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Thyroid hormone regulates protease expression and activation of Notch signaling in implantation and embryo development

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Abstract

A clinical association between thyroid dysfunction and pregnancy complications has been extensively reported; however, the molecular mechanisms through which TH might regulate key events of pregnancy have not been elucidated yet. In this respect, we performed *in vivo* studies in MMI-induced hypothyroid pregnant mice, evaluating the effect of hypothyroidism on the number of implantation sites, developing embryos/resorptions and pups per litter, at 4.5, 10.5, 18.5 days post-coitum (dpc) and at birth. We also studied the expression of major molecules involved in implantation and placentation, such as the proteases ISPs, MMPs, TIMPs and Notch pathway-related genes. Our results demonstrate that hypothyroidism may have a dual effect on pregnancy, by initially influencing implantation and by regulating placental development at later stages of gestation. To further elucidate the role of TH in implantation, we performed *in vitro* studies by culturing 3.5 dpc blastocysts in the presence of TH, with or without endometrial cells used as the feeder layer, and studied their ability to undergo hatching and outgrowth. We observed that, in the presence of endometrial feeder cells, TH is able to anticipate blastocyst hatching by upregulating the expression of blastocyst-produced ISPs, and to enhance blastocyst outgrowth by upregulating endometrial ISPs and MMPs. These results clearly indicate that TH is involved in the bidirectional crosstalk between the competent blastocyst and the receptive endometrium at the time of implantation.

Key Words

- ▶ blastocyst
- ▶ thyroid hormone
- ▶ placenta
- ▶ proteases
- ▶ implantation

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Introduction

Embryo implantation is one of the earliest and critical events of the mammalian reproduction. It occurs in the maternal uterus and allows the mother to nourish and

protect the embryo during its subsequent development, determining whether pregnancy will develop successfully. Once arrived in the uterus, the embryo has reached

the blastocyst stage and it is still protected by the zona pellucida (ZP), which prevents extra-ectopic implantation. In order to implant, the blastocyst has to hatch from the ZP. Hatching needs to occur in a well-defined timeframe, the window of implantation, when the molecular program regulating growth and differentiation of the embryo synchronizes with the molecular program regulating endometrial receptivity. Blastocyst hatching is a finely regulated process of crucial importance for the subsequent development of the embryo, allowing attachment of the blastocyst to the maternal tissue. Any dysregulation in the hatching process can lead to implantation failure and infertility (Petersen *et al.* 2005).

The hatching process is subject to both cellular and molecular controls and it begins with the formation of a nick in the ZP, in which the mechanical pressure exerted by the expanding blastocyst is involved. However, it has been demonstrated that hatching is not just a passive process that follows blastocyst expansion, but it also involves several regulatory molecules, which include growth factors (e.g. EGF, HB-EGF, TGF- β and LIF) (Mishra & Seshagiri 2000, Seshagiri *et al.* 2002), and the local production of potential zona-lysin, involved in the digestion of the ZP, produced by both the embryo and the endometrium (Vu *et al.* 1997, Mishra & Seshagiri 2000). These proteases may play a crucial role in the formation of the nick into the ZP. Recently, studies on mouse implantation have identified two new proteinases associated with hatching: Strypsin (ISP1) and Lysin (ISP2) (O'Sullivan *et al.* 2001, Sharma *et al.* 2006). ISP1 and ISP2 are two related S1-family serine proteinases localized on mouse chromosome 17. Isp1 and Isp2 genes are co-expressed in pre-implantation embryos and in the mouse uterine endometrium during the 'window of implantation' (O'Sullivan *et al.* 2001, Sharma *et al.* 2006, 2011). Sharma and coworkers have demonstrated a crucial role of ISPs, as antibodies against ISP1/ISP2 have been found to block murine embryo hatching and outgrowth (O'Sullivan *et al.* 2001).

Once escaped from the ZP, the embryo attaches to the uterine endometrium and trophoblast cells invade the endometrial tissue (Aplin *et al.* 2000, Kaneko *et al.* 2013) and contribute to the formation of the placenta, which is indispensable for the survival and growth of the embryo. Molecules involved in trophoblast invasion and placentation include growth factors and cytokines (EGF, HB-EGF, LIF and IL-1), hormones (hCG and progesterone), MMPs (which degrade the extracellular matrix of the uterine endometrium) and TIMPs (which limit the extent of trophoblast invasion). The balance between MMPs

and TIMPs strongly influences the process of invasion (Staun-Ram & Shalev 2005).

Among the hormones involved in the regulation of mammalian reproduction, thyroid hormone (TH) has been one of the most debated. THs are essential for the correct development, growth and metabolism of the body, in particular during fetal development and early childhood (Schwartz *et al.* 1983, Contempre *et al.* 1993, Chan & Kilby 2000, Dittrich *et al.* 2011, Moeller & Broecker-Preuss 2011). Interestingly, TH function during development is not limited to the stages during which fetal thyroid gland is present and functional, but also when it is absent or developing (Thorpe-Beeston *et al.* 1991, Krassas *et al.* 2010), implying a role for maternal TH on fetal development. Both hypothyroidism and hyperthyroidism have been associated with subfertility and recurrent miscarriage (Obregon *et al.* 1984, van den Boogaard *et al.* 2011). Several studies have demonstrated that producing and maintaining optimal levels of THs are fundamental during pregnancy for both mother and fetus and that TH-metabolizing enzymes are detectable already during the implantation stage, suggesting a putative regulatory role during early embryo development (Thorpe-Beeston *et al.* 1991, Ashkar *et al.* 2010); however, TH functions during the early stages of embryo development are not clearly understood.

It has been recently demonstrated that supplementation of TH has a beneficial effect on bovine embryo development *in vitro* (Aghajanova *et al.* 2011, Costa *et al.* 2013). *In vivo*, thyroid hormone receptor (TR) and thyrotropin receptor are expressed in the endometrium, and their expression in endometrial cells is concomitant with the appearance of pinopodes and the establishment of the endometrial receptivity (Idris *et al.* 2005).

The placenta, responsible for the exchange of oxygen, nutrients, hormones, growth factors and their waste products between mother and fetus and essential for a normal fetal development, regulates the transfer of maternal THs to the fetal circulation and protects the developing fetus from toxic concentration of THs. Besides facilitating transplacental TH transport, the placenta is a target organ for TH action and is sensitive to TH for the regulation of its metabolism, differentiation and development. Maternal hyperthyroidism and hypothyroidism may associate with pregnancy complications including miscarriages, reduced birth weight, intrauterine growth restriction (IUGR), pre-eclampsia and spontaneous abortion (Kumar & Chaudhuri 1989, Abalovich *et al.* 2002, Mestman 2004, Casey *et al.* 2005, LaFranchi *et al.* 2005, Kilby *et al.* 2005, Pillar *et al.* 2010, Luewan *et al.* 2011). Studies

using animal models of hypothyroidism have provided further evidence for the importance of THs in placental development. In rats, maternal hypothyroidism resulted in impaired reproduction and IUGR (Ashitaka *et al.* 1988, Nazarpour *et al.* 2015). The human placenta expresses a range of TH transporters, DIO and TR, which modulate TH availability and metabolism (Banovac *et al.* 1986, Kilby *et al.* 1998, Chan *et al.* 2003). *In vitro*, stimulation of placental explants by TH causes an increased secretion of progesterone, estradiol-17B and hCG (Maruo *et al.* 1991). These data suggest that the physiological level of TH may be essential in regulating placental endocrine activity during pregnancy. Based on these pieces of evidence, in the present work, we performed *in vivo* studies, evaluating the effect of hypothyroidism on implantation, placentation and the expression of the major molecules involved in these processes, such as MMPs, TIMPs and Notch target genes. In parallel, we performed *in vitro* studies to investigate if TH may be involved in embryo implantation, focusing on the regulation of blastocyst hatching from the ZP and its outgrowth.

Materials and methods

Animals

6-week-old CD1 mice were obtained from Charles River. Mice were housed and mated under standard conditions with controlled light cycle (lights-on between 07:00h and 19:00h) and temperature (20°C), and treated using humane care in order to inflict the least possible pain. All experiments were approved by Institutional Animal Care and Use Committee of the Tor Vergata University and the Italian Ministry of Health (approval date October 17th 2013; prot. n.32/2013), and carried out according to the Italian and European rules (D.L.116/92; C.E. 609/86; European Directive 2010/63/EU).

In vivo experiments

To induce hypothyroidism, groups of 10 CD1 females were treated with 2mg/mL Methimazole (MMI) (Sigma-Aldrich) dissolved in drinking water for 8 weeks. The MMI solution was replaced thrice a week. To evaluate TH-circulating levels, serum was collected after retro-orbital bleeding and assayed using an ELISA commercial kit, following the manufacturer's specification (Sigma-Aldrich). Hypothyroid and control females were housed overnight with males of proven fertility, and the presence

of a vaginal plug assessed the following morning. Before mating, the phase of the estrus cycle of each female was determined by visual inspection of the external genitalia, and only females in proestrus or estrus were selected. Midday of the day on which the vaginal plug was found was considered day 0.5 of pregnancy (0.5dpc). Pregnant animals were killed at 4.5, 10.5 and 18.5dpc by cervical dislocation, or allowed to give birth. MMI treatment was maintained until they were killed. For the visualization of implantation sites (4.5dpc), 100 µL of 10mg/mL Chicago blue dye (Sigma-Aldrich) in saline solution was injected in the retro-orbital plexus of pregnant females. After 10 min, females were killed and the uterus was analyzed for the presence of blue spots. Implantation and inter-implantation sites were independently collected, flash-frozen and stored at -80°C for further analysis. Similarly, placentas from 10.5- and 18.5 dpc females were collected.

Preparation of endometrial cell cultures

Endometrial cells were obtained by enzymatic digestion of competent uteri from 3.5 dpc pregnant females. In detail, after flushing the embryos out, uterine horns were squeezed using forceps and the resulting tissue incubated with a solution of 1 mg/mL collagenase (Sigma-Aldrich), 0.5 mg/mL hyaluronidase (Sigma-Aldrich) and 0.05 mg/mL DNase (Sigma-Aldrich) in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12, Lonza, Milan, Italy) (1:1) supplemented with 2 mM L-glutamine, 50 U/mL penicillin, 50 U/mL streptomycin, 20 mM Hepes (Lonza) and 10% (v/v) fetal bovine serum (Lonza), for 30 min at 37°C. After centrifugation and washing with phosphate buffered saline (Lonza), endometrial cells were cultured in 4-well Nunc plates (Thermo Fisher Scientific) in DMEM/F12 complete medium at 37°C and 5% CO₂.

In vitro hatching and outgrowth assays

Blastocysts were collected from 3.5 dpc pregnant females by flushing the uterine horns with DMEM/F12 complete medium.

For each experiment, an average number of 30 blastocysts was cultured in presence or absence of endometrial cells in DMEM/F12 complete medium (Lonza) at 37°C and 5% CO₂, in 4-well Nunc cell culture multi-dishes (Thermo Fisher Scientific). Blastocysts and endometrial cells were treated with 100 ng/mL L-thyroxine (T₄) (Sigma-Aldrich) for 16, 48 and 72 h. T₄ was dissolved in DMSO (Sigma-Aldrich) at the concentration of 2.5 mg/mL (stock solution). The working solution was

prepared with DMEM/F12 complete medium. The untreated group was administered with an equal volume of DMSO as control.

Blastocyst hatching and outgrowth was monitored at the indicated time points and photographed using a Leitz Diavert microscope connected to a Nikon DS-Fi1 camera. Hatching was calculated as the number of hatched blastocysts after 16, 24 and 48 h of culture on the total number of cultured blastocysts, and reported as a percentage. Blastocysts' outgrowth was estimated in terms of covered area (pixels), using the ImageJ Software.

RNA isolation and quantitative RT-PCR

Total RNA from tissues and cell cultures was prepared using the TRIZOL Reagent (Roche Diagnostics GmbH) according to the manufacturer's protocol. Possible DNA contamination was removed by DNase treatment. RNA quality was examined on agarose gels. mRNA was reverse-transcribed using Quantitect Reverse Transcription Kit (Qiagen) following the manufacturer's protocol. Specific primers for murine *Mmp2*, *Mmp3*, *Mmp9*, *Mmp10*, *Mmp11*, *Isp1*, *Isp2*, *Hes1*, *Hey1*, *Hey2*, *Timp1*, *Timp2*, *Timp3* and *Gapdh* were designed using the Primer Express software (Applied Biosystems in Life Technologies). Primer sequences are listed in Table 1. The gene expression analysis was performed using Real Master Mix SYBR ROX (Kapa Biosystems, Boston, MA, USA). Differences among the gene expression were quantified using the $\Delta\Delta C_t$ method with normalization to *Gapdh*.

Statistical analysis

Data were collected from at least three independent experiments and analyzed with Graph-pad Prism 6.0 software. Data are presented as mean $\pm$ S.E.M. *P* value was determined by *T*-test and Chi-square test. Statistical significance was based on *P* value: **P* < 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, *****P* < 0.0001.

Results

TH effect on pregnancy outcome

To evaluate the role of TH on pregnancy, we induced hypothyroidism in CD1 females by administration of the thyroid peroxidase inhibitor MMI. Reduction of circulating levels of T_4 was confirmed by ELISA assay. In control non-MMI-treated mice, the serum level of T_4 was

Table 1 Primer sets for qRT-PCR.

Gene	Direction	Sequence	Product size
<i>Mmp2</i>	Forward	CCCATGAAGCCTTGTTACCA	85 bp
	Reverse	TTGTAGGAGGTGCCCTGGAA	
<i>Mmp3</i>	Forward	TGATGAACGATGGACAGAGGAT	100 bp
	Reverse	AGCCTTGGCTGAGTGGTAGAGT	
<i>Mmp9</i>	Forward	GATGTTTTTGATGCTATTGCTGAGA	64 bp
	Reverse	AGTACCAACCGTCCTGAAGAAAT	
<i>Mmp10</i>	Forward	CCACTCACTTTCTCCAGGATCTCT	70 bp
	Reverse	CGTGTTCTCAACTGCAAAGG	
<i>Mmp11</i>	Forward	TCCCTAAGACCCACCGAGAA	83 bp
	Reverse	CAGGTCAGTTCCTGGTTGTC	
<i>Isp1</i>	Forward	AACTCCTGGGTGCACATCTGT	88 bp
	Reverse	CGGCGTCCTGACTTTGGAT	
<i>Isp2</i>	Forward	GGGCACACCCACATCTTTTG	93 bp
	Reverse	TCAGCATCTTGGCTCCAGAA	
<i>Hes1</i>	Forward	AGAAAGATAGCTCCCGGCATT	65 bp
	Reverse	GTGCTTCACAGTCATTCCAGAAT	
<i>Hey1</i>	Forward	GACGGAGAGGCATCATCGA	65 bp
	Reverse	TCTCAGCTCAGACAACTGTTATTGA	
<i>Hey2</i>	Forward	TTACCCTGGGCACGCTACA	72 bp
	Reverse	CTTGCCATAATCTGAGAGG TAGTTGT	
<i>Timp1</i>	Forward	CCCAGAACCAGTCAGTGAAGAG	68 bp
	Reverse	GGCACTGATGTGCAAATTTCC	
<i>Timp2</i>	Forward	GCAATGCAGACGTAGTGATCAGA	59 bp
	Reverse	CCCGGAATCCACCTCCTT	
<i>Timp3</i>	Forward	CACACGGAAGCCTCTGAAAGT	82 bp
	Reverse	CATACACGCGCCCTGTCA	
<i>Gapdh</i>	Forward	AACTTTGGCATTGTGGAAGG	222 bp
	Reverse	CACATTGGGGGTAGGAACAC	

6.6 $\pm$ 1.7 μ g/dL, while in mice treated with MMI, the level was reduced to about 1/3 (2 $\pm$ 0.26 μ g/dL; Fig. 1A).

After confirming that hypothyroidism was induced, females were mated and pregnancy was monitored up to delivery, when the number of pups per litter was counted. A clear association between reduced circulating levels of T_4 and a significant reduction in the number of pups with respect to control was observed (CTRL = 12.50 $\pm$ 0.97; MMI = 6.4 $\pm$ 1.66; Fig. 1B). To identify the developmental stage at which hypothyroidism was affecting embryo development, we killed pregnant females at different

Figure 1

MMI treatment induces hypothyroidism in CD1 females and reduces the number of pups/litter. (A) Dosage of total T_4 in the serum of controls and MMI-treated females, and (B) average number of pups/litter in control and MMI-treated hypothyroid females (number of animals analyzed, *n* = 10). ***P* $\leq$ 0.01.

Figure 2

Hypothyroidism influences pregnancy outcome. (A) Average number of implantation sites in control and MMI-treated females at 4.5 dpc; (B) Percentage of pregnant vs non-pregnant females in control and MMI-treated mice assessed at 4.5 dpc; $P=0.052$ ($n_{\text{ctrl}}=25$; $n_{\text{MMI}}=22$). (C, D, E and F) Comparison of the number of embryos and resorptions per female in controls and hypothyroid females at 10.5 dpc (C and D; $n_{\text{ctrl}}=18$; $n_{\text{MMI}}=15$) and 18.5 dpc (E and F; $n_{\text{ctrl}}=7$; $n_{\text{MMI}}=8$); (D and E) $P<0.05$. (G, H and I) Maternal hypothyroidism affects embryonic development at 18.5 dpc, influencing crown-rump length (G) fetal weight (H) and placental weight (I). ** $P\leq 0.01$.

gestational stages, from implantation (4.5 dpc) up to 18.5 dpc. First, we investigated if decreased circulating levels of T_4 affected implantation. To this end, we mated females, verified the presence of the plug and counted the number of implantation sites in the uterus at 4.5 dpc. At this developmental stage, we did not observe a significant difference in the number of implantation sites in the uteri of hypothyroid females (CTRL=9.5±0.9; MMI=8.6±0.9; Fig. 2A); however, the percentage of non-pregnant mice in the MMI-treated group was about five-fold higher than that of the control group, although the difference did not reach statistical significance (CTRL=3.7±3.7%; MMI=18.67±4.1%; Fig. 2B). To investigate if hypothyroidism influences placental development, pregnant females were killed at 10.5 dpc, a critical time point for mouse placentation (Watson & Cross 2005). We observed a slight, but non-significant decrease in the number of embryos (CTRL=14.22±0.75; MMI=12.25±0.81; Fig. 2C), accompanied by a concomitant significant increase in the number of fetal resorptions (CTRL=0.33±0.13; MMI=1.31±0.40; Fig. 2D). At a later developmental stage, close to parturition (18.5 dpc), we detected a significant reduction in the number of fetuses in the hypothyroid group (CTRL=10.71±2.2; MMI=4.37±1.72; Fig. 2E), which was associated with an increase in fetal resorptions

(CTRL=0; MMI=1.13±0.99; Fig. 2F). We also observed a significant reduction in fetal weight (CTRL=1.5±0.01 g; MMI=1.2±0.03 g; Fig. 2G) and crown-rump length (CTRL=2.3±0.01 cm; MMI=2.03±0.01 cm; Fig. 2H) and a slight increase in placental weight in the hypothyroid

Figure 3

Hypothyroidism induces a significant reduction of (A and C) *Lsp1* and (B and D) *Lsp2* gene expressions in implantation (A and B) and inter-implantation (C and D) sites. *** $P\leq 0.001$, **** $P\leq 0.0001$; Data are the mean of five independent experiments±s.e.

Figure 4

Maternal hypothyroidism affects placental expression of MMPs (A, B, C, D and E) and TIMPs (F, G and H) and of Notch target genes (I, J and K) at 10.5 dpc. (A) Expression of Mmp2, (B) Mmp3, (C) Mmp9, (D) Mmp10, (E) Mmp11, (F) Timp1, (G) Timp2, (H) Timp3, (I) Hes1, (J) Hey1 and (K) Hey2 in placentas from euthyroid and hypothyroid females. * $P < 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Data are the mean of four independent experiments $\pm$ s.e.

females compared to controls (CTRL=0.12 $\pm$ 0.002 g; MMI=0.14 $\pm$ 0.005 g; Fig. 2I).

Molecular pathways affected by reduced levels of TH

Based on the observation that hypothyroidism influences embryo/fetal development, we investigated molecular pathways involved in implantation and placental development, which responded to the reduced circulating levels of TH. To this end, we performed the gene expression analysis of implantation sites (4.5 dpc) and of 10.5 dpc

and 18.5 dpc placentas. In 4.5 dpc implantation and inter-implantation sites, we analyzed the expression of Isp1 and Isp2, two mouse-specific serine proteinases expressed by both the blastocyst and the endometrium during the 'window of implantation'. Quantitative RT-PCR analysis revealed a significant reduction in the expression of Isp1 and Isp2 in both implantation and inter-implantation sites collected from hypothyroid females compared to euthyroid females (Fig. 3). In 10.5 dpc placentas, we investigated the expression of matrix metalloproteinases 2, 3, 9, 10 and 11 (MMPs), involved in placental

Figure 5

Hypothyroidism reduces placental expression of Timp3 and Notch target genes at 18.5 dpc. Relative expression of Timp1 (A), Timp2 (B) and Timp3 (C) and Notch target genes Hes1 (D), Hey1 (E) and Hey2 (F) in placentas collected from 18.5 hypothyroid and euthyroid CD1 females. * $P < 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Data are the mean of three independent experiments $\pm$ s.e.

Figure 6

TH supplementation stimulates mouse blastocyst hatching *in vitro*. (A) Schematic representation of the *in vitro* model developed to assess TH role in implantation. (a) Co-culture of murine blastocysts and endometrial primary cells as the feeder layer; (b) blastocysts cultured on plastic; (c) endometrial cells cultured without blastocysts. (B) Representative images of the cultures. Scale bar 50 μm. (C and D) Graphs summarizing the results shown in B: percent of hatched blastocysts after co-culture on endometrial cells (C) ($n_{\text{ctrl}} = 771$; $n_{\text{T}_4} = 762$) or on plastic (D) ($n_{\text{ctrl}} = 154$; $n_{\text{T}_4} = 162$). * $P < 0.05$.

morphogenesis, tissue inhibitor of metalloproteinases 1, 2 and 3 (TIMPs), which function as modulators of tissue remodeling, and Notch target genes Hes1, Hey1 and Hey2, required for trophoblast function, and placental angiogenesis. All MMPs, TIMPs and Notch target genes analyzed resulted significantly downregulated in 10.5 dpc placentas collected from hypothyroid pregnant females (Fig. 4). Timp3 and Notch target genes were also significantly downregulated in placentas collected from 18.5 dpc hypothyroid females (Fig. 5).

TH role on blastocyst implantation *in vitro*

We further investigated the role of TH on embryo implantation by *in vitro* culturing blastocysts collected from 3.5 dpc control pregnant females, either on plastic or on endometrial primary cells, with or without supplementation of 100 ng/mL of T₄ (Fig. 6A, B, C and D). The number of blastocysts hatching from the ZP was counted after 16, 24 and 48 h of culture. Our results show that supplementation of the blastocyst/endometrium

Figure 7

Blastocyst–endometrium interaction is indispensable for TH-mediated increase in the expression of the serine proteases Isp1 and Isp2. Isp1 and Isp2 expressions in blastocysts cultured with or without T₄ supplementation on endometrial feeder layer (A and B) or plastic (C and D). Isp1 and Isp2 expressions in endometrial cells co-cultured with blastocysts (E and F) or cultured alone (G and H), with or without T₄ supplementation. * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Data are the mean of four independent experiments $\pm$ s.e.

Figure 8

TH induces a significant increase in the expression of MMPs in endometrial cells cultured in the presence of blastocysts. Relative expression of (A) Mmp2, (B) Mmp3, (C) Mmp9, (D) Mmp10 and (E) Mmp11 in endometrial cells co-cultured with blastocysts. Relative expression of (F) Mmp2, (G) Mmp3, (H) Mmp9, (I) Mmp10 and (J) Mmp11 in endometrial cells cultured without blastocysts. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are the mean of five independent experiments $\pm$ s.e.

cultures with T_4 significantly increases the percentage of hatching blastocyst after 16 h (CTRL=5.45 $\pm$ 2.5; T_4 =18.9 $\pm$ 2.8) and 24 h (CTRL=20.54 $\pm$ 4.1; T_4 =40.52 $\pm$ 5.5), while no differences were observed after 48 h (CTRL=81.42 $\pm$ 14.2; T_4 =97.9 $\pm$ 2.1) (Fig. 6C). Interestingly, T_4 supplementation of blastocyst cultures in the absence of endometrial feeder layer did not increase the percentage of hatched blastocysts at all time points studied (CTRL 16h=10.4 $\pm$ 3.3; T_4 16h=10.5 $\pm$ 3.66; CTRL 24h=21.167 $\pm$ 6.099; T_4 24h=23.267 $\pm$ 6.96; CTRL 48h=51.66 $\pm$ 3.38; T_4 48h=50.933 $\pm$ 2.69) (Fig. 6D). Since the serine proteases Isp1 and Isp2 have been demonstrated to be important regulators of blastocyst hatching in mice (O'Sullivan *et al.* 2001, Sharma *et al.* 2006), we cultured blastocysts in the presence of T_4 on either endometrial feeder layer or alone for 16 h and then analyzed the expression of these two proteases by qRT-PCR. When blastocysts were cultured on endometrial cells and treated with T_4 , the expression of Isp1 and Isp2 was significantly upregulated by about four-fold (Fig. 7A and B). Culture of blastocysts on plastic in the presence of TH induced a low but significant increase in the expression of Isp1, while it had no effect on Isp2 (Fig. 7C and D). Similar results were observed analyzing endometrial cells for the expression of Isp1 and Isp2 after 16 h of culture. Remarkably, in endometrial cells co-cultured with

blastocysts, T_4 supplementation induced a significant increase in both Isp1 and Isp2 (Fig. 7E and F), while T_4 supplementation of endometrial cells cultured without blastocysts induced a much less increase, which reached statistical significance only for Isp1 (Fig. 7G and H). We also investigated by qRT-PCR the endometrial expression of the major metalloproteinases expressed during the implantation window. When the endometrial cells were co-cultured for 16 h with blastocysts, T_4 supplementation induced a significant three- to four-fold increase in all MMPs studied. Similarly to what observed for Isp1 and Isp2, supplementation of T_4 induced a much lower increase in the expression of MMPs (which was statistically significant only for Mmp9) if endometrial cells were cultured without blastocysts (Fig. 8). These data suggest that TH regulates the bidirectional crosstalk between the receptive endometrium and the ready-to-implant embryo.

To investigate if TH might also be involved in blastocyst attachment and invasion of the endometrial tissue, blastocysts were cultured for 72 h either on endometrial cells or on plastic, with or without T_4 supplementation. At the end of the culture, the number of blastocysts that attached and underwent outgrowth was counted, and the area occupied by the trophoctodermal cells was measured. Our results show that T_4 supplementation does not influence

Figure 9

TH is able to positively influence blastocyst outgrowth. (A and B) Quantification of the area covered by trophoectodermal cells in the presence (A) or absence (B) of the endometrial feeder layer. In three independent experiments, twenty randomly chosen blastocysts were measured for each condition. (C and D) Representative images of blastocyst outgrowth in (C) control and (D) TH-treated blastocyst–endometrium cell co-cultures; (E and F) blastocysts outgrowth in (E) control and (F) TH-treated blastocysts cultured without the feeder layer. Scale bar 50 µm; * $P < 0.05$, ** $P \leq 0.01$.

blastocysts' attachment, as at the end of the culture, all blastocysts had adhered and exposed their inner cell mass; however, blastocyst outgrowth, measured as surface occupied by trophoectodermal cells, was significantly increased in cultures supplemented with TH (CTRL = 1493 ± 165 ; T_4 = 2705 ± 276 Pixels). Increased outgrowth of the trophoectoderm was also observed for blastocysts cultured with T_4 on plastic (CTRL = 579 ± 149 ; T_4 = 805 ± 130.6 Pixels), although the difference in the covered area between T_4 -treated and non-treated cultures was much lower than that measured after the outgrowth of blastocysts cultured on endometrial feeder cells (Fig. 9).

Discussion

Implantation and embryo development are finely regulated processes, characterized by highly orchestrated interactions between the competent-blastocyst and the maternal receptive endometrium. The hallmark events of implantation are represented by tissue remodeling and angiogenesis (Dey *et al.* 2004, Plaisier 2011), which are regulated by the activity of MMPs and MMP inhibitors (TIMPs), as well as activation of the Notch pathway. A clinical association between hypothyroidism and infertility and/or implantation failure has been extensively reported, although the molecular mechanism underlying this association remains unclear (Kilic *et al.* 2008, Stagnaro-Green & Pearce 2012, Granfors *et al.* 2013, Colicchia *et al.* 2014).

To study the effect of TH in implantation and embryo development, we have used an *in vivo* model of hypothyroidism, by treating CD1-outbred mouse females with the thyroid peroxidase inhibitor methimazole (MMI). To date, few *in vivo* experimental models of hypothyroidism have been developed, which include treatment with MMI, sodium perchlorate, propylthiouracil (PTU) and the use of transgenic strains, e.g., MCT8 knockout mice. In the present study, to induce hypothyroidism in pregnant mice, the use of MMI was preferred to the use of transgenic strains. In fact, mouse models of hypothyroidism obtained after ablation of genes involved in TH pathway, such as MCT8 knockout mice, display severe phenotypes which complicates the analysis of pregnancy-related pathways (Trajkovic-Arsic *et al.* 2010). Under our experimental conditions, MMI was able to significantly decrease the levels of circulating TH. Hypothyroid pregnant females had a significantly reduced average number of pups/litter, clearly indicating that decreased maternal circulating levels of TH are able to affect implantation and/or embryo development, confirming the many clinical observations reported in humans. To identify the critical developmental stage at which hypothyroidism affects pregnancy outcome, we have killed pregnant females at implantation, at the early stages of placentation, and one day prior to birth, when organogenesis is completed. At implantation, we observed no statistically significant differences in the number of implantation sites between hypothyroid and euthyroid females, but we detected a higher (although non-significant) percentage of non-pregnant females in the hypothyroid group, suggesting

the possibility that reduced levels of TH might affect ovulation and/or oocyte quality, impairing fertilization, or might regulate embryo–endometrium interaction. To further clarify this, we studied the expression of ISP1 and ISP2, two proteases that are key regulators of mouse embryo implantation, which are produced by both the blastocyst and the receptive endometrium and their action is indispensable for efficient implantation (O'Sullivan *et al.* 2001, Sharma *et al.* 2006, 2011). At implantation, levels of both ISP1 and ISP2 were significantly reduced in uteri from pregnant hypothyroid females compared to euthyroid females, suggesting that TH regulates the expression of factors involved in the preparation of the uterus to embryo implantation. However, our data do not rule out the possibility that TH might also regulate earlier stages of pre-implantation development, as well as oocyte maturation and fertilization. Further studies are needed to settle these points. Since implantation abnormalities may result in altered placentation and consequent embryo loss, we performed the gene expression analysis for genes involved in placental development at 10.5dpc, a stage at which labyrinth is expanding and critical events of organogenesis are still ongoing, and 18.5dpc, characterized mainly by fetal growth. In particular, we analyzed the expression of MMPs and TIMPs, which are important modulators of spongiotrophoblast migration into maternal decidua and of decidual extracellular matrix remodeling. Our results show an overall downregulation of MMPs and TIMPs at 10.5dpc, and a significant downregulation of Timp3 at 18.5dpc. We have also investigated activation of the Notch signaling pathway, a recognized key determinant of placental development. All Notch target genes studied were significantly downregulated at both 10.5 and 18.5dpc. Altogether, our results point toward a role of TH in the regulation of many of the key events of placentation, including trophoblast invasion and remodeling of decidual tissue, and possibly angiogenesis. Interestingly, morphological observation of the pregnant uteri demonstrated that at 10.5dpc, the number of embryos per female was similar between euthyroid and hypothyroid females, although a slight increase in the number of resorption was detected. On the contrary, a significant reduction in the number of fetuses per dam was observed at 18.5dpc in hypothyroid females, and was associated with significantly reduced fetal crown-rump length and weight and increased placental weight, suggesting that low levels of maternal TH induce in utero growth restriction and that compensatory placental expansion occurs. These results indicate that TH may play a critical role on fetal development in a window comprised

between 10.5 and birth, and that possible alteration in placental development may be the underlying causal mechanism.

Altogether, our *in vivo* study suggests that hypothyroidism may have a dual effect on pregnancy, by influencing implantation at very early stages of gestation, and by regulating placental development at later stages.

We further investigated the role of TH in implantation by culturing blastocyst and endometrial cells *in vitro* to recapitulate embryo implantation. After monitoring the cultures up to 48h, we observed that medium supplementation with TH significantly increased the number of hatching blastocysts already after 16h, possibly as a consequence of the observed upregulation of the two main proteases involved in mouse blastocyst hatching, namely Isp1 and Isp2. A translational application of these results might be foreseen in the development of complex media for application in *in vitro* reproductive technologies. Interestingly, we observed that the co-culture of blastocysts and endometrial feeder cells is indispensable for TH-mediated induction of Isp1 and 2 and other implantation relevant proteases, and for the increased hatching, suggesting that TH is involved in the bidirectional crosstalk between the competent blastocyst and the receptive endometrium at the time of implantation. The observed endometrial-dependent effect on blastocyst hatching might be the consequence of TH-stimulated release of proteases by the endometrial cells, which may act on both the endometrium itself and the blastocyst. On the other hand, we cannot exclude that TH stimulation of endometrial cells might result in the expression of factors other than proteases (e.g. LIF). The transcriptome analysis of TH-stimulated endometrial cells would give insights into this respect. At further support to the hypothesis of TH as a regulator of the bidirectional crosstalk between the endometrium and the blastocyst, we evaluated the ability of TH to modulate the initial phase of implantation. To this end, we cultured blastocysts *in vitro* and studied their outgrowth on the endometrial feeder layer or on plastic. After 72h of culture, trophectodermal cells attached and expanded on both endometrial cells and plastic, mimicking *in vitro* the invasion of maternal tissue; however, the surface occupied by trophectodermal cells was greater in the presence of the feeder layer. Of note, T₄ was capable of significantly increasing the outgrowth of trophectoderm cells under both culture conditions.

In conclusion, our results identify molecular mechanisms regulated by TH that may link hypothyroidism to implantation failure and recurrent miscarriage, events often observed under

this pathological condition, offering a mechanistic explanation to the clinical observations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

D P and E B performed most of the experiments; M M and R B helped in some of the experiments; A C and A S helped in the interpretation of the data and critically read the manuscript; M C helped in the interpretation of the data; S U and C M contributed to the study design and to the interpretation of the results; L C designed the study, interpreted the data and wrote the manuscript.

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