3. The detection and confirmation of 11β-hydroxysteroid dehydrogenase type 1 transcripts in human luteinized granulosa cells using RT–PCR and plasmid pUC18

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The activity of 11β-hydroxysteroid dehydrogenase (11β-HSD) in human granulosa cells has been shown to be associated with the outcome of treatment following in-vitro fertilization and embryo transfer. There are two known isoforms of 11β-HSD which differ significantly in their actions and co-factor requirements. The net activity of 11β-HSD within the human ovary is unclear, but may be of particular importance within the ovarian follicle in regulating possible glucocorticoid influences on the oocyte. This study presents preliminary information regarding establishment of techniques to identify transcripts of the 11β-HSD isoforms within human granulosa cells and human cumulus cells using reverse transcription–polymerase chain reaction. In view of the high expression of the type 1 11β-HSD isoform and the possibility of other 11β-HSD isoforms in the ovary, plasmid technology was used to confirm the technique specifically identifying the known isoforms. Key words: glucocorticoids/granulosa cells/11β-HSD/plasmid pUC18

Introduction
The enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) is expressed in many different human tissues and is important in determining glucocorticoid access to glucocorticoid and mineralocorticoid receptors. Abnormalities of 11β-HSD activity have been observed in a number of conditions including essential hypertension (Walker et al., 1993) and polycystic ovarian syndrome (PCOS; Rodin et al., 1994). The two known isoforms of the enzyme (type 1 and type 2) differ in their actions and co-factor requirements and their genes share only 14% sequence identity (Albiston et al., 1994). The human type 1 isoform was cloned and characterized from a testis cDNA library (Tannin et al., 1991) and type 2 from a kidney cDNA library (Albiston et al., 1994). Interest in the role of this enzyme in reproductive endocrinology was heightened by the findings of Michael et al. (1993) who observed that the absence of 11β-HSD activity in cultured granulosa–lutein cells was predictive of pregnancy following in-vitro fertilization (IVF) whereas patients with measurable 11β-HSD activity did not become pregnant. In this preliminary study we sought to establish the methodology to enable us to determine whether mRNA for either isoenzyme was transcribed in human granulosa cells using plasmid technology to help confirm the findings.

Materials and methods
Granulosa cells were collected by aspiration of mature preovulatory follicles, at oocyte retrieval from patients undergoing IVF treatment at the University of Bristol IVF unit using a standardized treatment protocol of pituitary desensitization using a gonadotrophin-releasing hormone agonist (GnRHa), ovarian stimulation and embryo culture methods as described by Hull et al. (1992).

The cells were purified by centrifugation for 30 min at 500 g on a 50% Percoll gradient and any remaining erythrocyte contamination was removed by incubation with lysis buffer. Leukocytes were then removed through a 30 min incubation with CD45-coated antibody beads (Dynabeads, Dynal Ltd, Norway). After counting, the granulosa cells were snap-frozen in liquid nitrogen and stored until ready for use. Total RNA was extracted using Trizol Reagent™ (Gibco BRL, Paisley, UK) and purified by DNase I treatment and a series of phenol/chloroform extractions and ethanol precipitations (Frayne et al., 1997). The concentration of total RNA was then estimated following gel electrophoresis and a 2 µg aliquot of this RNA used as the template for avian myeloblastosis virus (AMV) reverse transcriptase-directed cDNA synthesis using oligo (dT)₁₂₋₁₈ as a primer. cDNA was then amplified by polymerase chain reaction (PCR) using oligonucleotide primers specific for type 1 (5′-CATGCTGAGCAGCATGAGGA-3′ and 5′-CTGCTACCTCTCTGCATGATGGAT-3′) and type 2 (5′-GCATCTGTCATGATGGCAAGTAC-3′ and 5′-CTTCGCTAGCTGCATAGGCTG-3′) human 11β-HSD, using human placental tissue as a positive control. The resulting 11β-HSD-specific PCR products were then identified by agarose gel electrophoresis.

Verification of the identity of the PCR products was achieved by 5′-phosphorylating the PCR product with T4 polynucleotide kinase and subsequent blunt-ended ligation into Smal-cut, dephosphorylated, pUC18 plasmid DNA (Pharmacia Biotech, St Albans, UK). The resulting plasmids were then introduced, by electroporation, into Escherichia coli XL1-blue cells and plated out onto nutrient agar medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactoside (IPTG) for blue/white colour selection of recombinants. After overnight incubation at 37°C, plasmid DNA was isolated from putative recombinants (shown as white colonies) using an alkaline lysis method (Birnboim and Doly, 1979). The samples were then sequenced using an ABI 377 automated DNA sequencer, which confirmed the expected sequence of the cloned type 1 11β-HSD PCR product.
Figure 1. Gel electrophoresis showing type 1 11β-HSD (363 bp) and type 2 11β-HSD (434 bp) from (A) human term placenta which was used as a positive control; and (B) human cumulus cells, peripheral leukocytes, human granulosa cells, human cumulus cells from fertilized and unfertilized oocytes and human term placenta as a positive control.

Results
Types 1 and 2 11β-HSD were cloned and sequenced from human term placenta using RT–PCR and pUC18 which served as a positive control (see Figure 1A). A PCR product of the expected size (363 bp) was consistently detected by RT–PCR using total RNA from granulosa–lutein and cumulus cells, with primers specific for type 1 11β-HSD (see Figure 1B). However, type 2 11β-HSD transcripts were not convincingly and consistently detected in granulosa–lutein or cumulus cells.

Discussion
This study presents an example of the use of plasmid technology in current research. Plasmids are autonomously replicating extra-chromosomal DNA molecules found in numerous bacterial species. They were originally identified as naturally occurring episomes (distinct elements of DNA that are ‘not integrated into the genome of the recipient bacterium’) (Marmur et al., 1961). However, they do rely on enzymes and proteins encoded by the host for replication and transcription. Their ability to confer resistance (R) to specific antibiotics and mating factor (F) to the host bacterium led to a great deal of investigation. It was found that the properties of these episomes facilitated ready identification and ‘physical separation from other (non-circular) DNA species’ (Cohen and Miller, 1969). Further, it was soon found that the phenotypes conveyed to the host by plasmids included antibiotic resistance, the breakdown of complex organic compounds and the production of restriction and modification enzymes (Sambrook et al., 1989).

Plasmid replication is effected by the same enzymes used by the bacterial chromosome although different plasmids have different ‘copy numbers’ from the minimal level of a single plasmid per cell to upwards of 700 per cell. Plasmids can be introduced into bacteria in the laboratory through the process of transformation, where the bacteria are treated with divalent cations to achieve temporary permeability to small DNA molecules. Successful transformants can then easily be selected through resistance to any one of a number of specific antibiotics; virtually all plasmids carry some sort of antibacterial resistance through encoded gene sequences and many carry resistance to several types of antibiotics.

The use of plasmids as cloning vectors dates back to 1973 (Cohen et al., 1973) when it was found that specific genetic sequences could be incorporated into the plasmid genome within a particular restriction site by the in-vitro joining of restriction endonuclease-generated fragments from separate sources creating new chimaeric plasmid species. However, these early cloning vectors had low copy numbers and had selectable markers of only limited use. One such early multi-purpose cloning vector was pBR322 (Bolivar et al., 1977) constructed to give efficient cloning and selection in E.coli. It was extensively used as it was simple and its nucleotide sequence was widely available. However, as with many early cloning vectors it suffered from a lack of a direct selection scheme for successful recombinants. This led to the development of further vectors, some using the basic pBR322 sequence as a framework. Of special interest were vectors that would give high copy numbers within the host cell and easy recombinant selection. One such group are those that convey α-complementation of β-galactosidase activity to the host giving rise to blue/white colour selection where successful recombinants give white colonies of the host and transformants comprising the vector sequence alone give blue colonies. The pUC series of vectors (Vierra and Messing, 1982), including pUC18 used in this study (Norrander et al., 1983), convey this property and have been constructed to contain a polylinker with multiple restriction sites allowing for greater cloning possibilities. They are also ideal for DNA sequencing as the M13 universal and reverse priming sites (Yannish-Peron et al., 1983) flank the polylinker region.

In this study RT–PCR has provided a convenient, sensitive technique to establish the presence of 11β-HSD type 1 tran-
scripts in human granulosa cells, confirmed through the use of plasmid technology. The two known isoforms of 11β-HSD regulate the interconversion of cortisol and cortisone. Type 1 11β-HSD acts predominantly as a low affinity NADP(H)-dependent oxidoreductase generating cortisol from cortisone, whereas type 2 is a unidirectional, high affinity NAD-dependent dehydrogenase which converts cortisol to cortisone. The link between 11β-HSD activity and human fertility was suggested by the studies of Michael et al. (1993) who observed that conception in IVF cycles only occurred in the absence of 11β-HSD activity and that the presence of activity was associated with a high chance of the cycle being unsuccessful. This raised the possibility of being able to identify patients or even oocytes with the potential to lead to pregnancy.

Evidence is accumulating that glucocorticoids may have a role in oocyte maturation. Natural cycle IVF studies have shown higher follicular fluid cortisol concentrations in conception cycles (personal unpublished data). However, studies from stimulated cycles have observed higher total cortisol concentrations in follicular fluid from follicles whose oocytes did not fertilize (Fateh et al., 1989; Jimena et al., 1992).

Rising progesterone concentrations in follicular fluid following the onset of the luteinizing hormone (LH) surge may potentiate the effect of cortisol by displacing cortisol from its binding protein and increasing the free cortisol concentration in follicular fluid. Yding Andersen and Hornnes (1994) found no relationship between cortisol concentrations in individual follicles and the resulting embryos ability to cleave or implant. Exogenous glucocorticoids have been used as co-treatment with clomiphene citrate for ovulation induction and have been observed in some studies to increase ovulation and pregnancy rates in patients resistant to clomiphene alone (Lobo et al., 1982; Daly et al., 1984). In IVF cycles without pituitary desensitization prednisolone co-treatment resulted in significantly higher pregnancy rates (Kemeter et al., 1986), but whether co-treatment is beneficial when the pituitary is desensitized is not clear from published studies (Jenkins et al., 1993; Bider et al., 1996; Rein et al., 1996).

It has been suggested that 11β-HSD may be inappropriately regulated in polycystic ovarian syndrome (PCOS) and that increased oxidation of cortisol to cortisone may result in increasing levels of corticotropin which increases adrenal androgen synthesis (Rodin et al., 1994). The increased oxidation may result from increased type 2 expression or to reduced type 1. 11β-HSD influences the relative concentrations of cortisol and cortisone and the expression of the two isoforms in different ovarian cells (oocyte, cumulus and granulosa cells) may be important.

It is likely that there is cell specific metabolism of steroids and that different ovarian cell types express different 11β-HSD activity. Benedicksson et al. (1992) showed high expression within the rat ovary located mainly in the immature oocytes but were unable to identify 11β-HSD in rat granulosa cells. One may speculate that 11β-HSD expression in the human oocyte influences final maturation of the oocyte through alterations in glucocorticoid concentrations within the follicle. It has been suggested that 11β-HSD could be developmentally regulated and that other steroids such as 11β-hydroxyandrostenedione should be considered as possible substrates for 11β-HSD (Hillier 1994).

Although 11β-HSD is known to be important in ovarian function, the exact role is not yet clear. The presence of type 1 11β-HSD transcripts is confirmed in granulosa cells, but in addition there may be other, possibly as yet unknown, 11β-HSD transcripts in these and other ovarian cells playing significant roles. RT-PCR coupled with plasmid technology offers a highly sensitive molecular approach, which when combined with biochemical studies of human oocytes, cumulus cells and granulosa cells may help provide a better understanding of the important relationship between glucocorticoids and oocyte maturation.

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References

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