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Fine scale quantification of HCG beta gene transcription in human trophoblastic and non-malignant non-trophoblastic tissues

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Abstract

BACKGROUND: HCG is produced by syncytiotrophoblast of placenta. It delays the apoptosis of corpus luteum and functions in implantation. Its possible role in male reproduction has been raised. HCG beta subunit is encoded by *CGB*, *CGB5*, *CGB7* and *CGB8* genes located at 19q13.3 in a common genome cluster with beta subunit non-coding *CGB1* and *CGB2*. We conducted a sensitive quantification and comparison of *CGB* gene expression in human trophoblastic (blastocysts, n=6; normal/failed pregnancy, n=51) and non-malignant non-trophoblastic tissues (15 different tissue types, samples n=241).

METHODS: Real-time RT-PCR.

RESULTS: We showed a wide transcriptional window of *CGB* genes in normal pregnancy, a significant reduction in recurrent miscarriages, and a high expression (especially *CGB1/CGB2*) in ectopic and molar pregnancies. Expression was several orders of magnitude lower in the non-placental tissues, with the highest *CGB* levels being seen in testis, prostate, thymus, skeletal muscle and lung samples. The contribution of *CGB1/CGB2* to the summarized expression of six *CGB* genes was not proportional to their gene dosage: 1/1000 to 1/10000. An interesting exception was the testis exhibiting a much higher *CGB1/CGB2* to total *CGB* mRNA ratio of ~1/3, corresponding to gene dosage.

CONCLUSIONS: The expressional profile of *CGB* genes, activated already in blastocyst stage, is associated with the status of pregnancy. The presence of *CGB* transcripts in testes, and in particular *CGB1/CGB2* transcripts, may indicate a role in male reproductive tract.

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Declaration of authors' roles:

KR, RKC and ML designed the study; KR, MP and AS coordinated for the recruitment the study material; KR, PH, LU and JJ contributed to the acquisition of the data; KR, ML, JJ and RKC analyzed and interpreted the data; KR and ML drafted the manuscript and PH, LU, JJ, MP, AS and RKC revised it critically for substantial intellectual content. All the authors have read and approved the submitted manuscript version.

Keywords

Chorionic gonadotropin beta genes; normal and failed pregnancy; trophoblastic and non-trophoblastic *CGB* expression; *CGB* in male reproduction

Introduction

Human chorionic gonadotropin (HCG) is an evolutionary young hormone arisen in primate lineage (Talmadge *et al.*, 1984; Maston and Ruvolo, 2002). HCG like other glycoproteins (thyroid stimulating hormone - TSH, follicle stimulating hormone – FSH, luteinizing hormone – LH) consists of two subunits. The alpha subunit is shared by all four glycoproteins, and the beta subunit is specific for each hormone. Unlike the other glycoproteins that are synthesized in the anterior lobe of pituitary gland, HCG is mainly produced by syncytiotrophoblast of placenta during pregnancy (Pierce and Parsons, 1981). The structure of anthropoid primate-specific hemochorial placenta enables the transport of the large glycoprotein into the maternal circulation, where it exerts effects for fetal benefit (King, 1993). In addition to its endocrine function of delaying the apoptosis of the corpus luteum during the first trimester of pregnancy, HCG has several paracrine effects essential in the process of implantation (Srisuparp *et al.*, 2001, Cameo *et al.*, 2004), angiogenesis and placentation (Toth *et al.*, 2001; Zygmunt *et al.*, 2002; Herr *et al.*, 2007) and development of maternal immunotolerance (Kaylisli *et al.*, 2003). In addition to its function in pregnancy, elevated levels of HCG or its free beta subunit in serum is found to be as a sensitive tumor marker for trophoblastic tumors and testicular germ cell tumors (Bates and Longo, 1985; Seppälä *et al.*, 1986; Braunstein 1990; Duffy 2007). HCG and/or CGB proteins are present in a subset of cases with other tumor types, but their association is sporadic and the proteins have not been established as having functional, diagnostic or case management relevance in these other settings. HCG beta subunit is encoded by four genes (*CGB*, *CGB5*, *CGB7*, *CGB8*) that reside in a common genome cluster with the evolutionarily ancestral *LHB* and two recently duplicated genes *CGB1* and *CGB2* at chromosome 19q13.3 (Talmadge *et al.*, 1984; Maston and Ruvolo, 2002; Hallast *et al.*, 2005; 2007). Unlike the HCG beta coding genes that each produce a single mRNA transcript, *CGB1* and *CGB2* have four splice variants as originally described by Bo and Boime (1992). The major transcript of *CGB1/CGB2* (marked hereinafter as a transcript of *CGB1/CGB2*) codes for a unique 5'UTR and exon 1, and one basepair shifted open reading frame (ORF) for exon 2 and exon 3 (Fig.1). This predicts a completely different protein from HCG. The alternative mRNAs (marked as *CGB1/CGB2*+47 bp, +166 bp, +176 bp, respectively) contain additional 47 bp, 166 bp or 176 bp of DNA sequence from intron 1 (Fig.1) that alter the amino acid sequence of transcript.

We have previously studied the expression profile of individual *CGB* genes during normal and complicated pregnancy using a semi-quantitative Gene Scan Fragment Analysis approach (Rull and Laan, 2005). In this study we have addressed the fine-scale quantification of *CGB* genes in trophoblastic tissues (samples n=51) including normal pregnancy from the blastocyst (n=6) stage and different scenarios of pregnancy failure (recurrent miscarriages, ectopic and molar pregnancies). We also studied the transcriptional profile of *CGBs* in non-malignant non-trophoblastic human tissues (tissues n=15, samples n=39 for individual cDNAs and n=202 for tissue-specific pooled cDNAs). We enhanced the sensitivity of the transcript detection by using the real-time RT-PCR method, which enabled us to address separately the expressional profile of *CGB1/CGB2*. The screen for *CGB* expression in normal, non-malignant non-trophoblastic tissues was done to investigate whether these genes might have relevance outside of the blastocyst and pregnancy.

Materials and Methods

Experimental Subjects

The study was approved by the Ethics Committee of the University of Tartu, Estonia (protocols no. 117/9, 16.06.03, and 126/14, 26.04.2004), and a written informed consent was obtained from every patient. Blastocysts were kindly donated by six couples undergoing in vitro fertilization procedure at Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics (Dr. Peeter Karits). Chorionic villi/placental samples were obtained from females who underwent elective therapeutic abortion during first trimester of pregnancy (4-12 weeks of gestational age, n=10); therapeutic abortion during second trimester due to medical risks of pregnancy, where no fetal anomalies were detected (17-21 weeks of gestational age, n=8); normal delivery at term resulting from uncomplicated pregnancy (38-42 weeks of gestational age, n=12); surgical treatment of ectopic pregnancy (6-14 weeks of gestational age, n=8); cervical dilatation and uterine curettage because of recurrent (patients had 2 spontaneous abortions before the case) incomplete or missed abortion (6-17 weeks of gestational age, n=11); and because of molar pregnancy (9-10 weeks of gestational weeks, n=2). The autopsy material of selected tissues was obtained from Research Tissue Bank of Tartu University Hospital (cerebral cortex, liver, skeletal muscle, kidney, pancreas, spleen, small intestine, testes). Biopsy samples obtained during surgical therapeutic procedures upon informed consent were provided by Dr. Tõnu Vooder, Lung Clinic of Tartu University Hospital (lung and thymus) and by Dr. Margus Punab, Tartu University Hospital, Andrology Unit (testes). Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C or placed immediately into RNAlater solution (Ambion Inc, Austin TX) and kept at -20°C until RNA isolation. For all biopsy/autopsy samples histological examination was carried out to confirm the non-malignancy of the tissues. In addition to clinical samples the cDNA from Human Multiple Tissue cDNA Panel I and II (Clontech Laboratories Inc, Mountain View, CA) was used to broaden the range of studied tissues.

RNA extraction and cDNA synthesis

Total RNA from 30-1000 mg of tissue was extracted and quantified as described previously (Rull and Laan, 2005). The integrity of RNA was checked by 2100 Agilent Bioanalyzer using RNA 600 Nano LabChips (Agilent Technologies, Santa Clara, CA). RNA was reverse transcribed to cDNA using random hexamers and SuperScriptTM III Reverse Transcriptase according to manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA). Briefly, a 1 μg aliquot of DNase-treated total RNA was incubated with 50 ng random hexamer primers and 1 μl of a cocktail containing 10 mM of each dNTP at 70°C for 5 min. The reaction was cooled on ice and remaining reagents were added (2 μl of $10\times$ reaction buffer, 100 nmol MgCl_2 , 0.2 μmol dithiothreitol (DTT), 40 U RNaseOUTTM, 200 U SuperScriptTM III Reverse Transcriptase) and the reaction proceeded at 25°C for 10 min and 50°C for 50 min. The reverse transcription was inactivated by a 5 min incubation at 85°C and cDNA was treated with 2 U of *E.coli* RNase H at 37°C for 25 min to remove the RNA template from the cDNA:RNA hybrid molecule. The minus reverse transcription controls were treated as described above except that the reactions lacked SuperScriptTM III Reverse Transcriptase.

Primer and Taqman probe design, and real-time RT-PCR

All primers and probes (Table I) for real-time RT-PCR were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA) taking into account polymorphic positions (SNP) in *CGB* genes identified by the resequencing study (Hallast *et al.*, 2005). As the only splice-form discriminatory *CGBI* Taqman probe included a polymorphic position (A/G; exon II, pos. 233 bp from transcription start) we designed two alternative detection oligos specific to either allele (Table I, Fig. 1 probe 1,2). In subsequent calculations we

averaged the results obtained from two differentially labeled probes. The quantification of mRNA transcript *CGB1/CGB2*+166 bp was omitted because of an unfavorable sequence (ACCCCCA) on the form-specific exon I/exon II boundary for positioning either primers or a probe. The primer-probe mix of the reference genes (*GAPDH*, Hs99999905_m1, amplicon length 122 bp; *HPRT1*, Hs99999909_m1, 100 bp) were purchased from Applied Biosystems (Foster City, CA). *GAPDH* is expressed at approximately the same level with hCG beta subunit coding genes and *HPRT* is transcribed in the similar activity as *CGB1/CGB2* genes.

The real-time RT-PCRs was performed using Applied Biosystems 7900HT Fast Real-time PCR system in 384 micro-well plates. The 10 μ l PCR reactions consisted of 1 μ l cDNA product, 5 μ l TaqMan® Universal PCR Master Mix (2X) containing AmpliTaq Gold® DNA Polymerase and AmpErase® UNG (Applied Biosystems, Foster City, CA), as well as primers and a probe in following concentration: 500 nM forward and reverse primer, 100 nM probe for target genes; or 0.5 μ l ready-to-use endogenous control gene primer-probe mix (20X) for *GAPDH* or *HPRT1*. The PCR protocol was identical for all runs: 50°C for 2 min and 95 °C for 10 min to initiate the reaction followed by 50 two-step amplification cycles (15" 95 °C; 1' 60 °C). Each sample was run in triplicate. Statistical analysis included the data only from the reactions where minus cDNA gave the negative result.

Gene Scan Fragment Analysis was conducted as described (Rull and Laan, 2005), except that no discrimination of the transcripts for each individual hCG beta coding gene and *CGB1/CGB2* was carried out.

Construction of transcripts-specific and negative control plasmids

We cloned from normal first trimester placenta tissue RT-PCR amplified (primer sequences in Table I) and gel-puried cDNA fragments of *CGB8* (representing four HCG beta subunit coding genes), *CGB1* (= *CGB2*) and *CGB1*+176 bp splice-form, which were used for real-time RT-PCR optimization and preparation of standard curves for absolute quantification. All inserts were cloned into pTZ57R/T vector (InsTAclone™ PCR Cloning Kit, Fermentas Life Sciences, Burlington, Canada) and plasmid DNA was extracted with Nucleo Bond® PC 500 Plasmid Purification Kit (Macherey-Nagel GmbH&Co KG, Düren, Germany). As an additional negative control for the oligo specificity to mRNA and to assure the presence of *CGB1/CGB2* alternative forms (exclude genomic contamination) we used a cloned *CGB1* genomic fragment, which contained the regions targeted by Taqman primers and probes.

Data analysis

For quantification of the target genes two methods were used: comparative Ct (cycle threshold, cycle number at which the PCR amplification crosses the background fluorescence) method and standard curve method. The calculations by Ct method was carried out by two formulas differing in taking into account PCR efficiency (Livak and Schmittgen, 2001; Pfaffl, 2001). The calculated relative expression level of *CGB* genes by two methods was strongly correlated ($r^2=0.95-0.99$). Standard curve of serial dilutions of plasmid cDNA was run over eight logs covering 10^7-10^1 copies of target transcript per reaction. Copy number of *CGB* transcripts was quantified according to guidelines by Applied Biosystems (http://www.appliedbiosystems.com/support/apptech/#rt_pcr) and as described by Reimer *et al.* (2000).

For statistical analysis a Pearson correlation test and a linear regression model were used. All statistics were performed using R 2.4.1, a free software environment for statistical computing and graphics (<http://www.r-project.org/>)

Results

Real-time RT-PCR experiment validation

Reproducibility of the real-time RT-PCR experiments was validated by high amplification efficiency (100+/-10%) as well as low intra-assay (<1.9%) and inter-assay (<2%) variation coefficients. Although the expression of the two reference genes differed from each other (*GAPDH* > *HPRT1*) in trophoblastic samples (by average 6.8+/-0.7 cycles) as well as in non-trophoblastic tissues (from 2.0-2.7 cycles in testis to 8.6-9.0 cycles in skeletal muscle samples), the results were concordant using either of the endogenous controls.

Proportional expression of CGB transcripts

Consistent with previous reports the major sources of *CGB* mRNA transcription were HCG beta coding genes (*CGB*, *CGB5*, *CGB7*, *CGB8*). The contribution of *CGB1* and *CGB2* genes to the summarized expression of all *CGB* genes was not proportional to their gene dosage: in the first trimester of normal pregnancy these two of the total six *CGB* gene copies (1/3) provided only 1/1000 to 1/5000 of the *CGB* mRNA transcripts, during the second and third trimester the contribution was even lower (~1/10 000; Table II). In comparison to normal implantation process the trophoblastic tissue from the cases of ectopic pregnancy was characterized by higher transcriptional activity of *CGB1/CGB2* and/or lower expression level of HCG beta subunit coding genes: *CGB1/CGB2* contributed 1/500 to 1/3000 of summarized *CGB* transcripts. This expressional peak of *CGB1/CGB2* confirms the original finding by semi-quantitative analysis (Rull and Laan, 2005).

Interestingly, we detected approximately equal fractions of the originally described *CGB1/CGB2* transcript coding for a hypothetical protein and of the alternatively spliced *CGB1/CGB2* + 176 bp mRNA that contains a premature stop-codon (Fig.1-2). This observation contributes to the uncertainty of the function and role of *CGB1/CGB2* in human. The other reported alternative splice-form (+47 bp; Fig.1) was transcribed at the borderline (in trophoblastic samples) or even below (in non-trophoblastic tissues) the reliable quantitation limit of real-time RT-PCR. The transcription initiation of different splice forms of *CGB1/CGB2* correlated within a sample ($r^2=0.82-0.89$).

Transcription of CGB genes in trophoblastic tissue during the normal and complicated pregnancy

As expected, during normal pregnancy the highest summarized expression of all *CGB* genes was detected for the first trimester ($p=0.01$ and $p=0.11$ compared to the second and third trimester, respectively) (Fig. 3A). In cases of recurrent miscarriages (RM) the summarized transcriptional activity of all *CGB* genes was significantly reduced compared to normal pregnancy at same gestational age ($p=0.03$; Fig.3A). Inversely, in cases of ectopic pregnancy (EP), the total *CGB* mRNA level tended to be higher than the distribution described for normal intrauterine gestation ($p=0.06$). The expression of the three alternative *CGB1/CGB2* transcripts followed the same pattern: the strongest expression during first trimester compared to second and third trimester of normal pregnancy ($p<0.004$; $p<0.04$, respectively), a reduced transcriptional activity in cases of RM ($p<0.05$) and more abundant *CGB1/CGB2* mRNA transcripts in trophoblastic tissues from EP compared to normal pregnancy of same gestational age (0.02 p 0.09 , Fig.3B-D).

Expression of CGB genes in blastocysts and molar pregnancy

We explored the expression of *CGB* genes in trophoblastic tissues obtained from molar pregnancies ($n=2$) and from blastocysts ($n=6$). The transcription level of all *CGB* transcripts in molar pregnancy was at the upper range of the distribution obtained from normal first trimester pregnancy ($n=10$) (Fig.3A-D). In mRNA extracted from pooled blastocysts

material the summarized level of *CGB* transcripts was as high as the estimated median (Fig. 3A) for trophoblastic samples from the first trimester of normal pregnancy (10-fold lower compared to the calibrator) using relative quantification. However, low yield of the total mRNA extracted from blastocysts prevented the exact calculation of the amount of mRNA entering the cDNA synthesis and thus, no absolute quantification of *CGB* mRNA molecules was possible. This supports the suggestions that the full activation of *CGB* genes occurs in early preimplantation stage and is essential for normal implantation (Bonduelle *et al.*, 1988; Jurisicova *et al.*, 1999). Interestingly, despite the low general expression level of *CGB1/CGB2* and their alternative forms, their transcripts were also detected already in blastocyst material both by real-time RT-PCR (data not shown) as well as even by semi-quantitative analysis (Fig.4).

Expression of *CGB* genes in non-trophoblastic tissues

To avoid contamination and false-positive results facilitated by low expression level, we addressed the transcriptional profile of *CGB* genes in non-trophoblastic tissues by two alternative methods: a semi-quantitative RT-PCR combined with Gene Scan Fragment analysis (Bellet *et al.*, 1997; Miller-Lindholm *et al.*, 1997; Rull and Laan, 2005) and quantification with real-time RT-PCR using TaqMan method (Reimer *et al.*, 2000). Gene Scan Fragment analysis was used to screened *CGB* transcripts from commercial human tissue cDNA panels (15 pooled cDNAs from 1-45 subjects). For real-time RT-PCR experiments we used identical cDNA panels as well as analyzed additional RNAs (n=3-5) prepared from various human non-malignant tissues (Table II). The results obtained from tissue panel and analyzed selection of clinical samples and autopsies were concordant. As expected, real-time RT-PCR method proved to be more sensitive resulting in detection of *CGB* transcripts in 13 of the studied 15 non-trophoblastic tissues. The reliable expression of *CGB* genes was detected in testis, prostate, thymus, skeletal muscle and lung samples (160-722 cDNA copies per PCR reaction; Table II). However, the transcription level was still very low, from 1/2500 (testis) to 1/12500 (lung) of the *CGB* expression level in first trimester placenta. In several tissues (ovary, small intestine, kidney, spleen, liver, heart, brain and colon) *CGB* transcripts were present at the level of the borderline detection by the real-time RT-PCR method (9-86 copies per reaction; Table II). Finally, no *CGB* transcripts were detected in pancreas and peripheral blood leukocytes. Semi-quantitative Gene Scan Fragment analysis identified *CGB* transcripts only in four non-trophoblastic tissues (testis, prostate, thymus and lung). Thus, the detection limit of this method is >100 cDNA copies per PCR reaction.

Due to low expression *CGB1/CGB2* mRNA was amplified by real-time RT-PCR only in a limited number of non-trophoblastic tissues: testis, prostate, thymus, ovary, kidney and colon. Interestingly, *CGB1/CGB2* are ~16 times more transcribed in testis (453 copies per reaction) than in the second (15 copies per reaction) and third trimester of pregnancy (28 copies per reaction; Table II). Also, in testis the proportional contribution of HCG- β coding genes and *CGB1/CGB2* to the total *CGB* transcript pool is very different from placenta, ~66%/33% (722 copies versus 453 copies per reaction; Table II).

Discussion

This study reports a detailed mRNA transcription profile of *CGB* genes in trophoblastic (normal/complicated pregnancy) and in non-malignant non-trophoblastic human tissues. We chose the real-time RT-PCR method allowing sensitive quantification of the *CGB* transcripts in extra-uterine tissues expressing these placental genes at low level. In addition, the enhanced sensitivity of the experiment enabled to provide more confident data about the expression of low-transcribed *CGB1/CGB2* genes.

Both, semi-quantitative Gene Scan Fragment analysis (Miller-Lindholm *et al.*, 1997; Rull and Laan, 2005) and quantitative real-time RT-PCR experiments indicated a wide range of distribution of the transcriptional level for the six *CGB* genes in normal intrauterine pregnancy and a significant correlation between *CGB* mRNA abundance in placenta with HCG concentration in the maternal serum. There is evidence that in cases of recurrent miscarriages low HCG levels in urine/serum (Gerhard and Runnenbaum, 1984; Buyalos *et al.*, 1992; Letterie and Hibbert, 2000) may result as a direct consequence of a significant reduction in the expression of *CGB* genes. We are aware that ongoing biological processes to reject fetus from uterus during the miscarriage and induced therapeutic abortion may change the expression pattern of the genes in some extent, although decrease of hormone in serum after abortion considerably slow (Steier *et al.* 1984). Low transcriptional activation of *CGB* genes might result from an inadequate function of decidual cells and syncytiotrophoblasts producing the lactogenic hormones required for immunological adaptation of the embryo and trophoblast invasion (Lyll, 2002; Burton *et al.*, 2007). However, the low *CGB* mRNA level compared to normal gestational age-matched placentas may indicate not only maternal susceptibility to a miscarriage, but also chromosomal aberrations of the conceptus. In case of trisomy 18 (Edwards syndrome), decreased concentration of maternal serum HCG during first trimester is the consequence of impairment in the transcription of HCG beta subunit genes (Brizot *et al.*, 1996). The situation for trisomy 21 Down's pregnancies is more complicated. In vitro studies following the differentiation process of cultured cytotrophoblast cells showed a significant decrease in *CGB* mRNA transcription and consequently the secretion of HCG for trisomy 21 placentas causing a defect and/or a delay formation of syncytiotrophoblast formation (Massin *et al.*, 2001). But it is well known that Down's syndrome is associated with elevated, not reduced maternal serum concentrations of HCG as well as free HCG beta subunit (reviewed by Knöfler, 1999).

In contrast to recurrent miscarriages, the expression of the *CGB* genes in extra-uterine pregnancy (EP) is up-regulated and has a narrower expressional window (Fig.3). The *CGB* transcriptional activity in molar pregnancy falls into the same range as in EP and is responsible for high serum HCG levels (this report; Feng *et al.*, 2006). In cases of EP, the discrepancy between the HCG concentration in serum and *CGB* mRNA transcription level in trophoblastic tissues contrasts the trisomy 21 pregnancies: low hormone versus high transcriptional activity. In both physiological conditions, post-transcriptional processes like mRNA stability, translation initiation, posttranslational modifications, hormone assembly, activity and stability determine the fine regulation of HCG molecule biosynthesis and properties. Indeed, in Down syndrome the defective differentiation leads to the accumulation of cytotrophoblasts producing the structurally and functionally distinct hyperglycosylated hormone variant HCG-H instead of the regular HCG (reviewed by Cole, 2007). The abundance of HCG-H might be one of the reasons why the fetuses with trisomy 21 unlike most other chromosomal anomalies are not aborted during the gestation. In EP, the low hormone levels refer to the deficient hormone assembly and the high expressional activity of *CGB* genes might reflect the failure of a normal negative autoregulatory feedback. Hormonal effects on the stability of a specific mRNA can profoundly alter its steady-state concentration (e.g. reviewed by Staton *et al.*, 2000; Ing, 2004).

In addition to the detection of summarized transcription of all *CGB* genes we analyzed isolated expression of the most recently duplicated, the African great ape specific *CGB1/CGB2* and their alternative products both in trophoblastic and non-trophoblastic tissues. We have recently shown that the human and chimpanzee *CGB1/CGB2* genes have no ORF disrupting mutations (Hallast *et al.*, 2007), as well as there are signals that these novel genes evolve under positive selection (Hallast *et al.*, unpublished data). The expressional activity of *CGB1/CGB2* within a particular sample was correlated with the total *CGB* transcript

level suggesting a concerted transcriptional activation of the gene cluster. The concerted manner of the expression might be explained by either the transcriptional activation of the genome cluster through simultaneous chromatin modifications (e.g. demethylation of the abundant CpG-island within the cluster) and/or by highly homologous DNA sequence (>95%) in the promoter regions of all *CGB* genes. The low level of the transcription of *CGB1* and *CGB2* could result from modified promoter region lacking functionally important binding sites for two Ets-2 transcription factors (Fig.1; Ghosh *et al.*, 2003). The central role of Ets-2 in trophoblast differentiation and function has been found in several other studies (Roberts *et al.*, 2003; Chakrabarty *et al.*, 2007). It is still an open question whether the *CGB1/CGB2* genes are pseudogenes producing mRNA transcripts as a consequence of general expressional activation of the *CGB* gene cluster or code for a functional protein. The pseudogene status is supported by extremely low transcription level compared to HCG beta genes (with the exception of testis) and no structural similarity of the predicted hypothetical protein to other known proteins. However, there is growing evidences that transcribed pseudogenes are not entirely without purpose. For example, they can act as potential regulators of gene expression and effectors of the function of known genes (Hirotsune *et al.*, 2003, Korneev *et al.*, 1999). As concluded by Khaitovich *et al.* (2006) these types of regulatory transcripts tend to be expressed at low levels, sometimes at or below detection limit and the transcripts are located close to annotated genes or even within genes, in introns or on the opposite DNA strand. The transcripts produced by the pseudogenes located between protein coding genes show conserved tissue-specific expression patterns concordant with the protein-coding transcripts of known genes. Still, we cannot exclude the possibility that *CGB1* and *CGB2* code for a functioning protein – similarly to the HCG beta peptides (163 amino acids, 12 Cys), the predicted hypothetical *CGB1/CGB2* peptide is enriched in cysteins (132 amino acids, 5 Cys) relative to regular globular proteins. Thus, concordant to HCG synthesis, Cys-Cys bridges (forming a cystein-knot) may be critical for the 3D structure and function of the hypothetical protein involving *CGB1/CGB2* peptide (Ceslovas Venclovas, personal communication).

Despite the historic reports of *CGB* expression by individual cases of many tumors, to our knowledge there are only two larger previous studies addressing the transcriptional profile of *CGB* genes in normal non-malignant non-trophoblastic tissues (Bellet *et al.*, 1997: semi-quantitative detection; Reimer *et al.*, 2000: real time quantification with TaqMan). Consistent with these reports our data show that the expression of *CGB* genes varied among tissues. In all three studies the strongest non-trophoblastic *CGB* transcriptional activity was detected in testis, prostate and skeletal muscle, which is also concordant with the available gene expression microarray data (<http://expression.gnf.org>, 1153_f_at; Affymetrix Annotation). There is overlap between the two real-time experiments confirming reliably the transcription of *CGB* genes in thymus, colon, lung and kidney. Among the tissues studied in literature, *CGB* expression was detected also in uterus, mammary, thyroid, adrenal and pituitary glands (Bellet *et al.*, 1997, Reimer *et al.*, 2000, Dirnhofer *et al.*, 1996).

The question about the biological function of the *CGB* transcripts in testis (Berger *et al.*, 1994) and prostate (Dirnhofer *et al.*, 1998) was raised already more than 10 years ago when all the different mRNA variants (including three alternative splicing products of *CGB1/CGB2*) were identified in the cDNA pool from these tissues. Interestingly, compared to the placental tissue, the proportion of *CGB1/CGB2* transcripts was increased in testis (Table II). The presence of *CGB* transcripts in testes and prostate indicating a possible role male reproductive tract is in agreement with a recent study showing that HCG alpha and HCG beta free subunits, but much less the alpha/beta dimers (HCG) are produced in high amounts in the prostate and testis, and are subsequently observed in seminal plasma (Berger *et al.*, 2007). In testicular Leydig cells exclusively HCG beta free subunits were identified. It has

been suggested that HCG-derived molecules might act in auto-/paracrine or even transcrine ways in the prostate and the uterus.

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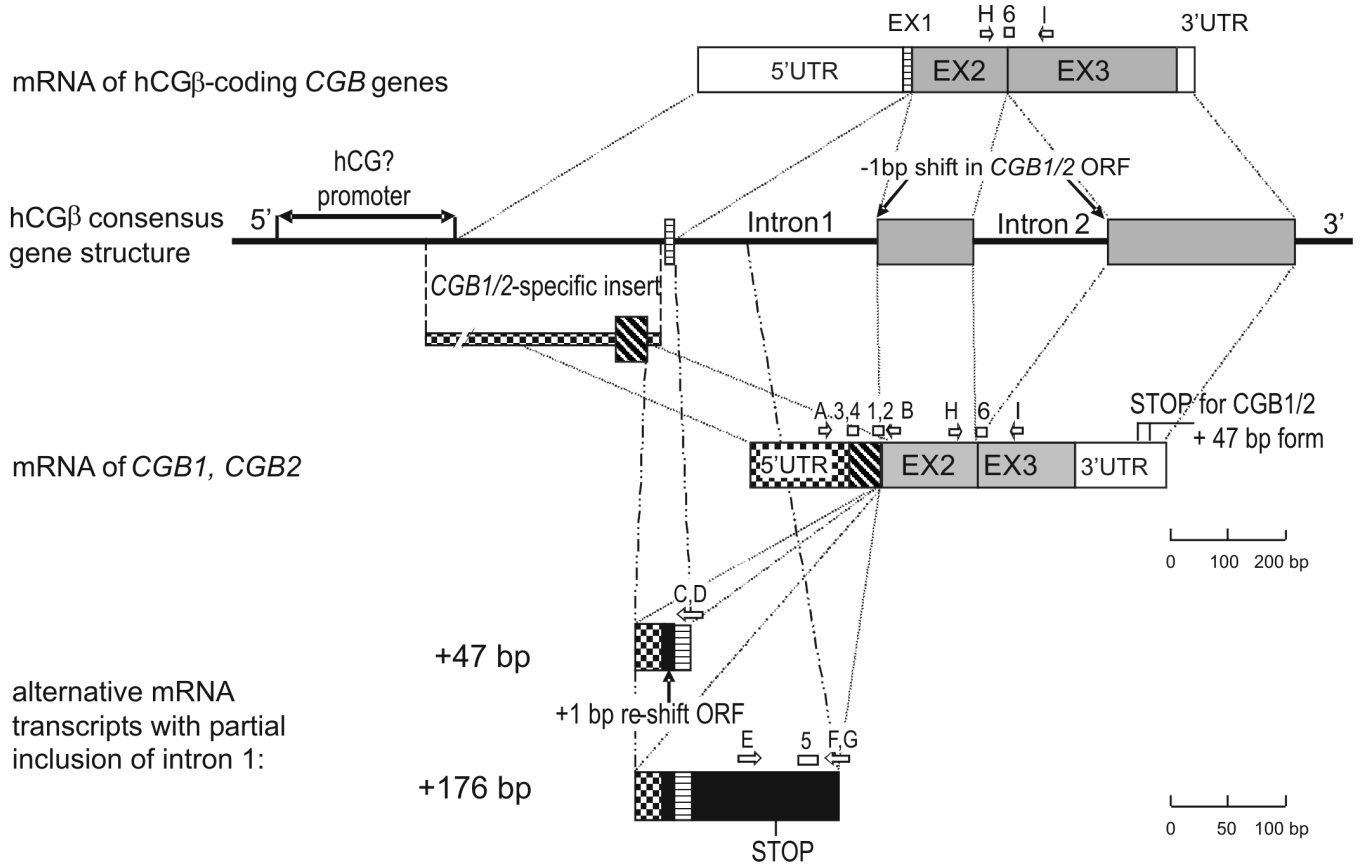


Figure 1. Schematic representation of the structure of the *CGB* genes and transcripts as well as localization of primers and Taqman probes used in the study. The coding segments of all *CGB* genes are marked on the consensus gene structure with grey boxes, except for the exon I for hCGβ-coding genes (horizontal stripes) and for *CGB1/CGB2* (diagonal stripes). The 5'UTR of mRNA transcribed from hCGβ-coding genes (white box) differs from the 5'UTR for *CGB1/CGB2* (chequered box); 3'UTR of the two groups of gene is of variable length (white box). Alternative +47 bp *CGB1/CGB2* mRNA forms contain additional sequence from *CGB1/CGB2*-specific intron I (22 bp, black box), including the fragment corresponding to the hCGβ 5'UTR (10 bp, chequered box) and exon I (15 bp, horizontal stripes), resulting in a re-shift the *CGB1/CGB2* ORF to the ORF of hCGβ-coding transcripts. Due to sequence divergence in 3'UTR of *CGB1/CGB2*, the predicted STOP codons for +47 bp *CGB1* and +47 bp *CGB2* differ by 7 aminoacids. Alternative transcripts +166 bp (not shown in the figure) and +176 bp contain additional 119 bp/129 bp sequence identical to the intronic part of all *CGB* genes (black box). The predicted STOP codon for +166 bp/+176 bp forms is located at position 355 from transcription start (hypothetical polypeptide 60 amino acids). Primer and probes use for real-time RT-PCR experiments are shown with white arrows or boxes and marked with letters or numbers, respectively. The oligonucleotide sequences are shown in Table I.

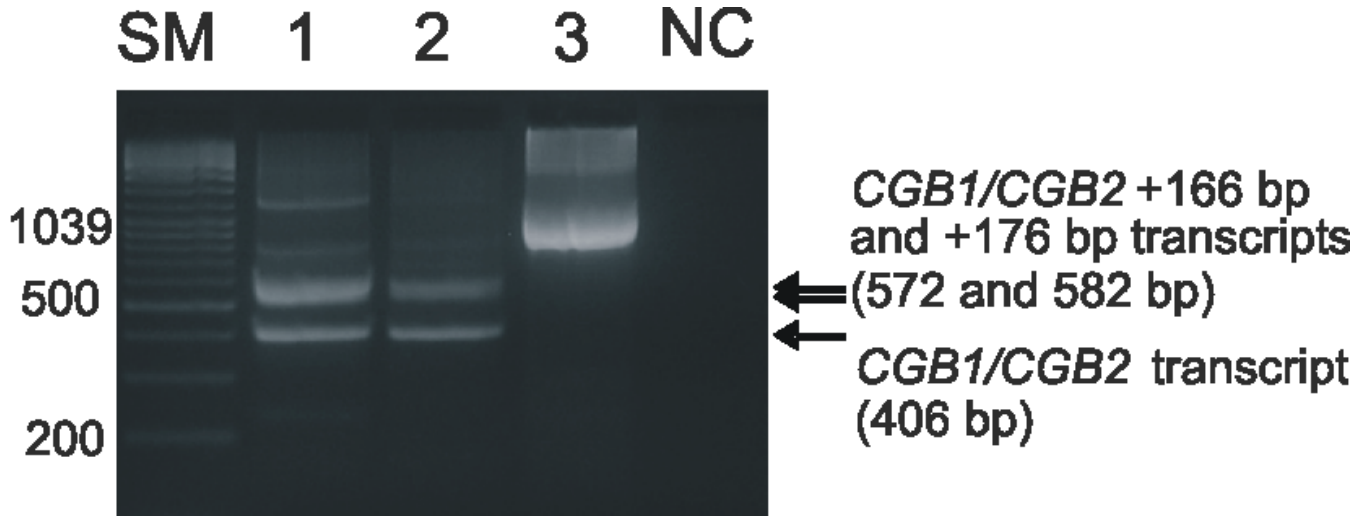


Figure 2.

Detection of *CGB1/CGB2* mRNA in trophoblastic tissue from the first trimester of a normal intrauterine pregnancy (Lane 1) and an ectopic pregnancy (Lane 2). Alternatively spliced transcripts *CGB1/CGB2*, *CGB1/CGB2 + 166bp* and *CGB1/CGB2 + 176 bp* are represented by the RT-PCR amplified fragments sized 406 bp, 572 bp and 582 bp, respectively. The *CGB1/CGB2 + 166bp* and the *CGB1/CGB2 + 176 bp* transcripts are indistinguishable from each other at the 3% agarose gel. The detection sensitivity of EtBr stained DNA separated on agarose gel is not enough for the visualization low expressed *CGB1/CGB2 + 47 bp* splice-variant. The amplified genomic DNA (Lane 3) is 1040 bp. NC, negative control with water as a template; SM, size marker (Gene Ruler™ 100 bp DNA Ladder Plus, Fermentas Life Sciences).

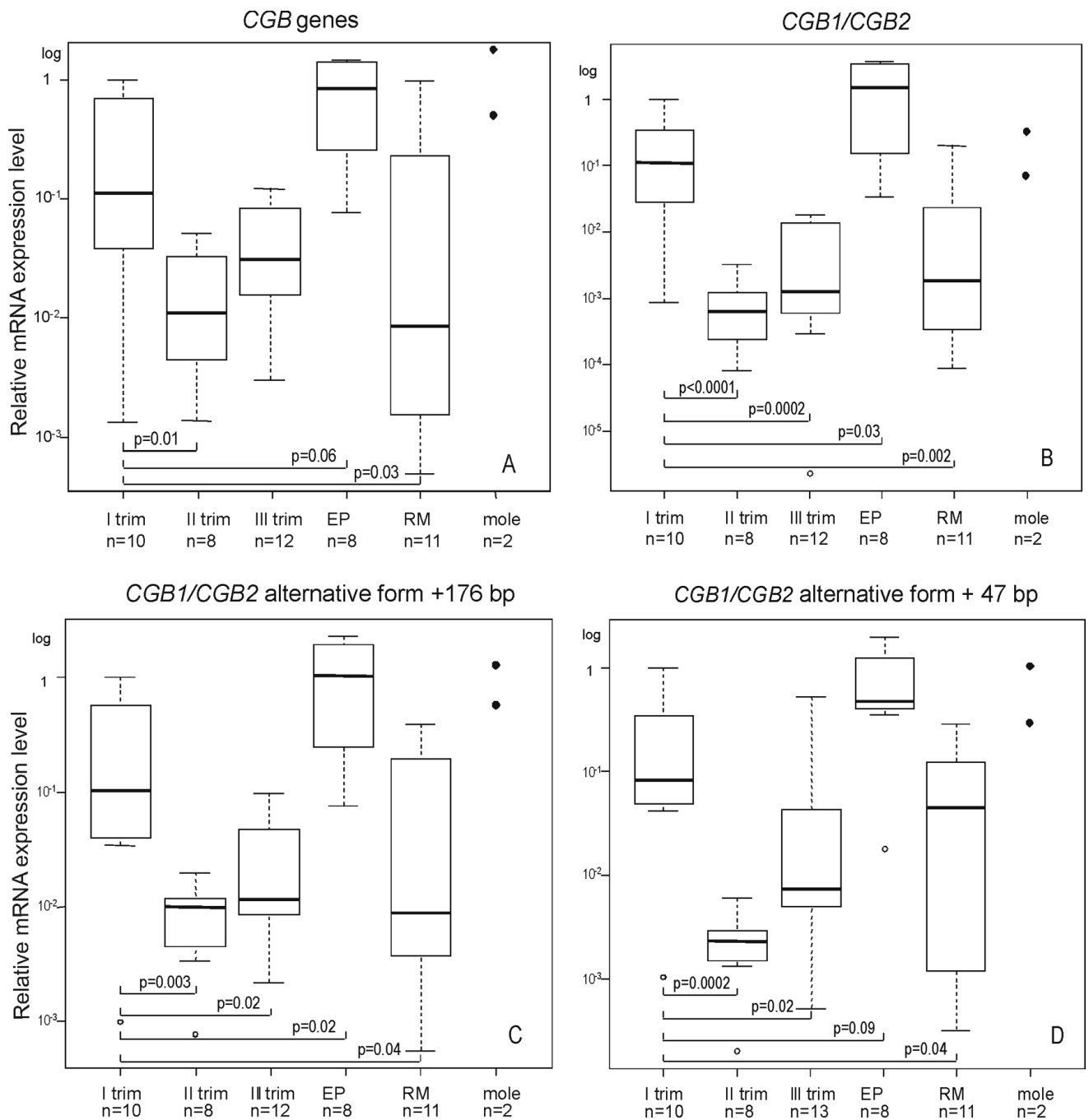


Figure 3.

Notched box plot for the distribution of the relative expression level of *CGB* transcripts in placenta during the first, second and trimester of normal pregnancy, in case of recurrent miscarriage (RM), ectopic pregnancy (EP) and molar pregnancy. The boxes represent the 25th and 75th percentiles. The median is denoted as the line that bisects the boxes. The whiskers are lines extending from each end of the box covering the extent of the data on 1.5 X interquartile range. Circles represent the outlier values. P-values reflecting the differences between groups are shown.

Relative mRNA quantization with real-time RT-PCR comparative Ct method for **A**, summarized *CGB* genes transcription; **B**, *CGB1/CGB2* transcripts; **C, D** *CGB1/CGB2+176 bp* and *CGB1/CGB2 +47 bp* alternative forms. The trophoblastic sample with highest summarized transcription activity of *CGB* genes from first trimester normal pregnancy is used as calibrator sample.

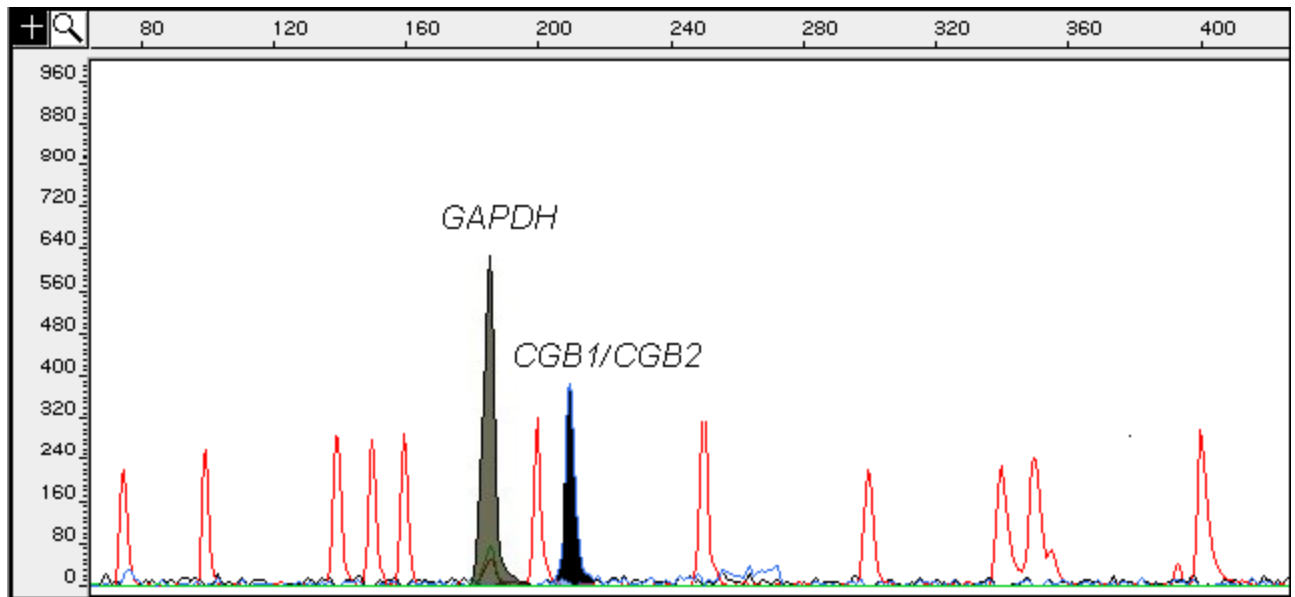


Figure 4.

Gene Scan Fragment Analysis electrophoretogram showing the amplified fluorescent-labeled products of *CGB1* and *CGB2* (6-FAM; 207 bp, black peak) gene and *GAPDH* (HEX; 196 bp, grey peak) from cDNA of a single blastocyst material. The x-axis shows the size of the detected fragments in base pairs (bp), and the y-axis represents the relative intensity of fluorescence (RFU). The empty peaks mark the GeneScan-500 TAMRA internal size standard.

Table I

The DNA sequences of the primers and probes for real-time RT-PCR experiments

Expression of <i>CGB</i> gene transcripts				
Real-time primer	Fig.1 Label	Sequence with Fluorescent Dye	T _m (°C)	Product size
<i>CGB1</i> /2F	A	5'-AACACCCCTCACTCCCTGTCT-3'	58.1	139 bp
<i>CGB1</i> /2R	B	5'-ATGCTCAGCAGCAGCAACA-3'	57.8	
alt47C1majR	C	5'-GCAGCAGCCTCTGGAACATCT-3'	58.6	167/ 165 bp
alt47C1min/C2R	D	5'-AGCAGCCCCTGGAACATCTC-3'	58.3	
alt176C1/C2intF	E	5'-GCCATCACTGGCATGAGAAG-3'	57.8	96 bp
alt176C1majR	F	5'-AGCAGCAGCCTTGAAGCTTACT-3'	58.6	
alt176C1min/C2R	G	5'-AGCAGCAGCCCCTGAAGCTTAC-3'	59.8	
<i>CGB</i> allF	H	5'-TCACCGTCAACACCACCATCT-3'	59.8	121 bp
<i>CGB</i> totalR	I	5'-ATGGACTCGAAGCGCACATC-3'	59.8	
Taqman probe				
<i>CGB1A</i>	1	5'-FAM-ACATGTCAAAGAGGCTG-MGB-3'	69.2	
<i>CGB1G</i>	2	5'-VIC-CATGTCAAAGGGGCTG-MGB-3'	69.9	
<i>CGB1</i> †	3	5'-FAM-CATGTCCACATCCCAGTG-MGB-3'	68.2	
<i>CGB2</i> †	4	5'-FAM-CATGTCCACATCCCCAGT-MGB-3'	67.9	
alt176C1/2	5	5'-FAM-CTCTTTCTGGAGGAGCGT-MGB-3'	68.3	
<i>CGB</i> all	6	5'-FAM-CCACCATGACCCGCGT-MGB-3'	68.6	
Primers for plasmid construction				
<i>CGB8</i> F		5'-AGCACCTTTCTCGGGTCAC-3'	60.2	791 bp
<i>CGB8</i> R		5'-GGCCTTTGAGGAAGAGGAGT-3'	59.5	
<i>CGB1</i> F		5'-GAGGAAGGGAACTGCATCT-3'	60.6	686/ 689 bp
<i>CGB2</i> F		5'-AGGGAGGAAGGGAACTGTA-3'	59.9	
<i>CGB1</i> , <i>CGB2</i> R		5'-TGCGGATTGAGAAGCCTTTA-3'	60.8	
<i>CGB1</i> Fnested		5'-GAAGGGAACTGCATCTGAG-3'	59.8	406 bp*
<i>CGB1</i> Rnested		5'-GGTAGTTGCACACCACCTGA-3'	59	

T_m, melting temperature; F, forward primer; R, reverse primer;† the probe was used to detect *CGB1/CGB2* + 47 bp alternative mRNA form* the primers amplified *CGB1* + 176 bp mRNA form, 581 bp and *CGB1* + 166 bp mRNA alternative form, 571 bp.

Table II

Expression of *CGB* genes in human tissues determined by Gene Scan Fragment Analysis and Taqman Real-Time methods

	No of samples		Summarized <i>CGB</i>		<i>CGBI/CGB2</i>	
	cDNA panel ^a	Biopsies/ autopsies	Gene Scan ^c	TaqMan ^d	Gene Scan ^c	TaqMan ^d
Trophoblastic tissue:						
Calibrator, I trim ^b	-	1	+	11873350	+	15000
Normal, I trim	-	10	+	2308383	+	879
Ectopic pregnancy	-	8	+	13163945	+	15067
Miscarriage, I trim	-	11	+	194371	+	134
Placenta, II trim	-	8	+	147295	+/-	15
Placenta, III trim	8	-	+	161600	nd	28
Testis	45	5	+	722	+	453
Prostate	32	-	+/-	491	+/-	17
Thymus	18	5	+/-	280	+	4
Skeletal muscle	8	3	nd	254	nd	nd
Lung	4	5	+/-	160	+	nd
Small intestine	32	3	nd	86	nd	nd
Ovary	14	-	nd	50	nd	3
Kidney	5	3	nd	28	nd	5
Spleen	3	3	nd	23	+/-	nd
Heart	3	-	nd	20	nd	nd
Brain	2	3	nd	20	nd	nd
Liver	1	3	nd	28	nd	nd
Colon	20	3	nd	9	nd	6
Pancreas	15	3	nd	nd	+/-	nd
Peripheral blood leucocytes	NA	-	nd	nd	nd	nd

NA – not available, trim – trimester, nd – not determined

^a pooled cDNAs from two tissue panels (see Materials and Methods),^b the calibrator sample (first trimester normal pregnancy) selected based on the highest expression of the target gene in the group and used in relative quantification

c detection of the target gene in at least in two runs of three (+); one run of three (+/-)
d mRNA molecules of the target gene/50ng of total RNA used for cDNA synthesis