

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

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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: T₄ and T₃) 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: T₄ and T₃) 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: TIMPs) 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 (HIF1 α), 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, HIF1 α 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 1 Thyroid-dependent molecules at the endometrium, ovary and blastocyst level around the implantation window.

Localization	Molecules	Evidence and possible role (PR)	Species	Ref.
Ovary	TR α 1, TR α 2, TSHR, TR β 1, DIO2, DIO3 (mRNAs)	T3 = dose-dependent mRNA expression of inflammation-associated genes: COX-2, MMP9, 11 β HSD1, ER α .	Human	Du and Li (2013)
OSE			Human	Fedail et al. (2013)
Primordial follicle:	TR α 1, TR α 2, TR β 1, TSH (proteins)	PR: Chronic hyperthyroidism might contribute to low-level of inflammation rendering OSE more susceptible to neoplastic transformation.	Pigs Cattle	Sun et al. (2010)
Primary follicle:	O: TSHR, TR α 1, TR β 1 (mRNAs, proteins)		Mice	Aghajanova et al. (2009)
Secondary follicle:	c-erbA α -2 (mRNA)	TSH = production of cAMP by luteinized GC	Bovine	Rae et al. (2007)
Antral follicle:	O:TSHR, TR α 1, TR β 1 (mRNAs, proteins)	T4 = production of ERK 1/2 in luteinized GC (maturation of pre-ovulatory follicle and OCC; meiotic oocyte maturation)	Rat Human	Zhang et al. (1997)
Follicular fluid:	GC: TSHR (mRNAs, proteins)	Gonadotrophins = increase TSHr	Human	Su et al. (2003)
Oocyte:	O: TSHR, TR α 1, TR β 1 (mRNAs, proteins)	Estradiol = decrease TSHr	Human	Tomek et al. (2002)
	GC: TSHR, TR β 1 (mRNAs, proteins)	Thyrostimulin acts through TSHr to increase cAMP	Human	Cecconi et al. (1999)
	O: TR α 1, TR β 1, TR β 2, c-erbA α -2 mRNAs	T3 = stimulation of	Porcine	De Silva (1994)
	GC: TSHR, TR α -1, TR β -1 (mRNA, proteins)	GC proliferation and hCG-induced cAMP in GC; synergy with FSH to induce differentiation of GC increasing LH receptors and progesterone secretion by GC from porcine follicles ; synergy with FSH to inhibit apoptosis of GC	Mice	Wakim et al. (1993)
	TR β -2, c-erbA α -2, DIO2, DIO3 mRNAs	T3 inhibits in a time- and dose-dependent manner FSH-induced aromatase activity in GC	Mice	Goldman et al. (1993)
	CC: TR α -1, TR β -1, TR β -2, c-erbA α -2 mRNAs	high T3 concentrations can impair preantral follicle development	Mice	Maruo et al. (1992)
	FT3, FT4, TSH	PR: T3, T4 in FF = regulation of human GC and steroid production	Mice	Maruo et al. (1987)
	Thyrostimulin	PR: Thyrostimulin = paracrine modulator of ovarian function controlled by a different system from HPT axis.		
Blastocyst (in vitro)	TH receptors mRNA and proteins DIO2 and DIO3 mRNAs (T3 produced in the bovine follicular fluid is an indirect indicator of DIO1 mRNA) TR α = 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) (in matured oocytes higher than in the germinal vesicle stage oocyte)	TH on Early Embryo development = increase blastocyst formation and hatching rate; improve embryo quality (greater total cell counts and reduced proportions of apoptotic cells); improve post-cryopreservation viability.	Bovine Human	Costa et al. (2013) Degrelle et al. (2013) Loubiere et al. (2012) Ashkar et al. (2010)
Endometrium	TSHR, TR α 1, TR β 1 (mRNA and proteins),	Mifepristone: down-regulation of TR α 1 and TR α 2; up-regulation of TR β 1 and DIO2 mRNA (regulation by PR)	Human	Li et al. (2014)
Glandular epithelium	TSHR, TR α 1, TR β 1 (mRNA and proteins)		Human	Scoccia et al. (2012)
Luminal epithelium	TSHR, TR α 1, TR β 1 (mRNA and proteins)	TSHR in the luminal epithelium and TR α 1 and TR β 1 in the glandular and luminal epithelium increase on LH days 6 to 9 (WOI, appearance of pinopodes)	Human	Dimitriadis et al. (2010)
Stroma	(TR α 2: +ve WB, -ve immunostaining, +ve RT-PCR)	Expression of DIO 1,2,3 mRNAs lower in the mid-secretory phase of the cycle	Human	Aghajanova et al. (2011)
Oviduct and uterine horn	NIS (sodium-iodide symporter) P4HB (a TG molecular chaperone), TG (thyroglobulin), TPO (Thyroid peroxidases), factors involved in thyroid hormone signalling DI 1, D2 (most abundant), D3 THR	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 T3 and GH = required for decidual response of the endometrium PR: endometrium site of thyroxine production PR: generation of appropriate T3 level to contribute to decidual response to implantation	Human Rat	Catalano et al. (2007) Kennedy and Doktorcic (1988)

Continued

Table 1 Continued

Localization	Molecules	Evidence and possible role (PR)	Species	Ref.
Other possible candidates			Mice	De Vito et al. (2011)
NK cells		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	Human	Weetman (2010)
Leptin		TSH releases leptin in human adipose tissue culture and leptin/leptin receptor system may be a delicate regulator of the implantation process	Mice	Provinciali et al. (1992)
Angiogenesis		TH = promotion of angiogenesis through genomic and nongenomic mechanisms. Transcription of bFGF and VEGF through MAPK and ERK1/2, binding on integrin α v β 3; Increase of Ang-2; PI3K activation through association with cytoplasmic TR β and p85 and transcription of HIF1 α , T4 induce FGF2 release through MAPK from endothelial cells activating STAT involved in vascular growth; tetraoc blocks T4 and T3 binding on the integrin receptor interfering with TH crosstalk with receptors for vascular growth factors (VEGF-R, bFGF-R).	Human	Migita et al. (1989)
			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)

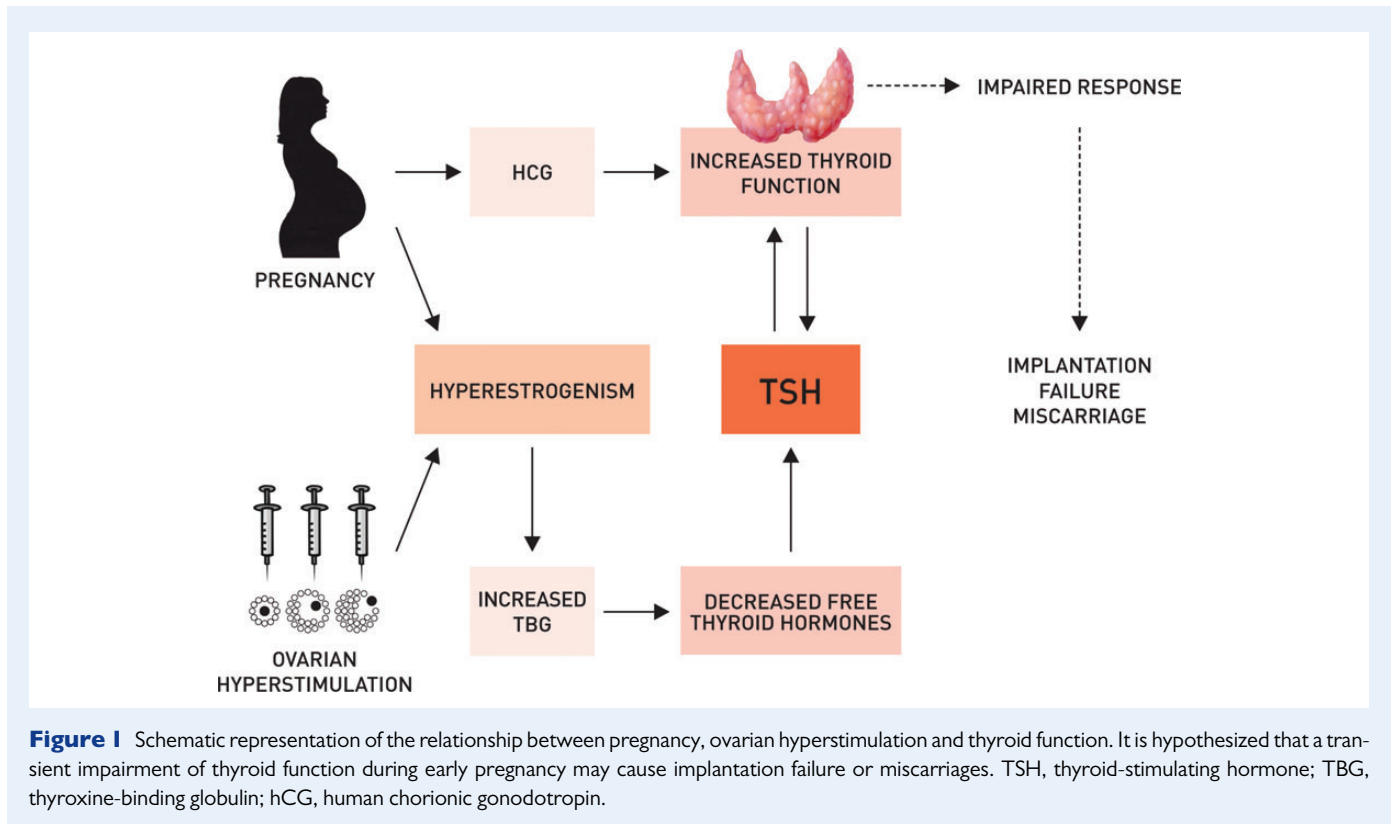
Ang-2, angiotensin 2; bFGF, basic fibroblast growth factor; bFGF-R, basic fibroblast growth factor receptor; cAMP, cyclic adenosine monophosphate; CC, cumulus cells; c-erbA α -2, alternative spliced product of TR α gene (non-T3 binding protein); COX-2, cyclooxygenase-2; D2, Type II iodothyronine deiodinase (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; ERK 1/2, extracellular signal-regulated kinase 1/2; ER α , 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; HIF1 α , Hypoxia-inducible factor 1- α ; 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; P3K, phosphatidylinositol 3-kinase; PR, progesterone; STAT, signal transducer and activator of transcription; T3, Triiodothyronine; T4, Thyroxine; TG, thyroglobulin; TH, thyroid hormones; TR α 1, thyroid hormone receptor α 1; TR α 2, thyroid hormone receptor α 2; TR β 1, thyroid hormone receptor β 1; 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; β 1, β -HSD1, β 1 beta-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 μ g/day for adults (Abalovich et al., 2007). Intake should be raised to 200–300 μ g/day during gestation because of the above described increased maternal T₄ requirement and of fetal thyroid function, and to compensate for the enhanced pregnancy-related urinary iodide excretion (Hetzl, 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 chorionicarcinoma 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 chorionicarcinoma 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 homeo-domain 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



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).

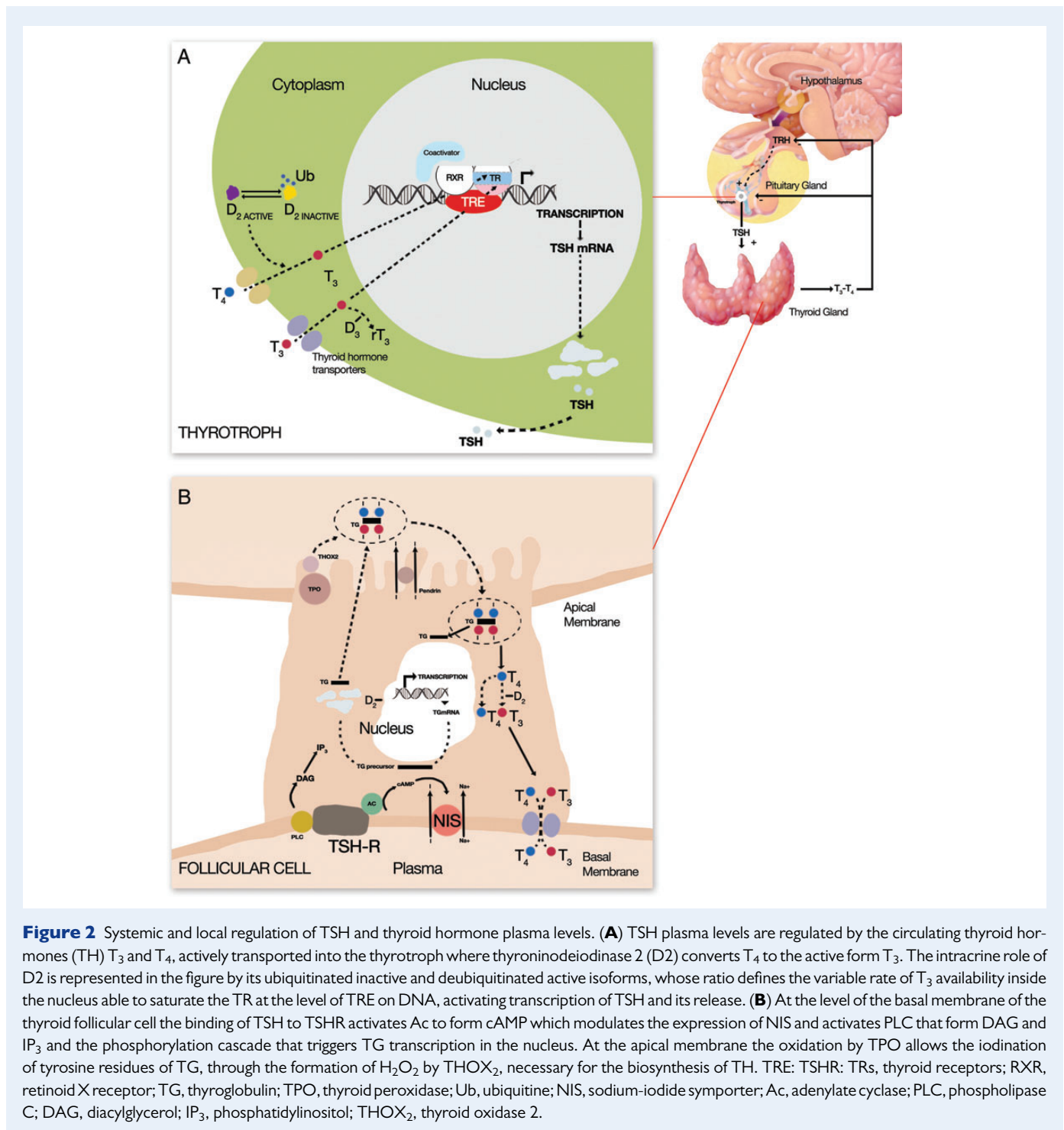
Additional regulatory factors, such as O_2 , are involved in NIS synthesis at placental level, but their role has not been fully elucidated. As mentioned above, during pregnancy O_2 tension gradually increases in the placenta from very low levels ($\sim 1\%$ in the first 6 weeks) to a plateau of 8% by 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



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

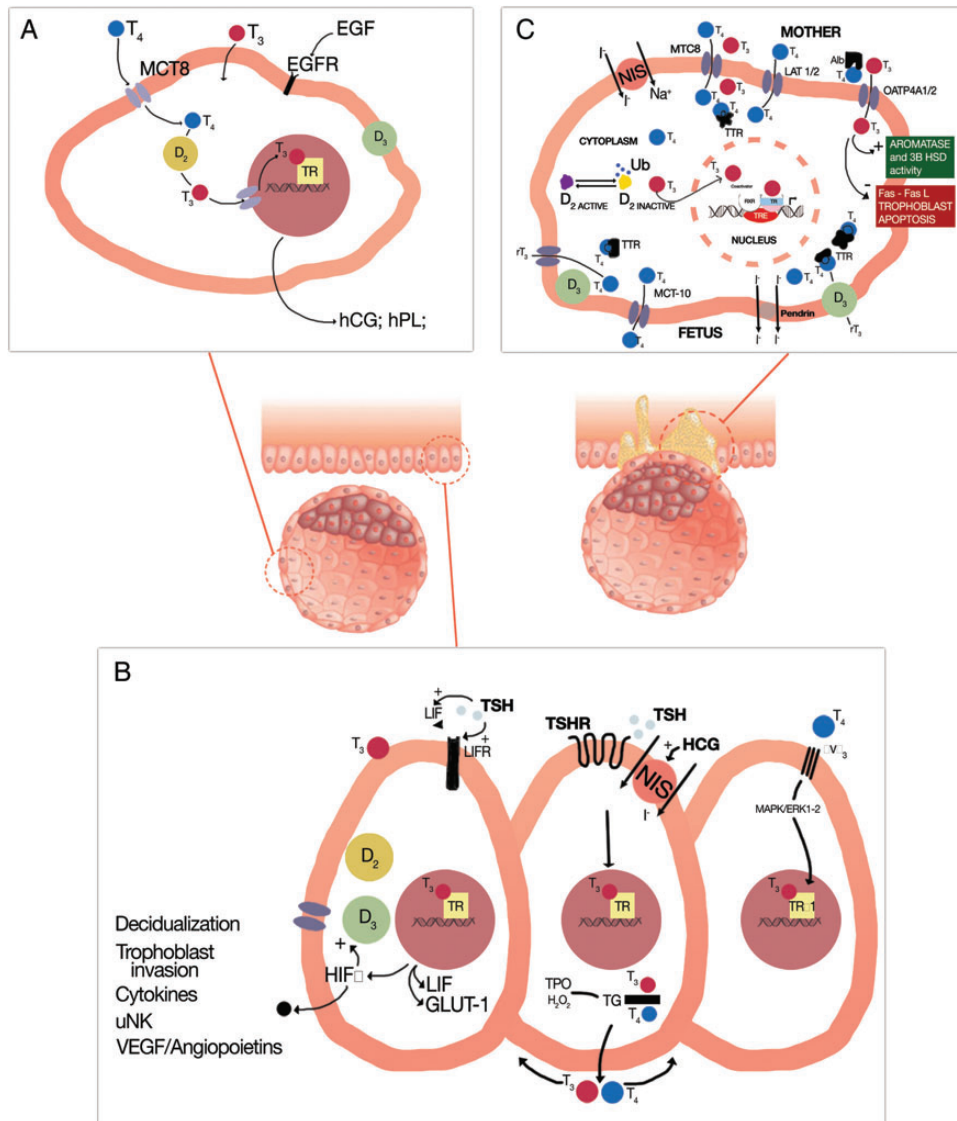


Figure 3 Thyroid hormone signaling is involved in the bi-directional dialogue between competent blastocyst and endometrium. **(A)** Before the window of implantation (WOI), in the early luteal phase, trophoblast cells express TRs, thyroid hormone transporters, mainly MCT8, and deiodinases. Both T_3 and 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 HIF α , possibly controlled by nongenomic pathways. **(C)** Past the WOI and during the early weeks of pregnancy, membrane polarization of NIS and Pendrin on syncytiotrophoblast cells resembles that present in the thyroid follicular cells. TH transporters, such as MCT8, MCT10, OATP4A1, LAT1 and OATP4A2, 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). rT_3 , reverse T_3 ; 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 amino acid transporter; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; GLUT-1, glucose transporter-1; HIF α , hypoxia-inducible factor α ; Ub, ubiquitin; TTR, transthyretin; 3 β HSD, 3 β hydroxysteroid dehydrogenase; 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 1 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 VSB-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 T₃ 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 fetoplacental unit (Table II) have been only partially demonstrated and further studies are needed.

Thyroid hormone receptors, coactivators, corepressors and modulators of T₃ 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 TR α 1 and TR α 2; THRB encodes TR β 1 and TR β 2. TR α 1, TR β 1 and TR β 2 bind TH, while the TR α 2 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 (9cis-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). T₄ 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 TR β 1 inducing its stabilization and reduction of affinity for CoR (Lin *et al.*, 2003). Davis and colleagues identified a structural plasma membrane protein, the integrin $\alpha\beta$ 3, 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, TR α and TR β conjugation with small ubiquitin-like modifier (SUMO) has been described (Liu *et al.*, 2012). In particular, it has been demonstrated that TR α and TR β sumoylation modulates T₃ action and TR activity (Liu *et al.*, 2012). SUMO1 has been shown to be essential for T₃-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 T₃-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). TR α 1 and TR β 1 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, TR α 1, TR α 2 and TR β 1 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, prollyl 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 antiprogesterin that makes the endometrium un-receptive and induces menstrual bleeding, reduces the expression of TR α 1, TR α 2 and Tg, while inducing that of TR β 1 and D2 (Catalano

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

Molecules	Localization	Evidence and possible role	Species	Ref.
<i>TH binding proteins</i>	ST	<i>Binding TH to prevent deiodination</i>	Hum	Landers et al. (2013a)
TTR (<i>HA</i>)	Synthesis (TTR, Alb, α IAT, β I gpa)	<i>Regulation of T3 access to the nucleus</i>	Hum	Landers et al. (2013b)
Albumin (<i>HA</i>)	Secretion (TTR, Alb)	(1) <i>Low oxygen = incr TTR</i>	JEG3	Patel et al. (2012)
α -1-antitrypsin (<i>LA</i>)	Internalization (TTR)			Patel et al. (2011a)
β -1-acid glycoprotein (<i>LA</i>)				Landers et al. (2009) McKinnon et al. (2005)
<i>Iodide transport</i>	ST	<i>Iodide influx into cells from mat. circulation</i>	BeWo	Degrelle et al. (2013)
NIS	Apical membrane (maternal side)	<i>Iodide efflux to the extracellular space towards the fetus</i>	Hum	Manley et al. (2005)
Pendrin	Basal membrane (fetal side)	(1) <i>TSH, hCG = incr NIS</i> (2) <i>Low oxygen = decr NIS</i>		Mitchell et al. (2001) Li et al. (2011) Arturi et al. (2002)
<i>Membrane transporters</i>	ST (apical), CT, EVT, decidual stroma	<i>TH uptake from maternal blood and passage through the cellular membrane/efflux to the fetus</i>	Hum	Visser (2013)
MCT 8		<i>alteration in IUGR</i>	Hum	Loubiere et al. (2012)
MCT 10	CT (basal), ST, EVT, decid strom,			Vasilopoulou et al. (2010)
LAT 1	villous strom			Loubiere et al. (2010)
OATPIA2	ST			Visser et al. (2008)
	ST (apical) CT EVT			Chan et al. (2006)
<i>TH receptors</i>	Endovascular/interstitial EVT	<i>Regulation of differentiated trophoblast (endocrine function, invasion and motility); interaction with factors involved in proliferation and stabilization.</i>	Hum	Maruo (2010)
TR α 1		(1) <i>T3 (10^{-8} M) = EGF-like molecules, hPL, hCG</i>	SGHPL-4	Barber et al. (2005)
TR α 2		(2) <i>T3 (10^{-8} m) / T4 (10^{-7} m): Progesterone; (incr 3βHsd) 17βestradiol; (incr aromatase)</i>	Hum	Oki et al. (2004)
TR β 1		(3) <i>T3 in synergy with EGF: incr motility and influence invasion SGHPL-4 (EVT cell)</i>	Hum	Maruo et al. (1991)
		(4) <i>T3 ($10^{-7/-9}$): decr apoptosis (inhibit Fas-FasL, Casp3 cleav and PARP)</i>	Hum	
		(5) <i>T3 (10^{-8} M): MMP2-MMP3 incr</i>		
<i>Deiodinases</i>	Villous CT ; ST (weak)	<i>Decrease during gestation</i>	Hum	Kurlak et al. (2013)
D2	Villous ST; CT (weak) uterine	<i>Conversion inactive T4 to T3</i>	Rodent	Patel et al. (2011b)
D3	decidua, umbilical cord vasa, perivascular myocyties of uterine arteria, fetus: umbilical vasa, respiratory and intestinal epithelium, urinary tract, skin	<i>Conversion T4 to rT3, T3 to T2</i> <i>Regulate maternal TH to fetal circulation. D3 protects the fetus from excessive mater TH</i>		Chan et al. (2009) Gereben et al. (2008) Wasco et al. (2003) Huang et al. (2003) Chan et al. (2003)

3 β HSD= 3 β hydroxysteroiddehydrogenase; 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 placental; 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, syncytiotrophoblast; T3, Triiodothyronine; TH, thyroid hormones; TR, Thyroid receptors; TSH, Thyroid-stimulating hormone; TTR, transthyretin; Villous strom, villous stroma; α IAT, α -1-antitrypsin; β I gpa, 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 Williams, 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, 11 β -hydroxysteroid dehydrogenase type 1, and also of ER α (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 TR α 1, TR β 1, and TR β 2 mRNAs in mature oocytes from IVF patients suggests that the human oocyte may be directly responsive to T_3 (Zhang *et al.*, 1997). TR α 1 and TR α 2 transcripts have been observed in immature oocytes as well (Zhang *et al.*, 1997). As above mentioned, TR α 2 is an alternatively spliced mRNA of the TR α 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 TR α 1, TR β 1, TR β 2, and TR α 2 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 (Ceconi *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; Ceconi *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/TSH β), LH (GPA1/LH β), FSH (GPA1/FSH β), and choriogonadotrophin (GPA1/CG β). More recently, a new heterodimeric glycoprotein hormone, composed of α 2 (GPA2) and β 5 (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/TSH β for TSH and GPA2/GPB5 for thyrostimulin have been quantified in rat ovaries and, as compared with the negligible expression level of the TSH β 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 $\alpha v \beta 3$, 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 TR β 1 and ER α thus creating a bidirectional molecular connection between an 'outside-in' (integrin – MAPK) and an 'inside-out' (MAPK – TR β 1, ER α) 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 $\alpha v \beta 3$ 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 HIF1 α , which is involved in angiogenesis through the activation of phosphatidylinositol 3-kinase after binding to cytoplasmic TR β 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, TR α and TR β 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 $\alpha v\beta 3$ 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 $\alpha v\beta 3$ in angiogenesis and immune regulatory activity of TH could create a link between thyroid and maternal-fetal dialogue, considering that functional blockade of $\alpha v\beta 3$ 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 $\alpha v\beta 3$ 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 O₂ 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. TR α 1, TR α 2, and TR β 1 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 T₃ and T₄, its up-regulation would create a sort of local hypothyroidism, reducing T₃ 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). T₃-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, T₃ 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 (onffFN) and integrin $\alpha_5\beta_1$, 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 onffFN 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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