Regulation of LH/CG receptor signalling in human endometrium

Camilla West [1], Stuart Lavery [1], Jan Brosens[2] & Aylin Hanyaloglu[1]

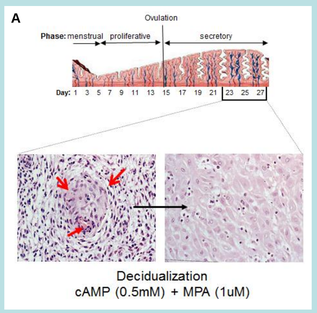
[1] Institute of Reproductive and Developmental Biology, Imperial College London, Hammersmith Hospital, London, United Kingdom. [2] Division of Reproductive Health, Warwick Medical School, Clinical Sciences Research Laboratories, University Hospital, Coventry, United Kingdom

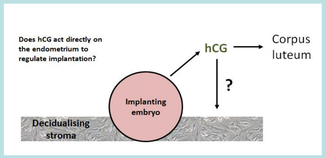
Introduction

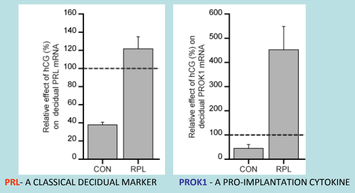
The interaction that takes place between an implanting embryo and the endometrium is essential for successful implantation. However, this process in humans is relatively inefficient and the frequency of subfertility is high, affecting one in six couples in developed countries. As well as a high frequency of subfertility, embryo wastage and pregnancy loss in humans is also prevalent, with an estimated 30% of embryos lost prior to implantation and a further 30% before 6 weeks gestation (Salker et al., 2010). The glycoprotein hormone human chorionic gonadotrophin (hCG) is one of the earliest signals secreted by the embryo, and may facilitate implantation. It is well established that hCG is an important factor at the time of implantation but the underlying signalling of its cognate receptor, the LH/CG receptor, in the endometrium is largely unknown. This research focuses on unravelling hCG signal transduction mechanisms and receptor trafficking in the human endometrium and how this may be important in the process of implantation and foeto-maternal communication.

Background

Human Endometrial Stromal Cells (HESCs) and hCG responses



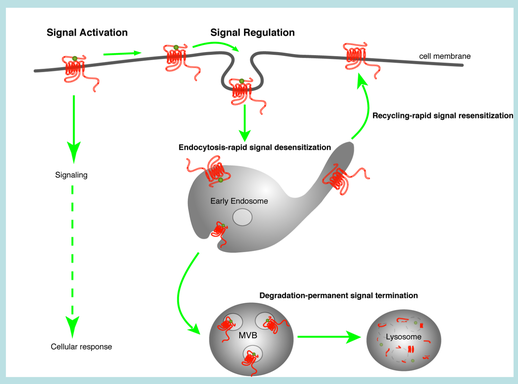




PRL- A CLASSICAL DECIDUAL MARKER PROK1 - A PRO-IMPLANTATION CYTOKINE

Under the influence of increased levels of cAMP and progesterone HESCs transform from elongated fibroblast like cells in to rounded epitheloid-like decidual cells and this can be recapitulated *in vitro (A).* Decidualisation is marked by a dramatic change in gene expression markers, such as prolactin (PRL) and prokineticin 1 (PROK1). hCG has been shown to directly act HESCs to attenuate decidual gene expression (right panal, Salker et al., 2010).

GPCR trafficking can modulate hormonal responsiveness



GPCRs are essential communicators of extracellular signals in all physiological systems. Classically, when bound to ligand, GPCRs undergo rapid endocytosis and desensitization. They may be subsequently sent for degradation by the lysosome or recycled back to the plasma membrane where they can undergo further ligand stimulation. This membrane trafficking has emerged as a system that is integrated also with cell signalling from GPCRs. Defects in endocytosis or cargo-sorting has been shown to result in various disease states.

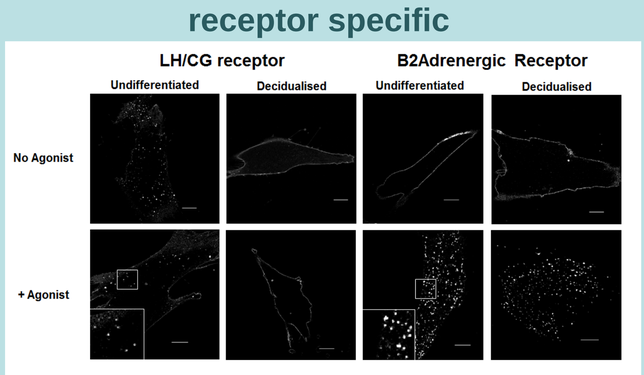
Aims

1) Identify the trafficking mechanism for the LH/CHR in HESCs, and investigate potential interacting proteins that may modulate this during decidualisation.

2) Define and characterize the LH/CGR signalling pathways that are activated in differentiated and non-differentiated HESCs both constitutively and in response to hCG.

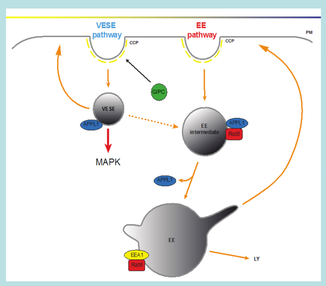
Results: LH/CGR Trafficking

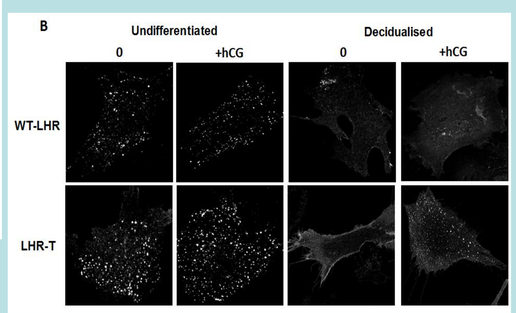
1. Trafficking of the LH/CG receptor alters between undifferentiated and decidualised HESCs and this is receptor specific



HESCs were seeded iimaging dishes and decidualised for 72 hours with cAMP/MPA while undifferentiated cells were cultured for the same period with medium alone. Cells were then transfected one day prior to imaging with Flag-tagged LH/CG or β2 adrenergic (β2AR) receptor constructs and labelled with fluorescently conjugated anti-Flag antibodies. 10 nM hCG and 10 mM isoproterenol (30 min) were used to activate each receptor respectively. Representative confocal microscopy images are shown from n=3. (20 cells analysed per condition). Scale bars = 10um.

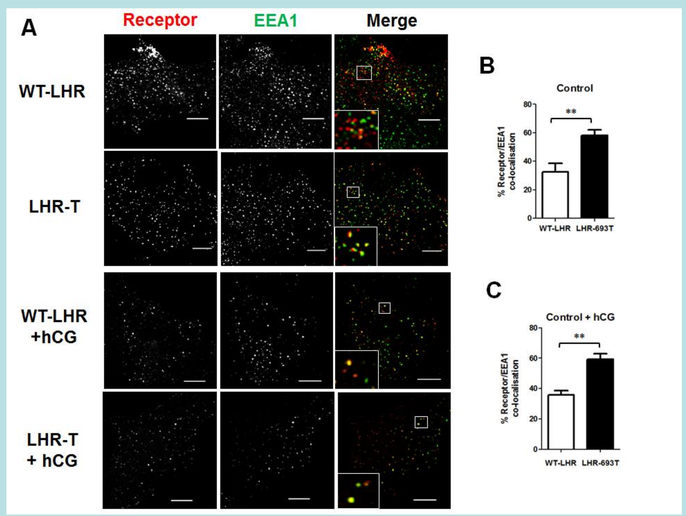
2. Truncation of the C-terminal tail of the LH/CGR does not affect receptor trafficking





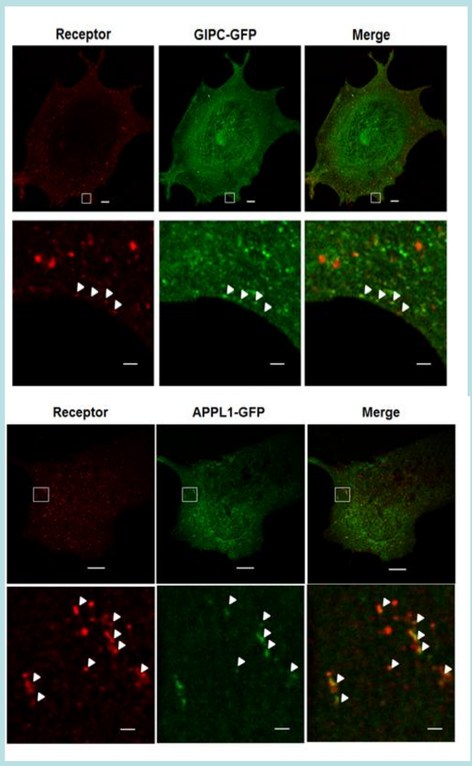
A. Schematic representation of the LHR C-terminal tail. Residues highlighted in red have been described to be essential for receptor recycling (Hirakawa et al., 2003; Galet et al., 2003; Galet et al., 2004). Arrow indicates the residue mutated to a stop codon to create truncation mutant LHR-T. *B.* HESCs were seeded onto coverslips and decidualised for 72 hours with cAMP/MPA and undifferentiated cells were cultured for the same period with medium alone. Cells were transfected with wild-type or truncated LHR Flag-tagged receptor 24 hrs before treatments. Surface receptor was then labelled with an anti-Flag mouse antibody prior to 20 minute stimulation with 10nM hCG, or left unstimulated. Cells were then washed, fixed and permeabilised before labelling with a fluorescent anti-mouse antibody

3. Truncation of the C-terminal tail forces the LH/CGR into a different subcellular compartment



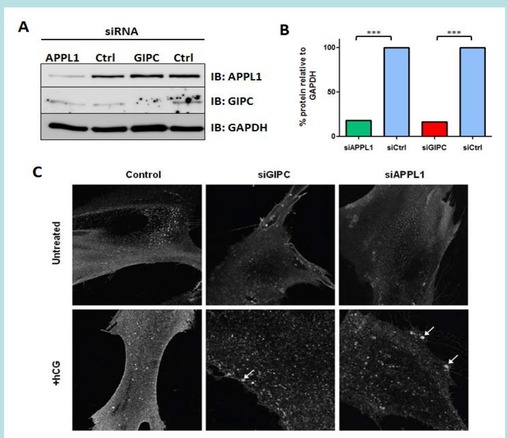
A. Undifferentiated HESCs were seeded onto coverslips and cells were transfected with wild-type or truncated LHR Flag-tagged receptors 24 hrs prior to treatments. Surface receptor was then labelled with an anti-Flag mouse antibody and cells were then stimulated for 20 minutes with 10nM hCG, or left unstimulated. Cells were washed, fixed and permeabilised before incubation with an anti-EEA1 rabbit antibody. Cells were then labelled with fluorescent mouse and rabbit secondary antibodies and imaged using confocal microscopy. *B & C.* Colocalization of LHR or LHR-T containing endosomes positive for EEA1 were quantified and the percentage calculated (n= 10 cells) A paired t-test was then carried out. **, $p < 0.01$. Scale bars = 10um.

4. The WT-LHR co-localises with APPL1 and GIPC in primary HESCs



Primary HESCs were plated onto coverslips and transfected with either GIPC (*A*) or APPL1 (*B*) constructs tagged with GFP and Flag-tagged wild-type LHR. Cells were then fixed, permeabilised and labelled with a fluorescently conjugated anti-Flag antibody. Images shown are undifferentiated HESCs only.

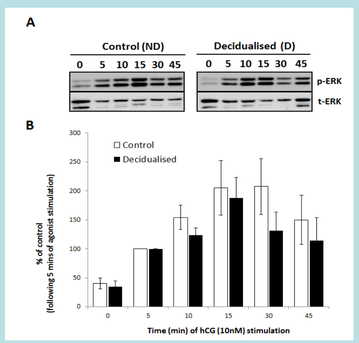
5. Trafficking of the LH/CG receptor in HESCs is altered following siRNA knockdown of GIPC and APPL1



A. Western blot of total cellular levels of APPL1 (top panel) or GIPC (middle panel) following siRNA-mediated knockdown as indicated and normalised to GAPDH (bottom panel). *B.* Bar graph representing the percent knockdown of APPL1 and GIPC analysed by densitometry analysis. Protein levels were normalised to GAPDH. A paired t-test was then carried out. ***, $p < 0.01$ *C.* Primary HESCs were plated in 35mm imaging dishes and transfected with siRNA against GIPC or APPL1 or left untreated, 72 hours prior to imaging. One day prior to imaging, cells were transfected with Flag-tagged LH/CG receptor and on day of imaging labelled with a fluorescently conjugated anti-Flag antibody to label surface receptor. n=2 patients

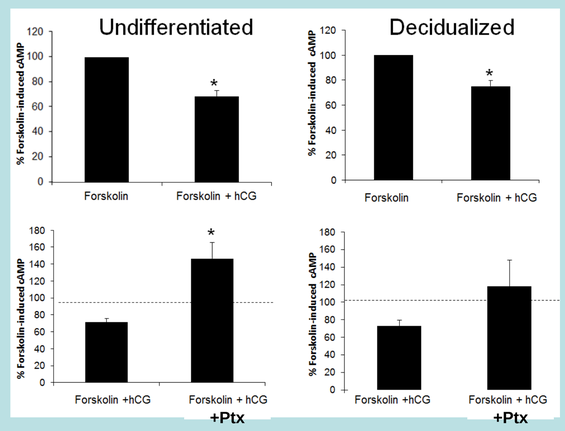
LH/CGR Signalling

6. hCG induces ERK1/2 phosphorylation in HESCs



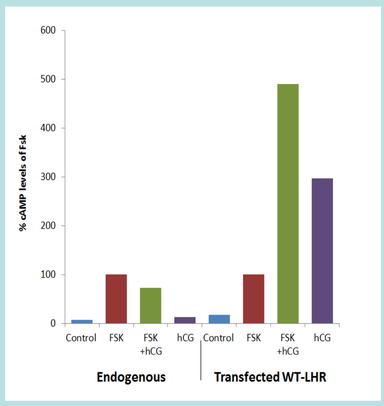
A. Primary HESCs which had been decidualised with cAMP and MPA for 72 hrs or left undifferentiated were treated with hCG (10nM) for the indicated times, and phosphorylation of ERK1/2 was determined by western blotting. Total ERK was used as a loading control. *B.* Densitometric analysis of ERK1/2 was normalised to 5 min stimulation and is shown in the bar graph. Data represents mean ± SEM, n=5 patients

7. hCG activates the non-classical Gαi signalling pathway to reduce levels of cAMP



HESCs were seeded into 6 well plates and grown until confluent. 24 hours prior to assay, cells were transfected with the wild-type LHR or left untransfected. On day of assaying cells were treated with Forskolin (3uM) in order to artificially raise levels of cAMP or left untreated, and were also stimulated using hCG (10uM) or left unstimulated. In some conditions, cells were treated overnight with the Gαi inhibitor Pertussis Toxin (Ptx). Cells were then washed and lysed and cAMP levels were tested using an ELISA-based cAMP assay kit (Enzo Biosciences). cAMP levels were then normalised to Forskolin (n=1 patient).

8. Overexpression of the LH/CGR alters its signalling profile



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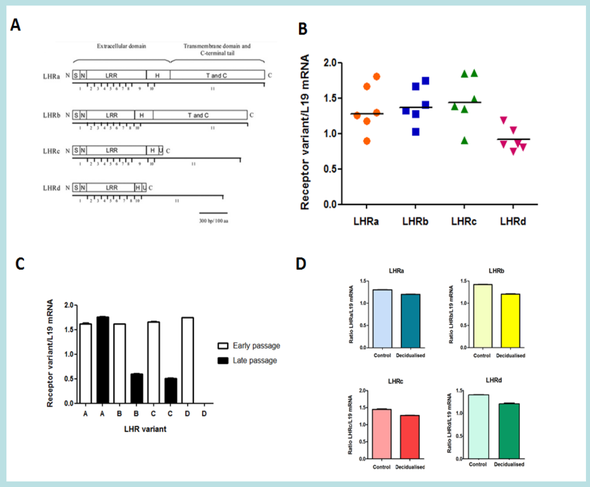
9. LH/CGR splice variants are expressed in HESCs



B. Expression of *LHR* splice variants in primary HESCs. RNA extracted from cultured HESCs at early passages from 5 patients was used as a template for RT-PCR using primers specific for each splice variant *LHRa*, *LHRb*, *LHRc* and *LHRd*. *C.* Expression of LHR splice variants is dependent on time in culture. *D.* Primary HESC cultures were decidualised with either 8-br-cAMP and MPA or left undifferentiated for 72 hrs and total LHRa, b and c transcript levels were measured. *A* is taken from Dickinson et al., 2009

Conclusions

1) HESCs display changes in receptor trafficking following decidualisation that is specific to the hCG/LH receptor

2) A truncated hCG/LHR unable to bind trafficking proteins such as GIPC changes the endocytic compartmentalisation of the receptor

3) Knockdown of APPL1 and GIPC may direct the LH/CGR to larger, EE-like compartments

4) The MAPK signalling pathway is activated in HESCs

5) hCG couples to the Gαi pathway in HESCs to decrease levels of cAMP. HESCs but the signalling profile changes when the receptor is over-expressed

6) Splice variants of the receptor are expressed in control and decidualised HESCs and may explain why this receptor activates the non-classical Gαi pathway in these cells

References

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