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Endometrial human chorionic gonadotropin (hCG) expression is a marker for adequate secretory transformation of the endometrium

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Abstract

Purpose Successful embryo implantation into the endometrium depends on embryonic characteristics and proper endometrial development. Reproductive medicine often focuses on embryo quality, whereas reliable diagnostic tests for endometrial receptivity are still needed. We previously found that human chorionic gonadotropin (hCG), one of the earliest proteins secreted by the embryo, was also expressed by the luteal phase endometrium around the implantation window. Here, we tested our hypothesis of endometrial hCG as an implantation marker.

Methods Endometrial biopsies and serum samples were taken from patients undergoing routine infertility diagnostics. Correlations of immunohistochemically detected endometrial hCG expression with adequate endometrial secretory transformation, the infiltration of CD45-positive leukocytes, clinical diagnostic parameters, and endometrial thickness were analyzed.

Results A highly significant correlation between the endometrial score, as a measurement for regular secretory transformation, and the intensity of hCG staining was found. The invasion of CD45-positive leukocytes increased with progressing endometrial secretory transformation and rising endometrial hCG expression. In addition, serum progesterone concentrations correlated with hCG expression by the endometrial glands.

Conclusions Our results suggest endometrial hCG as a possible diagnostic parameter characterizing the endometrial secretory transformation and, thus, possibly also its implantation capability.

Keywords Human chorionic gonadotropin hCG · Endometrium · Implantation marker · Reproductive medicine

The authors consider that Sindy Schug and Anja Baunacke should be regarded as joint first authors.

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Abbreviations

ART	Assisted reproductive technologies
CGB	Human chorionic gonadotropin beta subunit
ET	Embryo transfer
hCG	Human chorionic gonadotropin

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HE Hematoxylin–eosin
LH Luteinizing hormone
PAS Periodic acid-schiff

Introduction

Besides embryo characteristics, proper endometrial development is crucial for successful embryo implantation. Nevertheless, assisted reproductive technologies (ART) mostly focus on the quality of the embryo rather than on correct timing of the endometrium. Molecular markers describing implantation competence of the endometrium are needed, and would help to better assess the endometrial condition and increase success rates in assisted reproduction [1–3].

The histological evaluation of endometrial biopsies permits a descriptive statement concerning proliferation and secretion status of the endometrium relative to the menstrual cycle day. However, the usefulness and diagnostic value of this method has been controversially discussed [4–7]. To improve the diagnostic value, further methods to assess the regular endometrial transformation throughout the menstrual cycle were developed. In the previous studies, for example, conventional hematoxylin eosin (HE) staining was combined with the periodic acid-Schiff (PAS) reaction [8, 9], assuming a correlation between glycogen deposits in the endometrium and successful blastocyst implantation. As the PAS reaction also stains glycoproteins [8], we hypothesized the presence of human chorionic gonadotropin (hCG) in the endometrium. The expression of hCG and its receptor by Fallopian tubes in a cycle phase-dependent manner was another argument in favor of this hypothesis [10]. Indeed, we were able to demonstrate hCG production by the secretory endometrium of non-pregnant women during the normal menstrual cycle around the implantation window [11–13]. Furthermore, we also found hCG to be secreted by the decidua [14].

Human chorionic gonadotropin was originally thought to be produced only by the syncytiotrophoblast during early pregnancy. It belongs to the glycoprotein hormone family, together with luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyrotropin (TSH). These heterodimeric hormones share a common alpha subunit and possess distinct beta-subunits conferring their specific biological functions [15, 16]. Interestingly, six genes coding for the beta subunit of hCG (CGB) exist in a gene cluster together with the LH beta subunit (LHB), from which CGB evolutionary originated [16–18]. They differ mainly in an extended, highly glycosylated C-terminus, which results in increased stability of hCG in comparison to LH [19]. Due to their structural similarities, hCG and LH act via the same G-protein coupled receptor (LHCGR) [20, 21]. However, several recent studies demonstrated that LH and hCG, nevertheless, have specific functions and cannot substitute for each other in every case. They were shown to activate different downstream signaling pathways following receptor binding [22–24]. Hormone-specific interaction with the LHCGR extracellular hinge region was made responsible for this [25].

Whereas, in women, LH regulates ovulation, hCG plays an essential role in the establishment and maintenance of early human pregnancy. Its classical endocrine function is to rescue the corpus luteum to maintain progesterone production [15, 26]. However, over the last few years, a lot of further, mainly paracrine, functions of hCG have been detected. hCG is an important immune regulator supporting maternal immune tolerance towards the fetal semi-allograft by inducing, for example, the secretion of various cytokines and modulating infiltration of T lymphocytes, monocytes, and natural killer cells into the endometrium [16, 27–34]. hCG has also been described as an angiogenic factor inducing VEGF expression [16, 35–38]. In addition, hCG-mediated increased trophinin, and MMP expression, as well as decreased expression of TIMPs, facilitates implantation [39–43]. Altogether, hCG seems to be essential for decidualization, embryo implantation, and placentation. In this context, hCG production by the secretory endometrium at the implantation window, as observed by our group, fits well into this concept and could be regarded as the maternal contribution to early pregnancy establishment. Remarkably, we found only beta subunit variant CGB7 to be expressed by the endometrium, whereas the placenta mainly secretes CGB3, 5, and 8 [13]. We also detected simultaneous infiltration of CD45-, CD34-, and CD56-positive cells into the luteal phase endometrium and proposed hCG as a marker for endometrial receptivity [12, 13].

To test our hypothesis, in the present study, we have determined whether the immunohistochemical detection of endometrial hCG allows a diagnostic statement to be made about the adequate transformation of the secretory phase endometrium. Our results show a statistically significant correlation between endometrial hCG expression, CD45-positive leukocyte infiltration, serum progesterone concentration, and proper secretory transformation of the endometrium suggesting hCG as a possible diagnostic parameter for characterizing the luteal phase endometrium.

Results

hCG immunostaining correlates with endometrial secretory transformation

The aim of our study was to answer the question as to whether endometrial hCG expression correlates with proper secretory transformation of the endometrium and other diagnostic parameters. For this reason, endometrial biopsies as

well as serum samples from 41 women were analyzed. First, the endometrial biopsies were histologically examined and an endometrial score was allocated to each specimen [12] according to its glandular and stromal properties, as well as the degree of leukocyte infiltration (Table 1).

Figure 1 shows representative endometrial examples with endometrial scores from 1 (proliferative) to four (late-secretory). Score 0 (Fig. 1e) refers to asynchronous endometrial secretory transformation, in the depicted case because of the inadequate infiltration of leukocytes (detailed definition in the materials and methods section).

Immunohistochemical staining of the endometrial specimens with anti-CGB antibody showed that hCG expression is nearly absent in proliferative endometria (Fig. 2a). In the early secretory phase (Fig. 2b), hCG production by the glandular epithelium starts and increases during further secretory transformation (Fig. 2c, d). LH staining of the endometrial samples was negative demonstrating specificity of the hCG staining (Online Resource 1). In asynchronously transformed endometria, only a marginal hCG expression could be observed (Fig. 2e). Statistical evaluation established a clear and highly significant correlation between hCG staining intensity (hCG staining index) and the endometrial score (Fig. 2f). Thus, adequate secretory transformation of the endometrium is accompanied by hCG expression in the endometrial glands.

Table 1 Histological evaluation of the endometrial samples analyzed in this study using the modified endometrial score according to [12] ($n=41$)

Histological evaluation	Endometrial score	No. of cases
Asynchronous endometrium	0	5
Proliferative phase	1	5
	1.5	4
Early secretory phase	2	7
	2.5	4
Mid-secretory phase	3	12
	3.5	1
Late-secretory phase	4	3

Positive correlation between leukocyte infiltration and hCG expression

As hCG has been described as an important immune regulator during early pregnancy, for example by attracting regulatory T cells to the fetal–maternal interface [16, 33], we have specifically looked at leukocyte infiltration in relation to endometrial hCG expression. To this end, leukocytes were identified with anti-CD45 antibody recognizing leukocyte common antigen (Fig. 3). In the proliferative phase, no leukocytes were typically detected (Fig. 3a). With the beginning of secretory transformation, single leukocytes were stained whose number increased during further progression of the secretory phase (Fig. 3b, c). Leukocyte count was maximal in late-secretory/pre-decidual endometrium with ubiquitous distribution throughout the endometrial stroma (Fig. 3d). Consistently, our data show a statistically significant correlation between the CD45 score and the hCG staining index (Fig. 3f) as well as the endometrial score (Fig. 3g). Leukocyte staining in asynchronous endometria was inadequate compared to gland and stromal cell status (Fig. 3e). In agreement with its role as immune modulator, leukocyte infiltration into the endometrium increases with rising hCG expression.

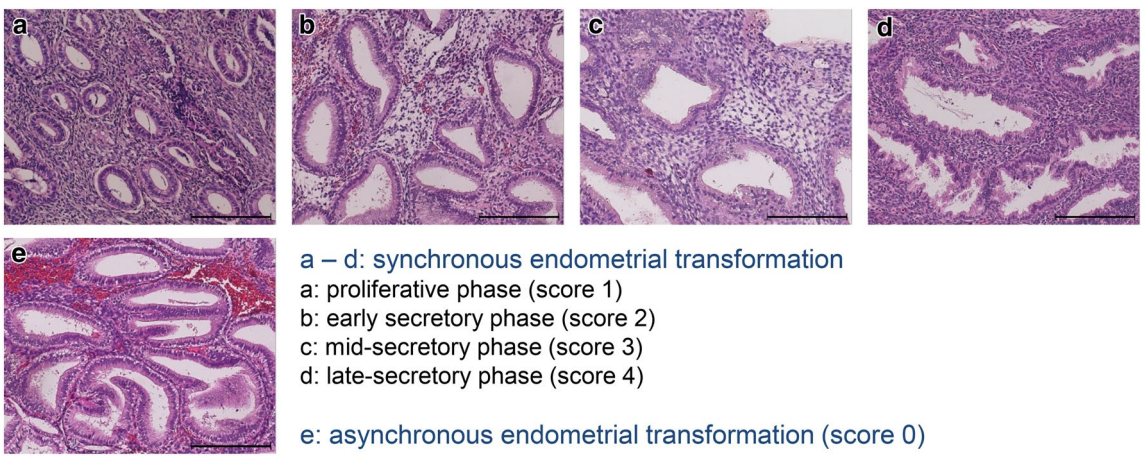


Fig. 1 Endometrial score. Examples of endometrial biopsies stained with hematoxylin–eosin in the different stages of endometrial development and the assignment of an endometrial score modified according to [12]. **a–d** Regular synchronous endometrial transformation: (**a**) proliferative phase = score 1; (**b**) early secretory phase = score 2; (**c**) mid-secretory phase = score 3, and (**d**) late-secretory phase = score 4. (**e**) Asynchronous endometrial transformation (score 0). Scale bar = 100 µm

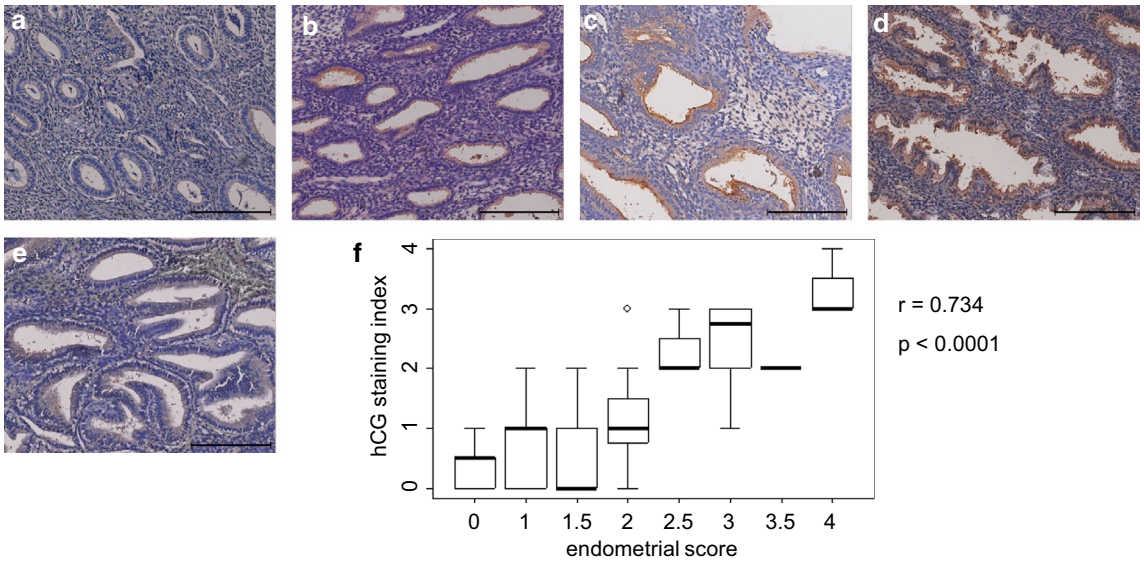


Fig. 2 hCG expression correlates with endometrial score. Endometrial biopsies were immunohistochemically stained with anti-CGB antibody (brown) and an hCG staining index was attributed to each specimen according to staining intensity [12]. Samples were counterstained with hematoxylin–eosin (blue). Representative examples are shown: (**a**) proliferative phase, endometrial score 1, hCG staining index 1; (**b**) early secretory phase, endometrial score 2, hCG staining index 2; (**c**) mid-secretory phase, endometrial score 3, hCG staining index 3; and (**d**) late-secretory phase, endometrial score 4, hCG staining index 4. **e** Asynchronous endometrial transformation, endometrial score 0, hCG staining index 0.5. **f** Correlation between hCG staining index and endometrial score ($n = 41$). Scale bar = 100 µm

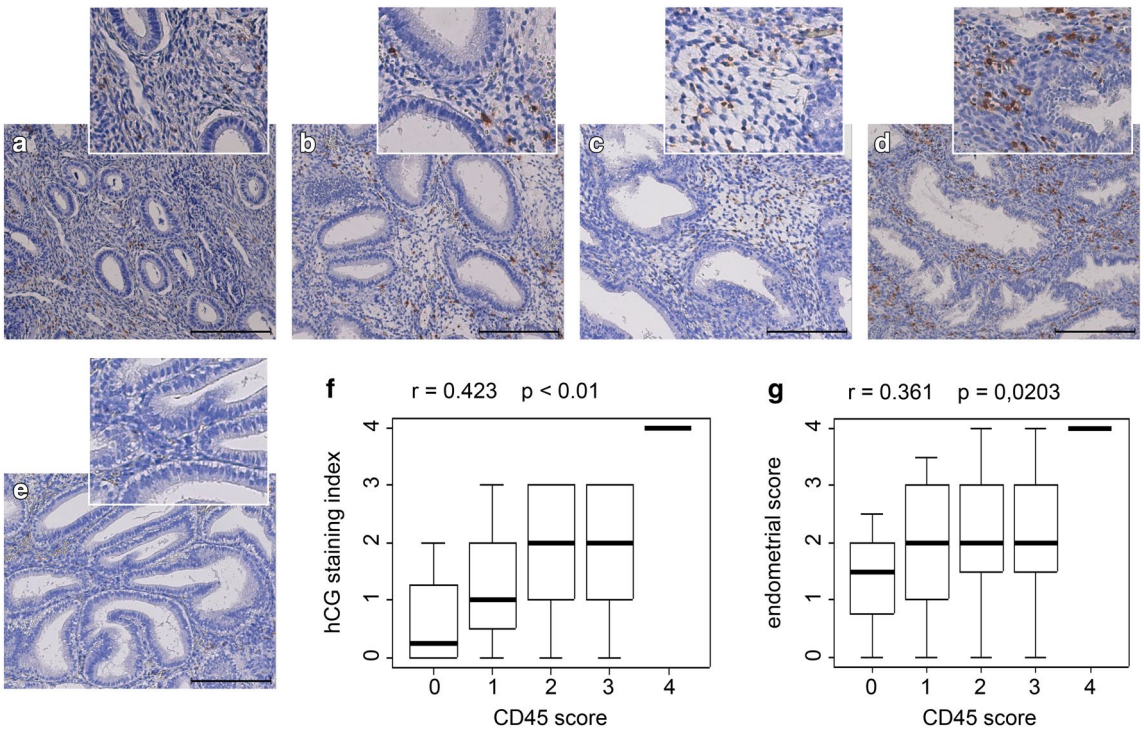


Fig. 3 Leukocyte infiltration correlates with hCG staining index. A CD45 score (modified according to [12]) was attributed to endometrial biopsies immunohistochemically stained with anti-CD45 common leukocyte antibody (brown), counterstained with hematoxylin–eosin (blue). Representative examples are shown: (**a**) proliferative phase, CD45 score 1; (**b**) early secretory phase, CD45 score 2; (**c**) mid-secretory phase, CD45 score 3; and (**d**) late-secretory phase, CD45 score 4. (**e**) Asynchronous endometrial transformation, CD45 score 1. (f + g) Correlation between CD45 score and hCG staining index (**f**) or endometrial score (**g**) ($n = 41$). Scale bar = 100 µm. Picture details are two times magnified

hCG staining index correlates with serum progesterone concentration

To further investigate the diagnostic value of hCG immunostaining, we analyzed the correlation of the hCG staining index with several commonly used parameters determined in infertility diagnostics. There was no significant correlation between hCG expression and serum estradiol concentration (Fig. 4a), PAS reaction (Fig. 4c), as well as cycle day (data not shown) and endometrial thickness (Fig. 4b). Interestingly, endometrial thickness did also not correlate with either cycle day or serum estradiol or progesterone concentrations, respectively. In contrast, a positive correlation of serum progesterone concentration, reaching a maximum in mid-luteal phase, with hCG staining index and endometrial score was observed (Fig. 5).

Altogether, we found that endometrial hCG expression, as determined by the hCG staining index, correlates significantly with secretory transformation of the endometrium, as well as with leukocyte infiltration and serum progesterone concentration, which are important criteria for successful embryo implantation.

Discussion

As the implantation rate following embryo transfer in infertility treatments is not only determined by the embryo quality but also by endometrial characteristics, there is a need for endometrium diagnostics to evaluate its receptivity [1]. Here, we show a statistically significant correlation between endometrial hCG expression, serum progesterone concentration, and adequate secretory transformation of the endometrium, suggesting endometrial hCG as a possible diagnostic parameter for characterizing the luteal phase endometrium.

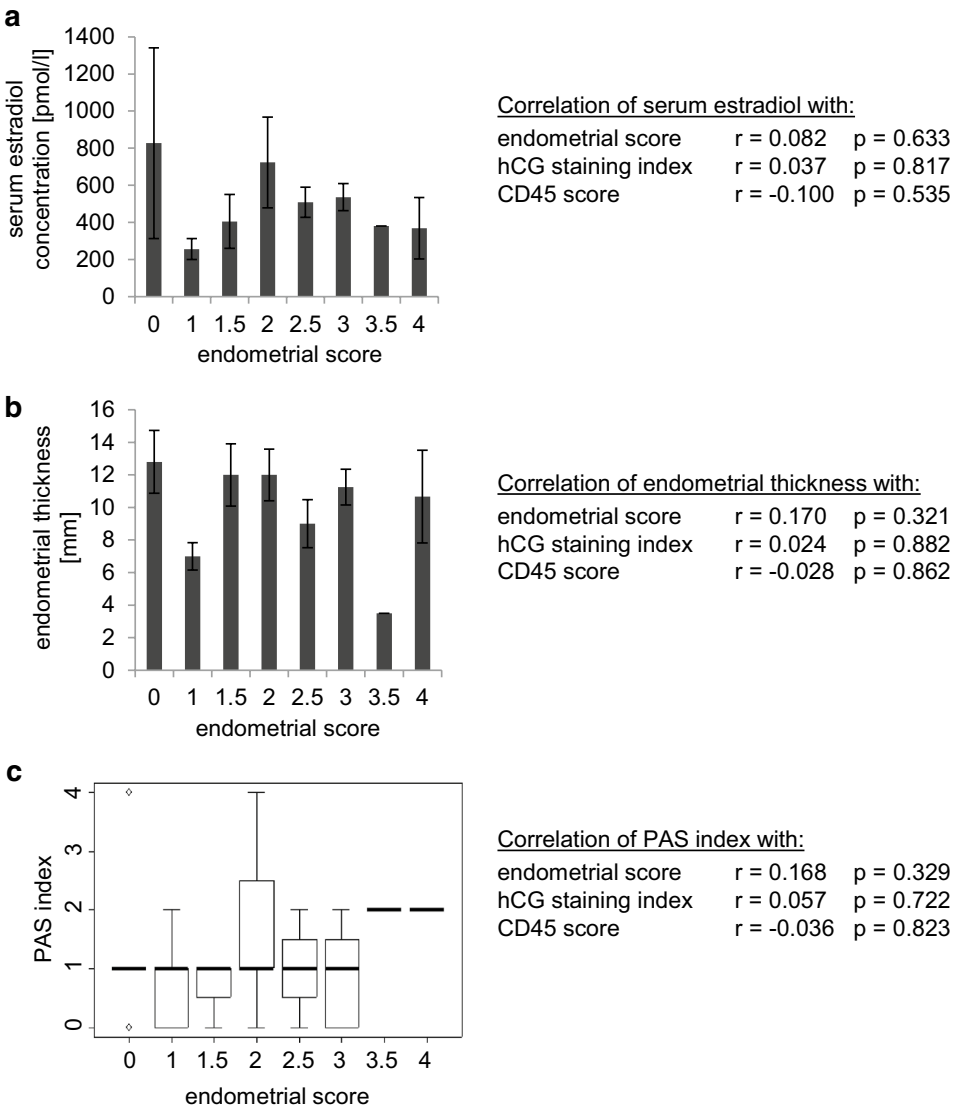
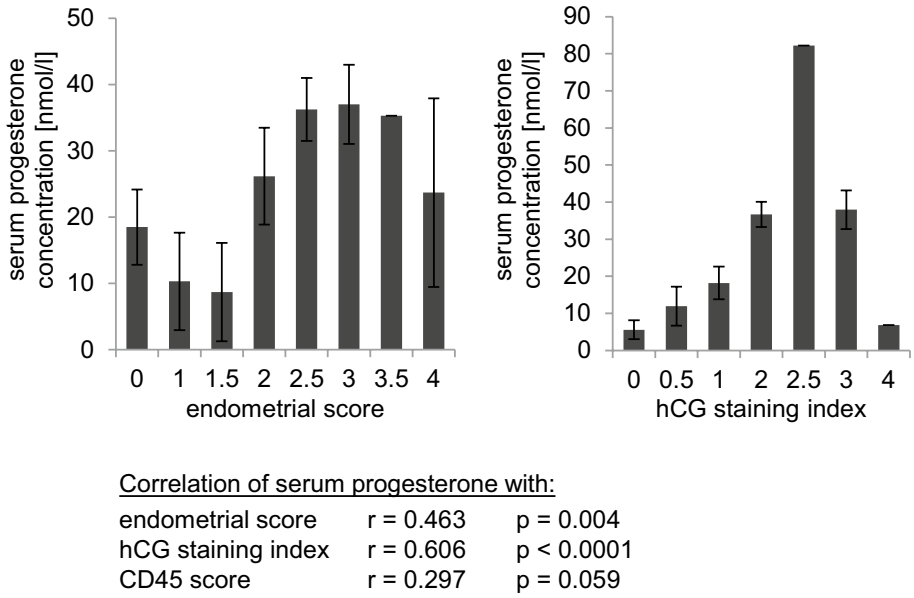


Fig. 4 Serum estradiol concentration, endometrial thickness and PAS index do not correlate with endometrial characteristics. Correlation coefficients (*r*) and *p* values for correlations with endometrial score, hCG staining index, and CD45 score are given, respectively. Relation between endometrial score and (**a**) serum estradiol concentration (mean ± SEM), (**b**) endometrial thickness (mean ± SEM), or (**c**) PAS index is shown graphically



Correlation of serum progesterone with:

endometrial score	r = 0.463	p = 0.004
hCG staining index	r = 0.606	p < 0.0001
CD45 score	r = 0.297	p = 0.059

Fig. 5 Serum progesterone concentration correlates with endometrial transformation and hCG expression. Correlation coefficients (r) and p values for correlations of serum progesterone concentration with endometrial score, hCG staining index, and CD45 score are given, respectively. Correlations between serum progesterone concentration (mean ± SEM) and endometrial score as well as hCG staining index are displayed graphically

We found a highly significant correlation between the endometrial score, as a measurement for regular secretory transformation of the endometrium, and the detected amount of hCG. hCG expression was diminished or absent in asynchronous or missing endometrial secretory transformation, which corroborates the assumption that endometrial hCG is an important fertility factor. The infiltration of CD45-positive leukocytes increased with progressing endometrial secretory transformation and rising hCG expression. This is in line with the fact that hCG acts as chemoattractant, e.g., for regulatory T cells, and has been found to modulate the local immune response at the feto-maternal interface [16, 33, 34]. In addition, serum progesterone concentrations correlated with hCG expression by the endometrial glands, as well as with secretory transformation of the endometrium. Shoupe et al. already described a significant correlation between serum progesterone concentration and endometrial dating [44]. Ghosh and Sengupta also emphasized the essential role of progesterone in the events leading to endometrial receptivity [45]. However, it remains open for future investigations whether a causal relationship is responsible for the biphasic correlation between serum progesterone concentration and hCG staining index shown here.

Besides attraction of immune cells mediating local immune tolerance, the endometrium itself might be a target of endometrial hCG. Expression of the LH/hCG receptor (LHCGR) in the endometrium was shown before [46–49]. Thus, it is likely that endometrial hCG acts on the endometrium to promote implantation, placentation, and decidualization in early pregnancy.

Interestingly, we found no correlation for our studied parameters with endometrial thickness. Thus, endometrial thickness does not seem to be suitable in predicting receptivity of the endometrium, as previously discussed in other studies [50–52]. However, an inverse relationship between early pregnancy loss and endometrial thickness has been shown with a thickness below 9.8 mm [53], suggesting that endometrial thickness is not a decisive parameter for embryo implantation as long as a critical value is exceeded [50]. Our observation that PAS reaction did not correlate with hCG staining is likely to be attributed to the fact that PAS reaction not only detects glycoproteins but also glycogen and glycolipids producing background staining. In addition, PAS reaction also did not reflect endometrial secretory transformation in our study and seems, therefore, obsolete in infertility diagnostics. This had already been concluded in the previous studies, which showed that glycogen production by the endometrium does not allow a statement to be made on its adequate secretory transformation and implantation competence [8, 54].

Furthermore, the endometrial score as well as the hCG staining index did not correlate with menstrual cycle day ($p = 0.304$ and $p = 0.278$, respectively; data not shown), which is probably attributable to individual cycle lengths and is consistent with the previous studies [55–57]. This emphasizes that evaluation of the luteal phase endometrium relative to cycle day is not optimal, as length of the proliferative phase and, thus, the ovulation time point and implantation window vary considerably between individuals. It would be preferable to determine the beginning of the luteal phase relative to ovulation time point on the basis of the LH surge [55, 57] or basal body temperature measurements.

As the adequate development of the endometrium is crucial for implantation success, there is a need for good endometrial diagnostics prior to embryo transfer. This has also been pointed out by Edgell et al., who underlined that

synchrony between the blastocyst and the endometrium is required for successful implantation and that a valid clinical test to evaluate endometrial receptivity is urgently needed [1]. Different biomarkers have already been discussed to describe endometrial secretory transformation [1, 58, 59]. Besides single biomarkers, also a wider approach using a customized microarray based on the expression of 238 genes was described. This so-called endometrial receptivity array (ERA) is coupled to a computational predictor to diagnose a functionally receptive endometrium. First clinical tests pointed out that a personalization of the window of implantation and, therefore, the individual day of embryo transfer might be possible with the ERA [60]. However, validations for routine clinical use with larger cohorts have still to be conducted. This would also be the case for hCG as a possible stand-alone molecular marker or in combination with other biomarkers. As our study includes a relatively small amount of patients, furthermore, larger studies should confirm our results and widen the spectrum of evaluated parameters. In particular, the correlation between successful implantation and endometrial hCG expression should be analyzed in future studies.

Endometrial receptivity tests would not only be helpful in screening infertile women to improve general diagnosis, but would also enable a decision as to whether to carry out fresh or frozen embryo transfer (ET) [1]. Impaired endometrial receptivity in fresh ET cycles after ovarian stimulation was found when compared to frozen ET cycles. This was attributed to an endometrium-embryo asynchrony in these hormonally stimulated cycles as artificial ovulation induction disturbs endometrial development [1, 3, 61]. Evans et al. have shown that the endometrial histology is altered following hormonal stimulation compared to natural cycles [2]. Embryos have a better chance of implantation if they are frozen for subsequent transfer in a natural, unstimulated cycle. Fresh versus frozen embryo transfer cycles prove that the endometrium plays a central role in implantation success. Therefore, a clinical test to determine endometrial receptivity would improve implantation rates.

Remarkably, only beta-hCG variant CGB7 is secreted by the endometrium [13], whereas the trophoblast mainly produces CGB3, 5, and 8. We also found that the decidua, as well as other epithelial tissues like retina and urothelium, express CGB7 [14, 62–64]. Furthermore, the tumor suppressor p53 induces CGB7 gene expression by directly binding to its promoter, whereas the other CGB variants remain unaffected [65]. These clearly different expression patterns raise the question of whether the different CGB isoforms possess individual functions or exert identical functions under different local and/or temporal conditions. This question needs to be addressed in further studies.

In summary, we conclude that endometrial hCG expression correlates with adequate secretory transformation of the endometrium. In our opinion, the hCG staining index might possibly allow a diagnostic statement about the implantation competence of the secretory phase endometrium. However, this hypothesis has to be addressed in more detail in future studies.

Materials and methods

Patients and biological samples

Biological samples (endometrial biopsies and peripheral blood) were taken from 41 women between 24 and 40 years of age who underwent hysteroscopy and diagnostic curettage within the routine infertility diagnostics at the Centre for Reproductive Medicine and Gynecological Endocrinology of the Department of Gynecology, University Hospital of Leipzig between December 2008 and October 2009. Samples were taken between days 14 and 28 of the menstrual cycle. Peripheral blood samples were obtained within 24 h before and after the endometrial biopsies, mostly at the same day. Endometrial thickness was determined by transvaginal ultrasound on the day before surgical intervention.

Ethical approval for the collection and use of human tissue

All human blood and tissue samples were obtained after informed consent from the patients. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Medical Ethics Committee of the University of Leipzig (Nr. 418-12-17,122,012).

Histological evaluation

Endometrial biopsies were stained with hematoxylin–eosin and initially routinely examined at the Institute of Pathology, University Hospital of Leipzig according to the criteria by Dallenbach-Hellweg et al. [66]. To simplify the classification, a modified endometrial score based on the proposed endometrial score from Zimmermann et al. [12] was determined, allowing quantification of the histological data. The endometrial specimens were morphologically characterized using the following four criteria: glandular shape (1: small and round; 2: elongated, tortuous; 3: dilated; 4: saw tooth-like), glandular epithelial nucleus configuration and localization (1: polystratified; 2: cylindrical, basal located, subnuclear vacuoles; 3: oval; 4: round), stromal cell density and shape (1: undifferentiated, dense; 2: spindle-shaped, broken up; 3: rounded cells, broken up, edematous; 4: pre-decidual), and endometrial leukocyte infiltration (1: without; 2: occasional; 3: numerous; 4: massive, subepithelial). Equal scores for the four features represent synchronous endometrial

development (endometrial score 1 = proliferative, 2 = early secretory, 3 = mid-secretory, and 4 = late-secretory). Score 0 was allocated for asynchronous secretory transformation when the four criteria differed in their individual scores. Table 1 shows the endometrial scores of the specimens analyzed in this study.

Immunohistochemistry and PAS reaction

Immunohistochemistry was carried out as described previously [12]. The following primary antibodies were used: polyclonal rabbit anti-CGB (Dako, Denmark, #A0231, 1:500) and monoclonal mouse anti-CD45 (leukocyte common antigen) (Dako, Denmark, clone 2B11 and PD7/26, # M0701, 1:500). Controls for secondary antibody binding were always performed in parallel.

The hCG staining index [12] was determined according to the degree of staining intensity (0: without; 1: starting; 2: existing; 3: strong; 4: massive) by two independent experimenters. In normal cycling endometrium, the hCG staining can be seen as follows: lack of hCG production in the endometrial proliferative phase (hCG staining index 0); beginning of hCG secretion in subnuclear glandular vacuoles (index 1); intensified and increasing glandular hCG secretion during the early secretory (index 2) and mid-secretory (index 3) phases, respectively; and strong glandular hCG secretion in functionalis and pre-decidual endometrium areas of the late-secretory phase (index 4). The staining intensities of the specimens analyzed in the present work were compared to standard samples used in the original paper [12]. Specificity of the hCG staining was verified by checking for LH staining with monoclonal rabbit anti-LH antibody (Zytomed Systems, Germany, #512-4320, 1:100) in the same samples. No LH was detected in all specimens (Online Resource 1).

The CD45 score semi-quantitatively describes the amount of CD45-positive cells (0: none; 1: occasional; 2: several; 3: numerous; 4: massive). It was also determined by two independent experimenters comparing the analyzed specimens to standard samples.

PAS reaction was carried out as follows: After deparaffinization and hydration, the endometrial tissue sections were rinsed for 5 min with pure water. The sections were then incubated for 5 min in periodic acid solution (1% Accustain, Sigma Diagnostics, USA) before being rinsed five times with water. After incubation (15 min) with Schiff´s Reagent Accustain (Sigma Diagnostics, USA) at room temperature, the sections were again five times rinsed with aqua dest and subsequently counterstained with hematoxylin (Gill no. 3, Dr. K. Hollborn & Söhne GmbH & Co. KG, Germany) for 90 s. Finally, the tissue sections were rinsed with water, dehydrated, and covered. According to the staining intensity, a PAS index from 0 to 4 was assigned.

Tissue sections were analyzed using the microscope Axioskop 2 plus with the camera AxioCam MRc 0.63 × and the Axiovision 4.5 software (Carl Zeiss Jena GmbH, Germany).

Determination of serum hormone concentrations

Serum progesterone and estradiol concentrations were determined during routine diagnostics using electro-chemiluminescence immunoassays (Roche, Cobas® system, Switzerland).

Statistical analyses

Statistical analyses were performed with the free software Statistical Lab© (http://www.statistiklabor.de/en/index.html) using the statistical programming language *R*. Correlation coefficients (r) and p values are given. Correlations of determined parameters with the endometrial score were calculated referring to regular endometrial transformation (scores 1–4). Score 0, indicating asynchronous endometrial transformation, was not included in these calculations as it builds a distinct group not linearly interrelated with scores 1–4.

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Author contributions SS: data analysis and manuscript writing. AB: Protocol/project development, data collection and management, data analysis, and manuscript writing/editing. MG: data analysis. LCH: data analysis and manuscript editing. GP: contribution of endometrial specimens. GZ: protocol/project development, data analysis, and manuscript editing. HA: protocol/project development and manuscript editing.

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Data availability The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest We declare that we have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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