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THE STUDY OF CELLULAR AND MOLECULAR ASPECTS OF THE HUMAN FALLOPIAN TUBE EPITHELIUM

A thesis submitted in partial fulfilment of the requirements of Kingston University for the degree of Doctor of Philosophy

in collaboration with

The University of London, Academic Department of Obstetrics and Gynaecology, St Bartholomew's and The Royal London School of Medicine and Dentistry.

Saeeda Sattar

Faculty of Science

School of Life Sciences

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Declaration

I hereby declare that whilst registered for a research degree at Kingston University, I have not been a registered candidate or enrolled student at any other academic or professional institution.

I declare that the materials contained in this thesis have not been used in any other submission for an academic award. All the sources of investigation have been duly acknowledged.

This thesis has been composed by myself and is a result of my own investigation.

Saeeda Sattar BSc (Hons)

Dedication

To my dearest and ever-loving parents, Sayeed and Jameela

and

my guardians, Asif and Carola

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ABSTRACT

The Fallopian tube provides physiological support to both gametes and the embryo. Experimental approaches to improve the outcome of infertility treatment have included co-culture systems, whereby sperm, ova and/or embryos are cultured in a monolayer of Fallopian tube epithelial cells. This approach has been shown to be superior to other methods based on co-culture with other cell types. In addition, the role of gap junction intercellular communication (GJIC) in the regulation of cell proliferation and differentiation is becoming increasingly recognised as one of the major cellular functions. GJIC plays an important role in fertilisation as well as in the normal development of both the embryo and foetus. It is also involved in the sexual maturation of the adult and in the maintenance of health throughout life. As such, GJIC may be the key element in the understanding of cellular functions especially during the reproductive process. Therefore, the aims of this thesis were to examine some of the cellular and molecular aspects of the human Fallopian tube epithelium in relation to infertility.

In the first part of this study, the gap junction proteins, connexins were examined by immunohistochemical and Western blotting techniques. Fallopian tubes were removed from women at different stages of the ovarian cycle. The results demonstrated the presence of gap junction proteins, connexins (cx) in the human Fallopian tube throughout the ovarian cycle. Fimbrial and ampullary regions were separated and subsequently processed for Western blotting analysis using a range of monoclonal or polyclonal antibodies directed against cx26, cx32 and cx43. The intensity of staining varied depending upon the hormonal status of the patients examined and appeared to be

upregulated during the secretory stage as opposed to the proliferative phase of the ovarian cycle. Human Fallopian tube did not express cx32, regardless of the anatomical site examined. In all cases though, the expression of both cx26 and 43 appeared to be more prevalent in the ampullary region.

The second part of this investigation focussed on transforming Fallopian tube epithelial cells into an immortal cell line. Normal cells cannot be sustained indefinitely, as cell degeneration occurs with continuous passaging. In most IVF treatments, sperm and ova are allowed to fertilise in a conventional medium but there is the potential that the Fallopian tube epithelial cells may be employed in IVF treatments in the future. Therefore, current techniques used to isolate and culture epithelial cells from the Fallopian tube were evaluated, in order to assess the best method that provides an optimal yield of Fallopian tube epithelial cells, both qualitatively and quantitatively.

The cells were isolated using (i) a mechanical technique, whereby the mucosal layer of the tube were minced finely using a pair of scissors and (ii) an enzymatic method which consisted of the enzymes, trypsin and pancreatin. The results showed that enzymatic isolation provided a large number of cells, but there was a significant detrimental effect on cell survival and their secretory status. In contrast, cells isolated by the mechanical method were fewer in number than those obtained by the enzymatic method but cell survival and secretory status were relatively better. Regardless, of the isolation technique employed, the Fallopian tube epithelial cells had a limited lifespan in culture. Bearing this in mind and coupled with the realisation that the interactions between the tubal epithelial cells and gametes/embryos cannot adequately be studied in vivo, the next part of the study investigated ways of establishing an immortalised human

Fallopian tube cell line. Such a cell line would be able to survive in culture indefinitely and therefore could be a potential model, which could mimic the in vivo milieu more closely. In order to obtain a 'bank' of Fallopian tube epithelial cells, cells isolated by either the mechanical or enzymatic method were cultured in growth medium for 24-48 hours prior to being transfected with a transforming gene, the SV40 Large T-antigen. Various techniques were used to transfect the epithelial cells in culture; these included well-established methods such as DNA co-precipitation as well as more advanced methods using cationic liposomes. The results from this study demonstrated that human Fallopian tube epithelial cells can be transiently transfected and could survive for over 102 days in culture, whilst still retaining many of the morphological characteristics of the original epithelial cell type.

By examining both the cellular and molecular aspects of the tube as described above, it was anticipated that further insight into the complex nature of this structure would be achieved. Furthermore, it was hoped to improve the current understanding and knowledge of the Fallopian tube's functions, thereby facilitating a greater awareness of the issues important in establishing and maintaining an in vitro model of the Fallopian tube.

LIST OF ABBREVIATIONS

AIJ	Ampullary-isthmic junction
Amp	Ampicillin
Amp^r	Ampicillin resistant
APH	Aminoglycoside phosphotransferase
ATP	Adenosine triphosphate
β-gal	Beta-galactosidase
BSA	Bovine serum albumin
CBF	Ciliary beat frequency
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
cx	Connexin
cx 26	Connexin 26
cx 32	Connexin 32
cx 37	Connexin 37
Cx 43	Connexin 43
cx 50	Connexin 50
DAB	3,3'-Diaminobenzidine
DEAE	Diethylaminoethyl
DMEM/ Nutrient F-12	Dulbecco's minimum essential medium/ nutrient F-12
DMRIE-C	1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethylammonium bromide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOPE	L-dioleoyl phosphatidylethanolamine
E	Oestrogen
E_2	Oestradiol
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidemal growth factor
EGP	Oestrogen-associated glycoprotein
ELISA	Enzyme-linked immunosorbent assay
E_R	Oestrogen receptor
ET	Embryo transfer
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Florescein Isothiocyanate
FSH	Follicle stimulating hormone
g	Relative centrifugal force
G418	Geneticin
GF	Growth factor
GFP	Green fluorescent protein
GIFT	Gamete intrafallopian transfer
GJIC	Gap junction intercellular communication
HEPES	N-(2-Hydroxyethyl) piperazine-N' –(2-ethanesulphoric acid)
HFTEC	Human fallopian tube epithelial cells

HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HTF	Human tubal fluid
IGF	Insulin growth factor
IVF	*In vitro* fertilization
kDa	Kilo Dalton
LB	Lennox L
LF2000	Lipofectamine 2000
LH	Luteinising hormone
MEM	Minimum essential media
ONPG	o-nitrophenyl-β-D-galactopyranoside
P	Progesterone
PAPP-A	Pregnancy associated plasma protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
P-D	Pancreatin-dispase
PEG	Polyethyleneglycerol
PP 5	Placental protein 5
PP 10	Placental protein 10
PP 14	Placental protein 14
P_R	Progesterone receptor
P-T	Pancreatin-trypsin
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPM	Revolutions per minute
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SFM	Serum free medium
SV40	Simian virus 40
SV40-LTAg	Simian virus 40 large Tumour-antigen
TBS	Tris buffer saline
TCA	Trichloroacetic acid
TE	Tris-EDTA
TEP-1	Tubal epithelial protein-1
TEP-2	Tubal epithelial protein-2
TGF-α	Transforming growth factor-alpha
TPS	Total protein secretion
Tris-HCL	Tris (hydroxymethyl)aminomethane hydrocholoric acid
TRITC	Tetramethylrhodamine isothiocyanate
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER 1

Literature Review

1.0 OVERVIEW

The Fallopian tube is the essential link between the ovary and the uterus and has many active roles in the process of reproduction. These include ovum pick-up, transportation and final maturation of both sets of gametes within the tube, fertilisation and maintenance of the pre-embryo, which is delivered to the uterine cavity at a time when conditions are optimal for nidation. Therefore, it can be said that the Fallopian tube is the natural habitat for fertilisation and very early embryogenesis. As such, the three key elements required for human life to continue are the ovum (egg), the spermatozoa and the provision of a suitable environment for their survival, union and subsequent development.

For the majority of couples, fertilisation occurs naturally. However, one in ten couples are affected by infertility or will experience some difficulty in conceiving during their reproductive lives (Hummel and Kettel, 1997). For a healthy couple of child-bearing age who are having unprotected intercourse, the probability of conceiving is one in four each cycle. The inability to conceive after one year of unprotected intercourse is defined as infertility (Brincat, 1994). Infertility may be attributed to one or more of the elements essential for fertilisation and subsequent human development i.e the spermatozoa, ovum and their milieu.

The review of the literature has been structured in a manner to reflect the nature of the work undertaken. It begins with a brief description of the importance of intercellular communication followed by some structural and functional aspects of the epithelium in relation to the reproductive process. In addition, connexins, the proteins that make up gap junctions which are involved in intercellular communication are highlighted and their role(s) in the reproductive system are examined. However prior to this, both the morphological and physiological aspects of the human Fallopian tube are discussed in detail, in order to understand the possible functional roles of connexins in the reproductive system. Furthermore, the tube and its relevance to the experiments undertaken in this project are emphasised, culminating with a brief conclusion from the literature review and the aims of the thesis.

1.1 INTERCELLULAR SIGNALLING

Intercellular signalling is a pre-requisite for co-ordinating biochemical functions in multicellular organisms and one pathway of communication includes secretion of signalling molecules, which are recognised by receptors at the target site and induce signal-specific reactions. One way such signalling is achieved is by gap junctions which are specializations of the plasma membrane consisting of intercellular channels that provide an aqueous path for the direct communication/interchange of ions and molecules <1kDa between the cytoplasm of adjacent cells.

In tissues, special junctions between neighbouring plasma membranes also exist and include tight junctions and desmosomes which are discussed in more detail later on. Currently, there is limited knowledge regarding the presence and roles of connexins in

the human Fallopian tube, though their expression has been reported in the endometrium (Jahn *et al.*, 1995).

1.2 EPITHELIA

Epithelia are derived from the three germ layers of the embryo. The ectoderm gives rise to the covering of the skin and body openings, the endoderm generates the lining and glands of the digestive and respiratory tract and the mesoderm gives rise to a variety of tissues, including the skeleton, muscle and kidney. Epithelia are tissues that serve as protective layers and/or secretory components of the body organs and systems.

The two established criteria used for classifying epithelia are the number of cell layers and the shape of cells which can be cuboidal, columnar or squamous. Most epithelia have a number of common characteristics that contribute to their functional capabilities. These include, a limited intercellular space, single or multiple layer of cells, free apical surfaces and avascularity.

The cells of an epithelium usually occur in sheets and are closely joined with little extra-cellular material between them. In many epithelia, the cells are riveted together by tight junctions, which are described in more detail in section 1.3.1. Indeed, this tight packing explains the function of the epithelium as a barrier, which protects against mechanical injury, invading micro-organisms as well as fluid loss. The free surface of the epithelium is exposed to the air or fluid, whereas the basal surface is attached to the basement membrane.

1.3 INTERCELLULAR JUNCTIONS

Epithelial cells are firmly linked to each other by intercellular junctions and are usually classified into three functional categories. These are a) occluding or impermeable junctions, b) adhering or anchoring junctions and c) communicating junctions.

1.3.1 Occluding junctions- Tight junctions

Human epithelia consist of highly organised layers of differentiated, polarised cells. Epithelial cells are linked by junctional complexes, providing a tight barrier between the lumen and the underlying tissues (Cozens *et al.*, 1992). Tight junctions generally 'seal' cells together in an epithelial cell sheet in such a way, as to inhibit small molecules from leaking from one side of the sheet to the other. Tight junctions maintain selective permeability barriers, separating fluids on each side that has different chemical compositions. They prevent the diffusion of membrane proteins and glycolipids between the apical and basolateral regions of the plasma membrane (Tsukita *et al.*, 1996).

1.3.2 Anchoring junctions- Desmosomes

These junctions function as robust structural units by connecting the cytoskeletal elements of a cell either to those of another cell or to the extracellular matrix. They occur in two structurally and functionally different forms namely; (1) adherens junctions, which are connection sites for actin filaments and (2) desmosomes, which are connection sites for intermediate filaments. Three types of desmosomes exist: (a) belt desmosomes, regions of enhanced adhesion just beneath the tight junctions; (b) spot desmosomes, regions of adhesion between neighbouring cells and (c) hemidesmosomes, which anchor the cell to the basal lamina.

1.3.3 Communicating junction- Gap junctions

Intercellular communication is vital for the function of the organism and it has profound importance for cell survival, differentiation, metabolism and morphogenesis (Sosinsky, 1996). Gap junctions occur randomly along the lateral surface of the cells and are characterised by the close apposition of adjacent cell membranes. Gap junctions are water-filled pores, composed of sub-unit proteins called connexins (Goodenough, 1975). Various types of connexins have been detected within an organism and there are differences in the expression of various connexin genes in different tissues.

1.4 CONNEXINS

The description of gap junctions and the discovery that adjacent cells connected by these structures directly exchanged cytoplasmic ions and molecules have led to a number of studies in this area. Before gap junction channels can function properly, their subunit protein, connexins, have to be synthesized and inserted into the cell membrane in their precise functional configuration and assembled into the intact channel structure (Falk, 2000). This ensures precise coordination and regulation. A number of studies have shown that there are variations in the composition, structure and regulation of gap junctions, suggesting that they are likely to perform various roles in different tissues (Kumar and Gilula, 1996). For example, recent studies have suggested that, any modifications of a specific connexin may be detrimental in the development and functioning of several tissues, and may eventually result in human diseases (Nicholson and Bruzzone, 1997).

1.4.1 Gap junctions- Structural considerations

Gap junctions are tightly packed aggregations of a few to over 10^5 intercellular channels that directly connect the cytoplasms of adjacent cells (Simon and Goodenough, 1998). Each cell of an adjacent pair contributes one hemichannel to form a gap junction channel (Figure 1.0a) and each hemichannel is composed of six connexins, a 'connexon' (Figure 1.0b) that spans the lipid bilayer. Therefore, one gap junction channel contains twelve sub-unit connexins

(Figure 1.0c) (Sosinsky, 1996). It is this end to end interaction of two connexons from adjacent cells that generates the intercellular aqueous channel which allows the exchange of nutrients, metabolites, ions and small molecules (Loewenstein, 1981). The molecular weight of these connexins range between 26-70 kDa and some eighteen connexin sub-units have been identified to date (Bennett *et al.*, 1991).

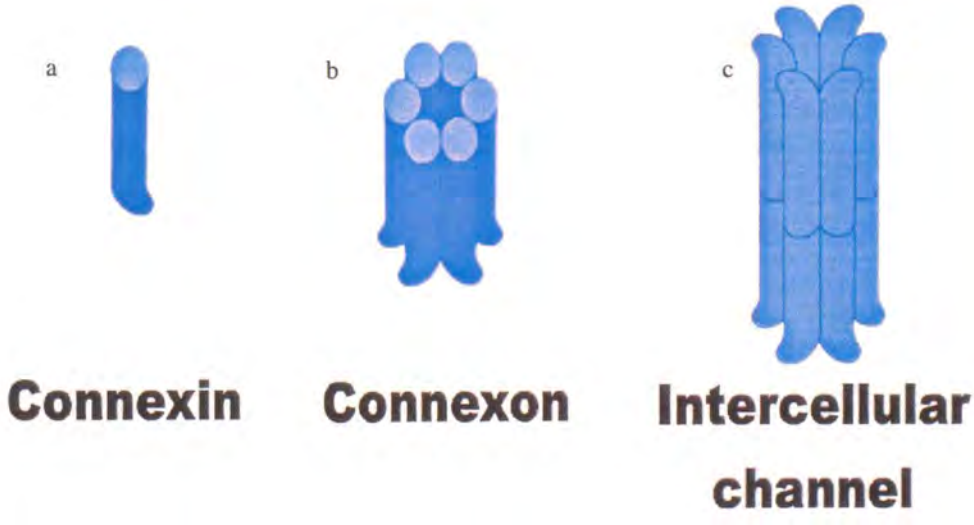


Figure 1.0: *Schematic drawing of (a) connexin, (b) connexon and (c) intercellular channel. Taken and modified from Simon and Goodenough, (1998).*

1.4.2 Synthesis of connexins and the formation of gap junctions

Most plasma membrane proteins, including connexins are synthesised at the endoplasmic reticulum (ER). They are co-translationally integrated into the ER membrane and transported by successive vesicle budding and fusion events from the ER, through the Golgi apparatus to the plasma membrane (Yeager *et al.*, 1998). It is during the process of integration into the ER membrane, that connexins adopt their final membrane topology (Falk and Gilula, 1998). A diagrammatic representation of the synthesis, assembly and degradation of gap junction membrane channels is shown in Figure 1.1.

1.4.3 Nomenclature

Gap junctions are composed of proteins from the connexin gene family, denoted by cx followed by the molecular mass in kilodaltons). These include, cx26, cx31, cx32, cx40, cx43, cx45.6, cx46, cx50, cx56 (Saez *et al.*, 1998) with cx43 being the most widely expressed connexin in tissues and cell lines.

1.4.4 Functional perspectives

The paramount function of connexin-mediated intercellular communication is to provide a pathway for the diffusional exchange of ions and small hydrophilic molecules from one cell to the next (Kumar and Gilula, 1996; Nicholson and Bruzzone, 1997). Overall, information on the specific cell biological details of how gap junctions serve to coordinate tissue functions is limited. This is partly due to the unavailability of highly specific inhibitors of gap junctional intercellular channels, and the relative non-specificity of the intercellular channels to the passage of a wide variety of small molecules (Simon and Goodenough, 1998). Although the intercellular passage of high-

energy metabolites has been documented (Goldberg *et al.*, 1998), the tissue-specific role(s) of this transfer cannot be elucidated from *in vitro* culture systems. Instead, the only way to acquire an understanding on the functional aspects of the possible role(s) carried out by the various connexins is by targeted disruptions of the genes that code for gap junction structural proteins (gene knockout experiments) and human mutations (Simon and Goodenough, 1998).

Characterisation of the diverse functions of gap junctions using 'gene knockout' is currently ongoing and a number of human diseases associated with gap junction abnormalities have been documented in the literature. For example, the first 'disease-associated' connexin was connexin 32 (cx 32), in the X-linked form of Charcot-Marie-Tooth disease, a neuropathy often associated with hearing loss (Bergoffen *et al.*,1993). Others, such as mutations in connexin 37 (cx37), have been shown to be implicated in reproductive dysfunction in mice, thus leading to infertility (Simon *et al.*,1997) whilst the lack of another gap junction protein, cx43 resulted in the failure of spermatogenesis in mice (Roscoe *et al.*, 2001). Such experiments cannot be carried out in humans and it is clear that gene knockout (KO) experiments in animals could potentially provide an insight into possible roles of connexins in the reproductive physiology or pathophysiology.

Connexin synthesis at the endoplasmic reticulum

↓

Oligomerization into gap junction connexons (hemichannels)

↓

Trafficing along the secretory pathways

↓

Intracellular storage within the Golgi apparatus

↓

Insertion of the gap junction connexons into the plasma membrane

↓

Formation of the gap junction plaque comprising many individual gap junction channels

↓

Internalisation of the plaque via formation of annular gap junctions

↓

Degradation of gap junction channels via lysosomal and/or proteasomal pathways

Figure 1.1: *A flow diagrammatic representation of the synthesis, assembly and degradation of gap junction membrane channels.*

(Taken from Yeager et al., (1998).

1.4.5 Expression of connexins in the endometrium during the menstrual cycle and its implications in infertility.

To date, the expression of connexins in the Fallopian tube throughout the menstrual cycle has not been studied, though it has been for the endometrium. Previously, it has been shown that the spatial and temporal patterns of gap junction connexins, in the endometrium appear to play a role in uterine receptivity as well as implantation in rodents and rabbits (Winterhager *et al.*, 1991, 1993). Since successful implantation in mammals requires a close interaction between the embryo and the uterus, direct cell to cell communication via gap junctions appears to be involved in the preparation of the uterus for embryo implantation and in the regulation of trophoblast invasion (Grummer *et al.*, 1996).

It is now well established that levels of steroid hormones can drastically and reproducibly modify tubal transport rates in the mammalian oviduct (Croxatto *et al.*, 1991). Similarly, the expression of gap junction connexins in the female reproductive tract of rodents and in the human endometrium is also regulated by the level of steroid hormones. During implantation in rodents, rabbits and humans the gap junctional proteins connexin cx26 and cx43 are suppressed. This loss of cell to cell communication seems to be important for the transition of the endometrium into the receptive phase when there is an increase in maternal progesterone (Jahn *et al.*, 1995; Grummer *et al.*, 1996). However, as implantation begins a spatial and temporal pattern of connexin expression is induced in response to embryo recognition. cx26 is locally expressed in the uterine epithelium of the implantation chamber whilst cx43 is found in the surrounding decidua prior to invasion. With progressing invasion, the decidual cells surrounding the invading trophoblast reveal cx26, in addition to cx43 that is already present. Previous studies of the endometrium revealed that both connexin proteins

(cx26 and cx43) react very sensitively to progesterone and oestrogen treatment (Jahn *et al.*, 1995; Grummer *et al.*, 1999). The initial suppression of connexin expression is a characteristic cell biological indicator of the endometrium's receptivity in different species and this, coupled with the limited induction of direct cell to cell communication (perhaps due to blastocyst signals) seems to be a pre-condition for embryo implantation (Jahn *et al.*, 1995).

A study carried out by Hermoso *et al.*, (1997), demonstrated that the content of both connexins (cx26 and cx43) in the oviduct depended on the cell type, state of maturation and hormonal status. They observed that the epithelial and smooth muscle cells of immature rat oviducts (<30 days) contained a low amount of connexin 43 and connexin 26 was undetectable. However, in mature oviducts (> 30days), connexin 26 was detected only in the isthmus and localised regions of the ampulla epithelial layer. The high content of connexins found between oviductal cells as well as their high responsiveness to hormone regulation, suggest that gap junctions might be involved in coordinating oviductal cell functions such as smooth muscle contraction and epithelial ciliary beat.

A number of cell biological markers have been defined as indicators of receptivity in various mammalian species in recent years. These include, rabbit secretory proteins, uteroglobin and β glycoprotein, which are controlled by progesterone (Beier *et al.*, 1991; Hegele-Hartung *et al.*, 1992). The presence of such markers, indicate that implantation may occur. Therefore, it is possible that delayed expression of a particular connexin(s) may be associated with infertility.

A study carried out by Simon *et al.*, (1997) demonstrated that female mice lacking connexin 37 resulted in infertility. It is known that at birth, the ovary contains primordial follicles consisting of meiotically arrested oocytes surrounded by a single layer of granulosa cells. However, following ovulation, oocytes resume meiosis whilst the granulosa cells which are retained in the follicle eventually form the corpus luteum. Simon *et al.*, (1997), also demonstrated that connexin 37 is present in gap junctions (which may influence aspects of follicular development) between oocyte and granulosa cells. Connexin 37 deficient mice however not only lacked mature follicles but also failed to ovulate and developed numerous inappropriate corpora lutea. It would appear that cell to cell signalling through the gap junctions critically regulated the highly co-ordinated sequence of cellular interactions necessary for successful oogenesis and ovulation. Currently, there is limited information regarding the presence of connexins in the Fallopian tube and hence their possible roles in reproduction remain unclear.

In order to appreciate the nature of the work undertaken in this study it is essential to have an overview of the structural and functional aspects of the Fallopian tubes and it's role in the reproductive process.

1.5 THE FALLOPIAN TUBE

The first anatomical description of the mammalian oviduct was published by Gabriele Fallopius in 1561, (Beck and Boots, 1974). The oviduct was originally described as the "tuba uteri" or 'trumpet of the uterus', after its resemblance to a brass musical instrument.

The Fallopian tube holds a fundamental position, and has many active roles in the process of reproduction. The different regions (fimbria, ampulla and isthmus) are specialised to perform different functions. Its transport mechanisms are now reasonably understood, and include ovum pick-up and transfer, and transportation of both sets of gametes to the site of fertilisation within well-defined time limits. It also provides an essential environment for final gamete maturation, fertilisation and embryonic development prior to implantation. In addition, both the composition and volume of the tubal fluid contribute to the environment in which these processes take place and also correlates with the hormonal status of the mother (Mastroianni and Go, 1979).

1.5.1 Morphology

1.5.1.1 Anatomical considerations

The Fallopian tube ranges between 6-15cm in length (Woodruff and Pauerstein, 1969; Pauerstein and Eddy, 1979) and is generally divided into four anatomical regions depending on the position, thickness of the smooth muscle, complexity of the mucosal folds and cellular composition of the mucosa. From the uterine end of the Fallopian tube the anatomical regions are termed the interstitial or intramural segment, the isthmus, the ampulla and the infundibulum, which is fringed by epithelial covered fimbriae. Each region of the tube has certain histological features that contribute to

their particular physiological and biochemical functions (Buhi *et al.*, 1997). The epithelial lining and mucosa of the Fallopian tube is convoluted into longitudinal folds, also known as "rugae" or "plicae" around the lumen. The size and complexity of these folds increases progressively along the lumen towards the fimbriated end where many secondary and tertiary folds are superimposed on the primary folds. The *muscle layer* or myosalpinx consists of both longitudinal and circular muscle fibres, which are continuous with those of the uterus. However, their thickness is dependent on the regional anatomy of the tube.

The isthmus is approximately 2-3 cm in length and consists of an inner longitudinal, middle circular and an outer longitudinal muscle layer (Woodruff & Pauerstein., 1969; Pauerstein *et al.*, 1970). The circular muscle layer is continuous with the outer longitundinal layer and is separated by a collagenous layer, which contains the blood vessels of the tubal wall. Figure 1.2a shows a cross section of the isthmic region.

The ampulla, is the longest portion of the Fallopian tube measuring between 5-8 cm in length. It usually follows a straight course, without abrupt angulations and convolutions, though, in some women it can be quite curved. The myosalpinx is arranged into an outer longitundinal layer and an inner circular layer, the former of which appears as scattered muscle bundles interspersed within the lamina propria of the complex epithelial folds (Figure 1.2b shows a transverse section of the epithelial folds of the ampullary region). The most obvious anatomical difference between the ampulla and the isthmus is the greater degree of invagination of the epithelium of the former and the greatly increased smooth muscle component of the latter. The zone between the

ampulla and the isthmus is known as the ampullary-isthmic junction (AIJ), whilst that between the isthmus and the uterus is referred to as the utero-tubal junction.

The infundibulum, is the trumpet shaped distal portion of the Fallopian tube. It consists of a thin muscle layer consisting of an outer longitundinal and inner circular layer. The internal surface of the infundibulum is also occupied by complex mucosal folds, which culminate to form numerous slender processes called fimbria (Figure 1.2c). In particular, the *fimbria ovarica*, is longer and is probably essential for ovum pick-up, during which the densely ciliated fimbria are brought into close contact to the tubal pole of the ovary thus capturing the ovum at ovulation, prior to its descent into the tubal ostium (Pauerstein and Eddy, 1979).

1.5.2 The layers of the Fallopian tube

The Fallopian tube consists of three layers: serosa, muscle layer and mucosa. The external layer or *serosa* is peritoneal and surrounds the Fallopian tube by a double fold of peritoneum which fuses to form the mesosalpinx, the primary supporting mesentry of the tube. Additionally, varying amounts of connective tissue coupled with nerves, vessels and lymphatics are dispersed between the mesosalphinx.

The internal layer of the tube consists of an intricately folded *mucosa* that surrounds the lumen. The mucosa is comprised of the epithelium and an underlying stroma, which, consists of mesenchymal cells embedded in an extracellular matrix (ECM). These cells form the stroma and are separated from the epithelial cells by a basement membrane (Lin and Bissell, 1993; Roskelley *et al.*, 1995).

1.5.3 Histology

1.5.3.1 The epithelial lining

The oviduct is lined by a simple columnar epithelium, which contains both ciliated and secretory cells. The normal Fallopian tube epithelium is composed of four cell types, which varies according to the location and hormonal status of the tube. These cells are (1) ciliated cells, (2) non-ciliated or 'secretory' cells, (3) intercalary or 'peg' cells and (4) un-differentiated or 'basal' cells (Pauerstein and Eddy, 1979).

Along the length of the human Fallopian tube there is a progressive decrease in the proportion of the ciliated cells (Figure 1.2d) from the fimbriated end to the isthmus (Critoph and Dennis, 1977a; Crow *et al.*, 1994). The ciliated cells constitute in excess of 50% of the entire cell population in the fimbriated end, whereas approximately 50% of epithelial cells are ciliated in the ampullary region and 27-35% in the isthmus (Crow *et al.*, 1994). In contrast, some investigators have observed a fairly uniformed distribution along the length of the tube (Patek *et al.*, 1972a, b; Ferenczy *et al.*, 1972).

1.5.3.2 Ciliated cells

The ciliated cells are cuboid in shape and contain a finely granular cytoplasm. The nucleus can be round or oval and is usually centrally located. These cells are further characterised by their vesiculated endoplasmic reticulum, long mitochondria and cytoplasmic droplets. These cells undergo little morphological and ultrastructural changes during the ovarian cycle and are commonly located at the apices of the epithelial folds. The cilia are attached to a refractile row of basal granules just beneath the cell membrane. They are approximately 5-7μm long and consist of two central filaments surrounded by nine double peripheral filaments (Salamonsen and Nancarrow.,

1994). Figure 1.3 shows a cross section of cilated cells with it's characteristics 9 + 2 arrangement.

1.5.3.3 Secretory cells

Tubal epithelial cells without cilia are sometimes referred to as secretory or 'non-ciliated' cells which, are also distributed along the length of the Fallopian tube. They have a more coarsely granular cytoplasm and oval nucleus, which is orientated along the long axis of the cell. The organelles present are typical of cells actively synthesising protein, with a well-developed endoplasmic reticulum, Golgi bodies and various secretory granules.

Secretory cells are most common in the ampullary region where they constitute approximately 50% of epithelial cells (Crow *et al.*, 1994), though the percentage present within the fimbria and isthmus have not been documented. Their shape and appearance also differ during different phases of the cycle as can be seen in the late proliferate phase where they form protruding 'dome-like' structures.

1.5.3.4 Intercalary peg' cells

The intercalary or 'peg' cells are usually present during the luteal phase of the ovarian cycle (Fredericks, 1986). These cells are long and slender with sparse cytoplasm, compressed between adjacent cells. They may represent exhausted secretory cells (Odor, 1974) that have discharged their secretions and henceforth gradually cast out into the lumen of the tube. Other authors believe that these are T-lymphocytes, predominantly of the cytotoxic/suppressor type (Morris *et al.*, 1986; Peters, 1986) that act as replacement cells evolving eventually into ciliated and secretory types.

1.5.3.5 Un-differentiated 'basal' cells

These undifferentiated 'basal' cells to be located in close proximity to the basement membrane. They are small and oval with a hyperchromatic nuclei and prominent nucleoli, and also contain a scant halo of cytoplasm (Odor, 1974). The author also concluded that such cells were not fibroblasts, macrophages, plasma cells or monocytes of connective tissue but lymphocytes. Nonetheless, the epithelial origin of these cells remains unclear.

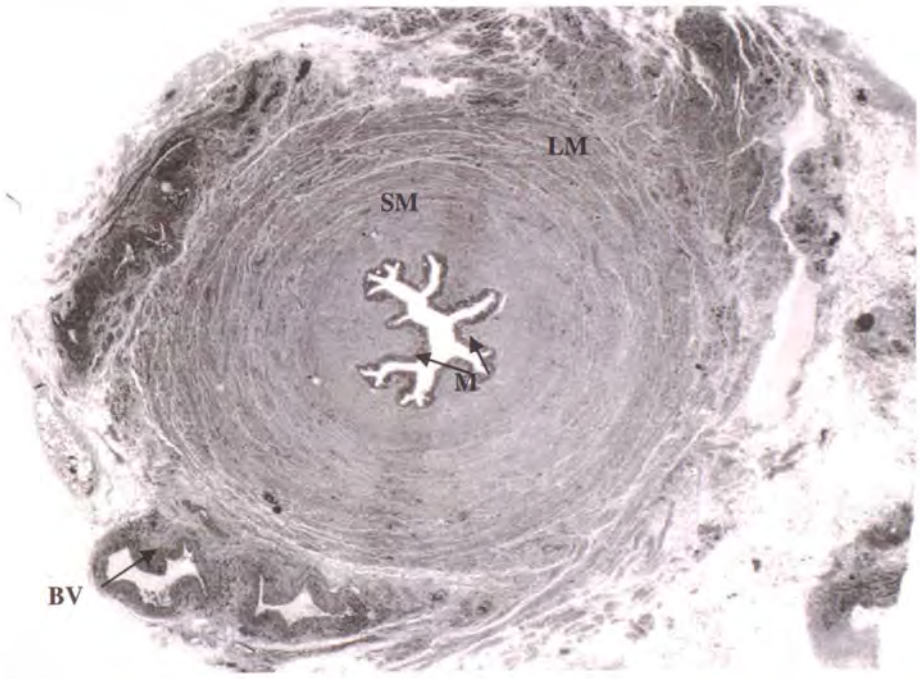


Figure 1.2a: *Light micrograph showing a cross section of the isthmic region of the fallopian tube showing (M) mucosal folds, (SM) smooth muscle layer, (LM) longitudinal muscle layer, (BV) blood vessel and (LV) lymph vessel. Original Mag (x100)*
Taken from Sattar (1995).

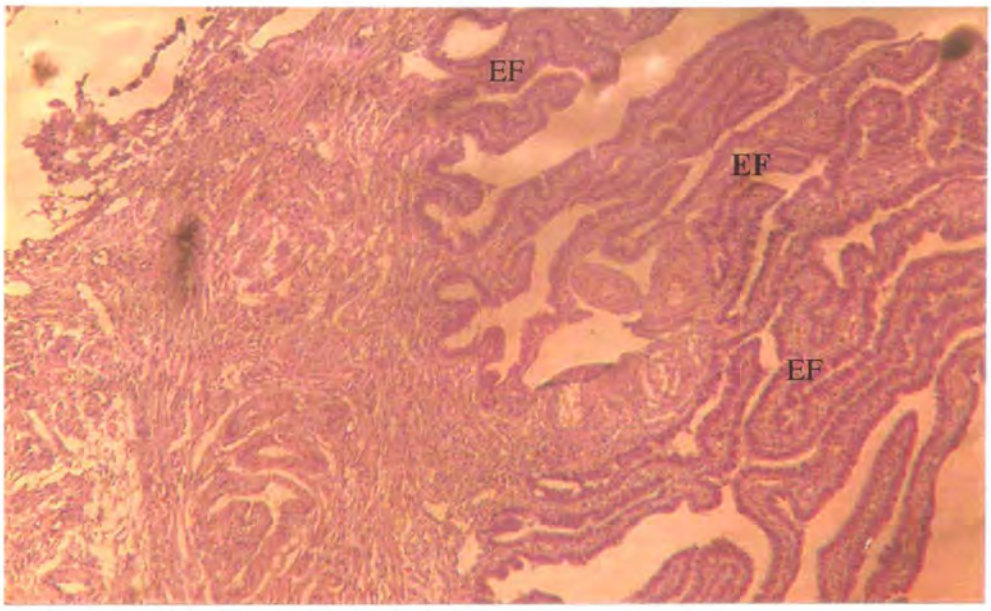


Figure 1.2b: *Light micrograph showing a cross section of epithelial folds (EF) from the ampullary region of the Fallopian tube. Original Mag (x400)*
Taken from Sattar (1995).

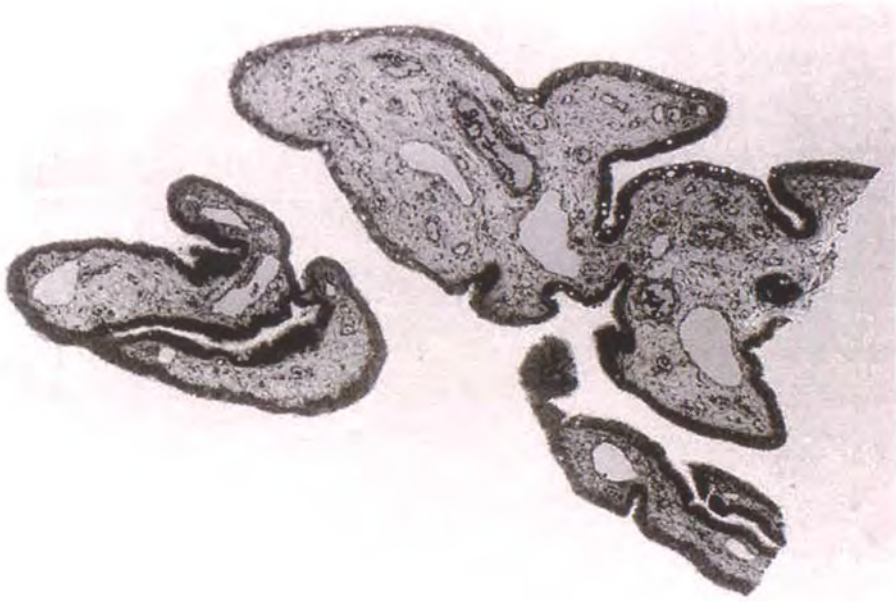


Figure 1.2c: *Light micrograph showing a cross section of the fimbrial region of the Fallopian tube. Original Mag (x100)*
Taken from Sattar (1995).

Figure 1.2d: *Light micrograph of mucosal fold showing ciliated epithelial cells (C) and blood vessels (BV). Original Mag (x400)*
Taken from Sattar (1995).

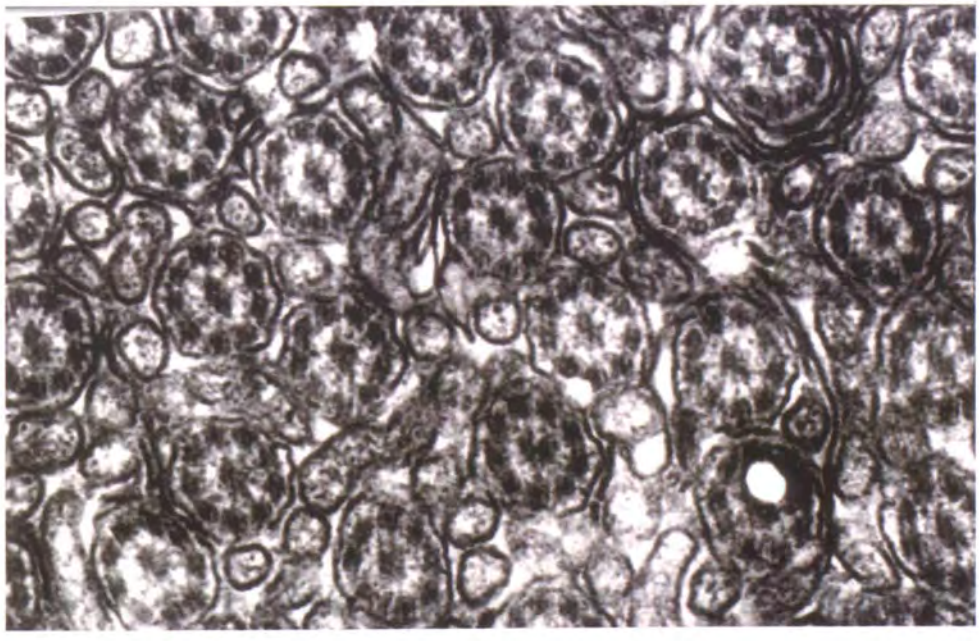


Figure 1.3: *Transmission electron micrograph showing a cross section of cilia with the characteristic 9 + 2 arrangement.*
Taken from Sattar (1995).

1.6 FUNCTIONAL ASPECTS OF THE FALLOPIAN TUBE

1.6.1 Oestrogen and Progesterone Receptors in the Fallopian tube

The Fallopian tube is considered to be under the influence of the ovarian steroids, oestrogen and progesterone. A study carried out by Batra *et al.*, (1980) demonstrated that the Fallopian tube is able to concentrate both oestradiol and progesterone at much higher levels than those found in the circulating plasma which suggests the presence of a number of highly specific tissue-binding proteins or receptors. Furthermore, the authors also reported that the highest concentration of both oestradiol and progesterone binding receptor sites was present in the ampullary region, a reduced number in the isthmic region and the least number of receptors were located in the infundibulum. Other investigations demonstrated that in the early follicular phase, there is a high concentration of oestradiol receptors in the cytoplasm, however, late in the follicular phase when the plasma oestradiol rises prior to ovulation there is an increase in the proportion of oestradiol receptors in the nucleus (Punnonen and Lukola., 1981; Pollow *et al.*, 1982).

1.6.2 The effect of ovarian steroids on tubal morphology

The cells of the Fallopian tube contain both cytosolic and nuclear receptors to oestrogen (E) and progesterone (P). These hormones accumulate up to 25 times that of plasma levels (Jansen, 1984) resulting in morphological changes of the Fallopian tube, which varies during the ovarian cycle. Verhage *et al.*, (1979) have demonstrated that the epithelial thickness is low during menstruation but increases during the follicular phase and thereafter, reaches its maximum height (30μm) during the late follicular phase. Scanning electron microscopic (SEM) studies of the mucosal surface of the Fallopian tube demonstrated the presence of both ciliated and non-ciliated cells and their

distribution at different stages of the ovarian cycle. There was no notable difference in terms of number, or distribution along the different anatomical regions of the tube (Ferenczy *et al.*, 1972). It is clear that the findings observed by Verhage *et al.*, (1979) and (Ferenczy *et al.*, 1972) are contradictory. Furthermore, Patek *et al.*, (1972b) reported that the ciliated cells present on the mucosal layer diminish in height during the menstrual cycle. During the luteal phase, the epithelium gradually loses its height to a minimum (10-15μm) during menstruation (Fredericks, 1986) but during the proliferative phase however, the height increases and the ciliated cells become covered by regularly distributed microvilli (Figure 1.4).

Support for the study carried out by Ferenczy *et al.*, in 1972, however, came from the investigators Jansen and Bajpai in 1982. They stated that maximum height is reached in the peri-ovulatory period, where both the ciliated and non-ciliated cells are more or less of equal height with 'domes' present on the non-ciliated cells between the tufts of cilia. As such, there was no absolute distinction between the two cells types and occasionally differentiating cells have been found to exhibit both cilia and secretory granules (Jansen and Bajpai, 1982).

Following ovulation, the cilia (Figure 1.4) become more prominent with the cells themselves becoming broader and shorter. The secretory cells also shorten but they are left with pedunculated apices during the luteal phase (Jansen, 1984). Verhage *et al.*, (1979), demonstrated cyclic changes both in the ampullary and fimbrial epithelial cells of the Fallopian tube. For example, during the luteal phase they described deciliation of 10-12 %, though the cells tended to regenerate in the early follicular phase of the next

cycle, where they became erect and vigorous. Critoph and Dennis, (1977b) observed that the rate of ciliary beat appeared to be the greatest at, or just after ovulation, which suggests that ciliary activity is related to circulating ovarian hormones.

The non-ciliated 'secretory' cells have been found to show the most noticeable cyclic changes. Prominent microvilli appear on the apices of the secretory cells during the early stage of the cycle though the cilia remain discrete. During the follicular phase, these secretory cells increase in height and are covered with tall, well-shaped, and regularly distributed microvilli. A number of cytoplasmic apical projections can be seen on the luminal surface. These are conspicuous both during the follicular and pre-ovulatory phase. These apical projections can sometimes become detached from the cell resulting in apocrine secretion. Here, secretory granules with large cell fragments, which, at times can include the nucleus and a number of whole cells tend to cover the cell surface thereby obscuring the cilia (Jansen, 1984).

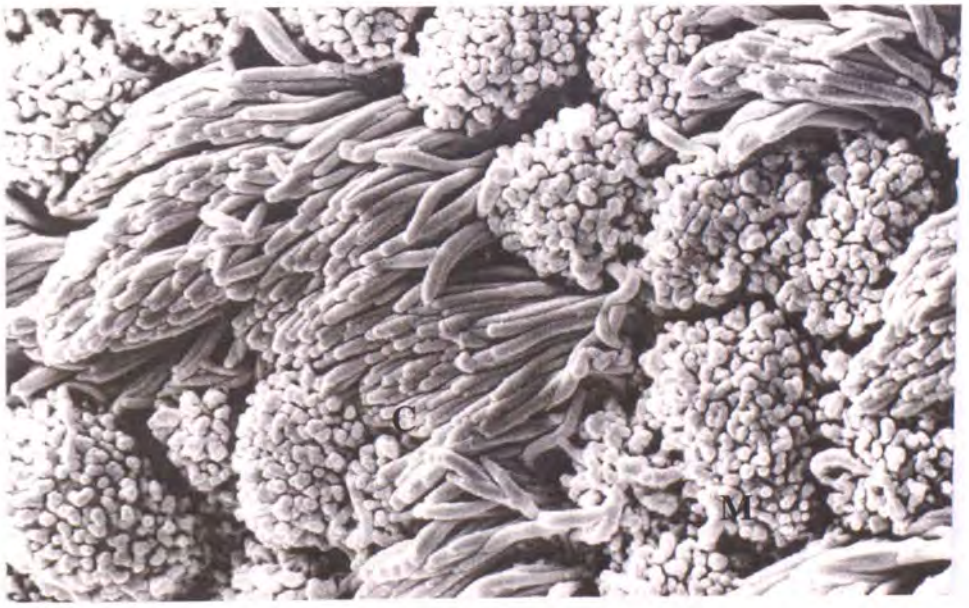


Figure 1.4: *Scanning electron micrograph showing (C) cilia and (M) microvilli on the mucosal fold.*
Taken from Sattar (1995).

Although, secretory activity was reported to be more pronounced in the isthmus throughout the ovulatory period (Jansen, 1980), it has also been observed in the ampulla (Pauerstein and Eddy, 1979; Ludwig and Metzger, 1976). Such evidence has been provided by ultrastructural studies carried out by these investigators. For example, at the onset of the proliferative phase, the Golgi bodies are compact with a reduced endoplasmic reticulum. These structures become more prominent during the follicular phase and around day 10 of the ovarian cycle, granules of ribonucleoprotein increase in number. However, at the end of the follicular phase these secretory granules appear beneath the cell membrane in close proximity to the lumen. In the luteal phase, the endoplasmic reticulum dilates, secretory granules appear, the Golgi apparatus expands and an increased number of liposomes appear (Ludwig and Metzger; 1976; Pauerstein and Eddy, 1979).

1.6.3 The effect of ovarian steroids on the epithelial ciliary beat frequency (CBF) in the human Fallopian tube

It is well-established that both the cilia and muscles of the Fallopian tube play a vital role in gamete transport. In addition, both progesterone and oestrogen are known to affect the mucosa of the Fallopian tube by causing deciliation and ciliation (Verhage *et al.*, 1979; Goldberg and Friedman., 1995). A study carried out by Mahmood *et al.*, (1998) investigated the effect of ovarian steroids on human Fallopian tube epithelial ciliary beat frequency *in vitro*. The authors demonstrated that cilia, from the ampullary segments of the tube beat significantly faster than those from the fimbrial segments. Furthermore, incubation with progesterone at a minimum concentration of 10 μmol/L or higher, suppressed human Fallopian tube epithelial ciliary beat frequency in the fimbrial, ampullary and isthmic regions. However, no effect was observed when

oestradiol at similar concentrations was used though it did prevent the reduction of CBF caused by progesterone.

1.6.4 Angiotensin receptors in the Fallopian tube and its effect on ciliary beat frequency.

The presence of angiotensin II (AT_2) receptors in the reproductive tissues suggest that they may play a role in the reproductive process (Lin and Goodfriend., 1970; Rouzaire-Dubois *et al.,* 1975; Hussain *et al.*, 1987). Both angiotensin-type I (AT_1) and angiotensin-type II AT_2 were demonstrated in the human Fallopian tube epithelial cells of the mucosa, as well as in the fimbrial, ampullary and isthmic regions (Saridogan *et al.,* 1996). The authors also demonstrated AT_2 had a stimulatory effect on tubal CBF though, this effect was inhibited by the specific AT_1 antagonist, Losartan.

In contrast, the AT_2 antagonist, CGP42112B had no inhibitory effect on CBF, an observation, which suggests that the effect of AT_2 on CBF is mediated through the receptors of AT_1. In a more recent study, AT_2 has been shown to be the primary receptor subtype in the Fallopian tube (Johnson *et al.*, 1998). Their preliminary observations suggest that there is an increase in angiotensin II-type 1 receptor binding in the proliferative phase of the cycle which subsequently decreases in the secretory phase. This is an indication that angiotensin II may act as an autocrine regulator that stimulates the cells themselves and/or paracrine regulator, which may exert an effect on neighbouring cells *in vivo*.

1.6.5 The importance of cell to cell contact in Fallopian epithelial cell co-cultures.

The interaction between sperm and the uterine tube has been of great interest in recent years. Both *in vivo* and *in vitro* studies have shown that this interaction improved sperm motility characteristics, induced capacitation and increased the fertilizing ability of these cells (Barratt and Cooke, 1991; Kervancioglu *et al.*, 1994b; Smith, 1998). The mechanisms involved in sperm-uterine tube interaction are poorly understood. However, it has been suggested that the components of the oviductal secretory fluids may induce sperm capacitation (Srivastava *et al.*, 1996). Furthermore, both *in vivo* and *in vitro* studies have shown that sperm cells become attached to the epithelial cells and hence it may be possible that such cell-to-cell contact may have a beneficial effect on sperm viability (Smith, 1998).

A recent study by (Kervancioglu *et al.*, 2000) investigated the mechanisms involved in the stimulatory effect of co-cultures of Fallopian tube epithelial cells on sperm movement characteristics. Here, a homologous co-culture model was used to investigate the functional significance of sperm-oviduct epithelial interaction by using a microporous membrane between the cell monolayers and spermatozoa. This prevented direct cell-to-cell contact with the epithelial cells and sperm plasma membrane but allowed the secretory products from the epithelial cells to the medium. The Fallopian tube epithelial cell co-culture increased sperm motility compared with the controls. However, this stimulatory effect was inhibited when the microporous membrane prevented the cell-to cell contact between the sperm and Fallopian tube epithelial cells. This finding suggests therefore, that the stimulatory effect of the cell monolayers was due to the direct interaction between the spermatozoa and the epithelial cells of the Fallopian tube. Indeed, such results are consistent with studies carried out in rabbit

(Smith and Nothnick, 1997) and human (Murray and Smith, 1997) oviductal apical plasma membrane fractions incubated with homologous spermatozoa.

The Fallopian tube synthesizes and secrete specific proteins which may vary according to anatomical site, stage of the ovarian cycle and even during pregnancy. Therefore, it is likely that tubal epithelial cells *in vitro* also secrete beneficial substances required for fertilisation and the subsequent development of preimplantation embryos. Since it is neither ethical nor feasible to conduct experiments of the Fallopian tube *in vivo*, it is important to establish a system whereby tubal epithelial cells can be cultured or sustained in an *in vitro* environment with minimum disturbance to both the cell's structural and functional characteristics.

1.7 CULTURE OF THE FALLOPIAN TUBE EPITHELIUM

The tube provides physiological support to both gametes and embryos as described in section 1.8. The results observed from co-culture experiments and from isolated tubal culture systems strongly suggest that the interactions between the tubal epithelial cells and gametes or embryos may play a vital role in the maturation of gametes, fertilisation and embryonic development. However, only a few investigators (Henriksen *et al.*, 1990; Thibodeaux *et al.*, 1991; Dickens *et al.*, 1993, 1996) have attempted to fully characterise these cells *in vitro*.

1.7.1 The isolation and culture of tubal epithelial cells

Tubal epithelial cells from a number of species have been successfully isolated and cultured using different isolation procedures. These include, mechanical isolation by mincing the mucosal layer (Kervancioglu *et al.*, 1994; Laird *et al.*, 1995) or by scraping (Bongso *et al.*, 1989; 1992; Piekos *et al.*, 1995; Walker *et al.*, 1997) thereby forming cell aggregates. Enzymes such as collagenase (Takeuchi *et al.*, 1991), trypsin (Thibodeaux *et al.*, 1991) or a mixture of trypsin and pancreatin (Dickens *et al.*, 1993; 1996) have also been used to isolate oviductal epithelial cells. These cells have been grown both on plastic surfaces (Takeuchi *et al.*, 1991; Kervancioglu *et al*; 1994; Mahmood *et al.*, 1998) and on basement membrane constituents such as matrigel (Joshi *et al.*, 1991; Kervancioglu *et al.*, 1994) or collagen (Dickens *et al.*, 1993; 1996).

The composition of the media is important for regulating cell proliferation and differentiation thereby maintaining a tissue-like architecture *in vitro*. A wide variety of tissue culture media supplemented with serum have been shown to support oviduct epithelial cell growth. These include Chang (Bongso *et al.*, 1989), RPMI 1640 (Henriksen *et al.*, 1990), minimum essential medium in Earle's salt with glutamine (Kervancioglu *et al.*, 1994), and a mixture of Ham's F12 and DMEM (Laird *et al.*, 1995). Takeuchi *et al.*, (1991; 1992), and Ouhibi *et al.*, (1989) supplemented their media with additional proteins such as transferrin and insulin or hormones such as progesterone and oestradiol. However, these supplements had no influence on the growth of oviductal epithelial cells *in vitro*. In contrast, a study carried out by Takeuchi *et al.*, (1991, 1992), demonstrated an increase in proliferation of human tubal cells in the presence of Epidermal Growth Factor (EGF).

1.7.2 Morphology of oviductal epithelial cells in vitro

Cultured epithelial cells are usually polygonal in shape with a some-what basal nuclei. Studies carried out by Henriksen *et al.*, (1990) and Dickens *et al.*, (1993) showed that the epithelial cells formed a monolayer with microvilli on their surface and desmosomes between adjacent cells. Polarity is an essential feature of epithelial cells in which the apical membrane is morphologically, functionally and biochemically distinct from the basal and basolateral membranes. Apical and basolateral components are prevented from intermixing by the formation of a barrier near the apical surface which consists of tight junctions between the lateral plasma membranes of adjacent cells (Gumbiner, 1987). The basolateral membranes contain protein pumps for acquiring nutrients diffused from the underlying blood vessels and receptors for hormones and neurotransmittors, which influence cell behaviour. A study carried out by Kervancioglu *et al.*, (1994) demonstrated that epithelial cells grown on Matrigel inserts retained polarity, as microvilli were observed to be present on the apical surface. The culture of cells on these extra-cellular matrices (eg Matrigel*see appendix 1) or collagen inserts encourages the formation of tight junctions between the cells that isolate the apical and basolateral compartments. The cells can then secrete proteins (Salamonsen *et al.*, 1992) and prostagladins (Schatz *et al.*, 1991). Indeed, both polarity and secretory characteristics can be attributed to the growth of cells on the extra-cellular matrix (ECM) and the porous membrane, which allows the exposure of the basal cell surfaces to the culture medium.

1.7.3 Characterisation of oviductal epithelial cells in vitro

A major cytoskeletal component of epithelial tissue is the intermediate filament called keratin, which differentiates epithelial cells from other cell types. The presence of these

cytokeratins represents a crude way to determine the epithelial origin of cultured oviductal cells. A number of studies have shown that at least 95% of confluent primary cultures of oviductal epithelial cells are cytokeratin positive regardless of the species (Henriksen *et al.*, 1990; Dickens *et al.*, 1993;.Laird *et al.*, 1995).

From the above account, it has been shown that epithelial cells from the Fallopian tube can be cultured and subsequently characterised. Of particular interest is the epithelial cells of the tube since the presence of these cultured cells have shown to be of significant importance in improving the current understanding of fertilisation and early embryo development *in vitro*.

1.8 SOME ASPECTS OF THE PHYSIOLOGY OF FERTILISATION AND EARLY EMBRYOGENESIS

1.8.1 Gametes and Embryo Transport in the Fallopian Tube

1.8.1.1 Ovum transport in the Fallopian tube

The human ovum is surrounded by follicular (granulosa) cells when it is released from the follicle at ovulation. A close interaction between the cilia on the fimbriated infundibulum and the follicular cells surrounding the ovum exists, prior to its 'capture' and subsequent journey down the tube. Oviductal gamete transport may be influenced by, smooth muscle contraction, ciliary activity, tubal fluid and the hormonal environment in the female. The activity of the cilia is greatest just after ovulation (Blandau, 1973), when the direction of the cilia beat is towards the ampullary isthmic junction (Blandau and Gaddum-Rosse, 1974). The rate of ciliary beat appears to be

oestrogen-driven and although the cilia beat in the direction of the uterus, spermatozoa are still able to move against this stream.

Ovum passage into the tube is also aided by the constant to- and fro movement of the ovaries relative to the fimbrial fronds of the infundibulum. Waves of contractile activity of both the circular and longitudinal muscles occur in the wall of the different tubal segments, which assist in the downward movement of the ovum towards the ampullary isthmic junction (AIJ).

1.8.1.2 Sperm transport in the Fallopian tube

In animal studies, it has been shown that contact between spermatozoa and oviductal epithelial cells are beneficial for sperm survival both *in vivo* (Smith and Yanagimachi, 1991) and *in vitro* (Pollard *et al.*, 1991). Pacey *et al.*, (1995) demonstrated that a strong interaction existed between the spermatozoa and the epithelial cells in humans. However, such close contact appeared to be random and there was no evidence of chemotaxis towards the epithelial cells. Furthermore, Kervancioglu *et al.*, (2000) showed that physical contact between sperm and epithelial cells in co-culture systems appeared to be the main factor in stimulating sperm movement characteristics.

1.8.1.3 Embryo transport in the Fallopian tube

In humans, following fertilisation in the ampulla region, the zygote, and subsequent cleavage to the morula or blastocyst stage (4-5 days), continues towards the isthmus before the embryo descends into the uterus for implantation. Both the time spent and the rate of transport of the embryo in the tube towards the uterine cavity is species

specific but this may reflect requirements for species survival and different endocrine environments in the proliferative phase of the ovarian cycle (Saridogan *et al.*, 1996).

1.8.2 The tubal fluid

The tubal environment (milieu) is constantly changing and may even vary between the different anatomical regions of the Fallopian tube at various stages of the menstrual cycle. Supporting evidence for this has in part, been described in section 1.6.2 whereby circulating ovarian steroids influence the fimbrial, ampullary and isthmic regions of the Fallopian tube.

The fluid present in the Fallopian tube provides the principal environment within the female duct system. Here, both the male and female gametes undergo final maturation and must retain full viability before fertilisation with the subsequent transport of the pre-implantation embryo towards the uterus. As such, the components and sources of the tubal fluid are of major interest in order to expand current understanding of reproductive events.

1.8.3 Composition of tubal fluid

The tubal fluid is a combination of secretory products from the oviductal epithelial cells and a transudate of serum (Malayer *et al.*, 1988; Leese, 1988). It is comprised largely of electrolytes, energy substrates, vitamins, lipids, growth factors, amino acids and proteins (Nancarrow and Hill, 1994). Proteins in the oviductal fluid are mainly derived from the blood (Oliphant *et al.*, 1978; Leese, 1988) and tend to remain relatively constant throughout the menstrual cycle (Lippes *et al.*, 1981). Non-plasma proteins have also been found in the tubal fluid of man (Rapisarda *et al.*, 1993) and sheep (Gandolfi *et al.*,

1989). However, both the synthesis and secretion of some of these proteins vary depending on the anatomical site, stage of the ovarian cycle and pregnancy status. In effect, both the quality and quantity, of the tubal fluid are influenced by the endocrine environment (Lippes *et al.*, 1972, 1981).

Glucose (Mastroianni *et al.*, 1961) and aminoacids, such as glycine (Miller and Schultz, 1987) and alanine (Nancarrow and Hill, 1994) have been shown to be present in tubal fluid. However, other constituents may also come from the contents of the graafian follicle released at ovulation, peritoneal fluid and the uterine cavity (Hunter, 1988).

The expression of certain proteins, polysaccharides, enzymes, and growth factors have also been demonstrated in various mammalian species. It has been proposed that the components of the glycocalyx of the oviduct plays an important role in the reproductive process (Abe, 1996). Glycoconjugates have an important role in mediating the interaction between male and female gametes and the oviductal epithelium in the reproductive processes of mammals.

The current of the tubal fluid is oriented towards the peritoneum at ovulation and the preceeding three days. Following this, it then changes and orients towards the uterus, at the time of embryo entry (Menezo and Guerin, 1997). The collection of human tubal fluid has proven to be a difficult task due to the small amount produced coupled with it's inaccessability.

Leese, (1988), suggested that changes in the volume and its control of tubal fluid may be explained in terms of changes in ion fluxes across the tubal epithelium. In particular,

chloride ions will alter the pH measurements at different stages of the menstrual cycle though, the precise mechanism by which this occurs across the oviduct remain unknown.

1.9 OVIDUCTAL PROTEINS

Typically, the protein concentration of tubal fluid is much lower than that of the plasma. It has also been reported that the different anatomical regions of the tube have different concentrations of proteins in the fluid (David *et al.*, 1969) and also secrete different volumes and types of glycoproteins (Hyde and Black, 1986). Fallopian tube mucosal explants or epithelial cells are used to study the *in vitro* synthesis and secretion of proteins. In these systems, radiolabelled amino acid precursors have been used to examine the biosynthetic activity of the oviduct. These precursors can be added to the culture medium, which is then incorporated in newly synthesised proteins. Using this technique, oestrogen-dependent high molecular weight oviduct secretory glycoproteins have been described in both animals, such as sheep and pigs (Murray, 1993; Buhi *et al.*, 1993) and human species (Rapisarda *et al.*, 1993).

A number of reviews have discussed the variety of proteins present in oviductal fluids (Hunter, 1988; Leese, 1988; Maguiness *et al.*, 1992). Proteins synthesised and secreted by the oviduct have been described for several species but one group of oestrus-associated glycoproteins (EGP) appear to have several common features for sheep, pigs, baboons and humans that may facilitate fertilisation or embryo development (Nancarrow and Hill, 1994). EGP has been shown to bind to the zona pellucida and blastomere membranes of baboons (Boice *et al.*, 1990), sheep (Gandolfi *et al.*, 1991) and cows (Wegner and Killian, 1991). Although such observations imply that these proteins may

regulate post fertilisation events, blastocysts that have been produced *in vitro* without the addition of oviduct specific proteins, still give rise to viable pregnancies (Nancarrow and Hill, 1994).

The total protein content, protein concentration and fluid volume in the oviductal luminal fluid varies in different species, during the ovarian cycle and early pregnancy (Lippes *et al.*, 1981; Hunter, 1988; Sutton *et al.*, 1984). Nevertheless, it is generally less than that in the corresponding serum. In humans, the protein concentration is 1.0-18.0 mg/mL and in sheep it is 9.3-30.0 mg/mL (Maguinesss *et al.*, 1992). Techniques employing the use of either Fallopian tube mucosal explants, or epithelial cells have been used to investigate the *in vitro* synthesis and secretion of proteins. These methods have contributed to the identification and characterisation of epithelial derived proteins in the oviduct environment.

1.9.1 Specific secretory proteins- Placental proteins

A number of placental proteins have been identified in the Fallopian tube using immunohistochemical localisation or radioimmunoassay. These include Pregnancy associated Placental Protein-A (PAPP-A) (Sjoberg *et al.*, 1986), Placental Protein 5 (PP 5) (Butzow, 1989), Placental Protein 10 (PP 10) (Tiitinen *et al.*, 1986), and Placental Protein 14 (PP 14) (Waites *et al.*, 1990; Maguiness et al., 1993). Other placental proteins such as PP4 and PP7 have been released into the growth medium by the Fallopian tube mucosal explants but the biological significance of these proteins are currently unknown (Verhage *et al.*, 1988).

In humans, tubal epithelial proteins (TEP-1 and TEP-2) of molecular weights 25 kDa and 17 kDa respectively were found to be secreted by the Fallopian tube but not the endometrium (Maguiness *et al.*, 1993). Furthermore, TEP-2 was not secreted by post-menopausal women, only pre-menopausal women which suggests a dependence on ovarian function. Such observations suggest that the Fallopian tube could be a potential target for contraception development.

1.9.1.1 Placental protein 14 (PP14)

PP14 is one of the major secretory proteins of the endometrium. Immunohistochemical studies, however, has confirmed its presence in the Fallopian tube epithelium during the luteal phase of the ovarian cycle and throughout pregnancy (Waites *et al.*, 1990). PP14 has been shown to occur in normal human ovarian tissue and is also produced by haemopoitic cells (Kamarainen *et al.*, 1994). This protein is sometimes referred to as glycodelin A (Kamarainen *et al.*, 1995) due to its glycoprotein structure.

A study carried out by Oehninger *et al.*, 1995 reported that the presence of PP14 reduces the capacity of the sperm to bind to the human zona pellucida. In another investigation, Clark *et al.*, (1996), suggested that this protein is involved in the human feto-embryonic defence system by providing an immunosuppresive environment which, protects the embryo from immediate attack from endometrial natural killer cells. A more recent study carried out by Rachmilewitz et al., (1999), suggested that PP14 functions as a direct T-cell inhibitor though the mechanism by which this occurs remain unclear.

In vitro de novo synthesis and the release of PP14 by the Fallopian tube mucosal explants has been observed during the ovarian cycle (Verhage *et al.*, 1988; Maguiness *et*

al., 1993). However, the study conducted by Verhage *et al.*, (1988) reported that the *in vitro* PP14 secretion was detected only in the late luteal phase but that of Maguiness *et al.*, (1993) demonstrated that both PP14 synthesis and secretion by the Fallopian tube mucosa remained relatively constant throughout the ovarian cycle. A study carried out by Saridogan *et al.*, (1997) examined the secretion of PP14 in epithelial cell cultures. Their results showed that polarised and non-polarised epithelial cultures secreted similar amounts of PP14 while frozen-thawed cells did not appear to secrete PP14. In addition, the time of the menstrual cycle at the time of removal of the Fallopian tube did not appear to have any effect on PP14 secretion by the cells.

1.9.1.2 Placental proteins and Pregnancy Associated Plasma Protein-A (PAPP-A)

Butzow *et al.*, (1989) demonstrated the presence of PP5 in all anatomical regions of the Fallopian tube during the proliferative and secretory phases of the ovarian cycle. PP10 was also detected in all the anatomical regions of the tube throughout the ovarian cycle (Tiitinen *et al.*, 1986). While PP5 is located primarily in the epithelium of the tubal mucosa, PP10 was localised in the subepithelial monocytic and lymphoid cells of the mucosa. Although, it has been hypothesised that PP5 may play a role in implantation, the significance of PP10 remains to be determined.

PAPP-A has also been shown to be present in the epithelial cells of the mucosa, throughout the tube during the menstrual cycle (Sjoberg *et al.*, 1986). Although, radioimmunoassay studies have shown that tubal samples obtained from the proliferative phase contained more than those of the secretory phase samples, the actual role of this protein in the reproductive process, remains to be elucidated.

1.9.2 Secretion of growth factors

The oviduct and the uterus may provide growth factors for embryo development either by direct secretion or through cell to cell contact (Nancarrow and Hill, 1994). The addition of transforming growth factor alpha and beta (TGF-α and TGF-β) and epidermal growth factor (EGF), *in vitro* have shown to improve embryonic development to blastocysts (Paria and Dey, 1990). Gardner and Kaye, (1991) have demonstrated that insulin and insulin-like growth factor (IGF-I) increase compaction, blastocyst formation rates and stimulate protein synthesis.

1.10 THE POTENTIAL VALUE OF FALLOPIAN TUBE EPITHELIAL CELLS IN ASSISTED REPRODUCTION

1.10.1 Assisted reproduction- Historical background

Sophisticated biological knowledge gained in the last century has led to significant advances in both human endocrinology and embryology. This has culminated in the development of a number of assisted reproductive techniques (ART) to assist sub-fertile couples. A number of studies were conducted during the 1960s, which included the use of ovarian stimulants to regulate follicle growth, oocyte maturation and ovulation, fertilisation and subsequent embryonic growth *in vitro*. It was a combination of these techniques coupled with embryo replacement in the uterus, which led to the introduction of *in vitro fertilisation* (IVF) for the alleviation of infertility.

Steptoe and Edwards, (1978) reported the first live-born conceived by IVF treatment. This pregnancy involved the aspiration of a single oocyte during a natural cycle and replacement of a cleaving embryo during the luteal phase. However, some other

techniques such as gamete intrafallopian tube transfer (GIFT), intracytoplasmic sperm injection (ICSI) and tubal embryo transfer (TET) have since been used.

1.10.2 In vitro fertilisation (IVF)

Assisted reproductive technologies are procedures, which involve the combination of sperm and eggs to treat infertility resulting in an improved probability of conception not otherwise possible. For those undergoing IVF treatment, the average success rate in the UK is just under 20 per cent for a single treatment cycle (Buxton, 2002). Since the first IVF in human, the number of patients using assisted reproductive technologies has increased tremendously. IVF was initially, specifically used for women with blocked Fallopian tubes, though nowadays it is used in many centres for other infertility problems, such as male factor infertility.

1.10.3 The co-culture concept

The oviduct provides various types of physiological support to both gametes and embryos. Interest in co-culture systems resulted from the introduction of gamete intra-fallopian tube (GIFT) and the realisation that embryo culture systems used in IVF remain far from ideal. Results from such co-culture experiments (Bongso *et al.*, 1990, Bongso and Fong, 1993) and from isolated oviduct culture systems, strongly suggested that interactions between oviductal epithelial cells and gametes might play important roles in the maturation of gametes, fertilisation, and embryonic development (Abe, 1996). As such, the fundamental purpose of co-culture is to imitate the oviductal function *in vitro*. A schematic diagram illustrating the co-culture is shown in Figure 1.5.

Although assisted reproductive techniques have contributed significantly to alleviating the problems of sub-fertility, the development of pre-implantation embryos *in vitro* is still generally compromised compared to those, which develop *in vivo.* The first co-culture attempt in human IVF were reported by Bongso and colleagues in 1989. They co-cultured human embryos on ampullary cells and found an improvement in embryo quality suggesting the beneficial effect of oviduct cell co-culture. Nevertheless, both the pregnancy and implantation rates have not been as high as those reported in farm animals, which are often greater than 60% per cycle (Iritani, 1994). It is well known that IVF can be used as a treatment for infertility and therefore demonstrates that the Fallopian tube is not essential for human reproduction. However, the success rate of IVF is relatively low, perhaps due to the inadequate conditions in which embryos are cultured. For this reason, it is feasible to suggest that the Fallopian tube, though not essential for reproduction, produces factors which aid both gamete and embryo development.

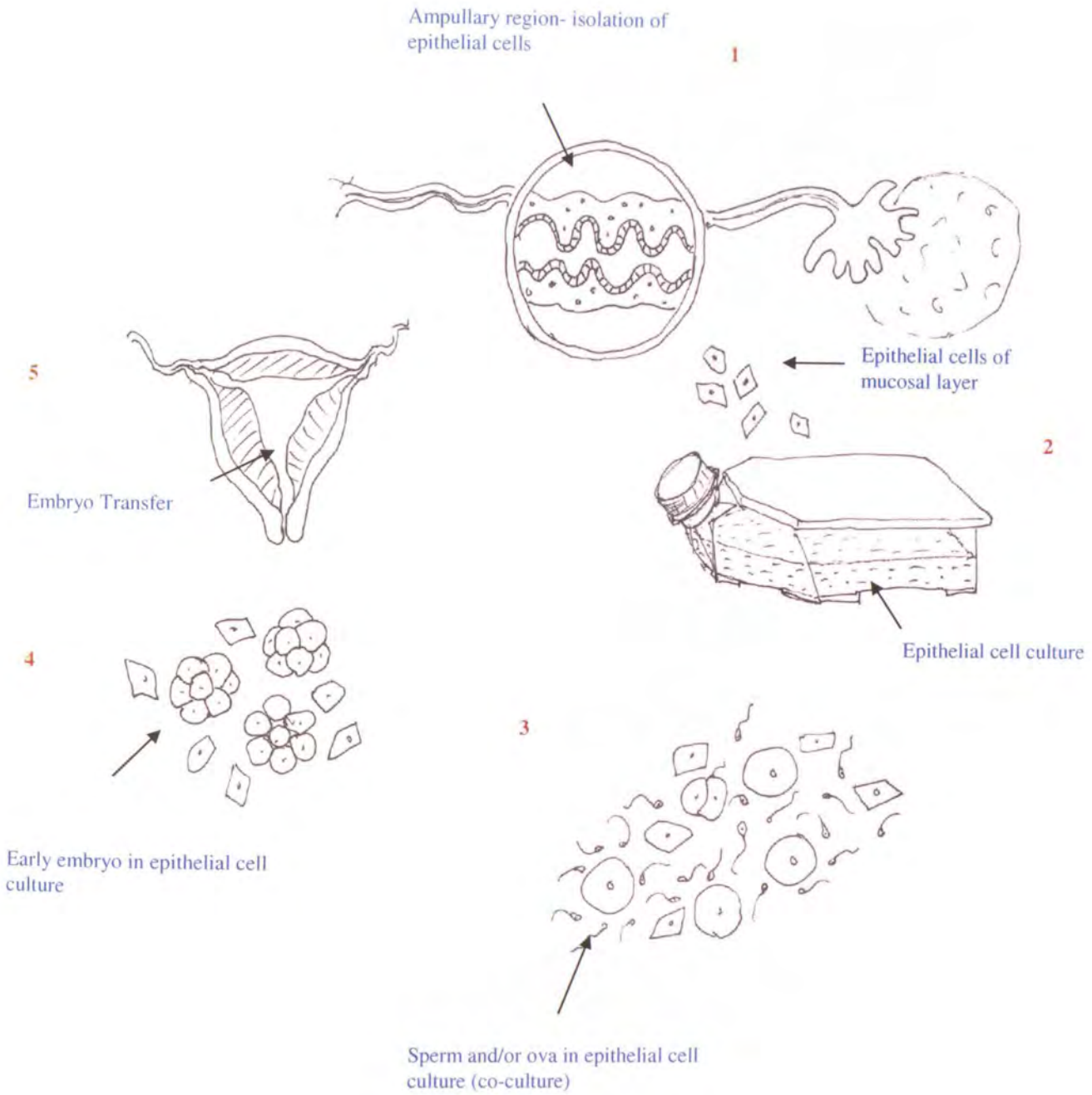


Figure 1.5: *A schematic illustration of the co-culture system. (1). Epithelial cells are isolated from the mucosal layer; (2). Cells are cultured in growth medium until confluence is reached (>70%); (3). Sperm and /or ova are placed in epithelial co-culture environment; (4) early embryonic development continues in co-culture and (5). Embryo is placed in the cervix. Taken and modified from Bongso & Fong (1993).*

Under existing laboratory conditions, the *in vitro* culture of human embryos up to the blastocyst stage remains sub-optimal, with only 17-25% of spare embryos cleaving normally to the blastocyst stage (Fehilly *et al.*, 1985; Bolton *et al.*, 1989). It is current practice in IVF to transfer human embryos 48 hours post-insemination at the relatively early 2-4 cell stage in an attempt to reduce any degenerative changes that may occur *in vitro*. However, this allows the embryo(s) to encounter an unsuitable environment, as they would normally be present in the Fallopian tube.

Usually, attempts to improve culture conditions have focussed mainly on the formulation and selection of appropriate media and the supplementation of a variety of additives (Walker *et al.*, 1997). However, a more promising method to improve the IVF laboratory conditions is to co-cultivate oocytes and sperm or early embryos with other 'helper' cells, which can be of tubal (specific) or of other origin (non-specific, e.g fibroblasts). Cultured tubal cells however, lose many of the morphological and perhaps, functional characteristics that they possess *in vivo*. The purpose of co-culture is to imitate the Fallopian tube function *in vitro* thereby, overcoming the developmental block and improve the cleavage stages of the developing embryo.

Several studies (Bongso *et al.*, 1991; Wiemer *et al.*, 1993) have highlighted the improved blastocyst formation and subsequent pregnancy rates from replacement of co-cultured embryos. In addition to their beneficial effects on early embryo development, co-culture systems have also been shown to improve sperm movement (Wetzels *et al.*, 1998; Bongso *et al.*, 1993; Pearlstone *et al.*, 1993), induce sperm capacitation and increase sperm survival (Kervancioglu *et al.*, 1994; Mansour *et al.*, 1995). It seems

therefore, that higher fertilisation rates would be expected in culture systems using helper cells.

1.10.4 Mode of action of co-cultures

It seems reasonable to suggest that if the physiological principles of early conception are to be followed, the ideal material for a co-culture system is the human Fallopian tube. These are available when women undergo hysterectomy for benign conditions or sterilisation. The exact mode by which these 'helper' cells exert their effect in a co-culture system remains unclear, although indirect evidence suggest a combined role of positive and negative conditioning of the medium (Bongso & Fong, 1993). Positive conditioning refers to the release of embryotrophic factors by the cells into the medium. These may include, (1) the secretion of the anti-oxidant taurine which improves embryonic development (Ouhibi *et al.*, 1990; Legge *et al.*, 1991), (2) glycoproteins released by the cells (Maguiness *et al.*, 1992) and (3) growth factors such as transforming growth factor and insulin-like growth factor (IGF) (Kane *et al.*, 1992). Negative conditioning involves the removal of deleterious components, which may result from their metabolic activities. These may include, (1) hypoxanthine which has been shown to cause the 2-cell block in mouse embryos (Loutradis *et al.*, 1987), (2) stabilization of the physico-chemical *in vitro* environment (pH, O_2, CO_2) and decreasing oxygen metabolic levels from the culture medium (Fukui *et al.*, 1991).

The widespread use of a human epithelial co-culture system has never materialised, perhaps due to the complexity of establishing a Fallopian tube epithelial cell line. Nevertheless, it is clear from the foregoing account that the concept of co-culturing gametes and or embryos in a Fallopian tube environment is logical and one that offers a

promising approach. As such, attempts to establish a 'bank' of epithelial cells which, initially can be used as a model for further experimental studies was undertaken.

1.11 THE SEARCH FOR A SUITABLE MODEL OF FALLOPIAN TUBE EPITHELIAL CELLS.

It is well documented that the oviduct is the physiological site for key events in reproduction such as, oocyte maturation, sperm capacitation, fertilisation and early embryonic development. However, due to the relative inaccessibility of the Fallopian tube, the interactions between oviductal epithelial cells and gametes or embryos cannot be studied adequately *in vivo*. In order to overcome this technical hurdle it is imperative to establish an *in vitro* model of the tube which mimics the *in vivo* environment.

1.11.1 Transfection and the induction of transformation

Transfection is the genetic modification of cultured cells by the uptake of DNA. The ability to introduce nucleic acids into cells has enabled the advancement of current knowledge of genetic regulation and protein function within eukaryotic cells, tissues and organs. Vaheri and Pagano, (1965) and Graham and Van der Eb, (1973) carried out pioneering studies with diethylaminoethyl (DEAE)-dextran and calcium phosphate-mediated transfection (non-viral) techniques. The DNA to be transfected is usually in the form of recombinant plasmids or other types of DNA vector containing the gene of interest. Plasmids are circular molecules of DNA which are capable of autonomous replication. As such, they can be isolated, manipulated and then re-introduced into bacterial cells.

1.11.2 Simian Virus 40 (SV40)

Simian virus 40 (SV40), is a simple virus with a small DNA genome, which encodes one major multifunctional regulatory protein, the large tumour antigen (LT-Ag). This is responsible for both the control of viral infection and the necessary alterations of cellular processes (Fanning and Knippers, 1992). SV40 is a papovavirus, which is found as a commensal in Asian macaques. It's genome is 5.2 Kb and can be divided into three regions; (1) a control region (*C*), (2) a region expressed early in infection (*E*) and (3) a region expressed late in infection (*L*), (Fanning and Knippers, 1992).

SV40 infects a variety of cultured mammalian cells, but initiates a productive infection cycle only in monkey cells, its natural host, and in cells of other primate species. Since most human cells are susceptible to infection with SV40 virus particles or can be transfected by purified DNA then the introduction of the SV40 genome into cells can extend the proliferative life span of such cells and increase the possibility of immortalization (Fanning and Knippers, 1992, Simmons, 2000).

The transformation and DNA synthesis of mammalian cells by SV40 requires the expression of the early (E) region of the genome, which encodes two proteins; the large T-antigen (100 kDa) and the small t-antigen (21 kDa). The former is necessary for the initiation and maintenance of the transformed phenotype whilst the latter, although not essential for transformation, may have an overall qualitative effect on the procedure.

Infection with SV40 is species dependent and although it can replicate efficiently in some monkey cell lines (eg Vero, CV-1) in others, such as rodents, infection is 'non-permissive' as it does not produce progeny virus but can express some of the viral genes and new

properties thereby becoming 'transformed'. Human cells are considered 'semi-permissive' because, although transformation is recognised by altered cell shape and growth properties, low but detectable levels of virus can also be produced. However, as mentioned before non-productive SV40 infection of permissive and human cells can be obtained by the introduction of defective viral genomes which cannot produce virus particles, as in the production of a recombinant DNA in a plasmid vector such as pMX1 or pCMV-SPORT. Efficient replication of DNA will occur but cells would not be expected to produce the extracellular virus.

1.11.3 Genetic Reporter Systems and Selection of Transfectants

Genetic reporter systems are most frequently used as indicators of transcriptional activity in cells (Rosenthal, 1987). Usually, a reporter gene is joined to a promoter sequence in an expression vector, which can then be used to monitor transfection efficiencies. Following transfer, the cells are assayed for the reporter by directly measuring the amount of mRNA, the reporter protein itself or the enzymatic activity of the reporter protein. β-galactosidase and green fluorescent proteins (GFP) are the most popular systems for monitoring genetic activity in eukaryotic cells but others include chloramphenicol acetyltransferase (CAT) and firefly luciferase.

1.11.4 Immortalisation

The ability of virtually all differentiated somatic cells to proliferate is finite. These cells whether present in tissue culture or in the organism itself display a limited capacity to divide and eventually reach a state of non-proliferation called cellular senescence. However, if provided, with forced signals to undergo continued division by trypsinization and replating at low density the cell population will eventually undergo cell death or crisis.

The ability to confer on primary cells the capacity for continued proliferation is commonly referred to as establishment or immortalization, an exceptionally rare spontaneous event in cultures of normal human cells (Freshney. 1993).

Chemicals (Dorman *et al.*, 1983), radiation (Namba *et al.*,1985) viruses and/or oncogenes (Linder and Marshall., 1990; De Silva *et* al., 1994) have all been used to transform human epithelial cells *in-vitro* (Figure 1.6). Chemical induced immortalization (eg. diethylnitrosamine (Emura *et al.*, 1985), aflatoxin B1 and β-propriolactone (Milo *et al.*, 1981) relies primarily on damage to the DNA. If the lesions of the DNA are not repaired, they can result in mutations for example, in genes that regulate cell growth so that cells may then expressed a transformed phenotype. ^{60}Co y-irradiation also aims at damaging the DNA and depending on the dose given, the growth state of the cells and other culture conditions, will determine whether transformation is achieved. Viral and plasmid transformation can be performed either by infection with oncogenic viruses which fuses with the cell membrane. The genetic material is then transferred to the cytoplasm and ultimately to the nucleus. DNA tumour viruses such as the SV40, adenovirus 5 and the high risk papilloma virus 16 and 18 are able to immortalize human cells by direct integration of their genetic material into the cellular DNA (Tooze, 1981; Chang, 1986).

Immortalization through transfection employs a recombinant DNA expression vector that carries a transforming gene (oncogene). Such constructs can still replicate DNA efficiently in human cells but cannot produce viral particles due to deletion or disruption of the L-region coding sequences. Cells infected with these viruses or transfected with plasmids containing their transforming genes frequently show an extension of their lifespan (Lomax, 1978; Ide 1984; Stein, 1985) at which time cells enter crisis. This is a

period of balanced cell growth and cell death followed by a decrease in the total number of surviving cells (Shay, 1989). In very rare instances, a focus of immortal cells arises which can be sub-cultivated as an established immortalized cell line.

The genes most often used for immortalization of epithelial cells include the large T-antigen of either the SV40 or polyoma and the E6 and E7 genes of the HPV. When the gene encoding the large T-antigen is introduced to normal dividing mortal cells, cellular programs that limit both the rate and the number of cell replications are inhibited and proteins that might participate in senescence are inactivated, resulting in an extended life- span for several generations.

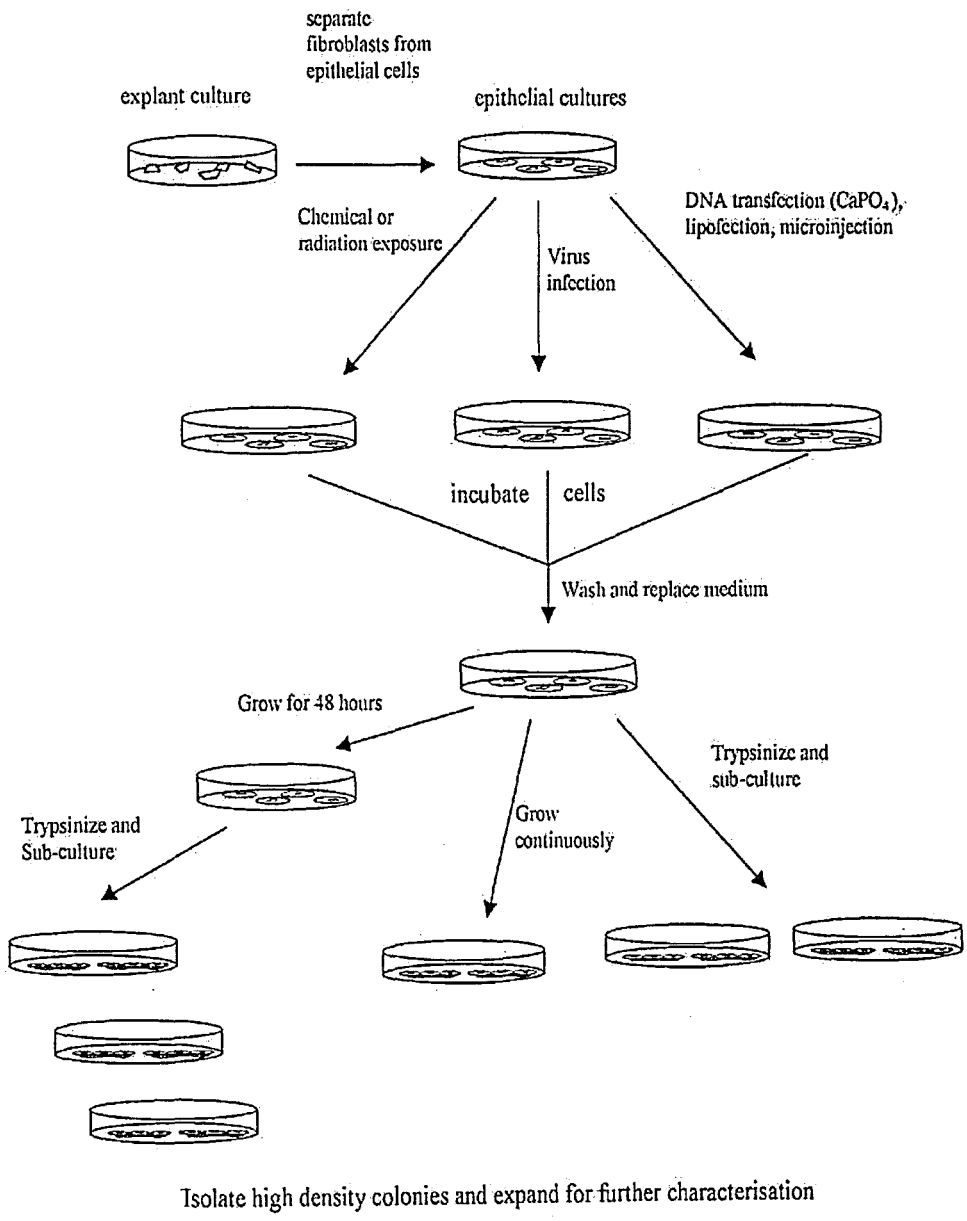


Figure 1.6: *A flow chart representing the numerous approaches to human epithelial cell transformation.*
Taken and modified from Gruenert (1987).

1.11.4.1 Characteristics of immortalised cells

Following immortalisation, cells may exhibit a number of characteristics that can be either similar or different from the normal cells. Some of these may include growth patterns, morphological and functional aspects as listed in Table 1.0

Growth	- high or indefinite saturation density - poorly oriented growth of cells - growth on monolayers of normal cells - tumour formation upon injection into susceptible animals
Surface	- absence of tight junctions - different staining properties
Intracellular	- disruption of the cytoskeleton - altered amounts of cyclic nucleotides
Evidence of virus	- virus-specific antigenic properties detectable - viral DNA sequence detected

Table 1.0: *Important characteristics of immortalised cells.*
Modified from Berg and Singer (1992).

1.12 CONCLUSIONS FROM THE LITERATURE REVIEW- AIMS OF THE THESIS

The preceeding pages of this chapter reviewed the literature with respect to the topics undertaken in this thesis. In multicellular organisms, the role of gap junction intercellular communication (GJIC) in the regulation of cell proliferation and differentiation is becoming increasingly recognised as one of the major cellular functions. Indeed, this begins as early as fertilisation, through normal development of both the embryo and foetus, to the sexual maturation of the adult and ultimately to the maintenance of health of the ageing individual (Trosko *et al.*, 2000). As such, communication or the lack of it may be the key element in the understanding important cellular functions, especially during the reproductive process.

The Fallopian tubes have many active roles in reproduction, which include the meeting of spermatozoa and the ovum under optimal conditions to encourage fertilisation, early cleavage and safe transport of the developing embryo down the uterus for implantation. However, such phenomena, their relation to the ovarian cycle and the interactions between the Fallopian tube and gametes/embryos are not well understood, although it is known that the Fallopian tube physiology is dependent on hormones, which vary throughout the ovarian cycle.

Experimental studies regarding the role of the Fallopian tube in the reproductive process has been hampered primarily by the relative inaccessibility of this tissue.. Therefore, in order to overcome this technical hurdle, it is imperative to establish an *in vitro* model of the tube, which mimics the *in vivo* environment. Indeed, cell culture provides both a simple and effective means to study the functional aspects of the Fallopian tube, though, an inherent disadvantage with this particular technique is that the original histology is lost together with any endocrine and paracrine influences which occur *in vivo* (Saridogan, 1996). For this reason, it is important to ensure that technique(s) used to isolate and establish an epithelial cell culture system should not impair the structure and function, but instead it should retain both the morphological and functional *in vivo* characteristics. Such a model would indeed facilitate and
expedite experimental work on the Fallopian tube. Therefore, one of primary aims of this thesis was to determine the best isolation procedure for obtaining epithelial cells from the Fallopian tube. This study was subsequently expanded by transfecting primary cultured cells with a transforming gene. This technique normally increases the life span of the cell and hence, another aim of this project was to establish an immortalised Fallopian tube epithelial cell line.

1.13 SUMMARY AND AIMS OF THE STUDY

Any organ is only as good as its constituent parts. This is particularly true of organs, whose function and morphology changes, not only over a period of years but also on a regular monthly cycle as is the case in female reproduction. In these structures, abnormalities or changes in even the smallest component can lead to a functional failure of the whole system. With respect to this thesis, any study that is intended to elucidate the role of the Fallopian tube either *in vivo* or *in vitro* must be designed in such a manner, to encompass various aspects of normal cell characteristics.

Therefore, this study has examined three diverse aspects of the Fallopian tube including, intercellular communication via gap junctions, morphology of the cells in an *in vitro* environment and the physiological status of cells harvested from the Fallopian tube. Specifically, the aims of the study were:

(1). To identify and quantify various gap junction proteins (connexins) in the different anatomical regions of the Fallopian tube at different stages of the ovarian cycle.

(2). To compare different isolation procedures for obtaining Fallopian tube epithelial cells by assessing their growth patterns, morphological features and secretory status using placental protein 14 (PP14) as a marker.

(3). To attempt to establish an immortalised cell line of Fallopian tube epithelial cells.

CHAPTER 2

Materials and Methods

2.0 Introduction

In this chapter, the methods used to carry out the various experiments in this project are described. Recruitment of patients for the study, the collection of the Fallopian tube tissue and the hormonal profile of the women at the time of tissue removal are detailed in sections 2.2 and 2.3. This is then followed by the techniques used to detect and quantify the presence of connexins in the different anatomical regions of the tube and include immunohistochemical staining of tissue sections and western blotting analysis of tissue pieces. Thereafter, sections 2.5-3.1 describes all the *in vitro* experiments, which involved the used of cell cultures coupled with the techniques used both in transformation and transfection procedures.

2.1 List of Materials

A list of various materials used for the experimental studies and the companies from which they were purchased are listed in Appendix 1.

2.2 Patients and tissue collection

Tissue samples were obtained from women undergoing sterilisation or hysterectomy for benign conditions, after informed consent and the local ethical committee approval. Detailed medical, obstetric and gynaecological histories were taken prior to operation. Immediately prior to surgery, a venous blood sample was obtained and follicle stimulating hormone (FSH), luteinising hormone (LH), oestradiol (E_2) and progesterone (P) were measured to determine the hormonal status or stage of the ovarian cycle in premenopausal

women. The stage of the endometrial cycle was confirmed by histological examination of the endometrium using the criteria described by Noyes *et al.*, (1950).

At operation, macroscopic appearance of the Fallopian tubes and the presence of any pelvic pathology, including uterine fibroids, endometriosis, pelvic adhesions and ovarian tumours were noted. Macroscopically abnormal looking Fallopian tubes were only used to establish techniques and data derived from experiments on these abnormal tubes were excluded from the final analysis. Data from women who had not received any hormonal treatment or oral contraceptives for three months prior to their operation were included in the study.

After removal, the tissue samples were immediately placed in ice cold minimum essential medium with Earle's salts and L-glutamine supplemented with heparin (1.8IU/ml), penicillin (100IU/ml), streptomycin (50μg/ml) and Hepes (10mM). The tissue samples were washed in this medium to remove excess blood. The tissue was then used either for morphological or immunohistochemical or cell culture experiments. The number of samples used for each of these techniques is stated under the relevant sections.

2.3 Hormonal assays

Serum FSH, LH, P and E_2 measurements were carried out by the Biochemistry Department at Newham General Hospital. The blood samples of the patients taken prior to the operation were centrifuged and the plasma stored at -20° C until required for analysis.

2.3.1 Serum LH assay

The assay was carried out automatically by the ACS:180® Plus Automated Chemiluminescence System where there is a direct relationship between the LH in the sample and the relative light units detected by the system. The assay measures LH concentrations up to 200IU/L with a minimum detectable concentration of 0.09IU/L.

2.3.2 Serum FSH assay

This was similar to the LH assay and its sensitivity was 0.3-200IU/L.

2.3.3 Serum Oestradiol (E_2 assay)

The serum oestradiol assay (Estradiol-6 assay), a competitive, chemiluminescent immunoassay was used. A highly specific anti-17β-oestradiol-6 antibody was used in this assay and E_2 in the patient's serum competes with E_2 labelled with dimethyl-acridinium ester for a limited amount of rabbit anti-oestradiol antibody. Rabbit anti-oestradiol antibody was captured by mouse anti-rabbit IgG, which was coupled to paramagnetic particles. An inverse relationship existed between the amount of E_2 in the patient's sample and the amount of light units detected by the ACS:180 system. The sensitivity of the assay was 36.7-3670 pmol/L.

2.3.4 Serum Progesterone assay

In this assay, progesterone in the patient's serum binds to an acridinium ester labelled mouse monoclonal antibody. Unbound antibody is bound to a progesterone derivative, which is coupled to paramagetic particles. An inverse relationship existed between the amount of progesterone in the patient's sample and the amount of relative light units detected by the ACS:180 systems. The sensitivity of the assay was 0.35-127.2 nmol/L.

2.4 IMMUNOHISTOCHEMISTRY AND WESTERN BLOTTING ANALYSIS OF CONNEXINS IN THE FALLOPIAN TUBE

In this study, immunohistochemistry and western blotting techniques were used to investigate the presence and level of expression of the gap junction proteins, 'connexins' in the fimbrial and ampullary regions of the Fallopian tube obtained from 10 women during the different phases of the reproductive cycle.

2.4.1 Histology

2.4.1.1 Tissue processing

Fimbrial and ampullary regions of the Fallopian tubes were dissected, rinsed in phosphate buffer and completely immersed in a 10% buffered formalin solution. Transverse sections of the Fallopian tube were placed in embedding cassettes (Tissue-Tek®II) and processed according to standard procedures as outlined in Table 2.0 using an automated tissue processor (Shandon Elliot, Shandon Hypercentre XP).

Procedure	Industrial Methylated Spirit (%)	TIME (hrs)
Rinsing	50	1
Dehydrating	50	1
"	70	2
"	80	2
"	90	2
"	100	2
"	100	2
"	100	1
Clearing	Histoclear	1
"	Histoclear	1
Embedding	Paraffin wax (55°C)	1
"	Paraffin wax (55°C)	5

Table 2.0: *Preparatory stages for immunohistochemical analysis of specimens.*

2.4.1.2 Tissue embedding

Processed tissues were finally embedded in paraffin wax (55 ±5°C) using a Shandon Histocentre and cooled on a cold-plate.

2.4.1.3 Section cutting

Tissues were then sectioned at 4-6μm using a Shandon hypercut rotary microtome. Ribbons of sections were obtained and floated onto a warm water-bath. Sections were collected onto clean labelled Opti-plus microscopic slides. The slides were left standing overnight in a slide holder at room temperature and thereafter in an oven maintained at 37°C until required.

2.4.2a Paraffin-Wax Embedded Sections

Prior to staining, sections were dewaxed/deparaffinized with xylene and rehydrated in a graded series of ethanol as outlined in Table 2.1.

Xylene	5 minutes (x 3)
Absolute alcohol	3 minutes (x 2)
95% alcohol	3 minutes (x 2)
Distilled water	30 seconds (x 2)

Table 2.1: *Stages for dewaxing and rehydration of tissue sections.*

Sections were then washed with two changes of PBS for 5 minutes each. Slides were wiped dry and placed in a humidity chamber. Endogenous peroxidase activity was quenched by incubating the hydrated sections with 0.5% hydrogen peroxide (BDH) in distilled water (1:200) for 10-15 minutes in a humidity chamber (0.2 mls H_2O_2 and 11.8 mls water). PAP pen was used to circle individual sections to be tested thereby forming a water-repellent barrier around the specimen. Sections were blocked with Triton-lysine

buffer for 30 minutes at room temperature to prevent non-specific antibody binding. The buffer was subsequently aspirated and the sections washed gently in PBS for five minutes.

Primary antibodies, cx 26, 32 and 43 were applied and left for one and half hours at 37 °C, four hours at room temperature or overnight at 4°C at (1-5μg/ml). Tissue sections were once again washed gently in TBS twice for 3 minutes each. The biotinylated secondary antibody was applied after the slides were wiped and placed in a humidity chamber for 30 minutes. Tissue sections were then washed gently in PBS twice for 3 minutes each. A fluorescence labelled (FITC or TRITC) secondary antibody was then applied and left for one hour in a humidity chamber. The sections were once again washed with PBS twice for five minutes, mounted with a coverslip and viewed under a fluorescent microscope.

2.4.2b Frozen Sections

Small pieces of fimbria and ampulla were sectioned (10μm) using a cryostat. The individual sections were placed onto clean Opti-Plus slides, wrapped in foil and stored at -20°C until needed. Prior to staining, the sections were allowed to warm up for 1-3 minutes, fixed in acetone, processed and examined as described in section 2.4.2a.

2.4.3 Western Blotting of connexins

In this experiment, Western blotting was used to (i) determine the presence and (ii) investigate the level of expression of the gap junction proteins, 'connexins' within the fimbrial and ampullary regions of the Fallopian tubes. Pieces of fimbria and ampulla were cut, placed in labelled cryovials and immersed in liquid nitrogen until required.

2.4.3.1 Homogenization and sonication of Fallopian tube regions

Pieces of tissue (eg ampulla) frozen in liquid nitrogen were retrieved, weighed and homogenised in homogenising buffer [0.05M Tris-HCl, pH 7.4; apoprotenin (0.1% of a 1mg/ml)] stock solution and trypsin inhibitor (0.1% of a 1mg/ml stock solution). For consistency, 10 mls of homogenising buffer was used per gram of tissue. In addition, the samples were further crushed using a manual homogeniser with a 'grounded' base for 5-10 minutes and sonicated for three times for 10 seconds in an ultrasonicator. They were then ultra-centrifuged at 11, 000 RPM for 20 minutes at 4°C and the resulting supernatant aspirated and subsequently recentrifuged at 40, 000 RPM for 60 minutes 4°C. At this stage, the supernatant was removed once again and the resulting pellet was dissolved in solubilising buffer. The membrane fraction (S9) was subsequently analysed for protein concentration using the Lowry method. (Lowry *et al.*, 1951). Following this, aliquots (50-100μl) were stored at –20 °C and used when required.

2.4.3.2 Gel electrophoresis

The following solutions were prepared using distilled water:

Upper gel buffer:	Tris-HCl [0.5 M, (pH 6.8) containing 0.4% (w/v) sodium dodecyl sulphate (SDS)]	
Lower gel buffer:	Tris-HCl [1.5 M, (pH 8.8) containing 0.4% (w/v) SDS]	
Electrode buffer:	Tris-HCl [25 mM, (pH 8.3) containing 192 mM glycine and 0.1% (w/v) SDS]	
Acrylagel (30%):	Commercially available	
Bis-Acrylagel (2%):	Commercially available	
Loading buffer: (store at 2-8°C)	Distilled water	12.4 ml
	Tris-HCl [1 M, (pH 6.8)	5.0 ml
	Glycerol (10%)	2.0 ml
	SDS (2 %)	0.4 g
	2% β-mercaptoethanol	0.4 ml
	Bromophenol blue (0.01% w/v)	0.002 g
Transfer Buffer:	Tris (20 mM), glycine (150 mM) and 15 % (v/v) methanol	

The mini-electrophoresis system was used for all Western blots. Glass plates, spacers and combs were cleaned with methanol. Spacers (0.75mm) were placed between glass plates and gently slotted into the plate holder. Screws were firmly tightened and plates were then checked for leakage.

The lower running gel (10%) was prepared as follows and was sufficient for four gels:

Acrylagel	8.11 ml
Bis-Acrylagel	3.38 ml
Lower gel buffer	6.25 ml
Distilled water	7.14 ml
Ammonium persulphate (10% w/v)	0.125 ml

TEMED (20 μl) was then added to initiate polymerisation of the gel. The solution was quickly mixed and poured between the glass plates using a pasteur pipette, leaving enough space for the upper gel. A small amount of saturated butan-1-ol was added onto the top of the gel solution to ensure a flat surface between the lower and upper gels and prevent evaporation. The gels were then left at 4°C for a minimum of 2 hours to ensure complete polymerisation after which the layer of butan-1-ol was gently removed using filter paper. Clean combs (10 line) were inserted between the glass plates and the stacking gel (3%) was prepared as follows:

Acrylagel	1.0 ml
Bis-Acrylagel	0.4 ml
Upper gel buffer	2.5 ml
Distilled water	6.0 ml
Ammonium persulphate (10% w/v)	0.1 ml

Polymerisation was once again initiated by the addition of TEMED (20 μl). The solution was mixed and gently poured into a small gap between the spacers and comb to ensure no air bubbles were present between the sample wells. This was left for at least 60 minutes to polymerise. The comb was gently removed and the sample wells were filled with electrode buffer to maintain their integrity. Equal volumes of loading buffer were added to the

diluted, solubilised membrane fractions and then placed in a boiling water-bath for 3 minutes. Samples were allowed to cool prior to centrifugation at 13000RPM for 1 minute using a microcentrifuge; 25μg of protein was loaded into the sample wells unless otherwise stated. A positive control and/or pre-stained molecular weight markers (10 μl) were run alongside the samples and they were as follows:

Band		*Approximate molecular weight (Da)*
1	Phosphorylase b (rabbit muscle)	97400
2	Bovine serum albumin	68000
3	Ovalbumin	46000
4	Carbonic anhydrase (human erthrocytes)	31000
5	Trypsin inhibitor (soybean)	20100
6	Lysozyme	14000

Electrophoresis was carried out immediately at a voltage of 120 V. When the loaded blue band had migrated to the lower rim of the lower gel (approx. 60-90 mins), the current was switched off.

2.4.3.3 Transfer of proteins onto nitrocellulose membrane.

Four blotting papers and one nitrocellulose membrane per gel were cut to size. Two scotchbrite pads and the blotting papers were left to soak in the transfer buffer for 5-10 minutes. The nitrocellulose membrane was soaked in methanol of 15-20 seconds prior to soaking in transfer buffer in order for the membrane to equilibrate. The plates were removed from the electrophoresis tank and the glass plates separated. The upper gel was

cut off and the gel transferred carefully onto the nitrocellulose membrane for immunoblotting.

The transfer cassette was placed in a tray containing transfer buffer (black side down). The membrane was sandwiched by using 1 scotchbride pad followed by 2 of the filter papers prepared earlier, the gel was then gently placed on top of the filter paper and was then covered by the nitrocellulose membrane. This membrane was then covered by a further 2 of the cut to size filter papers and was finally sandwiched by the addition of another scotchbride pad. The cassette was closed and placed in the transfer tank containing transfer buffer and the electrophoretic transfer of proteins was carried out overnight at a constant current of 100 mA and voltage of 120 V.

2.4.3.4 Immunodetection

The following reagents were prepared using distilled water:

Tris buffered saline (TBS), pH 7.6:	12.1g Tris-base and 40.0g sodium chloride dissolved in 5 litres of water
TBS-Tween:	0.1% Tween-20 in TBS

The following were prepared fresh and were sufficient for the immunodetection of four blots:

Membrane blocking reagent:	TBS containing 5% (w/v) milk powder (Marvel 2.0g marvel in 40 ml TBS
Diluent:	TBS containing 1% (w/v) milk powder 0.4 g Marvel in 40 ml TBS

The nitrocellulose membrane was gently rolled and placed in a 50 ml sterilin tube and was treated as follows using a total volume of 3 ml.

1. Block membrane	Membrane was incubated in the blocking reagent.	60 minutes

2. Wash	Membrane was washed with TBS-Tween 3 times.	1 x 10 minutes 2 x 5 minutes
3. Anti-connexin minutes (26, 32 or 43)	Membrane was incubated with primary antibody diluted in diluent.	90
4. Wash	Membrane was washed with TBS-Tween 3 times.	1 x 10 minutes 2 x 5 minutes
5. Secondary antibody minutes	Membrane was incubated with secondary antibody diluted in diluent.	60
6. Wash	Membrane was washed with TBS-Tween 3 times.	1 x 10 minutes 2 x 5 minutes
7. Incubated with STR-HRP	The lane containing the ECL marker was carefully cut out and incubated Separately from the rest of the membrane. Step 6 was repeated.	20-30 minutes
8. Detection	3 ml ECL detection reagent 1 was mixed with reagent 2. Membrane was removed from the sterilin tube and excess buffer was drained off. The membrane together with the lane containing the marker was placed on a glass plate and was gently covered with the membrane detection reagent.	1 minute
9. Exposure	Excess detection reagent was drained off. The membrane was placed on Saran Wrap membrane face down and was exposed to film	30-60 seconds
10. Processing	Each film was developed in trays until bands appear. This was followed by placing the films in fixer for 1-2 minutes and then washed thorougly in running tap water for 15-30 minutes	

2.5 Cell Cultures

All routine handling of cultures including the setting up of experiments and media preparation was carried out under sterile conditions in a class [2] Gelman (BH48) laminar air flow cabinet. Plastic tissue culture flasks and media components usually arrived pre-sterilised from the manufacturer, otherwise sterilization of solutions was carried out using a 0.20μm pore filter (Sartorius). Forceps for lifting coverslips, scissors, eppendorfs and microtips were autoclaved for 20-30 minutes at 121°C and 15psi to ensure sterility.

The different growth medium used were, Medium **A** (MEM-Earle's supplemented with penicillin (100IU/ml), streptomycin (50μg/ml); medium **B** (DMEM/Nutrient Mixture Ham's F-12 with GLUTAMAX1 supplemented with foetal bovine serum (15%), streptomycin (50μg/ml) and penicillin (100IU/ml); and medium **C** William's medium in Earle's salt, supplemented with foetal bovine serum (15%), streptomycin (50μg/ml) and penicillin (100IU/ml).

2.5.1 Establishment of Fallopian tube epithelial cell cultures

After excision, the tubes were transferred immediately to the tissue culture laboratory situated in the same building in sterile plastic tissue culture pots. These pots contained 20 mls of MEM-Earle's with L-glutamine supplemented with heparin (1.8IU/ml), penicillin (100IU/ml), streptomycin (50μg/ml) and HEPES (10mM). This medium was used for all tissue manipulation procedures. The Fallopian tubes were rinsed twice with medium to remove all visible evidence of blood. The lumen of the tube was exposed by incising it along the anti-mesentric border and the mucosal folds of the ampullary section dissected off under naked eye vision and placed in a separate dish which contained 15 mls of the appropriate tissue culture medium. The medium used for tissue culture depended on

whether the mucosal layer was isolated mechanically or enzymatically as described under section 2.5.1.1 and 2.5.1.2 respectively.

2.5.1.1 Mechanical isolation of epithelial cells

This method was carried out as described previously by (Kervancioglu *et al.*, 1994). The mucosal tissue was rinsed in 20-30 mls of medium A, cut into pieces $1mm^3$ and placed into $25mm^2$ sterile tissue culture flask containing 1 ml of medium A supplemented with 40% FBS. The primary cultures were then incubated at 37°C in an atmosphere of 5% CO_2:95% air and 95% humidity. After 3 days, a further 2 mls of growth medium A supplemented with 10% FBS medium was added (primary culture 1). Between day 7 and 10 of the primary culture the tissue explants were removed and the primary culture procedure was repeated (primary culture 2).

2.5.1.2 Enzymatic isolation of epithelial cells (Pancreatin-Trypsin)

This method has been previously described by (Leese *et al.*, 1996) but modified accordingly. The mucosal tissue was cut into $1cm^3$ pieces, then placed in 20mls of calcium and magnesium free Hanks balanced salt solution (HBSS) containing 0.14g Trypsin and 0.54g Pancreatin. The flask was then stored between 4-8°C for one hour and then brought to room temperature for another hour. The epithelial cells were then carefully removed, placed in a universal and centrifuge for 10 minutes at 1000 RPM. The supernatant was discarded and the cell pellet re-suspended in HBSS and re-centrifuged. This procedure was repeated for a further three times and the cells then plated onto $25cm^2$ flasks containing 5 mls of Medium B.

2.5.1.3 Enzymatic isolation of epithelial cells (Pancreatin-Dispase)

The mucosal tissue was cut into $1cm^3$ pieces, then placed in 20mls of calcium and magnesium free Hanks balanced salt solution (HBSS) containing 0.54g Pancreatin and 0.65g Dispase. The flask was left at room temperature for 30 minutes and at 37°C for further 30 minutes. The epithelial cells were then carefully removed, placed in a universal and centrifuge for 10 minutes at 1000 RPM. The supernatant was discarded and the cell pellet re-suspended in HBSS and re-centrifuged. This procedure was repeated twice and the cells then plated onto $25cm^2$ flasks containing 5 mls of medium **C**.

2.5.2 Subcultures

Fallopian tube cells isolated using either of the techniques described above were allowed to reach confluence. At this stage they were washed with calcium and magnesium free HBSS, then trypsinized using 3mls of Trypsin-EDTA. On some occasions adherent cells were detached from the substrate using 3 mls of cell dissociation medium. The cells were then centrifuged at 800 RPM for 5 minutes, and the supernatant discarded. Approximately 5×10^5 cells were subcultured in 3 mls of culture medium prepared as before and supplemented with 15% FBS. Subsequent cultures were passaged at confluence (5-6) days by transferring the same number of cells, using the same trypsinization technique, into identical culture dishes containing subculture medium.

2.5.3 Cell freezing and reconstitution procedure

Following trypsinisation or detachment using the cell dissociation medium, the cells were centrifuged at 800 RPM for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in either culture medium containing 50% FBS, 30% growth medium and 20% DMSO at a concentration of approximately 10^6 cells/ml. These were placed in

freezing vials and stored at -70°C for four hours and then immersed in liquid nitrogen. When reconstitution of cells was required frozen cryovials were retrieved from the liquid nitrogen store and immediately thawed in a 37°C water-bath for 1 minute. The contents were transferred into sterile universals containing 10mls of pre-warmed growth medium (depending on the method of isolation) with FBS (15%). The tubes were centrifuged at 800RPM for 10 minutes. The supernatant was removed and the cells were reconstituted in its specific growth medium, counted and diluted to a concentration of 2 x 10^5 cells/ml. They were then seeded in 25cm^2 tissue culture flasks or sterile glass coverslips (22mm x 22mm).

2.6 Microscopy

2.6.1 Light microscopy of monolayer cells

On reaching confluence, the cell monolayer was photographed using an inverted microscope (Nikon) at different magnifications, x10, x25 and x40. Fully or partially confluent primary cultures were, on two separate occasions, stained with haemotoxylin and/or eosin or fushin simply to give an idea of the morphological features within the cells.

2.6.2 Fluorescent microscopy

2.6.2.1 Cytokeratin staining

Cells from primary cultures and subcultures were seeded in chamber slides. At confluence, they were rinsed twice for two minutes each with PBS and then fixed in a fresh mixture of equal parts of chilled methanol:acetone for 10 minutes. This was discarded and the slides were allowed to dry briefly at room temperature with the cell monolayer surface facing up. The cells were washed in PBS (x 3) at 5 minutes intervals. The slides were then laid flat in a humidity chamber and 50-80μl of the diluted monoclonal mouse anti-pan cytokeratin

antibody (1:400 dilution in PBS) was pipetted over the cells. The chamber was then covered and the slides left undisturbed at 37°C for 60 minutes and once again washed in PBS (x 3). Following this, the slides were carefully wiped dried around the cell monolayer, incubated with FITC-conjugated anti mouse IgG antiserum (1:125 dilution in PBS) for 45-60 minutes. From this stage onwards they were protected from light and excess antibody was removed by washing twice in PBS at 5 minutes intervals. The cells were then viewed under a Zeiss fluorescence microscope at wavelength 525nm. All experiments included control slides in which the primary antibody was omitted.

2.6.2.2 Propidium Iodide and Hoechst 33258 staining

To confirm the integrity of the cell membranes both in primary cultures and subcultures, a mixture of propidium iodide (PI) and Hoechst 33358 was added to the cell culture medium to give a final concentration of 10μg/ml and 5μg/ml respectively. After 5 minutes at 37°C, the cells were examined under a Zeiss fluorescence microscope with a filter block giving excitation at 380nm and 480nm. The cells with disrupted membranes gave a strong red nuclear fluorescence with the uptake of PI whilst those cells with intact cell membranes gave a blue nuclear fluorescence due to the uptake of the permeable Hoechst 33358 fluorochrome.

2.6.3 Establishment of Growth Curve for HFTEC.

Confluent cells were passaged as described in section 2.5.3. After centrifugation at 800RPM for 5 minutes, much of the supernatant was discarded and 1 ml of pre-warmed growth medium was added to the cell pellet and mixed thoroughly. A cell count was carried out using a haemocytometer. The mixture was diluted with growth medium and cells seeded at an appropriate concentration. On days 2, 4, 6 and 8 cell counts were performed from individual flasks and recorded.

2.7 Cytogenetics

2.7.1 Chromosome Spreads

Cells were grown in 25cm^2 sterile flasks for 48 hours in either medium (B) or medium (E) depending on the method of isolation. They were then arrested in metaphase by the addition of colcemid at a concentration of 0.1μg/ml for 2-4 hours. The flask was shaken firmly (mitotic shake) to remove the metaphase cells and poured into a conical centrifuge tube. The solution was then spun in a centrifuge at 1000RPM for approximately 10minutes. The supernatant was removed and the cell pellet re-suspended by gentle agitation. The pellet was then treated with 5 mls of hypotonic solution, added drop-wise and left to incubate at 37°C for 10-15 minutes before being centrifuged at 1000RPM for 10 minutes. Once again the supernatant was removed leaving approximately 1 ml of liquid above the cell pellet. The cell suspension was fixed by adding 5mls of Carnoys fixative drop-wise to avoid clumping of the cells. The tube was then spun for 10 minutes in the centrifuge at 1000RPM. Much of the supernatant was removed and 2-3 drops of Carnoys (3:1 methanol:acetic acid) was slowly added but shaken vigorously to ensure no clumping of cells. One or two drops of the solution was then allowed to run down the length of a clean microscopic slide and allowed to air dry. The slides were then stained in a 5% Giemsa in phosphate buffer (pH 7.0) for approximately 5 minutes and then rinsed thoroughly in tap water. They were allowed to dry thoroughly (at least one hour) and were then mounted using a small amount of DPX and a glass coverslip. Metaphase spreads were then located, viewed under the light microscope and photographs taken at x40 and x100 magnifications. The preparations were used to count chromosome numbers (50 metaphases) and for detailed karyotype analysis (at least 25 metaphases) of each cell line.

2.8 Protein secretion

2.8.1 Monolayer cells

Tubal epthelial cells were isolated from Fallopian tubes and cultured (7 x 10^5) until they reached confluence. Following this, the culture media (3 mls) was removed and the cells rinsed in PBS. Fresh growth medium (3 mls) supplemented with FBS (15%) was added and the flasks returned to the incubator. After 24 hours the medium was aspirated, aliquoted and stored at -20°C until assayed for the presence of PP14.

2.8.2 Total Protein determination using the Lowry method

The total protein released into the culture medium was determined using the Lowry assay (Lowry *et al.*, 1951) a quantitative assay for determining protein content. Protein samples were diluted with 0.5 M NaOH to give a total volume of 1 ml. Bovine serum albumin was used as the protein standard in the range of 0-1000μg in order to determine the absorbance curve. Following this, 5 mls of freshly prepared Lowry reagent [2 mls of a 1% $CuSO_4$ solution and 2 mls of a 2% $KNaC_4H_4O_6$ solution mixed with 200 mls of a 2% Na_2CO_3 solution] was added to both the standards and samples in sequence, vortexed and allowed to stand for 10 minutes at room temperature. 0.5 mls of Folin-Ciocalteu's reagent was added to all samples and vortexed once again. After 30-45 minutes at room temperature the absorbance was read at 720nm and the difference between the zero standard and the SFM was taken into account when the protein content (μg/ml) was read off from the graph.

2.8.3 Radioimmunoassay (pp14 secretion)

Thawed samples of mechanically and enzymatically FTEC were assayed for PP14 by radioimmunoassay using [^{125}I] PP14 as a label and rabbit anti-human PP14 serum as described by Fay *et al.*, 1990. The bound fraction was separated from the free antigen

using polyethylene glycol-assisted anti-rabbit serum precipitation. Radioactivity was then measured in the bound fraction which was recovered as a precipitate.

2.9 Transformation of E.Coli bacterial cells

2.9.1 Growth, extraction and purification of plasmid DNA

2.9.1.1 Preparation of agar plates and nutrient medium

Prior to preparation of agar plates, two conical flasks each containing 2.0g of LB agar was dissolved in 100mls of distilled water. Similarly, another two conical flasks were used to prepare growth medium by dissolving 2.8g of Oxoid nutrient broth in 100mls in distilled water. All four flasks were autoclaved at 121psi for 15 minutes. The liquid agar was allowed to cool until 'hand-hot'. Sterile filtered, ampicillin (100μg/ml) and X-gal (50μg/ml) were then added to two of the flasks containing the liquid agar, prior to being poured into labelled, sterile petri-dishes. As such, plates containing ONLY agar and plates containing agar, ampicillin and X-gal were in effect, prepared. The dishes were then left at room temperature overnight, wrapped securely in parafilm and stored at 4-8°C until required. They were allowed to dry thoroughly in an aseptic drying cabinet prior to use.

2.9.1.2 Transformation of DH5α competent cells

Two microcentrifuged tubes containing 50μL each of DH5α competent cells were taken from the -70°C freezer and thawed on wet ice. To one of the tubes, 5μL (500pg) of control DNA, pUC19 was added by moving the pipette through the cells while dispensing. To the other tube, 10μL (500pg) of plasmid DNA pCMV-SPORT was added in a similar manner as above. Both tubes were then incubated on ice for 30 minutes, heat shocked for 20 second

at 37°C and placed on ice for 2 minutes. To each of the tubes, 950μL of LB was added and shaken at 225RPM for 1 hour at 37°C to allow for expression of the β-gal gene.

Following gene expression, both tubes were vortexed briefly and 100μl of the reaction containing the control DNA, pUC19, was added to 900μl of LB. In a similar manner, 100μL of pCMV-SPORT was also added to 900μL of LB. Following this, 100-200μLs of both the diluted and undiluted reaction mixtures were spread on the appropriate labelled agar plates and incubated at 37°C.

2.9.1.3 Growth of transformants for plasmid preparation/amplification of DNA

Two conical flasks containing 100mls of nutrient broth was prepared as described in section 2.9.1.1. DH5α competent cells, which have been transformed with the plasmids, pUC19 and pCMV-SPORT (transformants), should be observed from the respective agar plates. One colony from each were lifted using sterile toothpicks, placed in the nutrient broth and grown overnight in a shaker at 220RPM at 37°C.

2.9.1.4 Purification of plasmid DNA

This was carried out using the CONCERT high purity plasmid purification system (Figure 2.0). The column was equilibrated by adding 30 mls of equilibration buffer [600mM NaCl, 100mM sodium acetate (pH 5.0), 0.15% Triton X-100 (v/v)]. 100mls of an overnight culture containing pUC19 and pCMV-SPORT respectively, were centrifuged at 2000RPM for 5 minutes. The medium was thoroughly removed and 10 mls of cell suspension buffer [150mM Tris-HCL (pH 8.0), 10mM EDTA, 20mg/ml RNase A in water] was added and mixed thoroughly until homogenous.

Following this, 10 mls of neutralising buffer [3.1M potassium acetate (pH 5.5)] and mixed immediately by inverting the tubes five times. The mixture was then centrifuged at 15 000 x *g* at room temperature for 10 minutes. The resulting supernatant was pipetted into the equilibrated column and allowed to drain by gravity flow into a waste beaker. The flow through was discarded and the column washed by adding 60 mls of wash buffer [800mM NaCl, 100mM sodium acetate (pH 5.0)]. The flow through was discarded once again. At this stage a sterile falcon tube was securely placed under the column and 15 mls of elution buffer [1.25M NaCl, 100mM Tris-HCL (pH 8.5)] was added to the column and allowed to drain by gravity flow. Thereafter, 10.5 mls of isopropanol was added to the eluate, mixed and centrifuged at 15, 000 x *g* at 4°C for 30 minutes. The supernatant was carefully discarded, the plasmid DNA pellet was washed with 5 mls of 70% ethanol and centrifuged at 15, 000 x *g* at 4°C for 5 minutes. The ethanol was then carefully and fully removed and the DNA pellet allowed to air-dry for 10 minutes. The DNA was then dissolved in 500µLs of TE buffer [10mM Tris-HCl (pH 8.0), 0.1mM EDTA], assessed for purity and quantified.

2.9.1.5 Spectrophotometric analysis of DNA

A 1: 10 dilution of the resulting DNA (100µl DNA: 900µL TE) was prepared and analysed using a Unicam 8700 UV/VIS spectrometer at wavelengths 260nm and 280nm. The reading at 260nm allowed the calculation of the concentration of the DNA and the ratio between the readings 260-280nm (OD_{260}/OD_{280}) provided an estimation of the purity of the DNA.

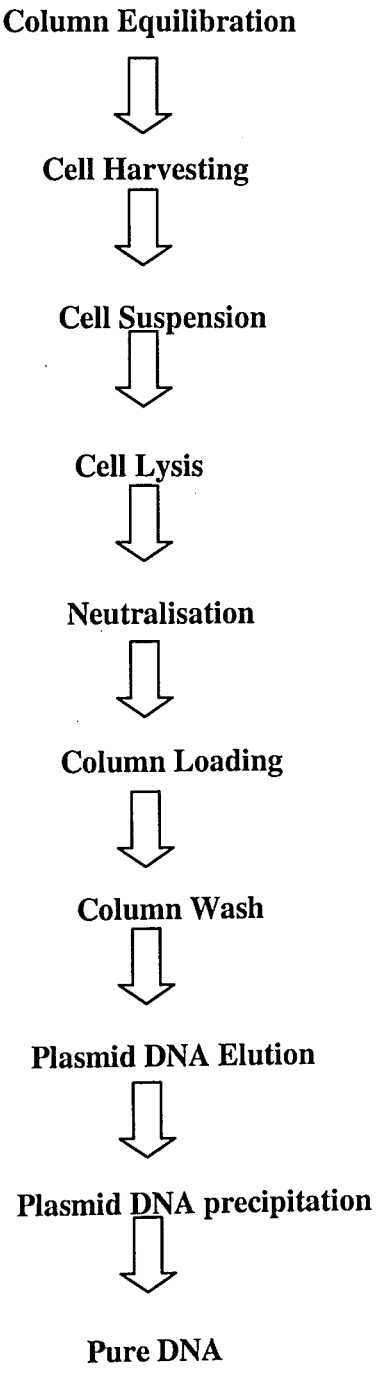


Figure 2.0: *A schematic outline of the various steps involved in plasmid purification using the CONCERT high purity plasmid purification system adapted from Life Technologies.*

2.10 Transfection of established cell lines

To establish whether the pCMV-SPORT plasmid DNA obtained in section 2.10.5 was successful, established cell lines Jurkat 6, CHO K1 and HRT18 cells were transfected using a range of cationic liposomes. Following successful transient transfection of these cells, both primary and subcultured human Fallopian tube epithelial cells were also transfected in a similar manner.

2.10.1 Transfection of adherent cells, HRT18 and CHO K1 cells.

This was carried out following the protocol described under section 2.10.5.3. Assessment of transfection efficiences using the plasmid vector pCMV-SPORT was performed by in-situ staining of β-gal or assaying the cell extracts. These procedures are described in detail under sections 2.11.1 and 2.11.3 respectively.

2.10.2 Transfection of Jurkat 6 suspension cells.

This method is essentially the same as for adherent cells. Once the DNA/liposomal complexes were prepared 50μl of cell suspension (4 x 10^5 cells/ml) was added. In order to assess the transfection efficiencies with the various liposomal reagents the cells were assayed for β-gal using cell extracts.

2.10.3 Transfection of Fallopian tube epithelial cells

2.10 .3.1 Selection of Stable transfectants using Geneticin (G418): Dose-response assay

The amount of Geneticin (G418) antibiotic required for selection purposes tended to vary due to differences in cell type, growth medium and serum concentration. A stock solution (50mg/ml), of the antibiotic was prepared as well as growth medium supplemented with FBS (15%) but without penicillin and streptomycin. The growth medium was added

equally to a 6-well plate and a volume within the range of 0-22μl of a 50mg/ml G418 antibiotic solution was added as outlined in Table 2.2.

A	0	2	4	6	8	10	12	14	16	18	20	22
B	22	20	18	16	14	12	10	8	6	4	2	0
C	0	100	200	300	400	500	600	700	800	900	1000	1100

Dose-response assay was carried out in duplicates for accuracy.

A-Volume of G418 (μl) B-*Volume of growth medium (mls)* C- *Concentration of G418 (μg/ml)*

Table 2.2: *Dose response assay to determine the concentration of G418 that caused cell death.*

A confluent monolayer of epithelial cells was trypsinized and diluted to a concentration of 4×10^3 cells/ml. To a 6-well plate, 100μl of the cell suspension was then added to each of the wells containing the 2 mls of medium and antibiotic. The plate was then incubated in a humidified incubator with 5% CO_2 atmosphere at 37^0C. The growth medium was replaced with fresh medium containing G418 twice a week for 10-14 days.

After this time, the medium was aspirated and discarded and the cells washed in PBS with calcium and magnesium. The cells were then stained with 0.5% methylene blue in 50% methanol for 15-20 minutes. Once the minimum concentration of G418 that killed the cells was determined the next higher concentration was used for the selection of stable transfectants.

2.10.3.2 Transfection and selection

Primary cultured cells and subcultured cells (P_1) in the exponential growth phase were transfected using different transfection reagents with plasmid vectors which coded for the antibiotic-resistant gene G418. Depending on the culture format used (60 mm flask, 24 well plate, 96 well plate etc.) the cells were passaged (1:3) 24 hours following transfection. After 2-3 days the cells were cultured in growth medium containing the concentration of G418 as determined by the dose response curve. This medium was changed twice a week for 14 days. Following this, any resistant colonies should be noticeable and the control cells should have died. The resistant colonies could then be fixed and stained for the SV40 large T-antigen or GFP, passage, or cloned to establish a cell line.

2.10.4 Transfection Techiques

2.10.4.1 Calcium phosphate co-precipitation technique

Cells from the exponential growth phase were seeded using growth medium into 25cm^2 tissue culture flask 24 hours prior to transfection. The cultures were placed in a 37^{0}C humidified incubator in an atmosphere of 5% CO_2. All solutions required for this process were sterilized by passage through a 0.22 micron filter (Millipore) and stored in 5ml aliquots. A solution of 250μl of 2.5M CaCl and 2μl of the pMX1 vector were diluted with 1/10 TE buffer to a final volume of 1ml. $CaPO_4$/DNA (250 μl) solution was quickly added to an equal amount of HEPES solution (x2). The mixture was incubated for approximately 20 minutes at room temperature to allow the formation of a fine precipitate and then agitated in order to re-suspend the precipitate. This suspension was then transferred onto the cell monolayer (0.5mls suspension for 5mls of medium) and the flask rocked gently to allow a thorough mixing of the medium. Fresh medium E was then added and the flasks of cells were incubated between 16-20 hours at 37°C in 5% CO_2.

The calcium phosphate/DNA co-precipitates were removed, discarded and the cell monolayer washed twice with PBS. Pre-warmed growth medium (5 mls) was added, the flasks of cells returned to the incubator until confluent. The medium was changed twice weekly and the flasks were left incubated until the presence of 'foci' was noticeable. Once these were observed, the cells were then trypsinized, centrifuged at 800RPM for 5 minutes, re-plated at high density and returned to the incubator. The cells were allowed to reach confluence and the medium changed every 2-4 days so that dead cells and other debris were removed.

2.10.5 Cationic Lipids

Cationic lipids have been shown to be suitable for the transfection of DNA into culture cells. The lipids interact spontaneously with the 'foreign' DNA to form a lipid-DNA complex (Felgner *et al.*, 1987). The fusion of the complex with the cultured cells results in an efficient uptake and expression of the 'foreign' DNA. The starting points for optimisation using various liposomal reagents are outlined in the following sub-sections and any changes are detailed in the appropriate results chapter.

2.10.5.1 Superfect reagent protocol

The day prior to transfection, primary cultured or subcultured cells (P_1) were seeded in appropriate growth medium in 35mm dishes. They were then incubated in a humidified incubator at 37^0C and 5% CO_2. In a sterile eppendorf, plasmid DNA was added to growth medium without serum and antibiotics and mixed gently. Superfect reagent was added to the DNA solution and incubated at room temperature for 5-10 minutes to allow complex formation. The growth medium from the culture dishes was aspirated and washed once in PBS. Growth medium containing serum and antibiotics was added to the DNA

transfection complexes and mixed thoroughly by gentle pipetting. This was then immediately transferred to the cells and incubated for 2-3 hours at 37°C and 5% CO_2.

Following this, the growth medium and the transfection complexes were removed by gentle aspiration and discarded. The cells were washed in PBS (x 3) and fresh cell growth medium (containing serum and antibiotics) was added and incubated as before, for 24-48 hours. Thereafter, the cells were passaged (1:3) and selective medium was added 24-48 hours post-transfection. Figure 2.1 shows a schematic representation of the method.

2.10.5.2 Effectene reagent protocol

A day prior to transfection, cells were seeded in the appropriate growth medium containing serum and antibiotics using 35mm dishes. The cells were incubated in a humidified incubator at 37°C and 5% CO_2. Plasmid DNA was dissolved with the DNA-condensation buffer (buffer EC) and enhancer was added and vortexed for 1 minute. The mixture was left at room temperature for 2-5 minutes and then spun down to remove drops from the top of the tube. The transfection reagent Effectene, was added to the DNA-enhancer mixture, vortexed for 10 seconds and incubated at room temperature for 5-10 minutes. Meanwhile, the growth medium was removed from the culture dish, the cells washed once in PBS and pre-warmed growth medium supplemented with serum and antibiotics. A volume of growth medium was also added to the tube containing the reaction mixture, mixed thoroughly 2-3 times and immediately added drop-wise onto the cells.

The cells were then returned to the incubator for 24-48 hours to allow for gene expression depending on the assay and gene used (eg GFP, β-gal). To assess for the GFP, the cells were sometimes seeded on chamber slides and transfected using the pEGFP plasmid vector and various liposomal reagents. Figure 2.2 illustrates a schematic drawing of this procedure.

2.10.5.3 Lipofectin, LipofectAMINE, Cellfectin and DMRIE-reagent

This sampler pack allowed for the comparison of four cationic lipids in order to determine which reagent is most for high efficiency transfection for HFTEC. This procedure is advantageous as it incorporates a dilution step to enable maximal transfection efficiency in the cells while avoiding problems relating to the cytostatic and cytotoxic effects caused by excessive lipid and DNA concentrations.

In a 24-well plate, HFTEC were seeded at a concentration of 3 x 10^4 cells/well in 1 ml of appropriate growth medium supplemented with serum. The cells were incubated at 37°C in a CO_2 incubator for 18-24 hours or until the cells are 60-80% confluent. A fresh 24-well plate was taken: each row was labelled A-D and each column 1-6. The plate containing the cells were labelled in a similar manner and 300μl of Opti-MEM I reduced serum medium was added to each well.

The following volumes of cationic liposomes were added into the appropriate wells as follows: 20μl of LIPOFECTIN to well A1; 10μl of LIPOFECTAMINE to well B1; 15μl CELLFECTIN to well C1; and 10μl of DMRIE-C to well D1.

The plates were incubated at room temperature for 30 minutes and for each 24-well plate and 1.5 mls of SFM containing 16μg of plasmid vector was prepared. 300μl of the DNA solution was then added to wells A1, B1, C1 and D1 and the plate was incubated at room temperature for 15 minutes.

The lipid-DNA complex was serially diluted by transferring 300μl of the solution in well A1 into A2; mix and transfer 300μl of the solution from well A2 to well A3. This was continued through to well A6, and the final 300μl of solution was aspirated and discarded from well A6. For rows B, C and D a similar procedure was carried out.

The cells were washed once with 1 ml of SFM and the lipid-DNA complex solutions (300μl) was then added to the cells. Care was taken to transfer to the corresponding wells between the two plates. Following this, the cells were then incubated for 5 hours at 37°C in a CO_2 incubator (Figure 2.3).

The DNA/lipid complex media was replaced with 1ml of growth medium supplemented with serum and returned to the incubator. After 24-48 hours, the cells were assayed for reporter gene activity depending on the plasmid used (GFP, pCMV-SPORT-β gal).

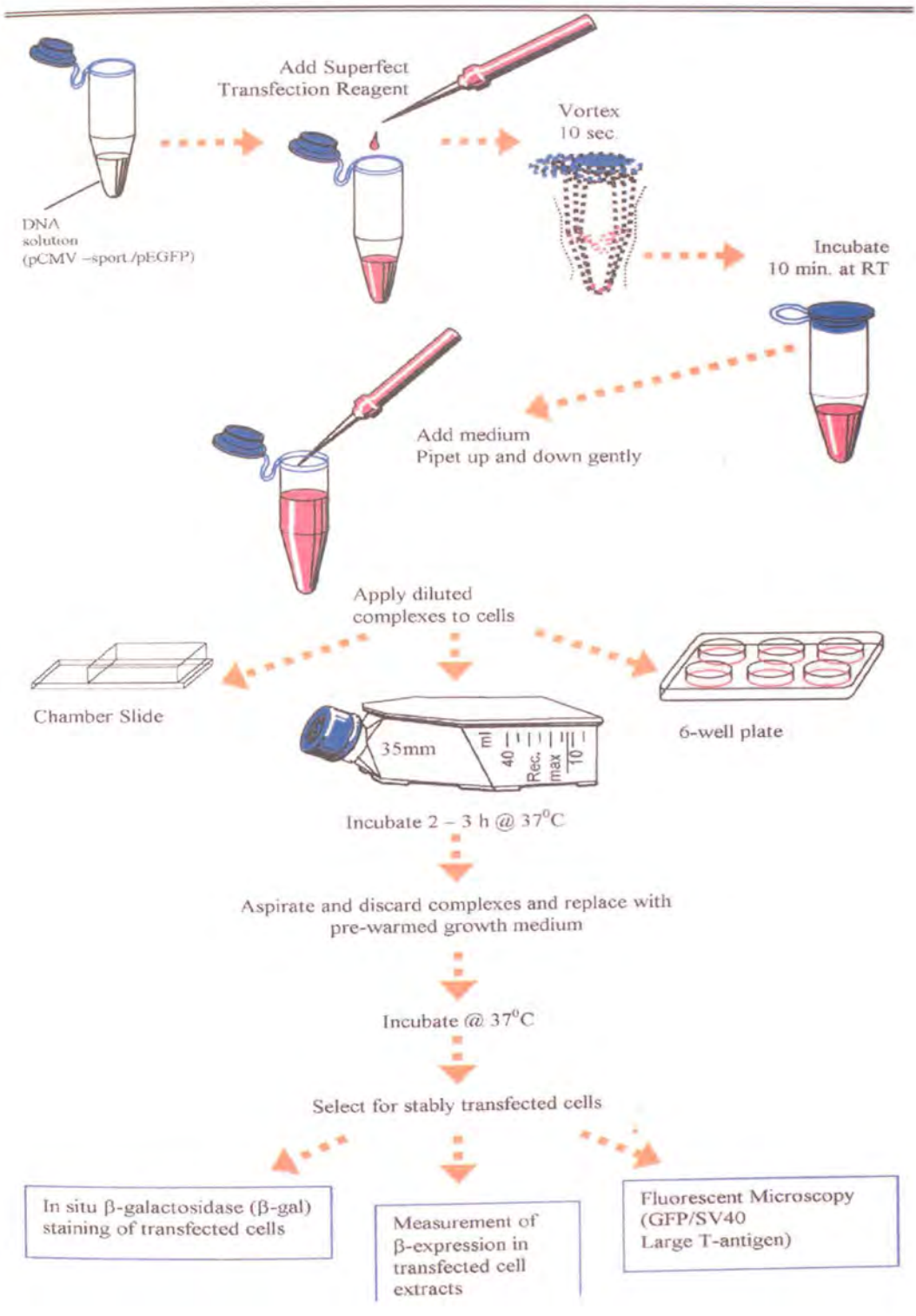


Figure 2.1: *A schematic representation showing the transfection procedure for Superfect transfection reagent.*

Adapted and Modified from The Qiagen Transfection Resource Book (1998).

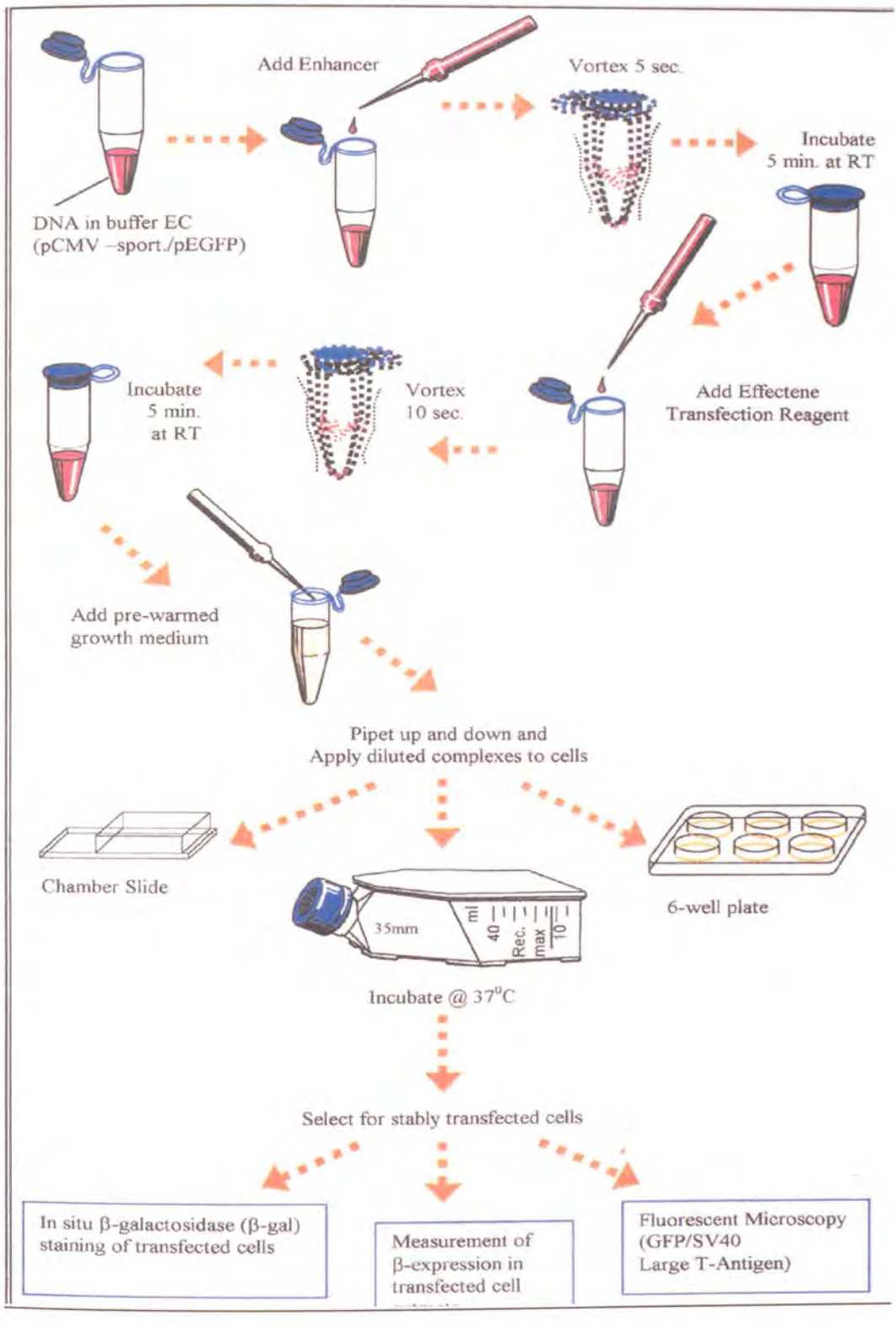
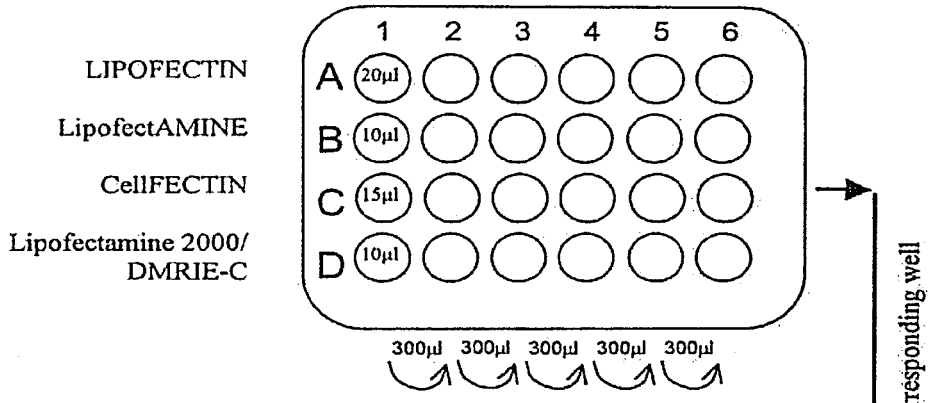


Figure 2.2: *A schematic representation showing the transfection procedure for Effectene transfection reagent.*

Adapted and Modified from The Qiagen Transfection Resource Book (1998).

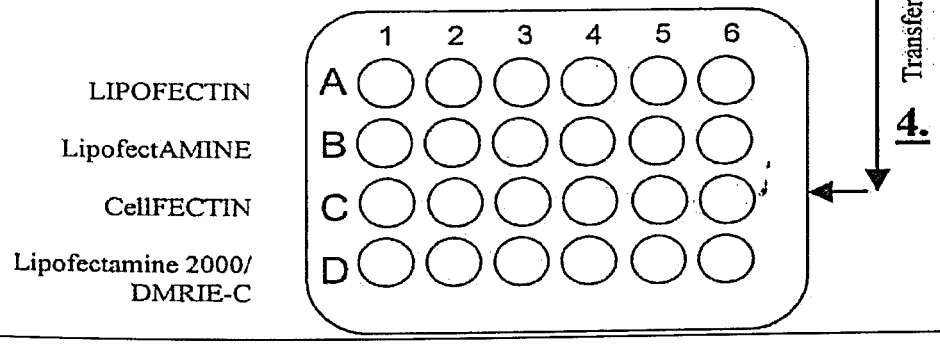
Diagram illustrates the preparation of DNA-lipid complexes and dilutions for comparative analysis of lipid reagents in an adherent cell line. Diluted DNA-lipid complex are then transferred to cells previously plated in 24-well plates.

1. Add 300µ of Opti-Mem to every well
Add cationic lipids to the corresponding wells in column No.1
Incubate 30 min. @ RT
Add DNA solution (PCMV-sport β-gal)
Incubate 15 – 30 min.



2. Transfer 300 µl to the next well in each row (2-fold dilution)

3. Plate 3 x 10^4 – 7.5 x 10^4 cells in each well the day before the transfection
Wash the cells 1 x with serum-free medium
Remove the serum-free medium



4. Transfer each lipid DNA complex to the corresponding well

Figure 2.3: *Schematic diagram showing the dilution stages for various liposomal reagents. Adapted from Life Technologies.*

2.10.5.3 Lipofectamine 2000 (LF2000)

A day prior to transfection, a confluent monolayer of primary cultured cells was trypsinised. The cells were plated in a 24-well culture dish at a concentration of 1 x 10^5 cells/well in 0.5 mls growth medium supplemented with FBS (15%). For each well of cells to be transfected, 0.8-1.0μg of plasmid DNA was diluted in 50μl of Opti-MEM I and was prepared in bulk according to the number of wells being used. For each well of cells, 1-3μL of LF2000 reagent was diluted in 50μL Opti-MEM I and incubated for 5 minutes at room temperature. Once again, this was prepared in bulk as before. Following dilution of the LF2000, the diluted DNA was added to it and left to incubate for 15 –20 minutes only to allow DNA-LF2000 reagent complexes to form. The DNA-LF2000 complexes (100μL) was directly added to each well and mixed gently by rocking the plate back and forth. The cells were then incubated at 37°C in a CO_2 incubator for 24-48 hours and then assayed for transient transgene expression (β-gal, GFP). Although it was not necessary to remove the DNA-LF200 complexes or change the medium, it was replaced with fresh growth medium without any loss of transfection activity. For stable expression of the DNA, cells were passaged 1:5 or 1:10 into fresh growth medium after 24 hours. The next day, selective medium containing G418 at an appropriate concentration was added to the wells and the medium changed twice a week. Once 'foci' were noticeable they were lifted and plated in a new culture plate, which contained fresh growth medium supplemented with serum.

2.11 Assessment of transfection

2.11.1 In-situ β-galactosidase staining of transfected cells

This procedure was adapted from (Sanes *et al.*, 1986). Prior to being assayed the cells were washed in each well once with 1 ml of PBS (containing calcium and magnesium) and discarded. The cells were fixed in 0.5 ml of fixative (PBS containing 2% formaldehyde, 0.05% glutaraldehyde kept at 4°C) for 5 minutes at room temperature. The cells in each well were washed in 1 ml of PBS (x 2). Into each well (0.5 ml) of substrate-stain solution (1mg/ml X-gal in PBS containing 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM magnesium chloride, stored at 4°C) was added to each well and the plate incubated for 2-24 hours at room temperature. Following this, the stain solution was removed, the cells were washed with 1 ml of PBS and observed under an inverted microscope in order to evaluate the proportion of blue (β-gal-positive) cells.

2.11.2 Measurements of β-gal expression in transfected cell extracts

An alternative to the staining method described above, β-gal activity in cell extracts was used to determine the activity from transfections in 24-well plates. The cells were transfected with the plasmid vector pCMV.SPORT-β-gal and incubated in 5% CO_2 at 37°C for 24-48 hours. Following this, each well was rinsed once in PBS with calcium and magnesium and 150μL of lysis buffer (0.1% Triton X-100, 0.1M Tris-HCl (pH 8.0)). The cells were lysed by freezing the plate at –70°C for at least one hour and thawed at 37°C.

The cells were pipetted up and down several times and centrifuged for 5 minutes at 9 000 x *g* in a microcentrifuge to pellet the insoluble material. The supernatant was transferred to a clean tube and stored at 20°C until assayed.

2.11.3 β-gal assay for transfected cells

β-gal standard at 10ng/μL was prepared by diluting 10μL of a 0.1mg/ml standard with 90μL of distilled water and stored on ice. Immediately prior to the assay, an ONPG cocktail with the following solutions was made up and used per well of a 96-well plate. 77μL (0.1M sodium phosphate, pH 7.5), 22μL (4mg/ml ONPG) and 1μL (100X Mg solution). A new 96-well plate was placed on ice and two columns were used for the standard curve.

0-7μL of the 10ng/μL βgal standard was added to eight wells and the cell extract (8-50μL) was added to the remaining wells. With the plate on ice, ONPG cocktail (100μL) was added to each well and incubated for 5-20 minutes at 37°C. The plate was checked every five minutes for the development of a yellow colour, although, the negative control wells which contained 0μL of the β-gal standard were colourless whilst those with 7μL of β-gal standard were a bright coloured yellow. Care was taken not to allow the plate to over-develop but instead a gradient of yellow colour was noticeable in the standard wells. At this stage, 150μL of a 1M sodium carbonate solution was added to each well and the absorbance measured at 405nm.

Larger volumes of cell extracts were assayed, by increasing the concentration and decreasing the amount of the ONPG cocktail used in the assay above, in order to maintain the final volume of 100μL. For example, for 30μL of cell extract, the ONPG cocktail was prepared as follows/well of a 96-well plate: 47μL (0.1M sodium phosphate solution), 22μL (4mg/ml ONPG) and 1μL of a (100X Mg solution). 100μL of a 5ng/μL β-gal standard was

prepared by diluting 5 μL of the 0.1mg/ml standard with 95μL of distilled water and store on ice.

Samples for a standard curve was prepared by adding 30μL/well of lysis buffer to the wells dedicated to the wells of a 96-well plate (eg rows 1-2 of columns A -H). To this, an appropriate volume (Table 2.3) of 5ng/μL β-gal standard was added accordingly. The β-gal assay was then carried out as described above.

β-gal (10ng/uL)	β-gal standard (μL)
0	0
10	1
20	2
30	3
40	4
50	5
60	6
70	7

Table 2.3: *Preparation of standard curve for β-galactosidase.*

3.1 INTRODUCTION

Cell to cell communication via gap junctions plays a vital role in embryogenesis and the maintenance of differentiated phenotype (Guthrie and Gilula, 1989; Warner, 1992). These gap junctions are specialised cell junctions which are found on the cell membranes of adjacent cells and permit the passage of small molecules between communicating cells. They are formed from a closely-related family of proteins called connexins (cx) which show tissue-specific patterns of expression (Monaghan and Moss, 1996) and are designated on the basis of their molecular weight. In the past, one of the primary methods used to detect the presence of gap junctions in tissues was by freeze-fracture. The gap junctions could be recognised from a distinctive pattern on both fracture faces provided the fractures had cleaved the intercellular space. In addition, ultra thin sections could be viewed under transmission electron microscopy but the gap junctions could only be seen if the junction ran perpendicular to the orientation of the section. In recent years however, a number of connexin-specific antibodies have been developed which allowed the identification of different connexin molecules that may be present in different cellular locations. The three commonly found and studied connexins are cx26, cx32 and cx43.

It has been shown that the expression of connexins is regulated by ovarian hormones in the female reproductive tract of rodents (Grummer *et al.*, 1999). In order to determine whether these hormones also affect the connexin expression in the human Fallopian tube, the presence and distribution patterns of cx26, cx32 and cx43 were investigated. This was done by immunohistochemical staining of paraffin-wax embedded and frozen sections of the fimbrial and ampullary regions of the tube, which were collected at various stages of the ovarian cycle. Western blotting was also used to identify connexins that may be present in the Fallopian tube.

This technique was first described by Towbin e*t al.*, (1979) and is sometimes referred to as immunoblotting. Essentially, this procedure involves the separation of polypeptides in gels followed by the electrophoretic transfer of the separated polypeptides to an immobilising matrix or membrane such as nitrocellulose (Burnette 1981). In this way, a 'replica' of the polypeptide separation profile from the gel electrophoresis step is created on the membrane, which can then be probed by cx (connexin) antibodies to identify specific polypeptide(s).

3.1.1 Objectives of the Study

The principal objectives of this study were:

1). To determine the presence of the gap junction proteins, connexins in the fimbrial and ampullary regions of the Fallopian tube throughout the ovarian cycle.

2). To quantify the expression levels of connexin 26, 32 and 43 in the ampullary and fimbrial regions of the Fallopian tube throughout the ovarian cycle.

3.2 METHODS

Briefly, Fallopian tubes were obtained from 10 women aged 31-67 following ethical approval and patients' consent, during a total abdominal hysterectomy for non-malignant gynaecological disease. The fimbrial and ampullary regions were separated, rinsed in PBS and immersed in liquid nitrogen. A venous blood sample was taken for each patient in this study and the plasma was subsequently analysed to determine the hormonal status of the women. Hormonal assays to detect oestradiol, progesterone, follicle stimulating hormone and luteinising hormone levels were carried out at the Biochemistry department at Newham

General Hospital. In addition, the stage of the ovarian cycle was assessed by histological dating of the endometrium.

3.3 RESULTS

3.3.1 Immunostaining of paraffin-wax embedded sections.

No staining was observed for cx26, cx32 and cx43 in the paraffin-wax embedded tissue sections of the fimbrial and ampullary regions. Table 3.0 highlights the various protocols used for antigen retrieval but despite these attempts, there was no indication that connexins were present.

3.3.2 Immunostaining of frozen sections.

No staining was observed for cx26, cx32 and cx43 in the frozen tissue sections of the fimbrial and ampullary regions examined.

Region	Antigen Retrieval System Used	1^0 Ab incubation time (dilution factor)	2^0 Ab incubation time (dilution factor)	Comment
A + F	none	60 minutes (1:1000)	60 minutes (1:250)	ns
A + F	none	90 minutes (1:1000)	60 minutes (1:250)	ns
A + F	Boiled in citric acid buffer	60 minutes (1:500)	60 minutes (1:500)	ns
A + F	Microwave	60 minutes (1:500)	60 minutes (1:500)	ns

Table 3.0: *Table Showing the Various Antigen Retrieval Procedures Used on Paraffin-Embedded Tissue Sections. A= ampulla, F= fimbria. ns= no staining*

3.3.2 Western Blotting of connexins

Positive results were obtained for cx26 and cx43 when homogenates of both the fimbrial and ampullary regions were blotted and probed with anti-connexin primary antibodies. However, in all cases, cx32 was not observed in either the fimbrial or ampullary region of the Fallopian tube.

3.3.3 Quantification of Cx26 and Cx43

Using scanning densitometry, the intensity of bands obtained from western blots was analysed. This provided a quantitative estimate of connexin (cx) 26, 32 and 43 expression within different anatomical regions of the tube examined in this study. Rat brain homogenate was used both as a positive control and standard.

3.3.4 Expression of connexin 26 in the ampullary region of the Fallopian tube.

Connexin 26 (cx26) was present in quantifiable amounts in all the samples investigated with the exception of two post-menopausal cases. Overall, there appeared to be a gradual increase in the expression of Cx26 from the early proliferative stages of the ovarian cycle and the early secretory phase appeared to have the highest expression of cx 26 (Figure 3.1). The level of staining intensity according to scanning densitometry analysis ranged from 26% for the early proliferative stage to 63% for a case in the early secretory stage. Following the latter stage, an apparent decrease in the staining intensity was noted for a patient at the mid-secretory stage.

3.3.5 Expression of connexin 43 in the ampullary region of the Fallopian tube.

Connexin 43 (cx43) was present in quantifiable amounts in all the cases examined. However, no particular trend was noticed in these cases. cx43 expression appeared to be highest both at the mid-cycle and mid-secretory stages at 72.5% and 71.3% respectively (Figure 3.2). Overall, the expression of cx43 seemed lower throughout the ovarian cycle compared to that observed for cx26 in the same region. In addition, cx43 was also expressed by the post-menopausal specimen with a staining intensity of 33%.

3.3.6 Expression of connexin 26 in the fimbrial region of the Fallopian tube.

Connexin 26 (cx26) was present in quantifiable amounts in all the samples investigated with the exception of a post-menopausal specimen. Overall, there seemed to be gradual increase in the expression of cx26 from the early proliferative stages of the ovarian cycle with the highest level noted at mid-cycle (Figure 3.3). At this point onwards the expression levels of Cx26 in the fimbrial region decreased.

3.3.7 Expression of connexin 43 in the fimbrial region of the Fallopian tube.

Connexin 43 (cx43) was present in quantifiable amounts in all the cases examined. However, comparatively low expression levels were noted both in the early and mid proliferative stages. There appeared to be an increase throughout the first half of the ovarian cycle, up until the late proliferative stage. This was then followed by a decrease in expression at mid-cycle but then increased once again at the early secretory stage before once again decreasing in expression levels (Figure 3.4).

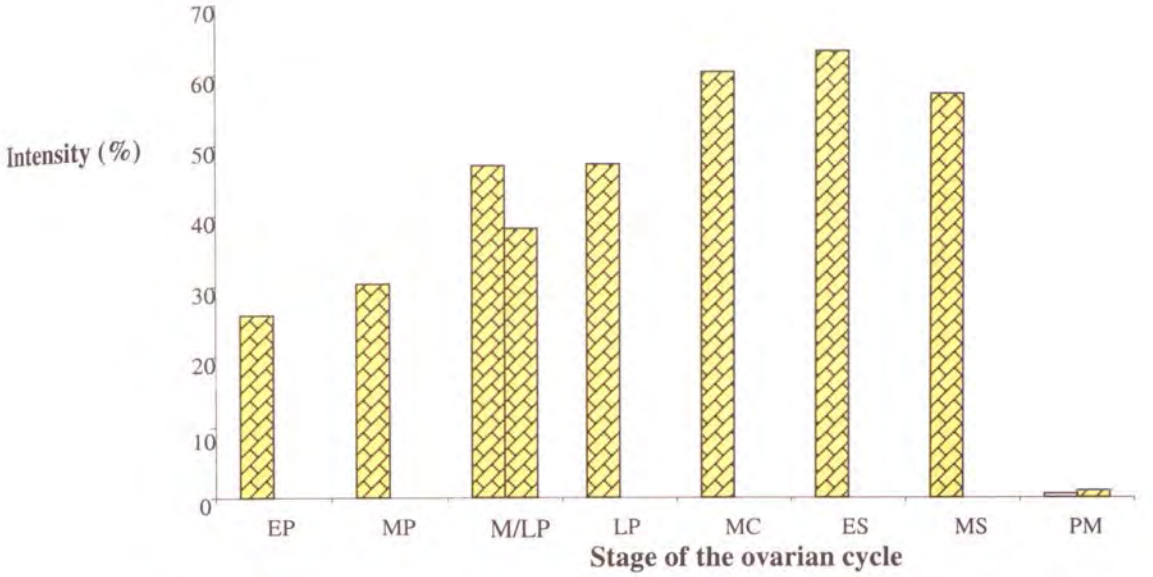


Figure 3.1: *The expression of connexin 26 (cx26) in the ampullary region of the Fallopian tube. EP (early proliferative), MP (mid proliferative), M/LP (mid/late proliferative), LP(late proliferative), MC (mid-cycle), ES (early secretory), MS (mid secretory) and PM (post-menopausal).*

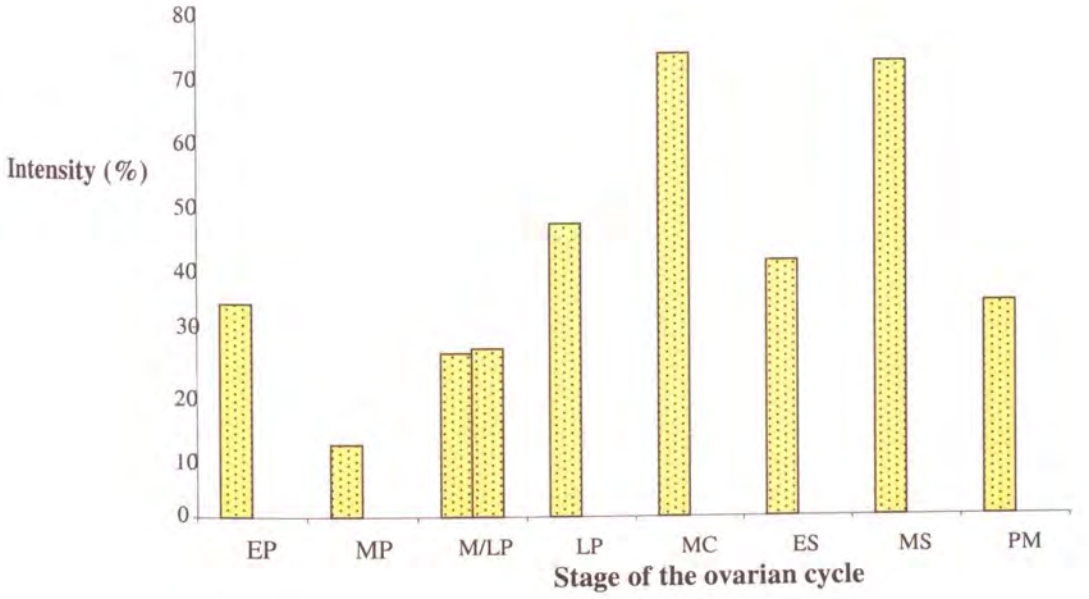


Figure 3.2: *The expression of connexin 43 (cx43) in the ampullary region of the Fallopian tube. EP (early proliferative), MP (mid proliferative), M/LP (mid/late proliferative), LP(late proliferative), MC (mid-cycle), ES (early secretory), MS (mid secretory) and PM (post-menopausal).*

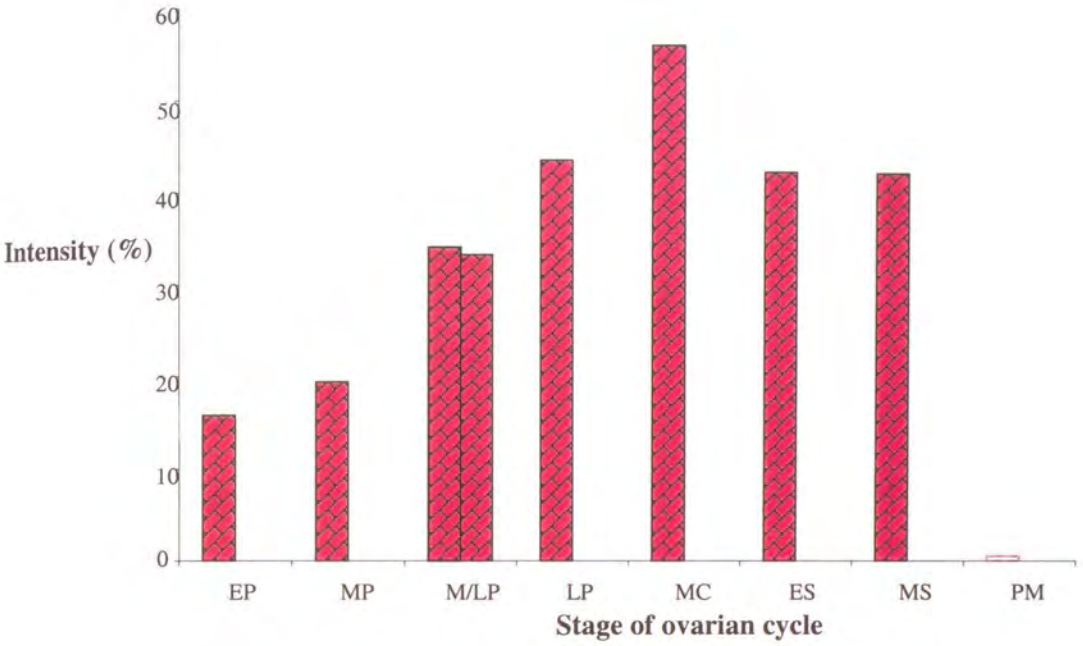


Figure 3.3: *The expression of connexin 26 (cx26) in the fimbrial region of the Fallopian tube. EP (early proliferative), MP (mid proliferative), M/LP (mid/late proliferative), LP(late proliferative), MC (mid-cycle), ES (early secretory), MS (mid secretory) and PM (post-menopausal).*

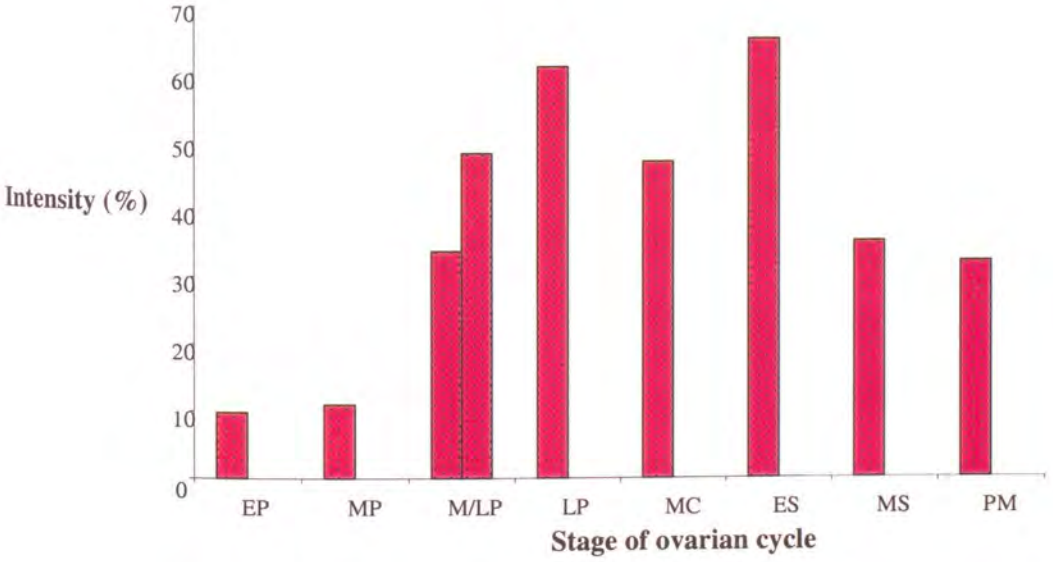


Figure 3.4: *The expression of connexin 43 (cx43) in the fimbrial region of the Fallopian tube. EP (early proliferative), MP (mid proliferative), M/LP (mid/late proliferative), LP(late proliferative), MC (mid-cycle), ES (early secretory), MS (mid secretory) and PM (post-menopausal).*

3.4 Discussion

The Fallopian tube plays a crucial role in human reproduction and is the site of gamete transport, fertilisation and early embryonic development. Indeed, it is a specifically timed sequence of events, which permit both sperm and ova to travel in opposite directions, interact with each other and develop, prior to being transported to the uterine cavity at an appropriate time. Both the musculature and ciliary activity are important factors in this regard which is not surprising, as the Fallopian tube epithelium is one of the hormonal targets and these cells show morphological alterations throughout the ovarian cycle (Jansen 1984).

In this study, the expression of cx26, cx32 and cx43 antigenic receptors in the fimbrial and ampullary regions of the human Fallopian tube at different stages of the ovarian cycle was demonstrated for the first time. Immunocytochemistry provides important information on the localization of antigens in both cells and tissues. Several attempts using various antigen retrieval procedures in this study to prepare tissues for immunocytochemical labelling have proved futile. It seems apparent that the cx antigens were 'masked' in the formalin fixative since it was quite difficult and impossible to detect following immunolabelling. Although, antigen retrieval procedures are widely used with paraffin-embedded material, on this occasion they did not seem to have unmasked the cx antigens effectively. Furthermore, connexins are probably expressed at specific times at short intervals and it is possible that they may not have been present at the time of sectioning. Although, microwaving is another commonly used method it may have destroyed the delicate gap junction structures.

To date there is very limited knowledge regarding the presence and roles of connexins in the human Fallopian tube. However, our findings in this study suggest that the expression of cx26 and cx43 is primarily under the influence of the steroid hormones.

In the cases studied, the expression of cx 26 and 43 appeared the highest at the mid secretory stage in the ampullary region. Perhaps, this may indicate a period whereby the ovum is temporarily in a 'resting' or 'maturation ' stage of development, prior to the process of fertilisation. Studies carried out by Croxatto *et al.*, (1978) and Diaz *et al.*, (1980) have shown that the ovum transport is arrested at the ampulla following the luteinising hormone peak.

A study carried out by Grummer *et al.*, (1996), in the endometrium, suggested that successful implantation in mammals require an intimate interaction between the embryo and the uterus. Hence direct cell to cell communication via gap junctions may play an important role in the preparation of the uterus prior to embryo implantation and eventual trophoblast invasion. Investigators have also demonstrated that during implantation in rodents and humans, both gap junction proteins, cx 26 and 43 are suppressed. Furthermore, it was suggested that this loss of cell to cell communication seems to be vital in preparing the endometrium into the receptive phase where there is a concomitant increase in maternal progesterone (Jahn *et al.*, 1995; Grummer *et al.*, 1996). However, as the invasive stage progressed the expression of cx 26 and cx43 gradually increased.

The muscle layer of the oviduct is in part responsible for the synchronised contractile activity and may be coordinated by gap junction intercellular communication between cells present in the muscle layer. Furthermore, the mucosal layer, composed primarily of epithelial cells may concomitantly be responsible for the synchronised ciliary activity, which may also be regulated by these gap junctions between epithelial cells. A study carried out by Hermoso *et al.*, (1997) demonstrated that the content of both cx26 and cx43 in the oviduct depended on the cell type, state of maturation and hormonal status. They also observed the both the epithelial and smooth muscle cells of immature rat oviducts (<30 days) contained a low amount of cx43 whilst cx26 was undetectable. In contrast, in mature oviducts (>30 days), cx26 was detected only in the isthmus and localised regions of the ampullary epithelial layer. The authors postulated that the high content of connexins found between oviductal cells as well as their high responsiveness to hormone regulation, suggest that gap junctions might be involved in co-ordinating oviductal cell functions such as smooth muscle contraction and epithelial ciliary beat frequency.

In summary, we have demonstrated that both cx 26 and 43 are expressed in the different anatomical regions of the Fallopian tube, though the intensity of the expression sometimes seem to vary with the ovarian cycle. It appears that cx32 is not expressed in the human Fallopian tube but it is quite possible that others may be present.

In view of the low number of patients at the various stages of the ovarian cycle in this study it is difficult to make any precise conclusion or define any statistical importance. Hence, this was more a preliminary study and further studies are definitely warranted in order to establish the exact role(s) of various connexins in the Fallopian tube. It has already been demonstrated that cx37 (KO) deficient mice are infertile and hence it will be

interesting to see whether the lack of a particular connexin(s) in the human Fallopian tube is responsible for some causes of infertility and even ectopic pregnancies. For obvious reasons, gene knockout experiments (KO) cannot be carried out on humans and hence experimental studies in both normal and abnormal Fallopian tubes are necessary in order to enhance the understanding of their role(s) both in the fertile and infertile couple.

4.1 INTRODUCTION

In an attempt to reduce existing gaps in the literature, this study examined mechanical and enzymatic methods of obtaining tubal epithelial cells and the effect(s) of these two isolation procedures on cellular growth patterns and morphology. In addition, while it seems plausible that the secretory activity of the HFTEC contributes to the provision of a suitable environment in the event of a pregnancy, it is important to establish whether this setting can be maintained *in vitro* when fresh or frozen-thawed HFTEC are used. If frozen-thawed cells provide similar results, then these can be stored as a stock and used when required.

Some investigators used fresh cells (Bongso *et al.*, 1992; Kervancioglu *et al.*, 1994; Kervancioglu *et al.*, 2000), while others employed frozen-thawed cell in co-culture experiments (Wetzels *et al.*, 1991; Pearlstone *et al.*, 1993). For this reason, it is necessary to observe whether the secretory function of cells isolated by the mechanical and enzymatic methods differ significantly from each other when used either as fresh or frozen-thawed cultures.

4.1.1 Objectives of the Study

The principal objectives of this study were:

1). To isolate human Fallopian tube epithelial cells using a mechanical and enzymatic method.

2). To determine the isolation procedure that would maintain both the morphological and functional characteristics of Fallopian tube epithelial cells *in vitro* by comparing cells isolated by enzymatic and non-enzymatic (mechanical) methods.

4.2 METHODS

An in-depth report on the various methods employed to obtain the results of this chapter are presented in chapter 2. The tubes of forty-three patients were used in experiments carried out in this experimental chapter. Tissue samples were obtained from women undergoing sterilisation or hysterectomy for benign conditions, after informed consent and the local ethical committee approval.

4.3.1 Isolation of Fallopian tube epithelial cells obtained by mechanical and enzymatic methods.

Cellular growth in culture was noted from mechanically isolated explants after 5-7 days, whereas, enzymatically (pancreatin-trypsin) isolated cells exhibited proliferation after 2-3 days (figures 4.1a,b). Both ciliated and non-ciliated tubal cells were observed regardless on the method of isolation. However, enzymatically (pancreatin-trypsin) isolated cells proliferated at a much faster rate (1-2 days) than mechanically isolated cells (3-4 days) but nevertheless they both eventually formed a relatively homogenous confluent layer consisting of polygonal cells with a centrally located nucleus (Figure 4.0c and 4.0d). Irrespective of the method employed, ciliary activity was observed in all primary cultures on the day of isolation. All tissue explants (mechanical isolation) in the culture vessel demonstrated rampant beating of the cilia. Similar observations were noted for tubal cells isolated enzymatically, though in this case the majority of cells were in small groups (5-15 cells) or as individually floating cells. Within 24-72 hours small clusters of isolated cells began to adhere and proliferate on the culture flasks thereby forming small round colonies. The cells that did not attach to the culture flasks simply continued to float around in the vessel either singly or in aggregates. Furthermore, following the initial seeding, ciliary activity was also observed in primary cultures up to 5-6 days in mechanically isolated cells and on days 1-3 for enzymatically isolated cells. However, overall, such activity diminished as the cells became attached to the base of the culture vessels.

Cells from the Fallopian tube were also isolated using pancreatin and dispase (pancreatin-dispase). Using haemocytometry, the number of cells obtained was greater

than those yielded by mechanical means but was not as high as those obtained with pancreatin and trypsin (pancreatin-trypsin). Nevertheless, these tubal epithelial cells could be sub-cultured for up to 5-8 passages.

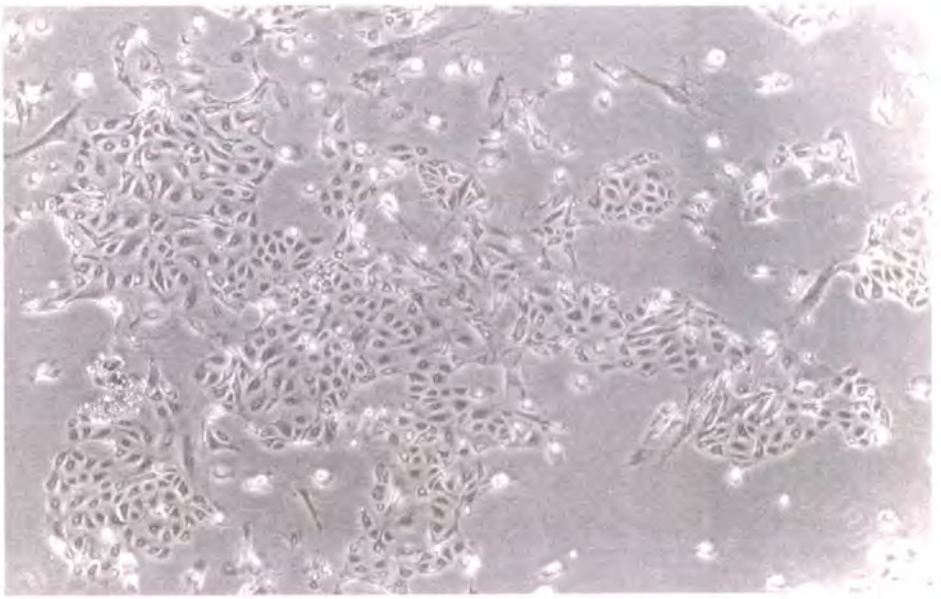


Figure 4.0a: *Phase-contrast micrograph of tubal epithelial cells isolated mechanically (day 5). Original Mag (x75)*

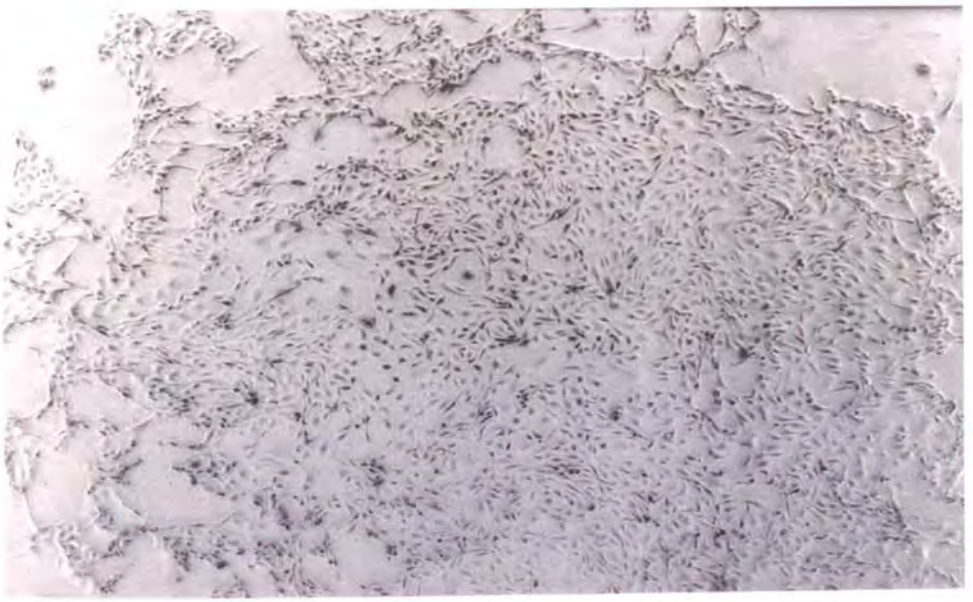


Figure 4.0b: *Phase-contrast micrograph of tubal epithelial cells isolated enzymatically (day 5) using Pancreatin-Trypsin. Original Mag (x75)*

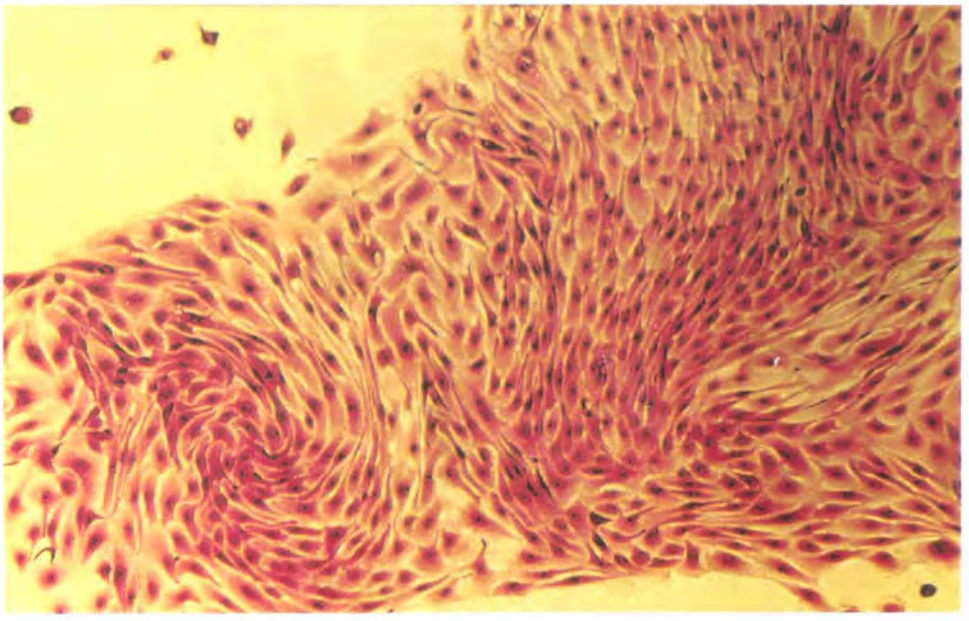


Figure 4.0c: *Confluent layer of Fallopian tube epithelial cells (day 8) isolated mechanically and stained with fushin and eosin. The cells were morphologically similar and appear quite uniform and cohesive with distinct cell boundaries. Original Mag (x100)*

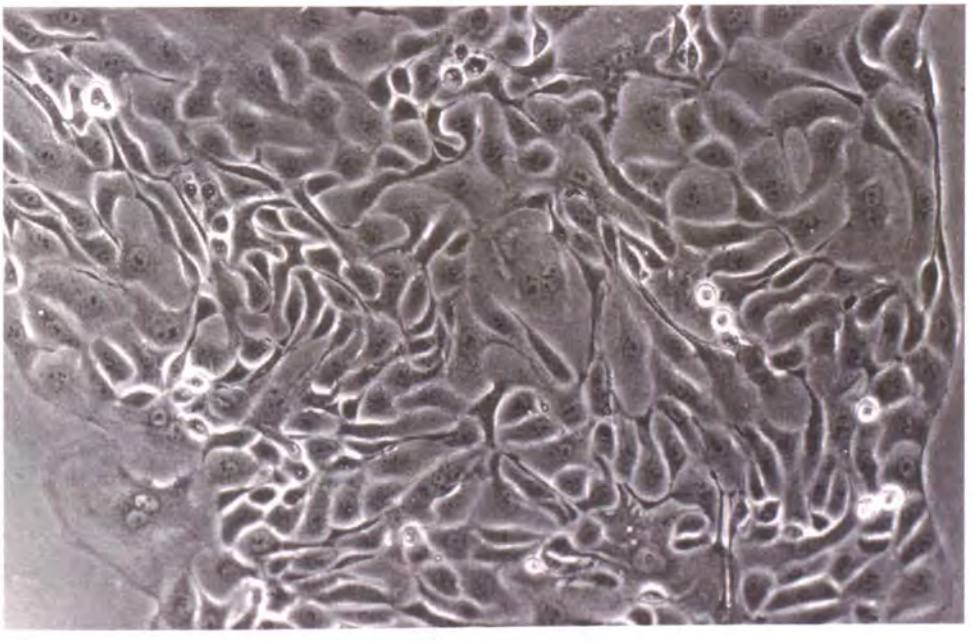


Figure 4.0d: *A confluent monolayer of enzymatically (P-T) isolated Fallopian tube epithelial cells at day 4. Original Mag (x 100)*

Enzymatically (pancreatin-trypsin) isolated cells resulted in a high number ($>10^5$) of epithelial cells initially. However, they did not survive sub-culturing for more than 2-3 passages. At low magnifications the cellular morphology appeared normal but examination at higher magnifications revealed that these cells possessed an irregular granular surface with numerous vacuoles. Such morphological appearances were regarded as evidence of cellular senescence. In contrast, mechanically isolated cells resulted in a lower cell number both at primary and sub-cultures but survived sub-culturing for over 8 passages.

Mechanically isolated cells did not appear vacuolar nor did they display irregular granular surfaces similar to that observed for enzymatically isolated cells. As such, it would seem that the former were more resistant to sub-culturing than enzymatically (pancreatin-trypsin) isolated cells, since the cells isolated mechanically continued to show signs of regeneration over 8 passages.

4.3.2 Cytokeratin staining in tubal epithelial cells.

Irrespective of the isolation method employed, both primary cultured and sub-cultured cells were positively stained for cytokeratins thereby confirming their epithelial originality (Figure 4.1). In all instances, a negative control was used whereby the primary antibody was omitted.

Immunofluorescent staining was also carried out on sub-cultures after passage 2-3 for cells isolated enzymatically and up to 9 paasages for cells isolated mechanically. This was carried out to ensure that the cells retained their epithelial origin following several passages.

These sub-cultured cells displayed a similar appearance to those cells in Figure 4.0c in that they were polygonal in shape with a centrally located nucleus and possessed numerous intermediate filaments, cytokeratins (Figure 4.1).

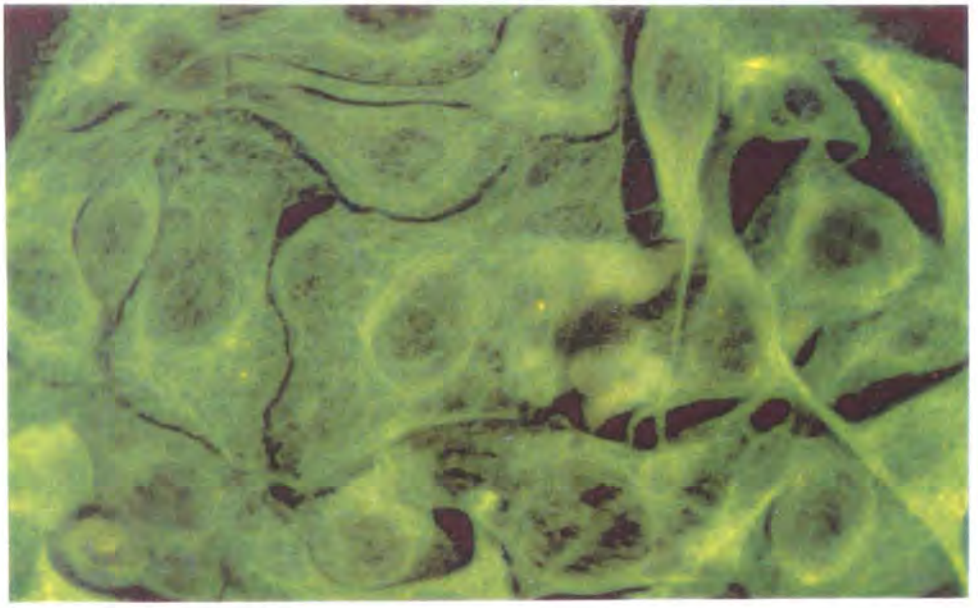


Figure 4.1: *Immunostaining of cultured Fallopian tube epithelial cells at day 5 for cytokeratins. Original Mag (x400)*

4.3.3 Structural integrity of tubal epithelial cells isolated by mechanical and enzymatic methods.

To confirm the integrity of the cell membranes both in primary cultures and sub-cultures, a mixture of propidium iodide (PI) and Hoechst 33358 was added to a sample of the cells after the first and third passage during their growth period. Generally, primary cultures of cells isolated using the mechanical method generally maintained intact cell membranes, which was indicated by the blue nuclear fluorescence (Figure 4.2a and 4.2b). However, as the number of passages increased during sub-culturing, the cells began taking in the PI, which was indicative that the cell membranes had leaked.

This was observed by the bright red fluorescence when viewed under a fluorescent microscope. At confluence, primary cultures of Fallopian tube epithelial cells isolated enzymatically had >98% of their cells stained with PI (Figure 4.2c) however these cells did not die but continued to divide when passaged and sub-cultured.

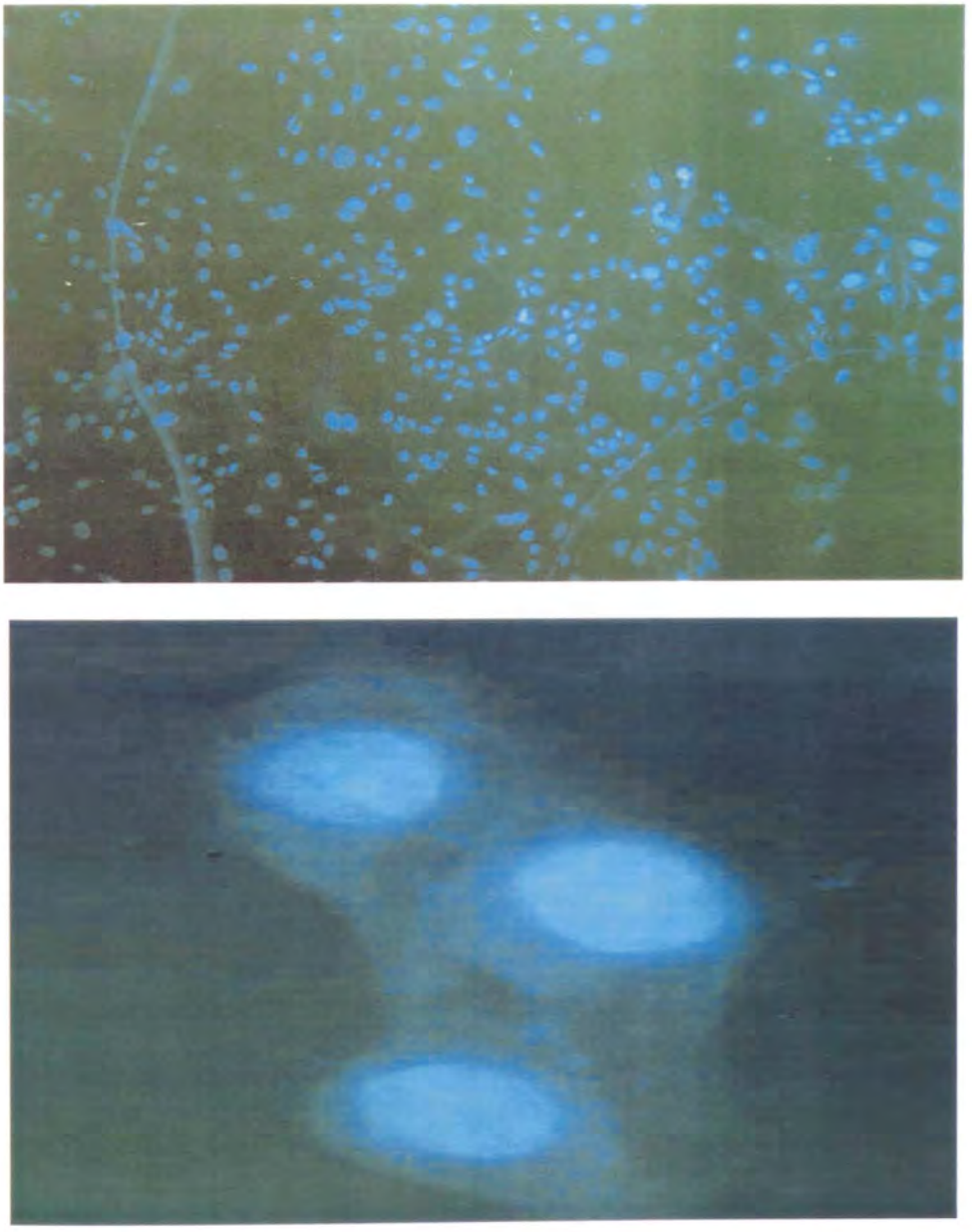


Figure 4.2a and 4.2b: *Light micrograph of a primary culture of epithelial cells isolated mechanically. The cell nucleus has absorbed the Hoechst 33358 stain indicative of an intact cell membrane. Original Mag (x100) and (x 400)*

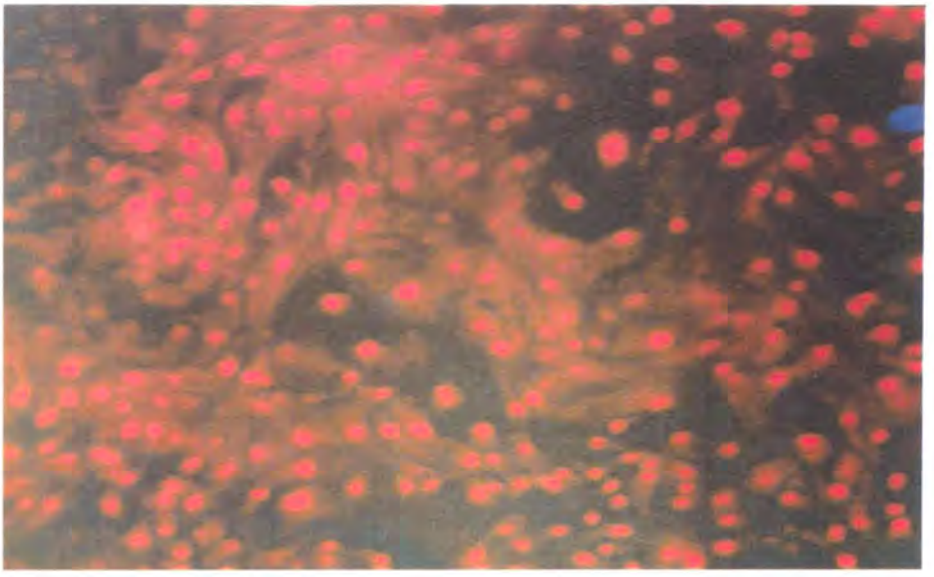


Figure 4.2c: *Light micrograph of an enzymatically isolated primary culture. Although the cells continue to proliferate when sub-cultured the cell membranes have been damaged as observed by the intake of propidium iodide (red) dye. Orginal mag (x 100)*

4.3.4 Frozen-thawed tubal epithelial cells.

Frozen-thawed epithelial cells took between 6-10 days to achieve confluence and their epithelial origin was confirmed by positive immunocytochemical staining for cytokeratins. However, on at least two separate occasions these cells were treated with a mixture of PI and Hoechst 33358 to determine whether the cell membrane integrity was compromised. In both instances, > 99% of the cells showed membrane damage (similar to that shown in Figure 4.2c).

4.3.5 Karyotypic stability of tubal epithelial cells in primary and sub-cultured cells.

Both primary and sub-cultured cells isolated by mechanical and enzymatic methods were analysed karyotypically to ensure that they retained the diploid characteristic. The chromosomes were analysed by adding Colcemid, (a spindle inhibitor that arrested the

dividing somatic cells in metaphase) 48-72 hours post culture. A hypotonic solution of potassium chloride (KCl) was added to allow nuclear swelling and the eventual rupture thereby allowing the separation of individual chromosomes (Figure 4.3). Chromosomal spreads of at least 50 cells were obtained from both primary and sub-cultures of epithelial cells isolated by mechanical and enzymatic techniques. Regardless of the method of isolation used, the chromosomal status never deviated from the normal 23 pairs.

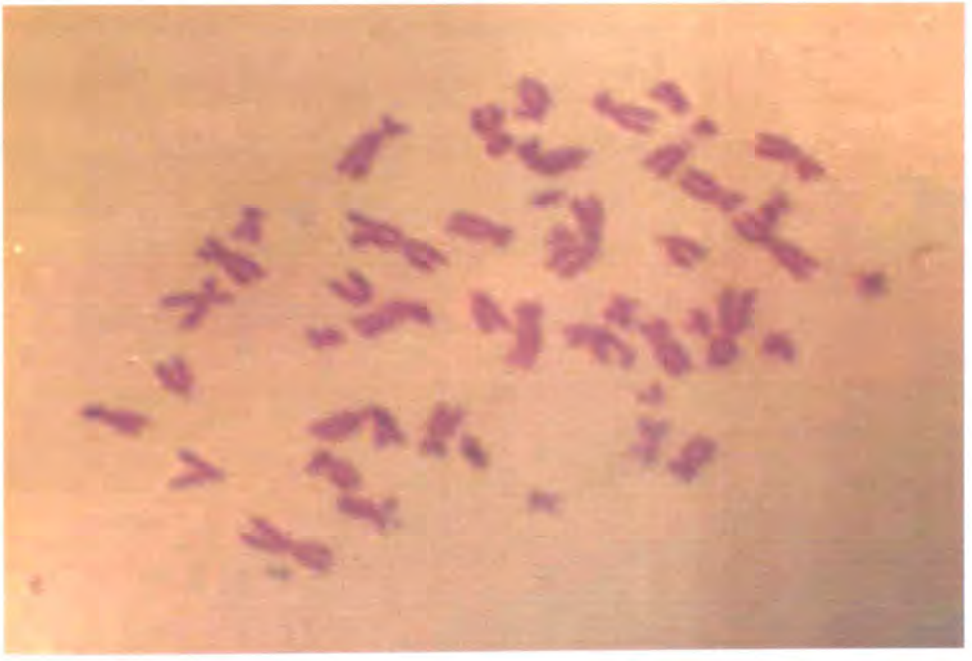


Figure 4.3: *A Giemsa-stained chromosomal spread of a Fallopian tube epithelial cell during metaphase. At this stage the chromosomes are highly contracted and each chromosome can be seen to consist of two identical chromatids joined at the centromere. Original Mag (x 100)*

4.3.6 Growth patterns of human fallopian tube epithelial cells obtained by mechanical and enzymatic methods.

Following isolation and confirmation that the cells isolated by both the mechanical and enzymatic methods were epithelial in origin, their rate of growth in culture was assessed. There were vast variations observed in terms of cell growth during the culture period and although tubal epithelial cultures appeared to be 'slow growing' at the beginning, their growth actually accelerated after day 4. Irrespective of the isolation method, a high seeding density ($>10^5$) was required in order to achieve a successful culture simply because lower cell concentrations normally failed to establish a healthy culture.

The Student t-test was used to analyse the data, in order to determine whether there were any significant difference(s) with respect to the cell number obtained from mechanically and enzymatically isolated cells in culture. The data confirmed that, with both isolation procedures there exist an exponential relationship between the number of days in culture (x) and the cell growth (y) in the first 8 days. However, in terms of cell yield/unit time, the enzymatic method provided a higher yield than the mechanical method (Figure 4.4). No further increase in cell number was found after day 8 though the cells remained viable for a further ten days after which the monolayer exhibited 'lifting off' from the base of the flasks (data not shown).

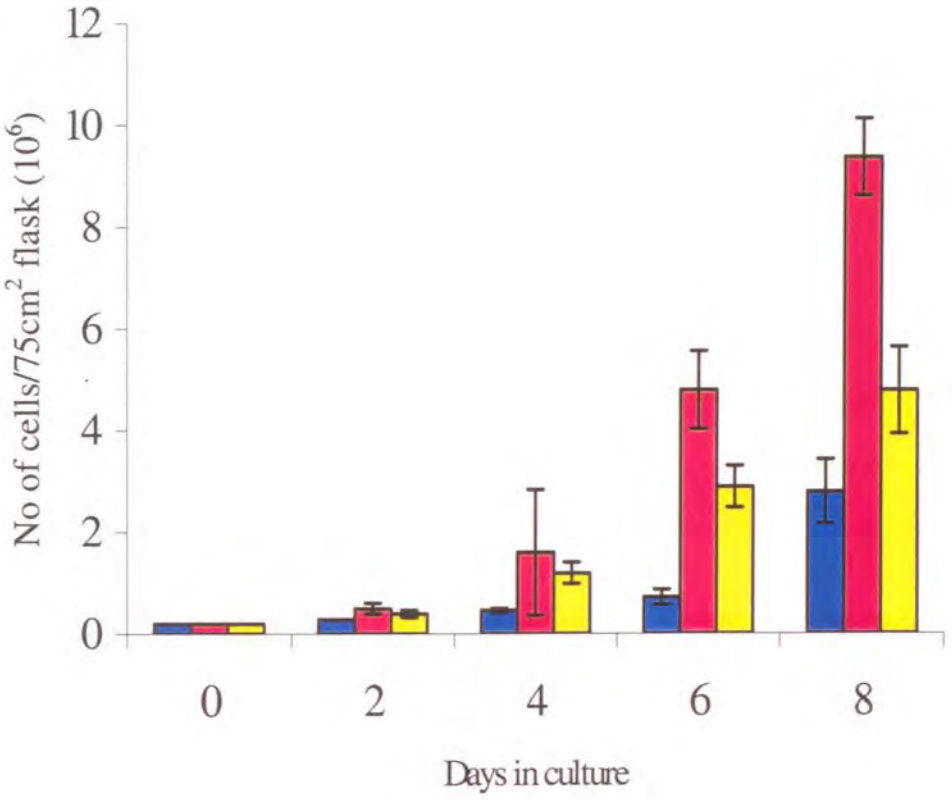


Figure 4.4: *The growth patterns of human Fallopian tube epithelial cells isolated by the mechanical method of fine mincing* ■ *the enzymes pancreatin/trypsin* ■ *and pancreatin/dispase* ■ *. The cell number at day 0 (day of seeding) was 1 x 10^5 cells/flask. The values represent the mean (n=9)± SEM. P>0.05.*

4.3.7 Total protein secretion of Fallopian tube epithelial cells isolated by mechanical and enzymatic methods

Although a similar number of cells (7 x10^5) were added to each culture flask, there was a decrease in the total protein secretion (TPS) observed from enzymatically isolated cells compared to those cells that were isolated mechanically (Figure 4.5). Frozen-thawed epithelial cells that were isolated either by mechanical and enzymatic methods had a reduction in the total protein secreted as compared to freshly isolated epithelial cells isolated in a similar manner. This strongly suggests, that freezing has a detrimental effect on the secretory properties of the cells.

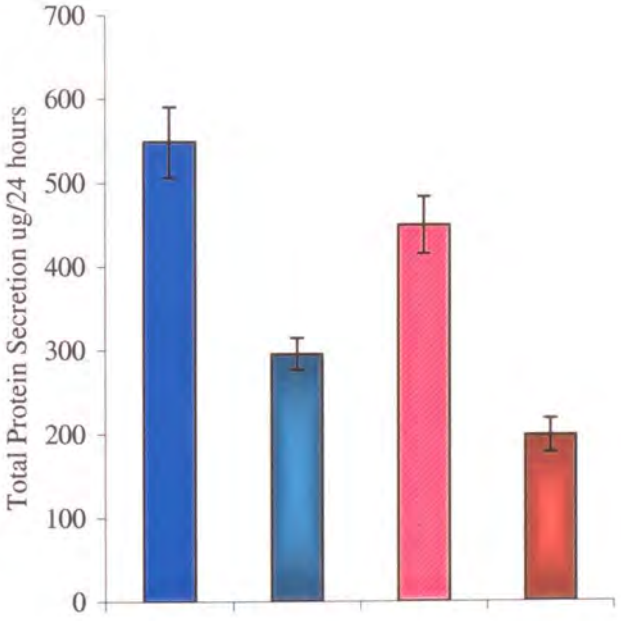


Figure 4.5: *Total protein secretion (μg/mL) from primary cultures of fresh and frozen-thawed Fallopian tube epithelial cells. Mechanically isolated fresh cells mechanically isolated frozen-thawed cells enzymatically isolated fresh cells and enzymatically isolated frozen-thawed cells . The values represent the mean (n=14) ± SEM. P>0.05.*

4.3.8 PP14 secretion from mechanically and enzymatically isolated Fallopian tube epithelial cells.

It is clear from Figure 4.6 that tubal epithelial cells isolated by the mechanical procedure produced a higher concentration of PP14 in comparison to the cells isolated by the enzymes, pancreatin and trypsin. No PP14 was detected frozen frozen-thawed tubal epithelial cells irrespective of the isolation method used. Once again, a similar number of cells (7 x 10^5) were used in all PP14 culture experiments and therefore the differences observed in the secretory properties may be attributed to the method that was used for their isolation.

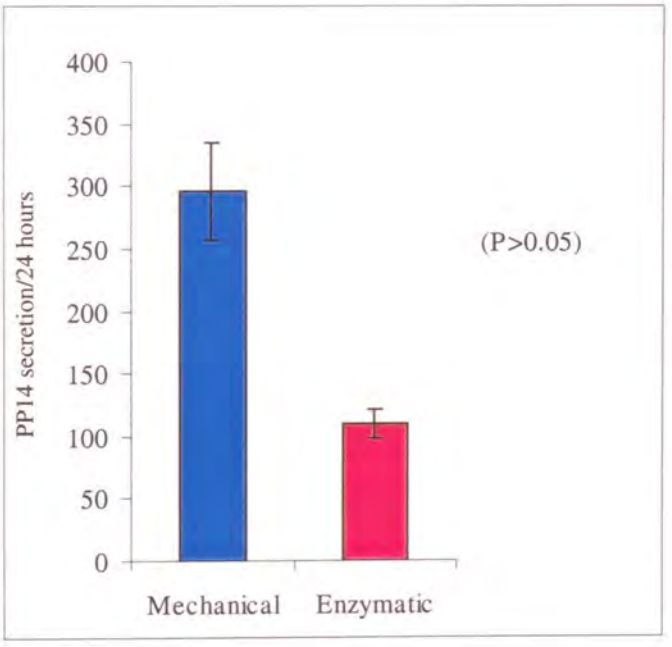


Figure 4.6*: PP14 secretion by primary cultures of Fallopian tube epithelial cells isolated by mechanical* ■ *and enzymatic* ■ *methods. The values represent the mean (n=14) ± SEM.*

4.4 DISCUSSION

Experimental studies regarding the role of the Fallopian tube in the reproductive process have been hampered by the relative inaccessibility of this organ. In order to overcome this technical hurdle, it is imperative to establish an *in vitro* model of the tube, which mimics the *in vivo* environment. Perhaps, the primary disadvantage of using cell culture systems is the loss of any paracrine influences that may contribute to their functions. As such, the isolation of these cells prior to conducting experiments involving co-culture is critical, since any damage to the cell's structural integrity may be detrimental to the functions they carry out and hence may not provide a functional working model.

To date, there is no documented evidence which describes comparative growth patterns of human Fallopian tube epithelial cells isolated mechanically and enzymatically. However, if such cells are to be used for co-culture in IVF programmes or for experimental studies, both their quantity and quality is of paramount concern. The results from the present study clearly demonstrated that a greater yield of epithelial cells was obtained from the Fallopian tube with enzymatic isolation. In contrast, mechanically isolated cells always resulted in a lower cell yield even if an abundance of tissue explants were placed in the culture flasks. Previous studies in this field have examined the effects of growth factors, ovarian hormones and proteins on the proliferation of Fallopian tube epithelial cells in culture (Henriksen *et al.*, 1990; Takeuchi *et al.*, 1991). In this study however, the addition of promoting agents other than FBS to the culture medium was purposely avoided so as not to influence the experiment. As such, no direct comparison with previous studies can be made regarding the data obtained in the initial part of this study.

Three methods used to isolate human Fallopian tube epithelial cells were compared in the first part of this investigation. These included mechanical isolation, pancreatin/trypsin and pancreatin/dispase treatment. All three methods provided a sufficient quantity of isolated epithelial cells for primary cultures though the mechanical method resulted in a lower yield of cells overall. The enzymatic method allowed for the rapid removal of cells from the explants unlike the mechanical method which required the cells to grow from the mucosal pieces. Furthermore, the mechanical isolation was easily performed and although the cells took longer to achieve confluence, they possessed a longer survival time than their counterparts obtained by enzymatic isolation. In addition, the possibility of cellular damage during the mechanical isolation was reduced since enzymes were not used. However, a limitation of the enzymatic method was that cells rarely survived sub-culturing after 2-3 passages. At this stage, the cell surface appeared highly irregular with numerous blebs. Such morphological features were suggestive of intra-cellular damage and loss of intercellular contacts. Perhaps, this could provide a reasonable explanation as to why the enzymatically isolated cells did not survive subsequent sub-cultures. Furthermore, the cells that were isolated enzymatically stained very strongly with the propidium iodide, a clear indication that cell membrane damage occurred. In the present investigation, trypsin was used both during the isolation procedure as well as for the detachment of the confluent cell monolayer though it has been suggested that the use of trypsin may cause detrimental effects such as proteolysis of the cell membrane proteins (Shiba *et al.*, 1998).

Both primary and sub-cultures revealed that the cells were epithelial in origin as indicated by the positive staining of the intermediate filaments, cytokerations. In addition, when the cells were processed for cytogenetic observation, the diploid chromosome number of 46 was retained throughout, regardless of the isolation procedure employed.

In this investigation, experiments were also carried out on the secretory characteristics of the Fallopian tube epithelial cells. PP14 was used as a marker to assess the secretory epithelial cell function. In addition, FBS is known to contain a very high protein content, which would have inadvertently affected any results obtained for the TPS and PP14 experiments. For this reason, it was necessary to supplement the growth medium with FBS until the cells reached confluence. It can be postulated therefore, that the TPS secretion was not indicative of the total *de novo* synthesis of the epithelial cells in culture, and may in fact have included proteins released by dead and/or ruptured cells as well as those cells that may have detached from the substrate. Both the TPS and the amount of PP14 secreted by enzymatically isolated cells were much lower in comparison with mechanically isolated cells. Perhaps, a plausible explanation for such observations was the extensive cell membrane damage noted when the cell's structural integrity was assessed. The use of enzymes may have hindered or disrupted cellular components necessary for secretory activity. Nevertheless, the precise role of PP14 in co-culture systems remains to be elucidated.

The results obtained in this study, both for TPS and PP14 secretion were slightly higher than those obtained by Saridogan *et al.*, (1997). However, this may simply have been due to the total number of cells used which was higher in these experiments. Usually most plasma membrane proteins are synthesized at the endoplasmic reticulum (ER) and co-translationally integrated into the ER membrane. Following this, the proteins are transported by successive vesicle budding and fusion events from the ER via the Golgi stacks prior to reaching the plasma membrane (Yeager *et al.*, 1998). This intracellular transport route is known as the secretory pathway and it seems likely that if the cell membranes are not intact, trypsin activity as used in this study could affect the enzymes that may be necessary for protein production. In addition, cells cultured *in vitro* are no longer under the hormonal influences that are normally present in the *in vivo* environment. Therefore, the cells may lose some of their secretory characteristics that are usually observed at different stages of the ovarian cycle. An interesting study carried out by Laird *et al.*, (1995), demonstrated that when epithelial cells from the Fallopian tube were cultured in growth medium supplemented with the ovarian hormones, progesterone and oestradiol, the production of PP14 was increased. This clearly indicates that the secretory nature of epithelial cells from the Fallopian tube is influenced by the ovarian hormones. Since primary cultures were used for the present study, cell de-differentiation or senescence is unlikely to have had any adverse effects of the secretory function. A more plausible suggestion may be the absence of specific growth factors, cytokines or paracrine influences in the cultures. Saridogan *et al.*, (1997) observed that the cell integrity following freezing was retained but suggested that the molecular events associated with protein synthesis and release may have been disrupted.

However, in this study, propidium iodide (red) and Hoechst 33358 (blue) stains were used to assess the cell integrity of confluent monolayers. In all cases though, over 99% of the frozen-thawed Fallopian tube cultures incorporated the propidium iodide stain, which was indicative of cell membrane damage.

PAGE NUMBERING AS ORIGINAL

5.1 INTRODUCTION

IVF procedures offer hope to those couples who seek to have children naturally but are limited by unsuccessful attempts to become pregnant. In recent years, co-culture systems have been used as a means to improve clinical pregnancy rates in human IVF programmes (Desai *et al.*, 1998). Briefly, this technique involves culture of human embryos on somatic cell monolayers for 3-6 days prior to embryo transfer to uterus.

It has been shown *in vitro*, that the co-culture of human embryos with various cellular monolayers such as, uterine fibroblasts (Wiemer *et al.*, 1989) and Vero cells (Menezo *et al.*, 1992), result in increased rates of embryo development, decreased fragmentation and improvement in implantation and pregnancy rates in IVF (Wiemer *et al.*, 1989; Menezo *et al.*, 1992; Freeman *et al.*, 1995). The exact mechanism whereby cells in co-culture exert their embryotrophic effect is still unclear. It has been suggested, that the somatic cells may help to dilute the effect of any potential growth-inhibiting substances in the embryo's micro-environment as well as secrete factors during proliferation, that may be beneficial to the embryo (Desai *et al.*, 1998). Although this evidence suggests a positive effect of co-culture cells upon IVF, the rate of development has been lower when the embryos have been cultured *in vitro* as compared with *in vivo* development (Feng *et al.*, 1996).

Co-culture systems were developed to mimic the *in vivo* environment thereby enhancing both gamete interaction and embryo development *in vitro*. However, although such experiments demonstrated beneficial effects, many of them were conducted using animal cell lines (Wiemer *et al.*, 1989; Sakkas *et al.*, 1994). In co-culture systems, no tissue or species specificity has been demonstrated (Freeman *et al.*, 1993; Desai *et al.*,

1998). For this reason, the establishment of epithelial cell monolayers *in vitro*, from the human Fallopian tube may represent a major breakthrough and could provide researchers with a powerful system for novel investigation of both the biochemical and physiological functions of the Fallopian tube.

As demonstrated in the previous chapter, the number of cells obtained during the isolation procedures are variable and the tubal epithelial cells that are grown in culture eventually lose morphological features associated with the epithelium *in situ* (e.g cilia). For this reason, an immortalised cell line derived from a single individual could provide an ideal experimental model. Once fully characterised, such a model would be preferable to primary cell cultures of Fallopian tube epithelial cells for carrying out various studies, as this will eliminate the need to rely on Fallopian tubes retrieved from women undergoing hysterectomy. Such a cell line will not only ensure that a "bank" of cells is available to be used if and when necessary, but also may also lead to the development of improved culture techniques.

In order to extend the life-span of normal cells, a transforming (foreign) gene (e.g large T-antigen) can be introduced into the nucleus. Both transformation and transfection are the simplest methods that are available for getting recombinant DNA into cells. The initial experiments undertaken for this chapter included the transformation of competent *Escherichia coli* (*E.Coli*) bacterial cells thereby allowing the uptake of plasmid DNA.

Transformation was first observed in 1928 by Fred Griffiths and involves the uptake of exogenous DNA by a bacterium, followed by its incorporation into the genome thereby transforming its genetic composition (Nicholl, 1994).

Following this, experiments involving transfection (the genetic modification of cultured cells by the uptake of DNA) were performed with the hope of obtaining immortalisation (infinite cell growth) of the Fallopian tube epithelial cells (HFTEC). The details of these experimental procedures have previously been described in sections 2.9 and 2.10 respectively.

In order to increase the quantity of the commercially purchased pCMV-SPORT β-gal and pUC19 plasmid vectors for transfection purposes, *E.Coli* DHα competent cells were transformed. Experimental studies were carried out on the established cell lines, CHO-K1, HRT18 and Jurkat 6 simply to ensure that the methods employed to transfect HFTEC were feasible/reproducible. In this study, HFTEC were transfected using a range of techniques in order to extend the current life span of these cells *in vitro*. This may then result in an immortalised cell line, which could subsequently be used as experimental model(s) in the field of reproductive biology.

5.1.1 Objectives of the Study

The principal objectives of this study were:

1). To transform *E.Coli* with the plasmid vectors pCMV-SPORT and pUC19 to increase the yield of DNA for transfection purposes.

2). To assess various transfection procedures on established cell lines and fresh/frozen-thawed primary cultured cells.

3). To attempt to establish an immortalised HFTEC line which can be fully characterised and used as a suitable model for further experimental studies.

5.2 METHODS

Fallopian tubes were obtained from thirty-three women aged 34-46 with normal reproductive history. The various methods used to achieve the results have been fully described in chapter 2 under the relevant sections, which include 2.9, 2.10 and 2.11.

5.3 RESULTS

5.3.1 Transformation efficiency of E.Coli DH5α competent cells

Agar plates containing the plasmid vectors pUC19, control DNA and the pCMV-SPORT but no ampicillin or X-gal, appeared cloudy after six hours of incubation at 37°C. After 24 hours however, all bacterial plates, which contained just the agar mixture were covered in small white colonies. Both the pUC19 control DNA and the pCMV-SPORT plasmid vector were successfully transformed and yielded in excess of 100 colonies. These were clearly visible on agar plates containing both ampicillin and X-gal because they formed blue colonies (Figure 5.0) in the presence of the chromogenic substrate X-gal. A single clone was then lifted and the plasmid DNA was amplified and purified. Following this, both the purity and quantity was estimated by spectrophotometrical analysis as described below.

5.3.2 Spectrophotometric analysis of plasmid DNA

The DNA (pCMV-SPORT and pUC19) (100μL) obtained was diluted in 900μL of TE and mixed gently. Using TE as a blank, the ratio between the readings at 260nm and 280nm (OD_{260} / OD_{280}) were read. The values obtained (Table 5.0) represented a relatively pure preparation of plasmid DNA, free of protein, RNA and other chemical contaminants. The plasmid DNA obtained was then used to carry out transfections using a range of techniques.

A

B

C

Figure 5.0: *Ampicillin-resistant (Amp^r) agar plates containing clones of the pUC and pCMV-SPORT plasmid DNA. A large number of clones (blue colonies) were obtained when the pUC19 (100µl) was spread onto the agar plates* ***(A)****. In contrast, a smaller number of clones were obtained for the pCMV-SPORT plasmid vector as shown in both diluted* ***(B)*** *and undiluted* ***(C)*** *reaction mixtures. Nevertheless, a single blue colony (red arrow) was sufficient to carry out amplification and purification of plasmid DNA, which was subsequently used in transfection experiments.*

Plasmid vector	OD at 260nm	OD at 280nm	OD_{260}/OD_{280}	Yield of DNA
pCMV-SPORT	0.239	0.125	1.912	119.5μg/ml
PUC19	0.282	0.144	1.95	141.0μg/ml

Table 5.0: *A table showing the optical densities obtained from the preparation of the plasmid vectors, pCMV-SPORT and pUC19, their purity and final yield.*

N.B *The total DNA yield was calculated based on the assumption that an OD of 1 corresponds to approximately 50μg/ml of plasmid DNA.*

5.3.3. Measurement of β-gal expression in transfected cell line extracts

The established cell lines, HRT18, CHO and Jurkat 6 were cultured and subsequently transfected. The amount of plasmid DNA added to the cells was diluted two-fold (Table 5.1) in order to establish how much DNA was required to give maximum transfection efficiencies. Established cell lines, HRT18, CHO-K1 and Jurkat 6 successfully incorporated the pCMV-SPORT vector, though the efficiency by which they did so varied (Figures 5.1, 5.2 and 5.3) respectively. The *E.Coli lacZ* gene encodes β-galactosidase, a tetrameric enzyme that catalyses the hydrolysis of β-galactoside sugars such as lactose. This enzyme was determined colorimetrically using the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG). It is the hydrolysis of ONPG by β-galactosidase, which yields o-nitrophenol, a yellow coloured product. The ONPG assay was carried out as described by Sambrook *et al.*, 1989 and the values were calculated from known concentrations of β-gal standards within the range 0-70ng/μL.

Denotion (X-axis)	1	2	3	4	5	6
Plasmid DNA (μg)	3.2	1.6	0.8	0.4	0.2	0.1

Table 5.1 *Plasmid DNA was diluted two-fold together with the various liposomal reagents in order to evaluate the maximal transfection efficiency in the cell lines used. These values are applicable to figures 5.2, 5.3 and 5.4.*

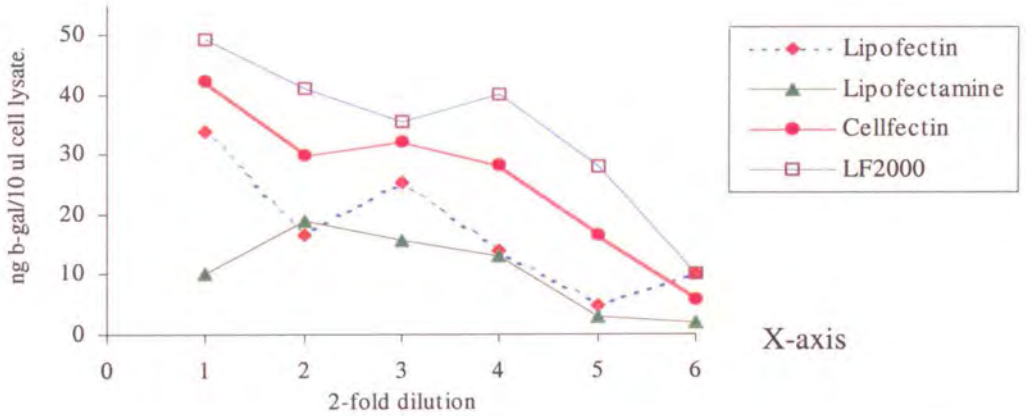


Figure 5.1 *Comparative analysis of lipid reagents in adherent cells HRT18 transfected with the pCMV-SPORT plasmid vector. Cells (4 x 10^4) were seeded per well in 1 ml of growth medium using 24-well plate. Once they were 60-80% confluent, the cells were transfected with 3.2- 0.1μg DNA and assayed for β-gal after 24 hours. Transfection efficiencies are given as β-galactosidase (μg/10μL cell lysate).*

The highest transfection efficiencies (49μg β-gal/10μl cell lysate) were obtained when HRT18 cells were transfected with 3.2μg pCMV-SPORT DNA and LF 2000 reagent (Figure 5.1). On the contrary, the lowest transfection efficiencies (2μg β-gal/10μl cell lysate) were obtained when HRT18 cells were transfected with 0.1μg pCMV-SPORT DNA and Lipofectamine. Irrespective of which lipid reagent was used, there was an overall decline in transfection efficiencies as the quantity of pCMV-SPORT DNA decreased.

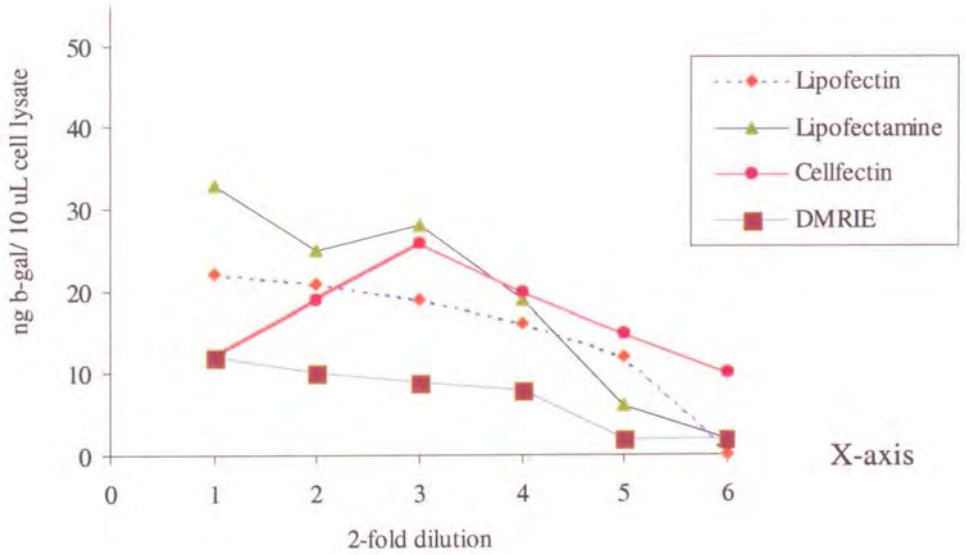


Figure 5.2 *Comparative analysis of lipid reagents in adherent cells CHO-K1 transfected with the pCMV-SPORT plasmid vector. Cells (4 x 10^4) were seeded per well in 1 ml of growth medium using 24-well plate. Once they were 60-80% confluent, the cells were transfected with 3.2- 0.1μg DNA assayed for β-gal after 24 hours. Transfection efficiencies are given as β-galactosidase (μg/10μL cell lysate).*

CHO-K1 adherent cells also successfully incorporated the pCMV-SPORT plasmid vector (Figure 5.2). Once again, there was a general decrease in the transfection efficiencies as the pCMV-SPORT DNA was diluted. Using Lipofectamine, 32ng β-gal/10μl cell lysate) was obtained when CHO-K1 cells were transfected with 3.2 μg pCMV-SPORT DNA. In contrast, DMRIE reagent provided the lowest transfection efficiencies overall. When 0.8μg pCMV-SPORT DNA was used with both lipofectamine and cellfectin, modest quantities of β-gal (27μg and 25μg) were obtained respectively. This demonstrates that even though the quantity of plasmid DNA is small, successful transfection can still be achieved.

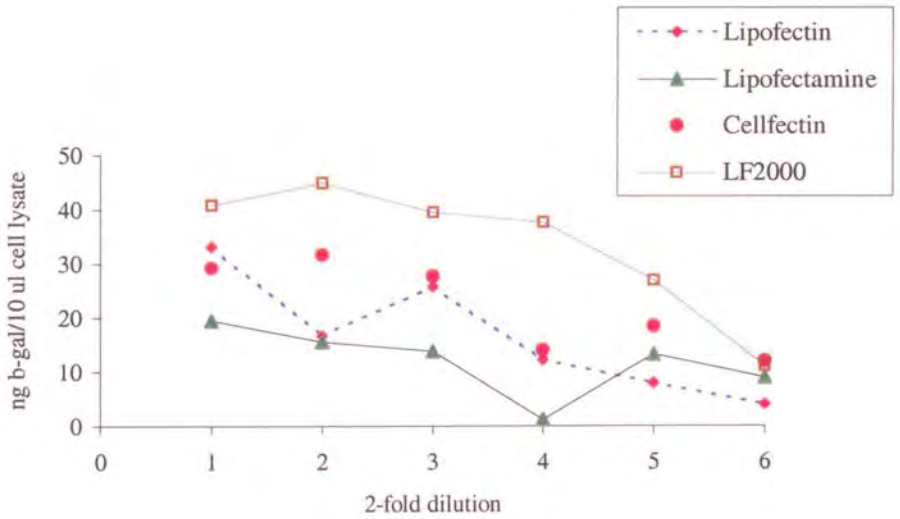
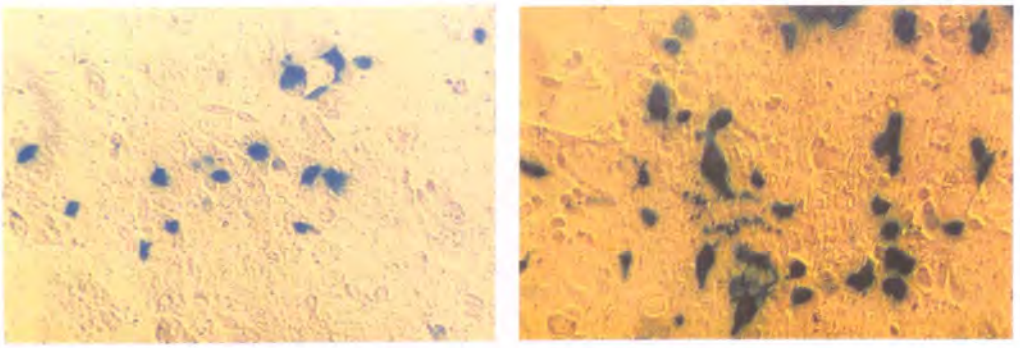


Figure 5.3: *Comparative analysis of lipid reagents in Jurkat 6 suspension cells, transfected with the pCMV-SPORT plasmid vector. Cells 4 x 10^4 were seeded per well in 1 ml of growth medium using 24-well plate. After 48 hours, the cells were transfected with 3.2- 0.1μg DNA assayed for β-gal after 24 hours. Transfection efficiencies are given as β-galactosidase (μg/10μl cell lysate).*

In Figure 5.3, Jurkat 6 cells (suspension cells) were transfected using the dilution step as previously described. The highest transfection efficiencies were obtained when 1.6μg pCMV-SPORT was used with LF2000, whilst the lowest transfection efficiencies were obtained with lipofectamine reagent. Although both lipofectin and cellfectin produced β-gal, the transfection efficiencies were not as high as those obtained when LF2000 was used. In addition, presence of β-gal was also apparent when small quantities of pCMV-SPORT (0.1-0.2μg) were used. Generally, as observed with the other cell lines, HRT18 and CHO-K1 there was a gradually decline in the efficiencies as both the DNA and lipid reagent was diluted.

5.3.4 In-situ X-gal staining of adherent HRT18 cells

This reporter gene (X-gal) can also be assayed by *in situ* histochemical staining using the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) whereby cells that express β-galactosidase are stained blue (Ausubel *et al.*, 1995). To confirm that HRT18 cells successfully incorporated the pCMV-SPORT vector, transfected cells were stained *in situ* to locate X-gal colonies (Figure 5.4).



(A) **(B)**

Figure 5.4: *X-gal (blue staining) in situ staining of HRT18 cells transfected with the pCMV-SPORT plasmid vector using the liposomal reagent LF2000. Original mag (100)*

5.3.5 HFTEC survival following G418 antibiotic addition

To assess successfully transfected HFTEC, a dose response study was carried out on non-transformed Fallopian tube epithelial cells, in order to determine the lowest concentration of G418 that killed the cells. A 100% cell death was observed when 600μg/ml G418 was added (Figure 5.5) and hence the next higher concentration (i.e 700μg/ml) was used to select for stable transformants following transfection.

5.3.6 Light microscopy of transfected HFTEC

Logarithmically growing cells were transfected using the calcium phosphate co-precipitation method. Calcium chloride was mixed directly with the pMX1 plasmid vector and phosphate buffer which resulted in a fine precipitate. This was then pipetted over the cultured cells and incubated under standard conditions. After 7-10 days in culture, the shape of the cells were not 'epithelial-like' as those observed in the control flasks. Instead the transfected cells appeared more 'needle-like' in culture. Fresh medium was added daily. Foci (mounds of cells) were subsequently observed in cultures transfected with the pMX1 plasmid vector (Figure 5.6). At this stage, the cells were trypisinised and grown in selective media, which contained the antibiotic G418. However, after only three days in culture with the antibiotic the cell monolayers showed signs of senescence. At high magnification, the cells appeared highly vacuolar and they also failed to adhere to the base of the culture flasks.

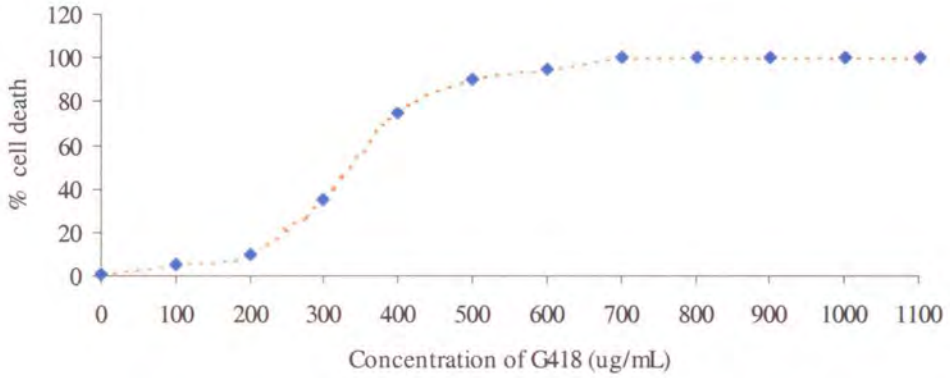


Figure 5.5: *Dose response curve for G418 selection.* ***Using*** *two 6-well plates, 100µl of Fallopian tube epithelial cells (4 x 10^3 cells/ml) were added to 2 mls of medium containing the diluted G418 and placed in a humidified 5% CO_2 atmosphere at 37° C. Fresh medium containing the diluted G418 was added twice a week. After 10-14 days, the medium was removed, the cells were washed in PBS and stained with 0.5% methylene blue in 50% methanol for 15-20 minutes. The minimum concentration that killed the cells was determined and the next higher concentration was used to select for stable transformants. The result represents the average cell death of duplicates samples.*

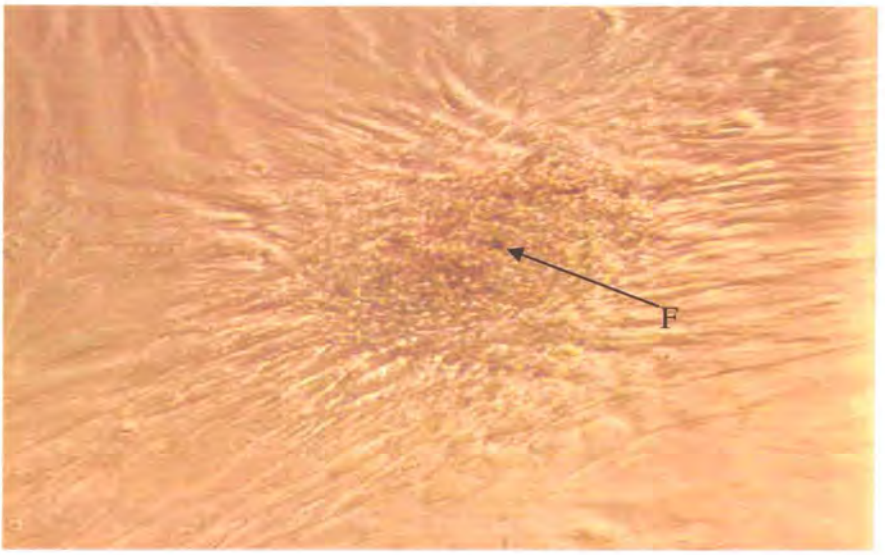


Figure 5.6: *A 'focus' of transfected Fallopian tube epithelial cultures. Epithelial cells were seeded in 25cm^2 flasks one day prior to transfection. The cells were transfected with a pMX1 vector using the calcium phosphate co-precipitation method. Following transfection, the cells were rinsed in PBS and fed with fresh growth medium on alternate days. On day 18, mounds of cells* ***(F)*** *were observed in two of the 25cm^2 flasks. Original Mag (x400)*

5.3.7 In-situ β-gal staining of transfected HFTEC

Initial observations under an inverted microscope confirmed that human Fallopian tube epithelial cells did not take in the pCMV-SPORT plasmid vector as efficiently as the HRT18 cells (Figure 5.4). Over 99% of the tubal epithelial cells resembled those observed in the control dish (Figure 5.7) following transfection (after 24 hours) regardless of the cationic liposomal reagent used. Due to the very low expression of X-gal it seemed futile to analyse cell extracts for β-galactosidase.

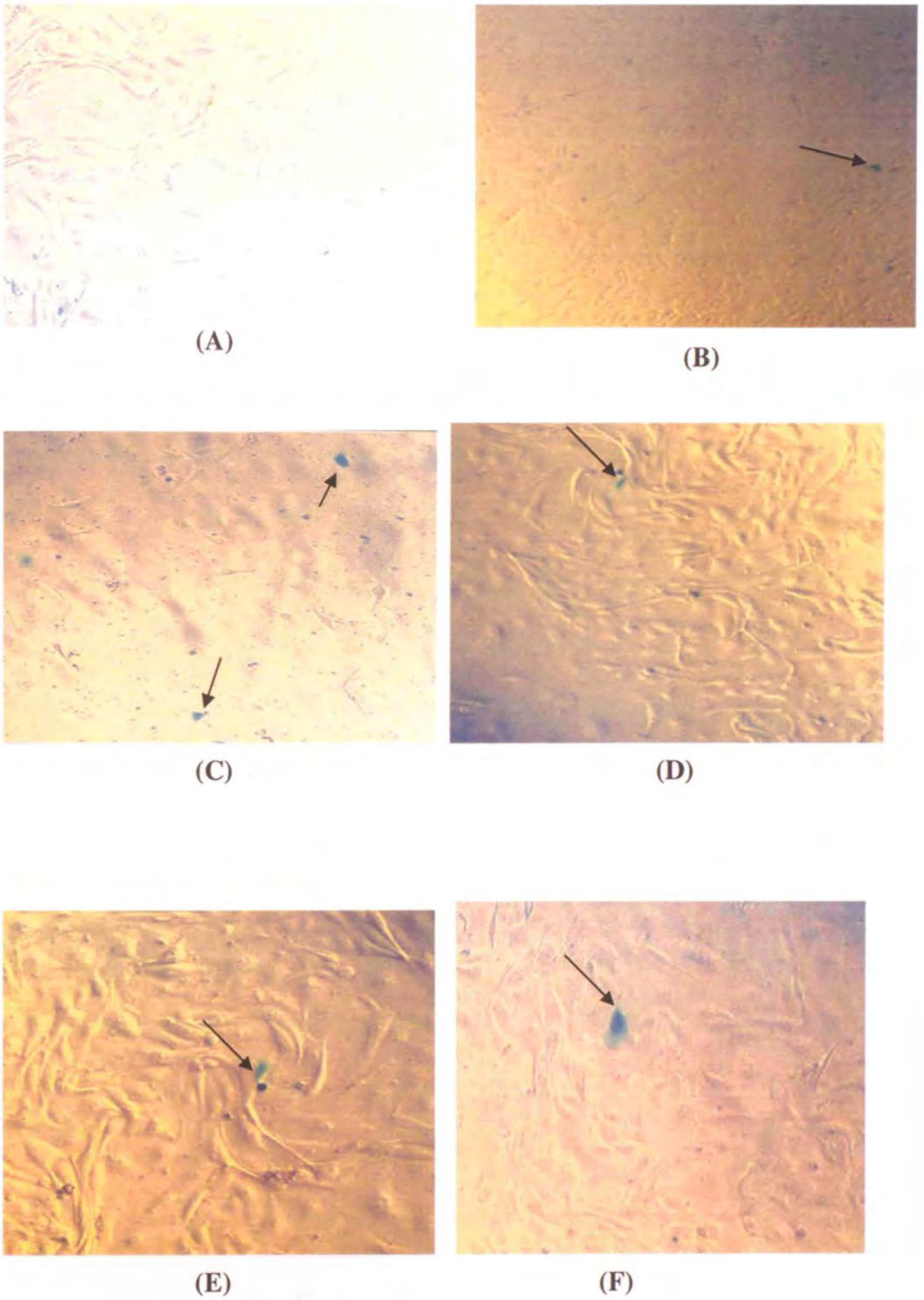


Figure 5.7: *In situ staining of X-gal in transfected HFTEC. Control epithelial cells (A) and cells that have incorporated the p-CMV-SPORT vector (B-F). Arrows indicate cells that have incorporated the β-gal gene. Original Mag (x 100)*

5.3.8 Transfection of HFTEC using Superfect, Effectene and LF2000.

Isolated Fallopian tube epithelial cells were routinely cultured as described in section 2.5.1. After 24 hours the cells were transfected using the liposomal reagents Superfect, Effectene and LF2000 and the transfection efficiencies are presented in Figure 5.8. The transfection efficiencies obtained for human tubal epithelial cells are significantly lower ($P > 0.5$) than those obtained for the established cell lines, HRT18, CHO-K1 and Jurkat 6. The maximum transfection obtained was 67μg β-gal/ml using Effectene reagent whilst both Superfect and LF2000 produced 49μg β-gal/ml. Although LF2000 proved to be detrimental to the Fallopian tube epithelial cells in previous experiments, in this instance the LF2000 reagent was removed after 5 hours instead of the usual 24 hours and replaced with fresh growth medium. This was carried out because excessive cell death was observed after 24 hours in culture. This suggests that LF2000 may be cytotoxic to normal cells and therefore a reduced exposure time for transfection was adopted.

In a subsequent experiment, 4 $x10^5$ cells/25cm^2 flasks were seeded. After 24 hours the cells were transfected with the Effectene reagent and the plasmid vector p-EGFP. These cells survived in culture for 92 days with routine feeding with DMEM/Nut F-12 medium and FBS (10%). At this stage the cells were trypsinized and cryopreserved until required. Upon reconstitution, the cells were plated on coverslips until confluence was reached.

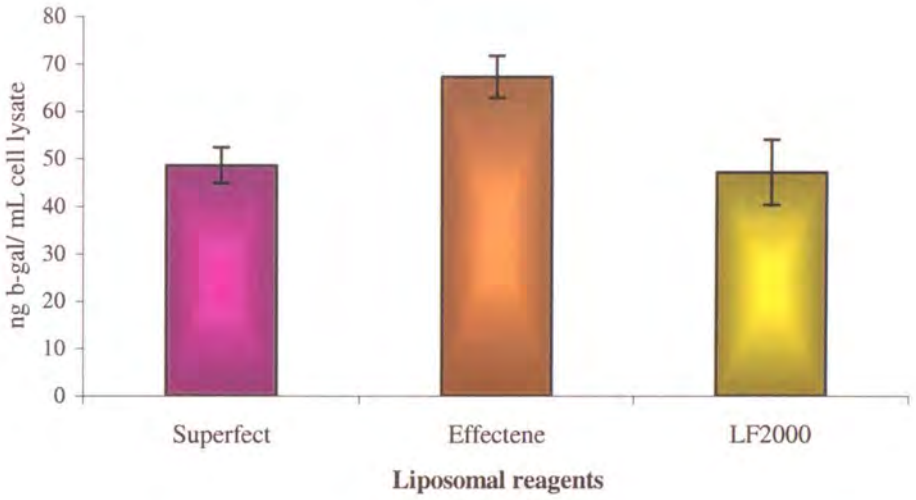


Figure 5.8: *Comparison of transfection efficiencies obtained using Superfect, Effectene and LF2000 liposomal reagents. Human Fallopian tube primary epithelial cells were transfected using 1.5μg pCMV-SPORT/35 mm dishes. 4 x 10^4 cells were seeded/dish one day prior to transfection. Transfection effciencies are given as β-galactosidase (μg/ml cell lysate) and each bar represents the average efficiency from duplicates samples.*

5.3.9 Fluorescent microscopy of GFP in transfected HFTEC

Human Fallopian tube epithelial cells were shown to have successfully incorporated the plasmid vector, p-EGFP. This was observed by the presence of the green fluorescent protein in some of the cells after 2-3 days in culture (Figure 5.9). The cells from a $25cm^2$ flask and three chamber slides were then trypinised and subsequently stained for cytokeratins, in order to confirm that the cell had remained epithelial in origin. The tubal monolayer cells demonstrated positive immunofluorescence staining for cytokeratins though no green nuclear staining was observed when the cells were stained for the large T-antigen.

(A) (B)

Figure 5.9: *HFTEC transfected with the p-EGFP plasmid vector. After 3 days in culture the cells display the GFP indicating successful transfection. Original Mag (x1000)*

5.3.10 Selection of transfectants using G418 antibiotic

Transfected cells were grown in selective medium (700μg/ml G418), but no new colonies of cells were observed despite continuous feeding at intervals of two days. This experiment was repeated and a lower concentration of antibiotic 600μg/ml G418 was added instead but this had no effect on cell survival.

5.3.11 Fluorescent microscopy of cytokeratins in transfected HFTEC

Transfected cells from three 25cm^2 flasks, which had not been exposed to the antibiotic G418 but which stained positive both for X-gal and GFP were passaged, cultured under routine conditions and stained for cytokeratins. This was carried out to determine whether the transfected cells had retained their epithelial nature.

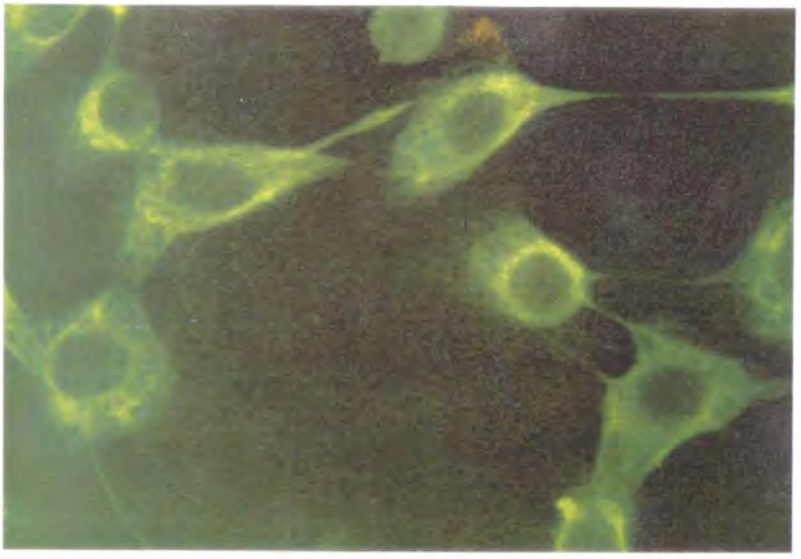


Figure 5.11: *Positive immunofluorescence staining of anti-cytokeratins in transfected HFTEC. This indicated that although these cells had incorporated a foreign gene, they retained their epithelial origin. Original Mag (x 400)*

5.3.12 Fluorescent microscopy of SV40 large T-antigen in transfected HFTEC

Despite staining positive for cytokeratins, no positive nuclear staining for SV40 large T-antigen was observed in the transfected Fallopian tube epithelial cells. This demonstrates that these cells did not achieve a stable or immortal status.

5.4 DISCUSSION

The principal objective of the present study was to extend the life span of cultured human Fallopian tube cells with a view to establish an immortalised cell line. Primary cultures of human tubal epithelial cells were transfected with a plasmid vector carrying the simian virus 40 (SV40) large T-antigen. Indeed this origin-defective SV40-Large T antigen have previously been used to immortalise various normal cells (Stoner *et al.*, 1991; Ishida *et al.*, 1995). In the present investigation, several techniques were employed in order to achieve this goal and the results were analysed using morphological and biochemical parameters.

Successful cloning experiments depend on being able to identify the desired gene sequence among the many different recombinants that may be produced. Bearing in mind that a large genomic library may contain a million or more cloned sequences, the identification of the target gene is not readily distinguishable (Nicholl, 1994). To overcome this hurdle, a number of selection methods have been developed. Genetic selection and screening methods rely on the expression (or non-expression) of certain traits which are usually encoded by the plasmid vector. One of the simplest genetic selection methods involves the use of antibiotics to select for the presence of vector molecules. In this study, the presence of plasmid DNA in these cells was detected by plating potential transformants on agar medium that contained ampicillin. Only cells that have taken up the plasmid DNA would be resistant and hence would grow in the presence of this antibiotic. In addition, a screening method using the chromogenic substrate X-gal was also used for further identification purposes. X-gal, is a colourless substrate for β-galactosidase which is normally synthesised by *E.Coli* cells when lactose becomes available (Nicholl, 1994).

On cleavage of X-gal, a blue coloured product is formed and thus the expression of the lac Z (β-galactosidase) gene can easily detected by the blue-coloured clones (Figure 5.0.

Normal mammalian cells (e.g fibroblasts) cultured *in vitro* will divide for a limited number of generations (Hayflick and Moorhead, 1961). The limit on cell divison is sometimes referred to as the 'Hayflick number or 'Hayflick limit, where having reached this limit, the cell continues to be metabolically active but cannot divide (Hayflick, 1974). At this stage, the cells begin to lose their potential to proliferate and will eventually reach a state of cellular senescence (McCormick and Campisi, 1991). Only primitive embryonic stem cells and embryonic germ cells seem to have an unlimited capacity for cell division in culture (Matsui *et al.*, 1992; Resnick *et al.*, 1992). *In vivo*, these cells differentiate ultimately into the various cell types of the body and thus, under normal circumstances they eventually acquire the finite life-span phenotype (McCormick and Campisi, 1991). In contrast, cells that have been genetically transformed (eg HRT18, CHO-K1, Jurkat 6 cells) appear to have lost the regulatory mechanisms of limited growth potential and may continue to divide indefinitely thus becoming immortal (Derventzi *et al.*, 1996).

Indeed, while cellular mortality is characterised by a progressive cessation of cell growth manifested in a cell culture by senescence, immortalisation is the escape from senescence as a result of multiple mechanisms involving genetic and epigenetic changes (Gonos *et al.*, 1992). Normal cells have multiple independent growth regulation mechanisms and there are several events needed to override these control elements to induce the numerous changes in cellular structure and functions that characterise the transformed phenotype (Derventzi *et al.*, 1996).

Normal differentiated human cells are destined to reach the stage of senescence and very rarely become immortalised spontaneously or by using treatment that are known to cause genetic alterations (Namba *et al.*, 1996). On the contrary, spontaneous immortalisation of cells from experimental animals such as the mouse, rat and hamster frequently occurs. Namba *et al.*, (1996) but human cells may be different from these rodent cell types in a way not yet fully understood. It has been suggested that the greater karyotypic stability of human cells may be the reason why such cells do not become immortalisation spontaneously. Namba *et al.*, (1996), stated that this may be due to the existence of DNA replication that is not only less prone to errors but due to the fact that the DNA repair systems in human cells are more efficient.

There have been a few reports of spontaneous immortalisation of human epithelial cells from various organs, such as skin (Weaver *et al.*, 1991), breast (Paine *et al.,* 1992) and parotid gland (Chopra *et al.*, 1995). It has been suggested that the causes of such spontaneity was probably, either a result of the epithelial cells being exposed to some carcinogenic agents (e.g UV, X-rays or chemicals) prior to being cultured or the cultures of epithelial cells may have contained some stem cells which are more prone to immortalisation (Namba *et al.*, 1996).

Over the years many transfection techniques have been developed to improve the efficient transfer of nucleic acids into the nucleus of the cell. In addition, newly designed methods have been developed to minimize interference with the normal cell physiology, reduce toxicity, but increase reproducibility and ease of use. The diethylaminoethyl (DEAE)-dextran method, calcium phosphate method, electroporation

and liposome-mediated transfection technologies represent some of the more common methods employed.

In the present investigation a range of liposomal reagents have been used to transfect both established cell lines and HFTEC. Furthermore, the classic calcium phosphate co-precipitation method was also used to transfect HFTEC. Felgner *et al.*, (1987) introduced the use of synthetic cationic liposomes as a means of delivering DNA into cells. Liposomes typically contain a mixture of both cationic and neutral lipids (eg L-dioleoyl phosphatidyl-ethanolamine [DOPE]), and can be used for transient or stable longer-term experiments, which rely upon the integration of the DNA into the chromosome or episomal maintenance. Cationic lipids have an overall net positive charge at physiological pH, which associates with the negatively charged nucleic acids. This results in compaction of the nucleic acid in the liposome-nucleic acid complex, the uptake of which is believed to be mediated by endocytosis. Following this uptake, the liposome-nucleic acid complex, appear in structures called endosomes prior to entry in the nucleus. However, the mechanism by which the complex is released from the endosomes and the subsequent integration in the nucleus remain unclear. Farhood *et al.*, (1995) suggested that the neutral lipid DOPE can be considered as a 'fusogenic' lipid and as such, may release these complexes from the endosomes and expedite the fusion of the outer cell membrane with the liposome-nucleic acid complex.

From the results obtained in the present investigation, it is clear that the established cell lines HRT18, CHO-K1 and Jurkat 6 cells incorporated the plasmid vector pCMV-SPORT. This was observed both in the ONPG assay as well as in the *in situ* staining of X-gal. In marked contrast, HFTEC transfected with the same cationic liposomes did not take in the pCMV-SPORT DNA as efficiently as the established cells lines regardless of the techniques used.

Another transfection technique employed in the present study was the calcium phosphate method which was first used to introduce adenovirus DNA into mammalian cells (Graham and Van der Eb, 1973). The protocol involved mixing DNA with calcium chloride and then adding this to phosphate buffered saline (PBS), which resulted in the formation of a precipitate. The calcium-phosphate-DNA complexes then adhere to the cell membranes and enter the cytoplasm of the target cell by phagocytosis (Loyter *et al.*, 1982). Indeed the size, quality of the precipitate and the pH of the reagents are crucial to the success of $CaPO_4$ transfections.

The calcium phosphate co-precipitation method is commonly used both for transient and stable transfection of a variety of cell types. The components are readily available, affordable and easy to use. However, the disadvantage of using this method is the low reproducibility and the fact that some cells, including primary cells, resist this form of DNA transfer.

HFTEC were transfected using this technique and after 2-3 weeks in culture high density cell growth was observed. These 'foci', a characteristic of a transformed phenotype was observed (Figure 5.7). At this stage, colonies of transformants could

have been isolated and analysed to verify the stability of transformation and the epithelial origin of transformed cells. If the cultures had survived though, some of the cells would have been isolated from the high-density foci, maintained in culture and sub-cultured numerous times. Eventually, these cells would normally reach a stage where they cease to proliferate and may accompany a decreased growth rate. This phase of *in vitro* neoplastic progression is termed crisis (Defendi, Naimski & Steinberg., 1982) whereby most of the cells die and only cells that are stably transformed will survive. These post-crisis cells can have different growth and phenotypic properties than pre-crisis cells. Other epithelial cell types have been successfully transfected using this method. These include tracheobronchial gland epithelial cells (Cozens *et al.*, 1992) and intestinal epithelial cells (Brandsch *et al.*, 1998).

HFTEC were also transfected using cationic lipid reagent-mediated transfection as they have been shown to yield high and previously unattainable transfection efficiencies in a wide range of eukaryotic cells (Ciccarone *et al.*, 1999; Tilkins *et al.*, 1994; Hawley-Nelson *et al.*, 1993). In addition, unlike the calcium-phosphate method cationic lipid reagent-mediated transfection requires relatively small amounts of DNA and no carrier DNA. In addition, HFTEC cells transfected with Effectene and the p-EGFP were analysed after 24 hours. The advantage of using this plasmid vector was that following transfection, the cells did not need to be stained or assayed to determine whether transfection had occurred. Instead, the cells were visually monitored using a fluorescence microscope to observe the green fluorescent protein, which provided an indication that the cells had indeed taken in the foreign DNA.

A number of other parameters, which have been optimised for these experiments could also influence transfection. These include the cell culture, since a healthy cell culture is necessary for successful transfection. In addition, different cells or cell lines would have very specific media, serum and supplement requirements. The level of confluence of the cultured cells can also affect transfection efficiencies and hence it is important to allow normal cell metabolism to occur by subculturing cells 24 hours before transfection. Furthermore, both the quality and quantity of the plasmid DNA can also affect the efficiency by which cells can incorporate the foreign DNA.

Although the transformation of human epithelial cells is a rare event (Shay *et al.*, 1991) the efficiency of this process can be enhanced. Varying parameters such as the cell number of the cell cycle state, at the time of administration of the transforming agent, or varying the method used to induce transformation has been effective in increasing the number of transformants (Chang, 1986). In general, logarithmically growing cells are more susceptible to transformation than quiescent cells; therefore, sub-confluent cultures actively undergoing DNA synthesis were routinely be used for transformation.

From the results obtained (Figures 5.7), cells were transiently transfected. As such, the plasmid DNA vector was introduced into the nucleus of the cells but did not integrate into the chromosomes. If it had done so then the cells would have stained positive for the Large-T-antigen.

Nevertheless, many copies of the gene of interest (β-gal) were present, which led to high levels of the expressed protein of interest in the established cell lines HRT18, CHO-K1 and Jurkat 6 cells. Following the introduction of the DNA, expression of the

transfected gene can be typically analysed within 24-96 hours. However, the optimal time interval depends upon the cell type, the doubling time of the cells and the type of construct used. From the experiments carried out in the present investigation, established cell lines (HRT18, CHO-K1, Jurkat 6) that were transfected, were analysed after 24 hours. In all cases, either β-gal was present or the monolayer cells displayed the characteristic blue staining of X-gal. However, transfected HFTEC were analysed between 48-72 hours simply because no transfection appeared to have taken place after 24 hours period. Even after 72 hours post-transfection, HFTEC did not demonstrate high transfection efficiencies and very rarely were these cells analysed after 96 hours post-transfection. Usually, at this stage the cell monolayers appeared 'stressed' and exhibited morphological features associated with senescence such as a granular cytoplasm and a number of vacuoles.

In comparison to the calcium phosphate methods, liposome-mediated delivery has been shown to be advantageous as it allows higher transfection efficiency, better reproducibility and can also be used for the *in vivo* transfer of DNA and RNA to both animals and humans (Felgner *et al.*, 1995). However, two particular impediments of this technique are firstly, that the transfection efficiency is often reduced in the presence of serum in the growth medium during transfection. Although, serum is usually excluded from the medium, this increases the cytotoxity of the liposome. As such, experiments designed to determine the optimal transfection time is dependent upon the cell line and DNA used. Secondly, the results obtained can vary considerably depending on the cell type.

Therefore, for this reason the success of various liposomal reagents with a specific cell line or primary cells can be unpredictable.

All the lipid reagents used in these experiments have been used to transfect a number of different cell types successfully. Lipofectin reagent, has been used to transfect both human and porcine endothelial cells which line the surface of blood vessels (Tilkins *et al.*, 1994). However, endothelial cells have a finite *in vitro* lifespan and the age of the cells may have affected the transfection efficiency in the experiments conducted in this project. Lipofectamine2000 and Lipofectamine plus have been shown to offer high transfection efficiency for a broad range of cell types (Hawley-Nelson *et al.*, 1993).

The ultimate goal of stable transfection is to isolate and propagate individual clones, which have incorporated the transfected DNA. In contrast to transient transfection, the DNA in stable transfection is either integrated into the chromosomal DNA or maintained as an episome. In order to distinguish non-transfected cells from those that have taken up the 'foreign' or exogenous DNA, selectable markers such as the genes encoding aminoglycoside phosphotransferase (APH) or hygromycin B phosphotransferase (HPH) are used. In the experiments carried out in this project, the ability to select for transfected cells was made possible using genes that encode resistance to a lethal drug (eg Geneticin) (Berg and Southern, 1982). Transfecting the gene of interest (eg SV40 *ori*) along with a selectable marker for drug resistance is commonly used to establish and select a stably-transfected cell line. G418 blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside and the aminoglycoside phosphotransferase gene (APH or neo^r) confers

resistance to the selective antibiotic, G418 sulphate. Varying concentrations of G418 is usually tested using a dose-response assay since cells differ in the susceptibility to G418.

The integration of DNA into the chromosome, or stable episomal maintenance of reporter genes occurs with a relatively low frequency. Individual cells that survive in the presence of the drug treatment can then expand into clonal groups that can be individually selected, propagated and analysed. In the present study, 700μg/ml was used in the experiments initially (Figure 5.5), but due to extensive cell death, 600μg was then used in further transfection experiments to see whether cells could survived in a lower concentration of the antibiotic.

Following successful transfection, isolated clones would have different integration sites of the DNA and hence, the levels of expression of large T-antigen may be an important factor in determining the degree of transformation. In other words, the level of the immortalizing protein can be quite variable and could therefore change the cell physiology, which may result in the cells responding to signals in a fundamentally different manner from that which occurs in the normal counterpart of that cell. However, the general pattern is for cells to assume transformed growth characteristics while maintaining phenotypic markers that distinguish the parental line (Sack, 1981).

For example, SV40 transformed human parathyroid cells retain parathyroid hormone production (Deftos *et al.*, 1968). Human tracheal epithelial cells that were transformed by pSVori plasmid expressed cytokeratins, a characteristic of epithelial cells, retained both

microvilli and tight junctions and produced secretory proteins (Gruenert *et al.*, 1987) characteristic of the epithelium *in vivo*. With regards to IVF, a human Fallopian tube cell line for example, could secrete certain proteins that may be beneficial to the fertilisation process and the development of early embryos.

Ando *et al.*, (2000) published the first report of an established ciliated cell line from the human Fallopian tube. In an experiment similar to one carried out in this project, human tubal epithelial cells in primary culture were transfected with a Simian Virus 40 (SV40) large T-antigen plasmid using the calcium phosphate co-precipiation method. This group obtained an immortalised ciliated cell line without crisis and designated it as NT/T-S. Transmission electron microscopy showed that these cells had cilia, microvilli, junctional complexes, and hence retained morphological features associated with normal human Fallopian tube epithelial cells *in situ*. Furthermore, preliminary co-culture experiments with these cells using surplus embryos at the 4- to 8-cell stage had a positive effect on blastocyst formation when the fragments of the embryo were <30% of the surface area. The NT/T-S co-culture data also showed a positive effect on the formation of the expanded blastocysts but did not show any significant effect on the "hatching rate". It has been reported however, that 63% and 40% of embryos expanded and hatched in a sequential oviductal-endometrial co-culture system, compared with 41% and 9% in the oviductal system alone, respectively (Bongso *et al.*, 1994).

Various cytokines/growth factors such as leukaemia inhibitory factor (LIF), interleukin (IL)-6, IL-8 and basic fibroblast growth factor were also secreted by both normal tubal epithelial cells in primary culture as well as the established NT/T-S cell line. It is clear that

this established ciliated cell line would undoubtedly provide a valuable resource for further studies of the Fallopian tube in the early events of pregnancy.

The ultimate goal of co-culture should be the elucidation of physiologically important substances that are necessary for the development of improved culture media from the point of fertilisation to subsequent blastocyst formation. It has been suggested that co-culture systems will be obsolete clinically in a few years time and would be replaced with improve culture conditions (Ando *et al*, 2000). For this reason, any experimental studies undertaken with oviductal cells may increase current knowledge in this field.

Optimal culture conditions are of paramount importance for the *in-vitro* development of gametes and pre-implantation embryo(s). The culture conditions for blastocyst stage transfer have been improved by using sequential culture media. This is simply a combination of two different media used before and after genomic activation since the nutritional requirements required by the developing embryo(s) would be dependent on the stage of development (Quinn, 1994).

To summarise, these experiments have shown that human Fallopian tube epithelial cells can be transiently transfected, as the cells are capable of incorporating foreign DNA when transfected under the appropriate conditions. Cultured human Fallopian tube epithelial cells can achieve an extended life-span *in vitro* (102 days) and has the potential to achieve immortalisation. Generally, primary cells are known to be very difficult to transfect and perhaps for this reason an established Fallopian tube epithelial cell line have not been reported in the past. However, established cell lines of the human Fallopian tube could provide excellent models to study both morphological and

functional roles of the tubal epithelial cells on gamete maturation, fertilisation and early embryonic development *in vitro*.

6.1 General Discussion and Summary

This chapter is intended to give a general summary outlining the main conclusions drawn from the work undertaken in this thesis as well as suggestions for future studies. More detailed discussions have been dealt with in the relevant experimental chapters.

The Fallopian tube is a dynamic structure that serves as a conduit for the ovulated egg to enter the uterine cavity following fertilisation. Its functional epithelium undergoes cyclic variation and contains both secretory and ciliated cells. It is well known that the Fallopian tube has many active roles in reproduction, however, the interaction between the tube and gametes/embryos are not well understood. For this reason, any study that is intended to elucidate the role of the tube either *in vivo* or *in vitro* must be designed in such a manner to encompass the various aspects of a normal cell environment.

In order to conclude whether the aims of this study were met, it is necessary and helpful to remind one about the nature of the work undertaken in this thesis. Three diverse aspects of the human Fallopian tube were examined and therefore the study adopted a three pronged approach. The primary objectives were as follows;

a) to identify and quantify various gap junction proteins (connexins) in the ampullary and fimbrial regions of the Fallopian tube at different stages of the ovarian cycle. This basic knowledge is required to increase the understanding of cell to cell communication and hence, tissue function in this important organ.

b) to compare different isolation procedures for obtaining tubal epithelial cells, by assessing their growth patterns, morphological features and secretory status using PP14 as a marker. It is important to ensure that the techniques used for cell isolation does not impair the epithelial structure and function so that further studies can be carried out in order to obtain a better understanding of the underlying mechanisms in the reproductive process.

c). to attempt to establish an immortalised cell line of Fallopian tube epithelial cells. This will undoubtedly alleviate the need to rely on Fallopian tubes following surgical procedures. Fully characterised immortalised cell lines can be stored in liquid nitrogen and used whenever required for coculture experiments and IVF procedures.

It is becoming increasingly recognised that gap junctions and their intercellular communication function play a key role in the regulation of cell proliferation and differentiation (Trosko *et al.*, 2000). GJIC (gap junction intercellular communication) not only plays an important role in fertilisation and the normal development of both the embryo and foetus but it is also involved in the sexual maturation of the adult and in the maintenance of health throughout life (Trosko *et al.*, 2000). As such, GJIC may be the key element in the understanding of tissue functions especially during the reproductive process.

The gap junction proteins, connexins, were examined by immunohistochemical and Western blotting techniques. The immunoblotting results demonstrated the presence of gap junction proteins, connexins (cx) in the human Fallopian tube throughout the ovarian cycle. Fimbrial and ampullary regions were separated and subsequently processed for Western blotting analysis using a range of monoclonal or polyclonal antibodies directed against cx26, cx32 and cx43. The intensity of staining varied depending upon the hormonal status of the patients examined and appeared to be upregulated during the secretory stage as opposed to the proliferative phase of the ovarian cycle. Human Fallopian tube did not express cx32, regardless of the anatomical site examined. In all cases though, the expression of both cx26 and 43 appeared highest in the ampullary region.

Furthermore, no convincing evidence to suggest the presence of connexins on histological sections were found despite using a range of antigen retrieval methods commonly used in immunohistochemistry. Therefore, further work in this area is crucial to ascertain whether the results observed reflect genuine tissue characteristics or, were simply artefacts of the technology used.

To date, knowledge on the physiological aspects of the Fallopian tube has come from several sources. These include experiments carried out on (1) animal oviducts (Walter, 1995), (2) tubal specimens removed during operations (Saridogan *et al.*, 1997), (3) tubal fluid obtained by cannulation and (Lippes *et al.*, 1989), (4) epithelial cell culture or short-term explants of the tube (Dickens *et al.*, 1993; Maguiness *et al.*, 1993).

Human epithelia consist of highly organised layers of differentiated, polar cells (Cozens *et al.*, 1992) and hence the task of reproducing this system *in vitro* constitutes a significant challenge. It is well known that the epithelial lining of the Fallopian tube is a simple columnar epithelium which contains both ciliated and mucus-secreting cells (Verhage *et al.*, 1979; Crow *et al.*, 1994). In order to study the human Fallopian tube environment with regard to secretory activity, the influence of hormones and growth factors and to investigate the mechanism(s) of embryo-maternal communication a cell culture system is required. Although these cells have been isolated from the tissue and cultured *in vitro*, they characteristically divide a finite number of times and eventually senesce or become terminally differentiated (Gruenert *et al.*, 1990).

The use of normal human cells for research, biotechnology and therapeutic purposes has been restricted in part by their limited proliferative potential. However, in many applications it would be ideal to have an unlimited number of well-characterised cells with the desired phenotype. This would undoubtedly eliminate variations that may arise when different cell strains are used (Yeager and Reddel, 1999). To this end, the present investigation examined different methods that could be used to extend the life-span of the human Fallopian tube epithelial cells in culture.

As mentioned earlier, the primary aim of this particular section was firstly to isolate human Fallopian tube epithelial cells using a mechanical and enzymatic method. Following this, the isolation procedure that would be most suitable for establishing a viable Fallopian tube epithelial cell culture system in terms of their practical feasibility, cell yield and cellular characteristics was determined.

Furthermore, for reasons described above, the study naturally progressed to incorporate and examine different methods that could be used to extend the life span of the human Fallopian tube epithelial cells in culture. In doing so, such a model may well be used to facilitate and expedite experimental work on the Fallopian tube and its secretions.

In this study, it was shown that regardless of method of isolation (mechanical or enzymatic), over 95% of the cells obtained stained positive for cytokeratins thereby confirming that cells were indeed epithelial in nature. In addition, when the integrity of the isolated cells was assessed, it confirmed that there was minimum damage to the cell membrane of mechanically isolated cells which was corroborated by the nuclear staining with Hoechst 33358. In contrast, there was extensive cell membrane damage to cells isolated by the enzymatic method as noted by the red propidium iodide staining of the nucleus. However, it is important to note that these cells continued to proliferate in culture despite such membrane damage. Furthermore, in both primary and sub-cultures the chromosomal status of the tubal epithelial cells in culture remained diploid.

Using morphology and growth pattern as parameters, the mechanical method is preferable to the enzymatic method for the purpose of establishing a Fallopian tube model *in vitro*. Although both methods provided sufficient amounts of isolated epithelial cells, the mechanical method was the most reliable, easily performed, inexpensive and possible cellular damage during treatment with enzymes was avoided.

The study was subsequently extended in order to determine the secretory nature of the tubal cells. PP14 was used as a marker to assess the secretory function of primary cultures of tubal epithelial cells isolated by mechanical and enzymatic methods. It was demonstrated that PP14 secretion was reduced in cells isolated by enzymatic means.

The enzymatic procedure, though initially more laborious, resulted in a higher yield of epithelial cells and allowed cell counts to be carried out within three hours following the isolation. The large number of oviductal epithelial cells that can be obtained enzymatically could be advantageous in experimental studies, such as those which aim to investigate the effects of growth factors, hormones or drugs. However, although mechanically isolated cells can also be used for such experiments, the total cell number obtained was relatively low.

The number of cells obtained during the isolation procedures can be variable and the tubal epithelial cells that are grown in culture eventually lose morphological features associated with the epithelium *in situ* (Ando *et al.*, 2000). For this reason, an immortalised cell line derived from a single individual could provide an ideal experimental model. Once fully characterised, such a model would be preferable to primary cell cultures of Fallopian tube epithelial cells for carrying out various studies, as this will eliminate the need to rely on Fallopian tubes retrieved from women undergoing hysterectomy.

As such, the principal objective of the second part of the cell culture experiments focussed on various ways to extend the life span of cultured human Fallopian tube cells with a view to establish an immortalised cell line. Primary cultures of human tubal epithelial cells were transfected with a plasmid vector carrying the simian virus 40 (SV40) large T-antigen. This origin-defective SV40-Large T antigen have previously been used to immortalise various normal cells (Stoner *et al.*, 1991; Ishida *et al.*, 1995). In the present investigation, several techniques were employed in order to achieve this goal and the results were analysed using morphological and biochemical parameters.

Various techniques were used to transfect the epithelial cells in culture; these included well-established methods such as DNA co-precipitation as well as more advanced methods using cationic liposomes. The results from this study demonstrated that human Fallopian tube epithelial cells can be transiently transfected and could survive for over 102 days in culture, whilst still retaining many of the morphological characteristics of the original epithelial cell type.

Ando *et al.*, (2000), were the first investigators to obtain a stable human Fallopian tube cell line. The primary differences in their study compared to the one carried out in this investigation was that an additional enzyme (collagenase) was used to isolate the tubal cells and a different plasmid vector (pMK16-SV40 Large T-Ag (*ori*$^-$) was employed for the transfection. It is important to note that the manner in which a cell is affected by a transforming agent cannot be predicted with absolute certainity (Gruenert, 1987) and the point of integration of a transforming virus or the site of mutation by a chemical or physical agent will vary from cell to cell (Gruenert, 1987).

This will not only impact the gene where the virus is integrated or the DNA damage occurs but will also influence the karyotype of the cells (Gruenert, 1987).

It is well known that the Fallopian tube epithelium is one of the hormonal targets. The cells show morphological changes throughout the ovarian cycle suggestive of secretory activity. Therefore, in order to study more specifically the activity of the Fallopian tube epithelial cells in terms of the components they produce and how such production is controlled or regulated it is important to isolate the cells under controlled measures or conditions.

Indeed, the availability of continuous (immortal) cell lines has many advantages over primary (mortal) cell cultures both for research and diagnostic purposes. For example, immortal cell lines can be continuously passaged *in vitro* without the need to return to the tissue as the source of cells. Indeed the isolation of epithelial cells from the Fallopian tube can be a tedious and time-consuming technique and obtaining sufficient tissue prior to establishing healthy cell cultures routinely can also be problematic due to the limited availability of the Fallopian tube.

In addition, primary cell cultures that are obtained from tissue explants are often heterogeneous. In contrast, continuous or immortal cell lines are relatively homogeneous with respect to both the genotype and phenotype and can be cultured within a short time period if necessary. They can be stabilised in liquid nitrogen for several years as they have an indefinite and stable life-span, unlike primary cells.

In the past, continuous cell lines have often been derived from abnormal tumours or mutants arising from treatment with toxic chemicals or radiation and as such may not express a number of the key characteristics of the original tissue. The co-culture of embryos with non-tubal epithelial cells (Schillaci *et al.*, 1994) and skin fibroblasts (Wetzels *et al.*, 1998) suggest that the effect of tubal epithelial cells on embryo development is neither species nor tubal specific. Cells from the human Fallopian tube however, may be superior to other cell types and could produce factors which facilitate fertilisation and early embryo development. For this reason, epithelial cells from the Fallopian tube would seem to be the ideal choice since many of the physiologically important substances could be extracted, purified, fully characterised and subsequently incorporated into new and improved culture media that can be used in IVF procedures. The use of cell monolayers to culture gametes or embryos may not be a practical and efficient method in a busy IVF clinic since these cells would have to be reconstituted from liquid nitrogen, placed in suitable vessels and allowed to grow for a few days prior to any co-culture activity. However, if the beneficial substances that are secreted by these cells can be used to formulate new culture media then this could be easily available on a global scale. Furthermore, additional and vital insight about the functional role(s) of the Fallopian tube and its secretions on gamete maturation, fertilisation and early embryonic development could also be obtained.

6.2 Future Studies

In women, the endometrium undergoes a monthly cycle which is controlled by the ovarian hormones and prepares the uterus for implantation. Successful implantation requires both the attachment and adhesion of the blastocyst to the endometrial luminal epithelium and the penetration of the trophoblast into the endometrium (Jahn *et al.*, 1995). The expression patern of gap junction channel proteins (connexins), is directly related to this process (Grummer and Winterhager, 1998). Whether these proteins also play a role in the human Fallopian tube remains to be established.

The investigations undertaken in this thesis address to some extent the possible importance of connexins, since both cx26 and cx43 have shown to be present within the Fallopian tube ampullary and fimbrial regions. However, further immunohistochemistry work is required on both paraffin-embedded and frozen sections of the fimbrial, ampullary and where possible, the isthmic regions of the tube at various stages of the ovarian cycle. Although a number of antigen retrieval systems exist, it was not possible to explore all of them. As such, future studies should evaluate different immunohistochemical techniques to determine which method is best suited for the identification of the connexion proteins.

Furthermore, the tubes from a greater number of women, including post menopausal ones should be assessed. Only then (in the case of pre menopausal women) can more ideas regarding the mechanism(s) involved during fertilisation and early embryo development emerge. Once the presence of various connexins in the different anatomical region of the tube have been identified, experiments can be carried out to determine whether they are upregulated or downregulated in the presence of the

ovarian hormones, as intercellular communication must be vital during these processes.

In IVF, coculture of embryos with somatic cells is one of the methods used to promote embryo development *in-vitro* (Lee *et al.*, 2001). However, despite the success of coculture, it has not been the primary method for improving embryo development in human assisted reproduction programmes because of its complexity in the implementation as a routine service (Lee *et al.*, 2001).

The development of the ideal culture media for assisted reproductive technology is constantly evolving. Indeed, the ultimate goal is to emulate the *in vivo* environment, which represents a dynamic milieu in which fertilisation and early embryonic development occurs. The first IVF medium developed specifically for use in human IVF and embryo culture was human tubal fluid (HFT). This was formulated over 20 years ago and was a simple solution based on the chemical components present in the tubal fluid (Lippes *et al.*, 1972; Borland *et al.*, 1980). However, it is now commonly accepted that this formulation is not the ultimate medium for human IVF and as such it has been modified and improved over the years (e.g by the addition of amino acids and the removal of glucose and phosphate)(Quinn, 1994., 1995).

For some years now, there has been a strong interest in using co-cultures in human *in vitro* fertilisation (IVF) though this was originally developed in animal embryo research in order to overcome *in vitro* development block. Various cell types have been used in co-culture systems, such as human and bovine tubal cells (Bongo *et al.*, 1992; Wiemer *et al.*, 1993), Vero cells (Menezo *et al.*, 1990, 1992; Sakkas *et al.*,

1994) and granulosa cells (Freeman *et al.*, 1995). These studies reported positive effects of co-culture in embryo development and pregnancy rates.

Although IVF and embryo transfer has made extensive progress recently, there are still some set-backs especially with regards to the time of embryo transfer to the uterus. Currently in IVF/embryo transfer procedures two or three embryos at the 4- to 8-cell stage are usually transferred to the uterus on day 2 or 3 following insemination. This timing is at least 2 days earlier than when the embryos would have entered the uterine cavity following fertilisation *in vivo*. However, the development of the embryo to the blastocyst stage *in vitro* is at present limited and hence two different approaches have been used to improve the culture conditions for the blastocyst-stage transfer (Menezo *et al.*, 1998). The first, as mentioned previously, is the use of co-culture of human embryos with human and animal monolayer cells and the second is the use of sequential culture media; a combination of two different media. A study carried out by Gardner and Lane,(1998) reported that the use of sequential serum-free media can support >50% of blastocyst development. In light of these observations, the common goal of future studies should focus on the development of improved IVF culture media rather than using a co-culture system directly.

It is known that the Fallopian tube synthesizes and secretes specific proteins, which may vary according to anatomical site, stage of the ovarian cycle and even during pregnancy. Hence, it is also likely that the tubal epithelial cells *in vitro* also secrete beneficial substances for the development of preimplantation embryos. However, human tubal cells are not constantly available, and the cultured cells are usually non-ciliated due to the processes of deciliation or dedifferentiation *in vitro*.

Therefore, it is important to develop such a cell line as this could provide a valuable resource for the development of improved culture media. The secretion of important substances released by the tubal epithelial cells, coupled with their effect(s) on gametes and early embryos undoubtedly requires further investigation.

Controlled and systematic studies of specific protein synthesis and secretion in normal Fallopian tubes are lacking but these would be beneficial especially in the development of new contraceptive measures and in assisted reproduction procedures. Most drugs act by targeting proteins or protein receptors and therefore if the structure of a particular protein secreted by the Fallopian tube is known, then this may assist in understanding the function(s).

Normal human cells divide a finite number of times before reaching a state of senescence. However, continuous or immortalised cell lines overcome this problem and can used for a number research and therapeutic purposes. Although, new immortalised cell lines can be generated using a range of techniques (e.g electroporation, irradiation) it is unlikely that all the differentiated functions of the parental cell type would be retained. The 'foreign' (i.e transforming gene) can integrate with chromosomal DNA at different sequences and this may result in different properties being exhibited by the immortalised cells. Therefore, it is crucial that a thorough characterisation be carried out on newly developed cell lines. Details such as morphological features, stability, growth characteristics, protein secretions, immunocytochemical surface markers and karyology to name a few, are important details which will undoubtedly be useful for comparative purposes in the future.

Over the years there have a number of investigations into the potential roles of the tubal epithelium in modulating different aspects of sperm function (Ellington *et al.*, 1999., Zhang and Zhu., 2000). Indeed, sperm survival is essential both in an *in vivo* and *in vitro* environment in order to undergo events such as capacitation and the acrosome reaction. Investigations carried out by Ellington *et al.*, (1999) and Yao *et al.*, (2000) demonstrated that sperm maintained their motility for longer periods than in standard culture medium, when present in an oviductal cell culture environment. Because, the epithelium of the oviduct has continuous and complex secretory activities, it is quite possible that it may enhance sperm survival and fertilising ability. Therefore, sperm characteristics can be studied further using an immortalised cell line.

The ciliated epithelial cells of the tube are likely to be involved in gamete and embryo development but whether they have any direct effect on the development of early embryos remain unknown. In addition, it is possible that secreted cytokines and growth factors that may be beneficial to both gametes and pre-implantation embryos are retained and concentrated near the apical cell surface due to the presence of both cilia and microvilli. As such, a fully characterised established cell line(s) can be used to further investigate these structures during embryo growth. Secreted products can also be used enhance or improve commercially available culture media (conditioned medium) which may in turn benefit assisted reproductive technology.

To end, 'human proteonomics' is fast becoming an important area especially where tubal diseases is concerned and it can use its analytical techniques to determine what makes a diseased or 'mutant' protein different from a normal protein. Furthermore, since most drugs act by targeting proteins or protein receptors, then this field may aid

in the development of new drugs (e.g contraceptives). Such information will certainly contribute to the current understanding of this dynamic and important organ, the Fallopian tube.

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LIST OF MATERIALS

Materials	Purchased From
Acetic acid	BDH Chemicals, Poole, UK
Acrylamide	National Diagnostics, Aylesbury, Bucks, UK
Ammonium persulphate	Sigma-Aldrich Company Ltd, Poole, UK
anti-mouse IgG labelled with FITC	Sigma-Aldrich Company Ltd, Poole, UK
anti-mouse IgG-peroxidase conjugate	Sigma-Aldrich Company Ltd, Poole, UK
anti-rabbit IgG labelled with TRITC	Sigma-Aldrich Company Ltd, Poole, UK
Bis-acrylamide	National Diagnostics, Aylesbury, Bucks, UK
Bovine serum albumin	Sigma-Aldrich Company Ltd, Poole, UK
Calcium chloride	BDH Chemicals, Poole, UK
Cell dissociation solution	Sigma-Aldrich Company Ltd, Poole, UK
Chloroform	Sigma-Aldrich Company Ltd, Poole, UK
CHO-K1 cells	European Collection of Cell Lines
Citrate acid	BDH Chemicals, Poole, UK
Colcemid	Life Technologies Ltd, Paisley, UK
Concert High Purity Plasmid Purification System	Life Technologies Ltd, Paisley, UK
Connexin antibodies sampler pack	Cambridge Bioscience, Cambridge, UK
Coomassie blue	Life Technologies Ltd, Paisley, UK
Copper sulphate	BDH Chemicals, Poole, UK
Cytochalasin B	Sigma-Aldrich Company Ltd, Poole, UK
DAB	Sigma-Aldrich Company Ltd, Poole, UK
Dihydrogen orthophosphate	BDH Chemicals, Poole, UK
Disodium hydrogen orthophosphate	BDH Chemicals, Poole, UK
Dispase	Life Technologies Ltd, Paisley, UK
DMSO	Life Technologies Ltd, Paisley, UK
DPX mountant	BDH Chemicals, Poole, UK
Dulbecco's Minimum Essential Medium/Nutrient mixture Ham's F-12 with Glutamax I	Life Technologies Ltd, Paisley, UK
ECL *in vitro* translation streptavidin-HRP	Amersham-Pharmacia Biotech, Bucks, UK
ECL markers	Amersham-Pharmacia Biotech, Bucks, UK
ECL western blot detection kit	Amersham-Pharmacia Biotech, Bucks, UK
EDTA	Sigma-Aldrich Company Ltd, Poole, UK
Effectene reagent	Qiagen Ltd, Crawley, West Sussex, UK

Materials	Purchased From
Eosin	BDH Chemicals, Poole, UK
Ethanol	BDH Chemicals, Poole, UK
Foetal bovine serum	Life Technologies Ltd, Paisley, UK
Folin-Ciocalteu reagent	Sigma-Aldrich Company Ltd, Poole, UK
Formaldehyde	BDH Chemicals, Poole, UK
Fungizone	Life Technologies Ltd, Paisley, UK
Geneticin (G418)	Sigma-Aldrich Company Ltd, Poole, UK
Giemsa stain	BDH Chemicals, Poole, UK
Glycerol	BDH Chemicals, Poole, UK
Glycine	BDH Chemicals, Poole, UK
Haematoxylin	BDH Chemicals, Poole, UK
Hanks Balanced Salt Solution without Calcium and Magnesium	Life Technologies Ltd, Paisley, UK
HEPES	Sigma-Aldrich Company Ltd, Poole, UK
Histomount	BDH Chemicals, Poole, UK
Hoechst 33258	Sigma-Aldrich Company Ltd, Poole, UK
HRT 18 cells	European Collection of Cell Lines
Hybond ECL	Amersham-Pharmacia Biotech, Bucks, UK
Hydrochloric acid	BDH Chemicals, Poole, UK
Hyperfilm	Amersham-Pharmacia Biotech, Bucks, UK
Igepal CA-630	Sigma-Aldrich Company Ltd, Poole, UK
Ilford XP2 film	AGFA Scientific Ltd
Ilford Hypam fixer	AGFA Scientific Ltd
Ilford microphen developer	AGFA Scientific Ltd
Jurkat 6 cells	European Collection of Cell Lines
Kodak GBX developer and replenisher	Sigma-Aldrich Company Ltd, Poole, UK
Kodak GBX fixer and replenisher	Sigma-Aldrich Company Ltd, Poole, UK
Lauryl sulphate	Sigma-Aldrich Company Ltd, Poole, UK
LB broth base	Life Technologies Ltd, Paisley, UK
Lipofectamine 2000	Life Technologies Ltd, Paisley, UK
Methanol	BDH Chemicals, Poole, UK
Minimum Essential Media in Earle's Salt with L-glutamine	Life Technologies Ltd, Paisley, UK

Materials	Purchased From
Monoclonal anti cytokeratin	Sigma-Aldrich Company Ltd, Poole, UK
Monoclonal anti-SV40T antibody	Chemicon International Ltd, Harrow, UK
Monoclonal mouse and polyclonal rabbit anti-connexins 26, 32 and 43	Cambridge Bioscience, Cambridge, UK
Nitrocellulose membrane	Amersham-Pharmacia Biotech, Bucks, UK
Opti-MEM reduced serum medium	Life Technologies Ltd, Paisley, UK
Oxoid nutrient medium	Oxoid Ltd, Basingstoke, Hants, UK
Pancreatin	Sigma-Aldrich Company Ltd, Poole, UK
pCMV sport-βgal	Life Technologies Ltd, Paisley, UK
p-EGFP-N1 plasmid vector	Clonetech Laboratories, Hampshire,UK
Penicillin/Streptomycin	Life Technologies Ltd, Paisley, UK
Phosphate buffer tablets	Life Technologies Ltd, Paisley, UK
Phosphate buffered saline with Calcium and Magnesium	Life Technologies Ltd, Paisley, UK
pMX1 plasmid vector	R &D Systems Europe Ltd, Oxon, UK
Potassium chloride	BDH Chemicals, Poole, UK
Profection mammalian transfection reagent kit	Promega, Southampton, UK
Rapitone photographic paper	AGFA Scientific Ltd
Sodium chloride	BDH Chemicals, Poole, UK
Sodium hydroxide pellets	BDH Chemicals, Poole, UK
Sodium potassium tartrate	BDH Chemicals, Poole, UK
Sterile pipettes and pastettes; bijous; universals; cryovials; multiwell plates; eppendorfs; centrifuge tubes and tissue culture flasks (Nunc).	Bibby Sterilin Ltd, staffs, UK
Subcloning efficiency DH5α competent cells	Life Technologies Ltd, Paisley, UK
Superfect reagent	Qiagen Ltd, Crawley, West Sussex, UK
TEMED	Sigma-Aldrich Company Ltd, Poole, UK
Transfection optimisation kit (a gift)	Qiagen Ltd, Crawley, West Sussex, UK
Transfection sampler pack	Life Technologies Ltd, Paisley, UK
Tri-reagent	Sigma-Aldrich Company Ltd, Poole, UK
Tris; tri-sodium citrate	BDH Chemicals, Poole, UK
Trypan blue solution	Sigma-Aldrich Company Ltd, Poole, UK
Trypsin	Sigma-Aldrich Company Ltd, Poole, UK

Materials	Purchased From
Trypsin-EDTA (1X) in HBSS w/o Ca and Mg with EDTA.4Na	Life Technologies Ltd, Paisley, UK
Tween 20	Sigma-Aldrich Company Ltd, Poole, UK
Vectastain Elite ABC Kit	Vector Laboratories Inc, Peterborough, UK
William's medium in Earle's salt	Life Technologies Ltd, Paisley, UK
X-gal substrate	Sigma-Aldrich Company Ltd, Poole, UK

Matrigel™ Matrix is a solubulized basement membrane preparation. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin. At room temperature, Matrigel™ Matrix polymerizes to produce biologically active matrix material resembling the mammalian cellular basement membrane. Cells behave as they do *in vivo* when they are cultured on Matrigel™ Matrix. It provides a physiologically relevant environment for studies of cell morphology, biochemical function, migration or invasion, and gene expression. Kleinman, H.K., et. al., Biochem. 21:6188 (1982).

AFFILIATIONS, RELATED ACADEMIC ACTIVITIES & PUBLICATIONS

- European Society of Human Reproduction and Embryology (ESHRE).
- British Fertility Society (BFS)
- British Andrology Society (BAS)
- Society for the Study of Fertility (SSF)
- Institute of Biomedical Science (IBMS)

Meetings, Conferences and Workshops attended

In vitro fertilisation **(IVF) and Semen Analysis Course-** Two day seminar at the Assisted Conception Unit at Newham General Hospital (London, United Kingdom: December 1995).

Joint International Symposium of Reproductive Medicine of the European Society of Human Reproduction and Embryology, American Society of Reproductive Medicine and Instituto Valenciano de Infertilidad. **"State of the Art of Human Implantation: basic and clinical aspects** (Madrid, Spain: May 1998).

The British Andrology Society: **Sperm Biology; New Techniques, New Insights.** The Medical Society of London (London, United Kingdom: September 1998).

Cellular and Metabolic Aspects of Epithelial Cells. The Royal London Hospital (London, United Kingdom: May 1999).

William Harvey Day. St Bartholomew's & the Royal London Hospital School of Medicine & Dentistry, London, United Kingdom: October 2000.

Qiagen Ltd.- Diagnostic Special- Workshop on nucleic acid isolation and PCR/RT-PCR at LGC, Teddington, Middlesex, United Kingdom: March 2001.

POSTER PRESENTATIONS, ABSTRACTS AND PUBLICATIONS

Poster presentation (Abstract): International Symposium of Reproductive Medicine (Madrid, Spain). The title: "*Morphological, karyotypical and immunohistochemical analysis of primary and sub-cultured human Fallopian tube epithelial cells*".

Sattar S, Jones L, Abbas B, Mahmood T, Saridogan E, Mehta J.G and Djahanbakhch O (1998).

Oral presentation: Scientific meeting entitled ***'Cellular and Metabolic Aspects of Epithelial Cells'*** at The Royal London Hospital, May 1999. "Isolation and growth aspects of Fallopian tube epithelial cells".

Oral presentation: Scientific meeting entitled "***Clinical Reproductive Medicine: The Fallopian tube and the Placenta***" at St Bartholomew's Hospital on the January 2000. "The clinical value of the Fallopian tube epithelial cells in assisted reproduction; the search for a suitable model *in vitro*".

Paper titled "*Comparison of the growth patterns and morphological characteristics of mechanically and enzymatically isolated Fallopian tube epithelial cells*". Saeeda Sattar, Babar Abbas, Lucy Jones, Ertan Saridogan, Tariq Mahmood, Jayant Mehta and Ovrang Djahanbakhch. *Cell Biology International* (1999); **23**: (5), pp.379-383.

Paper titled "*Fallopian tube ciliary beat frequency in relation to the stage of menstrual cycle and anatomical site.* R.A Lyons., O.Djahanbakhch., T.Mahmood., E. Saridogan., S. Sattar., M.T Sheaf., A.A. Naftalin., R. Chenoy. *Human Reproduction* (2002); **17**: (3), 584-588.

Article "*Trophoblastic invasion in vitro- The key to the start of life*". Djahanbakhch, O., Mahmood, T., Lyons, RA., Habib, A., Yacoub, S and Sattar, S. (2000). Accepted for publication.

Paper titled "*PP14 secretion as a marker of physiological activity in cultured human Fallopian tube epithelial cells.* Saeeda Sattar, Rachel Lyons, Babar Abbas, Lucy Jones, Ray Iles and Ovrang Djahanbakhch. *Cell Biology International* (2003)- To be submitted.

Paper titled "*The expression of connexins in the human Fallopian tube throughout the ovarian cycle*". S. Sattar-Sahibdeen, R.A. Lyons., T. Mahmood., B. Abbas., L. Jones and O. Djahanbakhch. *Human Reproduction* (2003)- In Final Preparation.