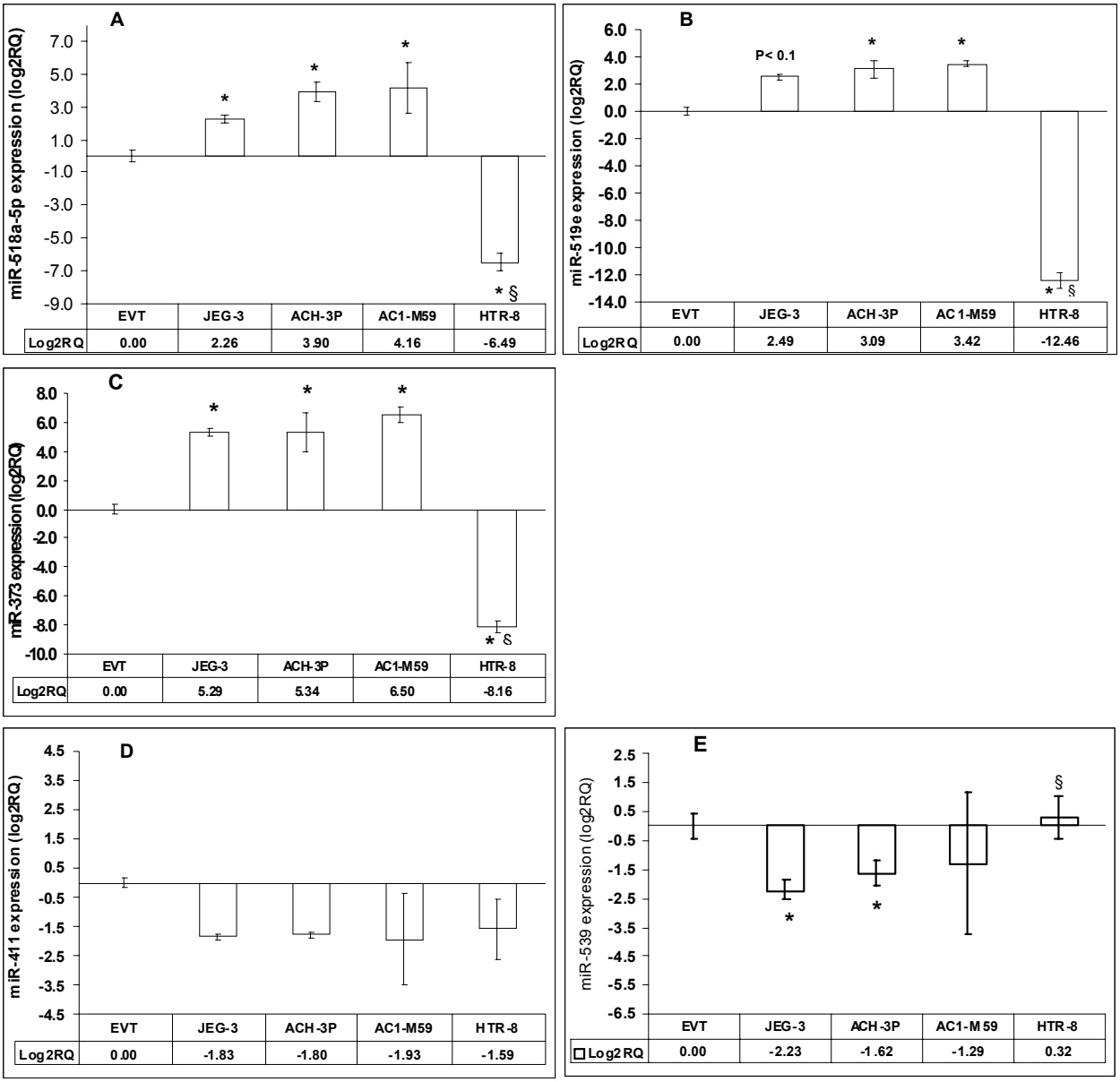
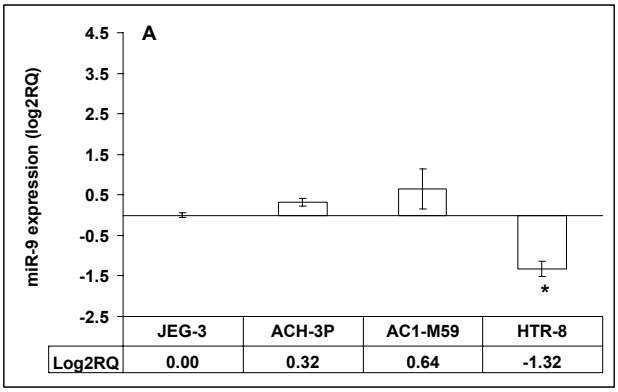
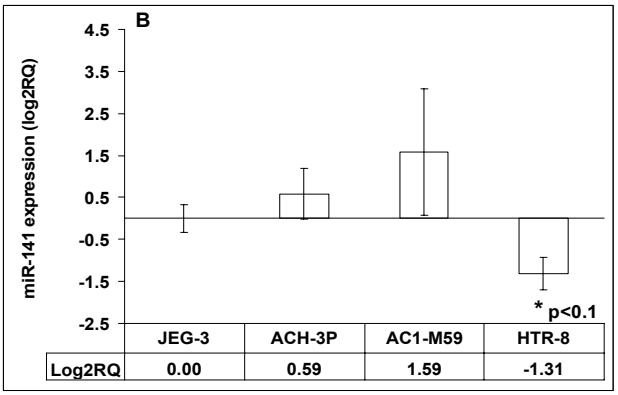
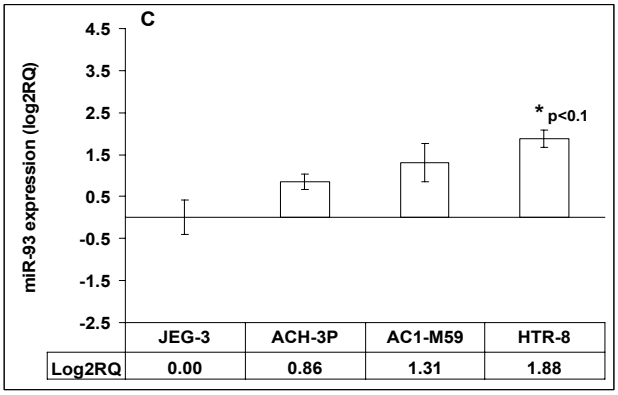
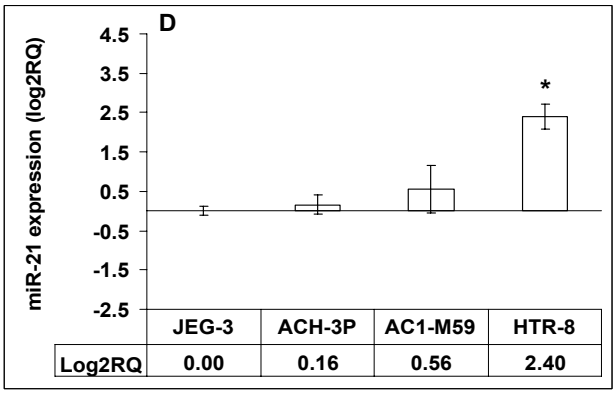

A
miR-518a-5p expression (log2RQ)
7.0
5.0
3.0
1.0
-1.0
-3.0
-5.0
-7.0
-9.0
* * * * §
EVT JEG-3 ACH-3P AC1-M59 HTR-8
Log2RQ 0.00 2.26 3.90 4.16 -6.49
B
miR-519e expression (log2RQ)
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
P< 0.1
* * * §
EVT JEG-3 ACH-3P AC1-M59 HTR-8
Log2RQ 0.00 2.49 3.09 3.42 -12.46
C
miR-373 expression (log2RQ)
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
* * * * §
EVT JEG-3 ACH-3P AC1-M59 HTR-8
Log2RQ 0.00 5.29 5.34 6.50 -8.16
D
miR-411 expression (log2RQ)
4.5
3.5
2.5
1.5
0.5
-0.5
-1.5
-2.5
-3.5
-4.5
EVT JEG-3 ACH-3P AC1-M59 HTR-8
Log2RQ 0.00 -1.83 -1.80 -1.93 -1.59
E
miR-539 expression (log2RQ)
2.5
1.5
0.5
-0.5
-1.5
-2.5
-3.5
-4.5
-5.5
-6.5
* * §
EVT JEG-3 ACH-3P AC1-M59 HTR-8
Log2RQ 0.00 -2.23 -1.62 -1.29 0.32

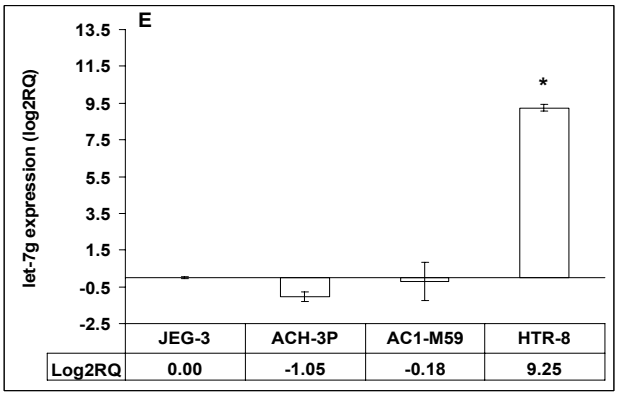
Figure 4











Average Ct	Troph	JEG-3	AC1-M59	ACH-3P	HTR8/SVNeo
hsa-miR-141	27.43	33.60	31.27	31.78	36.86
hsa-miR-21	24.71	22.77	21.74	22.02	20.56
hsa-miR-9	28.61	22.42	21.45	21.44	24.37
hsa-miR-93	27.86	23.52	22.92	23.22	23.82
hsa-let-7g	28.37	29.43	30.41	28.89	22.39
Fold Change					
hsa-miR-141		0.00	0.01	0.01	0.00
hsa-miR-21		1.01	1.31	1.30	25.50
hsa-miR-9		19.22	24.01	29.12	27.20
hsa-miR-93		5.32	5.16	5.03	23.53
hsa-let-7g		0.13	0.04	0.14	90.89

Leukemia Inhibitory Factor alters miRNome in trophoblastic cells

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Short title: MicroRNome after LIF in trophoblastic cells

Keywords: Pregnancy, trophoblast, LIF, microRNA, placenta.

Abstract

Background: MicroRNAs (miRNAs) are small single-stranded RNA molecules which are important post-transcriptional modulators of gene expression. The expression of miRNAs is distinct in primary trophoblast cells and among different trophoblastic cell lines. LIF is a pleitropic cytokine which induces a variety of effects on trophoblast cells, including proliferation and invasion, during implantation and placentation. It uses the Janus Kinase/Signal Transducer and Activator of Transcription 3 (JAK/STAT3) intracellular signalling pathway, which is regulated by Protein Inhibitors of Activated STATs 3 (PIAS3). Aim of this study was to identify miRNAs which are regulated by LIF in different trophoblastic cell lines and to identify a possible function.
Material and methods: After LIF stimulation, total RNA was isolated from the cell lines HTR-8/SVneo (immortalized trophoblast cells), JEG-3 (choriocarcinoma), ACH-3P and AC1-M59, which are choriocarcinoma cells fused with first and third trimester trophoblast cells, respectively. The expression level of 762 different miRNAs was quantitatively analzed by using a TaqMan Human MicroRNA Array. The results for 4 interesting miRNA were confirmed by individual qPCR. Finally, we silenced and overexpressed one out of those 4 miRNAs: miR-141 and analyzed PIAS1/3 expression by Western blotting.
Results: Approximately 10-30 % of miRNAs were affected by LIF stimulation of the different cell line. Three out of 762 miRNAs were significantly down-regulated in all cell lines after LIF treatment: miR-511, miR-550 and miR-885-5p, but only miR-641 was up-regulated in all tested cell lines. MiR-141 was differently affected by LIF in the different cell lines. Its silencing induced a decrease and its over-expression an increase of PIAS3 in HTR-8/SVneo cells, but not in JEG-3 cells, in which PIAS3 was not detectable.

Conclusion: LIF strongly affects miRNA expression in trophoblastic cell lines, but only a few miRNAs behave in a similar manner and, thus, may have crucial functions in the regulation of different subtypes of trophoblastic cells.

Introduction

The study of gene regulation beyond the DNA transcription has provided important insights into the field of genetics and has generated great interest in the field of human reproduction. Epigenetics is defined as the study of changes in gene expression that are not caused by changes in the DNA sequence [1, 2]. Epigenetic changes control differentiation in many tissues and are involved in the ability of a specie to response to environmental variations [2]. Remarkably, during embryogenesis, two critical time periods are controlled by epigenetic modification of genes: gamete development and preimplantation and trophoblastic growth [2]. Epigenetic regulation seems to be a key factor in the functional specificity of cytotrophoblast [3] thus, it to expect that alteration in the epigenetic regulation may be associated with a variety of pregnancy diseases. To date, four main mechanisms of epigenetic regulation have been described: DNA methylation, imprinting, histone modification, and small RNA-mediated control, specifically microRNAs (miRNAs) [1].

MicroRNAs are endogenous single-stranded RNA (~19-22nt) which repress gene expression transcriptionally [4]. Expressed in the nucleus as langer transcripts, miRNAs are sequentially processed by the RNAseIII enzymes Drosha and Dicer into a mature mRNA sequence, which by association with the RISC complex, has the ability to inhibit translation or cleavage target mRNAs, if partially or fully sequence complimentary occurs [5]. MiRNAs act as translational repressors controlling numerous cell procceses including fat metabolism, cell proliferation, apoptosis, and differentiation in several cell types [6-9].

Some microRNAs are universally expressed in normal human tissues, while some others are exclusively or preferentially expressed in a tissue-specific manner [10]. Interestingly, these last miRNAs tend to be organized into clusters [11, 12], commonly located in fragile sites of the chromosomes and also found frequently altered in cancer [13, 14]. Recent reports described two large microRNA clusters expressed almost exclusively in placenta: C19MC and C14MC [5, 9, 15]. Interestingly, C19MC is a primates' specific cluster and C14MC is found only within eutherian species [15], which suggests a pivotal role in the evolution and placenta development.

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine known to be indispensable for human reproduction. LIF controls uterine receptivity and influences trophoblast behavior by promoting proliferation, invasion and differentiation, and its aberrant expression is related with infertility and poor pregnancy outcome (Reviewed in [16]). Previously, we demonstrated that LIF stimulation is able to alter the expression of some microRNAs in JEG-3 choriocarcinoma cells [17], which suggested a role of miRNAs in the regulation of trophoblast behavior during pregnancy. In order to gain more insights into the regulation of miRNAs mediated by LIF, we used microarray analysis to elucidate the miRNome (768 genes miRBase version 13.0) of four trophoblastic cell lines (HTR-8/SV-neo, JEG-3, ACH-3P and AC1-M59), before and after LIF-induction. Thereafter, we decided to investigate the mRNA targets of some selected miRNAs and their association with proliferation and invasion of trophoblastic cells.

Materials and Methods

Cell lines

Four cell lines were used in this work: JEG-3 (DSMZ, Braunschweig, Germany), which is a human choriocarcinoma cell line preserving several trophoblast-like capacities including production of pregnancy related hormones and cytokines; two hybrids of JEG-3 with human first and third trimester trophoblast cells, ACH-3P and AC1-M59 cells [18-20], respectively (kind gift from G Desoye, Graz, Austria) and the immortalized human first-trimester trophoblast cell line HTR-8/SV40 [21].

Cell culture

Cells cultures were performed at 10^6 cells/175 cm^2 flask, and maintained under standard conditions (37ºC, 5% CO_2, humid atmosphere) in Ham's F-12 Nutrient Mixture with L-glutamine (GIBCO, Paisley, UK) or RPMI Medium (GIBCO) (HTR-8 cells) supplemented with 10 % heat-inactivated fetal calf serum (FCS; GIBCO) and 1 % penicillin/streptomycin antibiotic solution (GIBCO).

RNA isolation and array analysis

Cells were cultivated in 12-well plates and allowed to attach overnight. Afterwards, cells were deprived of serum for at least 2 hours and then challenged 4 hours with 10 ng/ml LIF (Millipore, Schwalbach, Germany). Total RNA was isolated with mirVana isolation kit (Life Technologies), according to the manufacturer's protocol. Thereafter, 100 ng of total RNA containing small RNAs was reverse transcribed using the specific Megaplex RT primers (Life Technologies) followed by a pre-amplification of the obtained cDNAs. Finally, the expression level of 768 miRNAs was performed

using the TaqMan® Array Human MicroRNA A+B Cards Set v3.0 (Life Technologies). Experimental data were analyzed by DataAssist v3.0 (Life Technologies) using RNU48 and RNU44 as endogenous controls.

Real-time quantitative RT-PCR

Arrays data was validated using individual TaqMan miRNA Assay (Applied Biosystems, Foster City, CA, USA) according to the protocol provided by the supplier. Briefly, cells were challenged as described above and total RNA was isolated by using miRVana kit (Applied Biosystems). RNA purity was assessed by the ratio of spectrophotometric absorbance at 260 and 280 nm (A260/280nm) using NanoDrop ND-1000 (NanoDrop Inc, Wilmington, DE USA). Reverse transcription were performed with miRNA specific stem-loop RT primer using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) followed by qRT-PCR using specific TaqMan Assays and TaqMAn Universal PCR Master Mix. 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 determined using the formula $2^{-\Delta\Delta Ct}$ relative to the expression of non-stimulated cells. Experiments were repeated independently three times and differences in the quantified gene expression were statistically assessed by using ttest and consider statistically significant if $p<0.05$.

Results

Trophoblast cells present distinct miRNome signatures after LIF

In order to investigate the miRNome of trophoblastic cells upon LIF-treatment, cells were serum-starved and then treated 4 hour with LIF (10ng/ml). MicroRNA expression was normalized to the one in non-treated cells and using RNU48 and RNU44 as endogenous controls.

Our results confirmed that LIF is able to induce changes in the miRNome of trophoblastic cells, however, this response seems to be cell-type dependent. ACH-3P cells present the highest change with a total of 237 miRNAs displaying more than 1.5-fold change (160 up-regulated and 77 down-regulated), followed by HTR-8 cells with 145 (66 up- and 79 down-regulated), AC1-M59 with 124 (68 up- and 56 down-regulated), and JEG-3 with 115 (60 up- and 55 down-regulated) (Figure 1 and Annexes).

A deeper analysis of the miRNAs found altered in 2 or more cell lines revealed that ACH-3P and HTR-8 had the most similar response to LIF with 35 miRs in common (18 up- and 17- down regulated) followed by ACH-3P and AC1-M59 which share 33 miRs: 9 up- and 24 down-regulated (Figure 1). Table 1 contains the complete list of miRNAs up- or down- regulated in two or more cell lines and the relative expression of these miRs before LIF treatment. Surprisingly, only 3 miRs were down-regulated in all cell lines after LIF treatment: miR-511, miR-550 and miR-885-5p, but only miR-641 appeared to be up-regulated in all tested cell lines (Figure 1 and Table 1).

Validation of miRNAs arrays data

In order to confirm our results, four miRNAs were selected and the expression was measured by qRT-PCR. We confirmed the up-regulation of miR-21 and miR-21# in all cell lines, as well as the down-regulation of miR-511 (Figure2). However, our results of miR-141 do not agree with the observed in the cards suggesting a problem during the measurement. In addition, the number of assays should be increased in order to minimize the error.

Knock-down of miR-141 correlates with decrease of PIAS3 in HTR-8 cells

MiR-141 was found to be altered simultaneously in most of the cell lines (Table 1). However, we observed a down-regulation of JEG-3 whilst in HTR-8SV Neo, AC1-M59 and ACH-3P it was up-regulated. Based on our previous report demonstrating an involvement of miR-141 in the regulation of JEG-3 proliferation [17], and also the fact that miR-141 in maternal plasma increases during pregnancy [22], we decided to investigate the possible targets of miR-141. Interestingly, PIAS3, a negative regulator of the LIF pathway, was found to be a putative target of miR-141 our results demonstrate that it is reduced after knock-down of miR-141 (Figure 3). This results in conjunction with our previous observations of changes in the JEG-3 proliferation after miR-141 inhibition, demonstrated that miR-141 is related with the JAK/STAT pathway and the changes in its levels may be a direct result of LIF stimulation.

Discussion

MicroRNA regulation of gene expression is one of the four main mechanisms of epigenetic regulation [1] and thus, alteration in the miRNAs expression levels may be associated with a variety of pregnancy diseases. Despite being demonstrated to be useful in the diagnosis and prediction of cancer [23], the study of miRNAs signatures during pregnancy remains incipient. Even though some miRNAs were found to be altered in preeclampsia [24], the biological functions and expression in placental cells remain unclear.

Several cell lines are used as a model for trophoblast invasion and proliferation, some of them derived from choriocarcinoma (e.g. JEG-3, ACH-3P and AC1-M59) or immortalized from isolated trophoblast (e.g. HTR-8) [18-21]. However the differences in their origin are also related with changes in the expression patterns of proteins and mRNA [25, 26], and recently, we have also reported several differences in the miRNome of those cell lines. These alterations correlate also with the invasiveness, proliferative rates and their response to external stimuli, for instance, cytokines stimulation [27]. Previously we demonstrated that LIF was able to induce proliferation and invasion in JEG-3 and HTR-8 cells and the mechanism involved changes in the activation of STAT3 and in the expression of several mRNAs including metalloproteinase 9 (MMP-9) and caspase 4(CASP4) [27, 28]. In addition, in JEG-3 cells we demonstrated that LIF reduces the levels of miR-141 that, in turn, is involved in the regulation of JEG-3 proliferation [17].

Here we analyzed the complete miRNome of four trophoblastic cells after LIF stimulation. Our results shown several differences in the cell-response to LIF, as only

three miRNAs were found down-regulated (miR-511, miR-550 and miR-885-5p), and one up-regulated (miR-641) in all cell lines after LIF treatment. Among them, miR-511 has been more studied due to its significantly lower expression in adenocarcinomas compared with normal tissues [29] and its potential role as modulator of human immune response [30]. On the other hand, miR-885-5p was also found down-regulated in primary neuroblastoma and seems to have a tumor suppressive role interfering with cell cycle progression and cell survival [31]. These associations allow us to hypothesize that these miRNAs may play an important role in the trophoblast response to external agents and thus, seek of their targets may be of great importance to understand the LIF-mediated invasion and proliferation and generate new approaches for future therapies.

Among the thousands of putative target genes of miR-511, miR-550 and miR-885-5p, and by using the microrna.org software, MAX interactor 1 (MXI1) and Wilms tumor 1 associated protein (WTAP) genes were predicted to be targets of all three miRNAs and were found to display the highest mirSVR score. Interestingly, both MXI1 and WTAP codify for proteins known to be tumor suppressors, however, to our knowledge, none of them have been associated with LIF. Based on the knowledge that LIF induces invasion in trophoblast cells, one may hypothesize that the reduction of miR-511, miR-550 and miR-885-5p may result in increased invasiveness of trophoblast cells, but this relation should be confirmed.

Knock-down of miR-141 correlates with decrease of PIAS3 in HTR-8 cells

We have recently reported that HTR-8 and JEG-3 cells differ in the expression of several proteins of the LIF intracellular cascade, including the basal levels of p-

STAT3 (Ser727), p-ERK [27] and PIAS1/3. PIAS are negative regulatory molecules of the JAK/STAT cascade, which in turn controls proliferation and invasion of trophoblast cells. PIAS shut down STATs effects by binding to STAT dimers preventing them from binding DNA [32, 33] and therefore they play an important role in the invasion capability of trophoblast cells. Interestingly, we found that HTR-8 cells expressed both PIAS1 and PIAS3, while in JEG-3 cells only the expression of PIAS1 was detected (Figure 3). However, we also demonstrated that both cell lines expressed mRNA for PIAS1 and PIAS3 and in similar levels (Grosse, et al In preparation). Since miRNAs regulation occurs postranscriptionally, we decided to investigate miRNAs that may potentially target PIAS3. Among them, miR-141 was found to be highly expressed in JEG-3 cells but almost absent in HTR-8 (Morales-Prieto, et al submitted), and here we demonstrated a deregulation in the levels in presence of LIF thus, suggesting a direct association with the PIAS3 protein levels.

The inhibition of miR-141 by knock-down in JEG-3 cells showed no alterations in the PIAS3 protein levels. Surprisingly, in HTR-8 cells, inhibition of miR-141 decreases significantly the expression of PIAS3 protein. As no changes in the PIAS1 protein expression were found, this result demonstrates that PIAS3 is indeed a target of miR-141. A recent study has demonstrated that miRNAs can switch to translation activation under growth-arrest conditions [34], since we have performed the protein expression experiments in serum-deprived medium we hypothesize that this may be the molecular mechanism responsible for our observations.

The molecular mechanisms underlying the regulation of gene expression by miRNAs are still unclear. This work correlates with recent publications demonstrating that miRNA alterations mediated by external factors seem to be cell-type depended [5,

14], but also that the microRNAs oscillate between repression and activation in coordination with the cell cycle [34]. We also highlight some miRNAs as potential regulators of the LIF-mediated cell response in trophoblastic cells including a direct implication in the control of trophoblast proliferation and invasion. These observations should be confirmed also in *in vivo* models, but our results open the possibility to use them as potential diagnosis markets.

Acknowledgement

The project has been supported by the German Research Foundation (DFG, project Ma1550/7-1). DMMP had a Ph.D. grant from the regional graduate academy of the Friedrich-Schiller-University Jena, Germany. WC has a DAAD grant.

Legends to Table and Figures

Table1. List of microRNA found up- or down-regulated after LIF-induction in more than two cell lines simultaneously. Expression levels are color-coded. Dark blue: Low expression, Light blue: High expression. Numbers within cells are the fold-change relative to non-stimulated cells. Boxes containing numbers but no colored display miRNAs also found deregulated but with fold-change < 1.5.

Figure 1. Venn Diagramas summarizing miRNAs altered after LIF treatment. Left: MicroRNAs up-regulated Right: MicroRNAs down-regulated after LIF, respectively.

Figure 2. Validation of microarray data by qRT-PCR. Expression levels of A) mir-511, B) miR-141, C) miR-21, D) miR-21# in all four tested cell lines n=3. Statistical analysis were performed in comparison with JEG-3 cells, calculated by Student-ttest and considered statistical significant when $p<0.05$.

Figure 3. Knock down of miR-141 decreases PIAS3 in HTR-8 cells. Cells were transfected either with pre- or anti- miRNAs and PIAS 1/3 protein levels were assessed by Western blot.

Tables

Table1.

miRNAs up-regulated					miRNAs down-regulated				
miRNA	JEG-3	AC1-M59	ACH-3P	HTR8	miRNA	JEG-3	AC1-M59	ACH-3P	HTR8
hsa- let-7c	2.86		18.25		hsa-miR- dme-miR-7			2.42	2.66
hsa- let-7e#		23743.00		1.85	hsa-miR- 1305	19.38	3.19		2.57
hsa-miR- 100#		1.66		1.58	hsa-miR- 155	35.21	2.88		2.03
hsa-miR- 1201	1.14	1.50	5.52		hsa-miR- 10a	20.28	35.97		
hsa-miR- 1243		3.57		9.28	hsa-miR- 10b#	62.50	1.06	4.47	
hsa-miR- 1256	1.12	50.73	1.86		hsa-miR- 1226#			10.00	2.08
hsa-miR- 1262		2.19	1.61		hsa-miR- 1255B			4.67	3.36
hsa-miR- 1276		1.90	2.90		hsa-miR- 1276	2.15			7.57
hsa-miR- 1282	2.21	1.04	28.06		hsa-miR- 136			7.67	8.31
hsa-miR- 1291		1.59	1.63		hsa-miR- 148a#		714.29	196.08	
hsa-miR- 130a#	1.12	72.80	1.99		hsa-miR- 192#	2.37		4.31	
hsa-miR- 141	1.27	3.09	3.55	11.36	hsa-miR- 206			5.35	4.07
hsa-miR- 142-5p	28.67		520.74	22.80	hsa-miR- 221#		2.79		3.26
hsa-miR- 148a#	1.72			72.70	hsa-miR- 23a#		243.90		39.84
hsa-miR- 152	2.24	31.27			hsa-miR- 23b#		625.00	9.81	
hsa-miR- 196b	2.02		7.52		hsa-miR- 302c#	71.43	5.34		
hsa-miR- 199a-3p	6.49	7.97			hsa-miR- 330-3p	2.00			2.35
hsa-miR- 206	1.91	3.00			hsa-miR- 33a			192.31	135.14
hsa-miR- 21#	3.19	2.91	23.47		hsa-miR- 378			3.50	2.07
hsa-miR- 217	1.02		35.95	5.96	hsa-miR- 429	3.89		6.48	6.40
hsa-miR- 221#	83.63		3.52		hsa-miR- 449b	2.18	1.99		
hsa-miR- 23a#	1.78		4.16		hsa-miR- 486-5p	42.19			4.63
hsa-miR- 23b	1.12	1.77	2.15		hsa-miR- 500		312.50		833.33
hsa-miR- 23a-2#		1.87	10.44		hsa-miR- 501-5p		2000.00		3.32
hsa-miR- 29a#	2.21	6.89	16.43		hsa-miR- 511	13.05	18.05	10.98	28.49
hsa-miR- 29b-1#		3.65	12.59		hsa-miR- 516-3p			2.79	3.10
hsa-miR- 30d#		1.77	8.32		hsa-miR- 548E		2.28	4.34	
hsa-miR- 31#		1.61	4.13		hsa-miR- 548I	15.43	1.08	4.32	
hsa-miR- 374b#	3.42	4.53	43.71		hsa-miR- 548J		20.08		10.88
hsa-miR- 450a		2.38	3.15		hsa-miR- 550	1.49	2.99	36.36	2.04
hsa-miR- 488	1.67	1.58			hsa-miR- 550			3.86	3.25
hsa-miR- 489	2.41			5.99	hsa-miR- 561		2.89	21.37	
hsa-miR- 505	958.80	1.68	2.36		hsa-miR- 570			4.53	10.17
hsa-miR- 518a-5p	1.62	1.32	2.23		hsa-miR- 580			2.72	2.50
hsa-miR- 519e	1.03		2.25	1.62	hsa-miR- 581			54.95	11.45
hsa-miR- 539	63.42	2.63	1.58		hsa-miR- 592			75.76	51.28
hsa-miR- 545#		6.19		3.73	hsa-miR- 606			114.94	24.75
hsa-miR- 545		3.58	4.70	1.93	hsa-miR- 654-3p		68.97	21.14	
hsa-miR- 548c-5p	1.92	2.87			hsa-miR- 672	20.16	1.06	80.00	
hsa-miR- 548E	41.70			4.74	hsa-miR- 708	50.25	2.87	42.74	
hsa-miR- 548K		25.73		2.81	hsa-miR- 885-5p	7.42	588.24	1.69	92.59
hsa-miR- 590-3P		1.73	5.89		hsa-miR- 922	7.39	8.35	0.02	
hsa-miR- 601	4.27			1.54	hsa-miR- 99b#	2.22	1.20	2.56	
hsa-miR- 618		6.76		19.28					
hsa-miR- 624			1.89	1.69					
hsa-miR- 628-5p	1.62	1.57	1.91						
hsa-miR- 635		3.28		9.10					
hsa-miR- 638			82.56	34.15					
hsa-miR- 641	1.82	1.05	5.06	1.60					
hsa-miR- 643	250.53	279.64							
hsa-miR- 645	2.09	3.71		1.67					
hsa-miR- 663B	8289.25	1.76							
hsa-miR- 744#			2.50	1.51					
hsa-miR- 767-3p	41.18	71.43							
hsa-miR- 892b	129.70			69.06					
hsa-miR- 938	4.84			9.19					
hsa-miR- 99a#	1.70	18.72		8.64					
hsa- U6 snRNA		1.74	7.12						
hsa-miR- 130b			1.70	2.59					
hsa-miR- 146b-5p	1.18	1.52	1.81						
hsa-miR- 202			1.73	2.18					
hsa-miR- 204			1.53	1.76					
hsa-miR- 24-2#	1.92	1.34	1.72						
hsa-miR- 34b	2.96	1.30	1.80						
hsa-miR- 373			1.66	5.97					
hsa-miR- 449a			1.81	1.52					
hsa-miR- 451	20.32	1.48	1.52						
hsa-miR- 627		1.59	1.94						
hsa-miR- 664			1.95	3.31					

<25	25-30	30-35	>35

Figures

Figure 1

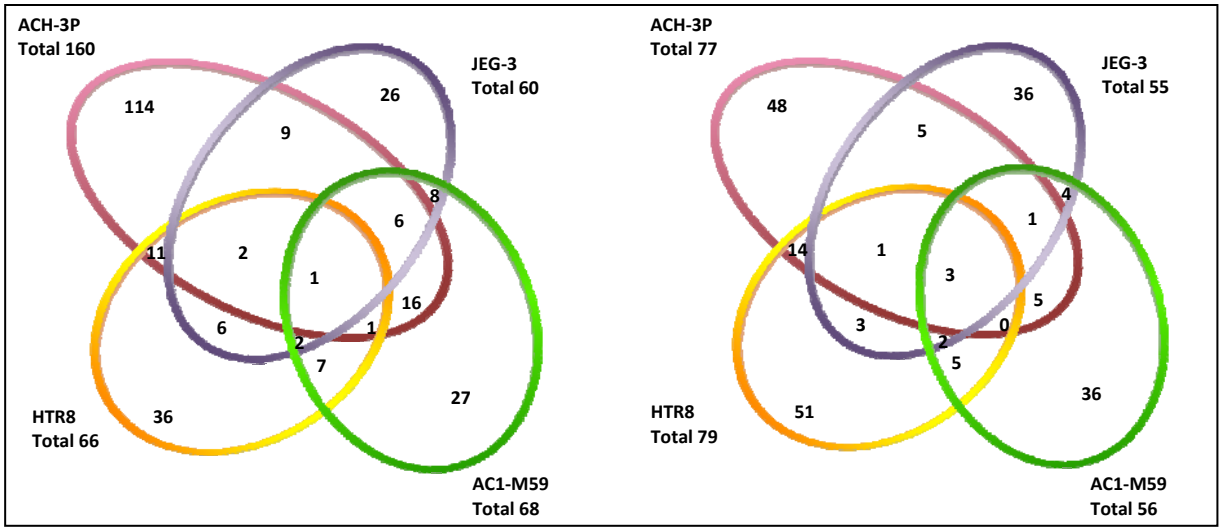


Figure 2

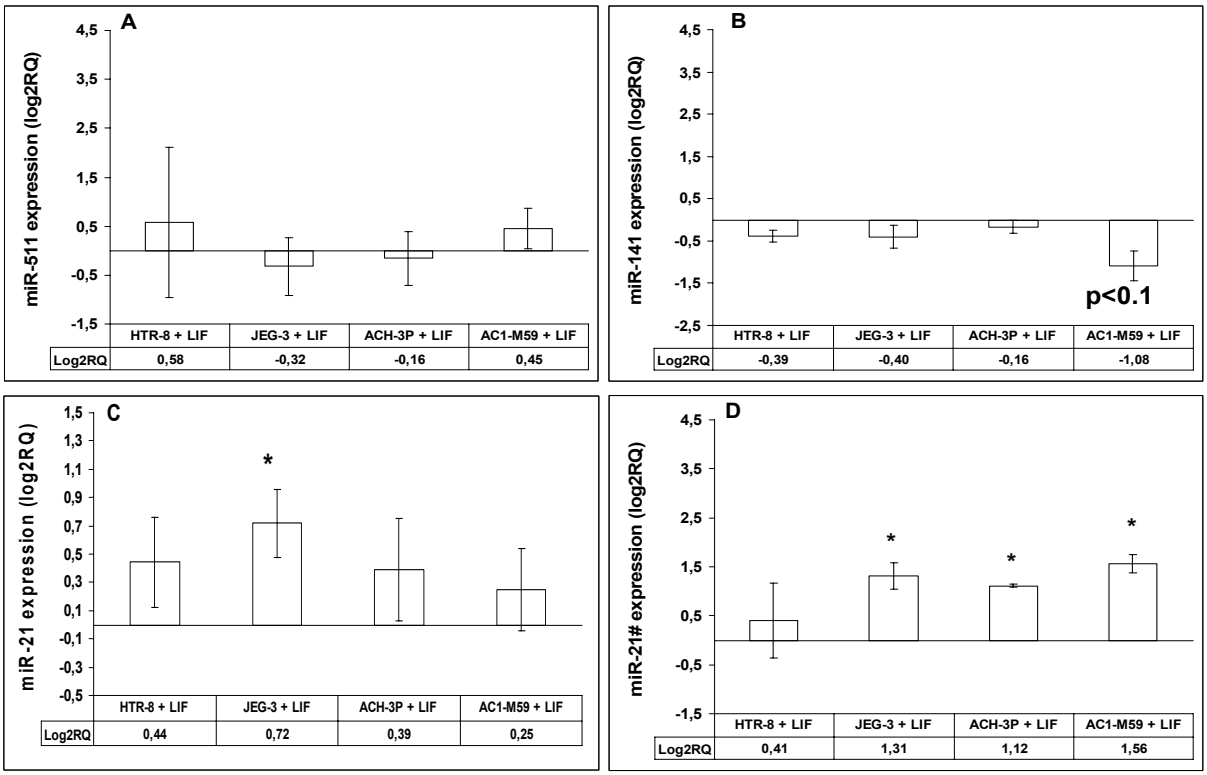
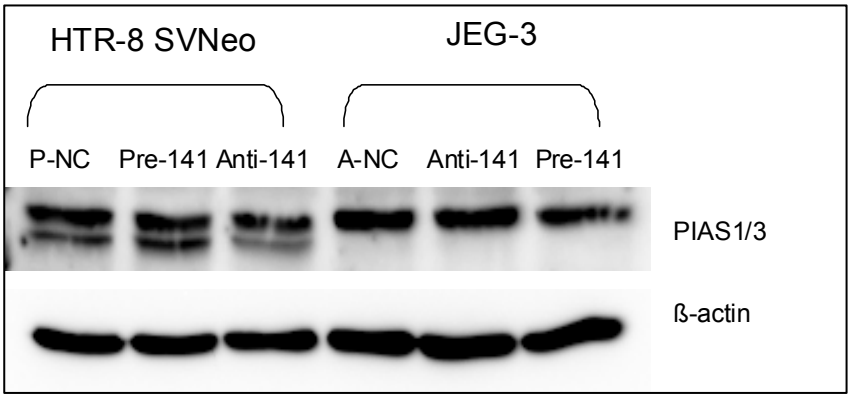


Figure 3


HTR-8 SVNeo
JEG-3
P-NC
Pre-141
Anti-141
A-NC
Anti-141
Pre-141
PIAS1/3
ß-actin

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AP-1 Transcription Factors, Mucin-Type Molecules and MMPs Regulate the IL-11 Mediated Invasiveness of JEG-3 and HTR-8/SVneo Trophoblastic Cells

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Abstract

This study examines the IL-11 mediated activation of downstream signaling and expression of effector molecules to resolve the controversies associated with the IL-11 mediated regulation of the invasiveness of two commonly used trophoblastic cell models viz. JEG-3 and HTR-8/SVneo cells. It has been reported that IL-11 increases the invasiveness of JEG-3 cells while, reduces the invasiveness of HTR-8/SVneo cells. Invasion assay performed simultaneously for both the cell lines confirmed the above findings. In addition, HTR-8/SVneo cells showed a higher basal invasiveness than JEG-3 cells. Western blot showed the IL-11 mediated activation of STAT3(tyr705) and STAT1(tyr701) in both the cell lines. However, IL-11 activated the ERK1/2 phosphorylation in JEG-3 cells but, inhibited it in HTR-8/SVneo cells. Within 10 min of IL-11 treatment, p-STAT3(tyr705) was localized inside the nucleus of both the cell lines but, there was enhanced co-localization of protein inhibitor of activated STAT1/3 (PIAS1/3) and p-STAT3(tyr705) in HTR-8/SVneo cells and not in JEG-3 cells. This could be reason for the poor responsiveness of STAT3 responsive genes like mucin 1 (*MUC1*) in HTR-8/SVneo cells and not in JEG-3 cells. Further, microarray analysis of the IL-11 treated cells revealed differential responsiveness of JEG-3 as compared to HTR-8/SVneo cells. Several family of genes like activator protein-1 (AP-1) transcription factors (*Jun* and *Fos*), mucin-type molecules, *MMP23B* etc showed enhanced expression in IL-11 treated JEG-3 cells while, there was no response or decrease in their expression in IL-11 treated HTR-8/SVneo cells. Expression of these molecules was confirmed by quantitative RT-PCR. In addition, HTR-8/SVneo cells also showed a significant decrease in the expression of *MMP2*, *MMP3* and *MMP9* upon IL-11 treatment. Hence, IL-11 mediated differential activation of signaling and expression of effector molecules is responsible for the differential invasive response of JEG-3 and HTR-8/SVneo cells.

Citation: Suman P, Godbole G, Thakur R, Morales-Prieto DM, Modi DN, et al. (2012) 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 7(1): e29745. doi:10.1371/journal.pone.0029745

Editor: Suresh Yenugu, University of Hyderabad, India

Received August 29, 2011; **Accepted** December 2, 2011; **Published** January 3, 2012

Funding: Financial support from the National Institute of Immunology, New Delhi and the Indian Council of Medical Research, Government of India is gratefully acknowledged. PS is recipient of Junior and Senior Research Fellowships of the Council of Scientific and Industrial Research (CSIR), Government of India, GG is grateful to Lady Tata Memorial Trust, Mumbai, India and CSIR, for Junior and Senior Research Fellowships respectively, and DM-P is supported by a Ph.D. grant from the Friedrich-Schiller University, Jena. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Invasion of trophoblast cells is one of the critical events associated with the embryo implantation as it helps in establishing the exquisite contact between the fetus and the maternal circulation. Aberration in invasive behavior of the trophoblast cells may lead to several pathological conditions which may range from pre-eclampsia (due to shallow implantation) to placental bed tumors (due to excessive invasion) [1,2]. Several cytokines and growth factors present at the implantation site regulate the spatial and temporal invasion of the trophoblast cells either by acting in autocrine or paracrine manner to achieve successful conception [3].

IL-11, a member of the IL-6 family, is present at the site of implantation and has been observed to be indispensable for the embryonic development [4]. The IL-11 receptor α (IL-11Rα) knockout female mice, are infertile because of defective decidualization of the endometrial stromal cells [5,6]. In humans, IL-11Rα is consistently expressed in the endometrium from proliferative and secretory phase to 7–9 weeks of gestation [7]. In contrast to this, IL-11 expression is barely detectable in the proliferative and secretory phase of endometrium but, its expression is significantly higher in the chorionic villi as well as in the decidua [5]. Further, defective production of IL-11 is associated with reduced fertility rate in human pregnancy [5]. Additionally, plasma level of IL-11 was low in women with spontaneous abortion [8].

Though, IL-11 plays a defined role in endometrial decidualization, its role in trophoblastic cell invasion has been held in controversy. Exogenous treatment of JEG-3 choriocarcinoma cells with IL-11 led to an increase in invasion [9]. The increase in the invasiveness of JEG-3 choriocarcinoma cells was associated with the activation of signal transducer and activator of transcription 3 (STAT3) as well as of STAT1 and extracellular signal regulated

kinases1/2 (ERK1/2) [9]. Further, silencing of STAT3 and gp130 (co-receptor for the IL-11 mediated signaling) expression in JEG-3 cells inhibits the IL-11 mediated increase in JEG-3 cells invasion [9]. However, using extra villous trophoblast (EVT) cells and HTR-8/SVneo cells (derived from human first trimester placenta explant cultures immortalized by SV40 large T antigen) as a trophoblast cell model, it was shown that, IL -11 reduces their invasiveness in spite of the activation of STAT3 dependent signaling pathway [10]. This decrease in invasiveness of HTR-8/SVneo cells was not associated with any significant changes in the expression of classical invasion associated molecules like matrix metalloproteinase 2 (MMP2), MMP9, tissue inhibitor of metalloproteinase 1 (TIMP1), TIMP2, TIMP3, plasminogen activator urokinase (PLAU), plasminogen activator urokinase receptor (PLAUR), and serpin peptidase inhibitors 1 and 2 (SERPINE1 and SERPINE2) [10]. Thus, the reason for inhibition of invasion of HTR-8/SVneo cells in response to IL-11 is not known.

The existing studies leaves behind several key questions which need to be addressed to resolve the ambiguities associated with the differential responsiveness of JEG-3 and HTR-8/SVneo cells towards the IL-11 treatment. 1) Are there differences in the IL-11 mediated activation of the downstream signaling in JEG-3 and HTR-8/SVneo cells? 2) What are the effector molecules whose alterations in response to IL-11 can explain the respective increase and decrease in the invasiveness of JEG-3 and HTR-8/SVneo cells? Keeping these key questions in mind, present study has been designed to provide evidences for the differential regulation of IL-11 mediated invasiveness of JEG-3 and HTR-8/SVneo cells.

Materials and Methods

Cell culture

JEG-3 (German collection of cell lines and microorganisms; DZMO, Braunschweig, Germany) and HTR-8/SVneo (kindly provided by Dr. Charles Graham, Queen's University, Kingston, ON, Canada) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Inc., St. Louis, MO, USA) or RPMI-1640 (Sigma-Aldrich Inc.) medium respectively, supplemented with 10% Fetal Bovine Serum (FBS; Biological Industries, Kibbutz beit Haemek, Israel) and an antibiotic-antimycotic cocktail [Penicillin (100 units/ml), Streptomycin (100 μg/ml) and Amphotericin B (0.25 μg/ml); Pen-Strep-Ampho sol, Biological Industries] under 5% CO_2 humidified atmosphere at 37°C [11].

Invasion assay

Invasion assay was performed as described before [9]. Briefly, $\sim 10^5$ cells were seeded onto the Matrigel matrix and incubated with or without an optimized concentration of IL-11 (200 ng/ml; Peprotech, Rocky Hill, NJ, USA) [9]. After 24 h of incubation,

Table 1. Primer sequences used for the real-time PCR.

Gene	Primers	Annealing temperature	Product size (bps)
Integrin αV	F: 5′ GCTCCATCTTCAGTGCCCTTA 3′ R: 5′ TTGGCAGACAATCTTCAAGCA 3′	60°C	274
Integrin α5	F: 5′ CGCAGCTCTGCTTCCTCGGG 3′ R: 5′ GCTGTGGCCACCTGACGCTC 3′	60°C	260
Integrin α6	F: 5′ TGCAGGCACTCAGGTTCGAGTGA 3′ R: 5′ AGCATGGTATCGGGGAACACTGTCA 3′	60°C	193
MMP2	F: 5′ ACCGCAAGTGGGGCTTCTGC 3′ R: 5′ CGTGGCCAAACTCGTGGGCT 3′	60°C	72
MMP3	F: 5′ TTGGCCCATGCCTATGCCCC 3′ R: 5′ ACAGGCGGAACCGAGTCAGG 3′	57°C	214
MMP9	F: 5′ CCGGCATTCAGGGAGACGCC 3′ R: 5′ TGGAACCACGACGCCCTTGC 3′	61°C	71
MMP23B	F: 5′ GCTGGTCGCCCTGTGCCTC 3′ R: 5′ GGAGTCAGCGTGTAGCGGCG 3′	60°C	177
TIMP1	F: 5′ TGACATCCGGTTCGTCTACA 3′ R: 5′ GTTTGCAGGGGATGGATAAA 3′	62°C	248
TIMP2	F: 5′ GATGCACATCACCCTCTGTG 3′ R: 5′ GTGCCCGTTGATGTTCTTCT 3′	62°C	196
TIMP3	F: 5′ CTGACAGGTCGCGTCTATGA 3′ R: 5′ AGTCACAAAGCAAGGCAGGT 3′	60°C	165
18S	F 5′ GGAGAGGGAGCCTGAGAAAC 3′ R 5′ CCTCCAATGGATCCTCGTTA 3′	60°C	171
Jun	F 5′ AGAGCGGTGCCTACGGCTACAGTAA 3′ R 5′ CGACGTGAGAAGGTCCGAGTTCTTG 3′	60°C	125
Fos	F: 5′ ATGGGCTCGCCTGTCAACGC 3′ R: 3′ GGAGATAACTGTTCCACCTTGCCCC 3′	60°C	284
MUC1	F: 5′ GTG CCC CCT AGC AGT ACC GA 3′ R: 5′ GAC GTG CCC CTA CAA GTT GG 3′	60°C	123
PDPN 1/3	F: 5′ AGCACAGTCCACGCGCAAGA 3′ R: 5′ CTTTAGGGCGAGTACCTTCCCGACA 3′	58°C	168
PDPN 2/4	F: 5′ GCCACCAGTCACTCCACGGAGAA 3′ R: 5′ GGGCCTTCCCGACATTTTTCGC 3′	58°C	230

doi:10.1371/journal.pone.0029745.t001

medium from lower chamber was aspirated and the excess of cells and Matrigel on the top of membrane of the transwell inserts were removed using moist cotton swab. Cells from the lower side of the membrane were fixed by chilled methanol for 7–10 min at 4°C, followed by staining with 0.2 µM Hoechst 33342 nuclear dye (Biotium Inc., Hayward, CA, USA) for 5 min at 37°C, washed with 50 mM PBS; pH 7.4 and visualized for counting using the fluorescent phase contrast microscope (Eclipse 80i, Nikon, Chiyoda Ku, Japan) under oil immersion.

Preparation of whole cell extract

Cells (10^5) were cultured in six well culture plates for 24 h and starved of FBS for at least 4 h before treatment with IL-11 (200 ng/ml) for 10, 30 and 60 min or for 24 h in the serum free medium. After each time point, the medium was aspirated and cells were lysed in 100 µl of lysis buffer (20 mM Tris-HCl, 10% glycerol, 0.2 mM EDTA, 0.137 M NaCl, 1% NP-40) supplemented with Complete protease and phosphatase inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). This was followed by 3 rapid freeze and thaw cycles to ensure the complete lysis of the cells. Cell lysates were centrifuged at 12,000× g for 10 min at 4°C and the supernatant was collected. The amount of protein in each sample was quantitated by BCA colorimetric assay using bovine serum albumin (BSA) as standard.

Western blot

About 40 µg of cell extract was electrophoressed and transferred onto the nitrocellulose membrane as described before [9]. Individual blots were incubated at 4°C overnight with 1:1000 dilution of rabbit polyclonal antibodies against phospho-STAT1 (p-STAT1)(tyr701), p-STAT3(tyr705), p-STAT3(ser727), p-ERK1/2(thr202/204), STAT1, STAT3 (All from Cell Signaling Technology Inc., Danvers, MA, USA) and mouse monoclonal antibody against ERK1/2 (Abcam, Cambridge, MA, USA) followed by incubation with 1:2000 dilution of HRP conjugated goat anti-rabbit/mouse IgG antibody (Pierce, Rockford, IL, USA) for 1 h at room temperature (RT). Intensity of bands on Western blots were quantified by LabWorks Software Version 4.5 (Ultra-Violet Products Ltd., Cambridge, UK).

In another set of experiment, cell lysates (~40 µg) prepared after 24 h of IL-11 (200 ng/ml) treatment to either JEG-3 or HTR-8/SVneo cells were resolved by SDS-PAGE and transferred onto the nitrocellulose membrane as described above. Blots were probed overnight at 4°C with goat polyclonal antibody against protein inhibitor of activated STAT 1/3 (PIAS1/3; 1:1000 dilution; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) followed by HRP conjugated donkey anti-goat IgG antibodies (1:2000 dilution) for 1 h at RT. Blots were developed by chemiluminescent substrate and further re-probed for actin as described before [9].

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Cells (~10^5) were seeded onto the 6 well culture plate and cultured for 24 h. Cell were serum starved for 4 h before addition of IL-11 (200 ng/ml) for 24 h, keeping appropriate vehicle control. Total RNA was isolated from cells using Tri reagent (Sigma-Aldrich Inc.) following the standard protocol employing chloroform-isopropanol-ethanol steps for its purification. Isolated RNA samples were quantitated by NanoDrop 3300 spectrophotometer (Thermo Scientific, NanoDrop Products, Wilmington, DE, USA) and were subjected to DNase I (Ferments International Inc., Ontario, Canada) treatment at 37°C for 15 min as per the manufacturer's instruction. The isolated RNA (1 µg) was used to prepare the cDNA using random hexamers, dNTP mixture, RT buffer and Superscript III reverse transcriptase following the manufacturer's protocol (Superscript III RT PCR System; Invitrogen, Carlsbad, CA, USA). qRT-PCR reactions were carried out in triplicates in 20 µl reaction mixture containing Maxima^{TM} SYBR green qPCR master mix (2×) (Ferments International Inc.), synthesized cDNA and gene specific primers (1 nm) on an ABI 7500 machine (Life Technologies Corp., Carlsbad, CA, USA). The primers used for real-time PCR and their respective annealing temperatures are listed in Table 1. The temperature profiles used for the amplification of target sequences were: initial denaturation for 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, amplification for 1 min at primer specific annealing temperature value (Table 1) and then a final melting curve analysis with a range from 60 to 95°C over 20 min. Gene-specific amplification was confirmed by a single peak in the ABI Dissociation Curve software. Average threshold cycle (Ct) values for 18S rRNA (run in parallel reactions to the genes of interest) were used to normalize average Ct values of the gene of interest. These values were used to calculate the average for each group, and the relative ΔCt was used to determine the change in expression between the groups.

Microarray

Total RNA was extracted using Tri reagent (Sigma Aldrich Inc.) and purified on RNeasy columns (Qiagen, Crawley, UK) according to the manufacturers recommendations. RNA quality was checked using an Agilant 2100 Bioanalyser (Agilent Technologies, Palo Alto, USA). Sense strand cDNA was prepared using the Ambion WT expression kit (Ambion Inc., Austin, Texas, USA) which was fragmented and biotin-labeled using the

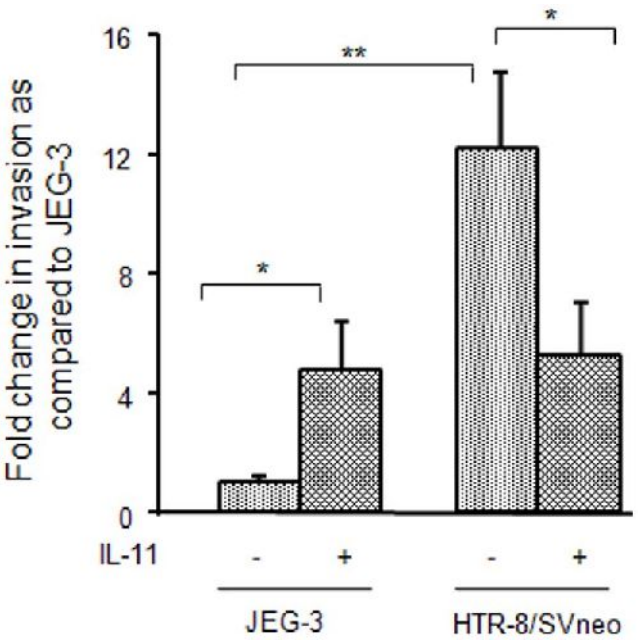

Figure 1. Effect of IL-11 on invasion of JEG-3 and HTR-8/SVneo cells. Invasion assay was performed as mentioned in *Materials and Methods*. Data is expressed as fold change in invasion following IL-11 (200 ng/ml) treatment as compared to untreated JEG-3 cells as control. Values are expressed as mean ± SEM of at least 6 experiments performed in duplicates. *$p<0.05$; **$p<0.001$.
doi:10.1371/journal.pone.0029745.g001

Affymetrix GeneChip WT terminal labeling kit (Affymetrix, High Wycombe, UK) according to manufacturers' recommendations. Fragmented and labeled cDNAs were hybridized to Affymetrix Exon 1 ST GeneChips (Affymetrix) at 45°C for 17 h in hybridization oven at 60 rpm according to Affymetrix protocols (Affymetrix). The washing and staining were performed using the Affymetrix Fluidics Station 450. The chips were read using a GeneChip Scanner 3000, and the resulting raw image was converted to signal intensities, detection calls, comparison files, signal log ratios, and change calls (Center for Genomic Application, New Delhi, India). Each of these pieces of data was generated independent of each other using algorithms from the Affymetrix GenChip Operating Software. For normalizing and summarizing probe-level intensity measurements from GeneChips, GCRMA was used which converts .CEL files into expression set using the Robust Multi-array Average (RMA) with the help of probe sequence and with GC-content background correction. Statistical (Student's *t* test) analysis was performed with Affymetrix Data Mining Tool software. The data were filtered on the criteria of 1.5 fold up- or 0.5 fold down-regulation taking into account the genes whose $p<0.05$. Basic information related to the microarray data has been submitted to the GEO database following the MIAME guidelines (Accession no. GSE31608).

Silencing of matrix metalloproteinase 23B (MMP23B) expression by siRNA

MMP23B siRNA contains the smart pool of 3 different siRNAs (Thermo Scientific Dharmacon, Lafayette, CO, USA). Cells were cultured in 6 well plates under standard conditions (37°C, 5% CO_2 humidified atmosphere). At 50% confluency, cells were washed twice with OPTI-MEM I medium and 800 µl of fresh OPTI-MEM I medium was added into each well. Annealed oligonucleotides (final concentration 100 nm) were mixed with OPTI-MEM I to make a total volume of 185 µl. In a separate tube, 4 µl lipofectamine 2000 was mixed with 11 µl OPTI-MEM I medium. Both the solutions were mixed and incubated for 20 min at RT. The mixed solutions were added carefully drop by drop in respective wells and after 4 h of incubation, complete medium was added to the cells in each well. Silencing experiments were performed by keeping transfection (transfected with non-genomic siRNA) controls. The extent of silencing following transfection with siRNA was accessed by RT-PCR after 72 h of silencing.

Immunofluorescence

Cells ($\sim 2\times10^4$) were grown on the cover slips in 24 well cell culture plates for 24 h. After 4 h of serum starvation, cells were

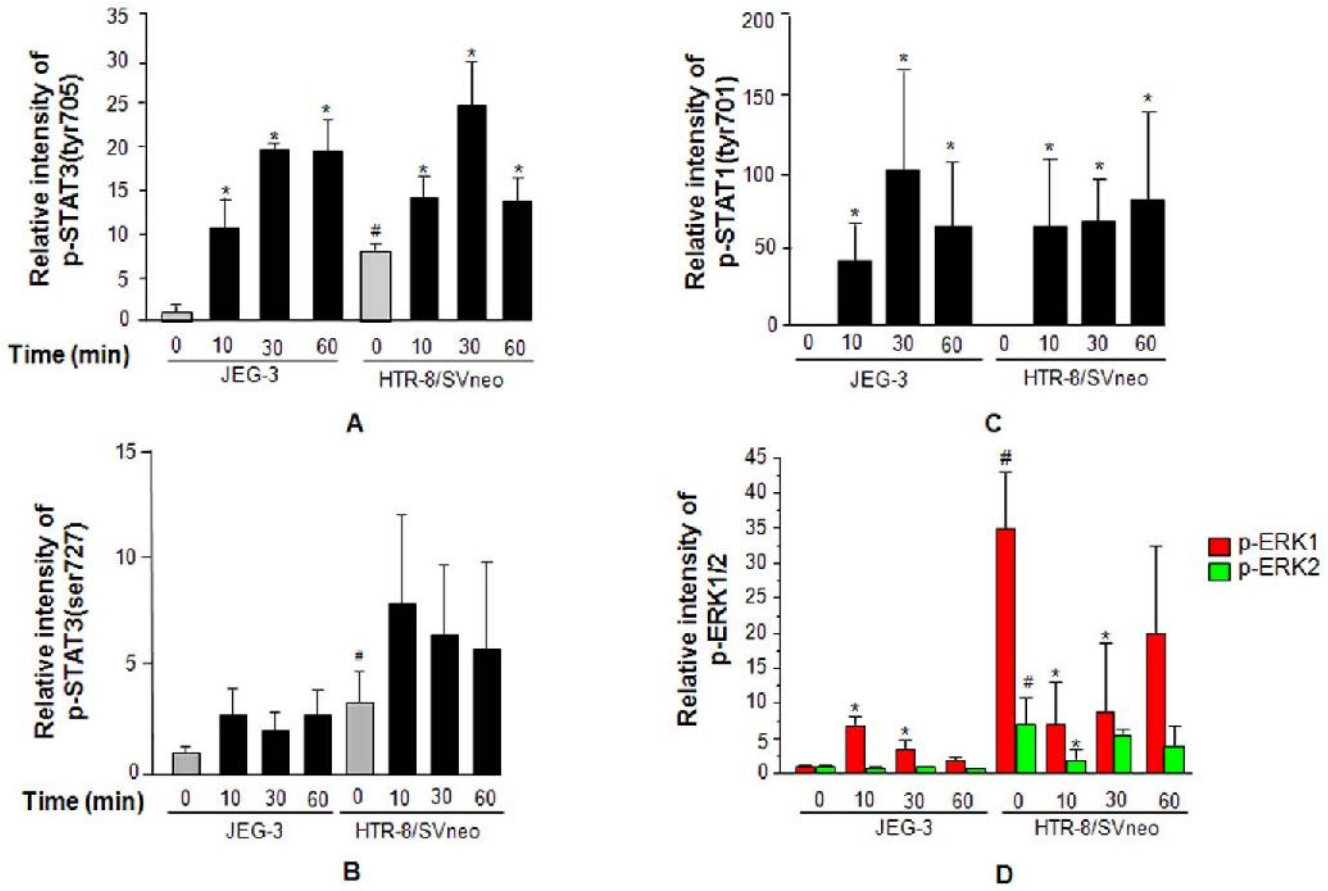

Figure 2. Activation of STAT and ERK1/2 dependent signaling pathway following IL-11 treatment. JEG-3 and HTR-8/SVneo cells were treated with IL-11 (200 ng/ml) for varying period of time and Western blots were done as mentioned in *Materials and Methods*. Panels A, B, C and D represent the densitometric plots of p-STAT3(tyr705), p-STAT3(ser727), p-STAT1(tyr705) and p-ERK1/2 respectively. Band intensities were normalized with respect to respective unphosphorylated proteins and the data is expressed as fold change with respect to JEG-3 control. The data is shown as mean ± SEM of at least 3 experiments. $^{\#}p<0.001$ between un-treated JEG-3 and HTR-8/SVneo cells; $^{*}p<0.05$ with respect to respective un-treated control cells.
doi:10.1371/journal.pone.0029745.g002

treated with IL-11 (200 ng/ml) for 10 min in serum free medium and fixed with chilled methanol for 5 min at 4°C. Cells were washed with 50 mM PBS; pH 7.4 and blocking was done for 1 h at RT using PBS containing 2% BSA. Cells were washed and incubated overnight at 4°C with rabbit polyclonal antibody against p-STAT3(tyr705) (1:100 dilution) and goat polyclonal antibody against PIAS1/3 (1:100 dilution). This was followed by washing of the cells with PBS for 3 times and incubation with 1:400 dilutions of Alexa Fluor 488 goat anti-rabbit IgG (H+L) and donkey anti-goat IgG-rhodamine for 1 h at RT to perform the double labeling. Cells were again washed with PBS (4 times) and mounted in dark onto the glass slide using Vectashield hard set mounting medium containing DAPI (1.5 μg/ml) (Vector Laboratories Inc., Burlingame, CA, USA). Slides were screened for immunofluorescence under a fluorescent phase contrast microscope (Nikon) and images were captured by using the Image Proplus software (Nikon).

Statistical analysis

All the experiments were performed at least three times and the results are expressed as mean ± SEM. For different sets of experiments like invasion assay and Western blot (densitometric analysis), the statistical analysis was done by comparing the means of the control and experimental sets by one-way ANOVA. A value of $p<0.05$ was considered to be statistically significant.

Results

Invasion of JEG-3 and HTR-8/SVneo cells under the influence of IL-11

At the basal level, a significantly higher (~12 fold; $p<0.001$) invasiveness of HTR-8/SVneo cells as compared to JEG-3 cells was observed (Fig. 1). As compared to untreated cells, almost five fold increase in invasion was observed in response to optimized concentration of IL-11 (200 ng/ml) in JEG-3 cells while, at the same concentration, IL-11 inhibited invasion of HTR-8/SVneo cells to almost half ($p<0.05$; Fig. 1).

Activation of downstream signaling molecules by IL-11 in JEG-3 and HTR-8/SVneo cells

JEG-3 and HTR-8/SVneo cells were treated with IL-11 (200 ng/ml) for varying time periods (0, 10, 30 and 60 min) and cell lysates collected at specific time points were subjected for Western blot. There was a significantly higher ($p<0.05$) basal levels of p-STAT3(tyr705) (Fig. 2A), p-STAT3(ser727) (Fig. 2B) and p-ERK1/2 (Fig. 2C) in HTR-8/SVneo cells as compared to JEG-3 cells but, no differences were observed in the levels of p-STAT1(tyr701) in both the cell lines (Fig. 2D).

As compared to untreated controls, following IL-11 treatment there was an increase in the activation of STAT3(tyr705) in both JEG-3 and HTR-8/SVneo cells, which was evident as early as 10 min and continued until 60 min of IL-11 challenge (Fig. 2A). However, no significant changes in the phosphorylation of STAT3(ser727) was observed in both the cells at all the time points studied (Fig. 2B). IL-11 treatment to JEG-3 and HTR-8/SVneo cells led to a significantly higher ($p<0.05$) phosphorylation of STAT1(tyr701) (Fig. 2C).

In JEG-3 cells, following IL-11 treatment, there was a significant increase ($p<0.05$) in activation of ERK1 by 10 and 30 min which was still far less than the basal level of activated ERK1 in HTR-8/SVneo cells (Fig. 2D). However, IL-11 treatment to HTR-8/SVneo cells led to a significant decrease ($p<0.05$) in the activated ERK1 and ERK2 from the basal level by 10 and 30 min (Fig. 2D).

Microarray analyses of the IL-11 treated JEG-3 and HTR-8/SVneo cells

JEG-3 and HTR-8/SVneo cells were treated with IL-11 (200 ng/ml) for 24 h and microarray analysis was carried out as

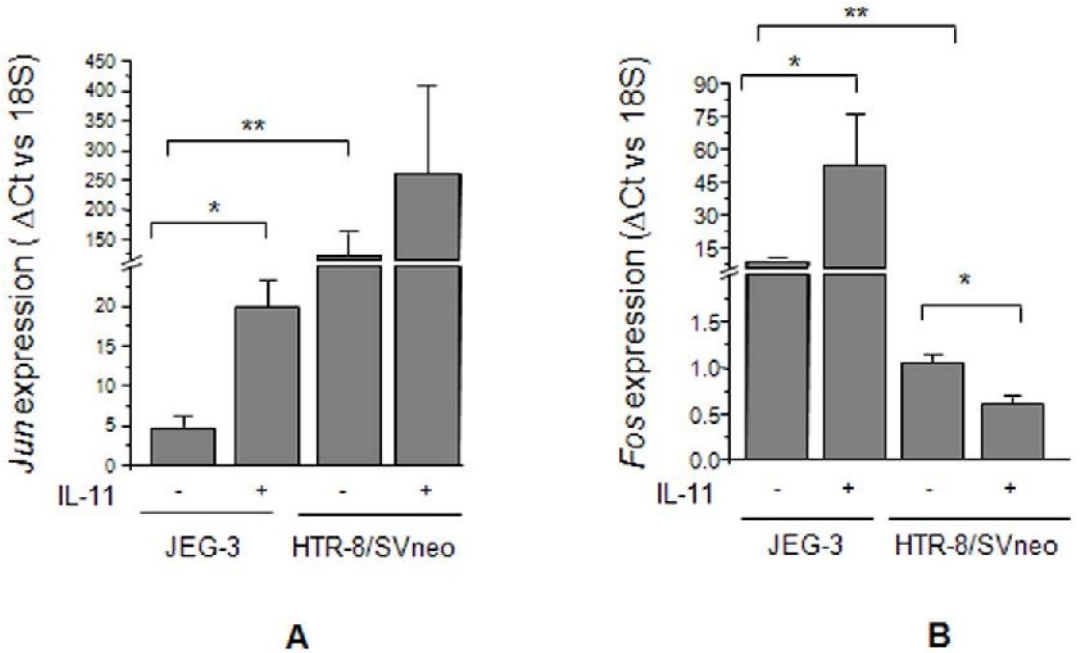

Figure 3. IL-11 mediated expression of *Jun* and *Fos* in JEG-3 and HTR-8/SVneo cells. Quantitative RT-PCR was done for the expression of *Jun* (Panel A) and *Fos* (Panel B) as mentioned in *Materials and Methods*. Each bar represents the ΔCt values after normalization with the 18S rRNA. The data is expressed as mean ± SEM of 3 experiments performed in triplicates. *$p<0.05$; **$p<0.001$.
doi:10.1371/journal.pone.0029745.g003

mentioned in *Materials and Methods*. We used the GeneChip® Human Exon 1.0 ST (Affymetrix, Santa Clara, CA) for these experiments. The array contains over 1.4 million probe sets and over 5 million probes. The probe sets are grouped into over 300,000 transcript clusters with over 90,000 transcript clusters containing more than one probe set. As compared to untreated controls, following IL-11 treatment to JEG-3 and HTR-8/SVneo cells, some distinct set of genes got up- and down- regulated. In JEG-3 cells, upon IL-11 treatment, 314 genes got upregulated by at least 1.5 fold while, 313 got downregulated by 0.5 fold. In contrast to this, in HTR-8/SVneo cells, 75 genes showed upregulation by at least 1.5 fold while, 54 showed downregulation by at least 0.5 fold following IL-11 treatment. Out of these, there were genes like *MMP23B*, *Jun*, *secretogranin II*, *dual specificity phosphatase 6*, *Wnt5A*, *homeobox A6*, *IL-1ζ* and *syntaxin 11*, which showed an increase in their expression in IL-11 treated JEG-3 cells while, a decrease in their expression was observed in IL-11 treated HTR-8/SVneo cells. Treatment of JEG-3 cells with IL-11 also led to an increase in the expression of genes like *mucin 1*, *cadherin 13* (*CDH13*), *defensin β1*, *insulin like growth factor binding protein 2* (*IGFBP2*) and *IGFBP5*, whose expression were not significantly altered in HTR-8/SVneo cells. Validation of some of the leads gained after microarray analysis have been carried out by performing qRT-PCR analysis on the RNA samples isolated from IL-11 treated JEG-3 and HTR-8/SVneo cells.

After this, we performed a comparative analysis of the gene expression in JEG-3 and HTR-8/SVneo cells. As compared to JEG-3 cells, in HTR-8/SVneo cells, there were about 1176 genes, which showed at least 2 fold increase while, about 1334 genes showed about 2 fold decrease in their expression. Amongst the differentially expressed genes, there were several molecules whose expression might influence the invasive capabilities of a given cell type. As compared to JEG-3 cells, HTR-8/SVneo cells showed over-expression of proteases [*MMP1*, *MMP2*, *MMP9*, *MMP23B*, *tissue palsminogen activator* (*TPA*), PLAUR], protease inhibitor like *TIMP1*, adhesion molecules [*CDH13*, *CDH2*, *integrin A2* (*ITGA2*), *ITGA3*, *ITGA4*, *ITGA11*, *MUC1*, *syndecan 2* (*SDC2*)], cytokines or their receptors (*IL-11*, *IL-32*, *IL-27A*, *MCSF1*, *IL-8*, *IL-1b*, *LIFR*, *NOTCH2*) and signaling intermediates [*Janus kinase 2* (*JAK2*), *STAT3*, *suppressor of cytokine signaling 3* (*SOCS3*), *SOCS5*, *human homologue of mothers against decapentaplegic 9* (*SMAD9*)]. However, there were several molecules like *MMP14*, *MMP19*, *TIMP4*, *CDH1(E-cadherin)*, *CDH3 (placental cadherin)*, *CDH5*, *CDH8*, *protocadherin beta 13*, *ITGB4*, *MUC15*, *CDH18*, *insulin like growth factor 2* (*IGF2*), *STAT1*, *FOS*, *SP6 transcription factor* (*SP6*) etc that showed a higher expression in JEG-3 cells as compared to HTR-8/SVneo cells.

Further, on the basis of the observed differences at the level of gene expression, we carried out pathway analysis by using DAVID functional annotation tool (DAVID Bioinformatics Resources 6.7, NIAID/NIH, USA) [12,13]. HTR-8/SVneo cells showed an over-expression of molecules associated with signaling pathways which promote the invasiveness of cells. These were MAPK signaling pathway, pathways in cancer, cytokine-cytokine receptor interaction, focal adhesion, chemokine signaling, ECM-receptor interaction, transforming growth factor β (TGF β) signaling pathway etc. As compared to HTR-8/SVneo cells, JEG-3 cells showed an increase in the expression of molecules associated with signaling pathways like renal cell carcinoma, thyroid cancer, insulin signaling, P53 signaling, tight junction etc.

Effect of IL-11 on the expression of activator protein-1 (AP-1) transcription factors

Jun and Fos are two main members of the AP-1 transcription factor. Microarray of IL-11 treated JEG-3 and HTR-8/SVneo cells suggested the upregulation of *Jun* expression in JEG-3 cells while, downregulation in HTR-8/SVneo cells. To further validate this observation, qRT-PCR was performed in IL-11 treated JEG-3 and HTR-8/SVneo cells. At the basal level, HTR-8/SVneo cells showed a significantly higher ($p<0.001$) level of *Jun* expression than that of the JEG-3 cells (Fig. 3A). Further, IL-11 treatment significantly increased the expression of *Jun* in JEG-3 cells while, the increase in its expression in HTR-8/SVneo cells was not significant (Fig. 3A). Analysis of the expression of *Fos* (one of the closely associated partners of Jun) was also carried out by qRT-PCR. Unlike *Jun*, at the basal level, there was a significantly higher ($P<0.001$) expression of *Fos* in JEG-3 cells as compared to HTR-8/SVneo cells (Fig. 3B). Treatment of JEG-3 cells with IL-11 led to a significantly higher ($p<0.05$) level of *Fos* expression while, there was a significant decrease ($p<0.05$) in the expression of Fos in HTR-8/SVneo cells (Fig. 3B).

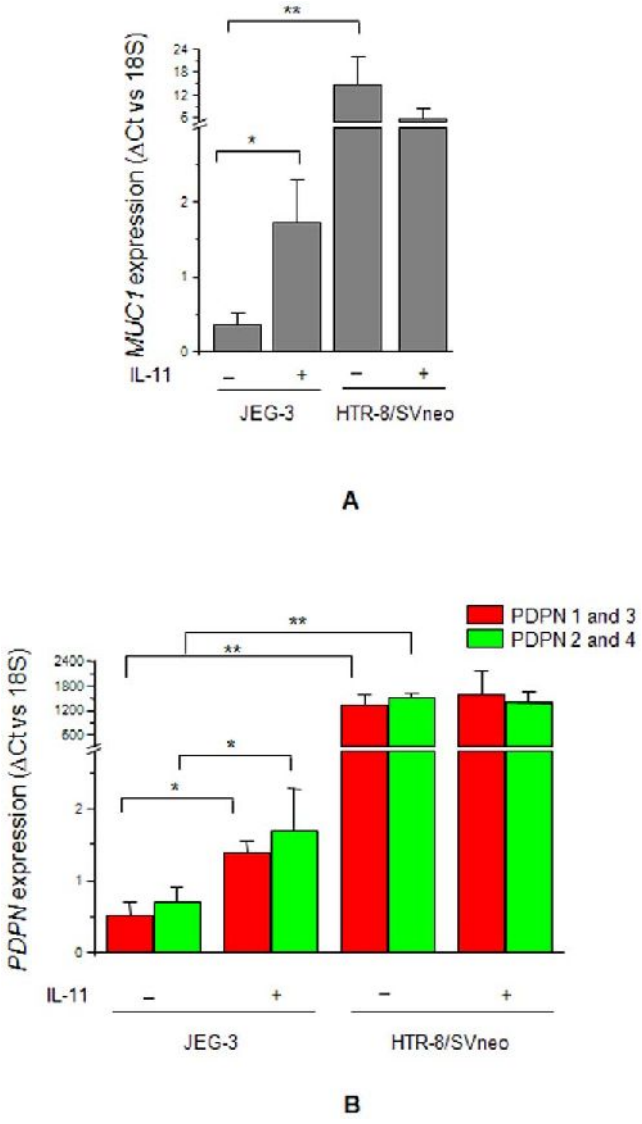

Figure 4. IL-11 mediated expression of *MUC1* and *PDPN* in JEG-3 and HTR-8/SVneo cells. Quantitative RT-PCR was done for the expression of *MUC1* (Panel A) and *PDPN* (Panel B) as mentioned in *Materials and Methods*. Each bar represents the ΔCt values after normalization with the 18S rRNA. The data is expressed as mean ± SEM of 3 experiments performed in triplicates. *$p<0.05$; **$p<0.001$.
doi:10.1371/journal.pone.0029745.g004

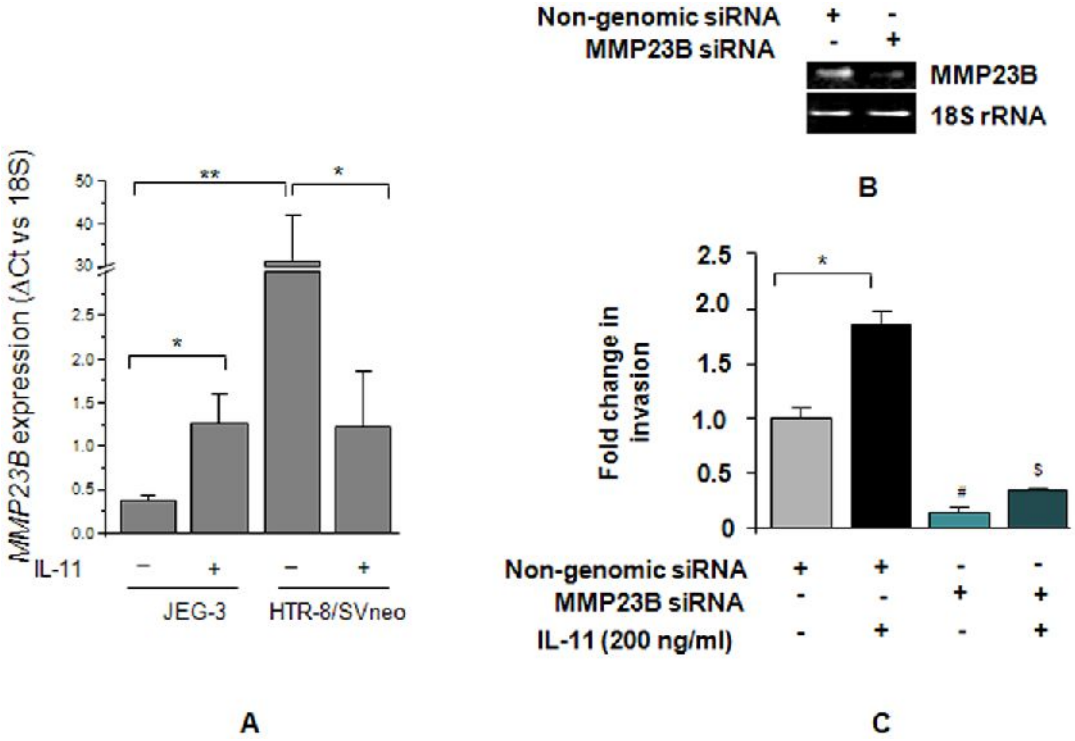

Figure 5. Role of *MMP23B* in IL-11 mediated invasion of JEG-3 and HTR-8/SVneo cells. Quantitative RT-PCR was done for the expression of *MMP23B* (Panel A) as mentioned in *Materials and Methods*. Each bar represents the ΔCt values after normalization with the 18S rRNA. The data is expressed as mean ± SEM of 3 experiments performed in triplicates. In another experiment, JEG-3 cells were transfected with either MMP23B siRNA or non-genomic siRNA for 72 h and end point RT-PCR was done to check the level of silencing in them, keeping 18S rRNA as internal control (Panel B). The transfected cells were used to study their invasive behavior in the presence or absence of IL-11 (200 ng/ml) as described in *Materials and Methods*. The results are expressed as mean ± SEM of fold change in invasion as compared to non-genomic siRNA transfected cells in the absence of IL-11, observed in 3 independent experiments. *$p<0.05$; **$p<0.001$; #$p<0.01$ between JEG-3 cells transfected with non-genomic and MMP23B siRNA; $^{\$}p<0.001$ between IL-11 treated non-genomic siRNA transfected and MMP23B siRNA transfected JEG-3 cells.
doi:10.1371/journal.pone.0029745.g005

Effect of IL-11 on the expression of mucin-type glycoproteins (mucin 1 and podoplanin) in JEG-3 and HTR-8/SVneo cells

Microarray analysis suggested an upregulation in the expression of *MUC1* in IL-11 treated JEG-3 cells while, its downregulation in IL-11 treated HTR-8/SVneo cells. This observation was confirmed by performing the qRT-PCR on the RNA samples isolated from JEG-3 and HTR-8/SVneo cells treated with IL-11 for 24 h. We observed an increase ($p<0.05$) in the expression of *MUC1* in IL-11 treated JEG-3 cells (Fig. 4A). However, decrease in the expression of *MUC1* in IL-11 treated HTR-8/SVneo cells was not statistically significant (Fig. 4A). Podoplanin (PDPN) is another mucin-like protein which is expressed as four splice variants. Though, its expression was not significantly altered in the microarray data but, considering its significance in the LIF mediated increase in invasion of trophoblast cells (unpublished data), qRT-PCR was performed to analyze the changes in its expression in IL-11 treated JEG-3 and HTR-8/SVneo cells. To analyze the expression of all the four splice variants, two sets of PCR primers were made for qRT-PCR (Table 1). As observed for *MUC1*, HTR-8/SVneo cells had a significantly higher ($p<0.001$) level of basal expression of *PDPN* than that of JEG-3 cells (Fig. 4B). Further, IL-11 treatment increased ($p<0.05$) the expression of *PDPN* in JEG-3 cells while; there was no significant change in the expression of *PDPN* in IL-11 treated HTR-8/SVneo cells (Fig. 4B).

Table 2. Effect of IL-11 on the expression of MMPs, TIMPs and integrins.

Gene	JEG-3		HTR-8/SVneo	
	Control	IL-11	Control	IL-11
MMP2	1±0.23	1.3±0.29	39.4±14.4$^{\#}$	14.4±6.2*
MMP3	1±0.34	0.8±0.56	19.3±7.18$^{\#}$	1.6±0.17*
MMP9	1±0.3	1.21±0.44	217.3±42.6$^{\#}$	83.5±19.6*
TIMP1	1±0.12	1.4±0.59	139±7.00$^{\#}$	111±25.00
TIMP2	1±0.38	0.9±0.03	3.5±0.30$^{\#}$	2.2±1.00
TIMP3	1±0.39	0.9±0.03	0.9±0.07	0.6±0.35
Integrin α5	1±0.45	1.1±0.10	2.9±1.55$^{\#}$	2.9±1.25
Integrin α6	1±0.24	0.7±0.15	0.9±0.05	0.6±0.04
Integrin αV	1±0.32	1.2±0.19	0.6±0.01	0.4±0.01

For each sample, ΔCt values were obtained after normalization with the Ct values for 18S rRNA. After that fold change in expression (ΔCt values) between the groups was calculated with respect to the untreated JEG-3 cells.
$^{\#}p<0.001$ between untreated JEG-3 and HTR-8/SVneo cells;
*$p<0.05$ between un-stimulated and IL-11 treated HTR-8/SVneo cells.
doi:10.1371/journal.pone.0029745.t002

IL-11 increases the expression of *MMP23B* in JEG-3 cells while, decreases its expression in HTR-8/SVneo cells

Microarray analysis of the IL-11 treated JEG-3 and HTR-8/SVneo cells showed an increase in the expression of *MMP23B* in JEG-3 cells while, a decrease in its expression in HTR-8/SVneo cells. To validate this observation, expression of *MMP23B* was analysed by qRT-PCR in RNA samples isolated from IL-11 treated JEG-3 and HTR-8/SVneo cells. In HTR-8/SVneo cells, there was a significantly higher basal expression ($p<0.001$) of *MMP23B* as compared to JEG-3 cells (Fig. 5A). Upon IL-11 treatment to JEG-3 cells, there was a significant increase in the *MMP23B* expression while, in HTR-8/SVneo cells, IL-11 significantly reduced ($P<0.05$) its expression (Fig. 5A).

Silencing of *MMP23B* expression abrogates the IL-11 mediated increase in JEG-3 cell invasion

To determine the significance of the increase in *MMP23B* expression in IL-11 mediated increase in invasive behavior of JEG-3 cells, its expression was silenced using siRNA. There was more than 70% silencing of the expression of *MMP23B* after 72 h of transfection with siRNA (Fig. 5B). Upon IL-11 treatment, there was a significant increase ($p<0.05$) in the invasiveness of non-genomic siRNA transfected cells (Fig. 5C). However, silencing of *MMP23B* expression by siRNA led to a significant decrease in the invasiveness of JEG-3 cells as compared to non-genomic siRNA transfected cells. Treatment of MMP23B silenced JEG-3 cells with IL-11 did not lead to a significant change in the invasion as compared to the control cells (Fig. 5C).

Expression of MMPs and TIMPs in JEG-3 and HTR-8/SVneo cells following IL-11 treatment

Expression of *MMP2*, *3*, *9* and *TIMP1*, *2* and *3* was analysed in JEG-3 and HTR/SVneo cells after 24 h of IL-11 treatment. In JEG-3 cells, there were no significant changes in the expression of these MMPs upon IL-11 treatment while, in HTR-8/SVneo cells, IL-11 treatment brought a significant decrease ($p<0.05$) in the expression of *MMP2*, *3* and *9* (Table 2). There were no significant changes in the expression of TIMPs in IL-11 treated JEG-3 and HTR-8/SVneo cells. At the basal level, HTR-8/SVneo cells had a significantly higher expression of *MMP2*, *MMP3*, *MMP9*, *TIMP1* and *TIMP2* than that of the JEG-3 cells (Table 2).

IL-11 mediated expression of integrins and other adhesion molecules in JEG-3 and HTR-8/SVneo cells

Switching in the expression of integrins like integrin α5, αV and α6 have been observed during the invasive differentiation of trophoblast cells. At the basal level, the expression of integrin α5 was significantly higher ($p<0.01$) in HTR-8/SVneo cells as compared to JEG-3 cells (Table 2). However, following IL-11 treatment no significant change in the expression of *integrin α5*, *αV* and *α6* were observed in both the cell lines as compared to respective controls (Table 2).

IL-11 decreases the expression of PIAS1 but, not of PIAS3 in HTR-8/SVneo cells

Western blots were performed for the analysis of PIAS1/3 expression in the cell lysates prepared after treatment of JEG-3 and HTR-8/SVneo cells with IL-11 for 24 h. At the basal level, both the cell lines expressed the PIAS1 while, PIAS3 was expressed only by HTR-8/SVneo cells. The level of expression of PIAS3 in JEG-3 cells was almost negligible as compared to HTR-8/SVneo cells. Upon IL-11 treatment, there was a significant decrease ($p<0.05$) in the expression of PIAS1 in HTR-8/SVneo cells while, there was no significant change in the expression of PIAS3 as compared to the control (Fig. 6). However, no significant changes in their expression were observed in JEG-3 cells after IL-11 treatment (Fig. 6).

IL-11 treatment to HTR-8/SVneo cells leads to nuclear co-localization of PIAS1/3 and p-STAT3(tyr705)

PAIS3 is a potential negative regulator of STAT3 signaling. If bound with the activated STAT3 molecules, PIAS3 can interfere with its transcriptional activity. To observe the IL-11 mediated nuclear localization of p-STAT3(tyr705) and its transcriptional activity which might get interfered by PIAS3, JEG-3 and HTR-8/SVneo cells were treated with IL-11 for 10 min and immunostained for p-STAT3(tyr705) and PIAS1/3. In untreated JEG-3 cells, fluorescence signal for PIAS1/3 was distributed throughout the cytoplasm and nucleus along with a typical punctate staining of PIAS1/3 at the cell boundary which would correspond to PIAS1 as JEG-3 cells have feeble expression of PIAS3 (Fig. 7). In untreated JEG-3 cells, there was no fluorescence signal for p-STAT3(tyr705) (Fig. 7). Upon treatment with IL-11, there was intense fluorescence signal for p-STAT3(tyr705) that was present well inside the nucleus. Though both activated STAT3 and PIAS1/3 were present in the nucleus of cells treated with IL-11; there were very few co-localization

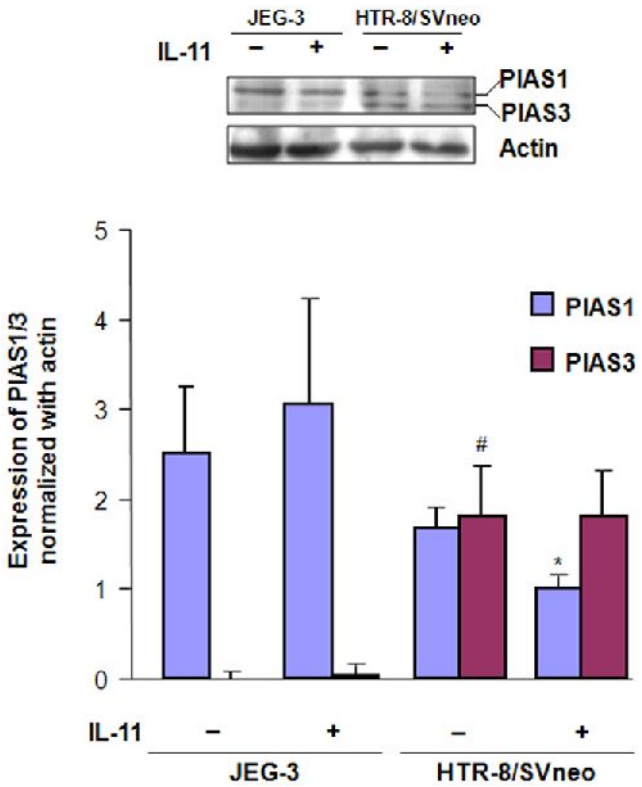

Figure 6. Expression of PIAS1/3 in JEG-3 and HTR-8/SVneo cells following IL-11 treatment. Cell lysates were prepared after treatment of JEG-3 and HTR-8/SVneo cells with IL-11 (200 ng/ml) for 24 h and Western blot was done for the expression of PIAS1/3 as mentioned in *Materials and Methods*. Band intensities were normalized with respect to actin and data is expressed as mean fold change in the expression ± SEM of PIAS1 and PIAS3 as compared to the JEG-3 control. *$p<0.05$ between untreated and IL-11 treated HTR-8/SVneo cells; #$p<0.001$ between untreated JEG-3 and HTR-8/SVneo cells.
doi:10.1371/journal.pone.0029745.g006

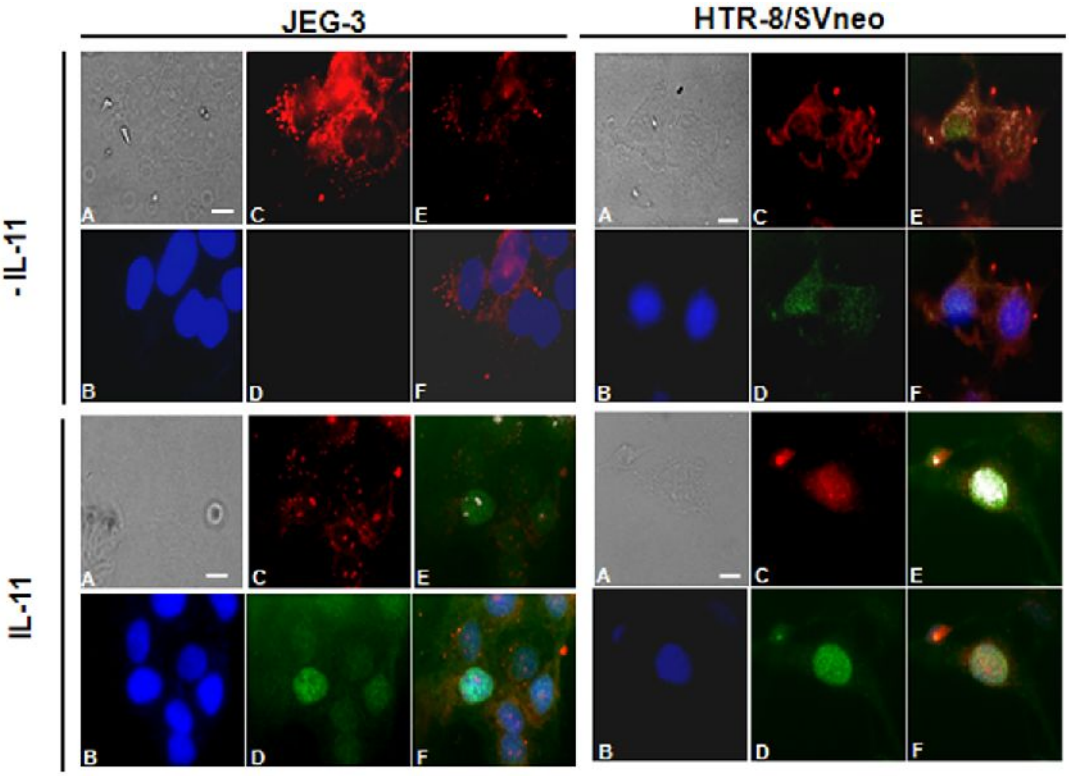

Figure 7. Immunolocalization of p-STAT3 (tyr705) and PIAS1/3 in JEG-3 and HTR-8/SVneo cells following IL-11 treatment. JEG-3 and HTR-8/SVneo cells were treated with IL-11 (200 ng/ml) for 10 min and then checked for the immunolocalization of p-STAT3 and PIAS1/3 followed by counter staining with DAPI. In the figure sub-panels are: A; phase contrast image, B; DAPI stained cells, C; staining for PIAS1/3, D; staining for p-STAT3(tyr705), E; co-localization of p-STAT3(tyr705) and PIAS1/3, F; merge image of the PIAS1/3, p-STAT3(tyr705) and DAPI images. Co-localization performed for PIAS1/3 and p-STAT3(tyr705) signals using "co-localization tool" of the ImageJ software. Two points are considered as co-localized, if their respective intensities are strictly higher than the threshold of their channels. Each co-localization point appears as white dot. Scale bar represents 20 μm size.
doi:10.1371/journal.pone.0029745.g007

points predicted by the software (Fig. 7). In un-treated HTR-8/SVneo cells, fluorescence signals for both PIAS1/3 and p-STAT3(tyr705) were distributed into the cytoplasm as well as inside the nucleus (Fig. 7). Upon IL-11 treatment, an increase in the nuclear localization of p-STAT3(tyr705) as well as of PIAS1/3 was observed (Fig. 7). The overlay of p-STAT3(tyr705), PIAS1/3 and DAPI showed co-localization into the nucleus (Fig. 7). In untreated cells, the co-localization of PIAS1/3 and p-STAT3(tyr705) was relatively less as compared to that observed after IL-11 treatment (Fig. 7).

Discussion

Amongst several trophoblastic cell models, JEG-3 choriocarcinoma and transformed EVT cells (HTR-8/SVneo) are the two cell lines, which have been widely employed to investigate the invasion and proliferation of trophoblastic cells [14]. However, as compared to EVTs, the JEG-3 cells show major differences in their responses to physiological ligands [15–17]. For example, TGF β decreases the invasiveness of EVT cells in Smad3 dependent manner while, JGE-3 cells could resist that effect due to the absence of Smad3 expression [15]. Decorin is a decidual product that acts in a TGF β independent manner to reduce the invasiveness of EVT but not JEG-3 cells [18–20]. Similarly, IL-11 (a decidua derived product) inhibits the invasiveness of EVTs and HTR-8/SVneo cells but, stimulates of invasion of JEG-3 cells [9,10]. However, the molecular basis of this differential effect is unknown. Under such situation, it is important to understand the mechanistic basis of the differential effects of IL-11 in these two cells lines, as it may give cues to understand the molecular basis of trophoblast invasion and an idea of how different extrinsic factors control the cellular invasion.

First trimester trophoblast cells, due to intrinsic mechanism have the invasive ability comparable to their malignant counterpart [21]. A comparative microarray analysis of JEG-3 and HTR-8/SVneo cells suggest that due to higher basal expression of pro-invasive molecules as well as the signaling pathways in HTR-8/SVneo cells, it has the higher invasive ability than that of the JEG-3 cells. At the molecular level, the increased expression of invasion related molecules require activation of transcription factors through activation of diverse signaling pathways. Of several pathways which can contribute to an increase in the invasion of cells, HTR-8/SVneo cells have a higher basal level of activation of STAT3 as well as ERK1/2 (Fig. 2). Activation of ERK1/2 and STAT3 may result into the higher level of basal expression of proteases as well as the cytokines/their receptors like *IL-11*, *IL-32*, *IL-8*, *IL-1*, *CSF1*, *LIFR*, *IGF1R* and *IL-4R* in HTR-8/SVneo cells as compared to JEG-3 cells.

Regulation of invasiveness of JEG-3 and HTR-8/SVneo cells by IL-11 has remained ambiguous, as it increases the invasiveness of JEG-3 cells while, decreases the invasiveness of HTR-8/SVneo cells [9,10]. In the present study also, IL-11 increased the invasiveness of JEG-3 cells while, reduced the invasiveness of HTR-8/SVneo cells. The active expression of invasion related

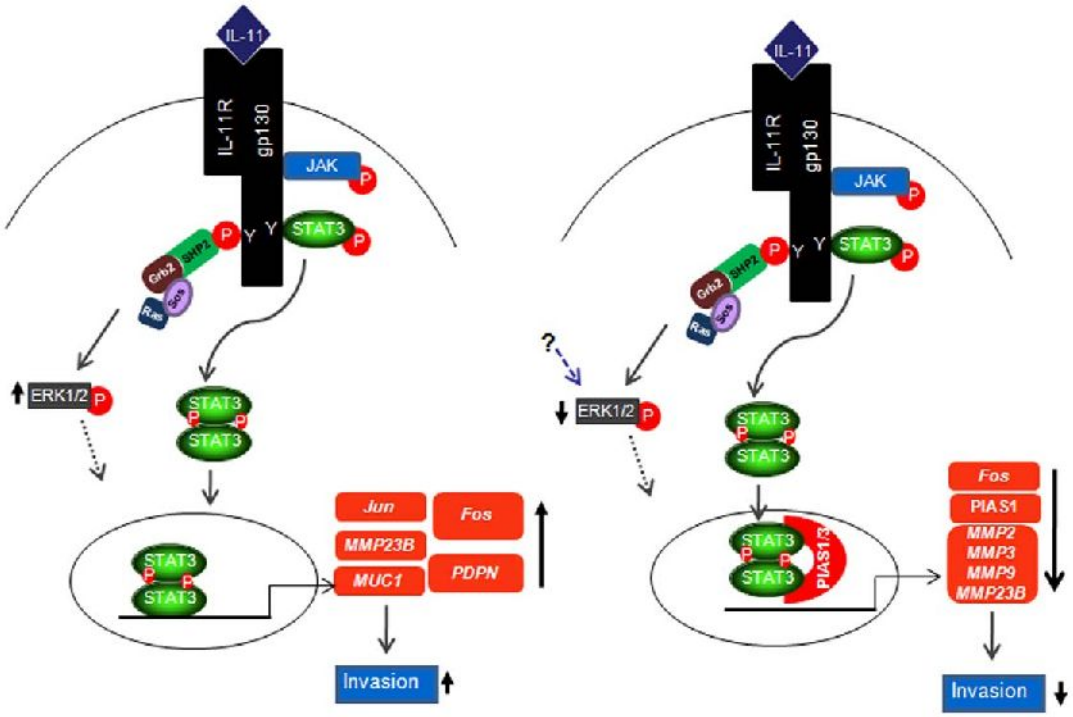

Figure 8. Schematic representation of the IL-11 mediated signaling and gene expression in JEG-3 and HTR-8/SVneo cells. IL-11 treatment to JEG-3 cells led to the activation of STAT1/3, which after dimerization moves into the nucleus. The activation of STAT3 was associated with a transient activation of ERK1/2. Activation of STAT3(tyr705) is associated with its nuclear localization. In effect to these, an increase in the expression of pro-invasive molecules like *Jun*, *Fos*, *MUC1*, *PDPN*, *MMP23B* etc have been observed. In HTR-8/SVneo cells, IL-11 treatment increases the activation of STAT1(tyr701) and STAT3(tyr705) while, decreases the activation of ERK1/2. The increase in p-STAT3(tyr705) was associated with its nuclear localization within 10 min of IL-11 treatment. However, upon IL-11 treatment, there was nuclear co-localization of p-STAT3(tyr705) with its inhibitory factor PIAS1/3. This could be the reason for the decrease in the expression of *Fos*, *MMP2*, *MMP3*, *MMP9* and *MMP23B*. (Solid arrows show the confirmed findings while, dotted arrows show the hypothetical links, which needs to be validated. Name of genes written in italics have been confirmed at the RNA level while, other have been confirmed at the protein level.).
doi:10.1371/journal.pone.0029745.g008

molecules is via activation of several signaling pathways including the mitogen activated protein kinase (MAPK) and STAT dependent signaling pathways [22]. Analysis of the IL-11 mediated activation of STAT and ERK1/2 dependent signaling pathways in both the cell lines was performed together on one blot to avoid the experimental variations in band intensities during Western blotting. IL-11 increased the activation of STAT3(tyr705) in both the cell lines, which is in agreement with the published report [9,10]. In addition to STAT3(tyr705), IL-11 also increased the phosphorylation of STAT1(tyr701) in both the cells lines without influencing the STAT3(ser727) phosphorylation. The major difference in the IL-11 mediated downstream signaling in the two cell lines was an increase in the ERK1 activation in JEG-3 cells while, a decrease in p-ERK1/2 in HTR-8/SVneo cells, which is consistent with the observed increase and decrease in IL-11 mediated invasiveness of JEG-3 and HTR-8/SVneo cells respectively. The observed decrease in ERK1/2 activation in HTR-8/SVneo cells could be due to the activation of phosphatases which dephosphorylate the activated ERK1/2 [23]. But, it needs further validation to specifically pin point the molecule which help in IL-11 mediated decrease in phosphorylation of ERK1/2 in HTR-8/SVneo cells.

AP-1 family of transcription factors is a family of proteins which controls the diverse biological processes like cellular proliferation, invasion and apoptosis. Jun and Fos are the transcription factors of AP-1 family which act as interacting partners for the activated STAT3 and cooperate in enhancing the STAT3 mediated transcriptional activity [24]. Upregulation of the expression of both Jun and Fos in IL-11 treated JEG-3 cells would be of paramount importance as they can enhance the transcriptional activity by cooperating with STAT3 as well as by forming Jun-Jun and Jun-Fos dimers and thereby facilitating the cellular invasion. Reduction in the expression of *Fos* in HTR-8/SVneo cells treated by IL-11 might be one of the factors associated with their reduced invasiveness.

After analyzing the expression of transcription factors, analyses of the IL-11 mediated expression of effector molecules (mucin-type molecules, MMPs, inhibitors of MMPs and integrins) were carried out in both the cell lines. Amongst mucin-type molecules, MUC1 and podoplanin (PDPN) gets upregulated in several tumors [25–27]. IL-11 upregulated the expression of *MUC1* as well as *PDPN* in JEG-3 cells while; there was no significant change in their expression in IL-11 treated HTR-8/SVneo cells. It was surprising to note that even after STAT3 activation in HTR-8/SVneo cells, there was no significant change in the expression of STAT3-responsive *MUC1*. Under such situation, it was plausible to analyze the expression and localization of PIAS1/3 in these cell lines as PIAS1/3 can inhibit the transcription activity of activated STAT3. In that direction, change in the expression of PIAS1 and PIAS3 was analysed after treatment of both JEG-3 and HTR-8/SVneo cells with IL-11 for 24 h. At a basal level, JEG-3 cells did not express PIAS3 while, HTR-8/SVneo cells expressed both PIAS1 and PIAS3 as previously observed [28]. IL-11 treatment reduced the expression of PIAS1 in HTR-8/SVneo cells while, it

had no influence on the PIAS1 expression in JEG-3 cells. A decrease in the PIAS1 expression in IL-11 treated HTR-8/SVneo cells would pose less hindrance to the p-STAT1(tyr701) directed anti-invasive transcriptional activity. So, this might be a contributory factor for the observed IL-11 mediated reduction in the invasiveness of HTR-8/SVneo cells. Further, extensive co-localization points for p-STAT3(tyr705) and PIAS1/3 in IL-11 treated HTR-8/SVneo cells as compared to JEG-3 cells would pose hindrance to the normal DNA binding and the transcriptional activity of activated STAT3. This could be the reason for the increase in the expression of STAT3-responsive *MUC1* in IL-11 treated JEG-3 cells but, not in the HTR-8/SVneo cells.

Trophoblastic cells express several proteases and their inhibitors but, the final outcome in terms of invasive behavior is governed by cytokine mediated shift in the fine balance between the activating and inhibiting molecules. Several cytokines and growth factors have been shown to increase the invasiveness of trophoblastic cells through changes in the expression of MMPs and TIMPs [29–32]. IL-11 reduced the expression of *MMP2*, *MMP3* and *MMP9* in HTR-8/SVneo cells but, not in JEG-3 cells. We observed an IL-11 mediated decrease in the expression of *MMP2* and *MMP9* after 24 h treatment of HTR-8/SVneo cells but, in an earlier study conducted after 48 h of IL-11 treatment, there was no significant change in their enzymatic activity [10]. This difference could be due to the differences in the time point for the analysis of the expression and activity of MMPs after IL-11 treatment. No effects of IL-11 were seen on TIMP expression in both the cell lines. *MMP23B* is unique membrane anchored MMP whose expression got significantly increased in IL-11 treated JEG-3 cells but, not HTR-8/SVneo cells, which was reflected by microarray as well as by qRT-PCR analysis [33,34]. Interestingly, silencing of *MMP23B* expression led to a significant decrease in the invasion of JEG-3 cells at the basal as well as after IL-11 treatment. This observation indicates MMP23B as a novel regulator of IL-11 mediated invasion of JEG-3 cells. Beyond MMPs and TIMPs, adhesion molecules like integrins and cadherins also play an important role in invasion of trophoblastic cells [35,36]. Treatment of JEG-3 as well as of HTR-8/SVneo cells with IL-11 did not show any significant change in the expression of *integrin* $\alpha 5$, αV and $\alpha 6$. However, microarray analysis of gene expression upon IL-11 treatment showed upregulation of the expression of cadherin 13 (*CDH13*) or H-cadherin in JEG-3 cells while, downregulation in HTR-8/SVneo cells. It will be of interest to study the role of CDH13 in trophoblast invasion.

From the above studies, following conclusions can be drawn. 1) In JEG-3 cells, IL-11 mediated activation of STAT and ERK1/2 signaling pathway is responsible for the increase in the expression of *Jun*, *Fos*, *MUC1*, *PDPN* and *MMP23B*, which ultimately leads to an increase in the invasiveness of JEG-3 cells (Fig. 8). 2) IL-11 mediated decrease in HTR-8/SVneo cells invasiveness was associated with a decrease in ERK1/2 activation, PIAS1/3 mediated activated STAT3(tyr705) sequestration and decrease in PIAS1 expression leading to a decrease in the expression of *Fos* and major families of MMPs (*MMP2*, *MMP3*, *MMP9* and *MMP23B*) (Fig. 8). 3) MMP23B has emerged as a novel regulator of the IL-11 mediated invasion of JEG-3 and HTR-8/SVneo cells. Thus, ERK1/2 and PIAS1/3 seems to be the critical factors that may be responsible for the differential effects of IL-11 on HTR-8/SVneo and JEG-3 cells. Taking cues from this study and that observed in case of TGF β, it appears that despite sharing gene expression signatures with EVT cells, the lack of crucial signaling components like Smad3 and PIAS3 in JEG-3 cells would bring about dramatic differences in the intricate regulatory mechanisms in response to external stimulus. Keeping in view of the observations described in this manuscript, it would be of interest to extend this study to analyze the gene expression and regulatory mechanisms associated with IL-11 mediated invasion of EVT cells.

Acknowledgments

The work reported in this manuscript was facilitated by Indo-German exchange program between the Department of Science and Technology (DST), Government of India and the Deutscher Akademischer Austausch Dienst (DAAD), Germany.

Author Contributions

Conceived and designed the experiments: PS SG. Performed the experiments: PS GG RT. Analyzed the data: PS SG. Contributed reagents/materials/analysis tools: PS SG. Wrote the paper: PS SG DM GG DM-P UM.

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IT'S A WOMAN THING: PART II - THE PLACENTA UNDER THE INFLUENCE OF TOBACCO

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KEY WORDS
Pregnancy, smoking, placenta, T cells, NK cells, trophoblast, villous, antioxidants, blood vessels, uterus, cigarette, tobacco, nicotine, CO, carbon monoxide

ABSTRACT

As exposed in part I, tobacco use during pregnancy is recognized by the WHO as one of the most important, preventable risk factors for developing a series of pregnancy pathologies. Many of these include those deemed to be mediated by the placenta: fetal growth retardation, preterm labor and stillbirth. Furthermore, preeclampsia, a hypertensive pregnancy disorder considered to be placenta-associated, seems to be subverted in women who smoke during pregnancy.

In general, smoking is considered a risk factor for cardiovascular diseases, and is associated with vascular dysfunction. Elevated vasoconstriction status, signs of altered uteroplacental blood flow and impaired endothelial nitric oxide synthase expression and activity have been demonstrated in pregnant women who smoke. Modifications in the maternal immune system have been proposed, including increased adhesion molecules expression and elevated leukocyte number, with increased T lymphocyte percentage, although the impact of these events is not completely understood.

The above changes seem to take a toll on the placenta. Prenatal smoking promotes adverse effects on trophoblast cells, with evident morphological and biochemical changes in early and term placenta. Altered apoptosis rate in trophoblasts and decreased expression of biochemical markers directly related to placental functional status have been described. Several modifications are also observed in villous development. Considered together, this data supports the concept that cigarette smoking is strongly associated with histological and physiological modifications during pregnancy, with subsequent impairment of placental and fetal development.

In part I, we identified and compared the placenta-associated disease profiles linked with individual types of nicotine exposure (cigarette, smokeless tobacco, nicotine replacement, secondhand smoke). Now, we review the literature on tobacco constituent effects on the placenta. We believe there are lessons to be learned from comparing epidemiological and laboratory data, since after we finally revisit the relevant pregnancy diseases, we can identify clarifying points and expose contradictions or research gaps.

MATERNAL SMOKING AND TROPHOBLAST CELLS

Trophoblast cells are the most exposed compartment of the placenta to toxic insults induced through chemicals such as nicotine, cotinine, benzo(a)pyrene, cadmium and others cigarette constituents/ by-products which are usually present in the circulating blood of pregnant women who smoke. Smoking during pregnancy has pleiotropic effects on trophoblast cells, with evident morphological and biochemical changes in early and term placenta. Some of these effects on trophoblast cells are summarized in this section.

Trophoblast Apoptosis

There is not a unified consensus about the effect of smoking on trophoblast apoptosis. Nicotine (see Table 3) has been proven to have cytoprotective effects, with apoptosis inhibition in fibroblasts, thymic cells and some cancerous cell lines. Women who smoke show significantly weaker labeling for apoptosis in syncytiotrophoblasts when compared to normal healthy women [1]. On the other hand, some reports convincingly show increased trophoblast apoptosis in women who smoke during pregnancy [2]. As described in the earlier section, both placentas from smokers as well as from snuff users display signs of increased trophoblast necrosis and apoptosis. This indicates that nicotine, the common product of both tobacco consumption forms, would be the main instigator of this effect. The increased apoptosis in trophoblast might be correlated with smoking-induced hypoxia, reduced intervillous blood flow and altered Epidermal Growth Factor (EGF) expression in the placenta. As mentioned in the earlier section, smoking is also associated with hyperplastic changes in the villous trophoblasts, with subsequent changes in the gas exchange between mother and growing fetus - which might result in intrauterine growth retardation [1].

Interestingly, carbon monoxide is able to inhibit syncytiotrophoblastic apoptosis and necrosis events that were mediated through hypoxia and reoxygenation in an in vitro model [3]. This observation indicates that carbon monoxide has potent antiapoptotic properties within the human placenta.

Considering the above information, it seems paradox that smokers' placentae show such morphological differences indicating increase in apoptosis and necrosis. Thus, it is interesting to compare placentae of cigarette smokers and snuff users. To

our knowledge, there is no work dedicated specifically to this theme, however, Ashfaq et al published two separate papers comparing the respective pathological placentae with normal placentae using identical measured endpoints and methods. A comparison of these sets of results makes apparent that the placentae of smokers, compared to that of snuff users, seem more heavily affected in terms of collagen content of chorionic villi and syncytial knots per unit area, but not in terms of morphologically apparent apoptosis rate [4, 5]. It should be mentioned here that the method of apoptosis measurement (morphological signs) was different from the study by Bainbridge [3], in which apoptotic markers as well as morphological observations on the electron microscopic level were investigated. In any case, it seems that there are dissimilarities between the placentae of tobacco users depending on consumption form, which indicates that nicotine and combustion products also mediate their effects differentially. Additional research is required to determine the underlying causes of these differences.

Trophoblast Proliferation and Differentiation

Maternal smoking is associated with reduced proliferation and abnormal differentiation (see Table 2) [6]. The non-progression in S phase of the cell cycle has been suggested to play an important role in this mechanism. It seems that especially the balance between cytotrophoblast proliferation and differentiation is altered in smokers' placentae, since explantation experiments of chorionic villi derived from smoking gravidas revealed that these cytotrophoblasts had a marked inability to differentiate into the invasive phenotype (reviewed by [7]; [6]).

Apart from nicotine, cigarette smoke is also a source of heavy metal-like cadmium (approximately 1-2µg per cigarette), which is, as mentioned above, detrimental to placental development mostly due to cadmium's characteristic as an endocrine disruptor, which alters placental steroidal and hormonal synthesis (reviewed also in [8]). Calmodulin is the intracellular calcium-binding protein which is affected by high doses of cadmium in trophoblast(ic) cells, subsequently decreasing trophoblast(ic)cell proliferation [9]. In vitro studies also indicate that B(a)P negatively influences the transcription of trophoblast stem cell proliferation and differentiation regulating proteins ins a manner which correlates high levels of B(a)P to declined trophblast stem cell proliferation and differentiation, and possibly to implantation failure [10].

Trophoblast Function

The general functional status of the trophoblast can be assessed in many manners, and often depends on the subtype under scrutiny. All trophoblast are capable of producing hormones, but is a main accomplishment of the syncytiotrophoblast, while cytotrophoblast and intermediate trophoblast are known mainly for their invasive capabilities.

- Trophoblast Invasion

Some of the in vivo and in vitro findings in the literature support the idea aroused from the epidemiological data that especially trophoblast invasion is negatively altered when pregnant women use tobacco. Many of the effects that indicate faulty placentation or trophoblast invasion while using tobacco products during pregnancy can also be explained through the alterations of proliferation and differentiation, particularly of trophoblast cells entering the invasive pathway (reviewed by [6]). However, there are some hints that trophoblast invasion is also directly impacted by tobacco usage, as described below.

An increased percentage of cytotrophoblast columns of anchoring villi fail to reach the uterus and tend to degenerate in the intervillous space, instead of invading the decidua [11] albeit this altered anchorage has also been made attributable to a nicotine mediated down-regulation of the l-selectin adhesion system [12]. In vitro exposure to nicotine inhibits the expression of fibronectin, integrin fibronectin receptor α5β1 and the 92 kDa type IV collagenase in cytotrophoblasts [11, 13], all factors that are normally associated with migration and invasion of the trophoblast cell. The migration capability of the macaque trophoblast cell is inhibited when cultured in the presence of medium conditioned with cigarette smoke [14]. B(a)P significantly inhibits trophoblastic choriocarcinoma cell invasion of the basement membrane in vitro [15]. It remains to be investigated whether a purported reduction of trophoblast invasion within maternal smokers' placentae actually results in deficient spiral artery remodeling, which is associated with preeclampsia and IUGR.

- Hormone and Growth Factor Production

Functional integrity is often assessed by expression of several biochemical markers, such as human chorionic gonadotropin (hCG), human placental lactogen (hPL), placental growth factor (PlGF), oestriol and oestradiol expressed by

trophoblasts (reviewed by [16]; [17]; [18]). The expression of these markers is altered in pregnant women who smoke (see Table 2) [19, 20].

HCG, oestriol and oestradiol are classical hormones used in the observation of placental or even gestational integrity. These hormones are implicated in a plethora of responsibilities associated with the maintenance of pregnancy. Listing all of them would go far beyond the scope of this review. Some of the relevant vital functions associated with some or all of the above mentioned hormones are: trophoblast fusion, proliferation and differentiation, placental hormone production or lipoprotein uptake (for a more specific review: [21]). Especially HCG-level in maternal serum has often been correlated with several pregnancy pathologies, and is commonly used to monitor gestational well-being (reviewed in [22, 23]). High levels are registered in some chromosomal deviations (such as trisomy 21) and gestational trophoblastic disease [23, 24], while erratic levels are found in ectopic pregnancies ([22, 25]). Too low levels have also been described for early detection of preeclampsia, IUGR and gestational diabetes [26], although not all of these results could be corroborated in a wide-spread manner (for example for preeclampsia: [27]). Reduced maternal serum levels of all three hormones are registered in cigarettes smokers [46, 47][28].

HPL and PlGF are factors more recently described in playing a role especially in pregnancy pathologies. PlGF is secreted by the trophoblast, its secretion rises during the course of gestation, shares 53% sequence homology with VEGF (Vascular Endothelial Growth Factor) and probably mediates a part of its angiogeneic activity by forming heterodimers with VEGF (all reviewed in [29]). Indeed, PlGF has been put forward as a determinant of placental vascularity [18]. PlGF concentration is elevated in the serum of prenatal smokers as compared to non-smokers, and low levels of this protein even during early gestation in maternal serum is associated with preeclampsia [27, 30]. HPL is also a growth hormone derived from the placenta that is normally synthesized by fully differentiated syncytiotrophoblast and intermediate (invasive) trophoblast cells located at the implantation site (reviewed in [31]). Secretion of HPL, unlike ßHCG which peaks in the first trimester, is continually produced throughout gestation and may constitute an indicator for syncytial well-being (reviewed in [32]). It is associated with maternal insulin resistance, thus raised maternal blood glucose levels, and other metabolic processes geared to ensuring fetal nutrition (reviewed in [17]). Reduced HPL levels at around midgestation are associated with growth restricted pregnancies (Bersinger, Odegard, 2004, Acta

Obstet Gynecol Scand). Smoking cigarettes negatively influences maternal blood levels of this protein [46, 47].

- Protein Synthesis

In addition to the histological view described in an earlier section, maternal smoking is also associated with increased alkaline ribonuclease (RNase) levels, possibly resulting in disturbed protein synthesis. This is accompanied by biochemical evidence of significantly increased cellularity (increased DNA/gm of placental mass and decreased protein/DNA ratios) (reviewed in [33]). Leucine Amino Peptidase (LAP), Alanine Amino Peptidase (APP) and membrane-associated ACE (Acetyl Choline Esterase)-like activities are significantly higher in smokers' trophoblast cells, which might be responsible for altered peptide hormone metabolism in the placenta [34]. Additionally, metalloproteinases play an important role in trophoblast invasion and its production and activation is downregulated with high doses of nicotine (see Table 2) [35].

- Generation of Energy

There are indications that prenatal smoking is associated with perturbed mitochondrial function with the placenta, which might limit the amount of available energy in cells (reviewed in [7]). A reduction by 30% of a mitochondrial membrane bound cytochrome was recently described in the placental mitochondria of smokers as compared to that of non-smokers [36]. Whether this placental dysfunctionality contribute to the pathogenesis of IUGR in prenatal smokers' remains to be further investigated. The uptake of glucose, at least by cells of the human choriocarcinoma cell line, BeWo, was not altered through nicotine [37].

- Oxidative Stress Management:

Free radicals are highly reactive molecules mainly generated by endogenous metabolic pathways, which include oxidative phosphorylation, DADPH (lactaldehyde reductase) oxidase and xanthine oxidase. Two types of free radicals have been extensively studied: reactive oxygen species (ROS) and reactive nitrogen species (RSN) [38]. These oxidative factors have been shown to impair most of the processes present during pre-implantation, such as oocyte fertilization and endometrial receptivity. Implantation and placental development are also negatively

affected [38]. The underlying mechanisms seem to be associated with changes in some ROS targets, such as DNA, protein and lipids, which are extremely relevant molecules in the fertilization process [39]. One study conducted a set of experiments dedicated to examining markers for oxidative damage on placental and cord blood DNA, lipids and proteins of prenatal smokers [40]. Here, maternal cotinine levels correlated with markers for DNA oxidation. Furthermore, maternal oxidative stress markers correlated to the level of DNA-adducts in the placenta, which in turn correlated to protein carbonylation in cord blood plasma. Interestingly, oxidative DNA damage and DNA-adduct level (in the placentae of smokers) negatively correlated to placental lipid peroxidation. Lipid peroxidation can change the property of a cell membrane, such as its fluidity or the activity of a membrane-bund property, and this can negatively alter the cell's function. This observation would indicate that smoking cigarettes might convey DNA and thus protein damage through oxidative stress, but also protect placental cell membranes.

Placental antioxidant systems:

In order to prevent oxidative conditions that might promote embryo injury or death, cells activate protective antioxidant mechanisms that degrade oxidative factors [39] . Four main antioxidant systems have been described in placenta: cooper/zinc superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and heme oxigenase (HO)[41]. Their function is to inactivate toxic substances that may lead to oxidative stress. SOD inactivates superoxide anion (O_2^-), CAT acts on hydrogen peroxide (H_2O_2), GPx inactivates H_2O_2 and lipid peroxides, and heme oxygenase enzymes (HO-1 and HO-2) degrade heme to CO, biliverdin (bilirubin) and Fe2+ [42].

Recent reports have shown some differences in smokers' placenta concerning the expression and function of antioxidant enzymes. Studies in vivo have demonstrated increased expression of HO-1 and HO-2 in placental basal plate from smokers. No differences regarding this expression were observed in anchoring villi (AV). Additionally, no changes in CAT, GPx and SOD levels were identified [41]. On the other hand, in vitro experiments using cigarette smoke extract (CSE) on a trophoblast choriocarcinoma hybrid cell line (HTR-8SVneo) showed a dose-dependent increase in HO-1 expression, but no changes in HO-2, SOD, GPx and CAT expression [41]. HO-1, besides being an anti-oxidant, is also anti-inflammatory

(reviewed by [43]). HO activity decreases during hypoxia, and HO protein is decreased in the placentae of preeclampsia patients (reviewed by [44]). HO-1 is able to negatively regulate the release of sFlt-1 and sEng in endothelial cells, and inhibition of HO-1 in placental villous explants potentiated the production of sFlt-1 and sEng [45]. Interestingly, the expression of HO is multifold elevated in pregnant myometrium compared to non-pregnant, and activation of HO significantly reduced both oxytocin-mediated, as well as spontaneous, uterine contractility [46].

Exogenous antioxidant system:

Cigarette smoke inhalation during pregnancy triggers activation of antioxidative systems through the generation of free radicals [47]. Oxidative stress might be reduced with supplementation of antioxidant compounds such as Vitamin C and E in diet. Increased vitamin deficiency in smokers has been shown [48]. In pregnant women who smoke, the concentration of vitamin E is decreased in plasma and erythrocytes when compared to non-smoking gravidas. Furthermore, Vitamin A, vitamin E, fS-carotene and total plasma antioxidant capacity were lowered in smokers' newborns [49]. The role that vitamins might play in embryo implantation, intrauterine growth and abortion control is controversial (as reviewed by Gupta et al [50]). Moreover, studies have implicated that vitamin C/E supplementation decreases the incidence of preeclampsia by decreasing the biochemical indices of oxidative stress [51], however, in a large prospective cohort study, the overall incidence of pre-eclampsia did not correlate with dietary vitamin C and E intake. In the same study, a decreasing trend in the incidence of severe pre-ecplampsia, eclampsia and HELLP with increasing dietary vitamin C intake was delineated [52]. Neither vitamin C nor E have an effect on sFlt-1 release [45].

- Effects of Specific Tobacco Constituents

Nicotine seems to restore endothelial dysfunction (as seen on experiments with umbilical endothelial cells) caused by excess antiangiogenic factors, such as soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng), both factors whose higher circulation in maternal serum is associated with onset of pre-eclampsia [53]. However, this restoration does not seem to be due to nicotinic acetylcholine receptor stimulation induced alteration of sFlt expression in placental cells, since neither nicotine nor its agonist alter expression of these factors [54]. Nicotine also

failed to affect sFlt-1, sEng, PlGF and TGF-ß mRNA expression in trophoblast cells. Smoking during pregnancy, however, is associated with lower circulating concentrations of sFlt1 and sEng [55] and exposure of placental villous explants to cigarette smoke extract lowers sFlt1 secretion [56]. Nicotine lowers sFlt-1 and s-Eng secretion in trophoblast cells [57]. Furthermore, in trophoblast cells, nicotine reduces PlGF release, but VEGF release seems unaffected [58]. The substance is also capable of suppressing placental cytokine production (TNF, IL6, IL8, IL1ß), although it is not yet corroborated which placental cell is responsible for this [54]. Nicotine activates phospholipase A2, which is implicated in prostaglandin E2 formation (a labor-inducing prostaglandin). Nicotine can also, however, activate placental nicotinic receptors and the release of placental acetylcholine, a vasodilator of placental arteries [59]. Nicotine has been described in elevating either sEng or sFlt-1 secretion in human umbilical cells ([57]; [58]). PlGF secretion appears to be stimulated by nicotine in human umbilical endothelial cells [58]. The subcutaneous applications of nicotine to gravid rats lead not only to a significant reduction of birthweight, but also an induction of cytochromes (CYP1A1, CYP2E2) indicative of oxidative stress [60].

B(a)P down-regulates the receptor expression of Epithelial Growth Factor (EGF), an important factor responsible for early implantation, and whose tyrosine kinase receptor activity has been demonstrated to be significantly decreased in smokers' trophoblast cells (see Table 3). Furthermore, benzo(a)pyrene reduces the expression of the proto-oncogene c-myc and of hCG (a positive regulator of trophoblast invasion), while it increases TGF-β1 expression (a negative regulator of trophoblast invasion) in first trimester placental villous explants and in choriocarcinoma cell lines [16, 23, 61]. Ethoxyresorufin-O-deethylase (EROD) is an enzyme that can be used as an indicator of placental hypoxia. Its activity is induced by exposure to polycyclic aromatic hydrocarbons found in tobacco smoke (see Table 3). There is a very high correlation between the plasmatic level of cotinine and placental EROD activity, indicating the direct impact of maternal cigarette smoking on placenta [62].

Cadmium (see Table 3) produces ultrastructural placental changes (lysosomal vesiculation, nuclear chromatin clumping, mitochondrial calcification) and decreases hCG secretion by trophoblast cells [63]. Animal experiments show that placental cadmium accumulation may interfere with the transfer of various essential minerals and other nutrients to the growing fetus [64]. Leptin mRNA in human trophoblast cells

declined dose-dependently to cadmium exposure [65]. Leptin is considered a pro-inflammatory cytokine/ adipokine that is associated with preeclampsia, because of the higher levels of this substance found in pre-eclampsia patients (reviewed in [43]). Leptin also probably stimulates trophoblast invasion, so cadmium might indirectly lower trophoblast invasion. Cadmium also interferes with trophoblast biosynthesis of progesterone, a steroid that plays a role, amongst others, in promoting uterine myometrial quiescence [66]. Acute exposure of high concentrations of cadmium to human placental explants may even lead to inhibited synthesis of thromboxane A2 and thus altered balance of thromboxane A2 to prostacyclin [64, 67]. In pre-eclampsia, the prostaglandin PGI2 is decreased, while the TXA2/PGI2 ratio is elevated so that lower TXA2 would lead to a more favorable TXA2/PGI2 ratio [68].

CO and CO-releasing molecules lower sFlt1 and sEng production in endothelial cells and placental villous explants derived from preeclamptics [45]. As mentioned earlier, CO also seems to down-regulate syncytiotrophoblast apoptosis and necrosis [3]. Pretreatment of HUVEC with CO releasing molecules results in a decrease of LPA-induced inflammation (as seen in through up-regulation of HO-1, inhibition of NF-kappaB and down-regulation of ICAM-1 and granulocyte adhesion [69].

Cotinine activates placental phospholipase-A2-like enzymes, resulting in the formation of prostaglandins (reviewed in [59]). Cotinine cannot, in contrast to nicotine, activate nicotinic and muscarinic receptors, and seems to enhance the effects of PGE2 [59]. Cotinine, as nicotine, reduces sFlt-1, sEng and, only at high dosages, PlGF release by trophoblast cells, while it increases PlGF and sFlt-1 release in HUVEC [58].

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