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Molecular basis of thyrotropin and thyroid hormone action during implantation and early development

Martina Colicchia[1], Luisa Campagnolo[2], Enke Baldini[3], Salvatore Ulisse[3], Herbert Valensise[4], and Costanzo Moretti[1,*]

[1]Department of Systems' Medicine, University of Rome Tor Vergata, UOC of Endocrinology and Diabetes, Section of Reproductive Endocrinology Fatebenefratelli Hospital, 'Isola Tiberina' 00187, Rome, Italy [2]Department of Biomedicine and Prevention, University of Rome Tor Vergata, Via Montpellier1, 00133 Rome, Italy [3]Department of Experimental Medicine, 'Sapienza' University of Rome, Rome, Italy [4]Department of Obstetrics and Gynaecology, University of Rome Tor Vergata, Fatebenefratelli Hospital 'Isola Tiberina', 00187 Rome, Italy

*Correspondence address. Department of Systems' Medicine, 'Tor Vergata' University of Rome, Via Montpellier, 1, 00133, Rome, Italy. Tel: +39-06-68370256; Fax: +39-06-72596663; E-mail: moretti@med.uniroma2.it

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BACKGROUND: Implantation and early embryo development are finely regulated processes in which several molecules are involved. Evidence that thyroid hormones (TH: T_4 and T_3) might be part of this machinery is emerging. An increased demand for TH occurs during gestation, and any alteration in maternal thyroid physiology has significant implications for both maternal and fetal health. Not only overt but also subclinical hypothyroidism is associated with infertility as well as with obstetric complications, including disruptions and disorders of pregnancy, labor, delivery, and troubles in early neonatal life.

METHODS: We searched the PubMed and Google Scholar databases for articles related to TH action on ovary, endometrium, trophoblast maturation and embryo implantation. In addition, articles on the regulation of TH activity at cellular level have been reviewed. The findings are hereby summarized and critically discussed.

RESULTS: TH have been shown to influence endometrial, ovarian and placental physiology. TH receptors (TR) and thyrotropin (thyroid-stimulating hormone: TSH) receptors (TSHR) are widely expressed in the feto-maternal unit during implantation, and both the endometrium and the trophoblast might be influenced by TH either directly or through TH effects on the synthesis and activity of implantation-mediating molecules. Interestingly, due to the multiplicity of mechanisms involved in TH action (e.g. differential expression of TR isoforms, heterodimeric receptor partners, interacting cellular proteins, and regulating enzymes), the TH concentration in blood is not always predictive of their cellular availability and activity at both genomic and nongenomic level.

CONCLUSIONS: In addition to the known role of TH on the hormonal *milieu* of the ovarian follicle cycle, which is essential for a woman's fertility, evidence is emerging on the importance of TH signaling during implantation and early pregnancy. Based on recent observations, a local action of TH on female reproductive organs and the embryo during implantation appears to be crucial for a successful pregnancy. Furthermore, an imbalance in the spatio-temporal expression of factors involved in TH activity might induce early arrest of pregnancy in women considered as euthyroid, based on their hormonal blood concentration. In conclusion, alterations of the highly regulated local activity of TH may play a crucial, previously underestimated, role in early pregnancy and pregnancy loss. Further studies elucidating this topic should be encouraged.

Key words: thyroid hormones / thyrotropin / pregnancy / implantation window / trophoblast

Introduction

Data from the literature support the hypothesis that thyroid hormones (TH: T_4 and T_3) play an important role during implantation and the early stages of embryo development. Successful implantation is the result of reciprocal interactions between the implantation-competent blastocyst and the receptive uterus (Franchi *et al*, 2008; Altmäe *et al.*, 2012; Koot and Macklon, 2013). During implantation, the free-floating blastocyst attaches to the endometrium through the trophoblast cell layer, invades the stroma and becomes intimately connected to the endometrial tissue (Aplin *et al.*, 2000; Kaneko *et al.*, 2013). Implantation involves spatiotemporally regulated endocrine, paracrine and juxtacrine modulators, and depends on a coordinated cellular and molecular cross-talk between the blastocyst and the receptive endometrium (Sharkey and Smith, 2003; Franchi *et al.*, 2008; Cuman *et al.*, 2013; Sharkey and Macklon, 2013). The embryo functions as an active unit, with its own molecular program of cell growth and differentiation and, in order to implant, it has to interact with the uterus during a well-defined period, which is called 'the implantation window'. This transient and unique period of uterine receptivity is of short duration, lasting ~4 days, and it is interposed between the pre-receptive and the refractory endometrial phases (Sharkey and Smith, 2003; Dey *et al*, 2004; Franchi *et al.*, 2008; Zhang *et al.*, 2013). Recently, *in vivo* and *in vitro* evidence has been reported concerning TH activity around implantation, a process that begins with apposition, continues through attachment and ends with the invasion. Apposition denotes the initial, still unstable, adhesion of the blastocyst to the uterine wall. Micro-protrusions of the apical uterine epithelium, named pinopodia, interdigitate with microvilli of the apical trophectoderm of the blastocyst (Norwitz *et al.*, 2001; Dey *et al*, 2004; Cheong *et al.*, 2013). Adhesion coincides with a localized increase in endothelial proliferation induced by angiogenic factors secreted by the blastocyst (Staun-Ram and Shalev, 2005; Demir *et al.*, 2010).

After implantation, stem cells of the trophectoderm fuse to form the peripherally located primitive syncytium. Then, cytotrophoblasts emanating from the trophectodermal layer generate primary villi by proliferation and invasion through the primitive syncytium, migrating up to the inner third of the myometrium (Hamilton and Boyd, 1960; Staun-Ram and Shalev, 2005; Huppertz *et al.*, 2013; Ji et al., 2013).

To allow implantation, morphological and biochemical reprogramming of the endometrial stromal compartment is needed (Gellersen *et al.*, 2007; Lee *et al.*, 2013; Palomino *et al.*, 2013; Pawar *et al.*, 2013; Shen *et al.*, 2013); such phenomenon, named decidualization, is controlled by the convergence of progesterone and cAMP signal transduction pathways, and consists of the transformation of stromal fibroblasts into epithelioid-like secretory cells, an increase in the number of local macrophages and lymphocytes, the elongation and thickening of spiral arteries and the production of molecules essential for blastocyst-endometrium interplay (Lea and Sandra 2007; van Mourik *et al.*, 2009). All steps of implantation are finely regulated by a plethora of cytokines, which includes leukemia inhibitory factor (LIF) and interleukin-11 (IL-11) (Marwood *et al.*, 2009; Paiva *et al.*, 2009; Salamonsen *et al.*, 2010; Sherwin *et al.*, 2010; Terakawa *et al.*, 2011; Pawar *et al.*, 2013; Wu *et al.*, 2013), adhesion molecules (Aplin, 1997; Singh and Aplin 2009; Lecce *et al.*, 2011; Sharma and Kumar, 2012; Taylor *et al.*, 2014), including the primary adhesion molecule Muc-1 (Carson *et al.*, 1998; Meseguer *et al.*, 2001; Goulart *et al.*, 2004; Marwood *et al.*, 2009; Margarit *et al*, 2010), and growth factors activating several signaling pathways (Raab et al, 1996; Lim and Dey, 2009; Altmäe *et al.*, 2012, 2013; Leach *et al.*, 2012). Interestingly, the overlapping expression of steroid receptors and several growth factors at the site of implantation suggests that redundant mechanisms might be at work, in order to achieve a successful implantation even if the expression of one or more of these factors is compromised (Boelen *et al.*, 2012; Leach *et al.*, 2012; Garrido-Gomez *et al.*, 2013; Vilella *et al.*, 2013).

Hallmark events during implantation and decidualization are represented by tissue remodeling and angiogenesis, considered as the rate-limiting steps of these processes (Dey *et al.*, 2004; Plaisier, 2011). Tissue remodeling depends on the critical balance between the activity of matrix metalloproteinases (MMPs) produced in the trophoblast and their inhibitors (tissue inhibitor of metalloproteinases: TIMP) produced in decidual stromal cells. Angiogenesis is strictly associated with tissue remodeling and plays a crucial role in successful implantation,

decidualization and placentation, with vascular endothelial growth factor (VEGF) and angiogenic factors (angiopoietins) being the main players (Cross *et al.*, 1994; Hess *et al.*, 2006; Kim *et al.*, 2013).

Molecular pathways connecting the above described mechanisms with (Sun *et al.*, 2010) TH signaling have been proposed. A failure of thyroid function often complicates pregnancy, causing a serious risk for maternal and fetal health; this event is generally a consequence of the physiologically increased demand for TH, which might make manifest a hitherto undetected subtle thyroid disorder. Subclinical hypothyroidism is frequently related to infertility and pregnancy loss, although the molecular mechanisms governing these events have not been elucidated yet. However, a clinical association between pregnancy complications and thyroid disease has been extensively reported (Kilic *et al.*, 2008; Stagnaro-Green and Pearce, 2012; Granfors *et al.*, 2013).

Studies on the spatio-temporal distribution of nuclear thyroid hormone receptors (TR), acting as ligand-dependent transcription factors, and the G protein-coupled TSH receptors (TSHR) have demonstrated their wide expression in the feto-maternal unit during the implantation window, suggesting a local action of TH and TSH on both the endometrium and the embryo. TH might directly regulate implantation or act through the regulation of a plethora of factors involved in the process (Aghajanova *et al.*, 2011; Stavreus, 2012).

The main focus of this review is to highlight the evidence indicating that the TH and TSH are new potential players in the implantation process and in early embryo development. The possibility that alterations of the highly regulated local activity of TH may play a crucial and underestimated role in early pregnancy and pregnancy loss will be discussed.

Methods

In preparation for this review, relevant and up-to-date studies focused on the involvement of TH in endometrium preparation for implantation and in placentation were identified by extensive PubMed and Google Scholar inquiries using the following key words: thyroid, implantation window, early embryo development, thyroid hormone, embryo implantation, trophoblast, placentation, deiodinases, coactivators, thyroid transporters and receptors, ovary, endometrium, sodium iodide symporter, pendrin, regulation, invasion, integrins, uterine NK cells, immune system, nongenomic, surface receptors, MAPK, ERK 1/2 pathways, SUMO, TRE, VEGF, bFGF. Almost 300 articles in English language published from 1960 to date were analyzed. Of these 250 are discussed in the present review.

Molecular Regulation of Thyroid Hormone Activity in the Female Reproductive System

While adequate TH serum levels are crucial to activate intracellular thyroid-dependent pathways, it is also fundamental that all membrane/nuclear receptors and signal transducers work at the appropriate time, to ensure their biological effect. Therefore, in order to understand the causes of an abnormal hormone action, it is important not only to consider TH serum levels but also to dissect what happens from secretion into blood to cellular access. The cellular metabolism and gene transcription linked to TH are influenced by the expression of several factors acting at multiple steps of TH-dependent pathways, including hormone blood transporters, deiodinases, nuclear transcription factors associated with the thyroid response element (TRE), TH receptors (TR), and coactivators and coinhibitors essential to TR-modulated transcription.

TH binding proteins in blood

The blood transport of the hydrophobic TH is accomplished by three binding proteins, synthesized and secreted by the liver: thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin (Schussler, 2000). All three binding proteins can transport both T_4 and T_3, although T_4 is bound with higher affinity (McKinnon *et al.*, 2005; Feldt-Rasmussen and Krogh Rasmussen, 2007; Richardson, 2009; Landers *et al.*, 2013a). TTR and albumin, together with the low-affinity TH binding proteins α-1-antitrypsin and β-1-acid glycoprotein, are also produced by human placental trophoblasts, and secreted into the maternal and fetal circulation (Landers *et al.*, 2009). These proteins may locally modulate the maternal-fetal hormonal transport, thus affecting TH uptake, efflux and deiodination (McKinnon *et al.* 2005; Landers *et al.*, 2013b). In addition, trophoblast cells are able to uptake maternal TTR-TH complexes and to transfer them from mother to fetus (Mortimer *et al.*, 2012; Landers *et al.* 2013a, b). It has been hypothesized that TTR-TH is internalized through a low-density lipoprotein receptor-dependent endocytic process, but further research is required to confirm this mechanism (Landers *et al.*, 2013b).

TTR appears to protect maternal TH from active deiodination within the placenta, allowing higher concentrations of TH to pass to the fetal circulation, as demonstrated by the increased D3 (type 3 deiodinase) activity after treating placental tissue with mefenamic acid, an inhibitor of TH-TTR binding (McKinnon *et al.*, 2005). During the first trimester, placenta and fetus are exposed to relatively low hypoxic conditions. The cellular response to changes in oxygen tension during normal development is finely regulated (Greer *et al.*, 2012). In mammals, the hypoxia-inducible factor α (HIF1α), a DNA-binding transcription factor that activates many genes by associating with specific nuclear cofactors under hypoxia, regulates the metabolic and phenotypic changes of the blastocyst before and after implantation (Greer *et al.*, 2012). In primary trophoblast cultures, HIF1α up-regulates TTR mRNA and protein levels (Patel *et al.*, 2012), a process critically involved in TH-mediated embryonic neurological development early in gestation (Morreale *et al.*, 2004; Bernal, 2005; Patel *et al.*, 2011b; Greer *et al.*, 2012).

Albumin, a protein with low affinity but high binding capacity for TH, can be detected in the trophoblast glycocalyx, where it might be part of a TH uptake pathway, although albumin binding to trophoblast appears weak (Douglas *et al.*, 1998; Landers *et al.*, 2013a, b). Albumin is internalized by placental explants, and in the syncytiotrophoblast it is either transferred to the maternal side of the explant or degraded (Lambot, *et al.*, 2006), suggesting that it might play a protective role for TH or act as a vehicle for the hormone in fetal circulation. However, the full role played by albumin at placental level remains to be elucidated. In summary, placental tissue has the ability to synthesize, secrete and internalize molecules involved in TH binding and transport, thus regulating, through a fine-tuned local mechanism, TH supply to fetus (Table I).

Active iodine uptake in thyroid follicle and placenta

As pregnancy occurs, several physiological changes influencing thyroid function take place (Krassas *et al.*, 2010). In particular, during pregnancy thyroid function increases by about 50% as a consequence of two stimuli:

Table I Thyroid-dependent molecules at the endometrium, ovary and blastocyst level around the implantation window.

Localization	Molecules	Evidence and possible role (PR)	Species	Ref.
Ovary	TRα1, TRα2, TSHR, TRβ1, DIO2, DIO3 (mRNAs)	*T3 = dose-dependent mRNA expression of inflammation-associated genes: COX-2, MMP9, 11βHSD1, ERα.*	Human	Du and Li (2013)
OSE	TRα1, TRα2, TRβ1, TSH (proteins)	*PR: Chronic hyperthyroidism might contribute to low-level of inflammation rendering OSE more susceptible to neoplastic transformation.*	Human	Fedail *et al.* (2013)
Primordial follicle:	O: TSHR, TRα1, TRβ1 (mRNAs, proteins)	*TSH = production of cAMP by luteinized GC*	Pigs Cattle	Sun *et al.* (2010)
Primary follicle:	c-erbAα-2 (mRNA)	*T4 = production of ERK 1/2 in luteinized GC (maturation of pre-ovulatory follicle and OCC; meiotic oocyte maturation)*	Mice	Aghajanova *et al.* (2009)
Secondary follicle:	O:TSHR, TRα1, TRβ1 (mRNAs, proteins)	*Gonadotrophins = increase TSHr*	Bovine	Rae *et al.* (2007)
Antral follicle:	GC: TSHR (mRNAs, proteins)	*Estradiol = decrease TSHr*	Rat Human	Zhang *et al.* (1997)
Follicular fluid:	O: TSHR, TRα1, TRβ1 (mRNAs, proteins)	*Thyrostimulin acts through TSHr to increase cAMP*	Human	Su *et al.* (2003)
Oocyte:	GC: TSHR, TRβ1 (mRNAs, proteins)	*T3 = stimulation of*	Human	Tomek *et al.* (2002)
	O: TRα1, TRβ1, TRβ2, c-erbAα-2 mRNAs	*GC proliferation and hCG-induced cAMP in GC; synergy with FSH*	Human	Cecconi *et al.* (1999)
	GC: TSHR, TRα-1, TRβ-1 (mRNA, proteins)	*to induce differentiation of GC increasing LH receptors and*	Porcine	De Silva (1994)
	TRβ-2, c-erbAα-2, DIO2, DIO3 mRNAs	*progesterone secretion by GC from porcine follicles ; synergy with FSH to inhibit*	Mice	Wakim *et al.* (1993)
	CC: TRα-1, TRβ-1, TRβ-2, c-erbAα-2 mRNAs	*apoptosis of GC*	Mice	Goldman *et al.* (1993)
	FT3, FT4, TSH	*T3 inhibits in a time- and dose-dependent manner FSH-induced aromatase activity in GC*	Mice	Maruo *et al.* (1992)
	Thyrostimulin	*high T3 concentrations can impair preantral follicle development*		Maruo *et al.* (1987)
		PR: T3, T4 in FF = regulation of human GC and steroid production		
		PR: T3, T4, TSH = oocyte maturation and development		
		PR: Thyrostimulin = paracrine modulator of ovarian function controlled by a different system from HPT axis.		
Blastocyst (in vitro)	TH receptors mRNA and proteins	*TH on Early Embryo development =*	Bovine	Costa *et al.* (2013)
	DIO2 and DIO3 mRNAs	*increase blastocyst formation and hatching rate; improve embryo quality (greater total cell counts and reduced proportions of apoptotic cells); improve post-cryopreservation viability.*	Human	Degrelle *et al.* (2013)
	(T3 produced in the bovine follicular fluid is an indirect indicator of DIO1 mRNA)			Loubiere *et al.* (2012)
	TRα = expressed in oocyte, zygote and all the cleavage stages from the 2 cell to 16 cell stage and blastocyst stage embryos in different culture condition (IVC medium alone and IVC medium supplemented with TH)			Ashkar *et al.* (2010)
	(in matured oocytes higher than in the germinal vesicle stage oocyte)			
Endometrium	TSHR,TRα1, TRβ1,(mRNA and proteins),	*Mifepristone: down-regulation of TRα1 and TRα2; up-regulation of TRβ1 and DIO2 mRNA (regulation by PR)*	Human	Li *et al.* (2014)
Glandular epithelium	TSHR,TRα1, TRβ1 (mRNA and proteins)	*TSHR in the luminal epithelium and TRα1 and TRβ1 in the glandular and luminal epithelium increase on LH days 6 to 9 (WOI, appearance of pinopodes)*	Human	Scoccia *et al.* (2012)
Luminal epithelium	TSHR,TRα1, TRβ1 (mRNA and proteins)	*Expression of DIO 1,2,3 mRNAs lower in the mid-secretory phase of the cycle*	Human	Dimitriadis *et al.* (2010)
Stroma	(TRα2: +ve WB, −ve immunostaining, +ve RT−PCR)	*TSH = increase LIF, LIFr mRNA in endometrial stromal cells, decrease LIF, LIFR in Ishikawa cells; increase Glucose transporter 1 in Ishikawa cells; increase secretion FT3 and T4 by Ishikawa cells IL-6 suppresses thyroid peroxidase gene expression and T3 secretion from cultured human thyrocytes*	Human	Aghajanova *et al.* (2011)
Oviduct and uterine horn	NIS (sodium-iodide symporter)	*T3 and GH = required for decidual response of the endometrium*	Human	Catalano *et al.* (2007)
	P4HB (a TG molecular chaperone),	*PR: endometrium site of thyroxine production*	Rat	Kennedy and Doktorcik (1988)
	TG (thyroglobulin),	*PR: generation of appropriate T3 level to contribute to decidual response to implantation*		
	TPO (Thyroid peroxidases),			
	factors involved in thyroid hormone signalling			
	D1, D2 (most abundant), D3			
	THR			

Other possible candidates	*Addition of TSH to NK cells augment their response to IL2, increasing proliferation and response to various stimuli and TH are involved in the regulation of inflammatory mediators in the endometrium*	Mice	De Vito *et al.* (2011)
NK cells		Human	Weetman (2010)
Leptin		Mice	Provinciali *et al.* (1992)
Angiogenesis	*TSH releases leptin in human adipose tissue culture and leptin/leptin receptor system may be a delicate regulator of the implantation process*	Human	Migita *et al.* (1989)
	TH = promotion of angiogenesis through genomic and nongenomic mechanisms. Transcription of bFGF and VEGF through MAPK and ERK1/2, binding on integrin αvβ3; Increase of Ang-2; PI3K activation through association with cytoplasmic TRβ and p85 and transcription of HIF1α, T4 induce FGF2 release trough MAPK from endothelial cells activating STAT involved in vascular growth; tetrac blocks T4 and T3 binding on the integrin receptor interfering with TH crosstalk with receptors for vascular growth factors (VEGF-R, bFGF-R).	Mice	De Oliveira *et al.* (2013)
		Chick	Yoon *et al.* (2005)
		Rat	Menendez *et al.* (2003)
			Mousa *et al.* (2006, 2008)
			Moeller *et al.* (2003)
			Bergh *et al.* (2005)
			Davis *et al.* (2004)

Ang 2, angiopoietin 2; bFGF, basic fibroblast growth factor; bFGF-R, basic fibroblast growth factor receptor; cAMP, Cyclic adenosine monophosphate; CC, cumulus cells; c-erbAα-2, alternative spliced product of TRα gene (non-T3 binding protein); COX-2, cyclooxygenases-2; D2, Type II iodothyronine deiodinase (protein); D3, Type III iodothyronine deiodinase (protein); DIO2, gene encoding Type II iodothyronine deiodinase or mRNA; DIO3, gene encoding Type III iodothyronine deiodinase or mRNA; ERK1/2, extracellular signal-regulated kinase 1/2; ERα, estrogen receptor α; FF, follicular fluid; FSH, follicle stimulating hormone; FT3, Free Triiodothyronine; FT4, Free Thyroxine; GC, granulosa cells; GH, growth hormone; hCG, human chorionic gonadotropin; HIF1α, Hypoxia-inducible factor 1-alpha; HPT, hypothalamus-pituitary-thyroid; IL2, interleukin 2; LH, luteinizing hormone; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; MAPK, Mitogen-Activated Protein Kinase; MMP9, matrix-metalloproteinase-9; NK cells, natural killer cells; O, oocyte; OCC, oocyte-cumulus-complex; OSE, ovarian surface epithelial cells; PI3K, phosphatidylinositol 3-kinase; PR, progesterone; STAT, signal transducer and activator of transcription; T3, Triiodothyronine; T4, Thyroxine; TG, thyroglobulin; TH, thyroid hormones; TRα1, thyroid hormone receptor α1; TRα2, thyroid hormone receptor α2; TRβ1, thyroid hormone receptor β1; TSH, thyroid stimulating hormone; TSHR, Thyroid-stimulating hormone receptor; VEGF, vascular endothelial growth factor; VEGF-R, vascular endothelial growth factor receptor; WOI,window of implantation; 11β-HSD1, 11beta-hydroxysteroid dehydrogenase type.

(i) the secretion by syncytiotrophoblast cells of hCG capable to bind and activate the TSHR in the thyroid gland; (ii) the increased secretion of TBG secondary to the hyperestrogenism occurring in pregnancy or to ovarian hyperstimulation during the assisted reproductive technologies. The increased TBG serum level leads in turn to a temporary decrease of free TH, resulting in increased TSH release which in turn stimulates thyroid function (Fig. 1). Iodine is essential for TH biosynthesis and, as recommended by the World Health Organization (WHO), the United Nations Children's Fund (UNICEF) and the International Council for the Control of Iodine Deficiency disorders (ICCIDD), its dietary intake should be 150 μg/day for adults (Abalovich *et al.*, 2007). Intake should be raised to 200–300 μg/day during gestation because of the above described increased maternal T_4 requirement and of fetal thyroid function, and to compensate for the enhanced pregnancy-related urinary iodide excretion (Hetzel, 1983; The Public Health Committee of the American Thyroid Association, 2006; Marchioni *et al.*, 2008). Iodine deficiency during pregnancy has been shown to cause maternal and fetal goiter, miscarriages, stillbirths, reduced fetal growth, neonatal hypothyroidism and reduced fertility in adult life (Delange, 2001; Ferri *et al.*, 2003), as well as inadequate mental development of the fetus, with severity varying from mild intellectual blunting to frank cretinism (Delange, 2001).

Iodine transport in the thyroid follicle and through placental tissue shares common features. Thyrocytes actively capture iodine through the sodium-iodide symporter (NIS) present on their basal cell membrane and release it into the follicle for TH synthesis through the apically located ionic transporter, pendrin (Manley *et al.*, 2005).

TSH increases NIS expression and iodine uptake. NIS is a membrane-bound glycoprotein and a member of the solute carrier family (SLC5A5) (Dohán and Carrasco, 2003; Darrouzet *et al.*, 2013), and mediates iodine transport from extracellular fluid into the cell through an active mechanism. Pendrin is an anion exchanger, activated by high concentrations of intracellular iodide (Yoshida *et al.*, 2004). Both NIS and pendrin are expressed in placenta, where they seem to carry out iodide transport from maternal to fetal circulation (Mitchell *et al.*, 2001; Chan *et al.*, 2009; Degrelle *et al.*, 2013). In the trophoblast iodine is taken up at the apical membrane and effluxes through the basal membrane. In BeWo human choriocarcinoma cells used as a placental model NIS is expressed, as expected, at the apical level while pendrin is expressed basally (Manley *et al.*, 2005; Karatas *et al.*, 2013). In thyroid follicular cells NIS is regulated by serum levels of TSH (Saito *et al.*, 1997) (Fig. 2B), while in syncytiotrophoblasts its expression is regulated by hCG (Fig. 3B). Exposure of JAR choriocarcinoma cells to hCG, which express high levels of NIS mRNA, leads to a further increase in NIS expression and iodide uptake (Arturi *et al.*, 2002). NIS mRNA and membrane protein are up-regulated by hCG in BeWo choriocarcinoma cells as well, and this is accompanied by increased levels of iodide uptake (Li *et al.*, 2007, 2011). Even though JAR and BeWo cells may represent an excellent *in vitro* model suitable to analyze iodine transport through placenta, it should be considered that the production of hCG by the trophoblast *in vivo* implies an autocrine control that malignant placental cells lack, so the self-regulation of hCG biosynthesis and the increase in NIS may differ (Li *et al.*, 2012; Akturk *et al.*, 2013).

The paired-domain transcription factor Pax8, along with the homeodomain thyroid transcription factor-1 (TTF-1), has a fundamental role in thyroid development and, in the adult gland, in the maintenance of the differentiated thyroid follicular cell phenotype, as it controls and activates the expression of thyroid-specific genes, such as those encoding

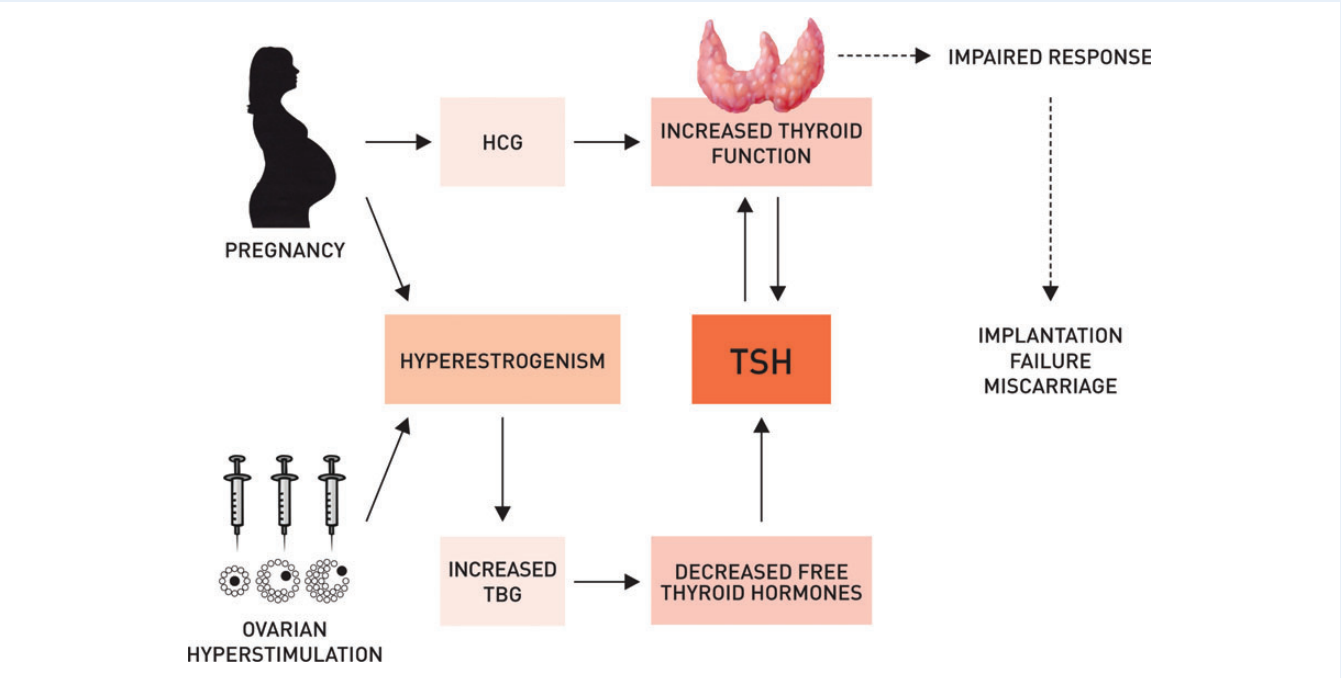

Figure 1 Schematic representation of the relationship between pregnancy, ovarian hyperstimulation and thyroid function. It is hypothesized that a transient impairment of thyroid function during early pregnancy may cause implantation failure or miscarriages. TSH, thyroid-stimulating hormone; TBG, thyroxine-binding globulin; hCG, human chorionic gonodotropin.

thyroglobulin, thyroperoxidase and NIS (Di Palma *et al.*, 2003). The expression of Pax8 in thyroid follicular cells is induced by TSH with a consequent increase in intracellular cAMP (Mascia *et al.*, 2002). PAX8 is expressed in the human placenta as well, in a cAMP-dependent fashion (in this case, hCG-mediated) (Kozmik *et al.*, 1993). However, at variance with the situation occurring in the thyroid, in which one of the main PAX8 functions is the regulation of NIS gene expression, variations in this transcription factor do not appear to have any effect on the expression of NIS in the JAR placental cell line (Ferretti *et al.*, 2005).

One more shared feature between thyroid and placental cells appears to be the feedback mechanisms regulating intracellular iodide concentration and iodide uptake. In BeWo cells intracellular iodide causes a down-regulation of NIS mRNA and protein expression, followed by a decrease in iodide uptake (Li *et al.*, 2007).

Additional regulatory factors, such as O_2, are involved in NIS synthesis at placental level, but their role has not been fully elucidated. As mentioned above, during pregnancy O_2 tension gradually increases in the placenta from very low levels (~1% in the first 6 weeks) to a plateau of 8% by the 16th week of gestation, a value which is maintained until birth. Li *et al.* (2011) have shown that in BeWo cells cultured under different O_2 concentrations (1, 8, 21%) the expression of NIS and hCG (mRNA and protein) and iodine uptake by the cells are directly related to O_2 concentration. In particular, cells grown in 1% oxygen do not respond to exogenous hCG by increasing NIS expression or iodine uptake suggesting that oxygen concentration plays a major role in regulating placental NIS expression. Thus, the O_2 increase at the end of the first trimester inducing NIS synthesis and iodine uptake by trophoblast is well timed to meet the increased iodide requirements of the developing fetal thyroid, beginning at 10–12 weeks of gestation (Di Cosmo *et al.*, 2006).

The iodine needs of the embryo are thus clearly ensured at the maternal-fetal interface by NIS and pendrin, and the fine regulation of their synthesis and activity by various factors shows analogies with the mechanisms operating in the thyroid gland. However, the evidence is still limited, and models other than the currently used choriocarcinoma cell lines are needed, considering that in neoplastic cells the ability to regulate the synthetic machinery and to respond to physiological feedback is generally altered (Orendi *et al.*, 2011).

Membrane TH transporters in placental cells

Thyroid hormone action in target tissues requires its active transport across the plasma membrane. Different types of TH transporters exist, showing different affinity for T_3 and T_4, namely, monocarboxylate transporters (MCT), L-type amino acid transporters (LAT) and organic anion transporting polypeptides (Oatp) (Fig. 3C). Among them, only MCT8, MCT10 and Oatp1c1 show a high degree of specificity towards TH (Visser *et al.*, 2008, 2013; Karapanou and Papadimitriou, 2011).

The mRNA for six different types of TH transporter are expressed at placental level, localized in different placental cell types: MCT8, MCT10, LAT1, LAT2, Oatp1a2 and Oatp4a1 (Loubière *et al.*, 2010; Patel *et al.*, 2011a). From 6 weeks gestation onwards, the whole set of TH transport proteins can be immunohistochemically detected in villous syncytiotrophoblasts and cytotrophoblasts, and also in extravillous cytotrophoblasts (EVT), with varying patterns of expression and intensity throughout pregnancy (Ritchie and Taylor 2001; Okamoto *et al.*, 2002; Sato *et al.*, 2003; Loubière *et al.*, 2010). It is worth noting that the maternal-facing apical microvillous plasma membrane of human syncytiotrophoblast, which serves as the first plasma membrane barrier to the transplacental

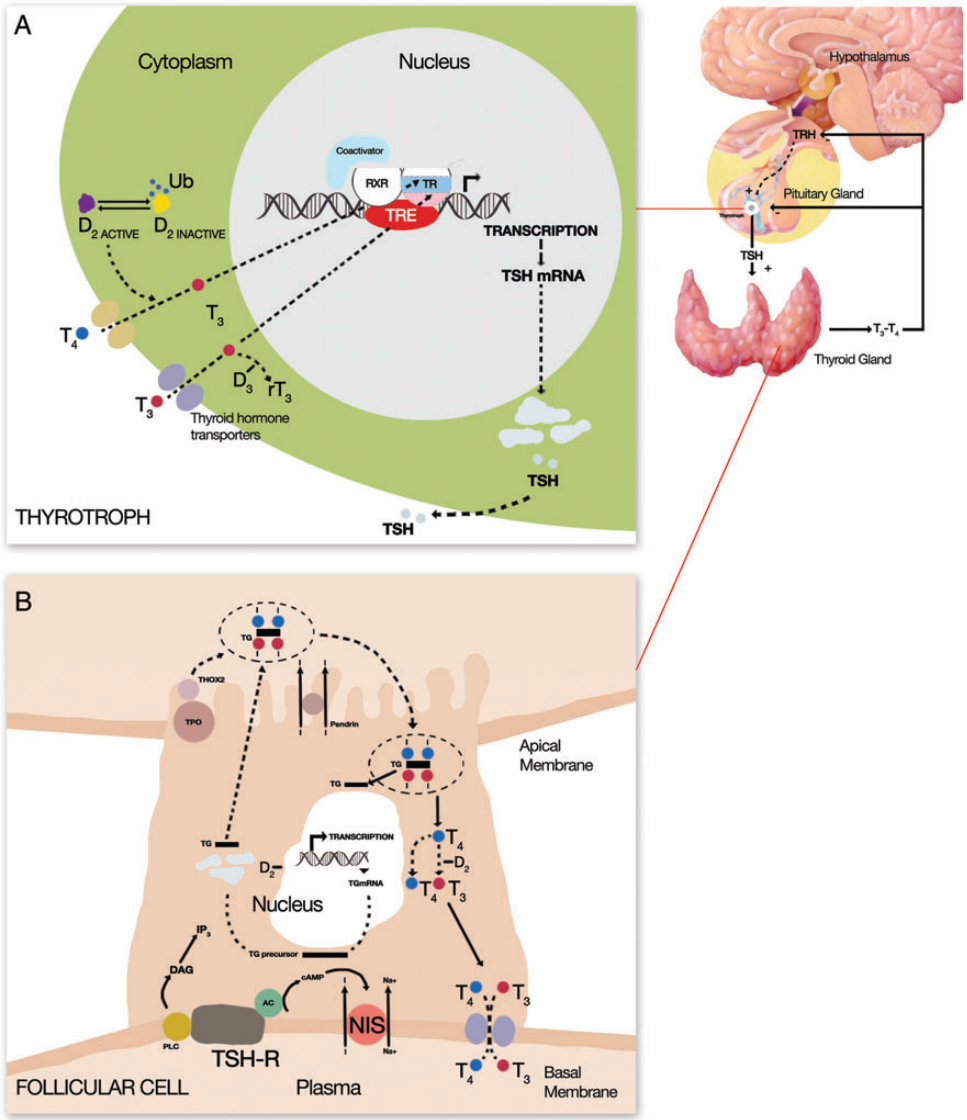

Figure 2 Systemic and local regulation of TSH and thyroid hormone plasma levels. (**A**) TSH plasma levels are regulated by the circulating thyroid hormones (TH) T_3 and T_4, actively transported into the thyrotroph where thyroninodeiodinase 2 (D2) converts T_4 to the active form T_3. The intracrine role of D2 is represented in the figure by its ubiquitinated inactive and deubiquitinated active isoforms, whose ratio defines the variable rate of T_3 availability inside the nucleus able to saturate the TR at the level of TRE on DNA, activating transcription of TSH and its release. (**B**) At the level of the basal membrane of the thyroid follicular cell the binding of TSH to TSHR activates Ac to form cAMP which modulates the expression of NIS and activates PLC that form DAG and IP_3 and the phosphorylation cascade that triggers TG transcription in the nucleus. At the apical membrane the oxidation by TPO allows the iodination of tyrosine residues of TG, through the formation of H_2O_2 by $THOX_2$, necessary for the biosynthesis of TH. TRE: TSHR: TRs, thyroid receptors; RXR, retinoid X receptor; TG, thyroglobulin; TPO, thyroid peroxidase; Ub, ubiquitine; NIS, sodium-iodide symporter; Ac, adenylate cyclase; PLC, phospholipase C; DAG, diacylglycerol; IP_3, phosphatidylinositol; $THOX_2$, thyroid oxidase 2.

passage of maternal TH, is capable of rapid, saturable T_4 and T_3 uptake from the maternal circulation (Loubière *et al.*, 2010) (Fig. 3A and C). In this site the expression of MCT8, MCT10, Oatp1a2 and LAT1 mRNA gradually increases with gestation, while that of Oatp4a1 and CD98 reaches a minimum at mid gestation, before increasing towards term. The preferential localization of Oatp4a1 and LAT2 at the apical surface of syncytiotrophoblast (Lewis *et al.*, 2007; Loubière *et al.*, 2010) suggests their direct involvement in the uptake of TH from maternal blood.

The redundant expression of TH transporters has been suggested to help ensure proper hormone availability to the fetus, and it may represent a critical point of regulation for the transplacental transfer of TH from mother to fetus (Loubière *et al.*, 2012). It should be underlined here that, in addition to TH, these transporters may mediate the delivery of other maternal molecules to the fetus, a role that needs to be better clarified. Nonetheless, MCT8, MCT10 and Oatp1a2 in villous cytotrophoblast and EVT have a higher affinity to TH than to other molecules

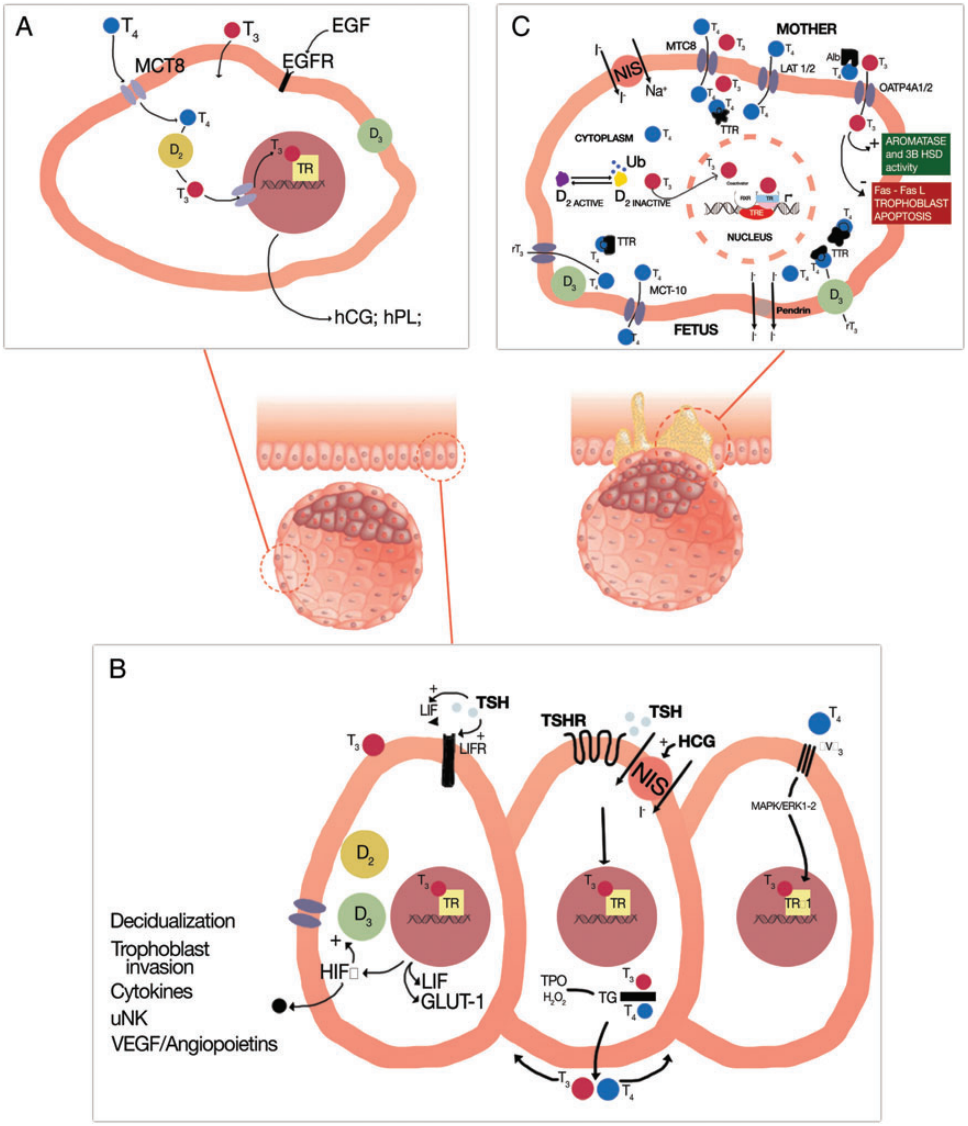

Figure 3 Thyroid hormone signaling is involved in the bi-directional dialogue between competent blastocyst and endometrium. (**A**) Before the window of implantation (WOI), in the early luteal phase, trophoblast cells express TRs, thyroid hormone transporters, mainly MCT8, and deiodinases. Both T_3 and EGF may act synergistically to regulate the production of hCG and, later, human placental lactogen (hPL). (**B**) Endometrial cells also express TRs, TG, TPO and deiodinases both D2 and D3, the former activating and the latter inactivating T_4, forming respectively T_3 and rT_3. During the WOI, TSH increases LIF and LIFR expression and regulates glucose transport increasing the expression of GLUT-1. TH may also influence angiogenesis and immune function during decidualization and implantation. At this time hypoxia induces D3 through HIFα, possibly controlled by nongenomic pathways. (**C**) Past the WOI and during the early weeks of pregnancy, membrane polarization of NIS and Pendrin on syncitiotrophoblast cells resembles that present in the thyroid follicular cells. TH transporters, such as MCT8, MCT10, OATP4A1, LAT1 and OATP1A2, can be detected in villous cytotrophoblasts and syncytiotrophoblast. Preferential localization of MCT8, OATP4A1 and LAT1 at the apical membrane of syncytiotrophoblasts suggests that these transporters play a key role in TH uptake directly from maternal blood while MCT10 at the basal membrane may have a key role in TH efflux from the trophoblast cell barrier to the fetus. TTR and albumin are present at trophoblast level protecting T_4 deiodination by D3. Furthermore, TH exert a protective mechanism at placental level, reducing trophoblast apoptosis and Fas/Fas-ligand expression (see text). rT3, reverse T3; MCT8, monocarboxylase transporter 8; MCT10, monocarboxylase transporter 10; EGF, epidermal growth factor; OATP4A1, organic anion transporting polypeptide 4A1; OATP4A2, organic anion transporting polypeptide 4A2; LAT, large aminoacid transporter; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; GLUT-1, glucose transporter-1; HIFα, hypoxia-inducible factor α; Ub, ubiquitine; TTR, transthyretin; 3βHSD, 3βhydroxysteroidodehydrogenase; VEGF, vascular endothelial growth factor.

(James *et al.*, 2009; Vasilopoulou *et al.*, 2013), suggesting a primary role of TH in early placentation.

The association between untreated maternal hypothyroidism and pregnancy complications, such as malplacentation, intrauterine growth restriction (IUGR), placental abruption and pre-eclampsia, has been described, while subclinical hypothyroidism is strongly associated with miscarriage and preterm labor (Abalovich *et al.*, 2002; Chan *et al.*, 2006; Vissenberg *et al.*, 2012). In the case of hypothyroxinemia, IUGR fetuses show high placental expression of MCT8 (Loubière *et al.*, 2010). Up-regulation of MCT8, associated with a down-regulation of MCT10, induces a sort of intracellular 'poisoning' by T_3 without changes in deiodinase activity. It is known that intracellular accumulation of T_3 induces apoptosis in trophoblastic cells (Vasilopoulou *et al.*, 2010). It is important to remember that the balance between proliferation and the apoptotic process is an important regulator of placentation, and is involved in syncytiotrophoblast renewal. We may speculate that the excessive T_3 placental influx and reduced efflux might be involved in the increased sensitivity to apoptotic stimuli. In conclusion, TH transporters appear to contribute to fetal-maternal exchange of TH in early pregnancy, and it can be safely hypothesized that they are involved in the fine regulation of trophoblastic activity (Loubière *et al.*, 2010; Vasilopoulou *et al.*, 2010).

Deiodinases

The modern paradigm of TH action is based on the evidence that hormone signaling in individual cells can change as a consequence of intracellular activation or inactivation of the hormone (Wondisford, 2003; Bianco and Cand Kim, 2006; Aranda *et al.*, 2013). Iodothyronine deiodinases are important mediators of TH action, being present in tissues throughout the body (Dentice *et al.*, 2013), where they catalyze T_3 production and degradation via outer and inner ring deiodination (Darras and Van Herck, 2012). In particular, type I deiodinase (D1) is responsible for generating most of the circulating T_3, type 2 deiodinase (D2) is considered as the source for intracellular T_3 and type 3 deiodinase (D3)—which deiodinates the inner ring of iodothyronines—is believed to be a TH-inactivating enzyme. Thus, independently of TH circulating levels, deiodinases may alter TH signaling in the target cells by regulating the cytoplasmic T_3 pool, nuclear T_3 concentration and TR saturation (Guadano-Ferraz *et al.*, 1997; Arrojo *et al.*, 2013). In the rat uterus the expression of D2 and D3 varies significantly during the estrous cycle and during early pregnancy in response, at least in part, to gonadal steroid hormone levels. In humans, after implantation, D3 expression increases by 200-fold over a period of 48–72 h (Huang, 2005). In rats, D3 activity maximally increases on Day 8–10 of pregnancy (Galton *et al.*, 1999; Wasco *et al.*, 2003), while the increase in D2 activity is minimal, and occurring over a more protracted time course (Wasco *et al.*, 2003). D2 is mainly controlled by estrogen, while D3 expression is modulated by the synergic action of estrogen and progesterone and by the implantation and decidualization processes themselves (Wasco *et al*, 2003). Uterine cytokines and growth factors, more than the presence of the blastocyst itself, seem to be involved in D3 stimulation (Wasco *et al.*, 2003).

The changes in uterine expression of deiodinases appear to provide the embryo with an optimal TH environment for early development. The main deiodinase isoform expressed at the beginning of human pregnancy is D3, which can be found in villous syncytiotrophoblast and cytotrophoblast. D3 is also expressed in uterine decidua, umbilical cord vessels, perivascular myocytes of uterine arteries and in non-pregnant endometrium (Huang *et al.*, 2003). The villous syncytiotrophoblast layer is directly exposed to maternal blood in the intervillous space and thus it is ideally placed to protect the fetus from excessive maternal TH transfer. On the other hand, during the first trimester the relatively undifferentiated villous cytotrophoblast layer expresses D2 protein more consistently than syncytiotrophoblasts, suggesting that at this stage D2 plays a primary role in supplying the fetoplacental unit (Chan *et al.*, 2003). In addition to placental tissue, TH can reach the fetus through the amniotic fluid, and T_4 can be exchanged through fetal respiratory epithelium, amniotic fluid swallowing and umbilical cord blood, but free T_4 concentration in amniotic fluid is higher than that in both maternal and fetal serum (Sack, 2003). Deiodinases are almost ubiquitous in the fetus, being present in umbilical vessels, respiratory and intestinal epithelium, urinary tract and the skin, reflecting a need to precisely control within narrow ranges the TH concentration during embryo development (Huang *et al.*, 2003). The coordinated expression of activating and inactivating deiodinases is thus crucial to protect the fetus against exposure to TH at inappropriate times or concentrations.

Intrauterine growth restriction (IUGR), usually caused by placental insufficiency in delivering oxygen and nutrients to the baby, is associated with mild neurodevelopmental deficits which have been partly attributed to reduced circulating fetal TH concentrations and decreased cerebral TR expression (Chan *et al.*, 2006). Despite the described up-regulation of placental TR expression in pregnancies complicated by IUGR (Kilby *et al.*, 1998), no difference in deiodinase expression in normal versus IUGR placentas was detected (Chan *et al.*, 2003), suggesting that these enzymes are not responsible for the hypothyroxinemia in circulating fetal TH observed in this condition (Chan *et al.*, 2003). It is interesting to note that in the rat uterus D3 mRNA can be detected prominently in the decidual tissue present in the central portion of the deciduoma, while D2 is seen at the periphery of the specimen, in a nearly circular pattern surrounding the D3-expressing decidualized cells, suggesting that active T_3 is involved more in placentation and uterine processes than embryo development (Wasco *et al.*, 2003). In the placenta the D2 gene has multiple transcription start sites and a transcriptional regulatory component involving cAMP, several cytokines and transcription factors (Kim *et al.*, 1998b; Bartha *et al.*, 2000, Canettieri *et al.*, 2000; Dentice *et al.* 2003; Kurlak *et al.*, 2013). In JEG3 cells, a human placental choriocarcinoma cell line highly responsive to cAMP treatment, the response to cAMP involves binding of the cAMP response element (CRE)-binding protein (CREB) to the CRE locus in the D2 promoter (Canettieri *et al.*, 2004). During its early development, trophoblast expresses epidermal growth factor (EGF) receptors and produces EGF, which induces D2 transcription (Song and Oka, 2003). Treatment of trophoblast cell cultures with EGF induces a threefold increase of D2 mRNA and an increase in enzymatic activity (Canettieri *et al.*, 2008). In JEG3 cells, EGF promotes the expression of D2 mRNA in synergy with cAMP, and this regulation occurs through a composite transcription factor module, which includes CREB, c-Jun, and c-Fos (Canettieri *et al.*, 2008). EGF regulation of D2 transcription resembles that played by the hCG α subunit transcriptional complex, recruiting c-Jun-c-Fos (AP-1) to the CRE binding complex (Robertson *et al.*, 2000).

D2 has a short half-life (about 40 min) and its substrate, T_4 at physiological concentration or T_3/rT_3 (reverse T_3) at high level, causes significant post-translational down-regulation through substrate-accelerated

selective proteolysis via the ubiquitin/proteasome pathway (Baqui *et al.*, 2000). The balance between ubiquitinated and non-ubiquitinated D2 regulates the concentration of available intracellular TH; in turn, several factors regulate this balance, including WSB-1, part of an E3 ubiquitin ligase which mediates ubiquitination and subsequent proteosomal degradation of D2, and VDU1/VDU2, D2 deubiquitinating enzymes (St Germain, 1988; Zavacki *et al.*, 2007; Gereben *et al.*, 2008). Thus, D2 activity appears to be controlled at various levels and the reason for such a fine mechanism is that it is responsible for T_3 availability to the nucleus. In addition, D2 activity is not only involved in thyroid metabolism, but it is a complex intracrine mechanism regulated by molecular events that might be involved in thyroid disease (Bianco and Cand Kim, 2006) and in the imbalance of thyroid function in the early pregnancy of healthy women experiencing recurrent miscarriage (Burrow *et al.*, 1994; Visser *et al.*, 1998; Kanou *et al.*, 2007; Dal Lago *et al.*, 2011; Kurlak *et al.*, 2013) (Fig. 2).

In conclusion, considering the implantation process as strictly controlled machinery, it can be hypothesized that deiodinase regulation is at work at the maternal-fetal interface to control TH activation and inactivation. However, identification and function of deiodinase regulatory factors in the feto-placental unit (Table II) have been only partially demonstrated and further studies are needed.

Thyroid hormone receptors, coactivators, corepressors and modulators of T_3 nuclear binding

In a given cell type TH may exert genomic and/or nongenomic actions (Cheng *et al.*, 2010). The genomic effects of TH are mediated by the nuclear TR which are encoded by two different genes, THRA, present on chromosome 17, and THRB, located on chromosome 3 (Lazar, 1993). By alternative splicing each gene generates two TR isoforms: THRA encodes TRα1 and TRα2; THRB encodes TRβ1 and TRβ2. TRα1, TRβ1 and TRβ2 bind TH, while the TRα2 does not (Harvey and Williams, 2002). The expression of the different TR is tissue-dependent and developmentally regulated (Cheng *et al.*, 2010). Nuclear TR act as hormone-dependent transcription factors by binding to a DNA motif located in the promoter regions of TH target genes, known as the TRE. TR may bind to the TRE as homodimers or as heterodimers with other members of the nuclear receptor superfamily such as RXR (9*cis*-retinoic acid receptor), vitamin D receptor and RAR (retinoic acid receptor). Heterodimerization of TR is thought to represent a means by which TR functions are modulated. For example, TR heterodimerization with RXR considerably increases TR binding to TRE, responsiveness to TH and its transcriptional activity (Zhang and Kahl, 1993). After binding to DNA, TR/RXR heterodimers alter the promoter transcriptional activity by interacting with corepressors (CoRs) or coactivators (CoAs) which connect the TR/RXR heterodimer with the basal transcription machinery (Cheng *et al.*, 2010). According to a widely accepted molecular model, in the absence of TH the TR/RXR complex binds to TRE where it interacts with CoR, thus inhibiting the basal transcriptional activity of the promoter. Following the binding of TH, structural changes in the TR occur resulting in the release of CoR and the recruitment of CoA and associated proteins capable of modifying the chromatin structure and to increase the transcriptional activity of the promoter (Cheng *et al.*, 2010).

In addition to the classical genomic pathway, TH can activate faster, nongenomic pathways (Moeller and Broecker-Preuss, 2011). T_4 promotes this non classical effect without entering the cell, by binding to a cell surface receptor and activating the mitogen-activated protein kinase MAPK-ERK1/2, which enters the nucleus and associates with TRβ1 inducing its stabilization and reduction of affinity for CoR (Lin *et al.*, 2003). Davis and colleagues identified a structural plasma membrane protein, the integrin αvβ3, as a TR capable of activating the ERK1/2 pathway, inducing angiogenesis and promoting cell growth (Davis *et al.*, 2000, 2004; Moeller *et al.*, 2006).

At variance with the genomic action of TH, this faster mechanism takes place in 10 min, reaching maximal activity in 30–40 min.

During embryo implantation an up-regulation of CoR and cofactors involved in TR transcriptional activity, such as TRIP, TR-associated protein 220 (TRAP220), nuclear receptor co-repressor (NCor) and silencing mediator for retinoid and TR (SMRT), occurs (Gereben *et al.*, 2008). Recently a new activator of TR, TRAM1 (Monden *et al.*, 1997; Lim *et al.*, 2004), has been described, which is highly expressed in placental tissue (Takeshita *et al.*, 1997). TRAP220, a subunit of the TRAP CoA complex, is essential for embryo survival, as evidenced by the fact that the placenta of TRAP $220^{-/-}$ null mice is histologically quite normal but there are villous alterations, blood circulation defects and growth defects (Takeshita *et al.*, 1997; Landles *et al.*, 2003).

As far as the post-translational modification of TR is concerned, TRα and TRβ conjugation with small ubiquitin-like modifier (SUMO) has been described (Liu *et al.*, 2012). In particular, it has been demonstrated that TRα and TRβ sumoylation modulates T_3 action and TR activity (Liu *et al.*, 2012). SUMO1 has been shown to be essential for T_3-induced recruitment of CoA CREB-binding protein (CBP) and release of NCoR from the TRE of TH positively regulated promoters. SUMO1 and SUMO3 are also required for T_3-mediated recruitment of NCoR and release of CBP from the TRE of TH negatively regulated promoters (Liu *et al.*, 2012). These observations are of interest as increased placental sumoylation has been hypothesized to contribute to the pathogenesis of serious placental pathologies that cause extreme preterm birth (Baczyk *et al.*, 2013).

Thyroid Hormone Activity Before the Implantation Window

TSHR and TR in the endometrium

TR and TSHR are present in endometrium and their expression varies during the menstrual cycle (Aghajanova *et al.*, 2011). TRα1 and TRβ1 are both expressed in the mid-luteal phase in glandular and luminal epithelium, and show an increase during the secretory phase and a subsequent dramatic decrease. It has been also demonstrated that TSHR, TRα1, TRα2 and TRβ1 expression in endometrial cells is increased at the same time as pinopodes appear and endometrial receptivity is established (Aghajanova *et al.*, 2011). Interestingly, transcripts involved in TH synthesis, including NIS, prolyl 4-hydroxylase beta (P4HB), a molecular chaperone involved in endocytosis of immature thyroglobulin (Tg) molecules, D2, thyroid peroxidase (TPO) and Tg, are also expressed in the endometrium (Catalano *et al.*, 2007). This suggests the hypothesis that TSH may induce TH secretion also in this tissue. The administration of mifepristone (RU486), an antiprogestin that makes the endometrium unreceptive and induces menstrual bleeding, reduces the expression of TRα1, TRα2 and Tg, while inducing that of TRβ1 and D2 (Catalano

Table II Intracellular regulation of thyroid hormone activity at placental level.

Molecules	Localization	Evidence and possible role	Species	Ref.
TH binding proteins TTR *(HA)* Albumin *(HA)* α-1-antitrypsin *(LA)* β-1-acid glycoprotein *(LA)*	ST Synthesis (TTR, Alb, α1AT, β1gpa) Secretion (TTR, Alb) Internalization (TTR)	*Binding TH to prevent deiodination* *Regulation of T3 access to the nucleus* *(1) Low oxygen = incr TTR*	Hum Hum *JEG3*	Landers *et al.* (2013a) Landers *et al.* (2013b) Patel *et al.* (2012) Patel *et al.* (2011a) Landers *et al.* (2009) McKinnon *et al.* (2005)
Iodide transport NIS Pendrin	ST Apical membrane (maternal side) Basal membrane (fetal side)	*Iodium influx into cells from mat. circulation* *Iodium efflux to the extracellular space towards the fetus* *(1) TSH, hCG = incr NIS* *(2) Low oxygen = decr NIS*	*BeWo* Hum	Degrelle *et al.* (2013) Manley *et al.* (2005) Mitchell *et al.* (2001) Li *et al.* (2011) Arturi *et al.* (2002)
Membrane transporters MCT 8 MCT 10 LAT 1 OATP1A2	ST (apical), CT, EVT, decidual stroma CT (basal), ST, EVT, decid strom, villous strom ST ST (apical) CT EVT	*TH uptake from maternal blood and passage through the cellular membrane/efflux to the fetus* *alteration in IUGR*	Hum Hum	Visser (2013) Loubiere et al. (2012) Vasilopoulou *et al.* (2010) Loubiere et al. (2010) Visser *et al.* (2008) Chan *et al.* (2006)
TH receptors TR α1 TR α2 TR β1	Endovascular/interstitial EVT	*Regulation of differentiated trophoblast (endocrine function, invasion and motility); interaction with factors involved in proliferation and stabilization.* *(1)T3 (10^{-8}M) = EGF-like molecules, hPL, hCG* *(2)T3 (10^{-8} m)/ T4 (10^{-7} m): Progesterone; (incr 3Bhsd) 17βestradiol; (incr aromatase)* *(3)T3 in synergy with EGF: incr motility and influence invasion SGHPL-4 (EVT cell)* *(4)T3 ($10^{-7/-9}$): decrem apoptosis (inhibit Fas-FasL, Casp3 cleav and PARP)* *(5)T3 (10^{-8}M): MMP2-MMP3 incr*	Hum SGHPL-4 Hum Hum Hum Hum Hum	Maruo (2010) Barber *et al.* (2005) Oki *et al.* (2004) Maruo *et al.* (1991)
Deiodinases D2 D3	Villous CT ; ST (weak) Villous ST; CT (weak) uterine decidua, umbilical cord vasa, perivascular myocities of uterine arteria, fetus: umbilical vasa, respiratory and intestinal epithelium, urinary tract, skin	*Decrease during gestation* *Conversion inactive T4 to T3* *Conversion T4 to rT3, T3 to T2* *Regulate maternal TH to fetal circulation. D3 protects the fetus from excessive mater TH*	Hum Rodent	Kurlak *et al.* (2013) Patel *et al.* (2011b) Chan *et al.* (2009) Gereben *et al.* (2008) Wasco *et al.* (2003) Huang *et al.* (2003) Chan *et al.* (2003)

3βHSD= 3βhydroxysteroidodehydrogenase; Alb, albumin; BeWO, choriocarcinoma cell line; Casp3, Caspase 3; cleav, cleavage; CT, cytotrophoblast; D, deiodinases; Decid strom, decidual stroma; decr, decrement; EVT, extravillous trophoblast; Fas-FasL, Fas-Fas ligand; HA, high affinity; hCG, human chorionic gonadotropin; hPL, human lactogen placentar; Hum, evidence on Human models; incr, increment; IUGR, intrauterine growth restriction; JEG3, Human placental choriocarcinoma cell line; LA, low affinity; LAT, L-type amino acid transporters; MCT, monocarboxylate transporters; MMP, metalloproteinases; NIS, sodium-iodide symporter; OATP, organic anion transporting polypeptides; PARP, Poly (ADP-ribose) polymerase; SGHPL-4=extravillous-like cell line; ST, syncitiotrophoblast; T3, Triiodothyronine; TH, thyroid hormones; TR, Thyroid receptors; TSH, Thyroid-stimulating hormone; TTR, transthyretin; Villous strom, villous stroma; α1AT, α-1-antitrypsin; β1gpa, β-1-acid glycoprotein.

et al., 2007). According to this evidence, progesterone seems to be important for the transcriptional regulation of factors involved in TR synthesis and metabolism, and explains, at least in part, the menstrual abnormalities and subfertility in women with primary hypothyroidism (Poppe and Velkeniers, 2004; Scoccia et al., 2012; Li et al., 2014).

TH may interfere with estrogen activity in its target tissues, including the reproductive tract. A possible explanation of this phenomenon could be derived from the observation of a crosstalk between estrogen receptor (ER) and TR on estrogen-responsive physiological promoters: interactions between different ER/TR isoforms have been shown to elicit different transcriptional effects (Vasudevan et al., 2001). Hypothyroidism is known to reduce the uterine cells' estrogenic response, resulting in development of reduced endometrial thickness (Inuwa and Wiliams, 1996).

It is also worth mentioning the well-known stimulatory effects of TH on hepatic expression of SHBG (sex hormone-binding globulin). In hypothyroidism conditions SHBG serum level is reduced leading to decreased level of total estradiol and increased level of free estradiol while, *vice versa*, in hyperthyroidism conditions the increased level of SHBG leads to an increase of circulating total estradiol, with normal or reduced free estradiol level (Redmond, 2004). In addition, the metabolic clearing rate of estradiol is reduced in both hypo- and hyperthyroidism conditions (Redmond, 2004). Thus, by modulating estradiol metabolism, TH could have profound effects on regulation of the hypothalamic-pituitary-ovarian axis, as well as on the proliferation and maturation of endometrial tissue and consequently on implantation and early development of the blastocyst.

TSHR and TR in the ovary

Both T_3 and T_4 have been found in human follicular fluid (Wakim et al., 1993). Moreover, granulosa cells and ovarian stromal cells express TR, thus representing a potential target for TH (Wakim et al., 1993, 1994; Zhang et al., 1997). Activation of the ERK1/2 pathway by a nongenomic TH action is involved in maturation of pre-ovulatory follicle and oocyte-cumulus cell (CC) complexes in mice (Su et al., 2003), as well as in meiotic maturation of bovine, pig and cattle oocytes (Tomek et al., 2002, Ellederová et al., 2008). *In vitro* exposure of human ovarian surface epithelial cells to T_3 was shown to cause a dose-dependent increase of expression of inflammation-related genes, i.e. cyclo-oxygenase-2, MMP-9, 11β-hydroxysteroid dehydrogenase type 1, and also of ERα (Rae et al., 2007). The evidence that severe hypothyroidism is associated with development of polycystic ovaries (Van Voorhis et al., 1994 Du and Li., 2013) and that treatment with levothyroxine can reduce the number, dimension and shape of ovarian cysts, and prevents their enlargement (Lindsay et al., 1983) indicates that ovarian follicles are influenced not only by LH, FSH and ovarian hormones but also by TH (Fitko et al., 1996). The presence of TRα1, TRβ1, and TRβ2 mRNAs in mature oocytes from IVF patients suggests that the human oocyte may be directly responsive to T_3 (Zhang et al., 1997). TRα1 and TRα2 transcripts have been observed in immature oocytes as well (Zhang et al., 1997). As above mentioned, TRα2 is an alternatively spliced mRNA of the TRα gene which cannot function as a TR but, when very highly expressed, acts as a ligand-independent inhibitor of TH action (Koenig et al., 1989). Considering that maturation of the oocyte involves complex interactions between oocyte and surrounding CC and that TRα1, TRβ1, TRβ2, and TRα2 isoforms are expressed in CC samples (Zhang et al., 1997), it may be hypothesized that T_3 may influence maturation of the oocyte and the secretion of hyaluronic acid causing pre-ovulatory cumulus expansion. However, T_3 has no detectable effects on the process of cumulus expansion and meiotic maturation of the oocyte in the mouse (Cecconi et al., 1999), and TSH or T_4 added to cultures of human ovarian tissue have no effect on the development of follicles and oocytes (Aghajanova et al., 2009). Nevertheless, TH potentiates FSH-induced granulosa cell (GC) survival by inhibiting cell apoptosis and promoting cell proliferation (Zhang et al, 2013). TR mRNAs have been detected in human GC (Wakim et al., 1994), and mural GC have important roles in folliculogenesis, steroidogenesis and synthesis of follicular fluid. T_3 increases proliferation of human luteinized GC *in vitro*, and T_3 and free T_4 in follicular fluid potentiate the hCG-induced cAMP response (Goldman et al., 1993) and synergize with FSH to increase LH receptors and progesterone secretion by GC from small porcine follicles (Maruo et al., 1992). Human GC stimulated by TSH show a significant increase in cAMP concentration, and exposure to T_4 results in increased ERK1/2 activation (Aghajanova et al., 2009).

TSH may also affect ovarian steroidogenesis as well as oocyte maturation, being present in human follicular fluid with levels positively correlated to blood concentration (De Silva, 1994). TSHR mRNA has been identified in oviducts and ovaries of mature and immature rats and the transcript increases immediately after the injection of pregnant mare's serum gonadotrophin or hCG, and may be regulated by steroid feedback, suggesting its function to be involved in the regulation of folliculogenesis and luteinization (Sun et al., 2010).

However, the findings that TSHR and TR are expressed by GC and the oocyte at different stages of follicular development, and that TSH and TH can be detected in follicular fluid have not been followed by a clarification of their local role. These molecules apparently have no role in oocyte maturation in the short term, but seem to influence GC survival and steroidogenesis (Wakim et al., 1994; Cecconi et al., 1999; Rae et al., 2007; Aghajanova et al., 2009; Zhang et al., 2013).

To conclude, clinical evidence that hypothyroidism deeply alters ovarian function and can be associated with polycystic ovary syndrome (Benetti-Pinto et al., 2013) or to ovulation alterations (Poppe and Velkeniers, 2004) confirms that TH play an important role in ovarian physiology. In neonatal and immature rats, TH seem to play an important role in the regulation of nitric oxide synthase (NOS) activity, whose signaling pathway is involved in ovarian follicular development (Fedail et al., 2013).

Thyrostimulin and ovarian functions

The receptors for TSH, FSH (FSHR) and LH (LHR), are all members of a G protein associated (GPA) receptor family that can be activated by heterodimeric glycoprotein hormones (TSH, FSH, LH, hCG). The latter share a common α subunit, which pairs with a unique β-subunit to establish receptor specificity, forming TSH (GPA1/TSHβ), LH (GPA1/LHβ), FSH (GPA1/FSHβ), and choriogonadotrophin (GPA1/CGβ). More recently, a new heterodimeric glycoprotein hormone, composed of α2 (GPA2) and β5 (GPB5) subunits, called thyrostimulin owing to its ability to activate TSHR *in vitro* and *in vivo*, has been added to the family (Nakabayashi et al., 2002). The exact role of thyrostimulin in thyroid physiology is still largely unknown. Transcripts GPA1/TSHβ for TSH and GPA2/GPB5 for thyrostimulin have been quantified in rat ovaries and, as compared with the negligible expression level of the TSHβ subunit, both GPA2 and GPB5 appear to be expressed in the ovary of gonadotrophin treated rats. Thyrostimulin is present in the oocyte and, as a paracrine factor, can activate cAMP and the *c-fos* nuclear

cascade in GC through TSHR (Sun *et al.*, 2010). The action of thyrostimulin can take place immediately after gonadotrophin stimulation, because gonadotrophins not only stimulate follicle growth and luteinization but also increase TSHR in GC via cAMP, while estradiol is likely to play the opposite role, by inducing a decrease in ovarian TSHR expression (Sun *et al.*, 2010). Hence, the finding that oocyte-derived thyrostimulin acts on GC-expressed TSHR indicates that a paracrine thyrostimulin-based system, tightly regulated by gonadotrophins, is present in the ovary and is involved in pre-ovulatory follicle maturation. In conclusion, since thyrostimulin is considered as the most ancestral glycoprotein hormone, its presence in the ovary may have some, yet to be discovered, primitive function in reproduction (Nakabayashi *et al.*, 2002; Sun *et al.*, 2010).

Thyroid Hormone Function During the Implantation Window

While direct evidence of the influence of TH on the implantation process is lacking, clinical findings indicate that TH could be involved in regulation of the implantation mechanism. Several data on TH involvement in early embryo development have been obtained in assisted reproduction technology studies. Supplementation of maturation, fertilization and culture media with TH increases bovine embryo cleavage, blastocyst formation and hatching rates (Ashkar *et al.*, 2010; Costa *et al.*, 2013). TH supplementation increases embryo quality as well, by reducing the level of apoptotic bodies. Furthermore, TH can increase the expansion rate of the blastocoel cavity of cryopreserved bovine embryos, probably by acting at a metabolic level (Ashkar *et al.*, 2010).

Concerning the role of TH in endometrial receptivity, an association between thyroid function and LIF expression has been highlighted (Stavreus, 2012) (Fig. 3B). An inverse correlation between grade of hypothyroidism and LIF levels has been proposed, since in the monkey administration of methimazole increases LIF and TSH serum levels, reducing at the same time T_3 and T_4 levels (Ren *et al.*, 1999). The expression of LIF and its receptor (LIFR) in endometrial stromal cells is increased also by TSH, which might be further involved in endometrial glucose transport, since TSH stimulation causes increased expression of the glucose transport protein GLUT1 in the Ishikawa cell line (Aghajanova *et al.*, 2011) (Fig. 3B). Furthermore, it should be recalled here that the leptin/leptin receptor system appears to be a fine regulator of the implantation process, as suggested by the differential expression of leptin receptors in implantation and inter-implantation sites (Yoon *et al.*, 2005), and the clinical association between a significantly lower expression of leptin and higher expression of leptin receptors in the endometrium of women with unexplained recurrent implantation failure, as compared with fertile women (Menendez *et al.*, 2003; Dos Santos *et al.*, 2012; de Oliveira *et al.*, 2013).

Several studies indicated that in primary hypothyroidism circulating leptin increases in parallel with TSH, while the opposite is true with primary hyperthyroidism. Leptin itself directly stimulates TRH and subsequently TSH and thyroid function, and it can stimulate T_3 production *via* an activation of T_4 to T_3. Nevertheless, leptin appears to have a direct inhibitory effect on several components involved in TH production by thyrocytes. Peripherally, both TH and leptin might be involved in adaptive thermogenesis, with TH acting as possible mediators of the effect of leptin on energy expenditure. In recent years the relationship between TH and leptin has been extensively studied but conflicting results have emerged, and further studies are still needed to clarify their combined effects on different tissues (Feldt-Rasmussen, 2007). TH may influence angiogenesis and immune function during implantation as reported for other systems (Pinto *et al.*, 2011; De Vito *et al.*, 2012). Angiogenesis is considered as a crucial event for successful implantation, decidualization and placentation. Increased vascular permeability and neoangiogenesis are mostly regulated by the VEGF, and genes encoding VEGF isoforms and their receptors are differentially expressed in the mouse uterus following a precise spatio-temporal regulation (Dey *et al.*, 2004). Their effects are complemented and coordinated by another class of angiogenic factors, the angiopoietins (Maisonpierre *et al.*, 1997; Dey *et al.*, 2004). While VEGF takes part in the attachment phase and the early stages of vessel development, the angiopoietins act later in the implantation process, to promote angiogenic remodeling, vessel maturation and stabilization (Smith, 2000; Cöl-Madendag *et al.*, 2014). It has been clearly demonstrated that TH, through both genomic and nongenomic mechanisms, exert a proangiogenic role in a variety of animal models, including (limb ischemia, myocardial ischemia, and the neovascularization required by tumor masses (Tomanek and Busch, 1998; Tomanek *et al.*, 2004; Yalcin *et al.*, 2010). The pro-angiogenic role of TH is initiated nongenomically at the cell surface through integrin αvβ3, which can bind at its Asp-Gly-Asp (RGD) recognition site a number of extracellular matrix (ECM) proteins and also TH (Bergh *et al.*, 2005). Through the MAPK ERK1/2, this binding induces the transcription of several angiogenesis-relevant genes, such as fibroblast growth factor (bFGF) and VEGF (Luidens *et al.*, 2010). In the chorioallantoic assay, the addition of anti-bFGF protein blocks the proangiogenic effect of TH (Davis *et al.*, 2009). MAPK is also known to activate TRβ1 and ERα thus creating a bi-directional molecular connection between an 'outside-in' (integrin – MAPK) and an 'inside-out' (MAPK – TRβ1, ERα) signaling' (Shen *et al.*, 2012). Furthermore, TH-activated ERK1/2 in turn activates members of the signal transduction and activator of transcription (STAT) family, which are involved in vascular growth, as demonstrated for STAT1, which transduces the VEGF signal, and STAT3, which is involved in VEGF gene expression. A TH analog, tetraiodothyroacetic acid, has been demonstrated to block T_4 and T_3 binding on the integrin receptor (Mousa *et al.*, 2008) and to interfere with crosstalk between αvβ3 and the adjacent plasma membrane receptors for vascular growth factors (VEGF-R and bFGF-R). It is interesting to note that T_3 has been shown to induce expression of HIF1α, which is involved in angiogenesis through the activation of phosphatidylinositol 3-kinase after binding to cytoplasmic TRβ and p85 (Moeller *et al.*, 2005, 2006).

Another important aspect of implantation in which the TH machinery might play a role, involves the immune system. Natural killer (NK) cells emerge as crucial modulators of implantation and placental angiogenesis (Xiong *et al.*, 2013). In women with recurrent spontaneous abortion or IVF failure the peripheral blood NK cell concentration and the level of NK cell cytotoxicity are higher than normal (Karami *et al.*, 2012). In addition, NK cell concentration is higher in patients with thyroid autoimmunity and recurrent spontaneous abortion or unexplained infertility (Kim *et al.*, 2011; Lazzarin *et al.*, 2012). Peripheral blood NK cells comprises 15% of blood lymphocytes (Robertson and Ritz, 1990), while uterine NK (uNK) cells are the predominant leukocyte population present at the time of implantation and early pregnancy (Fig. 3B), providing appropriate cytokine support and immunomodulation to regulate the process of decidualization (King, 2000), placental trophoblast growth (Jokhi *et al.*, 1994) and invasion (Saito *et al.*, 1993; Seshadri and sunkara, 2013).

Addition of TSH to NK cells augments their response to IL2, increasing their proliferation and response to various stimuli without modifying the basal level of cytotoxicity (Migita *et al.*, 1989; Provinciali *et al.*, 1992). Furthermore, the increased tumor necrosis factor-α release and TSH levels described in women with thyroid autoimmunity is accompanied by a 40% elevation in the peripheral mass of NK cells (Kim *et al.*, 2011). The importance of this evidence emerges from the consideration that peripheral NK cells, normally not present in substantial numbers in the uterus, could infiltrate the endometrium and, by altering the balance between NK and uNK cells, could compromise local immune and hormonal responses. A functional defect of a subpopulation of NK immune cells, involving both NK cytotoxicity and secretory activity, has been demonstrated in newly-diagnosed Graves' disease and Hashimoto thyroiditis patients (Solerte *et al.*, 2000) suggesting again their possible involvement in increased pregnancy losses (Konova, 2010). To make the issue more complex, TRα and TRβ are expressed on macrophages and dendritic cells (De Vito *et al.*, 2011), and TSH can be produced by T cells, B cells, splenic dendritic cells and bone marrow hematopoietic cells suggesting a role in the reciprocal modulation of immune system and thyroid function (Klein, 2006). Furthermore, nongenomic signals via the plasma membrane binding site αvβ3 may activate the mammalian target of rapamycin, mTOR, involved in immune regulation of chemotaxis, phagocytosis, generation of reactive oxygen species, and cytokine synthesis and release (De Vito *et al.*, 2012). The involvement αvβ3 in angiogenesis and immune regulatory activity of TH could create a link between thyroid and maternal-fetal dialogue, considering that functional blockade of αvβ3 on the day of implantation reduces in rabbit the number of implantation sites compared with controls (Illera *et al.*, 2003; Weetman, 2010; Carp *et al.*, 2012). Thus, integrin αvβ3 may play a critical role in the cascade of events regulated by TH leading to successful implantation.

Thyroid Autoantibodies and Implantation

As previously mentioned, transcripts required for TH synthesis, such as TPO and Tg, are expressed in the endometrium where they may be responsible for local thyroxine production (Catalano *et al.*, 2007). On the other hand, the expression of such thyroid-specific genes makes the endometrium susceptible to the action of anti-TPO and anti-Tg autoantibodies. The relationship between antithyroid antibodies and fertilization rate, implantation and pregnancy rate following IVF and embryo transfer (IVF-ET) in patients positive for antithyroid antibodies, in comparison to patients without thyroid autoimmunity, has been evaluated in different studies (Kim *et al.*, 1998a; Revelli *et al.*, 2009; Zhong *et al.*, 2012). The results obtained clearly indicate that patients with antithyroid antibodies show significantly lower fertilization, implantation and pregnancy rates as well as a higher risk for abortion following IVF-ET.

Thyroid Hormone Activity Past the Implantation Window

Thyroid hormone action on trophoblast cells

Differentiation of cytotrophoblast to syncytiotrophoblast or EVT cells is precisely controlled by different agents, such as specific genes, transcriptional factors, hormones, growth factors, cytokines and O_2 levels, whose altered expression increases the risk of developing pre-eclampsia, IUGR and premature rupture of membranes (Aplin, 1997). TH are now emerging as factors involved in the proliferation, stabilization, survival, endocrine and invasive function of trophoblast cells. TR are present in the villous placenta, within the nuclei of villous cyto- and syncytiotrophoblast, and their inappropriate expression is associated with obstetrical complications (Kilby *et al.*, 1998). Furthermore, deiodinases, which regulate TH effect within cells, have also been localized in villous syncytiotrophoblast cells. TRα1, TRα2, and TRβ1 isoforms can be immunohistochemically localized in both interstitial trophoblast and extravillous trophoblast, showing a more pronounced nuclear than cytoplasmic staining in extravillous trophoblast cells (Barber *et al.*, 2005) (Fig. 3C).

Differentiation and survival of the trophoblast

Implantation of the blastocyst, hemochorial placentation, and differentiation and invasion of the trophoblast cell lineage occur in a microenvironment with reduced oxygen concentration (Aplin., 1997, 2000). During the first trimester the trophoblastic villous layer is thicker compared with later in development and EVT cells invade spiral arteries and partially occlude them lowering the oxygen concentration to 2–3%, which has a protective role toward the embryo (Jauniaux *et al.*, 2003). Hypoxia induces deiodinase D3 *via* the HIF-1-dependent pathway (Simonides *et al.*, 2008) (Fig. 3B). Considering the D3 involvement in inactivation of both T_3 and T_4, its up-regulation would create a sort of local hypothyroidism, reducing T_3 dependent energetic expenditure (Simonides *et al.*, 2008). This D3 role is important not only during early pregnancy at uterine level but also in many other ischemic conditions, such as myocardial infarction, stroke and cardiomyopathies, when cells need to survive in a hypoxic environment (Paulding and Czyzyk-Krzeska, 2000; Lash *et al.*, 2002). HIF-1 stimulates *NOR1* transcription as well, which reduces the endothelial cell apoptotic rate in hypoxic environments (Martorell *et al.*, 2009). An additional way by which TH play a protective role against oxidative stress is by inducing mitochondrial antioxidant defenses (Chattopadhyay *et al.*, 2010). T_3-induced up-regulation of inducible nitric oxide synthase (iNOS) expression in rat liver protects against ischemia-reperfusion injury (Simonides *et al.*, 2008). In hypothyroid mice, in which mitochondria become susceptible to oxidative injury and the mitochondria-dependent antioxidant defence system is impaired, oxidative stress conditions in the testis heavily damages its function, leading to infertility (Zamoner *et al.*, 2008; Chattopadhyay *et al.*, 2010). The main protective mechanism by which TH act at placental level is, however, their influence on trophoblast apoptosis. Apoptosis is an important determinant in regulating placental growth, which is more evident in the invasive EVT than in its proliferative counterpart, and is associated with increased Fas and Fas ligand expression and reduced Bcl-2 protein expression (Murakoshi *et al.*, 2003) (Fig. 3C). At the physiological concentration of 10^{-8} M, T_3 suppresses apoptosis of early placental EVT in culture by inhibiting Fas and Fas ligand expression and caspase-3 and PARP cleavage (Laoag-Fernandez *et al.*, 2004).

An additional factor intervening in regulation of the placental apoptosis process is EGF. Human early placental trophoblast is capable of producing an EGF-like substance, and its local production is enhanced by TH (Matsuo *et al.*, 1993) (Fig. 3A). These observations suggest that an autocrine/paracrine control system, which includes TH, plays a role in placental growth and function in humans (Table I).

Production of steroids and glycoprotein hormones

Treatment with 10^{-8} M T_3 was shown to enhance the secretion of an EGF-like substance by cultured early placental explants (Barber *et al.*, 2005), while treatment with higher (10^{-5} M) or lower (10^{-10} M) concentrations had no stimulatory effect (Matsuo *et al.*, 1993). These results are interesting, considering that EGF, *via* its receptors on the syncytiotrophoblast, was found to stimulate the release of both hCG and human placental lactogen (hPL) in normal early placenta (Maruo *et al.*, 1991) and inhibit cytokine-induced apoptosis of primary trophoblasts (Garcia-Lloret *et al.*, 1996). Addition of T_3 (10^{-8} M) or T_4 (10^{-7} M) to cultures of trophoblasts obtained from normal early placentas raised daily secretion of progesterone, 17β-estradiol, hCG and hPL (Maruo *et al.*, 1991). With the concomitant addition of pregnenolone and testosterone to 10^{-8} M, T_3 further increases progesterone and estradiol secretion, respectively, suggesting that T_3 enhances 3β-hydroxysteroid dehydrogenase and aromatase activity in the placenta (Maruo *et al.*, 1991) (Fig. 3C). Higher or lower T_3/T_4 concentrations gave attenuated responses. Thus, TH stimulation of trophoblast endocrine function may not only be mediated through the induction of an EGF-like substance but also be a consequence of TH direct action. Unlike early placental tissues, term placental tissues in culture did not respond to the addition of T_3 or T_4 with increased endocrine activity, probably as a consequence of a lower binding capacity of their nuclear TR (Maruo *et al.*, 1991). Since TH concentrations stimulating trophoblast endocrine activity *in vitro* are within the physiological range of TH in human plasma, it is very likely that TH plays a physiological role as an enhancer of trophoblast endocrine function. The presence of such TH concentrations in the placenta then appears to be an important factor in the mechanisms regulating the increasing levels of progesterone and hCG that are required in early pregnancy. In conclusion, the frequent occurrence of spontaneous abortion in early pregnancy might, in certain instances, be a direct consequence of inadequate TH availability at placental trophoblast level (Maruo *et al.*, 1991).

Extravillous trophoblast invasiveness and metalloprotease expression

The establishment of anchoring villi and subsequent invasion of maternal uterine stroma and blood vessels play a critical role in pregnancy success, as inadequate vascular invasion is associated with common pathological conditions of pregnancy including pre-eclampsia and growth retardation (Robertson *et al.*, 1985). The invasion of maternal uterine tissues by EVT cells anchors the placenta and the fetus to the endometrium, enabling the conceptus to gain access to the maternal circulation (Robertson *et al.*, 1985). Invasion is based on the degradation of endometrial ECM and the expression of cell adhesion molecules by EVT (Bischof and Martelli, 1992; Aplin, 1997). The invasive potential of EVT begins with polar degradation of ECM in the direction of migration, followed by the suppression of the degradation to moderate the invasion, the binding of cells to ECM, and finally the active movement through the matrix (Oki *et al.*, 2004). ECM digestion is achieved by specific enzymes, the MMPs, and is limited by their tissue inhibitors (TIMPs). The distribution pattern of MMPs and TIMPs in invading EVT has been extensively investigated, and a pre-eminent role of MMP-2, MMP-3 and TIMP-1 has emerged (Maruo *et al.*, 1991; Takino *et al.*, 1995; Oki *et al.*, 2004). EVT anchoring to ECM is established through integrins, which are cell surface receptors for matrix proteins. The plasma membrane phenotypes of cytotrophoblast change during the invasion, with a decrease in $\alpha_6\beta_4$ integrin and a rapid increase in $\alpha_5\beta_1$ integrin (Oki *et al.*, 2004).

Using the *in vitro* Matrigel invasion assay it was observed that treatment with T_3 significantly increases the number of cell projections of invading EVT (Oki *et al.*, 2004). In addition, T_3 stimulates mRNA expression of MMP-2, MMP-3, oncofetal fibronectin (onfFN) and integrin $\alpha5\beta1$, and the synthesis of MMP-2 and MMP-3 (Oki *et al.*, 2004). This molecular deployment facilitates EVT migration through uterine ECM. MMP-2 hydrolyzes collagen IV, which is abundant in any ECM, while the main targets of MMP-3 are fibronectin, expressed more in the depths of ECM, and collagen IV. The onfFN is synthesized and deposited at sites of trophoblast-ECM contact and, together with integrins, favors cell migration into the matrix. The evidence that the expression of TIMP-1 protein in EVT cultures is not affected by T_3 treatment (Oki *et al.*, 2004) strengthens the hypothesis that TH promote trophoblastic invasion of maternal tissue. As a consequence, higher than normal TH concentrations during early pregnancy might be associated with uncontrolled and dangerous penetration of EVT into uterine stroma. As previously described, T_3 suppresses apoptosis by down-regulating the expression of Fas and Fas ligand (Laoag-Fernandez *et al.*, 2004), and these findings are consistent with the hypothesis that T_3 promotes EVT decidual invasion also by suppressing apoptosis in early pregnancy (Fig. 3C).

The EVT-derived cell line SGHPL-4 (immortalized EVT-like cell line), a suitable *in vitro* model for studying EVT invasion mechanisms, exhibits increased motility and invasive characteristics when subjected to stimulation with EGF, and T_3 seems to have modulating effects on SGHPL-4 migration and invasion stimulated by EGF (Barber *et al.*, 2005). However, the reduction of the number of invasive processes caused by T_3 on SGHPL-4 cells *in vitro* is in contrast with the evidence that T_3 increases the expression of molecules involved in invasion (Oki *et al.*, 2004).

Cell line models give contrasting results concerning TH effects on placental cell proliferation. SGHPL-4 cells become less proliferative when exposed to T_3, while JEG3 choriocarcinoma cells show increased proliferation; in addition, the survival of primary cultures of nonproliferative term cytotrophoblast is not influenced by T_3 (Barber *et al.*, 2005). In our opinion, the apparently conflicting data may be due to the different cell populations used as models for EVT cells: neoplastic cells and cultures from anchoring chorionic villi. In addition, TH might act late in the differentiated functions of nonproliferative EVT and villous syncytiotrophoblast, enhancing endocrine activity rather than proliferation, as previously described, and regulating invasion and motility.

Conclusion and Perspectives

The increased incidence of pregnancy loss in women with slightly abnormal TSH levels and hypothyroxinemia suggests the possibility that TH might be involved in endometrium preparation to pregnancy and initial trophoblast development. In the present paper we have reviewed data showing that TH may have a potential paracrine and intracrine role at uterine level during embryo implantation and early EVT development through their TR present in these tissues (Tables I and II). TH appear to be transported through placental tissue by membrane transporters detected on the syncytiotrophoblast. Molecules present in the cytoplasm or on the surface of the syncytium are involved in regulating access of T_3 to the nucleus, where it activates gene transcription. Among these are the deiodinases and molecules involved in

ubiquitination or inactivation of deiodinases, which determine the ratio between T_3, T_4 and rT_3, as well as the TH-binding proteins TTR and albumin, upon which depends the availability of free TH. Furthermore, placental transport of TH appears to be polarized from the maternal circulation to the fetus; different factors and molecules (e.g. oxygen) modulate this process, suggesting that a fine regulation of TH supply is essential during early pregnancy. Based on the presence of thyroid-specific genes involved in TH production (i.e. NIS, pendrin, Tg and TPO) in endometrial and syncytiotrophoblast cells, it may be speculated that a mechanism of local synthesis of TH, at the uterine level, exists. Other molecules important for implantation and early embryo development might regulate this machinery beyond the classical hypothalamic–pituitary–thyroid axis. Alteration of some of these factors may locally alter the TH effects even when systemic TH levels are normal and exerting their physiological role in other tissues. If this hypothesis is proven, TH blood concentrations would not necessarily indicate the real hormone availability and function at endometrial level, and different tests would be required to evaluate the local efficacy of TH. Another fundamental TH related event, angiogenesis, occurs in the endometrium during placentation, and alterations in the formation of the vascular network may be involved in obstetric complications such as pre-eclampsia and IUGR. Finally, based on the available data, a role of TH in the modulation of feto-maternal tolerance might be speculated, although this aspect needs to be further investigated.

In conclusion, all the experimental and clinical evidence reported in the present review clearly suggest that TH are essential players in the mechanisms regulating implantation and early fetal development. This warrants further studies aimed to better define the molecular details of TH action in these fundamental biological processes, which would help in solving infertility problems associated with thyroid dysfunction.

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Authors' roles

M.C. and C.M. contributed equally to determining the scope of the review. All authors contribute to the first draft of the manuscript and approved the final manuscript for submission.

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Conflict of interest

None declared.

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