Original Research

Human chorionic gonadotropin induces decidualization of ectopic human endometrium more effectively than forskolin in an *in-vivo* endometriosis model

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Impact statement

Impaired decidualization of endometrial stromal cells may contribute to the development of endometriosis, and an increased decidualization reaction may prevent or alleviate this prevalent gynecological disease. Human chorionic gonadotropin (hCG) has been shown to promote decidualization in eutopic endometrium. Up to now *in vitro* studies mainly used cAMP for successful induction of decidualization of isolated endometrial stromal cells. Here, for the first time, decidualization of ectopic endometrial lesions is induced in an experimental endometriosis mouse model, comparing the effectiveness of hCG with that of the direct adenylyl cyclase activator Forskolin. In this 3D-organ structure *in vivo*, hCG proved to be more effective in the induction of decidualization than forskolin. Particularly in case of progesterone resistance, alternative pathways inducing decidualization could alleviate endometriosis, and the sophisticated hCG action could constitute a therapeutical tool to induce terminal differentiation in ectopic endometrial lesions.

Abstract

Endometriosis, characterized by the presence of endometrial tissue at ectopic sites, is a leading cause of pelvic pain and subfertility in women. The stromal compartment of the endometrium is considered to play a pivotal role in the establishment and persistence of endometriotic lesions, thus impaired decidualization of these cells may result in enhanced invasion capacity at ectopic sites. Consequently, stimulation of decidualization may alleviate this disease. To analyze the effect of systemically applied compounds on decidualization of ectopic endometrial tissue, endometriosis was induced by suturing human eutopic endometrium to the peritoneum of 22 NOD/SCID mice. Each mouse received four tissue fragments from the same patient. Mice were randomly allocated either to one control and three experimental groups ($n = 4$/group) which were treated with progesterone alone or in combination with forskolin or human chorionic gonadotropin for seven days or to one control and one experimental group ($n = 3$/group) which was treated with progesterone and human chorionic gonadotropin for 10 days followed by 7 days without treatment. At the end of the experiments, lesions were measured and analyzed for markers of decidualization (FOXO-1, prolactin) and proliferation (Ki-67). Decidualization was induced in the ectopic lesions by systemic treatment *in vivo*. This induction was significantly stronger after treatment with progesterone in combination with human chorionic gonadotropin than with forskolin or with progesterone alone. Only the combination with human chorionic gonadotropin led to induction of FOXO1 protein expression and a significant physiologic transformation of the ectopic endometrial stromal cells after seven days of treatment. After termination of human chorionic gonadotropin treatment, the decidualization process continued, leading to a significant inhibition of proliferation. Thus, decidualization of human ectopic endometrial tissue can be induced in a humanized endometriosis mouse model *in vivo*. This model may help to decipher the signal pathways involved in this decidualization process and to develop novel therapeutical approaches to alleviate this painful disease.

Keywords: cAMP, endometriosis, decidualization, human chorionic gonadotropin, forskolin

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Introduction

Endometriosis is a leading cause of pelvic pain and subfertility, affecting up to 10% of reproductive-age women and even up to 50% of women seeking infertility treatment.[1,2] The symptoms are caused by endometrial tissue at ectopic sites outside the uterine cavity where it proliferates, induces neoangeogenesis, and invades surrounding structures.[3] The most accepted theory for the formation of peritoneal

endometriosis is retrograde menstruation of shed endometrial tissue via the fallopian tubes into the peritoneal cavity.[4] However, since not all women with retrograde menstruation develop lesions,[5] and endometriotic foci have also been described distant from the pelvis and even in male patients,[6] not all kinds of endometriosis can be explained by this theory. Additional theories of the pathogenesis of endometriosis have to be considered including genetical, immunological, and environmental factors[7] or an impaired differentiation of the urogenital tract.[8] Due to the severe clinical manifestation, endometriosis has a high impact on life quality and work productivity, causing a substantial economic burden on the health-care system.[9,10] Current therapies include the surgical removal of lesions and/or hormonal treatment, aimed at the establishment of a hypoestrogenic state in order to achieve a reduction in ectopic lesions and endometriosis-associated pain and to improve fertility.[11] However, recurrence rates are up to 50%.[12] Since there has been little research progress in the medical management of endometriosis over the last three years,[13] there is a definite need for developing more efficacious therapeutic alternatives.

One factor that may influence the severity of the disease is whether the cells undergo proliferation or differentiation at ectopic sites. Since it is assumed that the stromal compartment of the endometrium plays a pivotal role in the establishment and persistence of the endometriotic lesions,[14] one cause for this disease may be an impaired decidualization of endometrial stromal cells, resulting in enhanced invasion and proliferation capacity at ectopic sites.[15] Physiologically, the decidualization process of human endometrial stromal cells is initiated by progesterone during the luteal phase of the menstrual cycle[16] and continues throughout pregnancy under the influence of progesterone and human chorionic gonadotropin (hCG). If pregnancy does not occur, the endometrium is shed and may reach the peritoneal cavity by retrograde.[4] There is substantial evidence that the decidualization of stromal cells is impaired in endometriosis patients.[17,18] This may be due to a progesterone resistance in these patients[17,19,20] and may lead to an impaired differentiation of stromal cells and thus an enhanced proliferation and invasion capacity when reaching the peritoneal cavity. The involvement of additional pathways activated by hCG[21,22] or cAMP[15] has been described. The fact that hCG has an effect on ectopic endometrial tissue is supported by the finding that decidualization can be found in 77% of lesions in pregnant women.[23] This decidualization may induce atrophy of the ectopic tissue in humans,[24,25] and a regression of induced endometriotic lesions has been described after pregnancy in monkeys.[26] However, although numerous *in vitro* studies have investigated the pathways leading to decidualization, only a limited number of *in vivo* studies have been conducted and the pathways involved in the decidualization of ectopic lesions require further *in vivo* examination. We have here used an established humanized endometriosis mouse model[27] to investigate whether decidualization can be successfully induced in human ectopic endometriotic lesions *in vivo*, and whether there is a difference in the level of decidualization between cAMP/forskolin and hCG treatment, respectively.

Materials and methods

Human endometrial tissue

Endometrium was obtained from seven premenopausal women undergoing endometrial biopsy for diagnostic reasons due to benign indications at the Department of Gynecology, University Hospital Essen, Germany. Age of patients was 42.6 ± 3.0 years. All endometrial tissues were obtained from the proliferative phase of the menstrual cycle. Additionally, histological staging according to Noyes *et al.*[28] was performed to confirm the cycle stage. The patients received no hormonal treatment for at least three months before surgery. After removal, endometrial tissue was cut into fragments of 1.5 mm in diameter under sterile conditions and left for 1 h in culture medium (DMEM Ham's F12 1:1, Biochrom KG, Berlin, Germany) supplemented with Pen/Strep (Invitrogen, Karlsruhe, Germany) at 37°C and 5% CO_2 before transplantation into NOD/SCID mice.

Animals and transplantation of endometrial tissue fragments

Female non-obese diabetic (NOD) – severe combined immunodeficiency (SCID) mice (aged two to six months) were housed in a controlled pathogen-free barrier unit under a 12-h light/dark cycle with access to food and water ad libitum. All equipment and food entering the barrier was autoclaved. In all experiments, endometrial tissue of the same patient was transplanted in parallel into two or four cycling NOD/SCID mice, depending on the experimental approach. Each mouse received four endometrial tissue fragments of the same patient which were fixed with surgical sutures to the parietal peritoneum of the abdominal wall by laparotomy as described before.[27,29,30]

Ethical approval

Institutional ethical approval was given and written informed consent to the use of the human endometrial tissue for research purposes was obtained from all the women concerned. All the animal experiments were approved by the institutional animal care committee of the German government (LANUV 87–51.04.2010.A034).

Application of drugs and tissue processing

Endometrial tissue was transplanted in a total of 22 mice. In a first set of experiments, 16 transplanted mice were randomly allocated to one control group treated with vehicle only and three experimental groups ($n = 4$/group) which were treated with progesterone alone (50 µg/mouse/day s.c.; Sigma Aldrich, Munich, Germany) or in combination with forskolin (100 µg/mouse/day i.p.; Sigma Aldrich, Munich, Germany) or hCG (7.5 IU/mouse/day i.p.; Ovogest®, Intervet, Boxmeer, Netherlands) for seven days. In a second set of experiments, six transplanted mice were randomly allocated to one experimental group

($n = 3$/group) treated with progesterone in combination with hCG for 10 days and left untreated for an additional 7 days, and one control group which received an intraperitoneal injection of the vehicle only (benzylbenzoate-castor oil, 1:4) for the corresponding time period. After termination of the respective experiment, the size of the implanted endometrial tissue fragments was measured *in situ* before lesions were dissected. One lesion of each mouse was processed for paraffin histology and immunohistochemistry, and three lesions of each mouse were snap-frozen in liquid nitrogen and stored at −80°C for molecular analyses.

RNA preparation, cDNA synthesis, and quantitative real-time PCR

The frozen tissues were homogenized and RNA extracted using the E.Z.N.A total RNA midi kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's protocol. Following DNase treatment (Invitrogen), reverse transcription reactions were carried out as previously described.[31] Subsequently, quantitative real-time PCRs were performed in triplicate using an ABI Prism 7300 sequence detector (Applied Biosystems, Weiterstadt, Germany); 40 ng of cDNA was diluted in a final volume of 20 µl containing 40 ng of cDNA, 3.75 pmol gene-specific primers, and 10 µl of SYBR Green Master Mix reagent (Applied Biosystems, Weiterstadt, Germany). The primers were purchased from Invitrogen (Darmstadt, Germany). Melting curve analysis allowed determination of PCR product specificity. Quantification was performed by using a 10-fold series dilution of purified PCR products of each gene ranging from 1 pg to 0.1 fg as standards. The expression levels of the analyzed genes were normalized to actin-beta content. The primer sequences used for qPCR are summarized in Table 1.

Morphological and immunohistochemical analyses

The tissues were fixed in 4% formalin, dehydrated in a graded series of alcohol and embedded in Paraplast Tissue Embedding Medium (Mc Cormick Scientific, St. Louis, MO, USA). Sections of 7 µm were cut on a 2050 Supercut Reichert-Jung Microtom (Leica, Wetzlar, Germany) and two sections were mounted on each slide. For morphological evaluation, sections on every fifth slide were stained with hematoxylin and eosin. For immunohistochemistry, sections were deparaffinized, rehydrated in a series of alcohol and fixed in ice-cold absolute ethanol for 5 min. After washing with PBS, endogenous peroxidase activity was blocked with 2.5% hydrogen peroxide in methanol for 10 min in the dark followed by antigen retrieval by boiling slides in 0.01 M Natrium-citrate buffer (pH 6)/0.5% Tween-20 for 10 min. After cooling, sections were permeabilized in 0.1% Triton X-100 in PBS for 30 s. Non-specific binding sites were blocked with 0.5% BSA in PBS for 20 min. The primary antibodies used were anti-prolactin mouse monoclonal antibody (1:25; Zytomed, Carlsbad, CA, USA), anti-Ki-67 mouse monoclonal antibody (1:400; Dako, Hamburg, Germany), anti-Foxo1 rabbit polyclonal antibody (1:50; Cell signaling, Danvers, MA, USA), and anti-caspase-3 rabbit polyclonal antibody (1:200; Zytomed, Carlsbad, CA, USA). A suitable biotinylated secondary antibody (Dako, Hamburg, Germany) was used. The primary antibody was omitted in the negative controls. Positive controls were performed on paraffin-embedded decidual tissue of mature human placenta, since it has been shown that prolactin protein can be stained in decidual cells of term placentas.[32] Sections were counterstained with hematoxylin. Staining was analyzed using an Axiophot photomicroscope (Zeiss, Jena, Germany), and images were captured with a Nikon DS-U1 camera. The area of decidualized tissue and cell numbers were quantified with NIS-Elements BR software (Nikon, Düsseldorf, Germany). The proliferation rate was calculated as a percentage of Ki-67 stained cells per total amount of glandular epithelial cells and stromal cells, respectively. Evaluation of FOXO1 immunostaining was performed by semiquantitative classification as a percentage of stained tissue per total tissue with nine different staining intensity classes (0 = no staining to 8 = very intense staining). For each experimental approach, one representative section of an endometrial fragment of at least three different patients was analyzed.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 (IBM, Ehningen, Germany) using the Mann–Whitney U test for non-parametric analysis of variances. Results are represented as mean ± SEM. Probability values of ≤ 0.05 were considered statistically significant.

Results

Morphological differentiation of ectopic endometrial stromal cells is promoted by hCG

After intraperitoneal transplantation of human endometrial tissue, NOD-SCID mice were treated with progesterone

Table 1. Primer sequences used for qPCR.

Gene	Ref-Seq transcript ID	Primer sequence 5′-3′	Product size (bp)
FOXO1	NM002015.3	F GACAGCCCTGGATCACAGTT R AGATGGCGGGTACACCATAG	198
IGFBP1	NM000596.2	F CTATGATGGCTCGAAGGCTC R TTCTTGTTGCAGTTTGGCAG	156
PRL	NM000948.4	F CATCAACAGCTGCCACACTT R CGTTTGGTTTGCTCCTCAAT	213
ACTB	NM001101.3	F AGCACAGAGCCTGGCCTTTGCC R CACATGCCGGAGCCGTTGTCGA	108

alone or in combination with forskolin or hCG for seven days. The control mice only received the vehicle. At the end of the culture period, the peritoneal cavity was inspected. Only in few cases a weak adhesion of lesions to the gut was found which could be easily separated. No strong local and no distal adhesions were found. Histological examination revealed a well-preserved histomorphology of all lesions showing typical endometrial glands surrounded by stromal cells (Figure 1(a) to (d)). Decidualized stromal cells could be distinguished from non-decidualized stromal cells by their epitheloid appearance. While only very small spots of decidualized cells were observed in the lesions of the control group (Figure 1(a)) as well as in those treated with progesterone alone (Figure 1(b)), an increase in the size of the area of decidualized cells was seen after treatment with progesterone in combination with forskolin (Figure 1(c)). This effect was markedly increased after combination with hCG. Here nearly the whole area of the lesions consisted of decidualized cells (Figure 1(d) and (e)). After the seven-day culture period, no significant differences in the size of lesions were observed between the different treatment groups

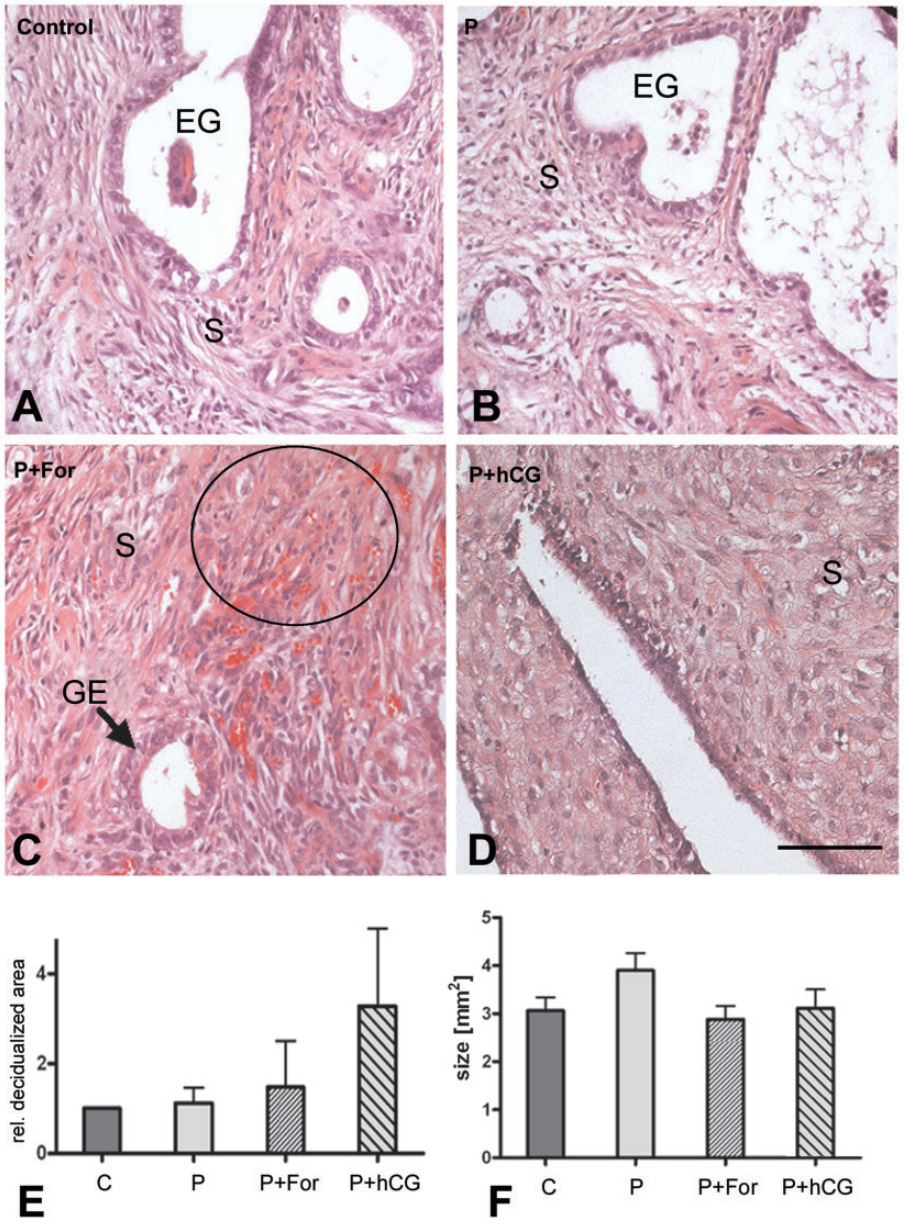

Figure 1. (a–d) HE staining of ectopic endometrial fragments after seven days of treatment in the peritoneal cavity of NOD-SCID mice. All lesions revealed a well-preserved histomorphology, showing typical endometrial glands surrounded by stromal cells. While hardly any spots of decidualized cells were observed in the lesions of the control group (a) or in those treated with progesterone alone (b), an increase in the size of the area of decidualized cells was seen after combined treatment with progesterone and forskolin (c, circle). After combined treatment with progesterone and hCG, nearly the complete stroma of the lesions consisted of decidualized cells (d). (e) Quantitative evaluation of areas within the lesions showing histological appearance of decidualized cells. Though not significant, in tendency there was an increase in size of area showing decidualized morphology. (f) No significant differences in the size of lesions were observed between the different treatment groups. C: control; P: progesterone; For: forskolin; hCG: human chorionic gonadotropin; EG: endometrial gland; S: stroma; bar for all figures = 50 μm. (A color version of this figure is available in the online journal.)

(Figure 1(f)). Thus, treatment with progesterone and hCG resulted in an extensive morphological differentiation of stromal cells of the ectopic endometrial lesions.

Functional differentiation of ectopic endometrial lesions is induced by hCG

Expression of the decidualization markers *PRL* and *IGFBP1* was investigated in the ectopic human endometrial lesions after seven days of treatment. qPCR analysis revealed that treatment with progesterone alone had no effect on endometrial *PRL* (Figure 2(a)) and *IGFBP1* (Figure 2(b)) gene expression compared to controls. Transcription of PRL and IGFBP1 was slightly enhanced after treatment with progesterone in combination with forskolin, while treatment with progesterone and hCG led to significantly enhanced endometrial *PRL* (Figure 2(a)) and *IGFBP1* expression (Figure 2(b)). To prove this decidual differentiation on protein level, the PRL protein was localized by immunohistochemical analysis. Lesions treated with the vehicle only (Figure 2(d)), progesterone only (Figure 2(e)) or in combination with forskolin (Figure 2(f)) showed no positive staining for PRL in any cell. In contrast, more than 30% of the lesions cultured in mice treated with hCG revealed cells positively stained for PRL (Figure 2(c) and (g)). PRL-stained cells were scattered within the decidualized areas of hCG-treated lesions (Figure 2(g)), correlating to the pattern seen in decidua of mature placentae (Figure 2(h)). In decidualized ectopic endometrial lesions as also in mature decidua, not all cells are stained positively for PRL as this protein is secreted and the amount is not high enough in all cells to be detected by immunohistochemistry. As a result, PRL protein was exclusively observed in those ectopic endometrial lesions treated with a combination of progesterone with hCG.

Since *FOXO1* is induced during decidualization,[33] the effect of the different treatments on the expression level of *FOXO1* was analyzed parallely in the ectopic

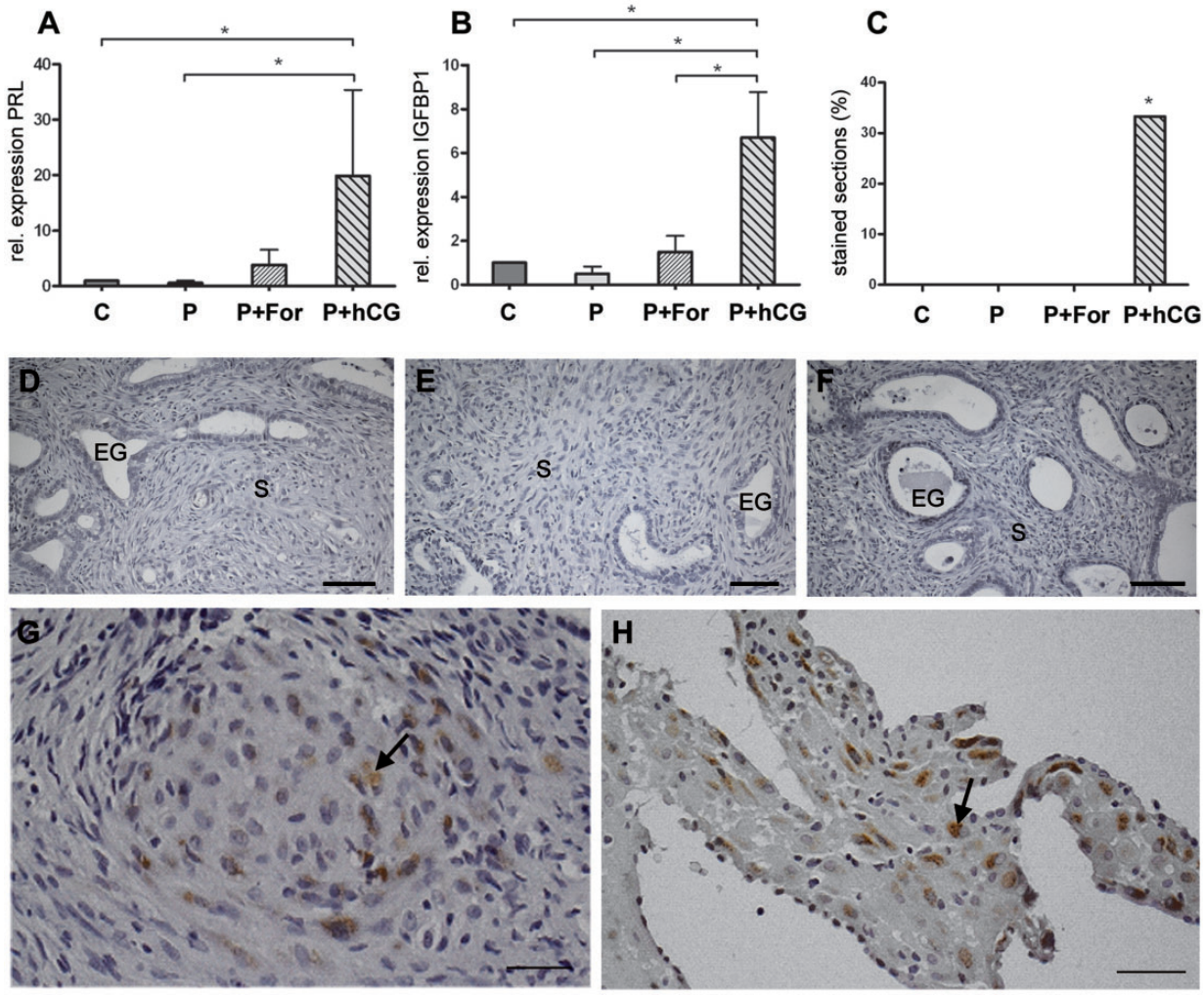

Figure 2. qPCR analysis of *PRL* (a) and *IGFBP1* (b) mRNA concentration. After seven days, only treatment with progesterone and hCG induced a significant increase in *PRL* and *IGFBP1* transcripts compared to all other experimental groups. (c) Percentage of lesions positively stained for PRL protein. PRL protein was only detected in those lesions treated with progesterone and hCG. (d–h) Immunohistochemical staining for PRL. While no staining was observed in lesions treated with the vehicle only (d), progesterone (e) or forskolin and progesterone (f), those treated with hCG revealed cells positively stained for PRL, as indicated by brown color (g, arrow) which correlated to the staining pattern seen in the decidual tissue of the mature placenta (h). C: control; P: progesterone; For: forskolin; hCG: human chorionic gonadotropin; EG: endometrial gland; S: stroma; bar in d–f = 50 μm, bar in g,h = 25 μm.

endometrial lesions after seven days of treatment. Combined treatment with progesterone and forskolin as well as with hCG significantly increased the transcription of the *FOXO1* gene compared to controls as well as to lesions grown in mice treated with progesterone only (Figure 3(a)). Only a faint immunohistochemical staining for the FOXO1 protein was observed in controls (Figure 3 (c)) as well as in those lesions treated with progesterone only (Figure 3(d)) or with progesterone and forskolin (Figure 3(e)). In contrast, lesions revealed an intense staining for the FOXO1 protein after combined treatment with hCG (Figure 3(f)), showing a pattern comparable to decidual tissue of the mature placenta (Figure 3(g)). Quantification of the staining intensity revealed a significant increase in FOXO1 protein expression only in those lesions treated with hCG (Figure 3(b)). In contrast to those decidualization markers, no staining for caspase-3 as an indicator of apoptosis was observed in any experimental group (not shown).

Thus, the increase in area of morphologically decidualized endometrial stromal cells in ectopic lesions treated with hCG corresponded to a significant increase in the production of the decidualization markers PRL and FOXO1.

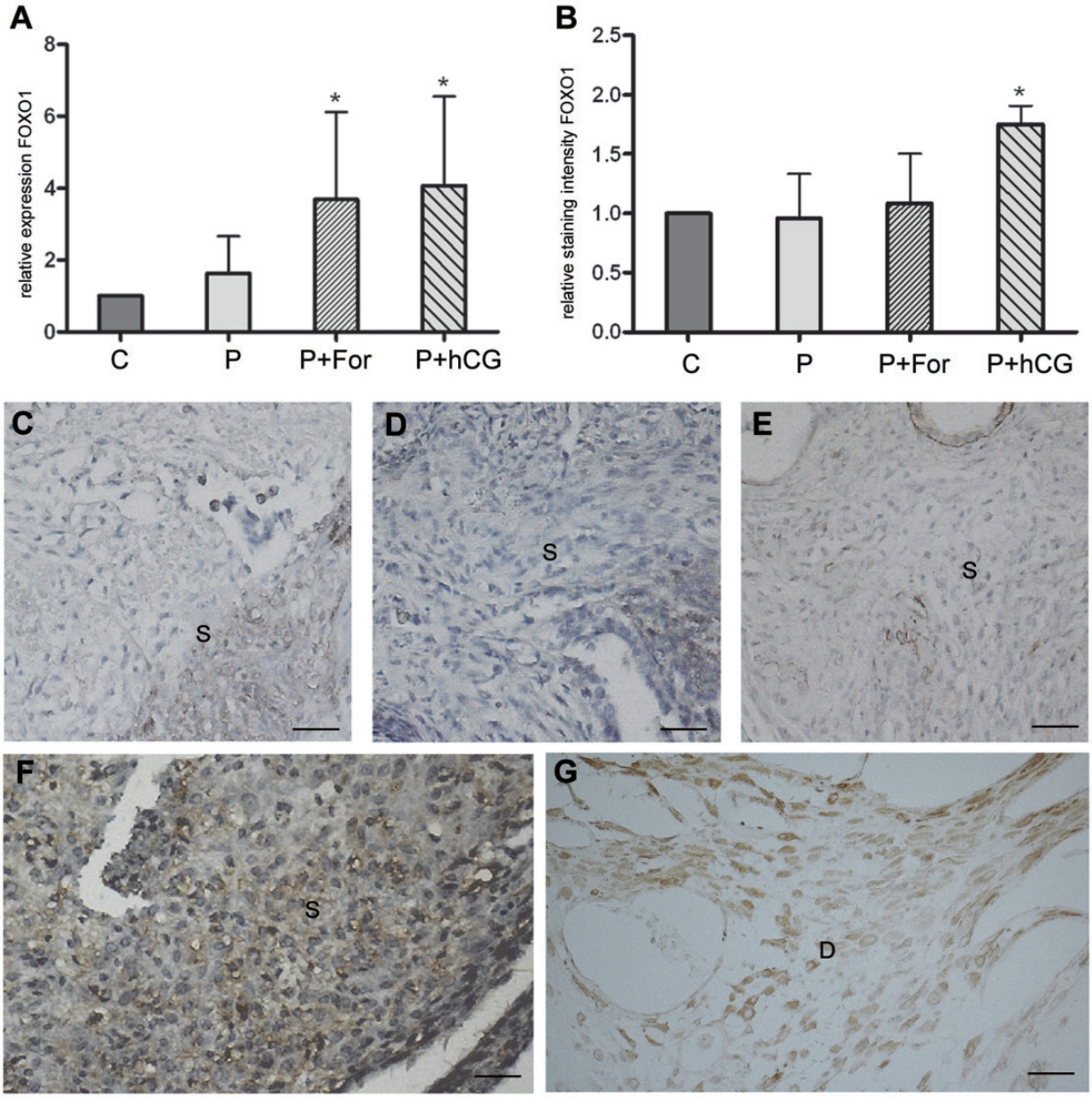

Figure 3. (a) qPCR analysis of *FOXO1* mRNA concentration. Treatment with progesterone in combination with forskolin or hCG induced a significant increase in *FOXO1* transcripts compared to controls or to treatment with progesterone after seven days of treatment. (c–g) Immunohistochemical staining for FOXO1 protein. While only a faint staining was observed in controls (c) as well as in those lesions treated with progesterone only (d) or with progesterone and forskolin (e), lesions revealed an intense staining for the FOXO1 protein after hCG treatment, as indicated by brown color (f) showing a pattern comparable to decidual tissue of the mature placenta (g). Quantification of staining intensity revealed a significant increase in FOXO1 protein expression only in those lesions treated with progesterone and hCG (b). C: control; P: progesterone; For: forskolin; hCG: human chorionic gonadotropin; EG: endometrial gland; S: stroma; bar in c–e = 50 μm, bar in g = 25 μm.

Proliferation in ectopic endometrial lesions

To analyze the effect of the different treatments on proliferation of the human ectopic endometrial lesions, the rate of proliferation of endometrial stromal cells was determined by quantification of immunostaining for Ki-67. While, in a comparison with controls after seven days (Figure 4(a) and (e)), there was a trend to an increase in the proliferation rate of ectopic endometrial stromal cells after treatment with progesterone alone (Figure 4(b) and (e)) or in combination with forskolin (Figure 4(c) and (e), such an increase was not observed after application of hCG (Figure 4(d) and (e)).

hCG has a sustainable effect on decidualization after termination of treatment

We have demonstrated that the combined treatment with progesterone and hCG proved to be a significantly stronger inductor of decidualization of the stromal compartment of the ectopic endometrial lesions than progesterone alone or in combination with forskolin. To evaluate if this hCG-mediated effect is maintained after termination of treatment, NOD-SCID mice transplanted with human endometrial tissue fragments were treated for 10 days with progesterone and hCG, followed by an additional period of 7 days without treatment. Subsequently, lesions were removed and analyzed. After termination of treatment, the expression of *PRL* continued to increase considerably, leading to a highly significant increase compared to controls (Figure 5(a)). In parallel thereto, a significant decrease in the proliferation rate of ectopic endometrial stromal cells was observed (Figure 5(b)). During this differentiation process, a trend towards a decrease in the size of the lesions was seen (Figure 5(c)), while no staining for ccaspase-3 as an indicator of apoptosis was observed

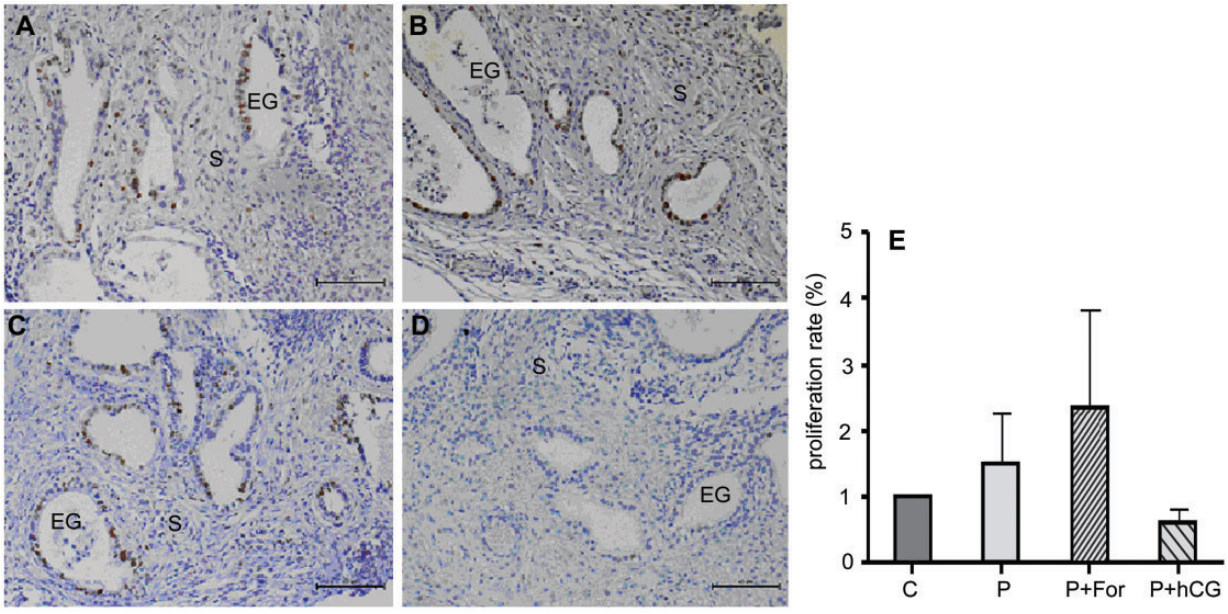

Figure 4. Immunostaining for Ki-67 of control (a) and of lesions treated with progesterone (b), progesterone and forskolin (c) or progesterone and hCG for seven days (d). (e) Quantitative evaluation of proliferation rate. Although there was no significant difference, proliferation after hCG application was lower than in the other treatment groups. C: control; P: progesterone; For: forskolin; hCG: human chorionic gonadotropin; EG: endometrial gland; S: stroma; bar in a–d = 50 μm.

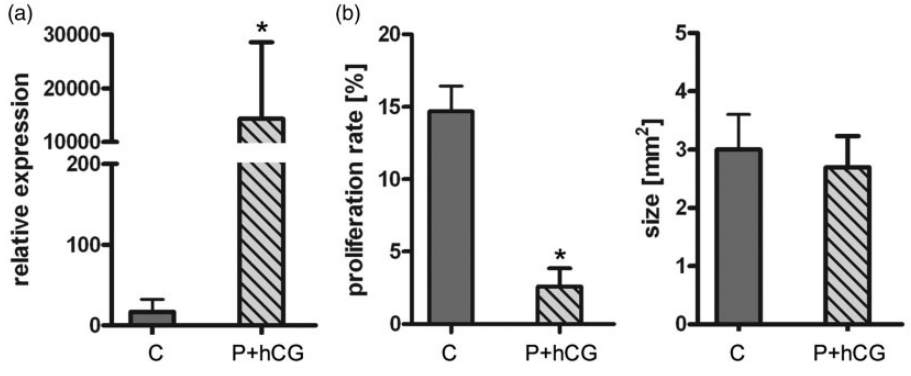

Figure 5. qPCR analysis of *PRL* mRNA concentration (a), proliferation rate (b) and size of ectopic endometriotic lesions (c) after 10 days of treatment with progesterone and hCG followed by an additional culture period of seven days without treatment. Decidualization increased after termination of treatment, resulting in a significant increase in prolactin secretion and a significant decrease in proliferation in the ectopic endometrial lesions.
C: control; P: progesterone; hCG: human chorionic gonadotropin.

(not shown). Thus, after initiation of the decidualization process by progesterone and hCG, this differentiation of the ectopic endometrial lesions also continued to progress after termination of treatment.

Discussion

In this study, we have demonstrated that decidualization of human ectopic endometrial tissue can be successfully induced in an endometriosis mouse model by systemic treatment *in vivo*. Using substances which had been shown before to be involved in the induction of decidualization of human endometrial stromal cells, it was shown that this induction of decidualization is significantly stronger when progesterone is applied with hCG rather than applying progesterone alone or in combination with the direct adenylyl cyclase activator forskolin.

Progesterone resistance is a common feature in endometriosis patients, potentially leading to impaired decidualization, and thus increased capability of ectopic endometrial tissue to proliferate and persist in ectopic sites.[17,18,20] Although progesterone is generally considered to be the key inducing factor for the decidualization process,[34] it is known to be a weak inductor of decidualization.[35,36] Thus, the enhancement of progesterone action could lead to an enhancement of the decidualization reaction. It has long been known that combining progestins with cAMP leads to a significantly increased decidualization of isolated human endometrial stromal cells *in vitro* compared to the application of progestins alone,[15,35] and that the activated cAMP/PKA-signal pathway may interact with the progesterone pathway.[35,37] Also hCG, a major early embryonic signal already produced by the pre-implantation blastocyst,[38,39] has been shown to modulate endometrial cell differentiation prior to blastocyst implantation in humans and non-human primates[21,40–42] and, in addition, is under discussion with regard to its effect on ectopic endometrial tissue.[23–26]

In the present *in vivo* study, hCG led to a significantly stronger induction of decidualization of human ectopic endometrial tissue in progesterone-treated mice than forskolin did. While both treatments initiated early steps in the decidualization process, such as the induction of gene expression of the transcription factor *FOXO1*, only hCG led to the induction of FOXO1 protein expression, which is important in the initiation of the decidualization process,[43–45] as well as in a significant morphological and physiologic transformation of the ectopic endometrial stromal cells after seven days of treatment. While forskolin enhances intracellular cAMP by direct activation of the enzyme adenylyl cyclase, hCG binds to the G-protein-coupled LH/hCG-receptor.[46–48] This receptor is well known to mediate the maintenance of progesterone production by the corpus luteum in the ovary during early pregnancy,[41,49] but is also found in human endometrial cells.[50–52] Here, hCG may induce *in vitro* decidualization of endometrial stromal cells via the cAMP/PKA pathway[22,53–55] but has also been shown to up-regulate the progesterone receptor in a PKA-independent manner in human endometrial stromal cells *in vitro*,[22] possibly increasing the effects of hCG on progesterone action in these cells, as has been observed previously.[56,57]

Although it has been shown that a continual application of progesterone and cAMP is necessary to maintain the decidual phenotype of isolated endometrial stromal cells *in vitro*,[35,58] in the present *in vivo* study, the decidualizing effect of progesterone and hCG on PRL secretion concomitant with an inhibition of proliferation even increased seven days after termination of treatment.

Published data regarding the effectiveness of hCG on decidualization induction *in vitro* have been contradictory.[53,59,60] In contrast to our study, however, these experiments have been performed with isolated endometrial stromal cells *in vitro*. The lack of a three-dimensional structure and of endometrial epithelial cells might have a considerable impact on the cellular pathways necessary for hCG-induced stromal cell differentiation. We here used an established humanized endometriosis mouse model in which the human endometriotic-like lesions resemble those found in patients.[27,61] When analyzing mechanisms of decidualization, it is paramount to use human endometrium instead of, for example, an autologous rodent model, since the process of decidualization differs considerably between rodents and primates.[33] In contrast to studies injecting minced endometrial tissue into the peritoneal cavity to analyze the degree of adhesion of the randomly attached lesions,[62] in our model, human endometrial fragments were fixed by surgical sutures to the abdominal wall. As described previously,[61] this allows a 100% recovery rate of fragments for analysis. Using this humanized endometriosis model, we have been able to demonstrate that systemic treatment with hCG induces decidualization of human ectopic endometrial lesions more effectively than forskolin.

In conclusion, in this study, we have proved that ectopic endometrial tissue can be decidualized in a humanized animal model *in vivo*. The factors and signaling pathways inducing the process of decidualization are only partly understood and are regulated by a complex interaction of multiple factors. It is still not fully clear how the distinct signal cascades are impaired in endometriosis patients. Though these may be altered by endometrial disorders like for example chronic endometritis,[63,64] especially in the case of progesterone resistance, alternative pathways inducing decidualization could alleviate endometriosis. The sophisticated hCG action could constitute a therapeutical tool to induce terminal differentiation in ectopic endometrial lesions. This model provides a tool to further investigate the effect of therapeutical compounds or compound combinations in regard to their decidualizing potential on ectopic endometrial tissue in an *in vivo* system.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; YK developed and performed the experiments, analyzed the data including statistical analysis, and revised the manuscript. PW provided administrative, technical and material support, and revised the manuscript. RG developed study concept and design, contributed to

analyzing the data and wrote the manuscript. All authors revised the manuscript and approved the final draft.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

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