Localization and variable expression of $\alpha_{i2}$ in human endometrium and Fallopian tubes

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BACKGROUND: Heterotrimeric G proteins take part in membrane-mediated cell signalling and have a role in hormonal regulation. This study clarifies the expression and localization of the G protein subunit $\alpha_{i2}$ in the human endometrium and Fallopian tube and changes in $\alpha_{i2}$ expression in human endometrium during the menstrual cycle. METHODS: The expression of $\alpha_{i2}$ was identified by Polymerase chain reaction (PCR), and localization confirmed by immunostaining. Cyclic changes in $\alpha_{i2}$ expression during the menstrual cycle were evaluated by quantitative real-time PCR. RESULTS: We found $\alpha_{i2}$ to be expressed in human endometrium, Fallopian tube tissue and in primary cultures of Fallopian tube epithelial cells. Our studies revealed enriched localization of $\alpha_{i2}$ in Fallopian tube cilia and in endometrial glands. We showed that $\alpha_{i2}$ expression in human endometrium changes significantly during the menstrual cycle, with a higher level in the secretory versus proliferative and menstrual phases ($P < 0.05$). CONCLUSIONS: $\alpha_{i2}$ is specifically localized in human Fallopian tube epithelial cells, particularly in the cilia, and is likely to have a cilia-specific role in reproduction. Significantly variable expression of $\alpha_{i2}$ during the menstrual cycle suggests $\alpha_{i2}$ might be under hormonal regulation in the female reproductive tract in vivo.

Key words: cilia/endometrium/fallopian tube/G protein/menstrual cycle

Introduction

Among the cell-surface receptors, G protein-coupled receptors are the most widespread and diverse, playing an essential regulatory role in cell growth, hormonal regulation, sensory perception and neuronal activity (Hepler and Gilman, 1992). In reproduction, G protein-coupled receptors have a neuroendocrine regulatory role in GnRH-induced secretion of LH and FSH from the anterior pituitary gland (Tsutsumi et al., 1992; Chi et al., 1993). In gonads, G protein-coupled receptors mediate gonadotrophin signalling (Loosfelt et al., 1989; McFarland et al., 1989; Minegishi et al., 1990, 1991; Sprengel et al., 1990), thus regulating the synthesis and secretion of sex hormones.

G protein-coupled receptors communicate via heterotrimeric G proteins, which are recognized as crucial elements in various types of membrane-mediated cell-signalling. Heterotrimeric G proteins consist of $\alpha$, $\beta$- and $\gamma$-subunits. For the $\alpha$-subunits, G proteins are divided into four classes ($G_\alpha$, $G_i$, $G_q$ and $G_{12}$) (Hepler and Gilman, 1992). Proteins of the $G_i$ family are the most diverse and interact with a wide variety of G protein-coupled receptors. For example, they take part in hormonal regulation via interaction with GnRH (Hawes et al., 1993; Stanislaus et al., 1998; Krsmanovic et al., 2001, 2003), FSH (Arey et al., 1997) and LH receptors (Herrlich et al., 1996). Moreover, the $G_i$ family of proteins play a role in the signal transduction of rapid, nongenomic actions of estrogen (Benten et al., 2001) and progesterone (Zhu et al., 2003; Karteris et al., 2006).

The dual balance between $G_i$ and $G_s$ signalling in the regulation of adenyl cyclase has been well established. Proteins of the $G_i$ family can inhibit adenyl cyclase and thus decrease intracellular cAMP concentration (Bokoch et al., 1984; Katada et al., 1984). Via this pathway, $G_i$-family protein $G_{i2}$ has been shown to take part in adrenergic signalling, controlling myometrium relaxation in the rat during pregnancy (Mhaouty et al., 1995). In the human myometrium, the levels of $G_{i2}$ have been shown to decrease during pregnancy, suggesting that the consequent, altered balance between $G_{i2}$ and $G_s$ could be responsible for maintaining the relaxation of uterus.

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during pregnancy (Europe-Finner et al., 1993). Although the role of Go₁₂ in myometrium has been thoroughly studied, the presence or the role of Go₁₂ elsewhere in the human reproductive tract remains unclear.

Immunohistochemical studies in the rat have shown that Go₁₂ is specifically localized in tissues having motile cilia with a characteristic 9+2 ultrastructure. Such a specific localization in rat oviductal, tracheal and brain ependymal cilia (Shinohara et al., 1998) implies that Go₁₂ may well serve a physiological function distinct from those of the other Go subunits. It is probable that Go₁₂ might play a cilia-specific physiological role. Interestingly, proteomic analysis has revealed Go₁₂ as a resident axonemal protein of the human bronchial cilia (Ostrowski et al., 2002). To date, however, there are no reports providing evidence of the localization of Go₁₂ in any other human ciliated tissues such as Fallopian tubes. In this study, we identify the presence and localization of Go₁₂ in tissues that are primarily in contact with gametes and provide the environment for fertilization, early development of the tissues that are primarily in contact with gametes and provide (Ostrowski et al.).

Materials and methods

Endometrial tissue collection and preparation for immunohistochemistry

The study was approved by the Local Ethics Committee and informed written consent was obtained prior to the collection of tissue samples. For immunohistochemical investigations, tissue samples were obtained from 6 fertile women, and for genomic studies, endometrial biopsies were obtained from 21 fertile women. All the women taking part in the study were 35 (range 24–40) years and each had had at least one previous successful pregnancy.

Endometrial biopsies for immunohistochemistry were immediately snap-frozen and stored in liquid nitrogen until processed. Cryosections were cut at 5 μm and stored at −70°C until use. For genomic studies, endometrial biopsies were immediately placed in RNA Later (Ambion, Huntingdon, UK), followed by immersion in liquid nitrogen until processed.

Fallopian tube tissue collection and preparation for immunohistochemistry

Human Fallopian tube tissues were collected from nine patients undergoing total abdominal hysterectomy for benign gynaecological conditions. The mean age of the women taking part in the study was 42 (range 33–56) years.

Fallopian tube tissue samples for immunohistochemistry were immediately fixed in 10% formalin overnight and embedded in paraffin. Paraffin sections were cut at 5 μm. For genomic studies, Fallopian tube tissue samples were immediately placed in RNA Later (Ambion) and stored for 24 h at 4°C, followed by immersion and storage in liquid nitrogen until processed.

Cell culture

Fallopian tube tissue samples for primary culture of epithelial cells were obtained as follows: Fallopian tubes were placed in Hank’s solution immediately after collection, cut open longitudinally and incubated 1 h with 0.25% collagenase (at 37°C, 95% O₂ and 5% CO₂). The cells were scraped gently using a sterile blade, washed with red blood cell lysing buffer (Sigma-Aldrich, Poole, UK) and then two to three times with Dulbecco’s modified Eagle’s medium (F12) (DMEM-F12, Invitrogen, Paisley, UK) culture media and plated into 75 ml flasks. Fallopian tube primary epithelial cells were cultured at 37°C in DMEM-F12 supplemented with 1% penicillin and streptomycin (Sigma-Aldrich), 10% fetal calf serum (Invitrogen) and 2 mM L-glutamine (Invitrogen) in 5% CO₂ atmosphere.

RNA isolation and cDNA synthesis

Tissues were removed from RNA Later and homogenized in 3 ml of TRI reagent (Sigma-Aldrich) using an Ultra-Turrax homogenizer for 2 min. Total RNA from the tissues and pelleted cells stored in TRI reagent was extracted following standard protocol supplied by the manufacturer. Total RNA (1–5 μg) was treated with Dnase I (DNA- free™, Ambion) to remove genomic DNA contamination from the samples. First strand complementary DNA (cDNA) synthesis was performed using oligo dT primers (Metabion, Martinsried, Germany) and RT by SuperScript II (200 U/μl, Invitrogen). RT controls were prepared without SuperScript II enzyme.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed with the cDNAs, Platinum Blue PCR Super Mix (Invitrogen) and primers from Meta bion. We used the following primer pairs: β-actin forward 5′-TGA CCC AGA TCA TGT TTG AGA CC-3′ and β-actin reverse 5′-GGA GGA GCA ATG ATC TTG ATC TTC-3′, Go₁₂ forward 5′-CTT GTC TGA GAT GCT GTG AAT GG-3′ and Go₁₂ reverse 5′-CTC CCT GTA AAC ATT TGG ACT TG-3′. The amplification was run for 35 cycles under the following conditions: 95°C 30 s, 58°C (β-Actin) or 65°C (Go₁₂) 30 s, 72°C 30 s. Amplified sequences were 643 and 212 base pairs for β-actin and Go₁₂, respectively. All experiments included RT controls as well as negative controls (no cDNA). PCR products were separated on 1.2% agarose gel.

Quantitative real-time PCR

Quantitative real-time PCR was performed with the cDNAs and the same primers that were used in PCR reactions. SYBR Green Jump Start Taq ReadyMix (S 4438, Sigma-Aldrich) master mix containing 10 μl SYBR Green, 7 μl water, 1 μl of each primer (20 pmol) and 1 μl cDNA was added to each well of PCR plate and amplification was performed under the following conditions: 50 cycles (95°C 30 s, 58°C or 65°C 30 s, 72°C 30 s). Amplified sequences were 643 and 212 base pairs for β-actin and Go₁₂, respectively. All experiments included RT controls and negative controls (no cDNA) and were performed in triplicate.

Results were analysed using iCycler (Biorad laboratories Ltd., Hemel Hempstead, UK). To compare relative quantities of Go₁₂ expression during the menstrual cycle, endometrial biopsies were divided into three groups: menstrual (LMP + 1–4; n = 3; LMP + 1, +4 and +4), proliferative (LMP + 5–14; n = 9; early proliferative LMP + 5, +5 and +7, mid-proliferative LMP + 8, +9 and +10, late proliferative LMP + 11, +12 and +13) and secretory (LMP + 15–29; n = 9; early secretory LMP + 16, +16 and +17, mid-secretory LMP + 20, +21 and +22, late secretory LMP + 26, +28 and +29). Relative Go₁₂ expression quantities were compared between these groups. The threshold cycle values were normalized against threshold value of human β-actin. The results were expressed as...
mean ± SEM. Statistical analysis was performed by using one-way analysis of variance with Tukey’s multiple comparison test. $P < 0.05$ was considered significant.

**Immunohistochemistry**

Cryosections of endometrium were thawed by immersion (15 min at 20°C) into fixative containing 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The slides were then washed with PBS (2 × 5 min) and further fixed by immersion in −20°C methanol (4 min) followed immediately by treatment with −20°C acetone (2 min). After 2 × 5 min washes with PBS, endogenous peroxidase activity was blocked by 5% H$_2$O$_2$ (in distilled water) treatment (5 min). The slides were then washed with deionized water (2 × 5 min) and PBS (2 × 5 min). After this, the protocol follows the same blocking and staining as described subsequently for paraffin sections.

Fallopian tube paraffin sections were first dewaxed in xylene, rehydrated through a series of ethanols and finally washed with PBS. Endogenous peroxidase activity was quenched by a 20 min incubation with 3% H$_2$O$_2$ (v/v) in methanol. Antigen retrieval was performed by microwave irradiation in 10 mM citrate buffer, pH 6.0 (12 min). The slides were allowed to cool in the buffer and then washed with PBS (2 × 3 min).

Vectastain Elite ABC Kit (Vector Laboratories, Peterborough, UK) was used according to the manufacturers instructions for both cryosections and paraffin sections, with the following modifications. Slides were blocked in buffer containing 250 μl avidin D m/l (1 h room temperature). Mouse anti-Gα$_{i2}$ monoclonal antibody, MAB3077 (Chemicon International, Temecula, CA, USA) was diluted (cryosections 1 : 1000, paraffin sections 1 : 500) in Dako antibody diluent (Dako UK Ltd., Cambridgeshire, UK) containing 250 μl biotin m/l and incubated overnight at 4°C. Primary antibody was omitted in negative controls. The slides were washed with PBS (5 min) and incubated with secondary antibody [1 : 200 biotinylated anti-mouse (Vector Laboratories)] for 30 min at 20°C. The slides were washed as before and incubated for 30 min with Vectastain ABC reagent (Vector Laboratories). After washing, binding was visualized by incubation with substrate 3,3′-diaminobenzidine (DAB) or DAB-Ni for 8 min (Vector Laboratories). The slides were rinsed with tap water (5 min) and PBS (3 min) and counterstained by using 10% haematoxylin (10 min). Following thorough rinse in tap water, slides were dehydrated through a series of ethanols, cleared in xylene and coverslipped with DePex mounting medium (VWR International, Lutterworth, UK).

The endometrial biopsy specimens were timed using LMP and morphology according to Noyes criteria (Noyes, 1950) and divided into three groups: menstrual, proliferative and secretory. The slides were viewed using a ×40 objective on an Olympus CKX41 microscope. Digital images were captured with a Nikon Coolpix 5400 camera and cut to same size and shape keeping their original scales in Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

**Results**

**PCR reveals the expression of Gα$_{i2}$ gene in human reproductive tissues**

We used human Fallopian tube tissue and human endometrial biopsies to study the expression of Gα$_{i2}$ by PCR. Our data revealed that Gα$_{i2}$ is expressed in human Fallopian tube and human endometrium (Figure 1A and B). Our studies also confirmed that Gα$_{i2}$ is expressed in primary cultures of Fallopian tube epithelial cells (Figure 1C). Control experiments with non-reverse transcribed RNA of each sample confirmed that there was no contamination of human DNA in the samples.

**Immunohistochemistry shows specific localization of Gα$_{i2}$ protein in Fallopian tube cilia and enrichment in endometrial glands**

Immunostaining on human Fallopian tube paraffin sections showed specific localization of Gα$_{i2}$ protein in Fallopian tube epithelial cells and the cilia (Figure 2C). Positive staining was also seen in the cytoplasm of epithelial cells, surrounding the nuclei. In endometrial tissue, Gα$_{i2}$ staining was enriched in endometrial glands, but was present also in stroma (Figure 2A and B).

**Quantitative real-time PCR shows alterations in Gα$_{i2}$ gene expression during the menstrual cycle**

We carried out quantitative real-time PCR experiment on endometrial biopsies spanning the menstrual cycle (Figure 3). Based on the phase of the menstrual cycle of each patient, the biopsies were designated in three groups, namely menstrual (LMP + 1–4), proliferative (LMP + 5–14) and secretory (LMP + 15–29).

Our results demonstrated that endometrial expression of Gα$_{i2}$ gene changed during the cycle. The expression of Gα$_{i2}$ reached its peak in secretory phase and this was significantly higher ($P < 0.05$) compared with that of the menstrual and proliferative phases.

**Discussion**

The present study demonstrates the existence and localization of Gα$_{i2}$ in human endometrium and Fallopian tube. Our data establish the specific localization of Gα$_{i2}$ in the Fallopian tube epithelial cells, particularly in the cilia of Fallopian tube

![Figure 1](https://academic.oup.com/humrep/article-abstract/22/5/1224/2914950/1226)
epithelial cells. In human endometrium, we have demonstrated that localization of Goα12 is enriched in endometrial glands. We have also shown that Goα12 expression in human endometrium changes significantly during the menstrual cycle with maximum expression in the secretory phase, providing evidence that expression of this G_i subunit might be under hormonal regulation in the female reproductive tract in vivo.

The presence of G protein subunit Goα12 in rat myometrial membranes was first reported by Milligan et al. (1989), and the finding was later supported by a study suggesting differential regulation of Goα12 and Goα13 in rat myometrium during gestation (Tanfin et al., 1991). In human myometrium, the levels of G protein subunits Goα1, Goα3, Goα4, and Goα11 have been shown to remain constant in pregnant and non-pregnant women, whereas levels of Goα12 decrease during pregnancy. The simultaneous, substantial increase in myometrial G_i suggested that the balance between Goα12 and G_i might be essential in regulating relaxation of the uterus during pregnancy (Europe-Finner et al., 1993). Besides this, G_i family proteins have been suggested to be functionally linked to α2 adrenergic signalling in human myometrium during pregnancy (Breuller et al., 1990). Later studies in the rat have confirmed the involvement of Goα12 and Goα13 in α2/β2 adrenergic signalling in the maintenance of uterus relaxation during rat pregnancy (Mhaouty et al., 1995).

Unlike the thoroughly studied myometrium, the presence and role of Goα12 in other regions of the reproductive tract has remained largely obscure. Although the presence of G_i family proteins has been described in human endometrium during artificial cycles of hormone replacement therapy, those studies rely solely on data from immunoblotting, using an antibody unable to discriminate between the closely related Goα11 and Goα12 (Bernardini et al., 1995, 1999). Therefore, prior to our study, cyclical changes in Goα12 expression have not been reported in humans. Quantitative PCR showed that Goα12 expression in human endometrium in vivo significantly increased in the secretory phase of the menstrual cycle. This suggested that sex hormones, such as estrogen or progesterone, might regulate the expression of this G_i subunit in human endometrium. Furthermore, immunostaining clearly demonstrated the main localization of Goα12 in endometrial glands and partially in endometrial stroma. In the future, studies clarifying the presence of Goα12 in endometrial epithelial and stromal cells would be interesting to carry out in order to reveal whether Goα12 is expressed differently in these cells.

It is likely that Goα12 is hormonally regulated in the human endometrium. Earlier studies on rat myometrium have shown that estradiol administration during rat pregnancy increases the levels of both Goα12 protein and Goα12 mRNA, whereas progesterone has no effect on Goα12 expression. Instead, progesterone was reported to cause a decrease in Goα4 subunit expression (Cohen-Tannoudji et al., 1995). Other studies in pregnant rat myometrium have suggested a regulatory role for progesterone in control of β2 receptors (Malter et al., 1989) and G_i proteins (Elwardy-Merezak et al., 1994), as well as in up regulation of β2 receptor expression (Vivat et al., 1992). Apart from the studies by Bernardini et al. (1995, 1999), the potential role for sex hormones in regulation of G_i proteins in the human has remained largely unexplored.

In the present study, we have reported for the first time the localization of Goα12 in Fallopian tube epithelial cilia. In Fallopian tubes, ciliary beat is essential for gamete transport in association with the tubal secretory flow and muscle contrac-
tility. Furthermore, Fallopian tube epithelial cells have also been demonstrated to preserve the viability of sperm (Kervancioglu et al., 1994; Murray and Smith, 1997; Kervancioglu et al., 2000).

The mean age of patients included in the present study for Fallopian tube tissue collection was higher in comparison to those included for collection of endometrial biopsies. Difference of the mean ages between the groups was 7 years. Although the age difference might involve reduced fertility in the older patients, the expression or localization of Goα12 was not significantly altered, as all the individual patients showed similar results in immunostaining.

Given the fact that Goα12 is specifically localized in rat tissue motile cilia with a characteristic 9+2 ultrastructure, namely in rat oviductal, tracheal and brain ependymal cilia (Shinohara et al., 1998), it seems evident that this G_i subunit might have a cilia-specific physiological role. Apart from proteomic analysis providing evidence of Goα12 as a resident axonemal protein of the human bronchial cilia (Ostrowski et al., 2002), there are no reports describing Goα12 in any other human ciliated tissue.
In addition to positive immunostaining of Fallopian tube cilia, we have reported here positive immunostaining surrounding the nuclei. This presumably represents pre-stage $\alpha_{i2}$, which is still undergoing synthesis, or alternatively, $\alpha_{i2}$, which is ready for transport into cilia by intraflagellar transport mechanisms. This intracellular machinery is vital for assembly and maintenance of the cilia, as it transports essential particles, such as proteins synthesised in the cytoplasm of cell, into the cilia and returns the turnover products to the cytoplasm (Rosenbaum and Witman, 2002).

Studies with $\alpha_{i2}$-knockout mice have established a crucial regulatory role for the $\alpha_{i2}$ subunit in immunological processes (Rudolph et al., 1995a,b; Jiang et al., 1997; Dalwadi et al., 2003; Fan et al., 2005; Han et al., 2005; Zhang et al., 2005). $\alpha_{i2}$ has been revealed to control regulation of T-cell proliferation (Zhang et al., 2005) and B-cell development (Dalwadi et al., 2003). Furthermore, $\alpha_{i2}$ has been suggested to mediate chemokine signalling (Han et al., 2005). However, reports of $\alpha_{i2}$-knockout studies have not provided any information on potential involvement of this $G_i$ subunit in modulation of mice fertility. Interestingly, a recent study on $\alpha_{i2}$-knockout mice showed $\alpha_{i2}$ to differentially regulate inflammatory mediator production in response to microbial stimuli and proposed a Toll-like receptor (TLR)-signalling regulating role for $\alpha_{i2}$ by an yet unknown mechanism (Fan et al., 2005). Regarding the potential link between TLR-signalling and $\alpha_{i2}$ in female reproductive tract, it is noteworthy that our previous studies showing the localization pattern of several TLRs (Fazeli et al., 2005) showed a pattern of localization similar to that we now report for $\alpha_{i2}$. Future studies should be directed towards understanding whether $\alpha_{i2}$ might share signalling pathways with TLRs and potentially have a TLR-signalling regulating role in human reproductive tract.

In conclusion, our studies reveal the presence of $\alpha_{i2}$ in human endometrium and Fallopian tube epithelium, especially the cilia of fallopian tube epithelial cells. To the best of our knowledge, this is the first report of the localization of $\alpha_{i2}$ in ciliated reproductive tissue in the human. We also report here, for the first time, the alterations in $\alpha_{i2}$ expression during human menstrual cycle. Our data imply this $G_i$ family subunit might be under hormonal regulation in the female reproductive tract in vivo. Further studies are required to clarify the physiological role of $\alpha_{i2}$ in the female reproductive tract.

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References

Goαi in human reproductive tissues


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