REVIEW ARTICLE

# Role of the Insulin-like growth factor axis and the Transforming growth factor-β in the regulation of the placenta and the pathogenesis of Gestational Trophoblastic Diseases

**Authors**
[1]Adriana Umaña-Perez, [2]Susana Novoa-Herran, [1]Juan Jose Castro, [1]Andres F. Correa-Sanchez, [1]Valentina Guevara, [1]David Alejandro Lopez-Gonzalez, [1*]Myriam Sanchez-Gomez

**Affiliations**
[1]Grupo de investigación en Hormonas. Departamento de Química, Facultad de Ciencias, Universidad Nacional de Colombia, Bogotá, Colombia
[2]Grupo de Fisiología Molecular, Subdirección de Investigación Científica y Tecnológica, Instituto Nacional de Salud, Bogotá, Colombia

***Correspondence**
Myriam Sanchez-Gomez
Emeritus Professor, Department of Chemisty, Faculty of Science, Universidad Nacional de Colombia
Email: mysanchezd@unal.edu.co

**Abstract**
During human pregnancy, the trophoblast develops as the fetal compartment, while in bidirectional communication with the maternal decidua. The trophoblast is responsible for the adequate implantation of the embryo, supply of nutrients and environmental protection of the fetus along the progress of pregnancy. To perform these functions trophoblast cells, undergo a complex and finely tuned differentiation into specialized groups of cells, in a process regulated by several hormones, growth factors and cytokines. Abnormalities in trophoblast function result in several pregnancy complications.

In this review, we focus our attention on two growth factors with pivotal roles during human pregnancy. The Insulin-like growth factor (IGF) family and the Transforming growth factor (TGF-β) axis are important regulators of the proliferation, differentiation, apoptosis, migration and invasion of human trophoblasts. We summarize what is described in the literature on how these factors and their receptors are expressed on the different subsets of trophoblasts, the signaling pathways that transduce their corresponding actions and functional biological effects. We describe the associations that have been found between these growth factors and the group of pathologies known as Gestational Trophoblastic Diseases (GTD).

**Key words:** Trophoblasts, Placenta, Transforming Growth Factor beta, Insulin-Like Growth Factor I, Insulin-Like Growth Factor II, GTD

## 1. Introduction

Normal placental development is dependent upon the differentiation and invasion of the trophoblast, a main cellular component of the placenta. Trophoblasts cells, which are of fetal origin, come in contact with maternal uterine decidua, thus initiating a bidirectional communication to ensure the progress and development of human pregnancy. The transfer of information involves a complex cellular signaling network, that regulates the production of many autocrine and paracrine factors in a spatiotemporal fashion, that intercalated with the hormonal network, finely tune specific mechanisms for trophoblast differentiation and function.

The trophoblast differentiates into three main subsets: the villous cytotrophoblast (CTB), the syncytiotrophoblast (STB) and the extravillous trophoblast (EVT), each one accomplishing specific function within the placenta. The external STB layer is responsible for transportation of oxygen and nutrients to the fetus. The CTB is known to differentiate either into STB or EVT along the invasive pathway. EVT invades the maternal decidua and a part differentiates into endovascular EVT, responsible of the remodeling of the spiral arteries, allowing optimal blood flow into the intervillous space. In this sense, the EVT cells behave as cancerous cells, however, trophoblast invasion is under control in terms of intensity, direction and time.

Several studies have shown that abnormalities in placental structural development can impair placental function and could lead to different pathologies. Insufficient placental invasion could lead to pre-eclampsia and intrauterine growth retardation, whereas uncontrolled invasion could result in diseases as placenta accreta, infertility or miscarriage. Trophoblast dedifferentiation can lead to forms of gestational trophoblastic diseases (GTD), either to benign or malign molar disease until choriocarcinoma.

Gestational trophoblastic disease (GTD) comprises a group of pregnancy-related pathologies characterized by abnormal trophoblast growth and invasion. GTD includes pre-malignant complete and partial hydatidiform moles and malignant lesions such as invasive mole and choriocarcinoma, the most rapidly progressive form of malignant GTD. With rare exceptions, complete moles are diploid and androgenic in origin, while partial moles are triploid, consisting of one maternal and two paternal sets of chromosomes. The incidence of choriocarcinoma after molar pregnancy appears to vary by population, but after uterine evacuation 10-20% of complete moles and up to 5% of partial moles undergo malignant change, mainly into choriocarcinoma. The clinical and pathologic diagnosis of molar pregnancy is imperfect due to overlapping clinical and morphologic features with a normal missed abortion.

Several growth factors and cytokines are expressed by the different subtypes of trophoblast cells in order to regulate placental function. Among them, the Insulin-like growth factors (IGFs) and the Transforming growth factor beta (TGF-β) display pivotal roles in the differentiation, proliferation and invasion of trophoblasts cells.

The Insulin-like growth factor (IGF) system is an important regulator of cell proliferation, differentiation, apoptosis and tissue growth. It has also been implicated in various pathological

 

conditions and play a critical role in tumorigenesis and cancer. All of the components of the IGF system so far investigated, are present in the placenta at some stage of pregnancy. The placenta is exposed to IGF-1 and IGF-2 from multiple sources, including those produced locally and those circulating within the fetus and the mother, and variations in both temporal and spatial expression suggest the presence of local regulatory factors, which have not been clearly defined yet.

TGF-β is a multifunctional cytokine that regulates various cellular functions, including cell proliferation, differentiation, apoptosis, migration and immune responses. TGF-β has also been suggested to be involved in the negative regulation of proliferation, differentiation and invasion of human trophoblasts, however, the molecular mechanisms underlying its actions have not been elucidated yet. In this review we focus on the possible roles of the IGF and TGF-β factors in the pathogenesis of GTD, on the premise of loss of control of their actions on the trophoblast cells. On one hand, an autocrine gain in growth promoting signals and IGF-dependent proliferation, and on the other, establishment of a refractory state to the TGF-β inhibitory signals resulting in choriocarcinoma and metastasis.

## 2. Physiology of the placenta

### 2.1 Trophoblast differentiation

In humans, in early events of development, a mass of totipotent cells called the morula, first differentiates into the blastocyst. The outer trophectoderm layer of the blastocyst encircles the blastocyst and generates all the extra-embryonic trophoblast cell types, contributing to the formation of the fetal placenta and extra-embryonic tissues. The placenta constitutes the fetal-maternal interface and has important roles in processes of early development, as well as nutritional and endocrine support during pregnancy. The trophoblast layer is involved in the initial adhesion, six or seven days after fertilization, of the blastocyst to the uterine wall and its subsequent implantation within the wall. This process can be divided into three phases: apposition, adhesion and invasion. The process starts with the contact between the blastocyst and the uterine wall followed by an increased physical contact between the blastocyst and decidua and then the penetration and invasion into the decidua, inner third of the myometrium and uterine vasculature.[1,2] Adherence is mediated by cell-surface receptors at the implantation site that interact with blastocyst receptors.[3,4] Cell invasion into the maternal uterine decidua is a critical process for normal placentation, pregnancy establishment and fetal growth continuance in humans.[5]

During the period of implantation, the trophoblast cells proliferate and become invasive as they differentiate.[6] Altered rates of cytotrophoblast proliferation are associated with different pathologies; enhanced levels are associated with increased fetal growth (macrosomia), while low levels are related to fetal growth restriction.[7]

Trophoblast stem cells follow two differentiation pathways, the villous and extra-villous pathways.[8] In the villous pathway, a group of villous cytotrophoblasts (vCTB) remain in the fetal compartment and fuse forming multinucleated syncytiotrophoblast cells (ST) which surround the chorionic villi. The external STB layer is in contact with

 

maternal blood and is responsible for transport of gases and nutrients from the maternal to the fetal circulation and represent the major endocrine unit of the placenta by secreting hormones, such as chorionic gonadotropin (hCG) and placental growth hormone (pGH).

The hyperglycosylated form of hCG (HhCG) is synthesized by the evCTB in early pregnancy during implantation of the embryo. In the extra-villous pathway, a set of vCTB differentiates into extra-villous cytotrophoblast (evCTB). These cells leave the basement membrane and form columns of cells denominated the anchoring villi, that attach and penetrate the uterine wall. Clusters of intermediate proliferating evCTB cells at the base of the anchoring villi, differentiate into intermediate invading evCTB, which in turn, gives up to two sets of cells: interstitial evCTB and endovascular evCTB (Figure 1). The differentiation of evCTB along the invasive pathway resembles that of transformed cells displaying a metastatic phenotype after malignant transformation.[9]

During interstitial invasion, interstitial evCTB cells, blend with decidual, myometrial and immune cells. The interstitial trophoblast population have the function to attach and adhere the placenta to the uterine wall: This is achieved by secretion of specific extra cellular matrix proteases, called matrix-type fibrinoid metalloproteases. As the cells move deeper into the decidua, the evCTB cells become multinucleated and terminally differentiated in placental bed giant cells.[8]

Endovascular evCTB invades the uterine spiral arteries and form plugs in the lumen of the vessels, that only allow blood plasma to seep through. During the first trimester of pregnancy, a first flow of fluids through the placental intervillous space is established, resulting in a physiological oxygen gradient between mother and fetus. The trophoblast plugs block spiral arteries until the beginning of the second trimester.[10] In parallel, endoglandular evCTB cells through a novel route, "break the basement membrane of uterine glands to open their lumen towards the intervillous space of the placenta, without showing the formation of plugs". It has been reported that these trophoblasts express the matrix metalloproteases MMP-1 and MMP-9 and Integrin β1.[10,11] This enables histiotrophic nutrition of the embryo in the first trimester, prior to onset of maternal blood flow into the placenta.

 

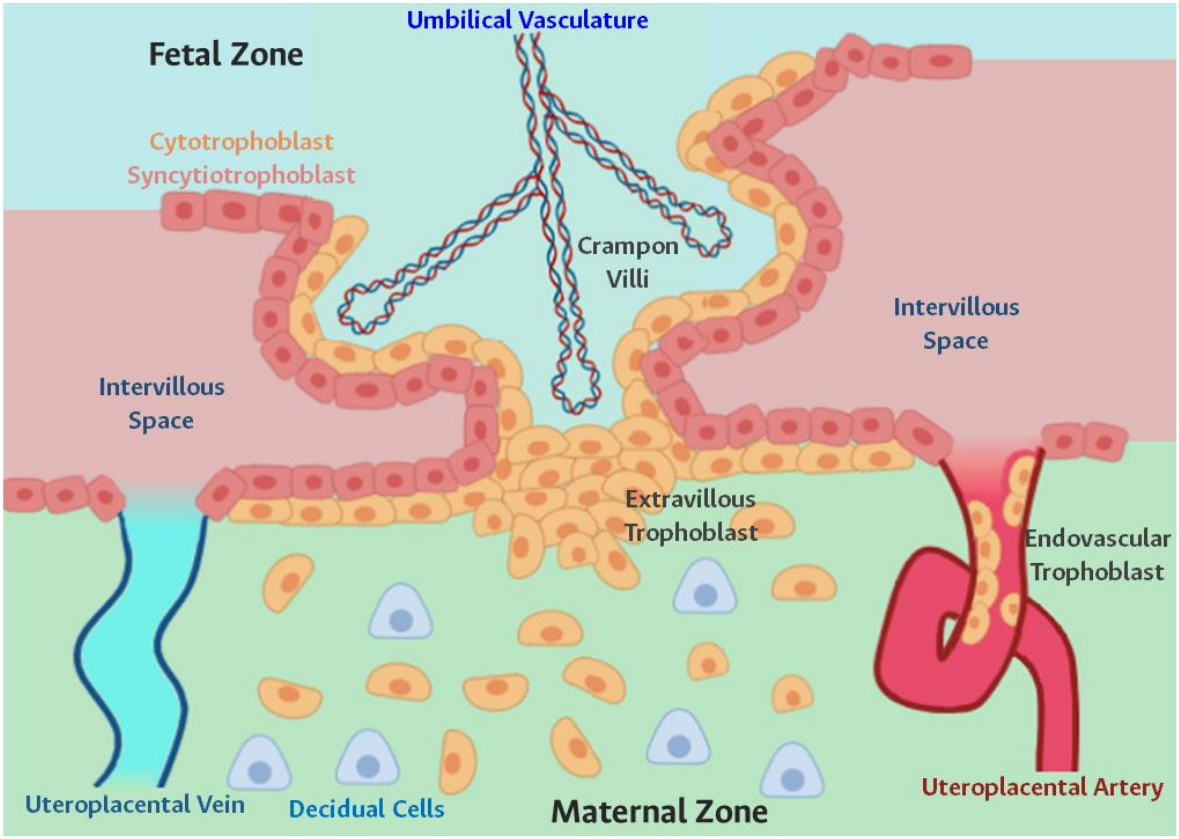


**Figure 1.** Types of functional populations after trophoblast differentiation

The invasion of endolymphatic trophoblast into the lymph vessels of the uterus has been described. After that, invasion of endoarterial trophoblasts into spiral arteries takes place, [11] enabling hemotrophic nutrition of the fetus starting with the second trimester of pregnancy. After dissolution of the plugs, the onset of maternal blood flow allows maternal blood to enter the intervillous space and oxygen concentrations rise up.[11,12] Failure of endovascular trophoblast invasion has profound effects on the oxygenation of the placenta. Interestingly, this does not lead to hypoxia, rather increased oxygen levels have been observed within the placenta in patients with a failure of spiral artery transformation. This finding adds important knowledge in better understanding pathological pregnancies as recurrent spontaneous abortions, fetal growth restriction and preeclampsia.[12,13]

### 2.2 Placental Epigenetics

DNA methylation is the best understood mechanism of epigenetic control as it has a fundamental role in diverse processes such as memory, aging, maintenance of the pluripotent potential of germ lines, maintenance of the somatic identity, tumor development and outstandingly, placental implantation and derived phenomena like embryonic development and tissue differentiation. This mechanism consists in the addition of a methyl group to position 5 of those cytosines forming cytosine-guanin dinucleotides (CpG sites). There are genomic regions with a high density of CpG sites called CpG islands (CGI), most of which collocate with promoter regions of many genes and whose hypermethylation prevents the access of the transcriptional machinery

 

through heterochromatin establishment, thus generating a decrease in gene expression. Regarding placental implantation, DNA methylation has been recognized as a modulator of several aspects of the placental invasive and proliferative phenotype promoting the expression of several genes, mediating the silencing of imprinted genes and it has also been related to several aspects of the outcome of pregnancy in diverse situations. Embryonic development depends on the correct function of the placenta for the exchange of nutrients and oxygen, the removal of waste products, besides protecting the embryo from the immune system of the mother and from environmental risks, as well as acting as major regulator of the intrauterine signaling environment, so that alterations in this delicate interplay may result in mortality and disease.

### 2.2.1 DNA Methylation in embryonic development

DNA methylation of gene promoter regions is probably the principal mechanism of modulation of the cellular function during development, permitting the silencing of genes in a tissue-specific manner. Considering that oocytes and spermatozoids are more "differentiated" cell types than the early embryo, when it reaches the morula stage, the early embryo suffers a reset of its methylome, allowing the potential activation of virtually all the genome (only some genes escape this demethylation process) and next, different promoters begin to be methylated in a specific manner according to diverse tissue differentiation requirements. For example, when the very first event of differentiation occurs, resulting cell populations in the blastocyst have completely different methylation landscapes: the inner cell mass that will form the fetus is hypermethylated while trophectoderm that will constitute the source of the different placental cell lineages remains hypomethylated, and these methylation landscapes are maintained throughout gestation.

## 2.3 Regulation of placental and trophoblast function by Growth Factors

A variety of growth factors, growth factor-binding proteins, proteoglycans and cytokines are implicated in trophoblast proliferation, migration and invasion.[1,14] Among them, we will focus in this review on two families of growth factors that have been demonstrated to be of pivotal importance in the control of human placentation and embryogenesis: the Insulin-like growth factor (IGF) family and the Transforming growth factor beta (TGF-β) axis.

### 2.3.1 The IGF axis and placenta

#### 2.3.1.1 The IGF signaling system and expression in placenta

The IGF family is organized in a complex regulatory network at the cellular and sub-cellular levels. In the human, the IGF system is essential in the development of the organism and maintenance of normal cellular function, both during fetal and postnatal life. In general, the IGF system consists of three ligands, IGF-1, IGF-2 and Insulin; three cell membrane receptors IGF-1R, IGF-2R and the Insulin receptor (IR); six high affinity IGF binding proteins, IGFBP-1 through -6, their specific proteases (IGFBP proteases) and membrane receptors (IGFBP-R).[15] IGF-1R and IR are members of the family of tyrosine kinase growth factor receptors and share high homology at the amino acid sequence level. The mature membrane

 

IGF-1R is a tetramer made of two α-chains and two β-chains with several disulfide bridges.[16] The extracellular α-subunits form the ligand binding domain and several lines of evidence suggest that the binding sites for IGF-1 and IGF-2 may be distinct.[17] IGF-1 and IGF-2 bind to IGF-1R with high affinity, however, ligand affinities may vary with cell type and experimental conditions. IGF-2 can also bind to the Insulin receptor isoform A (IR-A) with an affinity like that of insulin. IR-A is expressed in certain tumors and has a more mitogenic effect than the IR-B isoform, the latter having a more metabolic function.[18] The formation of hybrid receptors (IR/IGF-1R) in cells that co-express both receptors adds more complexity to the system and may play a role in receptor signalling in normal and abnormal tissues.[19] IGF-2 and, with a much lower affinity, IGF-1 can also bind to IGF-2R, which is a multifunctional single transmembrane glycoprotein, identical to the cation-independent mannose 6-phosphate receptor. It is composed of a large extra-cytoplasmic domain and a short cytoplasmic tail that lacks intrinsic cytoplasmic activity.[20]

IGF-1 and IGF-2 are important growth factors in fetal development. Both are synthesized in placenta and fetus with a considerable overlap in the location of both IGFs in the various placental cell types, in the mesenchymal cells such as macrophages and endothelial cells, with little change throughout gestation. However, there is a clear difference and developmental change in the trophoblast compartment. Whereas IGF-1 is present in syncytiotrophoblast and cytotrophoblast at all stages in gestation, IGF-2 is only found in cytotrophoblasts. It is unclear if the placenta derived IGFs serve local purposes by paracrine or autocrine regulation, or if they are secreted into the maternal or fetal circulation. IGF-binding proteins (IGFBPs) are key modulators of the ligand–receptor interaction. The six human IGFBPs described so far circulate in the plasma and bind IGFs with a higher affinity than the receptors. This interaction facilitates endocrine IGF transport and prolongs the half-life of circulating IGFBP-bound IGFs.[21]

The IGF-1R mRNA is expressed in all cell types of the placenta and receptors are localized in villous endothelium and stroma, trophoblast and decidua. The main IGF-1R expression site, however, is the basal membrane of the syncytiotrophoblast and the villous cytotrophoblasts.[22]

The activation of the IGF-1R by ligand binding leads to the activation of a complex signaling network through the two majors signaling pathways PI3K/Akt and Ras/Raf/MAPK.[23] Moreover, the IGF-1R also signals through the activation of Janus kinase (JAK) and Signal transducers and activators of transcription (STAT). This fact has important consequences for crosstalk between IGF-1R and cytokines signaling, because the JAK/STAT signaling system comprises a negative feed-back mechanism consisting of Suppressors of cytokine signaling (SOCS), enabling IGF-1R to block cytokines action.[24] Altered IGF signaling result in aberrant placental growth. It was reported that treatment of first trimester placental tissue with statins reduces IGF-mediated proliferation by inhibiting N-linked glycosylation of the IGF-1R and subsequent expression of the mature receptor at the placental cell surface.[25]

IGF-2 is one of the imprinted genes in mammals that are expressed from only one of the parental chromosomes and are

 

crucial for placental development and fetal growth. The IGF-2 is paternally expressed in the fetus and placenta and is a major regulator of the supply of maternal nutrients to the fetus.[26] Studies using animal models have demonstrated the functional importance of imprinting of H19, IGF-2 and IGF-2R genes during intrauterine development. However, the normal developmental patterns of imprinted genes expression in the human placenta are poorly understood.[27]

IGF-2R receptors perform diverse cellular functions related to lysosome biogenesis. The IGF-2R receptors recycle continuously between two cellular pools and at steady state most of them localize in endosomes.[20] The IGF-2R is expressed primarily on the maternal-facing microvillous membrane of the STB.[28] As the IGF-2R has no intrinsic kinase activity, it was suggested that the role of this receptor is to clear the extracellular IGF-2 concentrations and therefore, prevent excessive IGF-2 effects on the placenta. However, recent studies suggest that the IGF-2R also functions in signal transduction and may play an important role in tumour progression.[29,30] In experiments using the HTR-8/SVneo trophoblast cells, suitable as *in vitro* model of invasive EVT, showed that IGF-2 was able to stimulate EVT cell migration by signaling through IGF-2R independently of IGF-1R and involving signaling via inhibitory G proteins and the MAPK pathway.[31]

### 2.3.1.2 The IGF axis in the regulation of placental function

The IGF axis proteins play crucial roles in a wide variety of cellular processes in normal physiology and pathophysiology including growth, tumorigenesis, placental function and fetal growth. Many lines of evidence point to an important role for the IGF axis in embryonic and fetal growth in human pregnancy. IGF-1 and IGF-2 do not cross the placenta into the fetal circulation; however, they may be involved in placental growth. IGF-1 can be found in the intervillous space during pregnancy. The STB produces a variant of growth hormone (placental GH) that gradually replaces pituitary GH in maternal circulation and is thought to be responsible for the increase in maternal IGF-1 serum levels. An increase in maternal IGF-1 levels during pregnancy, with a rapid decrease after delivery indicates a significant placental influence. There is no major change in maternal IGF-2 levels throughout pregnancy.[32]

IGF-1 is important for fetal and postnatal development, but it also controls tissue homeostasis throughout life via regulation of cell proliferation and apoptosis. IGF-1 promotes CTB differentiation and IGF-1R is crucial for normal placenta function.[21] IGF-1 produced in the placenta, regulates transfer of nutrients across the placenta to the fetus, and thus, enhances fetal growth. IGF-1 is able to increase the expression of the glucose transporter GLUT1 at the basal membrane of SCTB, with significant effects on placental glucose transfer capacity and fetal circulating glucose.[33] There is also strong evidence for the IGF-1 involvement in the regulation of placental amino acids transporters,[34] and possibly fatty acid transporters.[35]

The bioavailability of IGFs is modulated by the IGFBPs, whose permissive or inhibitory effects are regulated in part by specific proteases.[36] IGFBP-1 is the main secretory product of the decidualized endometrium. IGFBP-1 modulates the metabolic effects of IGF-1 and IGF-2 and has been shown to increase the gelatinolytic activity of trophoblasts and

 

trophoblast invasiveness.[37] Persistently low IGFBP-1 in diabetic pregnancies is associated with relatively higher birth weight,[38] whereas decreased maternal serum IGF-1 levels were found in women who developed preeclampsia.[39]

Besides the role of the IGF axis in proliferation, migration an inhibition of apoptosis during placentation, many authors have shown that the IGF proteins stimulate EVT cell migration and enhance invasion of EVT cells to the maternal decidua. Irving and Lala[40] showed the role of cell surface integrins in the regulation of trophoblast migration. Furthermore, Hamilton et al[37] showed that trophoblast–derived IGF-2 and decidua-derived IGFBP-1 provide an autocrine/paracrine enhancement of trophoblast invasiveness. The migration stimulatory action of IGFBP-1 likely occurs by interaction with the RGD (Arg-Gly-Asp) binding domain of the α5β1 integrin, leading to stimulation of the MAPK pathway.[41] In a previous work in our laboratory using the HTR-8/SVneo cells, it was found that IGF-2 was able to stimulate the activity MMP-2 and MMP-9 metalloproteases, and in this way, enhancing EVT cell invasiveness.[42]

### 2.3.2 The TGF-β axis and placenta

#### 2.3.2.1 TGF-β signaling system

The transforming growth factor beta (TGF-β) is a cytokine, secreted as homodimeric glycoprotein by diverse tissues. The TGF-β signaling system involves three ligand isoforms, TGF-β1, -β2 and -β3, which signal through their binding to the hetero-tetrameric complex formed by their serine/threonine kinase receptors TGF-β receptor TβRII/TβRI, an interaction that many times is mediated by an initial binding of the ligand to the β-glycan co-receptor TβRIII, which forms complexes with the TβRII/TβRI receptors and mediates the presentation of the ligand to them. Activated TβRI (ALK5) phosphorylates intracellular effector Smad proteins. There are three functional classes of Smad: receptor-activated R-Smad (Smad1, 2, 3, 5, and 8), co-mediator or co-Smad (Smad4), and inhibitory or I-Smad (Smad6 and 7). The R-Smads are phosphorylated and activated by the TβRI, and after undergoing homodimerization they bind to the co-Smad to be translocated to the nucleus as a trimeric complex that will bind to transcriptional co-activators or co-repressors to regulate the expression of genes. Whereas, I-Smads competitively inhibit the phosphorylation of R-Smads by TβRI and its union to co-Smad, and recruit phosphatases that dephosphorylate and inactivate the receptor complex.[43,44]

TGF-β affects a wide variety of cellular processes during embryonic development and homeostasis of adult tissues. Current research is focused on the mechanisms that regulate Smad activity to generate cell-type-specific and context-dependent transcriptional programs.[45] TGF-β has been shown to inhibit proliferation and induce apoptosis in various cell types, acting as a tumor suppressor in early stages of tumorigenesis; although, on the other hand, many tumors overexpress TGF-β, whose autocrine actions promote cell invasion and metastasis in advanced tumor cells.[43] During pregnancy, TGF-β is a powerful regulator of the cellular functions of the villous and extra villous trophoblast, produced mainly by the maternal decidua and to a lesser extent by the placenta (mainly ST) and uterine Natural Killer (uNK) cells. It was one of the first identified regulators in the differentiation of the invasive trophoblast, and in addition to retarding the differentiation of the villous trophoblast, it inhibits the invasive process and reduces the proliferation of the

 

trophoblast of first trimester of pregnancy.[37]

#### 2.3.2.2 Expression of TGF-β in placenta and circulating levels in normal pregnancy

TGF-β is localized at the human fetal-maternal interface and is expressed by both maternal decidua and chorionic villi. Several reports agree that villous trophoblast express the TGF-β1/2, but its intensity vary throughout pregnancy. TGF-β was present in the cytoplasm of syncytiotrophoblast in both early and term placenta, but most intense levels were found in the first trimester trophoblast syncytial sprouts known to be an early stage in the development of placental villi.[46] Similarly, TGF-β1/2 was found more intense in the chorionic villi at first trimester and decreased in full-term villi, with alike levels in the cytoplasm of syncytiotrophoblast and cytotrophoblast.[47] A different result was showed by Karmakar and Das,[48] where TGF-β1 was predominantly expressed in term placental villi (>36 weeks). Graham and Lala observed that TGF-β is secreted in a latent form by decidua,[49] acting as a paracrine regulator. They reported that TGF-β presence was intense in the extracellular matrix (ECM) of the first trimester decidua and cytoplasm of term decidual cells, moderate on syncytiotrophoblast cell cytoplasm and the ECM in the core of the chorionic villi of both first-trimester and term placentas, and strong in the cytoplasm of term cytotrophoblastic shell.[50]

Unlike to maternal or circulating IGF-1 levels,[23,32] which increase during pregnancy, the circulating TGF-β levels increases only during the first trimester of pregnancy.[51] A cross-sectional study showed that maternal serum TGF-β1 levels were higher during all stages of pregnancy than those in normal healthy nonpregnant adults, suggesting that TGF-β1 levels rise during pregnancy and increased circulating levels come from the fetoplacental interface.[52] The mean TGF-β1 levels change over pregnancy, arising until 52.7 ± 5.5 ng/mL at 10-week, and then fell until full term, being significantly through to 26-week gestation, to 46.8 ± 5.5 ng/mL at 20-week gestation and to 40.5 ± 3.8 ng/mL at 26-week gestation, and without statistically significance from 32 to 38 weeks.[52]

#### 2.3.2.3 TGF-β regulates the trophoblast function and protein expression

TGF-β is one of several key regulators that control the placental function. Several studies have shown that it inhibits the proliferation, migration and invasion of the trophoblast and control differentiation and hormone production through multiple mechanisms. Due to its actions, TGF-β is considered a tumor suppressor in normal placenta.

Graham et. al. showed that TGF-β1/2 inhibit proliferation of first-trimester trophoblast cells; after 3-day-culture TGF-β1 stimulated formation of multinucleated cells by first trimester and term trophoblast cells, and found that endogenous TGF-β regulate the proliferation and differentiation of the trophoblast.[53] TGF-β1 inhibited cytotrophoblast differentiation, specifically EGF-induced syncytial formation, and secretion of human chorionic gonadotropin (hCG) and human placental lactogen (PL), in pure cytotrophoblast.[54] TGF-β inhibited production of hCG and aromatase activity, indicative of estrogen production, in purified placental trophoblasts.[55] TGF-β1 inhibited progesterone and estradiol production in trophoblast cells, possibly

 

by cholesterol transport and P450arom, respectively.[56] TGF-β reduce the hCG secretion in trophoblast types, including early and term trophoblasts,[57] and choriocarcinoma cells, but without showing a consistent effect on the cell cycle mRNA of any of the cell types.[58]

Several studies prove that TGF-β inhibits trophoblast invasion. Decidual-derived TGF-β, and to a lesser extent trophoblast-derived, controls first trimester trophoblast invasion, after being activated from its latent form by trophoblast-derived proteinases, and TGF-β induces expression of tissue inhibitor of metalloproteinases (TIMP-1), decreasing collagenase type IV activity and preventing ECM degradation and invasion.[49] Matrix metalloproteinase 9 (MMP-9) and urokinase-type plasminogen activator (uPA) are key proteases, required for trophoblast invasion. TGF-β reduces the secretion and activity of uPA, and up-regulates the expression and secretion of its inhibitor PAI-1 and -2 and the tissue inhibitor of metalloproteases TIMP-1 and -2, which inhibit the ECM degradation performed by the MMPs.[59,60] TGF-β reduced the expression and secretion of MMP-9 in first trimester primary trophoblast,[57] in explants of first trimester placenta,[61] in the choriocarcinoma cell line JEG-3,[60] and in the trophoblast cell line NPC.[62] On the contrary, other studies have shown that TGF-β stimulates expression and secretion of MMP-9 at short time (8 and 12 h) in primary cultures of first trimester cytotrophoblast.[63] In our group, we have seen that TGF-β1 affect in a dual fashion the MMP-9 and uPA (PLAU) expression in immortalized trophoblast HTR-8/SVneo cells. After TGF-β1 exposure, the mRNA levels of MMP-9 and PLAU vary over time, and 0,5% fetal bovine serum modified the nature the effects of TGF-b on uPA expression, from negative without serum to positive with it, showing opposite effects on MMP-9 expression.[64]

TGF-β also reduces migration, a key step in invasion process. Exogenous TGF-β increased fetal fibronectin (fFN),[57] upregulated integrin expression and reduced migration, conceivably due to increased cell adhesiveness to ECM.[65] TGF-β1 increased ezrin and E-cadherin expression, up-regulating the cell-to-cell adhesion, while reducing cell-to-matrix interaction, and these are associated with reduced invasiveness, along with an altered cellular morphology.[66] In a non-transformed cell-line representative of normal human trophoblast (NPC), TGF-β promoted intercellular adhesion, while inhibited cell invasion through repressing the expression and secretion of MMP-9 and up-regulated E-cadherin and β-catenin, expression involving Smad2 phosphorylation.[62] By MS/MS-based proteomic analysis, we found novel TGF-β-regulated proteins suggesting new regulatory effects in addition to the classical ones. In HTR-8/SVneo cells, TGF-β1 increased proteins levels, including: Phosphoribosylformyl-glycinamidine synthase (PFAS), CTP synthase 1 (PYRG1), Neutral alpha-glucosidase AB (GANAB), Kinesin-like protein (KIF11), Gelsolin, Serrate RNA effector molecule homolog (SRRT), LIM domain and actin-binding protein 1 (LIMA1), Talin (TLN1), Vinculin (VCL), and Annexin A2 (ANXA2). By other side, Acidic leucine-rich nuclear phosphoprotein 32 family member A (AN32A) protein and WD repeat-containing protein 1 (WDR1) were reduced.[67] The biological implications of these proteins and significance of protein changes is currently under assessment, but considering their reporter function, these results suggest that TGF-β may stabilize

 

the focal adhesion complex, by up-regulation of TLN1, VCL, LIMA1, and ANXA2 and may avoid actin filament disassembly and recycling by down-regulation of WDR1, and up-regulation of LIMA1, as a mechanism to inhibit cell migration and thus the invasion process. This agree with previous reports of reduced migration and increased cell adhesion.[57,62,65,66]

### 2.3.3. Additional pathways regulating the trophoblast

Besides the role of growth factors activating signaling pathways such as MAPK and PI3K/ Akt during development and in some complications during the progression of pregnancy, there are many signals whose regulation could contribute significantly to the correct implantation and placentation. This is the case of the Wnt signaling pathway activation, which is associated with proliferation, migration, and invasion processes since a high expression of the ligands of this signaling pathway and its receptors was found in the first-trimester cytotrophoblast.[68] Wnt signaling pathway, where canonical activation allows the translocation of β-catenin to the nucleus, has been shown to have proliferative activity due to the presence of high levels of β-catenin in the extravillous cytotrophoblast,[69] dependent on the presence of estrogens.

The non-canonical Wnt signaling pathway independent of β-catenin, is a pathway with high potential for study in trophoblast due to the activation of small GTPases that are connected to MAPK and PI3K/Akt pathways, presumably to promote the invasive and migratory phenotype of trophoblast cells.[70]

Although Notch signaling pathway has been shown not to have a significant presence in first-trimester placental tissue,[71] its inhibition reduces invasive processes in trophoblast cells. Furthermore, exposure to harmful substances such as perfluoroalkyl substances alters the Notch signaling pathway and could lead to problems during placental development, mainly due to inadequate vascularization.[72]

Crosstalk between the Notch signaling pathway and the Wnt signaling pathway has been reported in several biological models, through the interaction between cleaved Notch proteins and β-catenin, promoting translocation to the nucleus and facilitating gene transcription with proliferative action.[73] However, such crosstalk is not yet evident in placental tissue and presents itself as a promising field of investigation for the understanding of implantation processes.

Another widely described signaling pathway is the JAK/STAT pathway, which in trophoblast is activated through the leukaemia inhibitory factor (LIF) and whose role is essential in the implantation of the blastocyst.[74] Blocking the activity of STAT proteins, such as STAT3 and STAT5, could lead to decrease invasion of trophoblasts.[70] STAT3 activation in trophoblast and choriocarcinoma cells have shown significant crosstalk in allowing ERK1/2 activation facilitating *in vitro* invasive processes.[75]

The studies mentioned above show a confluence of several signaling pathways that would be acting together under normal conditions of the embryonic and placental development, and whose alterations would be contributing to the acquisition of unfavorable phenotypes for the development of pregnancy.

 

### 2.3.4 Role of Metalloproteases in the regulation of placental invasiveness

The metalloproteases have the ability to break down the proteins of the extracellular matrix (ECM) making it possible for cells to renew it and transform it. Regulation of the proteolytic activity of the MMPs by post-translational modifications, modulation of gene expression and co-localization of tissue specific enzyme inhibitors is normally high. Tissue inhibitors of metalloproteases (TIMPs) reversibly arrest the enzymatic activity of MMPs in a 1:1 stoichiometric fashion in the tissue.[76],[77] MMPs catalytic domain is bound by TIMPs through the N-terminal region inhibiting their activity and proMMPs C-terminal hemopexin domain form a stable bond via the C-terminal region of TIMPs.[77] Nevertheless, the degree of MMP inhibition varies between each TIMP; TIMP-1 strongly inhibits MMP-9 but poorly inhibits MT1-MMP, MT3-MMP, MT5-MMP, and MMP-19, and TIMP-2 strongly inhibits MMP-2 and can inhibit other MMP members. TIMP-3 inhibits pro-MMP-2 activation while TIMP-4 forms a TIMP-4-pro-MMP-2-MT1-MMP complex, leading to inhibit the activation of pro-MMP-2 via inhibition of MT1-MMP.[78] The balance between the expression of MMPs and the expression of TIMPs is then important in the invasive capability of the trophoblast cell, and has a significant role in the transformation of normal placenta into cancerous tissue. Comparative studies on the expression in normal first-trimester placenta, partial and complete mole, choriocarcinoma, and placental site trophoblastic tumour of MMP-7, CD147, MMP-14, MMP-21, MMP-28, TIMP-3 and TIMP-4 showed that MMP-21 is overexpressed in choriocarcinoma compared to normal placenta, partial mole and complete mole; MMP-28 is overexpressed in choriocarcinoma compared to normal placenta and choriocarcinoma has significantly less expression of TIMP-3 and TIMP-4.[76] This showed that decreased expression of TIMPs and increased expression of MMPs in choriocarcinoma might contribute to the invasiveness of this pathology. Augmented CD147 expression may also cause higher MMP-1 and MMP-2 levels in choriocarcinoma and by this means increase its invasiveness.

As said before, cytokines also have an important role in trophoblast invasion. A variety of chemokines and cytokines are secreted and expressed by trophoblast cells to sustain the maternal–fetal tolerance during pregnancy. Interlukin 35 (IL-35) is constitutively expressed by human first-trimester trophoblast and some studies have shown the inhibition in an IL-35-dependant manner of the proliferation of human naïve the conventional of T cells ($T_{conv}$ cells) and convert suppressed $T_{conv}$ cells into $iT_R35$ by trophoblast cells. The balance between anti and proinflammatory cytokines is important for correct placentation. Studies showed that a cocktail of proinflammatory cytokines (tumor necrosis factor-α, IL-1β and interferon-γ) inhibited MMP-2 activity in JEG-3 cells and activated the PKR-like ER kinase (PERK)-eukaryotic translation initiation factor 2A (EIF2A). This showed that trophoblast invasion might be modulated by proinflammatory cytokines through ER stress pathway which regulates MMP-2 expression at both the transcriptional and translational levels.[79] The effect of chemokines is also relevant in trophoblast invasion, rhCXCL6 significantly decreases the migration of HTR-8/SVneo cells and the invasion ability of primary trophoblast cells and HTR-8/SVneo cells in a dose-dependent manner. Likewise, a significant

 

suppression in pro-MMP-2 levels in the supernatant of rhCXCL6-treated HTR-8/SVneo cells was found but levels of pro-MMP-9 were not significantly different in supernatants of rhCXCL6-treated HTR-8/SVneo cells while active MMP-9 and MMP-2 were not detected.[80]

MMPs also regulate the bioavailability of growth factors, such as EGF or IGF-2, in cell surroundings. The ECM proteins are able to bind a great variety of soluble growth factors thereby regulating their bioavailability and integrating multivalent signals to the cell in a timely and spatially organized manner, so on the ability of MMPs to break these proteins also regulates the binding of ligands to the trophoblast cells. This is the case for MMP-3 and MMP-9 which are able to proteolyze IGFBP-1.[8] This protein is involved in the control of trophoblast migration but its precise role remains controversial as there are reports of enhanced or restrained trophoblast migration mediated by IGFBP-1.[81]

Serine protease uPA, is another important molecule, which promotes matrix degradation and is required for activation of certain MMPs by the invasive extravillous trophoblast of the human placenta.[82,83] These cells require diverse molecular mechanisms for their invasive function, with multiple steps including: binding to ECM components, degradation of the ECM by production of MMPs, particularly -2 and -9,[84] and migration through the degraded ECM in the presence of Asn-linked complex type oligosaccharides as well as α5β1 integrin on the cell surface.[4]

### 2.3.5 Regulatory effects of nutrition on the placenta

During pregnancy course, the oxygen levels change dramatically, starting with a low-oxygen environment that is considered as hypoxic. Remarkably, the placental metabolism is mainly glycolytic, without affecting the energy of a highly proliferative trophoblast. These conditions apparently are required to an appropriate placental development, as show findings in pathological pregnancies.[85] The nutrition features also change, from histotrophic to haemotrophic, with profound impact in nutrients and oxygen levels and trophoblast physiology.[86] Likewise, nutrition also exert regulatory effects on the placenta. A proteomic differential study showed that serum depletion on the first trimester human immortalized trophoblast cell line, HTR-8/SVneo induces specific changes in protein expression concordant with main cell metabolic adaptations and the epithelial-to-mesenchymal transition (EMT), resembling the progression to a malignant phenotype. Specifically, we observed downregulation of keratin 8, and upregulation of vimentin, the glycolytic enzymes enolase and pyruvate kinase (PKM2) and tumor progression-related inosine-5'-monophosphate dehydrogenase 2 (IMPDH2) enzyme in serum-depleted proteome. The proteins regulated by total serum depletion, but not affected by growth in 0.5 % serum, are members of the glycolytic and nucleotide metabolic pathways and EMT, suggesting an adaptive switch characteristic of malignant cells.[87] As we showed, serum affects the trophoblastic cellular context and the response to a factor as TGF-β. [87,88]

## 3. Pathologies of the Placenta

### 3.1 Gestational Trophoblastic Disease

Gestational trophoblastic disease (GTD) comprises a group of pregnancy-related

 

pathologies characterized by abnormal trophoblast growth and invasion. GTD includes pre-malignant complete (CHM) and partial (PHM) hydatidiform moles and malignant lesions such as invasive mole, placental site trophoblastic tumor (PSTT) and choriocarcinoma.[88] Hydatidiform moles are the only pathologies of the group that can be recurrent in the same patient and indicate a genetic predisposition.

Three situations could explain its origin.[89]
1. Expulsion of the female pronucleus at the time of fertilization, followed by endoreplication of the male pronucleus leading to a complete hydatidiform mole (CHM).
2. A triploid zygote fertilized by 2 spermatozoa leading to a partial hydatidiform mole (PHM) 3 point apart. A nutritional defect during the differentiation of the oocytes or deterioration of the oxygen pressure during the first trimester of pregnancy (HM).

Complete moles are more frequently invasive than partial moles. After uterine evacuation 10-20% of complete moles and up to 5% of partial moles undergo malignant transformation, into invasive mole, choriocarcinoma and PSTT. Choriocarcinoma is an extremely aggressive tumor that rapidly spreads giving up to metastases in lung, brain, kidney and liver.[90]

It is well known that deregulation of the production of hCG is associated with hydatidiform mole. Particularly the hyperglycosylated form (HhCG) is increased by 5% in complete moles and by 4% in partial moles, compared to values for a normal pregnancy. The transition to an invasive tumor is associated with a significant increase in HhCG, up to 30-35% for an invasive mole and 100% for choriocarcinoma.[91] Therefore, the measurement of the β-hCG in serum and urine, in concert with clinical and radiologic tests are useful for making the diagnosis of GTD and follow-up of the effect of therapy.

The risk of CHM is higher than PHM, and it has been seen higher frequencies of molar pregnancies at the upper and lower extremes of maternal age.[92] According to the American Cancer Society,[93] the risk of complete molar pregnancy is high in women over age 35 years and younger than 20 years. The risk is even higher over age 45 years. Age is less likely to be a factor for partial hydatidiform mole. For choriocarcinoma, the risk is low before age 25 years and then increases with age after menopause.

In developed countries, the incidence of GTD is 1-3 per 1000 pregnancies, whereas the frequency in developing countries varies considerably, [94,95] and could be 10 times more likely in Asian and African countries.[89] In developing countries, due to the delay in diagnosis of HM, it is common that patients develop clinical complications with adverse outcomes. The early diagnosis of HM and prompt uterine evacuation are the only ways to prevent those outcomes. After uterine evacuation, patients need to be carefully followed to prevent the risk of development a trophoblastic neoplasia.

### 3.1.1 The IGF axis and the pathogenesis of GTD

The pathogenesis of GTD and its malignant change are still the subject of study. Several risk factors have been suggested, including maternal age, ethnicity, contraceptives, blood group and maternal nutritional state.[89] Among them, race/ethnicity and a previous occurrence of a CHM appear to have a clear relationship

 

with the incidence of GTD.[96] Several hypotheses have been suggested to explain the propensity to malignancy of CHM. Due to the complete absence of maternal genome in CHM, the function of some of the paternally expressed genes has been studied in relation to hydatidiform mole and choriocarcinoma development.[97,98] Among these, IGF-2 and H19 tightly linked on human chromosome 11, are of special interest because of their reciprocal imprinting and possible association with certain congenital abnormalities. Loss of imprinting in IGF-2 gene locus has been shown in hydatidiform mole concomitant to increased mRNA levels, deregulation of IGF-2 promoter usage and altered expression of IGF-2 and H19.[98,99]

Most of the actions of IGF-1 and IGF-2 in the placenta are mediated through binding to the IGF-1R, that is expressed in syncytiotrophoblast and the villous cytotrophoblasts. However, conflicting reports exist on how the IGF-2 regulates the behavior of choriocarcinoma cells. We found that IGF-2 signaling in JEG-3 choriocarcinoma cells, is initiated mainly by the activation of the Insulin receptor (IR) followed by downstream activation of Akt/ERK and enhancement of invasion. In contrast, IGF-1 activates the same pathway but through activation of the IGF-1R.[100] In addition, IGF-2 in JEG-3 choriocarcinoma cells, induces the expression of the MMP-9 metalloprotease through the activation of PI3K/Akt pathway.[101]

The IGF-2R/Mannose-6 phosphate receptor is expressed primarily in STB at term, however, its role in regulating as it was previously described.

Recent studies have shown that the receptor also functions in signal transduction [20,29] and is able to stimulate cell migration in an *in vitro* model of extravillous trophoblast.[30]

Several studies show that both IGF-2 and IGF-2R are associated in fetal growth restriction (FGR),[102-105] therefore studying the effects and processes modulated by these factors would help with the understanding of this pathology. The IGF-2 ligand shows differential affinities for the IGF family receptors interacting and mediating signaling pathways in the intracellular level.[106] This factor's mitogenic and invasive actions are mediated by the insulin-like growth factor type 1 receptor (IGF-1R),[107] consequently researchers use an analogous peptide Leu$^{27}$IGF-2 that bind exclusively IGF-2R, becoming its agonist. This peptide is soluble, doesn't interact with IGF binding proteins (IGFBPs) and shows low affinity for IGF-1R and the insulin receptor (IR).[30,108-110]

Leu$^{27}$IGF-2 subcutaneous constant infusion in mice with FGR, showed fetal growth recovery with conditions similar to normal, and infusion in mice with normal pregnancy reduced fetus with weight lower than fifth percentile of the studied population.[111] In early pig gestation, Leu$^{27}$IGF-2 infusion increased proportion and volume of the placental labyrinthine zone and the syncytiotrophoblast surface area, resulting in greater placental glucose and aminoacid transport to the fetus.[112] This shows the relevance of the IGF-2/IGF-2R interaction in fetal and placental development.

These results along with other studies, show that IGF-2R activates one or a variety of intracellular signaling pathways by transactivating sphingosine-1-phosphate G-protein coupled receptors,[29] or by its own coupling to a G protein.[113-115] Then, this ligand activates downstream

 

effectors as the protein kinase C and the Calcium/calmodulin-dependent protein kinase (CAMK2).[116,117] Additionally, cardiomyocyte cells induced with Leu[27]IGF-2, show an increase in apoptosis by activation of the previously mentioned effectors.[118]

Studies with HTR-8/SVneo induced with Leu[27]IGF-2, show a 2.5-fold increase in migration rate.[30] Meanwhile, IGF-2R knockdown BeWo cell line, derived from choriocarcinoma, induced with the analogous peptide reveals that IGF-2R activated by IGF-2 participates in the inhibition of apoptosis induced by nutritional deprivation, without affecting cell proliferation.[119] Nevertheless, in placenta explants IGF-2R knockdown shows that IGF-2R promotes cell proliferation, suggesting that in trophoblastic cells signaling mediated by IGF-2R would be involved in GTD development regulation processes.

The ability of IGF-2R to enhance cell survival by inhibiting apoptosis by serum starvation was demonstrated in BeWo choriocarcinoma cells and this effect was lost following IGF-2R knockdown. Reduced IGF-2R expression increases the bioavailability of IGF-2 and enhances survival signaling via the IGF-1R.[119] This finding may provide a basis to understand the effects of elevated IGF-2 in the induction of cell survival in trophoblast tumorigenesis and malignancy.

As already mentioned, molar pregnancy is characterized by the high levels of IGF-2, both in tissue and plasma, suggesting its potential in the early diagnosis of the disease.

### 3.1.2 The TGF-β axis and the pathogenesis of GTD

Some studies present diverse alterations of TGF-β signaling system expression and TGF-β serum levels in GTD, with a distinctive alteration according to TGF-β1/2 and TGF-β3. TGF-β3 mRNA was expressed in complete hydatidiform mole (CHM), normal first-trimester villi, the normal term placenta (after vaginal/abdominal deliver) and the preeclamptic placenta at term, while TGF-β1 and TGF-β2 mRNA were not detected; and TGF-β3 expression was higher in CMH than normal first-trimester villi (the expression levels of TGF-β3 in the preeclamptic placenta and the normal placenta after cesarean birth were higher than in the placenta after vaginal delivery).[120] Another study of partial hydatidiform moles (PHM), complete hydatidiform moles, and choriocarcinoma, using nonhydropic spontaneous abortions as controls, concurs with TGF-β3 expression in all groups with the highest level in CHM and choriocarcinoma, being stronger in complete moles than that of choriocarcinomas although difference was not significant, and for TGF-β1 expression was highest in controls, and reduced in PHM, CHM and choriocarcinoma, the last with a minimum value.[121] Summarizing, TGF-β1/2 expression is downregulated while for TGF-β3 is up-regulated in GTD.

A different behavior presents the circulating levels. Serum level of TGF-β1 was found significantly higher in GTD patients (20.29 +/- 10.68 pg/ml with 95% CI of 18.10-22.48 pg/ml), grouping 55 complete moles, 32 persistent moles, and 8 choriocarcinoma, compared with pregnant controls (10.26 +/- 11.84 pg/ml with 95% CI of 5.75-14.76 pg/ml) and non-pregnant controls (7.27 +/- 9.61 pg/ml with 95% CI of 3.01-11.53 pg/ml) ($P < 0.001$).[122]

However, a systematic study of the expression of TGF-β signaling proteins

 

(TβRI, TβRII, Smad2/3, and Smad4) reveals differences in TGF-β1/2 levels and signaling proteins between molar lesions and choriocarcinoma. In general, these proteins are altered in GTD, displaying a different profile between moles and choriocarcinoma (132 cases, including 51 normal placenta (20 first trimester, 11 second trimester, and 20 third trimester) and 81 gestational trophoblastic diseases (17 choriocarcinoma, and 64 hydatidiform moles: 39 complete, 6 partial, and 19 invasive)).[47] Except choriocarcinoma, TGF-β1/2 expression was upregulated in GTD, and complete mole had a higher protein level than in normal placenta, but in choriocarcinoma the expression was decreased, lower than in complete and invasive moles. Except Smad2/3, expression of the TGF-β signaling proteins, TβRI, TβRII and Smad4, was significantly higher in various moles than normal trophoblast, but all evaluated TGF-β signaling proteins were significantly downregulated in choriocarcinoma, compared to moles. Remarkably, TβRI and Smad2/3, whose levels were lower in choriocarcinoma than normal villous trophoblast (TβRI: $p<0.025$, Smad2/3: $p<0.001$). These findings suggest that the TGF-β signaling pathway is functionally enhanced in molar lesions and inactive in choriocarcinomas.[47]

TβRI and Smad2/3 are essential mediators of the TGF-β signaling pathway, and its low level in choriocarcinoma could explain the reported resistance of choriocarcinoma to the anti-proliferative and anti-invasive effects of TGF-β.[59] Additional studies concur with the loss of Smad3 expression in choriocarcinoma cells,[123] and prove that after Smad3 reconstitution in choriocarcinoma cells these respond to TGF-β by up-regulating PAI-1,[124] and TIMP-1,[125] which are implied in control of invasion process.

The biological effects also depend on the TGF-β isoform. A study showed that all TGF-β isoforms decreased proliferation of HRP-1 cells, derived from midgestation chorioallantoic placental explants of the outbred Holtzman rat, in a dose-dependent manner, whereas only TGF-β2 reduced proliferation of RCHO-1 rat choriocarcinoma cells, being resistant to growth-suppressive effect of TGF-β1 and β3,[126] suggesting a differential isoform-based response of choriocarcinoma cells.

These studies reveal that the expression of TGF-β signaling proteins is dynamic and dual over the range of normal pregnancy, moles and choriocarcinoma, raising and falling. Considering the regulatory actions of TGF-β, those alterations could have a profound impact in the pathogenesis and progression of GTD. The reduced expression of TGF-β signaling proteins on choriocarcinoma may imply a loss of response to negative regulatory action of TGF-β, contributing to malignant progression and choriocarcinoma invasiveness. Despite it is not clear the functional implications of increased TGF-β signaling molecules in moles, these allow to differentiate the moles against choriocarcinoma and is a distinctive molecular feature in malignant progression. Before an irreversible malignization to choriocarcinoma, the moles may respond to regulatory stimuli, as those of TGF-β, nonetheless these have several mitogenic signaling pathways activated, as those of IGFs, which determine its pathological phenotype. The later according to the fact that biological regulation is carried on by circuits of signaling pathways, where multiple signals are integrated inside the cell, and the output and cellular response depend on the balance of these contrary signals. Studies comparing several signaling

 

molecules between invasive moles and choriocarcinoma may be interesting and would allow to understand the malignant progression and loss of response to negative regulatory signals.

Despite choriocarcinoma cells are refractory to some regulatory actions of TGF-β, they still can respond to other effects. In studies with the choriocarcinoma cell line JEG-3, TGF-β markedly decreased the secretion of hCG from choriocarcinoma JEG-3 cells, although mRNA levels were not markedly altered,[58] and reduced the expression and secretion of MMP-9,[60] which suggest a partial response to anti-invasive actions of TGF-β. However, the scenario is much more complex: while in placentae the TGF-β act as a negative regulator, in some tumoral models it acts as a positive regulator of proliferation and invasion.

**3.1.2.1 Actions of TGF as dual factor and context-determinants**

TGF-β could act as either a tumor suppressor or a tumor promoter in a context-dependent way. At early stages of tumorigenesis, TGF-beta may act as a suppressor, by inhibiting cell cycle progression and promoting apoptosis; although, in advanced malignancy it promotes tumor progression by enhancing migration, invasion, survival and metastasis of the tumor cells.[127]

In some models, different TGF-β isoforms could increase the invasiveness of choriocarcinoma cells. Lafontaine et. al. showed that TGF-β2 and TGF-β3 increased the invasive capability of placenta derived HRP-1 cells, and all TGF-β isoforms increased the invasiveness of choriocarcinoma RCHO-1 cells.[126] In choriocarcinoma JEG-3 cells, TGF-β1 promoted the invasiveness depending on the downregulation of TβRI, TβRII, Smad4 and the upregulation of MMP-9 and TIMP-1, but with a MMP-9/TIMP-1 ratio higher than 1 which allows invasiveness, suggesting that a limited TGF-β1/Smad4 signal propagation may promote the tumoral invasiveness.[128] Similar to hypoxia effects, TGF-β3 enhanced the amount of intracellular cysteine-rich 61 (CYR61, CCN1) and nephroblastoma overexpressed (NOV, CCN3) proteins and enhanced the secretion of CYR61 under hypoxic conditions in first-trimester placental explants and in JEG3 choriocarcinoma cells, and both CCN proteins increased migration and invasion of JEG3 cells.[129]

These opposing effects of TGF-β depend on intra and extracellular context-determinants and may result from distinct epigenomes and crosstalk with other pathways.

TGF-β can act through Smad-independent pathways, including MAPK pathway. In cells lacking endogenous Smad3 as JEG-3 choriocarcinoma cells, TGF-β stimulated an early activation of the small GTPase RhoA and RhoB, Smad2/3-independent and involving Src and the Guanine Nucleotide Exchange Factor Vav2 [130]. When Smad3 was overexpressed, a TGF-β-induced transcriptional up-regulation of the RhoB gene was restored, revealing a novel mechanism of cross-talk between the classical TGF-β/Smad pathway and Rho GTPases.[131] In a study, where JEG-3 cells unusually expressed Smad3, TGF-β activated Smad3 and induced Smad3 translocation into the nucleus, promoted p38 and phospho-p38 protein levels, TβRI inhibition suppressed activation of p38 MAPK signaling pathway while p38 MAPK inhibition attenuated TGF-β1-induced Smad3 expression and suppressed the activation of Smad3, suggesting that

 

TGF-β can induce the activation of p38 MAPK.[132] Blockade of the TGF-β and p38 MAPK pathways attenuated the expression of Smad3, TβRI and TβRII, revealing that p38 MAPK contributes to the TGF-β pathway,[133] showing a crosstalk between p38 and Smad3 through TGF-β1 in JEG-3 cells. Additional to isoform-specific effects, each TGF-β isoform activates distinct pathways. In HRP-1 and RCHO-1 cells, ERK, MAPK14 (p38 MAPK), or Smad pathways were activated by TGF-β in an isoform-specific manner and MTT proliferation assays revealed that ERK pathway is partially implicated in TGF-β3 - reduced HRP-1 cell proliferation.[126] Based on available evidence, we hypothesized that distinct and additional actions of TGF-β may be executed through a crosstalk with Smad-independent pathways. In moles and choriocarcinoma, the epigenome status could determine the context-specific effects of TGF-β. In breast cancer, distinct epigenomes of breast-tumor-initiating cells (BTICs) directs transcriptional programs, where cell-type-specific patterns of DNA and histone modifications provide a modulatory layer by determining accessibility of genes to regulation by TGF-β/Smad3.[134]

#### 3.1.2.2 TGF- β and DNA methylation

The intimate crosstalk between DNA methylation and activity and operation of many signaling and molecular mechanisms has been described in depth for several processes including cancer and pregnancy.[135] In this context, it has been demonstrated that TGF-β mediates the epithelial to mesenchymal transition in ovarian cancer,[136] that it plays an important role in esophageal cancer through DNA methylation,[137] and that it plays major and diverse roles in breast cancer.[138] Furthermore, it has been reported that almost all genes with differential methylation are related to TGF-β in prostate cancer.[139] Regarding gestational diseases, connections between DNA methylation and TGF- β function has been mainly described in preeclampsia. In this condition, members of TGF-β pathway showed general hypomethylation levels, which were related to augmented expression of cytokines promoting this condition, suggesting that alterations in the methylation landscape are driving factors operating at the basis of preeclampsia origin through destabilization of TGF-β signaling.

### 3.2 Altered placental DNA methylation and clinical complications

Several studies describe alterations in the methylation patterns during development and as a consequence of environmental stimuli: prenatal maternal consumption of alcohol and cigarette, as well as the quality of the maternal diet have direct and important effects on the methylation landscape and function of the placenta.[140-143] In this way, many alterations in the DNA methylation landscape of the placental genome are related to alterations in the phenotype of the placental cell lineages and those, with alterations in placentation and with embryonic development disorders such as preeclampsia, mole and other gestational trophoblastic diseases.

Several studies show that DNA methylation landscape in placental tissues has a direct relationship with the outcome of the pregnancy, specifically with the weight and size of the newborn and with many clinic alterations, and it has been associated even with the socioeconomic status of the mother.[144-148] The study of the role of the DNA methylation in many gestational complications has been greatly

 

facilitated by the use of the Illumina Infinium methylation technology, which allows the identification of differential methylation between control groups and groups of patients with the gestational disease under investigation.[149-158] Some of these pathologies and the associated methylation findings are discussed below.

### 3.2.1 Gestational diabetes

Gestational diabetes is maybe the gestational disease in which alterations in the DNA methylation landscape have been more characterized. This disease is an intolerance to glucose diagnosed during pregnancy, mainly during second and third trimesters and it is one of the principal complications during pregnancy that contributes to metabolic disorders of the offspring through epigenetic mechanisms. Remarkably, maternal hyperglycemia during pregnancy is associated with an excessive fetal growth and with perinatal and developmental complications, relationship that is regulated by changes in the placental methylome.[151,159-161] Gestational diabetes is also related to an increase of adverse perinatal outcomes and with a future risk of the offspring to develop disorders such as obesity and type 2 diabetes mellitus. Several projects have studied the relationship between the epigenetic mechanisms and the impacts on the health of the offspring, being the most abundant those regarding DNA methylation.[162] A complete list of the studies in this regard can be found in Elliot et. al.[163] Some studies show that gestational diabetes has epigenetic effects mainly in genes involved in type 1 diabetes mellitus, major histocompatibility complex-associated immunology and neuronal development related pathways, with important consequences for fetal growth and development. Furthermore, some studies back the notion that the DNA methylation status of the placenta is related to maternal sensibility to insulin,[164] and that the glycemic response is related to the methylation status of placental genes under epigenetic control.[165]

### 3.2.2 Preeclampsia

Preeclampsia is a hypertensive disorder characterized by high arterial blood pressure and liver or renal damage that affects around 6% of all pregnancies worldwide, resulting in fetal morbidity and mortality. Alterations in the DNA methylation landscape of vascular tissues of mothers with preeclampsia had been previously identified, with the involvement of important genes for vascular function,[166] early notion that an alteration of DNA methylation could contribute to the pathogenesis of preeclampsia. Later, another study aimed to identify those genes whose methylation state was associated with preeclampsia,[152] and it was demonstrated that preeclampsia is related with altered placental methylation through the comparison of the methylation status of 24 mothers with preeclampsia and a control group of 24 healthy mothers.[167] Remarkably, it was found that the methylation status of the placenta is associated with augmented blood pressure through genes involved in metabolic-vascular diseases.[144]

### 3.2.3 Hydatidiform mole

A recent study shows that a pre-existent mutation in the gene *KHDC3L* is a cause for hydatidiform mole.[168] This mutation is associated with a genomic hypomethy-lation in the ovulum such that it causes alterations in the placental implantation process. Likewise, mutations in the gene *NLRPJ* have associated to this

 

phenomenon of deleterious hypomethylation of DNA. Also, It has been demonstrated that strategies based on the analysis of DNA methylation have a high prediction potential of cases of gestational trophoblastic neoplasms.

### 3.2.4 Intrauterine growth restriction

Intrauterine growth restriction (IUGR) is a disorder characterized by the limited growth of the fetus during pregnancy and it has long-term consequences on the health of the offspring because its implications in fetal growth and development. These deleterious effects have been extensively described in different tissues and species,[157,169-172] and it is believed that placental DNA methylation has an important effect in its onset and development so alterations in this mechanism can imply the occurrence of alterations during placental invasion and function. In this regard, 8 genes whose methylation status was identified as differential between twin pairs with highly discordant growth have been identified,[173] as well as 4 genes directly related with IUGR, while a very recent study explores the possible mechanisms through which the disease could be progressing from a methylomic point of view.[174]

### 3.2.5 Cancer

For the correct operation of the placenta it is necessary a correct invasion of the maternal decidua and the remodeling of its vasculature, both processes remarkably similar to tumor metastasis. Interestingly, somatic transition to cancer has been associated with reactivation of embryonic developmental programs for the consecution of its highly proliferative and invasive phenotype. In this respect, a now outdated but very complete review discusses all aspects of the still relevant knowledge of DNA methylation in cancer as well as the future directions of the field and the most important questions yet to be answered. Of remarkable importance are those aspects regarding DNA abnormalities in placental diseases and specially the characterization of the epigenetic phenomena taking place in the somatic transition to cancer. Years later two important papers highlighted several aspects of the similarities of the placental and cancerous phenotypes in terms of DNA methylation.[175,176] Taken together, these findings suggest there is a whole epigenetic regulatory network governing the placental phenotype which is used by the cancerous cells to switch to an invasive phenotype. Finally, a recent review by Vlahos et al,[2] summarizes the current knowledge of the interplay between placental health and disease from a DNA methylome perspective, including those aspects regarding cancer initiation and progression.

## 4. Conclusion

A significant body of evidence has accumulated over the last years supporting the pivotal roles of the IGF and TGF-β growth factors in the regulation of human placenta development. Whereas the IGF axis mainly induces proliferation and differentiation of trophoblast cells, the TGF-β axis fundamental role is to inhibit cell invasiveness. A delicate balance between these cellular events is spatially and temporally regulated to ensure normal trophoblast development (Figure 2). The progressive loss of control in proliferation, migration and invasion of trophoblasts in normal placenta, originate the transformation into hydatidiform mole, choriocarcinoma and characterize placental pathologies, like preeclampsia.

 

Although much knowledge has been gained over the last years regarding the molecular circuits associated with placental pathologies, there are still many aspects that are not fully understood. Additional research in this area will improve the diagnosis and treatment of gestational trophoblastic diseases.

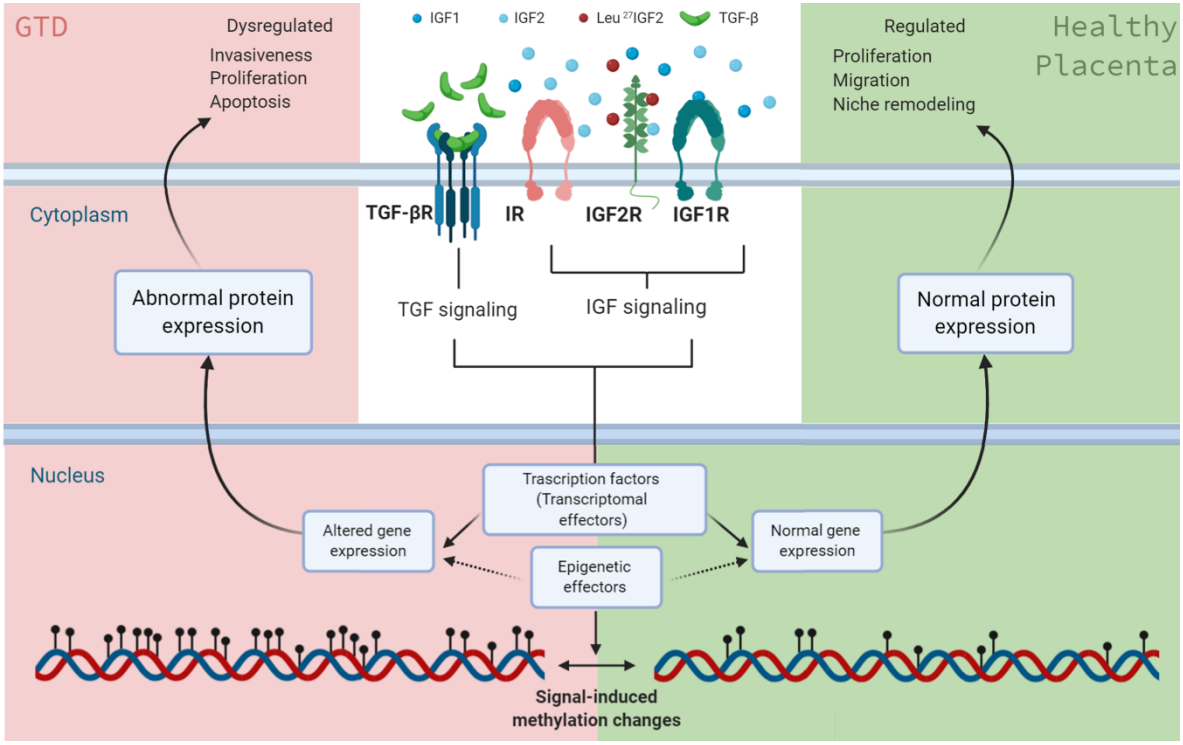


**Figure 2.** Altered protein expression in placenta and implications on GTD: transformation into hydatidiform mole and choriocarcinoma.

 

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