Dynamic changes in hyperglycosylated human chorionic gonadotrophin throughout the first trimester of pregnancy and its role in early placentation

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Submitted on September 5, 2014; resubmitted on December 17, 2014; accepted on January 16, 2015

STUDY QUESTION: What is the in situ localization and function of hyperglycosylated hCG (hCG-H) in first trimester pregnancy tissues?

SUMMARY ANSWER: HCG-H localizes to the syncytiotrophoblast, cytotrophoblast and invasive extravillous trophoblast within the maternal decidua and promotes invasion during the first trimester of pregnancy.

WHAT IS KNOWN ALREADY: Serum levels of hCG-H decline dramatically throughout the first trimester of pregnancy. As hCG-H is produced by choriocarcinoma cells, it is proposed to regulate trophoblast invasion.

STUDY DESIGN, SIZE, DURATION: Tissues were collected from elective first trimester pregnancy terminations. Placental villous and decidua basalis were collected from Week 6 to Week 12 of gestation (n = 49).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Tissues were collected from elective first trimester surgical pregnancy terminations to determine localization, abundance and function of hCG-H. Placental villous outgrowth studies determined the impact of neutralizing endogenous hCG-H on trophoblast function. Real-time proliferation, migration and invasion assays using JEG-3 choriocarcinoma cells further elucidated the role of hCG-H in trophoblast function.

MAIN RESULTS AND THE ROLE OF CHANCE: HCG-H localized to syncytiotrophoblast layer of the placental villous from gestational weeks 6–9; thereafter hCG-H localized as a discrete layer between syncytioc- and cyto-trophoblast layers. Immunoreactive hCG-H was also observed within the cytotrophoblast layer in Week 7–8 of gestation. HCG-H abundance decreased within placental villous from Weeks 6–12 of gestation (n = 3 placentas per gestational weeks 6–12). HCG-H also localized to anchoring villi within maternal decidua, extravillous trophoblasts invading into the maternal decidua and endovascular trophoblasts remodeling maternal blood vessels. Treatment of primary first trimester villous explants with hCG-H neutralizing antibody reduced trophoblast outgrowth (n = 3 placentas, P < 0.05). Treatment of a trophoblast cell line with neutralizing antibody reduced trophoblast invasion (n = 4, P < 0.05) but did not affect migration or proliferation.

LIMITATIONS, REASONS FOR CAUTION: Functional invasion and migration assays performed using cell lines. Not possible to perform such assays with primary human material.

WIDER IMPLICATIONS OF THE FINDINGS: HCG-H is an important autocrine factor facilitating trophoblast invasion in the first trimester of pregnancy. Targeting hCG-H may prove useful in the treatment of pathologic pregnancies, such as ectopic pregnancies, or pregnancy complications including pre-eclampsia and gestational trophoblast diseases.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the Victorian Government Operational Infrastructure Support Program. J.E. is supported by NHMRC project grant #1047756. L.A.S. and E.D. by NHMRC Fellowships #1002018 and #550905 respectively and E.M. by an NHMRC Early Career Fellowship #611827. The authors have no conflicts of interest relating to this work.

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Introduction

Trophoblast invasion into the maternal endometrium is essential for both initial implantation and subsequent placentation. The factors promoting or governing the invasive trophoblast ‘push’ are still largely unknown. However, an invasion-promoting hyperglycosylated form of human chorionic gonadotrophin (hCG) (O’Connor et al., 1998; Kovalevskaya et al., 1999) is a likely candidate as an early embryonic product with the potential to facilitate initial placentation.

HCG is one of the first embryonic products, expressed from the 8 cell stage (Bonduelle et al., 1988) and detectable in the human maternal circulation from Day 7 after fertilization, around the time of implantation (Ahmed and Klopper, 1983). It is a glycoprotein comprised of an α- and β-subunit held together by charge interactions. HCG is highly glycosylated, its β oligosaccharide side chains comprise ~30% of its molecular weight (Cole, 2010a). However, the degree of glycosylation and thus molecular size, of the hCG molecule can change dramatically with gestational age (Wide and Hobson, 1987; Skarulis et al., 1992).

For many years hCG was only considered as a hormone mediating maternal recognition of pregnancy, exerting a luteotrophic effect on the maternal corpus luteum to maintain progesterone production. However, it is now recognized that hCG is not a single entity. The hCG family comprises five independent variants including the ‘classic’ hCG which acts as a hormone, sulfated hCG, predicted to exert hormone-like effects, and hyperglycosylated hCG which acts as an autocrine signaling factor (Cole, 2012).

Hyperglycosylated hCG (hCG-H) has double sized O-linked oligosaccharides and extra-large N-linked oligosaccharides (Cole, 2012). This ‘over’ glycosylation results in a more acidic, larger molecule than ‘classic’ hCG, with a pI of 3.2 (hCG-H) versus 3.5 (hCG) and a molecular weight of 42 kDa (hCG-H) versus 37 kDa (hCG) (Sutton, 2004). This change in acidity is particularly relevant as in culture, human blastocysts at Day 11 of development secrete a more acidic form of hCG than those developed further (Lopata et al., 1997), suggesting a hyperglycosylated form is predominant during the implantation stages of pregnancy. These findings have been confirmed in assisted reproduction cycles, with hCG-H detectable in maternal serum from ~4 – 6 days after transfer of an embryo into the uterine cavity (9 days after oocyte retrieval) in women with ongoing pregnancies (Chuan et al., 2014). The pattern of hCG-H in maternal serum changes dramatically throughout the first trimester of pregnancy, transitioning from being the dominant form of hCG in maternal serum up to Week 5 of gestation, to comprising <2% of total hCG by Week 12 (Cole, 2010a,b).

A number of studies have suggested that hCG-H mediates trophoblast invasion, and that its dominance in early pregnancy facilitates early trophoblast invasion and placentation development (Handschuh et al., 2007). Particularly, hCG-H is produced by the invasive trophoblast of late stage (Week 9) first trimester placental tissues (Kovalevskaya et al., 2002; Guibourdenche et al., 2010). However, in the very early stages of pregnancy, up to approximately day 13 after fertilization, the syncytiotrophoblast comprises the invasive cell front. We therefore hypothesize that if hCG-H is the primary invasive factor at the initiation of pregnancy, the syncytiotrophoblast must either have produced this form of hCG at one stage, or in the first trimester have continued production of hCG-H after it differentiates from the villous cytotrophoblast.

While hCG-H has been measured in maternal serum throughout gestation (Cole, 2010a,b), peripheral levels have never been correlated with production within the source tissue, the first trimester placental villous. Additionally, while some steps have been made toward characterizing a role for hCG-H in trophoblast invasion, the model used thus far have focused on adding exogenous hCG-H to a system already maximally producing this autocrine invasive factor (Lee et al., 2013). Our aims were to determine: the localization and abundance of hCG-H in the placental villous across the first trimester of pregnancy; whether highly invasive trophoblasts within the maternal decidua produce hCG-H; and if blocking hCG-H in a human placental model impacts trophoblast migration and invasion.

Materials and Methods

Ethics statement

This study was approved by the Southern Health Human Research and Ethics Committee (#09317B; #06014C). Written and informed consent was obtained from each patient before surgical intervention.

Tissue collection for immunohistochemistry

Tissues were collected from women undergoing surgical termination of pregnancy (STOP) for psychosocial reasons during the first trimester (Weeks 6 – 12). Gestation was confirmed by ultrasound prior to STOP. Placental villi and maternal decidua were either fixed in 4% formalin for 24 h and processed to paraffin under standardized conditions or snap frozen for protein extraction.

Tissue collection for explant culture

Placental villi from first-trimester placentas (n = 3) were collected into DMEM/Ham’s F12 medium. Small pieces (1 × 1 mm) were dissected under the microscope and cultured in serum-free DMEM/Ham’s F12.

Immunohistochemistry

Hyperglycosylated hCG

HCG-H was immunolocalized in human first trimester placental villi and maternal decidua basalis using the B152 antibody (gifted by G. Kovalevskaya, Columbia University, New York). Fixed first trimester tissues from Week 6 (n = 5), Week 7 (n = 11), Week 8 (n = 10), Week 9 (n = 7), Week 10 (n = 7), Week 11 (n = 5) and Week 12 (n = 4) were sectioned (5 μm) onto Superfrost slides (Thermo Fisher Scientific, Scoresby, VIC, Australia). Sections were dewaxed in histosol (Sigma Chemical Co; St Louis, MO, USA), rehydrated through descending grades of alcohol (95% – 70%) to distilled water (dH2O) and antigen retrieval performed by microwave heating for 5 min in 0.1 M citrate buffer with 20 min of cooling. Sections were washed in Tris-buffered saline with 0.2% Tween 20 (TBS-T) and endogenous peroxidase activity blocked with 3% hydrogen peroxide in methanol for 10 min at room temperature. Non-specific binding was blocked with non-immune blocking serum (10% horse serum, 2% human serum, TBS) for 45 min in a humidified chamber at room temperature. B152 (anti-hCG-H) or mouse IgG (Thermo Fisher Scientific) were applied at 2.5 μg/ml and incubated overnight at 4°C in a humidified chamber. Sections were washed
Extensively in TBS-T/TBS with horse anti-mouse biotinylated antibody (1:200, Dako Australia, Sydney, NSW, Australia) then applied for 1 h at room temperature, re-washed in TBS-T and antibody detected using an avidin-biotin peroxidase system (ABC-HRP, Dako), followed by peroxidase substrate 3, 3′-diaminobenzidine (DAB, Dako), which produces a brown precipitate. Sections were counterstained with hematoxylin, dehydrated through ethanol (70–95%) and histostained and mounted with DPX. Images were acquired using an Olympus BX53 microscope and Olympus DP73 digital camera.

**Western immunoblotting**

Snap frozen placental villous samples across all weeks of gestation were homogenized in universal immunoprecipitation buffer (50 Mm Trizma base, 150 Mm NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 0.2% Triton X-100, 0.3% NP40, 25 mM β-glycol phosphate) for 4 min and centrifuged to remove cellular debris. Lysates (equivalent protein concentration) and molecular weight markers (Life Technologies, Mulgrave, VIC, Australia) were run on a pre-cast 4–20% gradient gel (Biorad, Gladesville, NSW, Australia). Proteins were immunoblotted onto PVDF membrane (Life Technologies, Mulgrave, VIC, Australia). The protocol was followed as described above with the following modifications: primary antibody was HLA-G (0.5 μg/ml, #557577 Pharmingen, Becton Dickinson Pty Ltd, North Ryde, NSW, Australia).

**Results**

**hCG-H localizes to the syncytiotrophoblast and villous cytotrophoblast layers of the first trimester placenta**

Intense immunostaining for hCG-H is observed within the syncytiotrophoblast (STB) layer of the placent villous from Weeks 6 to 9 of gestation (Fig. 1A–D, respectively). In Week 10 and 11 gestation samples, hCG-H presents as a defined layer between syncytiotrophoblast and villous cytotrophoblast (CTB) layers of the first trimester placenta (Fig. 1E and F, respectively). By Week 12 of gestation, hCG-H immunostaining was weaker, but localization between syncytiotrophoblast and cytotrophoblast layers was maintained (Fig. 1G). At higher magnification, hCG-H presents as a defined layer between syncytiotrophoblast and villous cytotrophoblast layers, was maintained (Fig. 1H). At higher magnification, hCG-H immunostaining was seen in individual villous cytotrophoblast cells (closed arrow, Fig. 1I, Week 6), between the villous cytotrophoblast cells (open arrows, Fig. 1J, Week 7), and within the villous cytotrophoblast layer (Arrows, Fig. 1K, Week 8). These data suggest that hCG-H may be produced and very rapidly exported/secreted by the villous cytotrophoblasts resulting in mainly negative immunostaining within these cells. From Week 10–12 of gestation hCG-H is again observed as a discrete band of immunostaining between the syncytiotrophoblast and villous cytotrophoblast layers (Fig. 1M–O, respectively). Within the floating chorionic villous, hCG-H is localized to the syncytiotrophoblast

**Migration**

The RTCA cell invasion migration (CIM) plate allows real-time measurement of cell migration through a gold electrode in a modified Boyden chamber assay. DMEM with 10% FCS (as chemoattractant) was pipetted into the top chamber before placement of the top ‘cell chamber’. Serum-free DMEM media containing 5 μg/ml B152 or mouse IgG was placed into quadruplicate top wells and a background reading taken. Jeg-3 cells (4 × 10⁶ cells/well) were seeded (diluting the B152 and IgG to 2.5 μg/ml). Readings (‘sweeps’) of electrical impedance were taken every 15 min for up to 40 h. N = 4 independent experiments.

**Invasion**

The experimental procedure was as above with modifications. Prior to addition of media containing B152 or mouse IgG, wells were coated with 25 μl of a 1:1 mix of growth factor reduced Matrigel™ (Becton Dickinson) and serum-free DMEM. The Matrigel™ was allowed to set for 30 min at 37°C before addition of media for background readings and Jeg-3 cells. N = 4 independent experiments.

**Statistical analysis**

All statistical analyses were performed using PRISM version 4.03 for Windows (PRISM, La Jolla, CA, USA). For placental explant studies, a paired t-test was applied. For western immunoblot analysis of hCG-H protein abundance and real-time xCelligence cell behavior an unpaired t-test performed on the cell index at specific time points. Significance was taken as P < 0.05. All data presented as mean ± SEM except where SD indicated.
layer at Week 6 (Fig. 1P), with intense immunostaining of the cytotrophoblast at Week 7 (Fig. 1Q) and localization to both syncytiotrophoblast and villous cytotrophoblast at Week 8 (Fig. 1R). From Weeks 9–12 of gestation, immunostaining within the chorionic villi again localized as a discrete band between the syncytiotrophoblast and cytotrophoblast layers (Fig. 1T–V, respectively). Mouse IgG treated sections were negative (Fig. 1W). Western immunoblot analysis of placental villi demonstrated the same trend observed by immunohistochemistry, with

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**Figure 1** hCG-H localizes to the syncytiotrophoblast and cytotrophoblast with staining intensity decreasing throughout gestation. Staining for hCG-H observed in the syncytiotrophoblast (STB) of Week 6 – 12 gestation placental villi (A – G, respectively), but no staining was observed within the stromal core (sc). In the Week 7 placental villi hCG-H is localized to the extravillous cytotrophoblast (ev-CTB, B). Very intense immunostaining for hCG is observed in the Week 12 placental villous localized to the syncytiotrophoblast (H). At a higher magnification staining for hCG-H can be observed between cells of the villous cytotrophoblast (v-CTB) layer (arrows, J) of the Week 7 placenta, with limited localization also observed in the v-CTB of the Week 8 placenta (arrows, K). From Week 10 – 12 hCG-H intensity is progressively weaker and immunostaining presents as a discrete layer between the syncytiotrophoblast and cytotrophoblast layers (M – O). In the Week 7 floating chorionic villi, hCG-H immunolocalized to the v-CTB layer (Q). In the Week 9 – 12 floating chorionic villous hCG-H presented as a discrete layer between the syncytiotrophoblast and cytotrophoblast layers (red outline, S (Week 9), T – V, Week 10 – 12, respectively). Negative control, mouse IgG inset panel W. Scale bars: A – H and W, 100 μm, I – V, 20 μm.
hCG-H elevated at Week 6 and levels progressively declining up to Week 12 (*P < 0.05, Fig. 2A and B).

**hCG-H immunostaining of placental anchoring villous within the maternal decidua**

Floating chorionic villi in close proximity to the maternal decidua at Week 6 of gestation immunolocalize hCG-H to the syncytiotrophoblast (STB) and extravillous cytotrophoblast (ev-CTB, Fig. 3A) and in anchoring villi attached to the maternal decidua (Fig. 3B). At Week 7, intense immunoreactive hCG-H localizes to the syncytiotrophoblast layer and the extravillous cytotrophoblast cells invading into the maternal decidua (Fig. 3C, highlighted area at a higher magnification 3D). In Week 8 decidua basalis, the placental anchoring villous within the decidua is surrounded by faint hCG-H immunostaining suggesting the placental cells are secreting hCG-H (Fig. 3E and F); immunoreactivity is also observed within extravillous trophoblasts within the tissue (asterisk, Fig. 3E). At Week 9 intense hCG-H immunoreactivity is again observed within the anchoring villi within the maternal decidua (Fig. 3G and H). Mouse IgG control sections were negative (inset, Fig. 3H).

**hCG-H immunostaining of invading EVT’s within the maternal decidua**

HCG-H localizes to the trophoblast column (tc) invading the maternal decidua (Fig. 4A), while in adjacent chorionic villi hCG-H localizes to both the STB and cytotrophoblast (CTB, Fig. 4B) in the Week 7 chorionic villous. Abundant hCG-H positive cells are observed throughout the decidua basalis from Week 7 to Week 12 of gestation (Fig. 4C–H; C, 4E, and F: Week 8, 4G Week 9, 4I Week 10, 4K and M Week 11, 4O Week 12) particularly in close association with the maternal blood vessels (mbv) within the decidua (Fig. 4E and F). These hCG-H positive cells have a similar abundance and staining pattern to HLA-G positive extravillous trophoblasts (Fig. 4D, 4H, and J: Week 9, 4L and N Week 11, 4P Week 12) within the same tissues suggesting that invasive EVT’s express hCG-H. Indeed, serial 3 μm sections of maternal decidua demonstrated hCG-H (Fig. 4A, I, K, M and O) and HLA-G (Fig. 4H, J, L, N and P) localization to a large number the same cells in the same tissues (marked by # in Fig. 4G–P) in Week 9–12 gestation tissues, indicating that a large proportion, but not all, invasive extravillous trophoblasts express hCG-H. Mouse IgG control sections were negative (inset, Fig. 4P).

**Blocking hCG-H inhibits first trimester explants outgrowth and trophoblast cell invasion**

First trimester placental villous tips on collagen droplets were treated with 2.5-μg/ml mouse serum or 2.5-μg/ml B152 antibody. Treatment with B152 decreased the area of explant outgrowth after 48 h (Fig. 5C and D; filled square, E, n = 3 placenta, P = 0.06) versus treatment with mouse IgG (Fig. 5A and B; open square, E). Similarly, treatment of Jeg-3 cells with 2.5-μg/ml B152 significantly decreased invasion (open square, Fig. 6A) at 20, 24, 30 and 40 h (**P < 0.05) compared with 2.5-μg/ml mouse IgG (filled square, Fig. 6A). Treatment with b152 did not significantly affect migration at any time point (Fig. 6B), proliferation or apoptosis of Jeg-3 cells (data not shown).

**Discussion**

This study clearly defines changes in localization and abundance of hCG-H during the first trimester of pregnancy and provides functional evidence of its role in trophoblast invasion and establishment of a viable placenta. HCG-H localizes to both STB and CTB of the placental...
hCG-H positive anchoring villous and placental contacts with maternal decidua. Immunostaining for hCG-H localized to the syncytiotrophoblast (STB) and the extravillous cytotrophoblast (ev-CTB) with no staining observed in the maternal decidua (MD) at Week 6 gestation (A). Immunolocalization for hCG-H observed within the syncytiotrophoblast layer of the invading floating villous (B). An anchoring villous in the maternal decidua (MD), demonstrates hCG-H immunostaining within the STB and invading ev-CTB (C and higher magnification of highlighted area D). Further anchoring villi within Week 8 (E and F) and Week 9 (G and H) gestation tissues demonstrate intense hCG-H immunostaining. Extravillous trophoblasts within maternal decidua (asterisk) observed at Week 8 of gestation (E). Negative control, mouse IgG inset panel H. Scale bars: A and C, 100 μm, B, D–G, 20 μm.

hCG-H is the predominant form of hCG present within the maternal urine and serum from the first 2 weeks after implantation (Chuan et al., 2014) to the sixth week of gestation (Cole, 2010a,b). After this time, hCG-H levels increase up to Week 10–11 of gestation, but it comprises a lesser proportion of measured total hCG as the less glycosylated syncytiotrophoblast-derived isoform becomes dominant (Cole, 2010a,b). After Week 11, hCG-H proportions decline dramatically to comprise just 1% of total hCG for the remainder of pregnancy, suggesting that hCG-H is required for invasive placenta establishment in the first trimester.

The data presented here demonstrate that the placenta is the likely source of serum hCG-H, and reported serum levels (Cole, 2010a,b) appear to correlate well with immunostaining intensity reported herein. Within placental villi of Week 6–8 gestation placentae, intense immunostaining for hCG-H is localized to the STB, the CTB and EVT within the decidua. This is the phase of gestation when hCG-H comprises 20–42% of total hCG within the maternal serum (Cole, 2010a,b). However, from Weeks 9–12, there was a change in the pattern of hCG-H immunoreactivity within the placental villi, with less intense staining and a more discrete pattern of localization with hCG-H predominantly localized between the STB and CTB layers. This decrease in hCG-H is particularly marked at Week 12 of gestation when immunostaining is faint to absent, corresponding to a time when hCG-H comprises just 3% of total hCG in serum (Cole, 2010a,b). This is in marked contrast with hCG, which displays extensive, intense immunostaining at Week 12 gestation. However, while placental hCG-H immunoreactivity declines by Week 12, immunostaining of EVT within the maternal decidua remains intense, suggesting that these actively invading cells maintain production of hCG-H to facilitate their remodeling of the maternal decidua and spiral arteries in the first trimester.

While hCG-H localization across all weeks of the first trimester of pregnancy (for which samples are available [Weeks 6–12]) has been extensively characterized herein, the cells that produce hCG-H in vivo are still unknown. In one report (Kovalevskaya et al., 2002), it was demonstrated that CTBs produced hCG-H in vitro, but STBs, which they demonstrated were immunopositive in vivo, were not examined. In contrast, Guibourdenche et al. (2010) using the same antibody, localized hCG-H to the CTB layer of floating chorionic villous, and in vitro demonstrated that invasive trophoblasts secrete hCG-H. In a series of functional studies, Handschuh et al. (2007) demonstrated that CTBs produce hCG-H and conditioned media from these cells promotes invasion. However, neither the specific function of hCG-H in invasion, nor the specific localization of hCG-H were examined. The evidence presented here is in agreement with these reports, with localization of hCG-H to both STB and CTB layers of the placental villi. However, given the highly specific localization of hCG-H between the CTB and STB layers in the later stages of the first trimester, when hCG-H secretion is known to decline, it seems likely that the main site of hCG-H production is the CTB. From careful observation of the immunostaining and knowledge of hCG-H action, it is likely that hCG-H is very rapidly secreted by the source cells (CTB) in the early stages of pregnancy, but accumulates in the STB. At later stages of the first trimester, when the ‘invasive push’ is less active, hCG-H secretion is known to decline. The importance of hCG-H as an invasive factor has been reinforced by recent reports from Keikkala et al. (2013, 2014). These data demonstrate the utility of hCG-H measurements in the first trimester as a diagnostic marker for early onset pre-eclampsia: hCG-H (expressed as % total hCG) is reduced in pregnancies complicated by pre-eclampsia. This is likely associated with the presumed etiology of the disease, in which the placental trophoblast cells are less invasive and thus do not adequately remodel the maternal vasculature. Investigation of placental and decidual hCG-H immunostaining is unfortunately impossible in this group of
patients as the disease manifests later than 20 weeks and termination tissues are limited to the first 13 weeks gestation. However, it is interesting to speculate on the therapeutic potential of local or placental-targeted administration of a stable form of hCG-H to women with low hCG-H in the first trimester to potentially facilitate further trophoblast invasion. Given the ethically sensitive nature of such interventions and that hCG-H likely stimulates invasion of human trophoblasts only (rendering commonly used non-primate animal models redundant), such a treatment is likely a long way off.

While hCG-H localization in the placental villous has previously been examined in a limited range of gestational weeks, the presence of HCG-H positive cells within the maternal decidua has never been studied. Here it is clearly demonstrated that placental villi making contact with the maternal decidua, and invasive EVT within the decidua (HLA-G positive cells), continue to express the invasion-inducing hCG-H. Interestingly, EVTs which have reached their ‘final destination’ and are remodeling the maternal spiral arteries, continued to express hCG-H. This form of hCG is known to act as an autocrine factor, in the manner of a chemokine or cytokine, rather than mediating the classic hormone actions ascribed to hCG. HCG-H is proposed to increase production of gelatinases (MMP2 and MMP9) by CTBs, hence contributing to their invasive properties (Lee et al., 2013). However, since hCG-H production is maintained even after the EVTs reach and remodel the maternal blood vessels, it may be speculated that hCG-H performs additional autocrine functions beyond promoting invasion via an autocrine mechanism. Possibilities for its actions within maternal blood vessels include: (i) secretion into the maternal circulation to facilitate immune tolerance, with hCG-H predicted to inhibit leukocyte adhesion via E-selectin (Stahn et al., 2005); (ii) enhancement of in vivo proliferation of the tolerogenic uNK cells essential for pregnancy success, very high doses of hCG can enhance proliferation of uNK cells via the mannose receptor (Kane et al., 2009), an effect abrogated by deglycosylation, suggesting the hyperglycosylated portion of the hCG mediates this effect; (iii) modulation of growth factor expression within the maternal decidua influencing the implantation process as demonstrated for hCG (Licht et al., 2001; Evans et al., 2009; Paiva et al., 2011); (iv) actions as an angiogenic factor to
further transform the maternal blood vessels into the low resistance, high-capacitance vessels of pregnancy, hCG-H can influence angiogenesis in an aortic ring assay (Berndt et al., 2013), but its effect on the highly specialized uterine microvasculature has never been examined. At present the downstream targets of hCG-H are largely unknown since clinical interest has primarily been its use as a diagnostic tool. Its mechanisms of action require elucidation, if we are to understand the underlying biology.

Previous studies have examined the impact of hCG-H addition to choriocarcinoma cells to determine its effect on cell invasion (Cole et al., 2006; Lee et al., 2013). However, it has been demonstrated that 100% of the hCG produced by choriocarcinoma cells is hyperglycosylated (Cole et al., 2006) and these cells have been used as the source of hCG-H for other studies (Berndt et al., 2013). HCG-H has a proposed autocrine action, to promote invasion by the cells which produce it. Given that the system is already swamped with endogenous hCG-H we instead blocked its action using the B152 antibody, proven to be efficacious in nude mice (Cole et al., 2006). This blocking antibody, decreased outgrowth from first trimester villous tip explants and potentially affected invasion, observed as decreased collagen gel digestion.

**Figure 5** Treatment with B152 reduces first trimester villous tip outgrowth. Treatment of first trimester villous tips with 2.5 μg/ml mouse IgG for 48 h allowed outgrowth and invasion of extravillous trophoblasts (A, B (degraded collagen areas indicated) and white column, E), whereas treatment with 2.5 μg/ml B152 inhibited the extent of extravillous trophoblast outgrowth and invasion (C, D and gray column E). Representative images presented from n = 3 individual placentas, with n = 5 replicates per placenta, *P < 0.05.
Further, Jeg-3 cells treated with B152, had decreased real-time invasion but not migration, likely due to inhibition of MMP production (Lee et al., 2013) thus inhibiting the invasive process but not affecting simple cell movement as measured by migration assays. These data reinforce the positive effect of conditioned media from invasive trophoblasts, that produce hCG-H, to promote invasion of a non-hCG producing trophoblast cell line (Handschuh et al., 2007). However, this study used an antibody blocking all forms of hCG not a specific hCG-H antibody to block function. Thus the specific influence of hCG-H is implied rather than proven. Additionally, in the same study the antibody depleted a 38 kDa form of hCG from the CTB conditioned media, rather than the 42 kDa hCG-H. Thus the data in the present study confirm that hCG-H mediates trophoblast invasion and that its function can be blocked using neutralizing antibodies. These data are also in agreement with the pro-invasive effect of hCG-H observed by Lee et al. (2013), and provide important future directions to investigate the utility of blocking hCG-H action. Clearly, trophoblast invasion is essential for appropriate placentation formation. However, there are situations in which blocking invasion may be of value. For example, the clinical consequences of inappropriately managed ectopic pregnancies can be life-threatening, with the major risk being rupture of the delicate Fallopian tube by the invasive trophoblast. Surgical intervention for ectopic pregnancies removes the affected Fallopian tube making therapeutic interventions highly desirable. Use of a hCG-H blocking antibody or a small molecule inhibitor could provide a new therapeutic intervention or adjuvant to current therapies. Additionally, gestational trophoblast disease (GTD), in which trophoblast cells remaining after childbirth become aggressively metastatic, is mediated by hCG-H, and use of this form of hCG in management of GTD is being actively pursued (Cole and Muller, 2010).

In conclusion, this paper has defined the localization and dynamic changes in hCG-H expression within the placental villi and maternal decidua throughout the first trimester of pregnancy and demonstrated that the changing placental levels reflect the levels in maternal serum. Importantly, a role of hCG-H in mediating cytotrophoblast invasion has been demonstrated, leading to a proposal that blocking hCG-H action could be effective in treatment of early stage ectopic pregnancies as a strategy in management of gestation trophoblast diseases.

Acknowledgements
We thank our research nurse Sister Judi Hocking for collection of samples and all women who donated tissues.

Authors’ roles
J.E. conceived the study, performed experimental work and wrote the manuscript. L.A.S. aided planning of the study, wrote and edited the manuscript. E.M. performed experimental work and edited the manuscript. E.D. wrote and edited the manuscript.

Funding
This work was supported by the Victorian Government Operational Infrastructure Support Program. J.E. is supported by NHMRC project grant #1047756, L.A.S. and E.D. by NHMRC Fellowships #1002018 and #550905 respectively and E.M. by an NHMRC Early Career Fellowship #611827.

Conflict of interest
None declared.

References