5α-Reductase Expression in the Human Prostate and the Effect of Permixon® on Isoenzyme Activity

Colin William Bayne

Thesis submitted for the degree of Doctor of Philosophy
Edinburgh, 1997
The expression, activity and control of 5α-reductase isoenzymes in human prostate and in various models of the prostate were examined along with the effect of the phytotherapeutic agent Permixon® on the activity of the expressed isoenzymes.

The available models for benign prostatic hyperplasia (BPH) suffer from a number of limitations including the absence of androgen receptor expression (except LNCaP cell line), the failure to secrete PSA, the inability to respond to androgens and the lack of 5α-reductase type II expression. It therefore became necessary to develop a novel in vitro model, which would reflect more clearly the physiological condition in man and present a suitable system to investigate the control of 5α-reductase type II expression. By culturing fibroblast and epithelial cells together in co-culture environment, utilising Millicell™ well inserts, it was possible to induce the mRNA expression of 5α-reductase type II in both epithelial and fibroblast cells as detected by reverse transcription polymerase chain reaction (RT-PCR). Furthermore 5α-reductase isozymes were shown to be functionally active in cultured cells by employing a pH assay, which exploited the different pH optimum of type I & II isozymes to distinguish between them. Western blot analysis of protein extracted from primary and co-cultured cells showed the expression of androgen receptors in co-cultured epithelial and fibroblast cells but not in primary cultured cells. Co-cultured epithelial and fibroblast cells also demonstrated increased growth in response to androgens and a capacity to express PSA as detected by ELISA and immunohistochemistry. The levels of PSA detected by ELISA, were increased following addition of androgens to the medium.

Attempts were also made to identify the mechanism(s), which controls the expression of 5α-reductase type II in the epithelial cells. Fractionation of fibroblast conditioned medium by size exclusion spin columns and ion exchange HPLC, demonstrated that an anionic protein of approximately 10kD in size, produced by prostate fibroblast cells, induced a stimulation of the expression of 5α-reductase type
II in primary cultured epithelial cells; Enzyme expression was reversed in the absence of this factor.

The role of the 5α-reductase isoenzymes in the prostate is somewhat controversial with no clear reason being elucidated for the expression of two isoenzymes, which appear to have the same function. However, by analysing the metabolites of testosterone, it was possible to identify separate and distinct roles for the isoenzymes within the prostate. Cells expressing only 5α-reductase type I demonstrate high levels of androstenedione following metabolism of testosterone and very low levels of DHT. Cells expressing both isoenzymes demonstrate much higher levels of DHT and lower levels of androstenedione. The implication from this data is that 5α-reductase type I has a higher affinity for androstenedione than for testosterone whereas the type II enzyme has a higher affinity for testosterone. This would suggest that these isoenzymes are responsible for two separate pathways for the metabolism of testosterone to DHT in the human prostate gland.

With the development of a BPH model, which expresses both isoforms of 5α-reductase and secretes PSA, the opportunity arose for investigation into the action and effect of Permixon® on 5α-reductase and PSA. Permixon® demonstrated an ability to inhibit the action of both isoforms of 5α-reductase without having any effect on androgen sensitive processes such as PSA secretion and androgen induced growth. Electronmicroscopy indicated that Permixon® has a disruptive effect on prostate derived cell membranes but has no similar effect on non-prostate derived cells.
DEDICATION

I dedicate this thesis to my parents Robert and Helen who have helped me in innumerable ways.
DECLARATION

I, Colin William Bayne, hereby declare, that unless otherwise stated the work embodied in this thesis is the result of my own independent investigation. This is in accordance with rule 3.4.7 of the University of Edinburgh Postgraduate Study Programme.
ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor Dr Fouad K. Habib for allowing me the opportunity to work in his laboratory and to turn some more of his hair grey. His patience and supervision made my time here very enjoyable and stimulating. I would also like to thank my co-supervisor, Dr Karen Chapman who provided new and interesting angles on many parts of the project.

I am also grateful to Margaret Ross, the laboratory technician, for all her help and for having to put up with the moaning. I would also like to thank Dr. Ewan Grant for his help when I first arrived and Anne Wilson for letting me take over her office to print this thesis off on her computer. I also wish to acknowledge the help of all my friends who put up with me over the last 3 years and never moaned (at least not to my face). I am very grateful to Frank Donnelly for his excellent electron microscopy work and to Neil Inglis for his assistance with the HPLC.

Finally, I wish to thank Pierre Fabre Medicament for funding this project.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Declaration</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vi</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xv</td>
</tr>
</tbody>
</table>

## 1 INTRODUCTION

### 1.1 The Prostate

1.1.1 Embryology..........................1  
1.1.2 Epithelial-Mesenchyme Interactions in the Embryo..................3  
1.1.3 Gross Anatomy..................................4  
1.1.4 Internal Anatomy of the Prostate..................................4  
1.1.5 Histology of the Prostatic Acini and ductal system..............7  
1.1.6 Function...................................11  

### 1.2 Endocrinology of the Prostate

1.2.1 Androgens and the Prostate..........................................11  
1.2.2 Control of Testosterone Production..................................14  
1.2.3 Mechanism of Action of Androgens...................................16  
1.2.4 Other Hormonal Influences on the Prostate........................19  
1.2.5 Stromal Epithelial Interaction in the Adult Prostate............21  

### 1.3 Benign Prostatic Hyperplasia

1.3.1 Incidence................................22  
1.3.2 Aetiology......................................23  
1.3.3 Morphology.....................................24  
1.3.4 Clinical course.................................27  
1.3.5 Development of BPH..............................27
1.4 5α-Reductase.................................................................30
  1.4.1 Actions of 5α-Reductase..............................................31
  1.4.2 5α-Reductase Isoenzymes..........................................32
  1.4.3 Substrate Specificity................................................32
  1.4.4 Distribution of 5α-Reductase........................................39
  1.4.5 The Role of 5α-Reductase in the Adult Human Prostate........42
1.5 Treatment of Benign Prostatic Hyperplasia.................................42
  1.5.1 Principles of Therapy................................................42
  1.5.2 Surgical Intervention.................................................42
  1.5.3 Pharmacological Therapy............................................43
  1.5.4 Endocrine Therapy..................................................44
  1.5.5 Phytotherapy..........................................................44
1.6 Permixon®.................................................................46
  1.6.1 Components of Permixon®.............................................46
  1.6.2 Action of Permixon®..................................................47
  1.6.3 Pharmacology of Permixon®........................................48
1.7 In-vitro Models of Prostatic Hyperplasia and 5α-Reductase expression...48
  1.7.1 Immortalised Prostatic carcinoma cell lines..........................50
  1.7.2 Primary Cultures of Prostatic Cells................................51
1.8 In-vivo Models of Benign Prostatic Hyperplasia............................53
1.9 Aims and Objectives..........................................................54

2 MATERIALS AND METHODS

2.1 Materials.................................................................55

  2.1.1 Cell lines.............................................................55
  2.1.2 Cell Culture..........................................................56
  2.1.3 Cell Proliferation....................................................57
  2.1.4 Preparation of LSESR (Permixon®).................................58
  2.1.5 Molecular Biology..................................................58
2.1 Methods

2.2.1 Cell Culture

2.2.1.1 Cell Lines

2.2.1.2 Culture of Epithelial and Fibroblast cells from Human Prostatic Tissue

2.2.1.3 Determination of Cell Numbers

2.2.1.4 Co-Culture of Epithelial and Fibroblast Cells

2.2.1.5 Measurement of Androgenic Response of Cultured Cells

2.2.2 Preparation and Fractionation of Conditioned Media

2.2.2.1 Preparation of Conditioned Medium from Epithelial and Fibroblast Monolayer Cultures

2.2.2.2 Preparation of Conditioned Medium from Human Prostatic Epithelial and Fibroblast Cell Co-Cultures

2.2.2.3 Fractionation of Conditioned Media from Fibroblast Monolayer Cultures

2.2.2.4 HPLC Analysis of Fibroblast Conditioned Medium

2.3 Analysis of 5α Reductase Activity

2.3.1 Preparation of Primary Cultured Prostate Epithelial and Fibroblast Cells for Analysis of 5α-Reductase Isozyme Activity

2.3.2 Preparation of Co-Cultured Epithelial and Fibroblast Cells for Analysis of 5α-Reductase Isozyme Activity

2.3.3 Preparation of Human Prostate Tissue for Analysis of 5α-Reductase Isozyme Activity

2.3.4 5α-Reductase Isozyme Assay

2.3.5 Analysis of 5α-Reductase Activity in Growing Cells

2.3.6 Separation of 5α Reductase Isozyme Activity Assay Metabolites by Thin Layer Chromatography (TLC)

2.4 Isolation of Nucleic Acids
2.4.1 Isolation of Total RNA from Primary Cultured Cells........................................77
2.4.2 Isolation of Total RNA from Co-Cultured Cells..................................................79
2.4.3 Isolation of Total RNA from Snap-Frozen Tissue.................................................80
2.4.4 Isolation of Messenger RNA from Total RNA.....................................................80

2.2.5 Analysis of Nucleic Acids.........................................................................................81
2.2.5.1 Reverse Transcription of Total RNA.................................................................81
2.2.5.2 Amplification of 5α-Reductase Type I (5αRI) and 5α-Reductase (5αRII) cDNA Sequences..........................................................82
2.2.5.3 PCR Reaction......................................................................................................83
2.2.5.4 Agarose Gel Electrophoresis of PCR Products..................................................83

2.2.6 Analysis of Proteins by Western Blotting...............................................................85
2.2.6.1 Isolation of Proteins from Cultured Cells.........................................................85
2.2.6.2 Isolation of Protein from Co-Cultured Cells.......................................................86
2.2.6.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins...............87
2.2.6.4 Transfer of Proteins from SDS-Polyacrylamide Gels to Nitrocellulose............88
2.2.6.5 Immuno-detection of Androgen Receptor Protein Immobilised on Nitrocellulose.......................................................................................89

2.2.7 Electronmicroscopy...............................................................................................90

2.2.8 Analysis of PSA Production in Cultured Cells.......................................................90
2.2.8.1 Immunohistochemical Analysis of PSA Production in Cultured Cells...........90
2.2.8.2 Measurement of PSA by ELISA...........................................................................92

2.2.9 Immunohistochemical Characterisation of Primary and Co-Cultured Epithelial Cells.............................................................................................................93

2.2.10 Analysis of effects of Permixon® (LSESR)............................................................93
2.2.10.1 Cytotoxicity of LSESR.......................................................................................93
2.2.10.2 Effect of LSESR on 5α-Reductase Activity.......................................................94
2.2.10.3 Effects of LSESR on Cell Membranes................................................................94

2.2.11 Statistical Analysis..............................................................................................94
3 RESULTS

3.1 5α-Reductase mRNA Expression in Cultured Prostate Cancer Cell Lines and Cultured Epithelial and Fibroblast Cells - Analysis by Reverse Transcription-Polymerase Chain Reaction

3.1.1 Analysis of Prostate Cancer Cell Lines for 5α-Reductase Isozyme RNA
3.1.2 Analysis of Prostate Tissue for 5α-Reductase Isozyme Expression
3.1.3 5α-Reductase Isozyme RNA Expression in Primary Cultured Prostate Fibroblast and Epithelial Cells
3.1.4 5α-Reductase Isozyme Expression in Co-Cultured Human Prostate Fibroblast and Epithelial Cells
3.1.5 5α-Reductase Isozyme Expression in Fibroblast Conditioned Medium Treated Human Prostatic Epithelial cells
3.1.6 Effect of Breast Fibroblast Cells on 5α-Reductase Expression in Prostatic Epithelial cells
3.1.7 Effect of Skin Fibroblast Cells on 5α-Reductase Expression in Prostatic Epithelial Cells
3.1.8 Further Characterisation of Fibroblast Secreted Factor Responsible for Effect on 5α-Reductase expression in Prostatic Epithelial Cells

3.2 Effect of Permixon® on Cell Growth

3.2.1 Growth of Prostate Cancer Cell Lines treated with Different Concentrations of Permixon®
3.2.2 Growth of Primary Cultured Prostate Epithelial and Fibroblast Cells Treated with Different Concentrations of Permixon®
3.2.3 Growth of Co-Cultured Prostate Epithelial and Fibroblast Cells Treated with Different Concentrations of Permixon®

3.3 Measurement of Functional 5α-Reductase Activity - Effect of Permixon®

3.3.1 5α-Reductase Isozyme Activity in Human Prostate Tissue
3.3.2 Effect of Permixon® on 5α-Reductase Activity in Human Prostate Tissue
3.3.5 5α-Reductase Isozyme Activity in Prostate Carcinoma Cell Lines........129
3.3.4 Effect of Permixon® on 5α-Reductase Activity in Prostate Carcinoma Cell
Lines....................................................................................................................129
3.3.5 5α-Reductase Activity in Primary Cultured Epithelial and Fibroblast
Cells.......................................................................................................................131
3.3.6 Effect of Permixon® on 5α-Reductase Activity in Primary Cultured
Prostate Epithelial and Fibroblast Cells..............................................................133
3.3.7 5α-Reductase Isozyme Activity in Co-Cultured Prostate Epithelial and
Fibroblast Cells.....................................................................................................133
3.3.8 Effect of Permixon® on 5α-Reductase Isozyme Activity in Co-Cultured
Epithelial and Fibroblast Cells...........................................................................134

3.4 Pattern of Testosterone Metabolism in Prostate Cancer Cell lines and Cultured
Prostatic Cells.......................................................................................................136
3.4.1 Testosterone Metabolism in Prostate Cancer Cell Lines..............................136
3.4.2 Testosterone Metabolism in Primary Cultured Epithelial and Fibroblast
Cells.......................................................................................................................137
3.4.3 Testosterone Metabolism in Co-Cultured Prostate Epithelial and Fibroblast
Cells.......................................................................................................................140

3.5 Growth Response of Primary and Co-Cultured Fibroblast and Epithelial Cells to
Androgens..............................................................................................................140
3.5.1 Response of Primary Cultured Cells to Androgens......................................140
3.5.2 Response of Co-Cultured Cells to Androgens.............................................144
3.5.3 Response of Co-Cultured Epithelial and Fibroblast Cells to Androgens in
the Presence of Permixon®................................................................................147

3.6 Expression of Androgen Receptor Protein in Cultured Human Prostate Cells.....147
3.6.1 Androgen Receptor Protein Expression in Co-Cultured Epithelial and
Fibroblast Cells as Detected by Western Blot Analysis.........................................147

3.7 Characterisation of Co-Cultured Prostatic Epithelial and Fibroblast Cells by
Electronmicroscopy and Immunohistochemistry ..................................................149
3.7.1 Electron Microscopy of Co-Cultured Epithelial and Fibroblast Cells...........149
3.7.2 Immunohistochemistry of Primary and Co-Cultured Epithelial Cells for Basal Cell Markers ................................................................. 153

3.8 PSA Expression in Cultured Human Prostate Cells, Response to Androgen Stimulation and the Effect of Permixon® .............................................. 156
   3.8.1 Immunohistochemical Localisation of PSA in Primary and Co-Cultured Epithelial Cells ................................................................. 156
   3.8.2 Effect of Permixon® on Immunohistochemical Staining of Co-Cultured Epithelial Cells for PSA Expression ........................................... 159
   3.8.3 Detected Levels of PSA Expression in Primary and Co-Cultured Epithelial Cells ................................................................. 159
   3.8.4 Change in Detected Levels of PSA Expression in Primary and Co-Cultured Epithelial Cells in Response to Androgens ................................. 164
   3.8.5 Effect of Permixon® on Detected Levels of PSA Expression in Co-Cultured and Androgen Stimulated Co-Cultured Epithelial Cells ............... 165

3.9 Effect of Permixon® on Intracellular Membranes of Cultured Cells from Prostate, Skin and Breast ................................................................. 166
   3.9.1 Effect of Permixon® on Prostate Fibroblast and Epithelial Cell Membranes ................................................................. 166
   3.9.2 Effect of Permixon® on Skin Fibroblast Cell Membranes .................................................................... 170
   3.9.3 Effect of Permixon® on Breast Fibroblast Cell Membranes .................................................................. 170

3.10 Summary of Results ........................................................................ 176

4 DISCUSSION

4.1 In-vitro models of BPH .................................................................... 177
4.2 5α Reductase Isozyme Expression and Activity in Cultured Human Prostate Cells - Different Roles for Different Isoenzymes ......................... 181
4.3 Epithelial - Fibroblast Cell Interactions ........................................... 184
4.4 The mechanism of action of Permixon® and its role in the treatment of BPH ......................................................... 188
4.5 Future Studies .............................................................................. 192
ABBREVIATIONS

5αRI  5α-reductase type I
5αRII  5α-reductase type II
AR   Androgen Receptor
bp   base pairs
BPH  Benign Prostatic Hyperplasia
BSA  Bovine Serum Albumin
CaP  Carcinoma of the Prostate
cDNA complementary DNA
CM   Conditioned Medium
DAB  3,3′-Diaminobenzidine Tetrahydrochloride
DHT  5α-dihydrotestosterone
DNA  Deoxyribonucleic Acid
dNTP deoxynucleotide Triphosphate
EGF  Epidermal Growth Factor
FCS  Foetal Calf Serum
FGF  Fibroblast Growth Factor
HPLC High Performance Liquid Chromatography
IGF  Insulin-like Growth Factor
kD   kilo Daltons
LSESRLipidosterolic Extract of Serenoa Repens (Permixon®)
M    Molar
mAB  monoclonal Antibody
ml   millilitre
mRNA messenger Ribonucleic Acid
MTT  3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
OD   Optical Density
PAGE Polyacrylamide Gel Electrophoresis
PCR  Polymerase Chain Reaction
RNase Ribonuclease
RT   Reverse Transcription
SDS  Sodium Duodecyl Sulphate
T    Testosterone
TBE Tris-Borate-EDTA
TGF  Transforming Growth Factor
μl   microlitre
1 INTRODUCTION

1.1 The Prostate

1.1.1 Embryology

The fusion of the urorectal septum and the cloacal membrane during the sixth week of intrauterine life divides the hindgut of the embryo into anterior and posterior compartments with the urogenital sinus lying anterior to the rectum [1]. The narrowest part of the urogenital sinus, the pars pelvica, is marked by the entry of the mesonephric ducts into the sinus (fig 1).

It is in the pars pelvica that signs of the developing prostate can first be observed in the twelfth week of intra-uterine life [2]. Lowsley described five groups of epithelial buds growing into the mesenchyme surrounding the urogenital sinus. These five groups form an anterior lobe which degenerates before birth, a posterior lobe from the Mullerian tubercle and Wolffian ducts; a middle lobe from the cephalad to the Mullerian tubercle and right and left lateral lobes in the troughs on either side of the Mullerian tubercle.

This account does not relate to the modern idea of the zonal anatomy of the prostate. More recently, Timms et al (1994) have described the distribution of epithelial buds from the urogenital sinus, which corresponds, with the concept of prostatic zones [3]. Three groups were described, one arising from Glennister’s mixed epithelium around the Mullerian tubercle and two arising from lateral to the tubercle.

These buds grow into the surrounding mesenchyme, become canalised and ramify to form the epithelial components of the prostate.

Further development of the embryological prostate is dependent upon the action of testosterone (T) and the more active metabolite dihydrotestosterone (DHT). The primordial urogenital tract has an innate tendency to develop into the female genital tract. Male differentiation of the genitalia is dependent upon the activity of testicular androgens [4].
Figure 1.1 Diagramatic representation of a seven week gestation embryo (sagittal section). The urorectal septum has fused with the cloacal membrane thereby separating the urogenital sinus and the rectum.
This innate femininity of the urogenital tract is evident in the condition of testicular feminisation, which has been described in many species including humans [5,6]. Individuals with this condition demonstrate a deficiency of androgen receptors, which results in the feminisation of the body although they are chromosomally male. These individuals fail to respond to androgens and do not develop a prostate gland.

Another demonstration of the requirement of androgens for the differentiation of the male phenotype is the condition of 5α-reductase type II deficiency or pseudohermaphroditism. This disease is characterised by normal levels of androgen receptors and circulating testosterone but no DHT. This is due to an inability to metabolise T to DHT [7], a process dependent upon the action of 5α-reductase type II [8]. Other characteristics of this condition are the presence of normal Wolffian duct derivatives (epididymis, vas deferens and seminal vesicles) but the prostate, which derives from the urogenital sinus, does not develop.

This clearly shows that for the normal development of the prostate, androgens and more specifically DHT are essential.

1.1.2 Epithelial-Mesenchyme Interactions in the Embryo

Using epithelium isolated from the urogenital sinus and mesenchyme from seminal vesicle, urogenital sinus and Integumental mesenchyme from the back, snout, sole and preputial gland of the embryonic rats, Cunha et al [9] demonstrated the role of cellular interaction in the development of prostatic epithelium. Cunha et al [9] recombined urogenital sinus epithelium with urogenital mesenchyme (homotypic recombinants) and with any on of the non-urogenital mesenchymes (heterotypic recombinants). The recombinants were grown in-vitro for up to 3 days before being transplanted into the anterior chamber of the eyes of adult male rats where they were exposed to normal circulating levels of androgens.

The homotypic recombinants developed typical structures of the rat prostate, branching ductal acinar structures. Heterotypic recombinants failed to show any ductal structure development [8, 9]. Clearly demonstrating that urogenital sinus mesenchyme is necessary for normal prostate development.
1.1.3 Gross Anatomy

The adult prostate is a cone shaped fibromuscular, glandular organ, which is of a similar size to a walnut. The organ is orientated such that the base of the cone lies superiorly and the apex inferiorly. The organ surrounds the prostatic urethra and is located posterior to the lower margin of the pubic symphysis to which it is attached by the puboprostatic ligament. The prostate is separated from the pubic symphysis by the space of Retzius, which contains fatty tissue. The base of the prostate is related superiorly to the bladder neck where some of the glands muscular tissue is seen to be continuous with that of the bladder detrusor muscle. Posteriorly, the prostate gland is separated from the rectum by the remnants of the uro-rectal septum. The seminal vesicles are located along the superior, posterior border of the gland. Immediately medial to the seminal vesicles, the gland is penetrated by the vasa deferentia. Inferiorly the apex of the gland is related to the investing fascia of the urogenital sinus [10] (fig 2).

1.1.4 Internal Anatomy of the Prostate

The accepted model of the prostate is that proposed by McNeal (1981) [11]. This concept was derived from observations McNeal made of histological sections of more than 300 adult prostates. From the observations, McNeal was able to distinguish two distinct zones initially. These were the central zone and the peripheral zone. Further studies led to the description of a further two zones, the transition zone and the anterior fibromuscular zone [11] (fig 3). Although these zones have distinct histological appearances due to differences in their composition, they do not represent separate functional compartments of the gland. The zones however do demonstrate discernible differences in the pathological conditions, which affect the prostate.

The central zone is yellowish in appearance and comprises of frequently branching ducts with large acini. The central zone comprises only 25% of the prostatic volume and is separated from the peripheral zone by a thin fibrous stroma. Cancer arising from cells in the central zone accounts for only 8% of all prostate cancers.
Figure 1.2 Diagramatic representation of male genitourinary tract showing relations of the prostate.
Figure 1.3 Schematic representation of a sagittal section of the prostate, demonstrating the anatomical zones of the prostate. The central, transitional and anterior fibromuscular zones are indicated. (adapted from Algaba, 1992).
The peripheral zone is distinguishable from the central zone in that it has a grey appearance and at the histological level, the ducts display smaller acini and do not branch to the same degree as those seen in the central zone. The ducts from the peripheral zone open mainly into the prostatic sinuses but several open onto the lateral wall of the urethra. The peripheral zone constitutes almost 75% of the glandular mass of the prostate and accounts for 32% of the total prostatic volume. From a study conducted by McNeal et al in 1988 [12] 68% of 88 prostate cancers whose zone of origin could be determined, arose in the peripheral zone. A further 33% of the total prostatic volume is made up the fibromuscular zone, which constitutes the entire anterior surface of the prostate gland. The remaining 10% of the total prostate volume comprise of the transitional zone, which is comprised of a small group of ducts, which arise at the junction of the proximal and distal urethral segments. It is divided into two lobes by the urethra and displays ducts, which open onto the lateral wall of the urethra (fig 4). The ducts of the transitional zone are histologically similar to those of the central zone.

1.1.5 Histology of the prostatic acini and ductal system

The normal prostatic gland is divided into three components. These components are the proximal or secretory region, the distal or non-secretory region and the distal or proliferative tip [13] (fig 5). The glands are lined with tall columnar or pseudostratified columnar epithelium whereas the ducts are lined with cuboidal epithelium (fig 6). Both epithelial layers are separated from the basement membrane by a basal cell layer. The basement membrane effectively isolates the epithelia of the prostatic acini and ducts from the stromal tissue.

The stroma of the prostate is comprised predominantly of smooth muscle cells and fibroblasts although endothelial, nerve and inflammatory response cells can also be demonstrated histologically.
Figure 1.4 Schematic representation of the posterior wall of the prostatic urethra. The openings of the various prostatic ducts are shown in relation to the verumontanum (V). Central zone (CZ), peripheral zone (PZ), transitional zone (TZ) and urethral glands (UG).
Figure 1.5 Schematic representation of the internal structure of the prostate. This demonstrates prostatic urethra surrounded by the periurethral zone in relation to the transitional zone. The wedge shaped central zones surrounds the ejaculatory ducts.
Figure 1.6 Diagramatic representation of the normal architecture of prostatic glands.
(adapted from Tenniswood, 1993).
1.1.6 Function

The prostate gland secretes a thin, milky, alkaline fluid containing citric acid, calcium, acid phosphate, a clotting enzyme and a profibrinolysin. During emission, the capsule of the prostate gland contracts simultaneously with the contractions of the vas deferens so that the thin, milky fluid of the prostate gland adds to the bulk of the semen. The alkaline characteristic of the prostatic fluid may be quite important for successful fertilisation of the ovum, because the fluid of the vas deferens is relatively acidic owing to the presence of metabolic end-products of the sperm and, consequently, inhibits sperm fertility. Also the vaginal secretions of the female tract are acidic (pH 3.5 to 4.00). Sperm do not become optimally motile until the pH of the surrounding fluids rises to approximately 6 to 6.5. Consequently, it is probable that prostatic fluid neutralises the acidity of these other fluids after ejaculation and greatly enhances the motility and fertility of the sperm [14].

1.2 Endocrinology of the Prostate

1.2.1 Androgens and the Prostate

All C19 steroids are referred to as androgens despite not all of them being capable of stimulating the androgen receptor. Androgens are important for maintenance of the normal function and size of the prostate. This was demonstrated by Behre et al (1994) [15] in a study of men with abnormally low levels of circulating testosterone. It was found that these individuals had abnormally small prostates but on the administration of testosterone, the size and secretory activity of the prostate could be returned to normal levels. Androgens are synthesised mainly in the testis and adrenal cortex. The synthesis of androgens in these two sites occurs through the conversion of cholesterol via a series of enzymatic pathways, which were described by Ghandian (1982) [16]. The biosynthesis of androgens is illustrated in (fig 7). The prostate is particularly dependent upon testicular androgens. Oesterling et al (1986) [17] demonstrated that in men with panhypopituitarism (i.e. no testicular or adrenocortical synthesis of testosterone) and
Fig 1.7. Biosynthetic pathway of Testosterone from cholesterol. The numbered arrows represent the actions of the enzymes involved: 1. 20,22 desmolase; 2. Δ⁵-3β-hydroxysteroid dehydrogenase/isomerase; 3. 17α-dehydroxylase; 4. 17β-hydroxysteroid oxidoreductase.
men suffering from hypogonadism (i.e. no testicular synthesis but normal adrenocortical function) both had atrophic prostates. This indicates that adrenal androgens alone are not capable of maintaining normal prostatic growth and function.

The site of androgen synthesis in the testis is the Leydig cells [18]. These are clusters of cells found amongst the seminiferous tubules of the testis. Androgens are synthesised from cholesterol, which is mainly obtained from circulating lipoproteins although the Leydig cells can synthesise it. The initial step in the synthesis of androgens from cholesterol is the formation of pregnenolone by cleavage of the C-17 aryl side chain of the cholesterol molecule. This cleavage is catalysed by the enzyme 20,22 desmolase which is found on the inner mitochondrial membrane. All further steps in the synthetic pathway are carried out in the smooth endoplasmic reticulum and are detailed in. Obviously, a number of possible pathways for the synthesis of testosterone from pregnenolone exist. Data produced by Yanaihara and Troen in 1972 [19, 20] demonstrated that the \( \Delta 5 \) intermediates predominate in testicular androgen synthesis. However, it is clear that the testis produces \( \Delta 4 \) steroids including progestagens.

The major androgenic products of the adrenal glands are Dehydroepiandrosterone (DHEA) and its sulphated conjugate (DHEAS). The adrenal cortex is also the major site of androstenedione synthesis. DHEA and DHEAS influence prostatic growth and function through their conversion to androstenedione and subsequently testosterone. This conversion occurs mainly in adipose tissue, the adrenals and in the prostate itself.

Although testosterone is produced in significant quantities, 98% of the plasma testosterone is bound to plasma proteins [21, 22]. Several proteins are capable of binding testosterone but not all proteins bind it to the same degree. The distribution of the protein binding of testosterone reported by Vermuelen (1977) is as follows: testosterone binding globulin (TeBG) 56%, albumin 40% and corticosteroid binding globulin (CBG) 2%. After taking into account the binding of testosterone to plasma proteins, it is clear that only 2% of the total circulating testosterone is free. It was this 2% free portion which was for many years regarded as the biologically active portion [21]. However, during the 1980’s it became clear that the actual active portion was much larger than 2%. Work by Pardridge and Landaw in 1985 [23] demonstrated that this was due to dissociation of bound testosterone within capillary beds. In 1986,
Pardridge [24] demonstrated *in-vivo* that almost all the albumin bound testosterone was available for tissue uptake and that as a result, the actual levels of bioavailable testosterone was the sum of albumin bound and free i.e. approx. 42% of the total circulating concentration. The prostate however may be exposed to much higher levels of testosterone due to the expression of specific TeBG receptors on the cell membranes [25].

1.2.2 Control of Testosterone Production

The synthesis of testosterone from cholesterol in the testis is under the control of a glycoprotein, luteinising hormone (LH). This was demonstrated in hypophysectomised animals by Hall *et al* (1962) [26]. The animal's testis exhibited abnormally low levels of testosterone production, which could be increased upon the administration of LH. LH is synthesised by gonadotrope cells in the anterior pituitary under the influence of luteinising hormone releasing hormone (LHRH), an oligopeptide produced by the hypothalamus. The released LHRH from the hypothalamus reaches the anterior pituitary via the hypothalamo-hypophyseal portal system [27, 28].

By binding to the cell surface receptors, LH initiates the transport of cholesterol into the inner mitochondrial membrane within minutes, where it is converted to pregnenolone, as all ready described [29]. However, this short-term action is masked by the long-term effects, which sees an increase in the activity of the steroidogenic enzymes. This long-term effect is reflected in the production of testosterone, which shows a circadian rhythm, rather than the pulsatile rhythm that would mimic LH release from the pituitary.

The release of LH from the pituitary is inhibited by a negative feedback system in which testosterone directly inhibits the release of LH from the gonadotrope cells and also effects the release of LHRH from the hypothalamus (fig 8). Oestrogens also show a similar effect on the production of LHRH and LH [30]. The prostate itself has no identified means of feedback control on the production of testosterone.
Figure 1.8 Diagramatic representation of the inhibitory feedback loop involved in the control of testosterone production. Positive influences (+) are indicated by a solid line and negative influences (-) are indicated by a broken line.
1.2.3 Mechanism of Action of Androgens

The action of androgens in the prostate is mediated through the androgen receptor. The receptor acts by affecting the expression of various androgen dependent proteins through interaction with the cellular DNA.

The androgen receptor is a member of a super family of nuclear receptors. The androgen receptor binds two ligands, testosterone and DHT. The production and action of DHT will be discussed in a later section.

The nuclear receptor super family all demonstrate, a highly conserved DNA binding domain. The structure of the DNA binding domain is such that “zinc fingers” are formed as columnar protrusions (fig 9). It is these zinc fingers which are responsible for binding to the DNA structure. Towards the N terminal end of the receptor lie the sequences responsible for transcriptional activation. The androgen receptor has a hinge region, which links the C terminal hormone-binding domain to the DNA binding domain (fig 10). When no ligand is bound and the receptor is inactivated, the C terminal domain interferes with the transcriptional activity of the N terminal domain.

The action of the receptor is dependent upon the formation of a heterodimer with other receptor-ligand activated complexes. Testosterone will activate the androgen receptor but the testosterone-receptor complexes are less stable and have a lower affinity for DNA than the DHT-receptor complexes [31, 32].

Steroid receptors are generally found in the nuclei of responsive cells but the receptors are also found in the cytoplasm. It has been demonstrated that these receptors are actively transported back to the nucleus after ligand binding [33]. Nuclei of epithelial cells in the prostate have been shown by immunohistochemistry to have intense staining for androgen receptors [34]. This staining is found only in secretory cells and not in the basal epithelial cells of the prostate [35].

Other factors influenced by androgens include the epithelially produced proteins PSA and Prostate Specific Membrane antigen (PSM) [36, 37] along with other prostate secretory proteins. The PSA gene demonstrates an androgen response element at its 5' end [37a, 37b]. This is believed to confer a direct transcription ability in response to direct interaction with the receptor-ligand complex [38, 39, 40].
Figure 1.9 Diagramatic representation of the androgen receptor showing zinc finger structure of DNA binding domain. In bulbar muscular dystrophy, the polymeric glutamine region in the transcriptional activation domain is expanded from the normal number of glutamine residues (11-30) to more than 40; in male breast cancer, two different amino acid substitutions have been identified in the second zinc finger of the receptor; and in prostate cancer, several amino acid substitutions in the steroid binding domain have been associated with altered cell responsiveness to anti-androgens, progesterone and adrenal androgens.

The polymeric glutamine region is also important in the normal function of the androgen receptor. An increase in the length of the polyglutamine tracts is associated with reduced trans-activation and male infertility [30b]. (Adapted from Brown, 1996 [30a]).
Fig 1.10 Diagramatic representation of a protein belonging to the nuclear receptor superfamily. Numbers in brackets indicate number of amino acid residues in each region. Functional characteristics of each region are indicated below the region concerned. (Adapted from Jänne et al; 1991)
This is demonstrated in LNCaP cells, which show an increase in mRNA and protein of PSA in response to androgens [39, 41].

The actions of androgens in the prostate are through effects on the expression of different proteins. Several groups have investigated possible factors, which are regulated by androgens. These include transforming growth factor α (TGFα) in the prostate cancer cell line LNCaP [42], epidermal growth factor (EGF) receptor in the LNCaP cell line [43, 44], EGF receptor in the rat [45] and Fibroblast growth factor 2 (FGF2) in the LNCaP cell line [42].

As both cellular compartments in the prostate are sensitive to the actions of androgens, it is possible that the androgenic response of the epithelial cells may in part be mediated by the response of stromal cells to androgens and vice-versa.

This was the hypothesis investigated by Chang & Chung (1989) [46] when they investigated the differential effect of DHT on cell line cultures of prostatic fibroblast (NbF-1) and epithelial cells (NbE-1). The published results demonstrate markedly different responses of the two cell types to DHT in the presence and absence of the other cell type with prostatic fibroblast cells being more sensitive to the actions of DHT than epithelial cells alone and epithelial cells showing a mitogenic response to DHT pulsed fibroblasts. In another comparison, Orlowski et al (1991) [47] investigated the differential metabolism of testosterone by prostate epithelial and fibroblast cells. Their findings demonstrated that epithelial cells are more active in converting testosterone to DHT than stromal cells which are more active at forming inactive polar metabolites (fig 11). When these two studies are viewed in conjunction, the interaction of the prostatic epithelial and fibroblast cells in response to androgens becomes a much more plausible possibility.

1.2.4 Other Hormonal Influences on the Prostate

As discussed previously, there are other hormonal influences on the prostate in addition to androgens. Although androgens appear to be the most important hormones in the development and maintenance of prostatic size and function, prostatic cells also express receptors for prolactin and oestrogen.
Figure 1.11 Overview of principal pathways of androgen metabolism in the prostate. Abbreviations used: DHT, 5α dihydrotestosterone; Andro, 3α, 17β-dihydroxy-5α-androstan-3β-ol, 17α-HSOR, 17β-hydroxysteroid oxidoreductase; 3β-HSOR, 3β-hydroxysteroid oxidoreductase; 17β-HSOR, 17β-hydroxysteroid oxidoreductase; 5α-R, 5α-reductase; 6a/7a-HSOR, 6α/7α-hydroxysteroid oxidoreductase; 6α/7α-HSH, 6α/7α-hydroxysteroid hydroxylase; 6α-atriol, 6α-dihydroxy-5α-androstan-3β-ol; 7α-atriol, 7α-dihydroxy-5α-androstan-3β-ol; 6α-adiol-17-one, 6α-dihydroxy-5α-androstan-17-one; 7α-adiol-17-one, 7α-dihydroxy-5α-androstan-17-one.

Abbreviations for enzymes are: 5α-R, 5α-reductase; 17β-HSOR, 17β-hydroxysteroid oxidoreductase; 3β-HSOR, 3β-hydroxysteroid oxidoreductase; 6α/7α-HSH, 6α/7α-hydroxysteroid hydroxylase.
Oestrogen has been shown to enhance the effects of testosterone in the castrated rat [48] but whether this has any significance in the adult human male is unclear. The levels of circulating oestrogens in the adult human male are very low but there is the possibility that aromatisation of testosterone in the prostate may increase the local concentration of this hormone [49].

The situation with prolactin is equally complicated with prolactin receptors being expressed by prostatic cells [50] and the prolactin receptor mRNA being upregulated by testosterone and oestrogen [51] but the role of prolactin in the adult prostate is unclear. It has been suggested by Huggins & Russel (1946) [52] that prolactin is necessary for the uptake of testosterone by prostate cells. In the rat, it has been demonstrated that the volume of the ventral prostate is only restored in hypophysectomised animals by the co-administration of testosterone and prolactin [53].

Prolactin has been proposed as being involved in the maintenance of fertility through effects on citrate oxidation and is required for normal growth and development of the prostate [54]. In the rat prostate, prolactin has been shown to regulate citrate oxidation by influencing the biosynthesis of the enzyme aconitase [55].

1.2.5 Stromal-Epithelial Interactions in the Adult Prostate

An epithelial-mesenchyme relationship has been demonstrated in the embryo and a similar relationship has been postulated in the adult prostate. Cunha described a variation in growth and differentiation of adult prostatic epithelium when grown as a recombinant with embryonic urogenital sinus epithelium [56]. Further to this, Cunha’s group also demonstrated that by combining Dunning tumour epithelium with seminal vesicle mesenchyme, a reduction in the rate of tumour growth in the rat could be achieved [57].

A theory of the interaction of stroma and epithelium was detailed by Tenniswood (1986) [58]. Based on the regrowth of prostatic ducts and acini in the prostate of previously castrated rats, he postulated that androgenic influence on the gland was mediated via growth factors, which stimulated prostatic cell growth. Tenniswood considered the interaction of these cell types to be mediated by diffusible factors.
The theory of diffusible factors being involved in cellular interaction was further advanced by the findings of Yan et al (1992) [59]. By growing immortalised primary prostate epithelial and stromal cells in co-culture, they demonstrated an increase of keratinocyte growth factor (KGF) secretion from stromal cells. Epithelial cells were shown to increase expression of a splice variant of one of the fibroblast growth factor (FGF) receptors, which specifically binds KGF. Although the exact role of these growth factors in the adult prostate is not known, it is a demonstration of the possible cellular interactions, which may be involved in the growth and differentiation of the adult prostate gland.

1.3 Benign Prostatic Hyperplasia (BPH)

BPH is an extremely common disorder in males over the age of 50. It is characterised by the formation of large, fairly discrete nodules in the periurethral region of the prostate. When sufficiently large, the nodules compress and narrow the urethral canal thereby causing partial or sometimes virtually complete obstruction of the urethra.

1.3.1 Incidence

Although reports vary slightly, a careful examination of the prostate in an unselected series of autopsies disclosed BPH in approximately 20% of men 40 years of age, 70% in men 60 years of age and 90% of men by the eighth decade of life [60]. With this prevalence, it has been argued that BPH is not truly a disease but rather a normal ageing process. Clinically, significant BPH is much less prevalent. Not more than 5 to 10% of men with this condition require surgical treatment for relief of urinary tract obstruction; in the remainder, the condition is of little clinical significance.

In 1991, Garraway et al [61] described a method for diagnosing BPH combining the use of transrectal ultrasound to measure prostate size and urinary flow measurements. Using these parameters, BPH was defined as a prostate with weight greater than 20g and a flow rate of less than 15ml/sec. When applied to the male population, these parameters suggested that 138 out of 1000 men aged between 40 and 49 and 430 out of 1000 men aged between 60 and 69 were suffering from clinical BPH.
The incidence of BPH also shows racial variation with the disease appearing almost a decade earlier in blacks than it does in whites and Japanese males have among the lowest incidence. This is thought to be due to the higher levels of 5α reduced testosterone metabolites found in blood samples taken from blacks which are higher than the levels found in whites which are significantly higher than the levels of 5α reduced androgens found in Japanese men [62].

1.3.2 Aetiology

Although the cause of BPH is still uncertain, the available evidence suggests that both androgens and oestrogens are involved in its genesis [63]. Much evidence relating to the hormonal basis of BPH has been obtained in dogs, the only animal species that develops BPH with ageing. In both humans and dogs, hyperplasia of the prostate develops only in the presence of intact testes. In castrated young dogs it is possible to induce BPH by administration of androgens, an effect markedly enhanced by simultaneous administration of 17β-oestradiol [64], thus pointing to a possible synergism between androgens and oestrogens. Dihydrotestosterone (DHT), which is derived from plasma testosterone, is believed to be the ultimate mediator of prostatic growth [65]. Prostate epithelial cells express androgen receptors, which bind both testosterone and DHT; oestrogens enhance the expression of these receptors mainly in the stromal cells [66]. There is no difference between levels of plasma testosterone in patients with BPH and in age-matched controls; in fact plasma testosterone levels decline after the age of 60 years. With ageing, oestradiol levels increase in men (absolute or relative to testosterone), as does aromatase activity in the periurethral and transitional zones [67]. Thus it is conceivable that despite the declining output of testosterone associated with ageing, the increase in oestradiol “sensitises” the prostate to the growth promoting effects of DHT possibly through the effect of oestrogen on sex-steroid binding globulin (SSBG) production in the liver. Hryb et al (1989) [25] described receptors for SSBG and bound androgens in the prostate.

Although plausible, none of these possible actions of androgens and oestrogens in ageing men accounts for the fact that not all men develop clinical BPH. This may in part be explained by the study described by Partin et al (1991) [68] where BPH volume
at radical prostatectomy was correlated with testosterone and oestradiol concentrations. This study demonstrated that men in whom sex-hormone production remained high relative to their age showed an increased risk of developing BPH.

Another possible explanation is that propounded by Isaacs and Coffey (1989) [69] in which they suggest that all prostates will atrophy when androgens are removed but will subsequently re-grow when androgens are returned. Isaacs and Coffey describe a more rapid re-growth of the hyperplastic prostate than the normal prostate under these conditions. They go on to suggest that the increased growth of the hyperplastic organ is due to an increase in the number of stem cells as compared to the normal organ. By considering that the total number of stem cells is set at the end of normal prostate development by the circulating levels of androgens, this theory implies that men with high sex-hormones will have increased numbers of stem cells and therefore an increased risk of developing clinical BPH.

Once again, although plausible, this hypothesis cannot explain the characteristic development of BPH in regions of the gland, which are androgen independent during development.

1.3.3 Morphology

Careful studies by Franks [10a] and also by McNeal have demonstrated that BPH originates almost exclusively in the preprostatic region [11]. This area, which lies proximal to the verumontanum (fig 12), corresponds to the “inner” periurethral portion of the classically defined inner and lateral lobes. This distribution is in striking contrast to that of prostatic carcinoma, which usually involves the posterior lobe.

From their origin and location, the nodular enlargements may encroach upon the lateral walls of the urethra to compress it to a slit-like orifice while, at the same time, nodular enlargement of the middle lobe may project up into the floor of the urethra as a hemispheric mass directly beneath the mucosa of the urethra. Enlargement of the middle lobe may assume a long, slender, delicate, polyploid appearance attached by a narrow neck; in many instances, it appears to act almost as a ball-valve, obstructing the mouth of the urethra.
Figure 1.12 Histological sections of benign prostatic hyperplasia. The upper plate (a) shows the characteristic appearance of fibromuscular nodular hyperplasia consisting of a mixture of fibromuscular and glandular elements. In some nodules, the fibromuscular element proliferates to the exclusion of the glandular epithelium. The lower plate (b) represents nodular hyperplasia with papillary epithelial hyperplasia. This is the more common pattern in which there is papillary proliferation of epithelial cells lining enlarged glands (arrowed). Occasionally, cribiform structures may be formed which should not be confused with carcinoma.
On cross section of the affected prostate, the nodules are usually readily identifiable because of the compression of the remainder of the prostatic tissue around the nodule. As mentioned earlier in this section, they usually arise from the inner prostatic mass, and only rarely do they extend to the outer perimeter of the gland. The nodule itself varies in colour and consistency, depending on whether it is primarily due to fibromuscular stromal hypertrophy and hyperplasia or to glandular proliferation. In those that are primarily glandular, the tissue has a yellow-pink colour and a soft consistency, which is fairly discretely demarcated from the greyer, glistening colour and firm consistency of the compressed prostatic capsule. Usually a milky-white prostatic fluid oozes out of these areas. In those that are primarily due to fibromuscular involvement, the nodule itself is also pale grey and has a tough, fibrous consistency. It does not exude fluid and is less clearly demarcated from the surrounding prostatic capsule.

Microscopically, the nodularity may be due mainly to glandular proliferation or dilatation or to fibrous or muscular proliferation of the stroma. All three elements are involved in almost every case, although the epithelial element predominates in most cases. It takes the form of aggregations of small to large to cystically dilated glands, lined by two layers, an inner columnar and an outer cuboidal or flattened epithelium, based on an intact basement membrane (fig 12). The epithelium is characteristically thrown up into numerous papillary buds and infoldings, which are more prominent then in the normal prostate.

In certain cases, many small glands are formed that may simulate the pattern of adenocarcinoma. Usually the glandular size is sufficiently large to be visible on inspection of the tissue section with a hand lens. Frequently, these glands contain inspissated secretion; granular desquamated epithelial cells and numerous corpora amylacea. When the fibromuscular hypertrophy or hyperplasia predominates, it may produce aggregates of almost solid spindle cells free of glands. Not infrequently, aggregates of lymphocytes are found within the stroma, which probably relates to atrophic death of cells. Two other histological changes are frequently found: 1) foci of squamous metaplasia and 2) small areas of infarction. The former tends to occur in the margins of the foci of infarction as nests of metaplastic, but orderly, squamous cells.
1.3.4 Clinical Course

Although BPH is an extremely common condition, it has been pointed out already that in only a small percentage of those affected does the lesion produce clinical symptoms. Symptoms when produced, relate to two secondary effects: 1) compression of the urethra with difficulty in urination; and 2) retention of urine in the bladder with subsequent distention and hypertrophy of the bladder, infection of the urine and the development of cystitis and renal infections.

These patients have frequency, nocturia, difficulty in starting and stopping the stream of urine, overflow dribbling and dysuria (painful micturition). In many cases, sudden acute urinary retention appears for unknown reasons and persists until the patient receives emergency catheterisation. In addition to these difficulties in urination, prostatic enlargement results in the inability to empty the bladder completely. Due to the simple mechanics of raising the level of the urethral floor so that, at the conclusion of micturition, a considerable amount of residual urine is left. This residual urine provides a static fluid that is vulnerable to infection.

Many secondary changes occur in the bladder, such as hypertrophy, trabeculation and diverticulum formation. Hydronephrosis or acute retention with secondary UTI and even azotemia or urenaemia may develop.

1.3.5 Development of BPH

The precise causes of BPH have not been elucidated as yet and there doubtlessly exists an involvement of several factors. Some of these have been identified and will be discussed in the following section: DHT, oestrogen levels, abnormality of stem cell growth and stromal-epithelial cell interactions. Of these four mechanisms involved in the development of BPH each has its merits although none is capable of explaining BPH on its own.
1.3.5.1 DHT

The best understood of the theories is the role of DHT in the growth of the adult prostate. As DHT has been shown to be the most active androgen in the prostate and responsible for the growth and development of the gland in the embryo, it was logical that DHT would also be responsible for the continued growth of the gland throughout adult life. Perhaps the best example is that of human 5α-reductase type II deficiency or pseudohermaphroditism as detailed in section 1.1.1. In this disease, the patient at birth shows ambiguity of external genitalia due to incomplete male differentiation but with normal masculinisation of the internal structures (inguinal or labial testicular masses, labia like scrotum, a blind ending vaginal pouch and a clitoris-like phallus). At puberty, the patient develops a typical male phenotype: development of musculature, deepening of the voice, enlargement of the phallus and descent of the testicles, but they always have a vestigial prostate gland. It has been shown that these individuals have normal circulating levels of testosterone and no deficit of androgen receptors. Administration of DHT to these patients results in growth of the prostate which cannot be achieved by treatment with testosterone, demonstrating that DHT is the key androgen for prostatic growth [70].

1.3.5.2 Oestrogen

The role of Oestrogen in the growth of the prostate has been demonstrated in the canine prostate, which expresses large numbers of high affinity oestrogen receptors [66]. Trachtenberg et al (1980) [66] demonstrated that oestrogen induced the expression of androgen receptors in the canine prostate. Walsh and Wilson (1976) [71] showed that experimental hyperplasia cannot be induced in young castrated dogs by androgens alone, but does occur with co-administration of oestradiol and DHT. It should be noted that BPH in the dog differs from that in the man.

The role of oestrogen receptors in man however is still ambiguous. Although prostatic tissue expresses oestrogen receptors, the levels are lower than those detected in other peripheral tissues and despite the change in testosterone: oestrogen ratio associated with ageing, there must be other factors involved in the development of BPH.
Barrack and Barry (1987) [72] also working on the canine prostate demonstrated that oestrogens when given in the presence of androgens reduced the rate of cell death in the prostate. This gives rise to the theory that BPH might arise not from increased growth of cells but rather from decreased cell death.

1.3.5.3 Stem cell Theory

Another possibility for the development of BPH is that it results from an abnormality in the control of prostatic stem cells. By considering the role of stem cells in the self renewing organ, a better understanding of how this may be involved in BPH can be gained.

Dormant stem cells divide rarely. When they do divide a second population of transiently proliferative cells capable of undergoing DNA synthesis and division is produced. These transiently proliferative cells then mature to become terminally differentiated cells with specialised functions. Terminally differentiated cells cannot divide further and are destined to eventually die at the end of their life span [73]. Any abnormality of stem cell division could therefore result in an increase in the number of transiently proliferative cells, which in turn would lead to an increase in the number of differentiated cells and hence an increase in the size of the tissue concerned.

1.3.5.3b Apoptosis in the Prostate

Another possible role of the stem cell theory in the development of BPH is through an alteration in the rate of apoptosis. Apoptosis is defined as programmed cell death, which involves the active participation of the cell. The initiation of this process leads to several morphological changes within the cell, most notably, the fragmentation of the DNA which precedes the eventual breakdown of the cellular structure [74, 75]. The fragmentation of genomic DNA is an early event in the apoptotic process, which commits the cell to die. It is the result of activation of $\text{Ca}^{2+}/\text{Mg}^{2+}$ dependent endonucleases. Activation of the nucleases is triggered by a sustained elevation in intracellular $\text{Ca}^{2+}$ concentration [76, 77]. Cleavage of the DNA results initially in the
release of 50bp chromatin loop domains [78] followed by 180-200bp internucleosomal DNA fragments [75, 79].

Apoptosis in the prostate can be initiated by a reduction in circulating DHT levels. In the castrated rat, by twelve to twenty-four hours post castration, DHT levels decrease to approximately 5% of those in non-castrated animals. There is also a concomitant decrease in androgen receptors retained in isolated ventral prostatic nuclei [80]. The changes in the levels of androgen receptors lead to expression of a number of genes, which are normally repressed in the prostate. These genes include testosterone repressed prostate message-2 (TRPM-2) [81], glutathione S-transferase [82], TGFβ1 and TGFβ2 receptor [80, 83], C-CAM [84], and c-myc, hsp70 and Ca^{2+}-responsive c-fos [85]. The expression of these normally repressed genes is believed to lead to the initiation of the apoptotic pathway with consequent DNA fragmentation and cell breakdown. Any reduction in the rate of apoptosis in a self-renewing gland such as the prostate would result in an increase in the mass of the gland, due to an accumulation of cells.

1.3.5.4 Cellular Interactions

BPH tissue consists of a substantial quantity of stroma. It was suggested by Franks and Barton (1960) [86] that the epithelial cells may, in some way, be stimulated by this stromal component. As described previously in section 1.1.2 Cunha et al (1972, 1983) [56, 87] demonstrated the interaction of embryonic urogenital sinus mesenchyme and epithelium in the development of the prostate gland. These experiments clearly demonstrated the role of stromal mediators in the growth and differentiation of the prostate gland. Whether this mechanism is still in action in the adult prostate however is a matter for study and conjecture. Many mediators of stromal–epithelial interaction have been proposed including EGF, IGF’s, TGFα and FGF. But the role of each of these growth factors in the development of BPH remains unsubstantiated [88].

1.4 5α-reductase

As discussed in section 1.3.5.1 DHT is the major hormone involved in the development of BPH. The metabolism of testosterone to DHT in the prostate is
dependent upon the action of 5α-reductase. In order to understand better the
development of BPH, it is necessary to understand the action, expression and role of
5α-reductase in the human prostate gland.

1.4.1 Actions of 5α-reductase

In the prostate, testosterone is metabolised by a number of enzymes into mostly
polar -diol and -triol androgens. However, one enzyme converts testosterone into the
most important androgen for the normal function and development of the prostate, 5α-
dihydrotestosterone (DHT). The enzyme 3-oxo-5α-steroid:Δ4-oxidoreductase (5α-
reductase) is an NADPH dependent, membrane bound protein that catalyses the
reduction of Δ4,5 double bonds in a variety of steroid substrates [89]. Similar to T, DHT
activates responsive genes by binding to the androgen receptor [90] this two ligand one
receptor theory is supported by observation that mutations to the androgen receptor
gene lead to developmental abnormalities in both testosterone and dihydrotestosterone
dependent processes [91].

Embryological studies have shown that DHT is essential for the virilisation of
the male urogenital sinus i.e. the formation of the male external genitalia, urethra and
prostate [92, 93]. It is also necessary for the development and maintenance of the male
secondary sex characteristics and maintenance at and after puberty Wilson (1978) [93].
Although there are no known effects of DHT deprivation in the adult human male, it is
well known that a lack of DHT during embryogenesis results in the development of a
condition known as male psuedohermaphroditism. This condition is characterised by
normal male levels of testosterone and male Wolffian structures but predominantly
female external genitalia at birth [94, 95].

It has also been demonstrated in patients with BPH, that a reduction in the
activity of 5α-reductase leads to a decrease in the levels of DHT in the gland and a
reduction in the size of the gland [96, 97].

The conversion of T to DHT is dependent on the presence of the enzyme 5α-
reductase which was first described in the rat but has since been cloned and sequenced
from human tissue [98]. The distribution of 5α-reductase is mirrored by its’ function
with high levels being detected in the accessory male sex organs, the liver and the skin
5α-reductase is also present in female tissues. In mice, it has been demonstrated that a lack of 5α reduction of testosterone leads to foetal death due to an excess of oestrogen. The conversion of T to DHT prevents aromatisation of T to oestrogen by the enzyme aromatase [100].

1.4.2 5α-reductase Isoenzymes

Expression cloning in *Xenopus laevis* oocytes and cultured mammalian cells isolated cDNA encoding 5α-reductase [101, 102]. These studies revealed two genes in both humans and rats, which encoded different 5α-reductase isoenzymes. These 5α-reductase isoenzymes were designated type I and type II, reflecting the chronological order in which the cDNAs encoding them were cloned.

The human type I gene contains 5 exons and 4 introns (fig 13) and encodes a hydrophobic protein of 259 amino acids [102, 103, 104] has a neutral to alkaline pH optimum and has been localised to the short arm (band p15) of chromosome 5 (fig 14) [103, 105]. The human type I isozyme has 60% homology with the rat type I isozyme (fig 15). There is also a non-functional untranscribed pseudogene for 5α-reductase type I which has been located on the long arm (q24-qter) of the X chromosome (fig 16).

The human type II 5α-reductase gene contains 5 exons and four introns (fig 17) and encodes a hydrophobic protein of 254 amino acids [102]. The amino acid sequence of the type II isozyme has 45% homology with the human type I isozyme and 77% homology with the rat type II isozyme (fig 15). It has an acidic pH optimum and has been localised to the short arm (p23) of chromosome 2 (fig 18). 5α-reductase type II has also been shown to exist in the form of an O-glycosylated sialoglycoprotein with a molecular weight of 42kDa comprising a peptide moiety of 36kDa [106].

1.4.3 Substrate Specificity

Although both isoforms of 5α-reductase are capable of reducing testosterone to DHT, differences in the substrate-binding domain means that they have varying affinities for different steroids.
Figure 1.13 5α reductase type I gene demonstrating introns and position of primer sequences for RT-PCR. Bottom diagram shows mRNA product of gene and site of endonuclease digestion of RT-PCR product.
Figure 1.14 Map of chromosome 5 showing location of 5α-reductase type I gene. (adapted from Russell et al, 1994).
Figure 1.15 Homology of amino acid sequences between 5α-reductase isoenzymes. 5α-reductase isoenzymes are represented by circles and the sequence homology is indicated by interconnecting lines.
Figure 1.16 Map of chromosome 2 identifying position of non-transcribed 5α-reductase type I pseudogene (adapted from Russell et al, 1994).
Figure 1.17 Representation of 5α reductase type II gene showing position of intron spanning primers utilised in PCR. Lower diagram shows RNA product of gene and endonuclease restriction cleavage site of PCR product.
Figure 1.18 Map of the X chromosome identifying the position of 5α-reductase type II gene (adapted from Russell et al, 1994).
The substrate binding domain at the amino terminus of type I 5α-reductase differs by 4 amino acids from that of the type II isozyme (fig 19) [107]. This region is encoded by exon 1. This difference in the substrate-binding domain may account for the observations of Normington and Russell (1992) [108] who reported differences in substrate affinities for the two isoforms of 5α-reductase. They hypothesised that these differences suggested that type II played an anabolic role whereas type II played a catabolic one. This hypothesis was further enhanced by Boudou et al (1994) [109] who demonstrated distinct androgen reduction pathways in prostate epithelial and stromal cells. The data suggested that stromal cells preferred the androsterone pathway via the oxidative formation of androstenedione (fig 20), whereas epithelial cells preferred the reductive DHT pathway (fig 21). Comparisons between human, rat and cynomolgus monkey carried out by Levy et al (1995) [110] also demonstrated a difference in Km and Vmax values between the human 5α-reductase isozymes for different substrates. Data from this study indicates that 5α-reductase type I has a lower affinity for testosterone than type II but has a much higher Vmax value for androstenedione than the type II isozyme. It also demonstrates an increased Vmax for androstendione in comparison to testosterone.

1.4.4 Distribution of 5α-reductase

5α-reductase type I has been shown to be expressed at low levels in androgen target tissues such as the prostate, liver [111, 112, 98, 103, 104]. The human type II 5α-reductase has been shown to be expressed at high levels in the prostate in the adult. The pattern in the human foetus is similar with androgen target organs expressing type I and the prostate expressing high levels of type II 5α-reductase [113]. Although both isozymes are expressed in the male reproductive tissues, in the peripheral tissues type I 5α-reductase is exclusively expressed [114].

Within the cell, both isozymes of 5α-reductase have been localised to the outer nuclear membrane of the nuclear envelope and to the rough endoplasmic reticulum, which is continuous with the nuclear membrane [115, 116].
Figure 1.19 4 amino acid sequence which confers Finasteride resistance on human 5α reductase type I. Human type I 5α reductase has a high IC₅₀ of 64nM. A chimeric protein containing a 4 amino acid residue from rat type I 5α reductase substituted for the corresponding human residues, assumes the pharmacological properties of the rat type I 5α reductase.
Figure 1.20 & 1.21 Two possible pathways of metabolism of testosterone in prostate cells.

1.20: Testosterone can be metabolised to Androstenedione via $17\beta$-hydroxysteroid dehydrogenase (3) and hence to androstanedione via $5\alpha$ reductase type I (1).

1.21: Alternatively testosterone can be metabolised directly to DHT via $5\alpha$-reductase type II (2)
1.4.5 The Role of 5α-reductase in the Adult Human Prostate

There has been no clear role elucidated for 5α-reductase in the adult prostate other than the continued growth of the organ throughout life. Drugs, which inhibit 5α-reductase activity, elicit a reduction in prostate size [117, 118]. It is however worth considering that as the two forms of 5α-reductase display different kinetic characteristics and that stroma and epithelia in the prostate also display different levels of 5α-reductase activities, there is a possibility that there is a paracrine system between the two cell types.

1.5 Treatment of BPH

1.5.1 Principles of Therapy

The principles behind the treatment of BPH are to reduce the symptoms to a manageable level, which does not interfere with the patients' life style to too great a degree while minimising the discomfort to the patient. This is achieved by reducing the size of the prostate and clearing any obstruction to the flow of urine through the urethra. This is achieved in 3 main ways: 1) Surgical intervention 2) Pharmacological therapy and 3) Endocrine therapy.

1.5.2 Surgical Intervention

Other methods of surgical intervention are available but the most common form of surgical intervention for relief of BPH is Trans Urethral Resection of the Prostate (TURP). Ambrose Pare performed the first TURP in the 16th century with a number of techniques being developed in the 19th century for the removal of obstructive prostate tissue. These techniques were limited in their efficacy due to the inability to effectively visualise the operative field.

With the development of the resectoscope in the early 20th century, along with the invention of electrocautery, the surgeon can now resect the prostate and control
haemostasis to such an extent that approximately 95% of benign prostates are removed by this method with morbidity and mortality rates below 0.5% [119].

The decision to elect a patient for surgical intervention is dependent upon the symptoms suffered, any secondary complications (i.e. haematuria, UTI or bladder stone formation) and a functional evaluation of urinary flow and residual volume [120].

1.5.3 Pharmacological Therapy

In the 1970’s with the discovery of alpha-adrenergic adrenoceptors in the prostate capsule, smooth muscle and bladder neck [121], there was an increased interest in the search for an effective pharmacological treatment for BPH. Investigations by Shapiro (1984) [122] demonstrated that both \( \alpha_1 \) and \( \alpha_2 \) adrenoceptors are present in the prostate, while Hedlund et al (1985) [123] showed that the receptors were restricted mainly to the stromal compartment. By exposing the prostate to \( \alpha_1 \) and \( \alpha_2 \) agonists, Lepor et al (1988) [124] demonstrated contraction of the prostatic smooth muscle. The contraction could be inhibited by the addition of specific \( \alpha_1 \) adrenoceptor antagonists but not by \( \alpha_2 \) antagonists [123].

The importance of the role of adrenoceptors in bladder outflow obstruction was demonstrated by Furuya et al (1982) [125] who showed that 40% of obstruction was from a reversible/dynamic element.

Since these discoveries, there has also been an interest in attempting to alleviate the symptoms of BPH and to try and prevent the onset of the disease. The drugs utilised in this respect have been 5\( \alpha \)-reductase inhibitors. The theory being that by reducing the levels of DHT in the prostate, the growth of the prostate will be reduced and BPH avoided. Initially, it was thought that only 5\( \alpha \)-reductase type II was active in the human prostate and drugs, which only inhibited 5\( \alpha \) RII, were developed. One of the first drugs developed specifically for this task was finasteride [126]. Finasteride is a neutral 4-azasteroid and functions as a 5\( \alpha \) R type II inhibitor. Finasteride has also been shown to inhibit the androgen receptor, which may account for some of the observed effects on BPH.

In the light of recent findings which have shown that 5\( \alpha \)-reductase type I is also active in the adult prostate [127] there has been an increased interest in the development
of what are termed dual inhibitors (types I & II) as opposed to specific 5α-reductase type II antagonists.

1.5.4 Endocrine therapy

In the 18th century, John Hunter noted that the prostate underwent atrophy following castration. It was this observation which led Cabot in 1896 [128] to perform 79 bilateral orchidectomies for the treatment of outflow obstruction. He reported an improvement in symptoms in 80% of cases. Huggins and Stevens in 1940 [129] reported micro and macroscopic evidence of glandular atrophy in prostates of men treated by orchidectomy.

Using 17α-hydroxyprogesterone caproate, which is also a potent anti-androgen through its inhibitory effects on the pituitary resulting in reduced LH release, Geller et al (1965) [130] reported beneficial effects on the relief of BPH symptoms. Another anti-androgen in the form of Flutamide was utilised by Caine et al (1975) [131] to demonstrate an improvement in urinary outflow and a degree of glandular atrophy. A more in-depth study by Stone et al (1989) [132] using flutamide reported a 23% reduction in gland volume following a 3 month trial with a concomitant increase in urinary outflow. Another anti-androgen in the form of cyproterone acetate is also widely used. Unlike flutamide, cyproterone acetate resembles the androgens in structure (table 1.1) and binds irreversibly to the androgen receptor.

Another approach to androgen deprivation has been the use of LHRH analogues. Bosch et al (1989) [133] using the LHRH agonist buserelin, reported a 29% reduction in gland volume in 12 patients following 12 weeks of treatment but there was little improvement in urinary outflow.

The major problem with all endocrine therapies is their detrimental effect on the libido and can even induce impotency. Due to these side effects, endocrine therapy is reserved mainly for the treatment of malignant disease rather than BPH.

1.5.5 Phytotherapy
<table>
<thead>
<tr>
<th>Androgen/Anti-androgen</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (androgen)</td>
<td><img src="image" alt="Structure of Testosterone" /></td>
</tr>
<tr>
<td>5α-Dihydrotestosterone (androgen)</td>
<td><img src="image" alt="Structure of 5α-Dihydrotestosterone" /></td>
</tr>
<tr>
<td>Flutamide (anti-androgen)</td>
<td><img src="image" alt="Structure of Flutamide" /></td>
</tr>
<tr>
<td>Cyproterone Acetate (anti-androgen)</td>
<td><img src="image" alt="Structure of Cyproterone Acetate" /></td>
</tr>
<tr>
<td>Cholesterol (basis of phytosterol structure which has anti-androgenic properties)</td>
<td><img src="image" alt="Structure of Cholesterol" /></td>
</tr>
</tbody>
</table>

Table 1.1 Structures of androgens and anti-androgens.
Phytotherapy is the use of plants or plant extracts for medicinal purposes. It has been described since ancient times. The use of plant extracts for the treatment of the symptoms of BPH was described as early as 15th century BC in Egyptian manuscript [134]. Most of the phytotherapeutic agents in use are plant extracts as opposed to the actual plant itself. There are about 30 phytotherapeutic compounds, which are currently available in Europe for the treatment of BPH. Most of these compounds are derived from 8 plants (table 1.2). In 15 of these marketed compounds, the active ingredient is derived from the dwarf palm Serenoa repens. The most widely used of the compounds are Permixon®, a lipidosterolic extract of the dwarf palm, Harzol, derived from African star grass, Hypoxis rooperi, Sabal extract from the dwarf palm, Tadenan from the African prune, Pygeum africanum and Cernilton which is a derivative of rye-grass pollens. These extracts are a mixture of multiple chemical compounds, which in the vast majority of cases have been poorly characterised. In fact, most of the phytotherapeutic agents available have undergone little or no formal testing to determine composition, active ingredients and efficacy. This is despite many of them having been used in Europe for many hundreds of years.

Work that has been done in characterising the active ingredients in phytotherapeutic agents has reported that the effect of such medications is attributable to compounds called phytosterols, which are related to cholesterol. Of the phytosterols, the most important agent is believed to be sitosterol [135]. The mechanism of action of sitosterol is unknown although various mechanisms have been proposed. These include anti-inflammatory effects through interference with prostaglandin metabolism [135], alteration of cholesterol metabolism [136] inhibition of prostatic growth by a direct mechanism [135], anti-androgenic and anti-oestrogenic effects [136, 137, 138] and a decrease in availability of sex hormone binding globulin [139]. Many of these actions have been demonstrated in-vitro but there is limited evidence for any of these actions occurring in-vivo [134, 135, 136, 137, 138, 139].
<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Trade name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serenoa repens</td>
<td>American dwarf palm, sago palm</td>
<td>Permixon, Strogen S, Prostaselect, Prostavigol, Remigeron, Talso</td>
</tr>
<tr>
<td>(sabal serrulatum)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxis rooperi</td>
<td>South African star grass</td>
<td>Harzol</td>
</tr>
<tr>
<td>Pygeum africanum</td>
<td>African prune</td>
<td>Tadenan</td>
</tr>
<tr>
<td>Secale cereale</td>
<td>Rye pollen</td>
<td>Cernilton, Adenoprostal, Prostabrit</td>
</tr>
<tr>
<td>Utrica dioica, Utrica urens</td>
<td>Stinging nettle (leaves &amp; roots)</td>
<td>Bazoten, Prostaherb, Utrica plus</td>
</tr>
<tr>
<td>Cucurbita pepo</td>
<td>Pumpkin seed</td>
<td>Cystofink</td>
</tr>
<tr>
<td>Populus tremula</td>
<td>Aspen</td>
<td>Eviprost</td>
</tr>
<tr>
<td>Echinacea purpurea</td>
<td>Purple cone flower</td>
<td>Azuprostat</td>
</tr>
</tbody>
</table>

Table 1.2 (adapted from Buck, 1996 [170])

1.6 Permixon®

1.6.1 Components of Permixon®

Permixon® is a n-hexane lipid/sterol extract of the dwarf palm (Serenoa Repens). The dwarf palm is a member of the Arecaceae family, which is native to the West Indies and the south-eastern United States. American Indians are known to have used the berries of the dwarf palm for the treatment of genitourinary disturbances. The berries are also believed to increase testicular function and relieve irritation of the mucous membranes.

The lipid soluble fraction of the berries is believed to contain the active ingredients. This consists mostly of sterols (structurally similar to cholesterol (table 1.1)) and fatty acids. The sterol fraction is made up of β-sitosterol, stigmasterol, cycloartenol, lupeol, lupenone and methylcycloartenol. Also present in the extract are free fatty acids and long-chain alcohols. Of the sterol components, the sitosterols are believed to be the active components (vs). Although these compounds are chemically related to cholesterol, their mechanism of action is poorly understood.
1.6.2 Action of Permixon®

Permixon® has been reported to exert a number of effects in in-vitro studies. Studies on human foreskin fibroblasts in cell culture have demonstrated that Permixon® can competitively inhibit the binding of DHT to cytosolic and nuclear androgen receptors and inhibition of androgen binding in the rat prostate (IC₅₀ ≈ 370mg/L). Data is has also been published which demonstrates that Permixon® decreases the number of nuclear oestrogen and androgen receptors in the prostate tissue of BPH patients treated with the drug by 600 and 800% respectively[140]. It has also been shown to interfere with the prolactin receptor signal transduction in Chinese Hamster Ovary cells causing a reduction in the basal activity of the K⁺ channel and of protein kinase C [217]. In addition, inhibition of 5α-reductase type I activity by Permixon® has been demonstrated in human foreskin fibroblasts and DU145 cell lines. The inhibition of 5α-reductase type I and II have been demonstrated in insect cell expression systems transfected with the 5α-reductase isoforms [141] with Permixon® inhibiting both isoforms at similar concentrations (Ki 7.2mg/L for type I and 4.9mg/L for type II).

However, studies carried out by Rhodes et al (1993) [142] produced results conflicting with the studies mentioned previously. Rhodes data showed that Permixon® did not inhibit testosterone-stimulated growth of the prostate of castrated rats and in-vitro studies using human prostate cells showed only minimal inhibition of 5α-reductase activity by Permixon® in comparison to finasteride.

Moreover, when tested in direct comparison to finasteride in a clinical trial involving 1098 patients, Permixon® treated patients (320mg once a day) demonstrated a similar level of relief in symptoms associated with BPH to the finasteride (5mg once a day) treated patients while reporting fewer side effects. Permixon® also demonstrated little effect on PSA levels whereas Finasteride caused a marked decrease in PSA levels [143].

Other effects of Permixon® include actions on the inflammatory response. These effects are attributed to the flavonoid component of Permixon®, which has been shown to inhibit the formation of certain products of the metabolism of arachidonic acid which are involved in inflammatory responses by inhibiting the enzymes cyclo-
oxygenase and lipoxygenase (fig 22) [144, 145, 146]. These anti-inflammatory actions have also been linked to the free-radical scavenging properties of flavones [147].

1.6.3 Pharmacology of Permixon®

Permixon® is a complex mixture of several compounds and as such has had little pharmacokinetic data gathered on it. Plasma concentrations of one of the components in Permixon has been measured in healthy male volunteers receiving one 320mg dose. The mean peak plasma concentration of 2.6mg/ml was achieved after 1.5 hours after administration. The elimination half-life was 1.9 hours [148].

The tissue distribution of Permixon® has been investigated in rats where it was demonstrated that the uptake of several of the components of Permixon® (Oleic acid, lauric acid and β-sitosterol) was higher in the prostate than in the liver or other genitourinary tissues [149].

1.7 In-Vitro Models of Prostatic Hyperplasia and 5α-reductase Expression

Several models exist for the study of BPH and 5α-reductase expression. Although the prostatic carcinoma cell lines are in fact derived from cancers, they are sometimes used as a model for BPH as they express some of the characteristics of non-cancerous prostate tissue and they express 5α-reductase type I. The cell lines however have several limitations. All the cell lines arose from metastasis rather than from primary tumours, they have been immortalised and are all clones. Another limiting factor is the fact that all cell lines are a homogenous population whereas the prostate gland is a heterogenous population of cells. All these factors result in models, which although usefull are not ideal for the study of BPH or indeed prostate cancer.

A most valid model for the in-vitro study of BPH is the primary cultures of cells obtained from BPH tissue but this has certain drawbacks and limitations, which will be discussed.
Figure 1.22 Arachidonic acid metabolism cascade. Prostaglandins, thromboxanes, leukotrienes and hydroxy acids, HETE (hydroxyeicosatetraenoic acid), HPETE (hydroxyperoxyeicosatetraenoic acid) are the oxygenated metabolic products of free arachidonic acid.
1.7.1 Immortalised Prostatic Carcinoma Cell Lines

Several human prostatic cancer cell lines are currently available for study. These include LNCaP, DU145, PC3, PC-EW, Honda, PC-82 and EB-33 to name a few. There have been a few more additions to this list in the form of immortalised primary cultured epithelial and fibroblast cells. But only the characteristics and origins of the three most common cell lines, which are dealt with in this thesis, namely LNCaP, PC3 and DU145 are outlined in the following sections.

1.7.1.1 LNCaP Cell Line

The LNCaP cell line derives its name from its site of origin i.e. Lymph Node carcinoma of the Prostate. The cell line was established by Horoszewicz et al (1983) [150] from needle biopsies taken from the supraclavicular lymph node of a 50-year-old patient with metastatic stage 2 disease. The LNCaP cell line is sensitive to stimulation by androgens, which cause an increase in growth and also induce the secretion of acid phosphatase and PSA. The androgen receptor expressed by the LNCaP cell line has been characterised as a high affinity receptor [150, 151, 152, 153, 154, 155]. Although expressing an androgen receptor, the LNCaP cells are not truly androgen dependent as the receptor contains a mutation which alters its ligand specificity [156] to such a degree that androgen antagonists such as hydroxy flutamide can in fact function as agonists and stimulate the cells [157]. LNCaP cells also only express 5α-reductase type I and show no 5α-reductase type II expression or activity.

The karotype of LNCaP cells is pseudodiploid in the majority of cases with a typical chromosome number of 46 but a small sub-population of cells display a tetraploid karotype [158]. In common with DU145 and PC3 cell lines, the LNCaP cells line demonstrates 7 chromosome markers (m1-7) arising from 5 separate chromosomal translocations.

Several subclones of the LNCaP cell line have been established including The resistant variant LNCaP-r which was first characterised by Hasenson et al (1985) [159], who demonstrated unresponsiveness to androgens, oestradiol and flutamide although androgen receptor appears to be expressed. A similar sub-clone has since been
characterised by Pousette et al (1997) [160] and used as a model for elucidating the actions of a drug used in advanced CaP.

1.7.1.2 DU145 Cell Line

The Du145 cell line was derived from a metastatic brain lesion from a patient who had undergone oestrogen therapy for advanced CaP [160, 161]. The DU145 cell line has been shown to be neither androgen dependent nor androgen responsive. Androgen receptors cannot be detected at the protein level or at the level of mRNA expression [155].

Analysis of the karotype of the DU145 cell line demonstrates aneuploidy i.e. a modal chromosome number of 64 [162]. Immunohistochemical analysis demonstrates only weak staining for acid phosphatase and negative staining for PSA [162].

DU145 cells only express 5α-reductase type I and do not express 5α-reductase type II.

1.7.1.3 PC3 Cell Line

The PC3 cell line originates from a bone metastasis of a patient who had been treated for advanced CaP by castration and oestrogen therapy. The originating tumour was androgen independent and the PC3 cells demonstrate the same characteristics. The cells are typically aneuploid (chromosome number between 55 and 62), demonstrate no androgen sensitivity or dependence [163, 164] and demonstrate no detectable androgen receptors [156, 164]. Similar to the DU145 and LNCaP cell lines, the PC3 cell line does not express 5α-reductase type II and demonstrates only 5α-reductase type I expression and activity.
1.7.2 Primary Cultures of Prostatic Cells

With the shortcomings of the use of long-term cultured cell lines bringing into question reliability as representative models of the in-vivo situation, it seems sensible to look “closer to home” as it were for a more reliable model. In theory, short-term primary cell cultures should not be as far removed from their tissue of origin in terms of function and expression and should therefore provide a better model for the study of the in-vivo gland.

Organ culture is the growth or maintenance of the whole or whole part of the organ in-vitro. This approach is useful in that it allows the study of interactions between different cell types within the organ while maintaining a degree of tissue architecture. However organ culture has the problem of ensuring an adequate oxygen and nutrient supply to the central areas of the cultured organ preventing necrosis.

Primary cultures are somewhere between organ culture and truly replicative cell culture as they are derived from outgrowths of cells from tissue fragments or from dispersed tissue. This system offers advantages in that the problem of oxygen and nutrient supply is overcome by the formation of monolayer cultures and that the resulting cells are homogenous and are very similar to the tissue of origin. However, due to the loss of extracellular matrix and stromal-epithelial interaction in the culturing process, the cells demonstrate a decreased ability to differentiate and organise into structures observable in the organ itself.

The first demonstration of outgrowth of epithelial cells from an adenocarcinoma was by Burrows et al in 1917 [165] and since then, several groups have reported on differing methods for the establishment of primary cultures. The various protocols available, in general, demonstrate small variations around a core concept. Basically the main points to be considered are 1) The method of separation of the tissue (either mechanical or enzymatic) and 2) the choice of medium for growth of the different cell types.

The use of collagenase for the separation of the tissue as opposed to mechanical disruption does appear to be less damaging to the cells [166, 167] but there is no real evidence to favour one method over the other, as neither offers a substantial improvement in the resulting cell growth or characteristics.
1.7.2.1 Characterisation of Primary Cultured Stromal and Epithelial Cells

Stromal and epithelial cell cultures prepared from collagenase digestion of tissue obtained from TURP of patients suffering from BPH were characterised by Tsugaya et al (1996) [168]. The cultures were characterised by Immunohistochemical means employing a number of antibodies for epithelia, smooth muscle, striated muscle and fibroblast cells. The cells obtained from this procedure demonstrate the characteristics of normal fibroblast cells and basal epithelial cells. The epithelial cells however are not secretory and as such do not offer an adequate representation of the gland. Other characteristics of the cells are lost with culturing. It has been noted that primary cultured epithelial and fibroblast cells demonstrate high levels of androstenedione production from the metabolism of testosterone but only very small amounts of DHT are detectable [205].

1.8 In-Vivo Models of BPH

There are two basic models of in-vivo BPH available to be studied. These are the dog which develops BPH spontaneously with age the same as man and the transgenic mouse which was developed and described by Tutrone et al (1993) [169].

Both these models offer advantages and disadvantages over the in-vitro models. As they are developed in the live intact animal, they obviously preserve all normal cellular interactions within the gland and are susceptible to the changing hormonal milieu that is the living body. However, these advantages are also a disadvantage. Animals are difficult and expensive to keep and maintain and do not lend themselves easily to manipulation of the microenvironment or to observations of drug effects upon the prostate cells. There is also growing pressure from animal welfare organisations to limit the use of animal models in experimentation.
1.9 Aims and Objectives

The control and function of 5α-reductase isozymes in BPH of the human prostate is poorly understood and there is a great deal of conflicting evidence concerning the expression of the two isozymes in the human prostate.

At present, the models, which are available for the evaluation of the expression of 5α-reductase isozymes, are inadequate in respect to their ability to mimic the in-vitro system. It is necessary to develop a model, which is more indicative of the prostate gland in order to understand better the mechanisms, which control the action of 5α-reductase types I & II. The development of such a model would also prove useful in understanding the role of 5α-reductase inhibitors such as Permixon®, which is a phytotherapeutic agent used in the treatment of BPH. The precise method of action of this drug is however not well understood as the models of BPH which are available for study, behave and respond to therapeutic agents in a manner which is different to the prostate gland itself. Permixon® has been shown to inhibit the action of both isoenzymes of 5α-reductase in expression systems but its actions in the prostate remain to be elucidated.

The aim of this work was to investigate the control of 5α-reductase isozyme expression in the hyperplastic prostate and to investigate the actions and effect of Permixon® on these isozymes and other markers of prostatic disease. Consequently, a new model for the study of BPH has been developed which offers advantages over the conventional models in that it expresses more of the characteristics of the in-vivo prostate gland. By investigating possible interactions in the novel model system, a possible modulator of the expression of 5α-reductase type II has been partially identified. The effects of Permixon® on the prostate and on PSA have been investigated and a possible mode of action elucidated which may give a better understanding of the role of Permixon® in the treatment of BPH.
2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Lines

Three immortalised prostatic carcinoma cell lines were utilised in the experiments described within this section.

The LNCaP cell line was obtained from the American Type Culture Collection (A.T.C.C.), Rockville, USA. This cell line was established from a metastatic lesion of a human adenocarcinoma and embodies many of the malignant properties which are characteristic of neoplastic epithelial cells in Prostate Cancer [151]. Cells of low passage number were used in all experiments.

The PC3 cell line was isolated from a poorly differentiated bone metastasis of prostatic carcinoma [164] and was obtained from the European Collection of Animal Cell Cultures (E.C.A.C.C), PHLS Centre for Applied Microbiology and Research, Porton, Salisbury, England. The passage number of these cells was unknown but for the purpose of our experiments, only cells passaged up to 10X were used.

The DU145 cell line was kindly donated by Dr. D. D. Mickey, Department of Urology, University of North Carolina, Chapel Hill, USA. The DU145 cell line was originally derived from a brain metastasis, which had been identified as a moderately differentiated prostatic adenocarcinoma [163]. In all experiments, low passage number cells were utilised.

The cell lines were regularly screened for mycoplasma contamination using a mycoplasma detection kit from Boehringer Mannheim UK. (Diagnostics and Biochemicals) Ltd., Lewes, East Sussex, UK.
2.1.2 Cell Culture

2.1.2.1 General consumables

All plasticware including tissue culture flasks, Universal flasks, Erlenmeyer flasks and pipettes was obtained from Bibby Sterilin Ltd., Staffordshire, UK. All media and solutions were sterile-filtered before use with a 0.2μm bottle filter and 0.2μm sterile Acrodiscs supplied by Sigma Chemical Company Ltd., Poole, Dorset, UK and Gelman Sciences, Northampton, UK respectively.

Oxoid Dulbeco ‘A’ phosphate buffered saline was purchased from Unipath Ltd., Basingstoke, Hampshire, UK. 10X Trypsin-EDTA (0.5% w/v trypsin; 0.2% w/v EDTA; 0.85% w/v NaCl) was purchased from Gibco-Brl, Paisley, Renfrewshire, UK.

Unless otherwise stated, all chemicals used in tissue culture procedures were obtained from Sigma Chemical Company Ltd.

2.1.2.2 Cell lines - media/supplements

RPMI 1640 medium was obtained from Gibco-Brl. Ham’s F 12 medium was purchased from ICN Biomedicals Ltd., High Wycombe, UK. All media were supplemented with foetal bovine serum (FBS), L-Glutamine (200mM), Penicillin (10 000 Units/ml) and Streptomycin (10 000μg/ml) which were acquired from Gibco Brl.

2.1.2.3 Epithelial and Fibroblast Primary Culture - media/supplements

Collagenase was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, USA.

WAJC 404 medium was purchased from Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan. HEPES, zinc stabilised insulin, cholera toxin, dexamethasone, sodium hydrogen carbonate and epidermal growth factor (EGF) were all purchased from Sigma.

56
Fungizone was obtained from Gibco Brl.

2.1.2.4 Conditioned Medium

Dialysis membranes were purchased from Membrane Filtration Products Inc., Texas, USA.

Microconcentrator spin columns were purchased from Amicon Ltd, Gloucestershire, UK.

All chemicals were obtained from Sigma as previously detailed.

2.1.2.5 HPLC

The MA75 cation exchange column was obtained from Bio-Rad Laboratories Ltd., Hertfordshire, UK. The TSK DEAE-5PW anion exchange column was purchased from Tosohass, Philadelphia, USA. All HPLC was performed on a Beckman Gold HPLC apparatus supplied by Beckman Instruments (UK) Ltd., Bucks, UK.

2.1.3 Cell proliferation

Cell culture media without phenol red were obtained from the suppliers listed previously.

The MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and dimethylsulphoxide (DMSO) were purchased from Sigma. The activated charcoal and Dextran T70 utilised in the removal of endogenous steroids from FBS were obtained from Sigma and Pharmacia Biotech Ltd., Milton Keynes, UK. respectively. Cell culture tested dihydrotestosterone (5α androstan-17β-ol-3-one) was purchased from Sigma.
2.1.4 LSESR (Permixon™).

LSESR was supplied by Pierre Fabre Medicament, Castres, France.

2.1.5 Molecular Biology

2.1.5.1 General Consumables.

The majority of reagents employed in molecular biology procedures were obtained from Sigma and were wherever possible of molecular biology grade.

NaCl, tri-sodium citrate, NaOH, 37% formaldehyde (12.3M, pH>4.0), ethanol, Tris-saturated phenol (20mM, pH8.0) and glacial acetic acid were all purchased from Fisons Scientific Equipment, Loughborough, Leicestershire, UK.

Isopropanol (2-propanol), isoamylalcohol (3-methyl-1-butanol) and formamide were obtained from the Aldrich Chemical Company Ltd., Gillingham, Dorset, UK.

Agarose NA and DNA gel loading buffer (Bio 101 inc.) were purchased from Pharmacia Biotech Ltd. And Stratech Scientific Ltd., Luton, Bedfordshire, UK. respectively.

Nuclease-free water was acquired from the Promega Corporation, Southampton, Hampshire, UK..

Sterile screw cap Eppendorf tubes were purchased from Sarstedt Ltd., Beaumont Leys, Leicester, UK. Sterile pastettes were obtained from Alpha Laboratories Ltd., Eastleigh, Hampshire, UK. Whatman 3MM paper was acquired from Whatman International Ltd., Maidstone, UK.

Hybond C extra (nylon supported nitro-cellulose) was obtained from Amersham International plc., Aylesbury, UK. Fastrad autoradiography cassettes (Hoefer Scientific Instruments) and intensifying screens were acquired from Scottish Biotechnology Instrumentation, Auchterarder, Perthshire, UK.
2.1.5.2 Isolation and Analysis of Nucleic Acids.

Guanidine isothiocyanate was purchased from Northumbria Biologicals Ltd., Cramlington, Northumberland, UK.

All materials utilised in first strand synthesis of cDNA were obtained from the Promega Corporation. PCR reactions were carried out utilising Promega Taq DNA polymerase and Taq 10X buffer and dNTP's. Mineral oil was supplied by the Sigma chemical company Ltd.

All primers were synthesised by Oswel DNA Service, Dept. of Chemistry, University of Edinburgh, Scotland.

2.1.5.3 Analysis of Proteins.

ECL Western blotting detection system and Hyperfilm™-MP was supplied by Amersham International plc.

The rabbit polyclonal anti-human androgen receptor antibody was purchased from Novocastra Laboratories, Newcastle-upon-Tyne, UK.

2.1.6 Thin Layer Chromatography (TLC).

ITLC™ SA polysilicic acid gel impregnated glass fibre plates were purchased from Gelman Sciences Inc., Michigan, USA.

Ultima Gold High flash point Liquid Scintillation Cocktail (LSC) was supplied by Packard Instrument B.V. - Chemical Operations, Groeningen, NL.

Diethyl ether and dichloromethane solvents were obtained from Fisher Scientific UK., Leicestershire, UK.

Phosphomolybdic acid was purchased from Sigma Chemical Company Ltd.

Microcaps disposable micro pipettes were purchased from Drummond Scientific Company, Broomall, Pasadena, USA.
2.2 Methods

2.2.1 Cell Culture.

2.2.1.1 Cell lines.

2.2.1.1a Cell Culture Media.

The DU145 and LNCaP cell lines were cultured in RPMI 1640 Medium. The media was supplemented with 10%v/v foetal calf serum (FCS), L-Glutamine (2mM) and with penicillin (100 units/ml) & streptomycin (100 µg/ml).

PC3 cell were maintained in Ham’s F 12 containing 10% v/v FCS, L-Glutamine (2mM), penicillin (100 units/ml) and streptomycin (100 µg/ml).

2.2.1.1b Culture of Cell Lines.

The cell lines were grown in monolayer culture in 75cm² tissue culture flasks in a humidified incubator (T305GF Assab; Kebo Assab AB, Solna, Sweden.) maintaining an atmosphere of 95% air and 5% CO₂ at 37°C. All tissue culture procedures were carried out in a laminar flow cabinet (Microbiological Class II; Howarth Air Engineering Ltd., Farnworth, Bolton, UK.) using aseptic techniques and utilising sterile, disposable plastic pipettes and pipette tips. To ensure that a sterile environment was maintained, the laminar flow cabinet was thoroughly cleaned with a solution of 70% ethanol before any operations were conducted. In addition, all handling of cell lines was performed using disposable latex gloves.

Continuous cell growth was maintained through regular sub-culture. Upon achieving approximately 90% confluence, cells were harvested through trypsinisation, diluted 1:3 in medium and delivered into 3 fresh flasks. The sub-culture process involved the aspiration of spent medium from cells followed by 2 washes with Dulbecco ‘A’ phosphate buffered saline (PBS) and a 3 minute
incubation at 37°C in 2ml 1xTrypsin-EDTA (0.2ml of 10x Trypsin in 1.8ml of Dulbecco ‘A’ PBS). The cells were then detached through gentle agitation and 18mls of the appropriate media were added to the cell suspension. Following centrifugation at 2000 rpm for 5 minutes, resuspension of the pellet in 30ml of medium, repeated pipetting ensured a single cell suspension. 10ml of this suspension was delivered to each flask. The cells were allowed to adhere over a 24-hour period before being supplemented with fresh medium. Thereafter, the medium was changed every 48 hours.

2.2.1.2 Culture of Epithelial and Fibroblast Cells from Human Prostatic Tissue.

The primary culture of epithelial cells from prostatic acini in serum-free medium was first described by Chaproniere et al (1986) [171]. The protocol described here for the autonomous culture of epithelial and fibroblast cells is based on the technique developed by Chaproniere.

Prostate tissue was obtained from patients undergoing transurethral resection of the prostate (TURP) for the treatment and relief of the symptoms of BPH. The prostate chips were immediately placed in 15ml of Transport Medium (RPMI 1640 supplemented with 5% v/v FCS) and were stored for up to 7 days at 4°C before use. Pathology was determined by routine examination of representative samples of excised tissue by the department of Pathology, Western General Hospital, Edinburgh.

2.2.1.2a Enzymatic Dissociation of Epithelial and Fibroblast cells.

The prostate chips were washed twice in 25ml of Dulbecco ‘A’ PBS, weighed and cut into approximately 1mm² cubes using sterilised scissors and forceps. The tissue was subsequently transferred to a sterile Erlenmeyer flask, washed with 20mls of Transport Medium and finally suspended in 5ml of Transport Medium per gram of tissue. A sterile filtered collagenase solution (600 units/ml) in Transport medium equivalent to 2.5 mls per gram of tissue was mixed with the tissue and the flask placed in a 37°C shaking incubator (Luckham R300; Luckham Ltd., Burgess Hill,
The tissue was incubated for 20 hrs with gentle agitation to maintain the integrity of the acini. The digest was triturated by repeated pipetting, decanted into sterile Universal flasks and centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted and the cell pellet resuspended by inversion in 25mls Dulbecco ‘A’ PBS to wash out the collagenase. The cells were again centrifuged at 2000 rpm for 10 minutes and washed in Dulbecco ‘A’ PBS. These steps of centrifugation and washing were repeated a further twice prior to suspension of the pellet in 10 mls of Transport Medium. The acini, consisting of epithelial cells were sedimented by centrifugation at 800 rpm for 20 sec. The supernatant contained aggregates of fibroblasts. The acini were carefully aspirated using a sterile Pasteur pipette and transferred to a fresh Universal. The acini were spun down twice more and collected in the same manner.

2.2.1.2b Primary/Secondary Epithelial Cell Culture.

The acinar deposits were resuspended in 5mls of epithelial growth medium (EGM) per gram of original tissue and plated out in 75cm² tissue culture flasks. One flask was used for every 5 grams of tissue. The cultures were incubated at 37°C for 72 hours before being supplemented with 5mls EGM. After a further 48 hours incubation, the epithelial cells could be supplemented with fresh medium.

Epithelial growth medium was prepared as outlined below.
Epithelial growth medium (EGM)  
1 litre  
1.104% w/v WAJC 404 medium  
0.67% w/v HEPES  
0.12% w/v sodium hydrogen carbonate  
2mls of 0.25 mg/ml zinc stabilised insulin  
2mls of 10μg/ml cholera toxin  
100μl of 3.92mg/ml dexamethasone in ethanol  
1ml of 10μg/ml epidermal growth factor  
0.5% v/v foetal calf serum  
10mls of penicillin/streptomycin  
(10 000 units/ml / 10 000μg/ml)  
10mls of Fungizone (250μg/ml), pH 7.6  

Made up to 1 litre using distilled H₂O  

The epithelial cell cultures were subcultured as per the immortalised cell lines. Following the detachment of the cells from the flask, the 0.1% trypsin solution was neutralised using RPMI 1640 supplemented with 10% v/v FCS (FGM). The cells were then centrifuged at 1500 rpm for 5 minutes and resuspended in 20mls EGM. The cell suspension was then divided equally into two 75cm² flasks. In general, it was possible to passage the cells once. Beyond passage one, the cells displayed a reduced growth rate and had difficulties adhering to the plastic flasks.

2.2.1.2c Primary/Secondary Fibroblast Cell Culture  

The supernatant from 2.2.1.2b was centrifuged at 2000 rpm for 10 minutes and the resulting cell pellet was resuspended in 10mls of FGM supplemented with 2.5μg/ml Fungizone per gram of tissue. The fibroblast cell suspension was seeded into one 75cm² flask per five grams of tissue and grown in monolayer culture at 37°C.
for 72 hrs. The cells were then supplemented with fresh medium until 90% confluent. Sub-culture was performed as described in 2.2.1.1b.

2.2.1.2d Long Term Storage of Cells

Cell lines and primary cultured fibroblast cells could be stored for long periods by freezing in a gas phase liquid nitrogen storage container. Cells were prepared for freezing by trypsinisation as described previously. The cells were then pelleted by centrifugation at 2000 rpm for 10 minutes. The pellet was then resuspended in 1ml of freezing medium, which consisted of 5% dimethyl sulphoxide (DMSO), in FCS. The cells were mixed with the freezing medium by tituration before being transferred to cryogenic ampoules and placed in the −70°C freezer overnight. The cells were then transferred to the gas phase, liquid nitrogen store.

2.2.1.2e Thawing of Frozen Cells

Frozen cells were thawed in a 37°C water bath. DMSO was washed out of the thawed cell suspension by transferring it to a universal containing 20ml of RPMI supplemented with 10% FCS. The cell suspension was thoroughly mixed by vortexing prior to centrifugation at 2000 rpm for 10 mins. to pellet the cells. The cell pellet was then resuspended in 10ml of appropriate growth medium and transferred to a T75cm² tissue culture flask.

2.2.1.3 Determination of Cell Numbers

2.2.1.3a Spectrophotometric Determination of Viable Cell Numbers.

The proliferative rates of cultured cells lines and primary epithelial cells was determined using the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The MTT assay relies on the presence of active mitochondrial
dehydrogenases to convert the soluble tetrazolium salt into insoluble formazan [172]. Dead cells do not cause this change.

Cells were grown in 96 well plates, supplemented with 100μl of the appropriate medium per well. 50μl of MTT solution at a concentration of 1.5mg per ml of culture medium was aliquoted into each test well using a multi-channel pipette (Titertek plus; ICN Biochemicals Ltd.) and the cells incubated at 37°C for 2 hrs. Subsequent to this incubation, 130μl of the assay mixture was carefully aspirated from each well. The formazan crystals formed as a result of the intracellular dehydrogenase activity were solubilised by suspension in 150μl of dimethylsulfoxide (DMSO) containing 0.5% v/v FCS. The plates were then shaken at room temperature on an orbital shaker for 30 minutes to ensure complete dissolution of the crystals and the absorbance of the resulting solution was measured at 540nm with a microplate reader (model 450; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK.).

The viability of the MTT assay as a determinant of cell number was assessed using the DU145 cell line.

DU1435 cells were seeded into 96 well tissue culture plates at an initial plating density of 1000 cells per well. 5 plates corresponding to days 0-4 were used for both the MTT assay and cell counts, with 8 wells employed for each. 48 hours post seeding, the medium was removed and replaced with 100μl of fresh medium. At this time and at 24 hour intervals thereafter, the cell number was assessed using the MTT assay and by manual counting using a haemocytometer.

The MTT assay was compared to manual cell counts whenever implemented.

2.2.1.3b Manual Determination of Cell Numbers

Cells to be counted were washed twice in Dulbecco ‘A’ PBS, incubated with 20μl of 1X trypsin-EDTA for 2 minutes at 37°C, mixed with 80μl of FGM and cell numbers determined using both chambers of a haemocytometer (five 1mm squares in each chamber).
Figure 2.1 demonstrates the growth pattern of DU145 cells as determined by both cell counting and by the MTT assay. Obviously both approaches demonstrate similar trends in cell growth but it is apparent that the experimental error associated with cell counting performed using the haemocytometer is considerably greater than that with the MTT assay.

2.2.1.4 Co-culture of Epithelial and Fibroblast Cells

Primary cultured epithelial and fibroblast cells which had been grown in monolayer cultures in isolation for 14 days, were harvested as described in 2.2.1.2b. The harvested cells were resuspended in FGM to neutralise the 1X trypsin-EDTA before being centrifuged at 2000 rpm for 5 min. The pellet was resuspended by gentle pipetting in medium comprising 1:1 v/v mixture of EGM:FGM. The resuspended cells were then counted manually using a haemocytometer before being seeded, at a density of 10000 cells/well for epithelial cells and 2000 cells/well for fibroblast cells, either into 6 well plates or into Millicell™ micropore (2μm pore size) inserts obtained from Sigma Chemical Company Ltd.. Subsequent to a 2hr incubation period which allowed the cells to sediment, the inserts containing the appropriate cell type were removed from the wells and placed, using sterile forceps, into the wells containing the other cell type. The plates were then returned to the incubator for a period of up to 12 days. Cells were supplemented with fresh medium every 3 days.

2.2.1.5 Measurement of the Androgenic Responses of Cultured Cells.

The response of primary and co-cultured epithelial and fibroblast cells to dihydrotestosterone was determined using manual cell counts as outlined in 2.2.1.3. All media employed were phenol red-free and the FCS supplements were rendered steroid hormone-free through treatment with Dextran Coated Charcoal (DCC).

All cells were grown in 6 well tissue culture plates.
Fig 2.1 Comparison between manual cell counting and MTT assay methods for the determination of cell proliferation.

Cells were plated out in RPMI 1640 with 10% FCS at an initial density of 1000 cells/well in 96 well plates. After 48 hours, the medium was replaced and cell numbers determined using a haemocytometer and the MTT assay. Subsequently, all cell numbers were assessed in this manner at 24 hour intervals for 5 days. Each data point represents the mean of 8 wells ± S.E.M.
2.2.1.5a Preparation of DCC-Stripped FCS

50ml of DCC suspension was pelleted by dividing into two equal 25ml aliquots and centrifuging at 4000 rpm for 20mins (4°C) in a Denley BS400 centrifuge.

DCC was prepared as outlined below.

DCC suspension (50ml)  
1% w/v activated charcoal  
1ml of 5mg/ml Dextran T70  
0.5ml of 1M Tris-HCl, pH7.5  
0.5ml of 0.1M EDTA  
Make to 50ml with distilled H2O

The charcoal pellets were mixed in an Erlenmeyer flask with 100ml of FCS pre-warmed to 37°C and the mixture shaken in a 37°C incubator for 1hr. Following this period of incubation, the charcoal was removed by centrifugation as described previously and the serum sterile filtered through a 0.2µm bottle-top filter.

2.2.1.5b Primary Epithelial and Fibroblast Cells

The growth of primary and co-cultured epithelial cells over a period of 4 days was assessed after exposure to two concentrations of testosterone (T) and dihydrotestosterone (DHT). The concentrations used were 100nM and 10nM with a control included which received 0nM T or DHT. A 1mM stock solution of DHT in ethanol was prepared and diluted down to 1µM. The addition of 200µl of T or DHT to 2mls of medium gave a final concentration of 100nM. The 1µM solution was diluted 10 fold to achieve the other desired well concentration. The control well was supplemented with 200µl of appropriate ethanol solution.

Four 6 well plates, for each concentration, corresponding to days 1-4 were employed. A total of 3 wells per plate were used for each concentration of T or DHT.
with an initial plate density of 10000 cells/well for epithelial cells and 2000 cells/well for fibroblast cells.

Cells grown in monolayer culture in 75cm² tissue culture flasks were harvested through trypsinisation as described in 2.2.1.1b, and resuspended in 10mls of their normal growth medium. The cell number was determined using a haemocytometer and the cells diluted to the desired final concentration per 2ml of medium in a total volume of 32mls. 2ml of the cell suspension were delivered to each test well and the cells allowed to adhere over 24 hrs in a 37°C incubator. The medium was subsequently aspirated from the test wells and replaced with 2mls of medium containing DCC-treated FCS. The cells were incubated for a further 48 hrs at 37°C prior to the addition of DHT to the desired concentration. The cell numbers were determined on Day1 using manual cell counts as described previously and measured subsequently every 24hrs up to 96hrs.

2.2.1.5c Co-cultured Epithelial and Fibroblast cells

The protocol for measurement of co-cultured epithelial and fibroblast cell proliferation in response to T and DHT was essentially the same as that described for the primary epithelial cells (2.2.1.5b) and fibroblast cells. 3 wells were used for each concentration of drug per plate and the cells were plated at the same density as described in 2.2.1.5b in a 1:1 v/v mix of EGM:FGM. After 24 hours at 37°C, the media was replaced with DCC-treated FCS medium. 48 hours later, the medium was supplemented with the appropriate concentration of T or DHT and the response measured as per 2.2.1.5b.
2.2.2 Preparation and Fractionation of Conditioned Media.

2.2.2.1 Preparation of Conditioned Media from Epithelial and Fibroblast Monolayer Cultures.

Cells, which had been grown for 10 days under normal culture conditions for monolayer cell cultures, were washed twice with 10mls of Dulbecco 'A' PBS. The cells were then supplemented with 10mls per flask of serum free medium and incubated at 37°C in 5% CO₂ for 3 days. Subsequent to this incubation in serum free medium, 100ml of the conditioned medium was collected by aspiration from the cell cultures and pipetted into dialysis membranes with a 1kD molecular weight cut off. The medium was dialysed against 5 litres of distilled water for 24 hours with the distilled water being changed 4 times during this period. The dialysed medium was then aliquoted in 10ml volumes into 50ml Falcon tubes (Corning). The dialysed medium was then snap frozen in liquid nitrogen before being lyophilised in a lyoprep freeze dryer. The lyophilised medium was stored in sealed falcon tubes at -20°C.

2.2.2.2 Preparation of Conditioned Media from Human Prostatic Epithelial and Fibroblast Cell Co-cultures.

Co-cultures of human prostate epithelial and fibroblast cells that had been maintained for 10 days were washed with Dulbecco ‘A’ PBS and supplemented with fresh medium. The co-cultures were then returned to the incubator and incubated at 37°C in 5% CO₂ for a further 3 days. Following this incubation, 24mls of medium was collected by aspiration and sealed in 1kD molecular weight cut off dialysis membranes. The medium was dialysed against 5 litres of distilled water as described in 2.2.2.1.. The dialysed medium was then dispensed into 50ml Falcon tubes and snap frozen in liquid nitrogen. The frozen, dialysed medium was then lyophilised as described in 2.2.2.1..
2.2.2.3 Fractionation of Conditioned Media from Fibroblast Monolayer Cultures.

Lyophilised conditioned medium was reconstituted in 1ml of autoclaved distilled water. The reconstituted samples were then fractionated by utilisation of microspin microconcentrator spin columns with molecular weight cut-off of 50kD, 30kD, 10kD, 3kD. The reconstituted samples were initially spun in the 50kD molecular weight cut-off spin column. The filtrate was collected in the catch tube and was spun in the 30kD cut-off column. The filtrate was once again collected and spun in the 10kD column. This was continued until the entire reconstituted sample had been fractionated. The filtrates and retentates of the spin columns were collected and stored in screw-cap Eppendorf tubes at -20°C. The filtrates and retentates collected by this method yielded fractions of conditioned medium in the range of greater than 50kD, 50 to 30kD, 30 to 10kD, 10 to 3kD and less than 3kD. The samples were spun in a microcentrifuge at room temperature for the following times and at the following gravitational forces.

<table>
<thead>
<tr>
<th>Molecular weight cut-off</th>
<th>Time (mins)</th>
<th>X g</th>
</tr>
</thead>
<tbody>
<tr>
<td>50kD</td>
<td>3</td>
<td>5000</td>
</tr>
<tr>
<td>30kD</td>
<td>3</td>
<td>5000</td>
</tr>
<tr>
<td>10kD</td>
<td>3</td>
<td>5000</td>
</tr>
<tr>
<td>3kD</td>
<td>2</td>
<td>7500</td>
</tr>
</tbody>
</table>

2.2.2.4 HPLC Analysis of Fibroblast Conditioned Medium

The proteins in fibroblast conditioned medium were fractionated by HPLC using anion and cation exchange columns on a Beckman System Gold HPLC apparatus utilising a 166 detector and a 126 solvent module by Neil Inglis, a research fellow at the Moredun Research Institute, Edinburgh. Briefly, HPLC was carried out described below.

Initially, proteins were separated on a Bio-Rad MA75 cation exchange column (5.0cm x 0.75cm). The sample was resuspended in 5ml of 25mM MES (2-
[N-Morpholino]ethanesulfonic acid) buffer at pH 6.0 and loaded onto the column in 1ml injections over a period of 20-30 minutes. Any bound flow through was collected in a universal and stored for analysis. Bound material was eluted over 30 minutes by applying a 0-1M NaCl gradient. The flow rate used was 0.5ml per minute. Events were monitored at 280nm and fractions were collected at 2 minute intervals.

Flow through from the cation exchange was dialysed against distilled water for a period of 48 hours using a 1kD dialysis membrane. The sample was then lyophilised (as described previously) subsequent to being resuspended in 5ml of 25mM Tris/HCl at pH 7.5. The sample was loaded onto a Tosohass TSK DEAE-5PW anion exchange column (7.5cm x 0.75cm) in a series of 1ml injections over a period of 20–30 minutes. Absorbed material was eluted using a 0-1M NaCl gradient over a period of 30 minutes. Events were monitored at 280nm and samples collected every two minutes as before.

All collected samples were dialysed against distilled water using a 1kD membrane to remove salt from the elution procedure. Dialysed samples were then lyophilised and stored at -20°C.

2.3 Analysis of 5α-reductase Activity.

2.3.1 Preparation of Primary Cultured Prostate Epithelial and Fibroblast Cells for Analysis of 5α-reductase Isozyme Activity.

Primary cultures of prostate epithelial and fibroblast cells were grown and maintained as described previously in 2.2.1. Following a 10-day incubation period, cells were harvested by trypsinisation, resuspended in 20ml of FGM and centrifuged for 10 min. at 2000 rpm. The resultant cell pellet was resuspended in 10ml of FGM and an aliquot used to determine cell number. The cells were again pelleted by centrifugation, resuspended in 1ml of FGM and transferred to a sterile Eppendorf tube. The suspension was centrifuged in a bench top centrifuge for 10 min. at 2000
rpm. Subsequent to pelleting, the supernatant was removed with a Pasteur pipette and the cell pellet was stored at -20°C until needed.

The frozen pellet was resuspended in 1ml of 4mM sodium phosphate buffer of appropriate pH containing 0.32M sucrose. The suspended cells were lysed by passing twice through a 23G needle. The specific pH sodium phosphate buffers were prepared as follows.

Sodium Phosphate buffer (0.1M; pH5.0) 1.5ml 1M Na₂HPO₄
98.5ml 1M NaH₂PO₄
Diluted to 1000ml with distilled H₂O

Sodium Phosphate buffer (0.1M; pH7.5) 81.5ml 1M Na₂HPO₄
18.5ml 1M NaH₂PO₄
Diluted to 1000ml with distilled H₂O

Stock solutions of 0.1M sodium phosphate buffer were diluted to 4mM with distilled H₂O and supplemented with 0.32M sucrose.

2.3.2 Preparation of Co-cultured Epithelial and Fibroblast Cells for Analysis of 5α-reductase Isozyme Activity.

Co-cultured epithelial and fibroblast cells which had been grown at 37°C in 5% CO₂ for 10 days were harvested by trypsinisation and resuspended in 20ml RPMI1640 supplemented with 10% FCS. The cells were pelleted by centrifugation at 2000rpm for 10 min. Subsequent to being pelleted by centrifugation the cells were prepared for analysis of 5α-reductase isozyme activity as described in 2.3.1..
2.3.3 Preparation of Human Prostate Tissue for Analysis of 5α-reductase Isozyme Activity.

Prostate tissue obtained from men undergoing TURP operations for BPH was prepared by snap freezing the tissue in liquid nitrogen and storing at -70°C. 1g of frozen tissue was placed into 50ml Falcon tubes. 30ml of 40mM sodium phosphate buffer (pH 5.0 or 7.5) containing 0.32M sucrose and 1mM DTT (dithiothreitol) was added to the tubes containing the tissue samples which were then homogenised using a Ystral blender (3 times for 15 sec at 15 000rpm). The homogenised tissue was then filtered through sterile gauze and transferred into Eppendorf tubes in 1ml aliquots. The protein concentration of each aliquot was determined colourimetrically by the method of Bradford (1976) [173] using a Bio-Rad protein assay reagent.

2.3.3.1 Determination of Protein Concentration

Protein concentration was determined using a Bio-Rad protein assay kit which followed the method of Bradford (1976) [173]. The protocol followed is detailed below.

10μl of the protein sample was transferred to a 96 well plate. To this sample, 25μl of reagent A and 250μl of reagent B were added. The plate was shaken on an orbital shaker for 15 min at room temperature. Subsequent to this incubation, the plate was read at 595nm using a microplate reader (model 450; Bio-Rad laboratories, Hemel Hempstead, Hertfordshire, UK) and the protein concentration assessed against Bovine Serum Albumin (BSA) standards ranging from 0.05mg/ml to 40mg/ml.

Samples were then prepared for 5α-reductase isozyme activity analysis as described previously in section 2.3.1. or stored at -70°C.
2.3.4 5α-reductase Isozyme Assay.

The 5α-reductase activity of primary cultured cells was determined by analysing the ability of the cells to convert of 3H testosterone to 3H DHT in the presence of a NADPH generating system. The protocol used is an adaptation of that described by Smith et al (1995) [174]. 200µl of suspended cells were added to a glass test tube containing 0.5mM NADP⁺, 0.1U/ml glucose-6-phosphate, 5mM glucose-6-phosphate dehydrogenase and 1mM DTT. Substrate in the form of 20nM testosterone containing 1µCi 3H testosterone (specific activity 105Ci/mmol) was added to the test tubes and the volume adjusted to 1ml. The assay mixture was incubated at 37°C for 30 min before the reaction was stopped by the addition of 2 volumes of diethyl ether containing 500cpm 14C DHT (specific activity: 50mCi/mmol) and 25µg each of unlabelled 3α-androstanediol, 3β-androstanediol, testosterone, DHT and androstendione. The resultant mixture was vortexed for 60 sec. The assay tubes were then transferred to a -70°C freezer for 5 min. until the assay mixture had frozen. The supernatant consisting of diethyl ether and extracted steroids was decanted into clean glass test tubes and evaporated to dryness in a vacuum oven at 40°C. This extraction process was repeated.

Co-factor reagents were prepared as detailed below.

10mM NADP⁺ 255mg dissolved in 30ml sodium phosphate buffer

100mM Glucose-6-phosphate 1.144g dissolved in 29ml sodium phosphate buffer
5U/ml Glucose-6-phosphate dehydrogenase  16ml sodium phosphate buffer
4ml glycerol
100μl of 1U/μl glucose-6-phosphate dehydrogenase

All co-factors were aliquoted and frozen at -20°C.

2.3.5 Analysis of 5α-reductase Activity in Growing Cells.

5α-reductase activity in growing cells was analysed by growing the cells in medium supplemented with DCC stripped FCS for a period of 24 hours. Prior to this, cells were trypsinised, counted manually utilising a haemocytometer and plated at known densities. The medium was then changed for 10mls of medium supplemented with DCC stripped FCS and 10nM testosterone. The testosterone substrate contained 1μCi of 3H-testosterone (specific activity; 105Ci/mmol). The cells were incubated for a period of 4 hours. 1ml samples were removed from the cells at period of 1, 2 and 4 hours. Steroids were extracted from the samples as described in section 2.3.4.. Negative controls, which had no cells growing in the flasks were included.

2.3.6 Separation of 5α-reductase Isozyme Assay Metabolites by Thin Layer Chromatography (TLC).

Following evaporation to dryness, the residue was resuspended in 50μl of absolute ethanol. The resuspended extract was spotted onto ITLC™ SA polysilicic acid gel impregnated glass fibre plates using Microcaps micropipettes (Drummond
Scientific Company). Each extract sample was allowed to dry fully before application of the next sample. The TLC plates were run in glass chromatography tanks for 40 min., or until the solvent reached the boundary of the chromatography plate, using a dichloromethane:diethyl ether (9:1 vol:vol) solvent. The separated steroids were visualised by spraying the TLC plate with phosphomolybdic acid reagent and heating the plate to 90 °C until colour development was observed.

Sections of the TLC plate corresponding to the DHT standards which were run alongside the samples were removed by scraping the appropriate area into scintillation vials with a clean scalpel and adding 6ml. of liquid gold scintillation cocktail (Canberra Packard). Radioactive metabolites in the sample were quantified using a Tri-Carb liquid scintillation counter (Canberra Packard).

Losses of metabolites due to the extraction procedure were calculated by analysing the recovery of $^{14}$C DHT from the samples. Samples where recovery was less than 70% were discarded and the experiment repeated. The same procedure was used for determining the losses of androstenedione and androstanedione utilising $^{14}$C labelled steroids as appropriate.

2.4 Isolation of Nucleic Acids.

2.4.1 Isolation of total RNA from Primary Cultured Cells.

Total RNA was extracted from cultured cells using a modification of the acid-guanidium-phenol-choroform (AGPC) method of Chomczynski and Sacchi (1987) [175].

In order to minimise RNase contamination of the RNA preparation; all solutions were prepared using RNase-free glassware, plasticware, water and chemicals. Wherever possible, the solutions were treated with 0.1% DEPC (diethyl pyrocarbonate) for 24 hours at room temperature and subsequently autoclaved for 20mins at 15lb/sq.in. on liquid cycle.
GTC was prepared as follows.

GTC solution

- 9.46g guanidine isothiocyanate
- 636μl 0.75M sodium citrate, pH7.0
- 11.08ml Ultra-pure H2O

Solution A was prepared by addition of 2-Mercaptoethanol to the GTC solution to a final concentration of 0.1M.

Cells were grown in monolayer culture to 90% confluency in 75cm² tissue culture flasks, washed in DEPC-treated Dubecco ‘A’ PBS and lysed in 1.9mls of solution A per flask. 100μl of 10% n-lauryl sarcosine was added to each flask and mixed by gentle agitation. The tissue culture flask was shaken at 4°C for 10mins to ensure complete lysis of the cells. 500μl aliquots of the cell lysate were pipetted into Eppendorf tubes and 50μl of 2M sodium acetate (pH4.0) was delivered to each tube. The tubes were vortexed for 10 sec. prior to the addition of 500μl of water-saturated phenol (pH4.0) and 100μl of chloroform-isoamylalcohol (49:1). The resulting suspension was vortexed for 10 sec. and then incubated on ice for 15 min. The samples were then centrifuged at 13 000rpm for 20 min. at 4°C in a microcentrifuge. Subsequently, the upper aqueous phase was transferred to a fresh Eppendorf tube where it was mixed with an equal volume of isopropanol. The tubes were then left overnight at -20°C to allow the RNA to precipitate. The RNA precipitate was sedimented by further centrifugation at 13 000rpm for 20 min. at 4°C. The RNA pellet was resuspended in 50μl of solution A and the samples pooled and precipitated by the addition of an equal volume of isopropanol and a further incubation at -20°C for 2 hrs. The RNA was centrifuged at 13 000rpm for 20 min. at 4°C and the resulting pellet was washed in 200μl of 75% ethanol prior to a further 10 min. centrifugation. The supernatant was carefully aspirated and the RNA allowed to air dry for 30 min. at room temperature before being resuspended in 50μl nuclease-free water.
A 5μl aliquot of the RNA solution was diluted in 2.995ml of nuclease-free water and the concentration and purity of the preparation determined spectrophotometrically. In order to quantify the amount of RNA in the sample, readings were taken at 260nm and 280nm. An OD_{260} of 1 corresponds to an RNA concentration of approximately 40μg/ml (Maniatis et al). The ratio between readings taken at 260nm and 280nm (OD_{260}/OD_{280}) provides an estimate of the purity of the sample. A ratio of 2 corresponds to a pure sample. If the OD_{260}/OD_{280} ratio was less than 1.7, the sample was re-extracted with phenol-chloroform.

45μl of phenol:chloroform (1:1) was added to the RNA solution and the suspension vortexed and spun at 13 000rpm for 5mins (4°C). The aqueous phase was transferred to a fresh Eppendorf tube and mixed with 45μl of chloroform:isoamylalcohol (49:1) prior to a further centrifugation at 13 000rpm for 5 min. (4°C). The aqueous phase was removed and the RNA precipitated by the addition of 3 volumes of ethanol and 3M sodium acetate to 0.3M final concentration. The precipitated RNA was pelleted and resuspended as previously described. The concentration of the RNA solution could then be re-assessed spectrophotometrically as before.

2.2.4.2 Isolation of Total RNA from Co-cultured Cells.

Total RNA from co-cultured cells was isolated using the same method as described in section 2.2.4.2 with the exception of the initial stages where solution A was pipetted into each well of 6 well plates in turn following the removal of the Millipore™ well inserts. Total RNA could also be isolated from cells grown on the Millipore™ well inserts by placing the Millipore™ well inserts into fresh wells and pipetting Solution A into the inserts in turn. Following lysis of the cells with Solution A, the procedure for the isolation of total RNA from co-cultured cells was carried out as per 2.2.4.2.
2.2.4.3 Isolation of Total RNA from Snap Frozen Tissue.

Prostatic tissue obtained by TURP was promptly washed in Dulbecco 'A' PBS and snap frozen in liquid nitrogen. The frozen tissue was either used immediately for RNA preparation or stored at -70°C for use within 7 days of resection. Up to 1 gram of frozen tissue could be used per preparation.

The frozen tissue was disaggregated using a MIKR-Dismembranator II (B. Braun Biotech International GmbH, Melsungen, Germany.) fitted with a 7ml Teflon shaking flask and accompanying stainless steel grinding ball, both of which were pre-chilled in liquid nitrogen. The Dismembranator was operated on full power for 20 sec. and the resulting tissue powder scraped into 5mls of solution A (2.2.4.1) using a pre-chilled micro-spatula. The stainless steel grinding ball was also placed into the aliquot of solution A since a significant amount of the powdered tissue adhered to the ball. The RNA preparation was continued as outlined in 2.2.4.1.

2.2.4.4 Isolation of Messenger RNA from Total RNA.

Total RNA samples from either cultured cells or from snap frozen tissue were purified to messenger RNA (mRNA) by employing a PolyA tract mRNA purification kit purchased from Promega. The purification of total RNA to mRNA was carried out as per manufacturer instructions.

0.1-1.0mg of total RNA was diluted in a total volume of 500μl of Rnase-free water in a sterile, Rnase-free Eppendorf tube. The RNA solution was heated to 65°C in a dry heating block for 10 min. Subsequent to this incubation, 150 pmol of biotinylated-oligo (dT) probe were added. 20X SSC was added to a final concentration of 0.2X. This solution was mixed and incubated at room temperature for 10 min. or until completely cooled to allow the hybridisation of the biotinylated-oligo (dT) probe to the mRNA in the solution. The Streptavidin-ParaMagnetic Particles (SA-PMP) were resuspended and washed three times with 0.5X SSC. Each time the particles were captured by use of the magnetic stand provided with the kit and the supernatant carefully aspirated with a fine tip Pasteur pipette. Following the
washes, the SA-PMPs were resuspended in 0.5X SSC. Once resuspended, the entire contents of the annealing reaction was added to the tube containing the SA-PMPs and incubated at room temperature for 10 min.. Upon completion of the incubation, the SA-PMPs were captured using the magnet and the supernatant was removed with a fin tip Pasteur pipette. The particles were then washed four times with 0.1X SSC each time removing as much of the supernatant as possible without disturbing the SA-PMPs.

The bound mRNA was eluted from the SA-PMPs by resuspending the pellet in Rnase-free water. The SA-PMPs were captured by the magnetic stand and the eluted mRNA transferred to a sterile, Rnase-free tube. The elution process was repeated and the eluates pooled. The concentration of the mRNA could then be assessed spectrophotometrically as previously described.

2.2.5 Analysis of Nucleic Acids.

2.2.5.1 Reverse Transcription of Total RNA.

The protocol for first strand synthesis of cDNA from samples of total RNA is a modification of that described in the Promega Protocols and Applications Guide (2nd Edition, 1991). A volume of total RNA solution corresponding to 1μg total RNA was aliquoted into a 500μl micro-test tube. The tube was heated to 85°C for 5 minutes and cooled on ice for 3 min.. The tube was then pulsed in a microfuge and the following solutions added to the RNA solution:

- 0.5μl of rRNasin ribonuclease inhibitor (40units/μl)
- 4μl of 25mM MgCl₂
- 2μl of 10X reverse transcription buffer
- 2μl of 10mM dNTP mixture
- 1μl of oligo(dT)₁₅ (0.5μg/μl)
- 0.6μl of AMV reverse transcriptase (HC; 25units/μl)

Made up to final volume of 20μl using ultrapure H₂O
The tube was vortexed, pulsed in a microcentrifuge and then incubated at 42°C for 60 min. Following the incubation, the reverse transcription mixture was heated to 99°C for 5 mins. followed by a 5 min. incubation on ice.

2.2.5.2 Amplification of 5α-reductase Type I (5αRI) and 5α-reductase type II (5αRII) cDNA sequences.

Primers.

All primer sequences are written 5’ to 3’ with their locations on the gene bracketed. All sequence information was obtained from Genebank EMBL and the primers were designed using the primer design program incorporated into Genebank. Melting temperatures were pre-set before the primer design was initiated.

2.2.5.2a 5α-reductase Type I

The set of primers used to analyse 5αRI cDNA located to exons 2 and 3 respectively. These primers span intron 2. The locations of the primers for the analysis of 5αRI cDNA are shown in figure 1.15. There is also a 5αRI non-transcribed psuedogene which demonstrates very high homology with the transcribed gene (>90%). To prevent detection of a false positive signal due to contamination of the total RNA sample with psuedogene DNA, samples used to analyse 5αRI RNA expression were either purified to mRNA or were subjected to a shorter number of PCR reaction cycles. Attempts to synthesise primers, which did not hybridise to the psuedogene sequence proved to be futile. Appropriate controls were included in all reactions involving 5αRI.

20 5'TGCTGATGACTGGTGTAACAG 3'
2.2.5.2b 5α-reductase Type II

The primer pairs used to analyse 5αRII cDNA located to exons 1 and 2 respectively. These primers span intron 1. The locations of the primers for the analysis of 5αRII are shown in figure 1.16.

4B 5'CCTTGTACGTCGGAAGC 3'

7B 5'CCACCCATCAGGTTATCG 3'

2.2.5.3 PCR Reaction

The procedure for the amplification of cDNA sequences of 5αRI and 5αRII was essentially the same, the reaction mixture being described below.

<table>
<thead>
<tr>
<th>PCR reaction mixture</th>
<th>20μl of reverse transcription reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10μl of sense primer (50μg/ml)</td>
</tr>
<tr>
<td></td>
<td>10μl of antisense primer (50μg/ml)</td>
</tr>
<tr>
<td></td>
<td>8μl of 10X reaction buffer</td>
</tr>
<tr>
<td></td>
<td>(containing 1.5mM MgCl₂)</td>
</tr>
<tr>
<td></td>
<td>16μl of dNTP mix (1.25mM)</td>
</tr>
<tr>
<td></td>
<td>0.2μl of Taq DNA polymerase</td>
</tr>
<tr>
<td></td>
<td>(5units/μl)</td>
</tr>
<tr>
<td></td>
<td>35.8μl of nuclease-free water</td>
</tr>
</tbody>
</table>

The components of the PCR reaction were thoroughly mixed through brief vortexing, the mixture overlaid with 50μl of mineral oil and pulsed in a microcentrifuge.
2.2.5.3a 5α-reductase Type I.

Samples to be analysed for 5αRI were subjected to the following reactions for a maximum of 30 cycles. Conditions were determined according to details set out in PCR Protocols-A guide to methods and applications [176].

- Denaturation: 96°C, 1 minute 30 seconds
- Annealing: 52°C, 1 minute
- Polymerisation: 72°C, 1 minute 30 seconds

2.2.5.3b 5α-reductase Type II.

Samples to be analysed for 5αRII were subjected to the following reactions for a maximum of 35 cycles. Conditions were determined according to details set out in PCR Protocols-A guide to methods and applications [176].

- Denaturation: 96°C, 1 minute 30 seconds
- Annealing: 56°C, 1 minute
- Polymerisation: 72°C, 1 minute 30 seconds

2.2.5.4 Agarose Gel Electrophoresis of PCR Products.

The PCR products were analysed by electrophoresis in 2% agarose gels (75ml) containing 1XTris-Borate-EDTA (TBE) buffer.

5X TBE buffer was prepared as follows.

- 5X TBE buffer: 5.4% w/v Tris-base
- 2.75% w/v boric acid
- 10mM EDTA (pH8.0)

Make up to a final volume of 1 litre using distilled H₂O
2.2.5.4a Preparation of Agarose Gels

The 2% agarose gel was prepared by melting 1g of Agarose NA in 75ml of 1XTBE using a microwave oven. The molten agarose was cooled to approximately 40°C before adding 1μl of 10mg/ml ethidium bromide. The agarose was then poured into a level tray containing a comb of well dimensions 5.5x1.5mm 16 well comb.

2.2.5.4b Electrophoresis of Samples in Agarose Gels

The gel was run in 800ml of 0.5XTBE in midi-gel apparatus (Northumbria Biologicals Ltd.). 18μl of each PCR reaction with 2μl of DNA loading buffer could be loaded per well alongside 20μl of 100bp ladder (approx. 0.1mg/ml). Electrophoresis was performed for 4 hours at 50 volts after which the gel was examined and photographed under U.V. transillumination.

2.2.6 Analysis of Proteins by Western Blotting.

2.2.6.1 Isolation of Protein from Cultured Cells.

Cells grown in monolayer culture to approx. 90% confluency in 75cm² tissue culture flasks were washed twice with 15ml of Dulbecco ‘A’ PBS, drained completely and lysed in 500μl of 1X Sodium Duodecyl Sulphate (SDS) gel loading buffer pre-heated to 85°C. SDS gel loading buffer was prepared as detailed below.

1X SDS gel-loading buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mMTris-HCl (pH6.8)</td>
<td>100mM dithiothreitol</td>
</tr>
<tr>
<td>2% w/v SDS</td>
<td>0.1% w/v bromophenol blue</td>
</tr>
<tr>
<td>2% w/v SDS</td>
<td>10% v/v glycerol</td>
</tr>
</tbody>
</table>

Make up to final volume of 100ml using distilled H₂O
The lysates were transferred to Eppendorf tubes and boiled in a water bath for 10 minutes. The tubes were placed on ice and sonicated in a Soniprep 150 (MSE; Fisons Scientific Equipment Ltd., Loughborough, Leics., UK.) set at full power for 10 sec. The tubes were then centrifuged in a microcentrifuge at 13 000rpm for 10 min. At room temperature and the resulting supernatants transferred to fresh tubes.

20μl of the protein preparation was mixed with 80μl of 100% ethanol and boiled in a water bath for 1min. The resulting precipitate was pelleted by centrifugation at 13 000 rpm for 5 min. in a microcentrifuge, dried at room temperature for 5 min. and resuspended in 20μl of autoclaved water. The protein was precipitated as before and finally resuspended in 80μl of autoclaved water. The protein concentration of this 80μl aliquot was determined colourmetrically by the method of Bradford (1976) [174] using a Bio-Rad protein assay reagent as detailed in section 2.3.3.. Protein concentration was assessed against bovine serum albumin (BSA) standards ranging from 40μg/ml to 1μg/ml of protein.

The protein samples were either used immediately for SDS- Polyacrylamide Gel Electrophoresis (PAGE) or stored at -20°C.

2.2.6.2 Isolation of Protein from Co-cultured Cells.

Epithelial and fibroblast cells were grown in co-culture in 6 well plates until they achieved 90% confluency. The cells were then lysed in 500μl of 1XSDS gel-loading buffer as per 2.2.6.1. The loading buffer was pipetted from the original well into each subsequent well. The rest of the protocol for the sample preparation was as explained in 2.2.6.1.

2.2.6.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins.

The procedure detailed is based on the discontinuous buffer system described by Laemmli (1970) [177]. Electrophoresis was performed using the Protean II vertical electrophoresis system (16cm configuration [Bio-Rad Laboratories Ltd.]) using 18.3x1.5mm spacers.
The electrophoresis plates were washed in a weak detergent solution, rinsed thoroughly and cleaned with 100% ethanol. The gel plates were assembled according to manufacturers instructions. The resolving gel was prepared by pouring 30mls of a 5% polyacrylamide solution into the gap between the plates. The resolving gel was then overlaid with isobutanol to a depth of approx. 0.5cm and polymerisation allowed to proceed for 30 min...

The resolving gel was prepared as follows.

5% Polyacrylamide Resolving Gel 40% w/v acrylamide/bisacrylamide
(30ml)
1.5M Tris-base (pH8.8)
10% w/v SDS
10% w/v ammonium persulphate
10µl TEMED
Make up to final volume of 30ml using autoclaved distilled H₂O

The isobutanol was removed using a paper towel and the top of the gel washed with deionised water to remove any unpolymerised polyacrylamide. 10mls of a 5% stacking gel was poured onto the surface of the resolving gel and a 10 well Teflon comb (10mmx1.5mm) was inserted, ensuring that no air bubbles were trapped. The stacking gel was allowed to set for 30 min. at room temperature

The stacking gel was prepared as follows.

5% Polyacrylamide Stacking gel (10ml) 7.25mls autoclaved water
1.275mls 40% w/v acrylamide/bis-acrylamide
1.25mls 1M Tris-base (pH6.8)
0.1ml 10% w/v SDS
0.1ml 10% w/v ammonium persulphate
10µl TEMED
After the completion of the polymerisation, the comb was removed and the wells washed with de-ionised water to remove any unpolymerised acrylamide. The gel could then be mounted into the gel apparatus and Tris-glycine electrophoresis buffer poured in the top and bottom reservoirs. Air bubbles trapped at the bottom of the gel between the glass plates were removed.

The preparation of the electrophoresis buffer is detailed below.

Tris-glycine Electrophoresis buffer  
(3 litres)

9.09G Tris-base

43.23g glycine

3g SDS

Make up to a final volume of 3 litres using distilled H₂O

The protein samples were boiled in a water bath for 10mins and incubated on ice for 5 min. prior to loading into the bottom of the wells using Prot/Elec tips (Bio-Rad Laboratories Ltd.). The samples were loaded alongside 15μl of Rainbow™ molecular weight markers (Amersham International plc.; molecular weight range 14 300-200 000). The Rainbow markers (15μl) were mixed with 15μl of 1xSDS gel loading buffer, boiled for 10 min. and loaded onto the gel.

The gel was initially run at 20mA until the bromophenol blue reached the interface of the stacking and resolving gels, whereupon the gel was run at 40mA until the bromophenol blue was seen to run off the bottom of the resolving gel.

2.2.6.4 Transfer of Proteins from SDS-Polyacrylamide Gels to Nitro-Cellulose.

The SDS-polyacrylamide gel was placed into 1L of transfer buffer and soaked for 30 min.. The gel could then be measured and nitro-cellulose (Hybond C extra) along with 4 pieces of Whatmann 3MM paper cut to the same dimensions. The gel, nitro-cellulose and 3MM paper were then equilibrated in transfer buffer for 30 min..

Preparation of the transfer buffer was as follows.
Transfer buffer (1 litre) 5.82g Tris-base
2.93g glycine
200ml methanol
3.75mls 10% w/v SDS, pH9.2

Make up to a final volume of 1 litre using distilled H₂O

Blotting was performed with a Transblot SD semi-dry transfer cell (Bio-Rad Laboratories Ltd.). The electrodes were carefully cleaned with 100% ethanol prior to the blotting procedures.

2 pieces of 3MM paper were placed on top of each other on the anode. The nitro-cellulose could then be positioned above the 3MM paper with the gel placed exactly above the membrane. The remaining pieces of 3MM paper were stacked on top of the gel. After carefully placing the cathode atop the stack, transfer was carried out for 16 hrs at 5 volts.

2.2.6.5 Immunodetection of Androgen Receptor Protein Immobilised on Nitro-Cellulose.

Protein detection was carried out using the ECL Western blotting detection system.

The nitro-cellulose membrane was washed in 500mls of washing buffer for 10mins and incubated in blocking solution (0.1ml per cm²) for 2 hours in a shallow tray at room temperature on an orbital shaker. The membrane was subsequently placed in a heat sealable bag and incubated with the polyclonal anti-human androgen receptor antibody diluted 1:50 in blocking solution (0.1ml solution per cm²). The membrane was incubated with the primary antibody for 2 hrs.
2.2.7 Electron Microscopy.

Primary and co-cultured epithelial and fibroblast cells were harvested by trypsinisation, resuspended in 20mls of FGM medium and centrifuged at 2 000 rpm for 5 minutes. The cell pellets were then resuspended in 1ml of FGM medium and transferred to an Eppendorf tube. The cells were then pelleted by centrifugation at 6500rpm for 2 min. in a bench top microfuge. The resulting pellets were then fixed by slowly adding 3% glutaraldehyde in 0.1M Cacodylate HCl buffer to the Eppendorf tube without causing any disruption to the pelleted cells. The fixed cells were then incubated at 4°C overnight.

The fixed cell pellets were then taken to the Pathology Department of the Western General Hospital where MLSO Frank Donnelly carried out all further preparation and analysis. The electronmicroscope utilised was a Jeol 100CXII transmission microscope operating at 60kV.

2.2.8 Analysis of PSA Production in Cultured Cells.

2.2.8.1 Immunohistochemical Analysis of PSA Production in Cultured Cells.

Co-cultured and primary cultured epithelial and fibroblast cells were grown in 24 well plates as described previously. After being maintained in culture for 10 days, cells were prepared for immunohistochemical analysis of PSA expression.

Cells were washed twice in Dulbecco ‘A’ PBS and drained. The cultured cells were then fixed for immunostaining by incubating the cells in 1% formaldehyde in PBS for 20 min at room temperature. The formaldehyde was carefully aspirated off the cells and replaced with 0.5 mls of 5% acetic acid in ethanol for a period of 10 min at room temperature. After fixation, the cells were then ready to be stained for PSA production using the Streptavidin-DAB method. The fixed cells were incubated with 250µl of 3% hydrogen peroxide for 5 min at room temperature to block any endogenous peroxidase activity in the cells. The Hydrogen Peroxide was aspirated from the cells and replaced with 250µl of 20% Sheep serum in 1% TBS-Bovine
Serum Albumin (BSA). After a 20 min incubation with the 20% sheep serum, the serum was aspirated from the fixed cells and 200μl of the primary antibody, a mouse anti-human PSA monoclonal (Unipath) diluted 1:50 000 was applied to the cells and incubated at room temperature for 1 hour. Following this incubation, the primary antibody was removed and the fixed cells washed in 3 volumes of TBS for 5 minutes each on a shaking incubator. All washes were carefully pipetted off before the following wash. After careful washing, the cultured cells were incubated with an anti-mouse secondary antibody (Boehringer Mannheim) diluted 1: 400. Fixed cells were incubated with the secondary antibody (anti-mouse IgG POD Fab Fragment; Boehringer Mannheim) for 1 hour at room temperature. Upon completion of the incubation, the secondary antibody was pipetted from the cells and the cells once again were washed with 3 volumes of TBS for 5 min. each. The washed cells were then incubated with 200μl of Streptavidin linked HRP for 30 min. at room temperature. Once again, upon completion of the incubation with the streptavidin, the cells were washed with 3 volumes of TBS for 5 minutes at room temperature. While the cells were being washed, an appropriate volume of DAB substrate was prepared using a DAB kit (Sigma). 200μl of DAB substrate was added to the freshly washed cells at room temperature for a period of 5 min.. The cells were washed in tap water before being counterstained using Mayer’s haematoxylin (Sigma). Haematoxylin was added to the stained cells in excess and incubated at room temperature for 3 min... The haematoxylin was then poured off the cells and an excess of a saturated solution of Lithium carbonate was pipetted onto the cells. Following a short incubation, the lithium carbonate was poured off the stained cells and the cells washed with tap water. The cells could then be stored at 4°C for a short period of time by pipetting 2mls of TBS into each well.

Immunohistochemically stained cells could then be photographed using an Olympus PM-10AK automatic exposure photomicrographic system.

TBS buffer was prepared as follows.
TBS (pH 7.6; 1 litre) 6.05g Trizma Base
8.2g Sodium chloride
1ml Triton-X 100

Make up to a final volume of 1 litre using distilled H₂O

2.2.8.2 Measurement of PSA by Enzyme Linked Immunosorbent Assay.

Conditioned medium from co-cultured and primary cultured epithelial cells was prepared as described in section 2.2.2. The 24mls of conditioned medium collected from the 6 well plates was dialysed against 5 litres of distilled water through 1kD molecular weight cut off membranes for a period of 24 hours with the distilled water being changed 4 times during this period. The dialysed medium was then snap frozen in liquid nitrogen before being lyophilised using a Lyoprep freeze dryer. The lyophilised medium was reconstituted in 240µl of autoclaved distilled water before being analysed for PSA production employing a Tandem-MP assay kit (Hybritech, Belgium). The ELISA was carried out according to manufacturer protocol.

PSA standards, which were compatible with the kit, were obtained from Hybritech. The ELISA was performed in the supplied 96 well plates. 50µl of standards and samples were pipetted into wells in the plate. Standards used were 0, 0.5, 2.0, 10, 25 and 50ng/ml. The standards were supplied ready made with the kit.

Subsequent to pipetting the samples and standards into the wells, 50µl of the assay conjugate (mouse monoclonal IgG conjugated to bovine alkaline phosphatase and mouse monoclonal IgG conjugated to biotin in a bovine/mouse protein matrix containing 0.1% sodium azide) was added to the wells and incubated at room temperature on a horizontal shaker. Following completion of the incubation, the samples were washed three times with the wash solution supplied in the kit each time aspirating as much liquid from the well as possible. 100µl of the substrate reagent (p-Nitrophenyl phosphate buffer containing 0.05% thimerosal and 0.1% sodium azide) could then be added to each well and incubated for 30 min at room temperature on a horizontal shaker. Subsequent to the completion of this incubation,
100µl of quench reagent (EDTA in buffer containing 0.1% sodium azide) was pipetted into each well. The absorbance of each well could then be read in a microplate plate reader (model 450; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) at a wavelength of 450nm within 1 hour of addition of the quench reagent.

Results were calculated using a commercial curve-fitting program. This was in accordance with the manufacturers instructions.

2.2.9 Immunohistochemical Characterisation of Primary and Co-cultured Epithelial Cells.

Primary and co-cultured epithelial cells were analysed for the expression of high molecular weight cytokeratins (1, 4, 10 &14) by immunohistochemistry. These cytokeratins are only expressed by basal epithelial cells and not luminal cells.

The antibody employed was a mouse anti-human high molecular weight cytokeratin obtained from Dako (Bucks, UK). Staining was carried out as detailed for PSA in section 2.2.8.1. Excluding the primary anti-body, all other procedures and reagents were identical. The primary anti-body was employed at a concentration of 1:50.

2.2.10 Analysis of Effects of Permixon (LSESR).

2.2.10.1 Cytotoxicity of LSESR.

Primary epithelial and fibroblast cells were grown in 96 well plates, supplemented with 100µl of the appropriate medium per well. After an initial incubation of 24 hours, the medium was aspirated from the wells and replaced with media supplemented with LSESR at concentrations of 10, 25, 50 and 100µg/ml. The cells were incubated for a period of up to 5 days at 37°C in 5% CO₂. Subsequent to this incubation, the number of viable cells was determined by the MTT assay method as detailed in section 2.2.1.
2.2.10.2 Effect of LSES R on 5α-reductase activity.

Cells grown in either monolayer or co-culture were harvested by trypsinisation resuspended in 20mls RPMI 1640 supplemented with 10% FCS and pelleted by centrifugation at 2000rpm for 10 min. Subsequent to pelleting, the cells were prepared for analysis of 5α-reductase activity as described in section 2.3.1. Upon addition of the cell lysate to the co-factor solution, LSES R was added to a final concentration of 10μg/ml. Thereafter, the assay was carried out as detailed in section 2.3.4.

2.2.10.3 Effect of LSES R on Cell Membranes.

The media was removed from cells grown in co-culture in 6 well plates for a period of 7 days and the cells were washed with 10ml of Dulbecco ‘A’ PBS. The co-cultured cells were supplemented with media containing 10μg/ml LSES R and incubated at 37°C in 5% CO₂ for a further 2 days. The co-cultured cells were then harvested by trypsinisation, resuspended in RPMI 1640 supplemented with 10% FCS and pelleted by centrifugation at 2000rpm for 10 min. The cell pellet was then fixed for electronmicroscopy as described in section 2.2.7.

2.2.11 Statistical Analysis.

All statistics were carried out using the unpaired Student’s T-Test for analysis of replicates.

Results are expressed as mean ± standard error of mean (SEM) and are the result of at least 3 experiments.
3 RESULTS

3.1 5α-reductase mRNA expression in Cultured Prostate Cancer Cell Lines and Cultured Epithelial and Fibroblast Cells - Analysis by Reverse Transcription-Polymerase Chain reaction.

3.1.1 Analysis of Prostate Cancer Cell lines for 5α-reductase Isozyme RNA

Although the expression of 5α-reductase in the DU145 cell line has been investigated and published, we felt it was necessary for these studies to determine the expression using the protocol and conditions described in section 2.2.5. The expression of 5α-reductase isozymes in the other two cell lines utilised (PC3 and LNCaP) has not been published. It was therefore necessary to determine this for ourselves.

The expression of 5α-reductase isozymes RNA by prostate cancer cell lines was investigated by RT-PCR. The results obtained from total RNA are demonstrated in fig 3.1. These results show a single band of 170bp, indicating the presence of 5α-reductase type I RNA, which agree with results published by Delos et al (1994) [179] for the DU145 cell line. There was no detectable expression of 5α-reductase type II in this cell line. The other two cell lines employed (PC3 and LNCaP) also displayed a similar pattern of expression of 5α-reductase as the DU145 cell line by expressing 5α-reductase type I RNA only (fig 3.1).

3.1.1.1 Elimination of 5α-reductase type I pseudogene contamination

The presence of a non-transcribed pseudogene for 5α-reductase type I was described by Jenkins et al [104]. The published sequence shows little difference with the transcribed, active form of 5α-reductase type I. As there is minimum difference between the two sequences, attempts at development of selective primers for RT-PCR proved to be futile. This posed a problem when utilising RT-PCR in that it could result
Fig 3.1 RT-PCR of DU145, PC3 & LNCaP cell lines for 5α reductase type I & II.

RNA extracted from the above cell lines was analysed by RT-PCR for expression of RNA encoding type I (170bp) & II (350bp) 5α reductase. PCR was performed at 30 cycles for type I and 35 cycles for type II. L- 100bp ladders, lane 1- DU145 type I, lane 2- DU145 type II, lane 3- PC3 type I, lane 4- PC3 type II, lane 5- LNCaP type I, lane 6- LNCaP type II. Negatives are shown in plate (b). L- 100bp ladders, lane 1- DU145 type I RT negative, lane 2- DU145 type I PCR negative, lane 3- DU145 type II RT negative, lane 4- DU145 type II PCR negative, lane 5- PC3 type I RT negative, lane 6- PC3 type I PCR negative, lane 7- PC3 type II RT negative, lane 8- PC3 type II PCR negative, lane 9- LNCaP type I RT negative, lane 10- LNCaP type I PCR negative, lane 11- LNCaP type II RT negative, lane 12- LNCaP type II PCR negative.
170bp-
in false positive results being obtained from total RNA samples. There were two methods of overcoming the problem, which were feasible. The first involved purifying total RNA samples to mRNA utilising a poly A tract mRNA isolation kit available from Promega. The poly A tract isolation kit had the drawback of requiring large numbers of cells to provide adequate total RNA to obtain useable amounts of mRNA. The preferred method was to alter the PCR methodology to only amplify mRNA and not any contaminating DNA. By reducing the number of cycles in the PCR process, it was possible to prevent the amplification of any contaminating DNA. As can be seen from fig 3.2, the optimum number of cycles for amplification of RNA without the concomitant amplification of contaminating DNA was 30. Fewer cycles resulted in no signal being obtained from the RNA and higher numbers of cycles resulted in amplification of contaminating DNA. All further RT-PCR was carried out using total RNA. This proved to be the case for RNA samples from all the cell types utilised.

3.1.2 Analysis of Prostate Tissue for 5α-reductase Isozyme Expression

Due to alterations in the genotype of the cancer cell lines, the cells differ from the originating tissue in terms of the expression of different enzymes and proteins. In order to compare the cell lines and the primary culture models to the original tissue, it was necessary to determine the expression of 5α-reductase isoforms in the prostate. There has been much controversy over the expression of 5α-reductase isoforms in the prostate over the last few years with many researchers maintaining that only 5α-reductase type II RNA was expressed in the prostate. Recent work however, has clearly demonstrated that both isoforms of 5α-reductase RNA are expressed in the prostate [128]. The results demonstrated in fig 3.3 are in agreement with this data in that RNA from both isoforms of 5α-reductase can be easily detected from total RNA samples from prostate tissue obtained from transurethral resection of the prostate (TURP) specimens. The figure shows an easily identifiable band at 170bp (lane-1) which corresponds to 5α-reductase type I and a band running at 350bp (lane-2) which corresponds to 5α-reductase type II. Negative controls in the form of no RT and no PCR for each isozyme of each sample were run and are shown in plate (b). Importantly, the no RT
Fig 3.2 RT-PCR of 5α reductase type I in fibroblast RNA at varying cycles.

In order to optimise PCR conditions for elimination of false positive results for 5α-reductase type I due to contamination of RNA samples with DNA encoding the non-transcribed pseudogene. Samples which had been reverse transcribed were subjected to PCR at 20 (lane 1), 25 (lane 2), 30 (lane 3) & 35 (lane 4) cycles. RNA samples which had not been reverse transcribed were subjected to PCR under the same conditions (b). L- 100bp ladders, lane 1- 20 cycles, lane 2- 25 cycles, lane 3- 30 cycles, lane 4- 35 cycles.
RNA extracted from prostate tissue obtained by TURP was investigated for the expression of 5α-reductase type I & II RNA. PCR for 5α-reductase type I was carried out at 30 cycles, 5α-reductase type II at 35 cycles. L- 100bp ladders, lane 1- type I (170bp), lane 2- type II (350bp). Plate b shows negative controls. L- 100bp ladders, lane 1- type I RT neg., lane 2-type I PCR neg., lane 3- type II RT neg., lane 4- type II PCR neg.
samples for 5α-reductase I were negative, indicating no genomic DNA contamination from the pseudogene.

3.1.2.1 Endonuclease Digestion of RT-PCR Products

The RT-PCR products of 5α-reductase type I and II primers were analysed by endonuclease digestion in order to ascertain the nature of the products and to demonstrate that they were not the result of mis-priming or arose from false positive results. The enzymes employed were Eco RII for 5α-reductase type I product digestion and Nci I for 5α-reductase type II product digestion. Both cleave the respective products only at one site, which are not common restriction sites. The results are shown in fig 3.4. 5α-reductase type I product is cleaved into two fragments of 60bp and 110bp respectively as anticipated. 5α-reductase type II product is cleaved into two products of 137bp and 213bp respectively. This demonstrates that the products are legitimate and not as a result of false priming.

3.1.3 5α-reductase Isozyme RNA Expression in Primary Cultured Prostate Fibroblast and Epithelial Cells

Primary cultured epithelial and fibroblast cells are a better model of BPH than the cell lines in that they are more representative of the tissue of origin. However, following analysis of the expression of 5α-reductase isozymes, it became evident that this was not an ideal model. The results shown in fig 3.5 show that both cell types although expressing both isoenzymes initially, stop expressing 5α-reductase type II very quickly (within 7 days) after they are set up as primary cultures (fig 3.6). Both primary epithelial and fibroblast cells continued to express 5α-reductase type I throughout the culture period.
Fig 3.4 Endonuclease digestion of RT-PCR products of 5α-reductase type I & II.

RT-PCR products of 5α-reductase type I & II were extracted from the gel and subjected to endonuclease digestion with Eco RII (type I) and Nci I (type II) respectively. Eco RII produced two fragments of 110 & 60 bp, Nci I produced two fragments of 137 & 213 bp.
Lane 1- 100bp ladders, lane 2- Eco RII digestion of type I PCR product, lane 3- Type I PCR product (170bp), lane 4- Nci I digestion of type II PCR product, lane 5- Type II PCR product.
Expression of 5α-reductase type I & II RNA in short term cultures of human prostate derived epithelial and fibroblast cells was investigated by RT-PCR. PCR for type I was carried out at 30 cycles, type II at 35 cycles. L- 100bp ladders, lane 1- epithelial cells type I, lane 2- epithelial cells type II, lane 3- fibroblast cells type I, lane 4- fibroblast cells type II.

Plate (b) demonstrates the negative controls for RT-PCR reactions. L- 100bp ladders, lane 1- epithelial type I RT negative, lane 2- epithelial type I PCR negative, lane 3- epithelial type II RT negative, lane 4- epithelial type II PCR negative, lane 5- fibroblast type I RT negative, lane 6- fibroblast type I PCR negative, lane 7- fibroblast type II RT negative, lane 8- fibroblast type II PCR negative.
Fig 3.6 Expression of 5α-reductase type I & II RNA in long term (in excess of 3 weeks in culture) cultures of human prostate epithelial and fibroblast cells.

Expression of 5α-reductase type I & II RNA in long term cultures of human prostate derived epithelial and fibroblast cells was investigated by RT-PCR. PCR for type I was carried out at 30 cycles, type II at 35 cycles. L- 100bp ladders, lane 1- epithelial cells type I, lane 2- epithelial cells type II, lane 3- fibroblast cells type I, lane 4- fibroblast cells type II.

Plate (b) demonstrates the negative controls for RT-PCR reactions. L- 100bp ladders, lane 1- epithelial type I RT negative, lane 2- epithelial PCR type I negative, lane 3- epithelial type II RT negative, lane 4- epithelial type II PCR negative, lane 5- fibroblast type I RT negative, lane 6- fibroblast type I PCR negative, lane 7- fibroblast type II RT negative, lane 8- fibroblast type II PCR negative.

Throughout the course of 50 other experiments the observed pattern of results was repeated in each case.
3.1.4 5α-reductase Isozyme Expression in Co-cultured Human Prostate Fibroblast and Epithelial Cells

5α-reductase RNA expression in co-cultured epithelial and fibroblast cells differed markedly from that in primary cultured cells in that both co-cultured cell types expressed RNA for both 5α-reductase isozymes in detectable quantities. 5α-reductase type II RNA could be induced in epithelial and fibroblast cells which no longer expressed type II RNA following a period in primary culture by introducing the cells into a co-culture system (fig 3.7; lanes 2&4, 350bp; lane 1&3, 170bp). Both co-cultured cell types continued to express both RNA isoforms of 5α-reductase for the duration of the period of culture. Once again, negative controls show no pseudogene contamination (plate b).

3.1.5 5α-reductase Isozyme Expression in Fibroblast Conditioned Medium Treated Human Prostate Epithelial Cells

Following the observation that primary cultured epithelial cells could re-express 5α-reductase type II following co-culturing with prostatic fibroblast cells, the effect of fibroblast conditioned medium on primary cultured epithelial cells needed to be investigated to determine whether there was a factor being secreted by prostatic fibroblast cells which could influence the expression of 5α-reductase type II RNA. It was postulated that it was a soluble growth factor that was responsible as a fluid filled space and a physical barrier separated the cells. This prevented any cell to cell contact, which could also theoretically have been responsible for these demonstrated changes in phenotype.

To determine the effect of fibroblast CM on primary cultured epithelial cells which had stopped expressing 5α-reductase type II RNA, cells were grown in fibroblast conditioned medium for a period of 6 days and the expression of 5α-reductase type II RNA was analysed by RT-PCR (fig 3.8; lane 2, 350bp). These results demonstrated that fibroblast CM was capable of inducing the re-expression of 5α-reductase type II RNA in primary cultured epithelial cells which had stopped expressing 5α-reductase type II RNA in primary culture. These cells failed to re-express 5α-reductase type II
Fig 3.7 Expression of 5α-reductase type I & II RNA in co-cultured human prostate derived epithelial and fibroblast cells.

5α-reductase type I & II RNA expression in co-cultured prostate derived epithelial and fibroblast cells was investigated by RT-PCR analysis. PCR for 5α-reductase type I was carried out at 30 cycles, type II at 35 cycles. L-100bp ladders, lane 1- epithelial type I (170bp), lane 2- epithelial type II (350bp), lane 3- fibroblast type I (170bp), lane 4- fibroblast type II (350bp). Negative controls are shown in plate (b). L-100bp ladders, lane 1- epithelial type I RT neg., lane 2 epithelial type I PCR neg., lane 3- epithelial type II RT neg., lane 4- epithelial type II PCR neg., lane 5- fibroblast type I RT neg., lane 6- fibroblast type I PCR neg., lane 7- fibroblast type II RT neg., lane 8- fibroblast type II PCR neg.
Type II 5α-reductase RNA expression in primary cultured prostate derived epithelial cells cultured in fibroblast conditioned medium was investigated by RT-PCR. PCR was carried out at 35 cycles.

Negative controls are shown in plate (b). L- 100bp ladders, lane 1- epithelial cells type I in fibroblast CM, lane 2- epithelial cells type II in CM, lane 3- epithelial cells type I in non-CM, lane 4- epithelial cells type II in non-CM medium.

Fig 3.8 Expression of 5α-reductase type II in primary cultured epithelial cells following culturing with fibroblast CM.
RNA when grown in non-conditioned fibroblast growth medium (fig 3.8). All cells investigated, continued to express 5α-reductase type I throughout the culture period.

3.1.6 Effect of Breast Fibroblast Cells on 5α-reductase Expression in Prostatic Epithelial Cells when Grown in Co-culture

Considering the results obtained by growing prostate fibroblast and epithelial cells in co-culture, it was decided to determine whether this effect was limited to prostate fibroblasts or whether it could be induced by fibroblast cells from other organs.

Primary cultured breast fibroblasts were provided by Professor Miller and grown in co-culture with prostatic epithelial cells using the method described in section 2.2.1.4. The epithelial cells were analysed for 5α-reductase type II RNA expression by RT-PCR as described in section 2.2.4. The results are shown in fig 3.9. The RT-PCR shows a band at 170bp indicating the presence of 5α-reductase type I RNA. There is no detectable expression of 5α-reductase type II RNA following co-culturing of prostate epithelial cells with breast fibroblast cells. Breast fibroblasts from 3 patients were used and each sample was used twice.

3.1.7 Effect of Skin Fibroblast Cells on 5α-reductase Expression in Prostatic Epithelial Cells when Grown in Co-culture

Following the results obtained from the use of breast fibroblasts in co-culture with prostatic epithelial cells, it was decided to repeat the experiment utilising primary cultured fibroblasts obtained from skin. Skin fibroblasts were provided by the MRC at the Western General Hospital. The effect on 5α-reductase type II RNA expression in prostatic epithelial cells was analysed using the same procedure as previously described. The gel displayed in fig 3.9 shows clearly the presence of a band at 170bp indicating the presence of 5α-reductase type I in the co-cultured epithelial cells but there is no detectable 5α-reductase type II RNA.

These results suggest that the observed effect on 5α-reductase type II RNA expression in co-cultured epithelia cells is confined to prostate derived fibroblasts.
Fig 3.9 RT-PCR of 5α-reductase type I and II RNA from prostate epithelial cells co-cultured with human breast and skin fibroblast cells.

Plate (a) L -100bp ladders; lane 1- epithelial cells co-cultured with breast fibroblasts type I (170bp); lane 2- epithelial cells co-cultured with breast fibroblasts type II; lane 3- epithelial cells co-cultured with skin fibroblasts type I (170bp); lane 4- epithelial cells co-cultured with skin fibroblasts type II.

Plate (b) L -100bp ladders; lane 1- epithelial/breast fibroblast co-culture type I RT neg; lane 2- epithelial/breast fibroblast co-culture type I PCR neg; lane 3- epithelial/breast fibroblast co-culture type II RT neg; lane 4- epithelial/breast fibroblast co-culture type II PCR neg; lane 5- epithelial/skin fibroblast co-culture type I RT neg; lane 6- epithelial/skin fibroblast co-culture type I PCR neg; lane 7- epithelial/skin fibroblast co-culture type II RT neg; lane 8- epithelial/skin fibroblast co-culture type II PCR neg.
3.1.8 Further Characterisation of Fibroblast Secreted Factor Responsible for Effect on 5α-reductase Expression in Prostatic Epithelial Cells

As the observed results from the experiments involving fibroblast conditioned medium indicated a secreted factor from prostatic fibroblast cells having an effect on the expression of 5α-reductase type II RNA in prostatic epithelial cells it was necessary to further characterise this factor.

Fibroblast conditioned medium was fractionated through Centricon concentrator spin columns and the fractions were tested by RT-PCR to determine the effect of the fractions on 5α-reductase type II RNA expression in primary cultured epithelial cells. The results are shown in fig 3.9. These results show that although stimulation of 5α-reductase type II RNA (350bp band) is achieved by fractions from >10kD, <10kD and >3kD samples, the best stimulation is achieved by using the >10kD fraction. The 'cross over' of activity is due to the imperfect nature of the spin columns, which do not have exact cut-off weights or possibly to dimerisation of the protein. All further studies were performed using the >10kD fraction.

The >10kD fraction of fibroblast conditioned medium was further characterised by treatment with heat or trypsin digestion and analysed for it’s ability to induce 5α-reductase type II RNA expression in cultured epithelial cells which had stopped expressing this isoform. Following heat treatment, the fractionated fibroblast CM failed to induce any response in the form of 5α-reductase type II RNA expression in the epithelial cells (fig 3.10; lane 4, 350bp). Subsequent to trypsin digestion, the fractionated fibroblast CM again failed to induce any 5α-reductase type II RNA expression in epithelial cells which had ceased to express 5α-reductase type II RNA (fig 3.10; lane 5, 350bp). This was in contrast to untreated fractionated fibroblast CM, which induced 5α-reductase type II expression in prostatic epithelial cells (3.1.5).

Fractionated conditioned medium was also characterised by HPLC. Passing through a cation exchange column further fractionated the >10kD sample. The resulting follow-through from the column proved to contain the active fraction, as indicated by RT-PCR analysis of RNA extracted from epithelial cells which had been grown in the conditioned medium, indicating that the protein in question was anionic. The follow-through from the cation exchange column was analysed utilising an anion exchange
Fig 3.10 Expression of 5α-reductase type II in primary cultured epithelial cells following culturing in fractionated and heat and trypsin treated fractions of fibroblast conditioned medium.

Fibroblast CM was fractionated through spin columns and the fractions tested for their ability to induce 5α-reductase type II (350bp) RNA expression in primary cultured epithelial cells. Plate (a.) L-100bp ladders, lane 1->10kD fraction, lane 2-<10kD fraction, lane 3->3kD fraction.

Negative controls, plate (b.) Lane 1- >10kD RT negative, lane 2- >10kD PCR negative, lane 3-<10kD RT negative, lane 4-<10kD PCR negative, lane 5->3 kD RT negative, lane 6->3kD PCR negative.

Heat and trypsin treated samples of the >10kD fraction were added to primary cultured epithelial cells and the expression of 5α-reductase type II RNA investigated by RT-PCR. PCR was carried out at 35 cycles.

Plate (c.) L-100bp ladders, lane 1-heat treated fraction type I, lane 2- trypsin treated fraction type I, lane 3- untreated control type I, lane 4- heat treated fraction type II, lane 5- trypsin treated fraction type II, lane 6- untreated control type II.

Plate (d.) Negative controls, lane 1- heat treated type I RT neg., lane 2- heat treated type I PCR neg., lane 3- heat treated type II RT neg., lane 4-heat treated type II PCR neg., lane 5- trypsin treated type I RT neg., lane 6- trypsin treated type I PCR neg., lane 7- trypsin treated type II RT neg., lane 8- trypsin treated type II PCR neg.,

Plate (e.) Flow through from cation exchange HPLC L-100bp ladders, lane 1- type I, lane 2- type II.

Plate (f.) Negative control of cation exchange fraction treated samples. L-100bp ladders, lane 2- type I RT neg., lane 2- type I PCR neg., lane 3- type II RT neg., lane 4- type II PCR neg.
column and the chromatogram shown in fig 3.11 obtained. The flow-through from the anion exchange column contained no detectable protein in the sample.

3.2 Effect of Permixon® on cell growth

3.2.1 Growth of Prostate Cancer Cell Lines Treated with Different Concentrations of Permixon®

As the cytotoxicity of Permixon® was unknown, it was decided to investigate this first on prostate cancer cell lines. The growth of the cells was determined using the MTT assay method.

DU145 and PC3 cells both showed similar responses to Permixon®. At concentrations below 50µg/ml there was little difference in the growth of the cells compared to controls. At concentrations of 100µg/ml Permixon® proved to be cytotoxic to the cells resulting in a statistically significant decrease in growth compared to controls (fig 3.12a & 3.12b). The cytotoxic effect was observable within 24 hours of administration of the drug. A similar pattern was observed for concentrations of 50 µg/ml with 25 µg/ml showing a smaller degree of cytotoxicity.

The LNCaP cell line displayed a different pattern of response to Permixon® with concentrations above 10µg/ml (100, 50 & 25 µg/ml) having a cytotoxic effect on the cells (fig 3.12a). Concentrations above 10µg/ml caused a statistically significant decrease in the growth of the LNCaP cells within 24 hours of administration of the drug in comparison to controls (fig 3.12b).

The controls for all concentrations of the drug were prepared using a similar concentration of alcohol as in the drug preparations. This was to demonstrate that the effect was drug induced and not as a result of the solubilising agent. The controls show no statistical effect of the alcohol on the cells utilised (figs 3.11b).
Fig 3.11 HPLC chromatogram of anion exchange column.

Fibroblast conditioned medium which had previously been fractionated by cation exchange chromatography was passed through an anion exchange column and eluted using a salt gradient 0-1M over a period of 40 minutes.
Fig 3.12(A) Growth response of DU145 (a), PC3 (b) and LNCaP (c) cells to 10, 25, 50 & 100µg/ml Permixon®.

Cells were plated at a density of 1000 cells per well in 96 well plates. After a 24 hour incubation period, the medium was replaced and Permixon added to a final concentration of 10, 25, 50, 100µg/ml. 4 wells were used with each concentration of Permixon®. The growth of the cells was assessed spectrophotometrically (540nm) by MTT assay. Each data point represents mean ± S.E.M. Controls supplemented with ethanol are shown in 3.12(B).

In the DU145 cells, Permixon® at concentrations of 100 and 50µg/ml showed a statistically significant decrease in the growth rate of the cells (p<0.05). Concentrations of 10 and 25µg/ml had no statistically significant effect on cell growth rate compared to controls (p>0.05).

Concentrations of Permixon® of 100 and 50µg/ml in PC3 cells showed a statistically significant decrease in the growth of the cells over the 5 day period (p<0.05). Concentrations of 25 and 10µg/ml showed a statistically significant increase in growth of the cells up to day 3 (p<0.05). From day 3 onward, there was a statistically significant decrease in the growth of the PC3 cells in response to Permixon® at concentrations of 25 and 10µg/ml. This is probably due to the cells becoming confluent.

LNCaP cells showed a statistically significant decrease in growth in response to all concentrations of Permixon® over the 5 day period (p<0.05) in relation to controls. Treatment with Permixon® at a concentration of 10µg/ml resulted in a statistically significant higher growth rate than Permixon® at concentrations of 100, 50 or 25µg/ml (p<0.05).
Fig 3.12(B) Negative controls of growth response of DU145 (a), PC3 (b) and LNCaP (c) cells to Permixon®

Cells were plated at a density of 1000 cells per well in 96 well plates. After a 24 hour incubation period, the medium was replaced and ethanol added to a final concentration of 0.1%, 0.25%, 0.5%, 1%. 4 wells were used with each concentration of ethanol. The growth of the cells was assessed spectrophotometrically (540nm) by MTT assay. Each data point represents mean ± S.E.M. The controls show no statistical difference in response to the different concentrations of alcohol (p>0.05).
3.2.2 Growth of Primary Cultured Prostate Epithelial and Fibroblast Cells Treated with Different Concentrations of Permixon®

Having determined the cytotoxicity of Permixon® in prostatic cell lines, the cytotoxic effects at different concentrations were investigated in primary cultures of epithelial and fibroblast cells. The results obtained were similar to the results obtained from the effect of Permixon® on the LNCaP cell line with concentrations in excess of 10μg/ml proving to be cytotoxic to both epithelial and fibroblast cells within 24 hours (fig 3.13 & 3.14). This effect was however noted to be dose dependent with the higher concentrations causing a much larger decrease in the growth of the cells. These results are in comparison to controls, which received a medium with the same concentration of ethanol as in the test samples (fig 3.13 & 3.14).

3.2.3 Growth of Co-cultured Prostate Epithelial and Fibroblast Cells Treated with Different Concentrations of Permixon®

As co-cultured epithelial and fibroblast cells had already displayed different characteristics compared to the primary cultured cells, it was necessary to determine the cytotoxic effect of Permixon® on the co-cultured cells. The results obtained from these experiments are displayed in fig 3.15 & 3.16. The co-cultured epithelial and fibroblast cells displayed a similar response to the DU145 cell line with only concentrations in excess of 50μg/ml proving to be cytotoxic to a significant degree when compared to controls (fig 3.15 & 3.16). This would seem to indicate some cell interaction in maintaining cell integrity and reducing the cytotoxic effects of Permixon®.

3.3 Measurement of Functional 5α-reductase Activity - Effect of Permixon®

3.3.1 5α-reductase Isozyme Activity in Human Prostate Tissue

The functional activity of both 5α-reductase isoenzymes in the prostate has been published in several papers with some degree of controversy. There is disagreement over which enzymes are expressed and active in the prostate. Due to this controversy
Fig 3.13 Growth response of primary cultured epithelial cells to varying concentrations of Permixon®.

Cells were plated at a density of 500 cells per well in 96 well plates. After a 24 hour incubation period, the medium was replaced and ethanol added to a final concentration of 10, 25, 50, 100μg/ml. 4 wells were used with each concentration of Permixon®. The growth of the cells was assessed spectrophotometrically (540nm) by MTT assay. Each data point represents mean ± S.E.M. Controls supplemented with ethanol are shown in (b). At concentrations of 25, 50 and 100μg/ml Permixon® caused a statistically significant decrease in growth rates of the cells (p<0.05) but at a concentration of 10μg/ml, Permixon® had no statistically significant effect on growth of the cells compared to controls (p>0.05). Controls showed no statistical difference (p>0.05).
Fig 3.14 Growth response of primary cultured fibroblast cells to varying concentrations of Permixon®.

Cells were plated at a density of 500 cells per well in 96 well plates. After a 24 hour incubation period, the medium was replaced and Permixon added to a final concentration of 10, 25, 50, 100μg/ml. 4 wells were used with each concentration of Permixon®. The growth of the cells was assessed spectrophotometrically (540nm) by MTT assay. Each data point represents mean ± S.E.M. Controls supplemented with ethanol are shown in (b). Permixon® at concentrations of 25, 50 and 100μg/ml caused a statistically significant decrease in growth of the cells (p<0.05) as compared with controls. At a concentration of 10μg/ml, Permixon® had no statistically significant effect on growth (p>0.05). Controls showed no statistical difference (p>0.05).
Fig 3.15 Growth response of co-cultured epithelial cells to varying concentrations of Permixon®.

Cells were plated at a density of 500 cells per well in 96 well plates. After a 24 hour incubation period, the medium was replaced and Permixon added to a final concentration of 10, 25, 50, 100μg/ml. 4 wells were used with each concentration of Permixon®. The growth of the cells was assessed spectrophotometrically (540nm) by MTT assay. Each data point represents mean ± S.E.M. Controls supplemented with ethanol are shown in (b). There was no statistically significant difference in response to different concentrations of Permixon® when compared to controls (p>0.05).
Fig 3.16 Growth response of co-cultured fibroblast cells to varying concentrations of Permixon®.

Cells were plated at a density of 500 cells per well in 96 well plates. After a 24 hour incubation period, the medium was replaced and Permixon added to a final concentration of 10, 25, 50, 100μg/ml. 4 wells were used with each concentration of Permixon®. The growth of the cells was assessed spectrophotometrically (540nm) by MTT assay. Each data point represents mean ± S.E.M. Controls supplemented with ethanol are shown in (b). There was no statistically significant difference with Permixon® concentration (p>0.05) on growth compared to controls.
and because the protocol used was new, it was decided to determine the isozyme activity in prostatic tissue.

The results are displayed in fig 3.17. From this it can be seen that both isoforms of 5α-reductase are active in prostatic tissue as determined using the protocol outlined in section 2.3. The results show that the most active isoform in the prostate is 5α-reductase type II (0.426 pmoles DHT/mg protein/hour) but under these assay conditions, 5α-reductase type I (0.127 pmoles DHT/mg protein/hour) is also still quite active indicating that both enzymes play an active role in androgen metabolism in prostatic tissue.

3.3.2 Effect of Permixon® on 5α-reductase Activity in Human Prostate Tissue

Permixon® is marketed across Europe as a treatment for BPH, several papers have been published on its effects but as with all plant extracts, these are wide and varied. It was therefore necessary to determine its effect on 5α-reductase isozymes in human prostatic tissue, which expresses both isoforms of 5α-reductase, before analysing its effects on cultured cells, which differ from the tissue. A similar response from the cultured cells to the drug as that obtained from the tissue would allow the use of the cultured cells as a comparable model for investigation of the actions of the drug.

The effect of Permixon® at 10 μg/ml on both isoforms of 5α-reductase in human prostate tissue was analysed. The results are shown in 3.17. From these results it can be seen that Permixon® is an effective inhibitor of both forms of 5α-reductase in the human prostate. The results also show that Permixon® reduces the activities of both isoenzymes to a similar level (RI 0.062 pmoles DHT/mg protein/hour; RII, 0.112 pmoles DHT/mg protein/hour) although the activity of type II 5α-reductase is initially much higher than that of type I. Results were compared to control samples, which contained no protein. These control samples showed no conversion of T to DHT.
Fig 3.17 5\(\alpha\)-reductase type I & II activity in prostate tissue. Prostate tissue was analysed for its ability to convert testosterone to DHT utilising pH specific assays (pH7.5, 5\(\alpha\)-reductase type I; pH5.5 5\(\alpha\)-reductase type II). Steroids were extracted and separate using TLC. The effect of Permixon® was also investigated. Both isozymes are detectable in the tissue and both isozymes are inhibited by Permixon®. The inhibition of the 5\(\alpha\)-reductase type II isozyme is statistically significant (p<0.05), but the inhibition of 5\(\alpha\)-reductase type I isozyme is not statistically significant (p>0.05). Each data set is the mean of 3 separate experiments. Results are expressed as mean ±S.E.M.
3.3.3 5α-reductase Isozyme Activity in Prostate Carcinoma Cell Lines

As the DU145 cell line only demonstrated 5α-reductase type I RNA following RT-PCR analysis, it was expected only to find type I activity in the cell line, as can be seen from fig 3.18 this was the case. DU145 showed a low level of 5α-reductase type I (0.037 pmoles DHT/10⁶ cells/30 min) activity and no 5α-reductase type II activity.

As with the DU145 cell line, the PC3 cell only expressed 5α-reductase type I RNA (fig 3.1). Consequently, the only activity detected was that of the type I enzyme (fig 3.18). In keeping with the DU145 cell line, the PC3 cell line showed a similar level of activity (fig 3.18) (0.029 pmoles DHT/10⁶ cells/30 min).

The LNCaP cell line, in keeping with the other two cell lines utilised, showed only 5α-reductase type I activity (fig 3.18). Once again the level of activity of the 5α-reductase type I isozyme detected was of the same order of magnitude as that found in the other two cells lines with low levels of 5α-reductase type I detected (0.03 pmoles DHT/10⁶ cells/30 min).

All results were calculated according to cell numbers and standardised to activity per 1x10⁶ cells.

3.3.4 Effect of Permixon® on 5α-reductase Activity in Prostate Carcinoma Cell Lines

The effect of Permixon® (10μg/ml) on the activity of 5α-reductase type I in DU145 cells resulted in a decrease in the activity of the enzyme (fig 3.18) (0.021 pmoles DHT/10⁶ cells/30min).

The effect of Permixon® (10μg/ml) on the activity of 5α-reductase type I isozyme expressed in PC3 cells was similar to that observed in DU145 cells. The activity of the enzyme was decreased (fig 3.17) to relatively the same level as in the DU145 and the PC3 cell lines (0.023 pmoles DHT/106 cells/30min).

The results obtained from the effect of Permixon® (10μg/ml) on the activity of 5α-reductase type I as expressed in the LNCaP cell line (0.019 pmoles DHT/10⁶ cells/30min) was similar to that observed for the other cell lines. The results detailed in fig 3.18 demonstrate that Permixon® inhibits the activity of 5α-reductase type I isozyme when compared to controls.
Fig 3.18 5α-reductase type I & II activity in cell lines (DU145, PC3, LNCaP) is shown in (a). Effect of Permixon® on 5α-reductase type I & II activity is demonstrated in (b). Results show that only 5α-reductase type I is active in these cell lines (a) and that Permixon® causes an inhibition of 5α-reductase type I activity at a concentration of 10μg/ml which is statistically significant (p<0.05). Each data set is the result of 3 experiments and results are expressed as mean ± S.E.M..
3.3.5 5α-reductase Isozyme Activity in Primary Cultured Epithelial and Fibroblast Cells.

3.3.5.1 5α-reductase isozyme activity in primary cultured epithelial cells

Primary cultured epithelial cells were analysed for the activity of 5α-reductase types I and II. After being grown in culture for a period of 2 weeks, the cells displayed only 5α-reductase type I activity (fig 3.19). This is in contrast to cells freshly prepared from prostatic tissue, which displayed activity by both isoforms of 5α-reductase (fig 3.17). The level of activity of the 5α-reductase isoenzyme type I (0.045 pmoles DHT/10^6 cells/30 min.) was of a similar magnitude to the levels of activity displayed by 5α-reductase type I in the cell lines DU145, PC3 and LNCaP (fig 3.18) when related to cell number. There was no detectable activity of 5α-reductase type II isoenzyme in the samples. Negative controls containing no cells demonstrated no conversion of T to DHT. These results indicate that growth of epithelial cells under primary culture conditions results in the loss of function of 5α-reductase type II as would be expected from the loss of 5α-reductase type II RNA expression (fig 3.5).

3.3.5.2 5α-reductase isozyme activity in primary cultured fibroblast cells

In the same manner as with the epithelial cells, primary cultured prostatic fibroblast cells were analysed for 5α-reductase isoenzyme activity. As the results in fig 3.19 demonstrate, primary cultured fibroblast cells display only 5α-reductase type I activity (0.031 pmoles DHT/10^6 cells/30 min.) following two weeks in culture. Once again this is contrary to the results obtained from freshly separated fibroblast cells from prostate tissue which show activity from both isoenzymes of 5α-reductase (fig 3.19). The level of 5α-reductase type I activity is comparable to levels displayed by primary cultured epithelial cells and the cell lines when corrected for cell numbers. Negative controls showed no metabolism of T to DHT.
Fig 3.19 5α-reductase type I & II activity in primary cultured epithelial (a) and fibroblast (b) cells.

Primary cultured cells were analysed for their ability to convert testosterone to DHT. The assays were carried out at pH7.5 (type I) and pH5.5 (type II). Steroid metabolites were extracted and separated using TLC. The effect of Permixon® at 10µg/ml was also investigated. Each data set is the mean of 3 separate experiments. Results are expressed as mean ± S.E.M.. The inhibition of 5α-reductase type I by Permixon® was shown to be statistically significant (P<0.05)
3.3.6 Effect of Permixon® on 5α-reductase in Primary Cultured Prostate Epithelial and Fibroblast Cells.

3.3.6.1 Effect of Permixon® on 5α-reductase isozyme activity in primary cultured prostate epithelial cells

As the primary cultured epithelial cells only expressed type I 5α-reductase, the effect of Permixon® on the activity of the isoenzyme was expected to be similar to that on the isoenzyme expressed in the cell lines. As can be seen from fig 3.19, this was indeed the case with Permixon® inhibiting the 5α-reductase type I to a level comparable to that demonstrated in the other cells analysed (0.03 pmoles DHT/10^6 cells/30 min.) when standardised for cell numbers. The level of inhibition was statistically significant (P<0.05).

3.3.6.2 Effect of Permixon® on 5α-reductase isozyme activity in primary cultured prostate fibroblast cells

Primary cultured fibroblasts like primary cultured epithelial cells showed an inhibition of 5α-reductase type I (0.02 pmoles DHT/10^6 cells/30 min.) to the same level as described for the cell lines (fig 3.19). The results are summarised in fig 3.19. Negative controls showed no metabolism of T to DHT. The level of inhibition demonstrated was statistically significant (P<0.05).

3.3.7 5α-reductase Isozyme Activity in Co-cultured Prostate Epithelial and Fibroblast Cells

3.3.7.1 5α-reductase isozyme activity in co-cultured prostate epithelial cells

The results from the RT-PCR analysis of 5α-reductase isozyme RNA expression indicated that RNA for both isoenzymes was expressed in these co-cultured cells. This however did not determine whether both isoforms were expressed as active enzymes. By analysing the conversion of testosterone to DHT at different pH conditions, it was
determined that both isoenzymes were active in the co-cultured cells. The results are summarised in fig 3.20. The activity of the 5α-reductase type I (32.9 pmoles DHT/10^6 cells/30 min) isozyme was similar to the levels demonstrated in the cell lines and primary cultured cells when standardised for cell numbers. 5α-reductase type II activity was 40.5 pmoles DHT/10^6 cells/30 min.

3.3.7.2 5α-reductase isozyme activity in co-cultured prostate fibroblast cells

The co-cultured fibroblast cells had also demonstrated RNA for both isoenzymes of 5α-reductase by RT-PCR analysis (fig 3.6). As with the co-cultured epithelial cells it was not known whether this would result in both enzymes being functionally active in the cells. As the data shown in fig 3.20 demonstrates, both isoenzymes are active in the co-cultured fibroblast cells. The levels of activity are however considerably lower than those detected in co-cultured epithelial cells. This indicates that the majority of the activity of 5α-reductase can be found in the epithelial cells. In keeping with the data obtained from co-cultured epithelial cells, the levels of 5α-reductase type II activity (40.5 pmoles DHT/10^6 cells/30min) detected in co-cultured fibroblast cells is much higher than detected activity levels of 5α-reductase type I.

All results were calculated according to cell numbers and time.

3.3.8 Effect of Permixon® on 5α-reductase Isozyme Activity in Co-cultured Epithelial and Fibroblast Cells

3.3.8.1 Effect of Permixon® on 5α-reductase isozyme activity in co-cultured epithelial cells

When treated with Permixon® at 10μg/ml, co-cultured epithelial cells demonstrated an inhibition of both isoenzymes of 5α-reductase (fig 3.20). 5α-reductase type I was inhibited by Permixon® to a statistically significant level (14.5 pmoles DHT/10^6 cells/30 min) (P<0.05). The degree of inhibition was comparable with the inhibition demonstrated in other cell types utilised. Permixon® inhibited 5α-reductase type II to a much greater degree than 5α-reductase type I. 5α-reductase type II activity
Fig 3.20 5α-reductase type I & II activity in co-cultured epithelial (a) and fibroblast (b) cells. Co-cultured cells were analysed for their ability to convert testosterone to DHT. The assays were carried out at pH7.5 (type I) and pH5.5 (type II). Steroid metabolites were extracted and separated using TLC. The effect of Permixon® at 10μg/ml was also investigated. Each data set is the mean of 3 separate experiments. Results are expressed as mean ± S.E.M.. Inhibition of both isoenzymes in both cell types by Permixon® was shown to be statistically significant (P<0.05).
was inhibited to 15.9 pmoles DHT/10^6 cells/30 min. which was statistically significant (P<0.05).

3.3.8.2 Effect of Permixon® on 5α-reductase isozyme activity in co-cultured fibroblast cells

Co-cultured fibroblast cells showed a similar response to Permixon® at 10μg/ml as the co-cultured epithelial cells, with both isoenzymes being inhibited by the drug (fig 3.20). In common with the co-cultured epithelial cells, 5α-reductase type II was inhibited to the greatest degree by Permixon® (1.22 pmoles DHT/10^6 cells/30 min.; statistically significant) but 5α-reductase type I also showed a degree of inhibition (0.68 pmoles DHT/10^6 cells/30 min.). This was expected as 5α-reductase type II is more active than type I in these cells as demonstrated in fig 3.20. Both results were statistically significant when compared to untreated controls (P<0.05).

3.4 Pattern of Testosterone Metabolism in Prostate Cancer Cell Lines and Cultured Prostatic Cells.

Testosterone is metabolised in living cells by one of two pathways. It can either be metabolised by 17β-hydroxysteroid dehydrogenase to androstenedione. Androstenedione can then be metabolised by 5α-reductase to androstanedione, which in turn can be converted to DHT by 17β-hydroxysteroid dehydrogenase, which is a reversible enzyme. The alternative pathway is via 5α-reductase directly to DHT. Whether both isoenzymes are equally active in both pathways is an area for future research and discussion.

3.4.1 Testosterone metabolism in Prostate cancer cell lines

The metabolism of testosterone by DU145 cells grown in culture shows a distinct pattern. Initially there are large amounts of androstenedione formed by the action of 17β-hydroxysteroid dehydrogenase. Following the metabolism of testosterone to androstenedione via 17β hydroxysteroid dehydrogenase, the androstenedione is
metabolised via androstenedione to DHT by 17β hydroxysteroid dehydrogenase and 5α-reductase type I respectively. PC3 and LNCaP cells show a similar pattern of testosterone metabolism as DU145 cells (fig 3.21). This is as expected since all three of these cell lines express functional 5α-reductase type I only. These results are in accordance with the data published by Levy et al (1995) [111] and Smith et al (1996) [174], which suggests that 5α-reductase type I utilises androstenedione, as it’s preferred substrate. Control samples, which contained no cells, showed no metabolism of testosterone. All results were expressed as percentage of testosterone metabolised in the prescribed time period.

3.4.2 Testosterone metabolism in primary cultured prostate epithelial and fibroblast cells

3.4.2.1 Epithelial cells

The metabolism of testosterone in intact primary cultured epithelial cells (fig 3.21) was identical to the pattern observed in the cell lines (fig 3.21) with testosterone being converted initially to androstenedione prior to DHT formation. This was an expected result as primary cultured epithelial cells express 5α-reductase type I only, after a period in culture. Negative controls demonstrated no metabolism of testosterone.

3.4.2.2 Fibroblast cells

As expected, the primary cultured fibroblast cells showed a similar pattern of testosterone metabolism to the cell lines and primary cultured epithelial cells, with androstenedione as the initial metabolite and DHT being detected after a period of several hours (fig 3.22). The fibroblasts show a lower production of DHT due to the lower activity of 5α-reductase type I in these cells compared to primary cultured epithelial cells. Negative controls showed no testosterone metabolism.
Fig 3.21 Testosterone metabolites in cell lines expressing 5α- reductase type I, after 2 and 4 hours incubation. Results are expressed as percentage of metabolites detected. Lane 1- DU145 cells 2 hours incubation; lane 2- Du145 cells 4 hours incubation, lane 3- PC3 cells 2 hours incubation, lane 4- PC3 cells 4 hours incubation, lane 5- LNCaP cells 2 hours incubation, lane 6- LNCaP cells 4 hours incubation. All 3 cell lines demonstrated an increase in formation of DHT with time which was statistically significant (p<0.05). The formation of DHT was dependent upon high levels of androstene- and androstane-dione.
Fig 3.22 Testosterone metabolites in primary cultured prostate epithelial and fibroblast cells expressing \(5\alpha\)-reductase type I, after 2 and 4 hours incubation. Results are expressed as percentage of metabolites detected. Lane 1- primary cultured epithelial cells after 2 hours incubation; lane 2- Primary cultured epithelial cells after 4 hours incubation; lane 3- Primary cultured fibroblast cells after 2 hours incubation; lane 4- Primary cultured fibroblast cells after 4 hours incubation. Both cell types demonstrated a statistically significant increase in DHT formation over time (\(p<0.05\)) which was dependent upon the presence of high levels of androstenedione and androstanedione.
3.4.3 Testosterone metabolism in co-cultured prostate epithelial and fibroblast cells

3.4.3.1 Co-cultured epithelial cells and fibroblast cells

The pattern of metabolism in co-cultured epithelial and fibroblast cells when investigated proved to be different in several respects from the pattern seen in primary cultured cells. The results shown in fig 3.23 show that although there is a low level of androstenedione production, the majority of metabolite produced is DHT. This can be explained by the presence of functional 5α-reductase type II in the co-cultured cells. 5α-reductase type II has a higher affinity for testosterone [111] than androstenedione and has a higher rate of activity than 5α-reductase type I. This results in more testosterone being converted to DHT directly without initially being converted to androstenedione by 17β-hydroxysteroid dehydrogenase. This represents a possible explanation for the presence of two isoforms of the enzyme 5α-reductase.

3.5 Growth response of Primary and Co-cultured Fibroblast and Epithelial Cells to Androgens

3.5.1 Response of Primary cultured cells to androgens

3.5.1.1 Response of primary cultured epithelial and fibroblast cells to androgens

In order to assess the presence of a functional androgen receptor in primary cultured epithelial and fibroblast cells, the cells were pulsed with either testosterone or DHT. The treated cells showed no proliferative response to either of these androgens (fig 3.24A&B), indicating a lack of any functional androgen receptor. This data is in agreement with work undertaken by Grant et al (1996)[158], which also demonstrated a negative response of primary cultured prostatic epithelial and fibroblast cells to androgen stimulation. On further investigation, it was also noted that following analysis by Northern blotting, there was no detectable androgen receptor RNA expressed in these cells [158].
Fig 3.23 Testosterone metabolites in co-cultured prostate epithelial and fibroblast cells expressing 5α-reductase type I & type II, after 2 and 4 hours incubation. Results are expressed as percentage of metabolites detected. Lane 1- Co-cultured cells after 2 hours incubation; lane 2- Co-cultured cells after 4 hours incubation. Co-cultured fibroblast cells demonstrated increased androstenedione formation as compared to co-cultured epithelial cells. The increased formation of androstenedione was statistically significant (p<0.05).
Fig 3.24a Growth response of primary epithelial cells to Testosterone (Test) and DHT (DHT) at concentrations of 10 & 100nM. Cell numbers were counted manually. All results are compared to controls, which received no androgens. There was no statistically significant difference between treatments and controls (p>0.05).
Fig 3.24b Growth response of primary cultured prostate fibroblast cells to Testosterone (Test) and DHT (DHT) at concentrations of 10 & 100nM. Cell numbers were counted manually. There was no statistically significant difference between treatments and controls (p>0.05).
3.5.2 Response of Co-cultured Cells to Androgens

3.5.2.1 Response of co-cultured epithelial and fibroblast cells to androgens

Co-cultured epithelial cells were assessed for response to androgens by investigating any difference in growth rate of the cells in relation to controls when pulsed with either testosterone or DHT. Co-cultured epithelial cells showed an increase in growth in response to testosterone and DHT (fig 3.25A&B) by 138% (10nM T), 169% (100nM T), 169% (10nM DHT) and 215% (100nM DHT) respectively. The increase in growth became statistically significant (P<0.05) when compared to controls after 4 days, with DHT producing a larger increase in cell number than testosterone.

Co-cultured fibroblast cells responded to stimulation by androgens in a similar manner as co-cultured epithelial cells. The co-cultured fibroblast cells showed a statistically significant increase in growth when pulsed with either testosterone or DHT compared to controls (fig 3.25A&B) 134% (10nM T), 157% (100nM T), 139% (10nM DHT) and 157% (100nM DHT). The increase in growth became significant after 4 days in culture (P<0.05). The co-cultured fibroblast cells showed a greater response to DHT than to testosterone.

The increased response to DHT by both cell types can be explained by a difference in affinity of the androgen receptor for the two ligands and the difference in mitogenicity of the two androgens used. The possibility also exists that the androgens were exerting their effect through an alteration in the rate of cell death of the cultured cells and not merely by inducing an increase in the rate of proliferation.

Although these results suggest the presence of a functional androgen receptor, whether it is expressed in the epithelial cells the fibroblast cells or both cannot be determined by interpretation of this data alone. The growth of one cell type may be in response to androgen induced production of growth factors from the other cell type.
Fig 3.25A Growth response of co-cultured epithelial cells to Testosterone (Test) (a) and DHT (DHT) (b) at concentrations of 10 & 100nM. Cell numbers were counted manually. All results are compared to controls, which received no androgens. After 4 days, both testosterone and DHT had stimulated a significant increase in growth of these cells (P<0.05) at both concentrations. Cells which received DHT showed a statistically significant increase in growth compared to cells which received testosterone (p<0.05). Permixon® did not have a significant effect on the androgen stimulated growth of these cells (p>0.05).
Fig 3.25B Growth response of co-cultured fibroblast cells to Testosterone (Test) (a) and DHT (DHT) (b) at concentrations of 10 & 100nM. Cell numbers were counted manually. All results are compared to controls, which received no androgens. After 4 days, both testosterone and DHT had stimulated a significant increase in growth of these cells (P<0.05). There was no statistically significant difference between DHT and testosterone treated cells (p>0.05). Permixon® did not have a significant effect on the androgen stimulated growth of these cells (p>0.05).
3.5.3 Response of co-cultured epithelial and fibroblast cells to androgens in the presence of Permixon® (10μg/ml)

As Permixon® has been reported to inhibit ligand binding to the androgen receptor, the effect of Permixon® on the growth of androgen stimulated co-cultured epithelial and fibroblast cells was investigated. The results obtained from these experiments are presented in fig 3.25. From this data, it can be seen that Permixon® at a concentration of 10μg/ml does not affect the growth of the co-cultured epithelial or fibroblast cells in response to androgen stimulation when compared to controls. Permixon® at a concentration of 10μg/ml therefore does not appear to inhibit the binding of ligands to the androgen receptor.

3.6 Expression of Androgen Receptor Protein in Cultured Human Prostate cells

3.6.1 Androgen receptor protein expression in co-cultured epithelial and fibroblast cells as detected by Western blot analysis

Expression of androgen receptor protein was investigated by Western blot analysis utilising a polyclonal antibody. Protein samples were prepared from co-cultured and primary cultured epithelial and fibroblast cells. Protein from LNCaP cells, which are known to express androgen receptors, was included as a positive control. The results of this analysis are shown in fig 3.26.

Samples prepared from primary cultured epithelial cells (lane 4) and fibroblast cells (lane 5) demonstrated no detectable androgen receptor protein using the polyclonal antibody when compared to protein samples from LNCaP cells (lane 1; 110kD).

Protein samples prepared from co-cultured epithelial cells (lane 2) and co-cultured fibroblast cells (lane 3) both demonstrated a band at 110kD which is indicative of androgen receptor protein.
Fig 3.26 Western blot analysis for androgen receptor protein in protein extracted from LNCaP (1), Co-cultured epithelial cells (2), co-cultured fibroblast cells (3), primary cultured epithelial cells (4), primary cultured fibroblast cells (5). 25µg/ml of protein from each source was loaded onto the gel. A mouse anti-human polyclonal anti-body was employed at a dilution of 1:50. A band of 110kD was detected in lanes 1, 2 & 3, indicating the presence of androgen receptor protein in these samples. Samples 4 & 5 were negative. Protein from LNCaP cells served as a positive control.
3.7 Characterisation of Co-cultured Prostatic Epithelial and Fibroblast Cells by Electron Microscopy and Immunohistochemistry

All experiments were repeated 3 times and 15 sections viewed from each sample.

3.7.1 Electron microscopy of co-cultured epithelial and fibroblast cells

3.7.1.1 Electron microscopy of co-cultured epithelial cells

When examined by electron microscopy, the co-cultured epithelial cells (fig 3.27) exhibited markedly different characteristics from the primary cultured epithelial cells (fig 3.27). Co-cultured epithelial cells exhibited an abundance of tonofibrils (T), possessed a large number of secretory vesicles (V) and displayed a complex structure of microvilli (Mi). The co-cultured epithelial cells also showed a greater degree of differentiation than the primary cultured cells and displayed a compact nucleus (N).

The primary cultured epithelial cells did not appear as well differentiated as the co-cultured epithelial cells and did not exhibit the same complex structure of microvilli. Primary cultured epithelial cells appeared to have larger nuclei and did not appear as well organised as the co-cultured cells. There was no evidence of fibroblast or smooth muscle cell contamination in the epithelial cell cultures.

3.7.1.2 Electron microscopy of co-cultured fibroblast cells

Primary (fig 3.29) and co-cultured (fig 3.28) fibroblast cells also displayed structural differences when analysed by electron microscopy. Co-cultured fibroblast cells demonstrated the characteristic spindle shape, abundant cilia (C) and Golgi (G). Co-cultured fibroblast cells also demonstrated collagen fibril production (F). The primary cultured fibroblast cells appeared more oval and showed limited evidence of cilia. There were however large amounts of Golgi. There was no evidence of any smooth muscle cell contamination in any of the fibroblast cultures in any of the sections viewed. These results indicate that the fibroblast cells are more highly differentiated in the co-culture system in comparison to primary cultured fibroblast cells.
Fig 3.26 Electronmicroscopy of co-cultured (A) (mag x3888) and primary cultured (B) (mag x2817) epithelial cells. Co-cultured epithelial cells (A) demonstrate compact nuclei, large amounts of microvilli (M) and lumen formation (L). The primary cultured epithelial cells (B) in contrast are shown to have larger nuclei, less well organised cytoplasm and to have fewer microvilli (M).
Fig 3.28 Electronmicrographs of primary cultured fibroblast cells (A & B) (mag x7914; mag x2817). Primary cultured cells demonstrate much rounder nuclei (N) and have more cytoplasm (C) than co-cultured cells (fig 3.28). Primary cultured fibroblast cells still demonstrate large amounts of cilia which are indicative of fibroblast cells.
Fig 3.29 Electronmicroscopy of co-cultured fibroblast cells.

Co-cultured fibroblast cells demonstrate cilia (Ci), a convoluted nucleus (N) and large amounts of Golgi (G) in the cytoplasm (C) are demonstrated in plate (A) (mag x3888). An immature collagen fibril (F) is visible in plate (B) (mag x86400).
3.7.2 Immunohistochemistry of primary cultured and co-cultured epithelial cells for basal cell markers

3.7.2.1 Immunohistochemistry of primary cultured epithelial cells

To determine the origin of the primary cultured epithelial cells, the cells were stained using a mouse monoclonal anti-human high molecular weight cytokeratin antibody from Dako. The antibody reacts with cytokeratins 1, 5, 10 and 14, which are markers of basal epithelial cells and are not expressed in other differentiated epithelial cells. As a control, a section of BPH prostate was also stained using the antibody to demonstrate only basal cell staining (fig 3.30a). From the figure it can be clearly seen that there is no luminal cell staining using this antibody in the tissue section. The primary cultured epithelial cells demonstrated positive staining using the anti-body indicating that the cells were basal in origin. Negative controls showed no staining (fig 3.30b).

3.7.2.2 Immunohistochemistry of co-cultured epithelial cells

Co-cultured epithelial cells were stained using the same anti-body to high molecular weight cytokeratins in order to determine whether they were basal or luminal. The results shown in fig 3.31a demonstrate positive staining of the cells similar to that shown by the primary cultured epithelial cells, indicating that these cells also show basal cell characteristics and have not differentiated into luminal cells. It should be noted however that there are a sizeable number of cells, which demonstrate no staining for high molecular weight cytokeratins (1, 5, 10 & 14). These cells may be luminal in nature having differentiated from the basal cell population. There was no demonstrable staining in negative controls (fig 3.31b). Co-cultured epithelial cells also were examined by MLSO Bill Neil using flow cytometry. This technique demonstrated the presence of two populations of cells in the co-culture model as compared to primary cultured epithelial cells, which demonstrated only one cell population. This data was not included as not enough samples were analysed.
Figure 3.30 Positive immunohistochemical staining of primary cultured epithelial cells with the basal cell marker high molecular weight cytokeratins (1,5,10 & 14) upper plate (a). Lower plate (b) demonstrates negative control.
Figure 3.31 Positive immunohistochemical staining of co-cultured epithelial cells with the basal cell marker high molecular weight cytokeratins (1,5,10 & 14) upper plate (a). The co-cultured cells also demonstrate a number of negative staining cells. Lower plate (b) demonstrates negative control.
3.8 PSA expression in Cultured Human Prostate Cells, Response to Androgen Stimulation and the Effect of Permixon®

3.8.1 Immunohistochemical localisation of PSA in primary and Co-cultured epithelial cells

3.8.1.1 Primary cultured epithelial cells

Immunohistochemistry was performed on primary cultured epithelial cells using a mouse anti-human PSA monoclonal antibody. The results of the immunohistochemistry were photographed and are shown in fig 3.32a. The primary cultured epithelial cells showed no staining for PSA using this antibody and there was no detectable difference between the sample cells and the negative controls (fig 3.32b). This indicates that these cells were not expressing any immunoreactive PSA. This is in keeping with the basal cell nature of these cells. Only differentiated luminal cells have been shown to express PSA.

3.8.1.2 Co-cultured epithelial cells

Immunohistochemistry was performed on co-cultured epithelial cells using the same mouse anti-human PSA monoclonal antibody. The results displayed in fig 3.33a demonstrate a clear difference between the co-cultured cells and the negative controls (fig 3.33b). The co-cultured epithelial cells show positive cytoplasmic staining for PSA although not all the cells appear to expressing immunoreactive PSA. This is somewhat confusing as co-cultured epithelial cells stained positively for basal cell markers as demonstrated previously in section 3.7.2.2. and show no luminal cell characteristics. This would seem to indicate that these basal epithelial cells are being induced to express PSA by the fibroblast cells. This would be in keeping with the high molecular weight cytokeratin immunostaining, which demonstrated a population of cells which did not stain positively for the high molecular weight cytokeratins.
Fig 3.32 Immunohistochemistry of PSA expression in primary cultured prostate epithelial cells.

Primary cultured epithelial cells were stained for PSA expression using a monoclonal mouse anti-human PSA antibody at a dilution of 1:50,000. Staining was detected using the streptavidin-DAB system. Nuclei were counter-stained using haematoxylin. An example of a negative control is shown in plate (b).
Figure 3.33 Positive immunohistochemical staining of co-cultured epithelial cells with the PSA mouse anti-human antibody (upper plate(a)). Lower plate (b) demonstrates the negative control. Cells have been counterstained using haematoxylin.
3.8.1.3 Primary and co-cultured fibroblast cells

Primary and co-cultured fibroblasts were stained using the mouse anti-human PSA monoclonal antibody. Neither form of culturing results in the expression of PSA by the fibroblasts, which remain negative in relation to controls (fig 3.34A & B). This is as expected as fibroblast cells do not express PSA.

3.8.2 Effect of Permixon® on immunohistochemical staining of co-cultured epithelial cells for PSA expression

Co-cultured epithelial cells, which had been treated with Permixon® at a concentration of 10µg/ml, were stained for the presence of PSA using the same monoclonal anti-body (fig 3.35). Permixon® was shown to have no effect on PSA expression in these cells. There was no detectable difference between treated and untreated cells with both samples showing positive staining for PSA.

3.8.3 Detected levels of PSA expression in primary and co-cultured epithelial cells

3.8.3.1 Detected levels of PSA expression in primary cultured epithelial cells

As the immunohistochemistry had shown negative staining for PSA in primary cultured cells, conditioned medium collected from primary cultured cells was analysed using the Hybritech Tandem-MP PSA assay kit, as a negative control for comparison to conditioned medium collected from co-cultured epithelial cells. PSA in conditioned medium from primary cultured epithelial cells was not detectable using the assay (fig 3.36).
Fig 3.34A Immunohistochemistry of PSA expression in co-cultured prostate fibroblast cells. Co-cultured fibroblast cells were stained for PSA expression using a monoclonal mouse anti-human PSA antibody at a dilution of 1:50 000. Staining was detected using the streptavidin-DAB system. Nuclei were counter-stained using haematoxylin. An example of a negative control is shown in plate (b).
Fig 3.34B Immunohistochemistry of PSA expression in primary cultured prostate fibroblast cells. Primary cultured fibroblast cells were stained for PSA expression using a monoclonal mouse anti-human PSA antibody at a dilution of 1:50 000. Staining was detected using the streptavidin-DAB system. Nuclei were counter-stained using haematoxylin. An example of a negative control is shown in plate (b).
Figure 3.35 Positive immunohistochemical staining of co-cultured epithelial cells with the PSA mouse anti-human antibody (upper plate (a)) following treatment with 10µg/ml Permixon®. Lower plate (b) demonstrates the negative control.
Fig 3.36 PSA levels in medium from co-cultured epithelial cells measured using Hybritech Tandem_MP ELISA. PSA concentrations were noted to increase when cells were pulsed with 100nM Testosterone. Permixon® had no effect on PSA production by these cells regardless of addition of androgens. The increase in PSA production was statistically significant. Primary cultured epithelial cells showed no measurable PSA production even on addition of androgens.
3.8.3.2 Detected levels of PSA expression in co-cultured epithelial cells

Conditioned medium from co-cultured epithelial cells was collected and analysed for PSA expression utilising the Hybritech Tandem-MP PSA assay. The results are shown in fig 3.36. PSA was detected in the CM at a concentration of 0.28±0.002 ng/ml. Although the levels are low, they are detectable with the kit employed. The low level of PSA detected can be justified by the low number of cells employed in preparation of the conditioned medium.

3.8.4 Change in detected levels of PSA expression in primary cultured and co-cultured epithelial cells in response to androgens

3.8.4.1 Change in detected levels of PSA expression in primary cultured epithelial cells in response to androgens

Primary cultured epithelial cells pulsed with androgens demonstrated no detectable change in PSA production. As expected, there was no detectable PSA production in the conditioned medium collected from these cells (fig 3.36). This reflects the lack of detectable androgen receptor protein and the lack of proliferative response to androgens.

3.8.4.2 Change in detected levels of PSA expression in co-cultured epithelial cells in response to androgens

When pulsed with androgens (10 nM testosterone) there was a marked increase in the expression of PSA by the co-cultured epithelial cells (fig 3.36). The measured concentration increased to 0.4115±0.003 ng/ml. This represents a statistically significant increase (P<0.05) of nearly 100% over untreated controls (0.28±0.002 ng/ml).
3.8.5 Effect of Permixon® on detected levels of PSA expression in co-cultured and androgen stimulated co-cultured epithelial cells

3.8.5.1 Effect of Permixon® on detected levels of PSA expression in co-cultured epithelial cells

The effect of Permixon® (10µg/ml) on PSA production from co-cultured epithelial cells was investigated using the same assay method. The results are shown in fig 3.36. This data suggests that Permixon® has no effect on the production of PSA by co-cultured epithelial cells. The concentration of PSA measured in the conditioned medium from treated cells (0.22±0.0015 ng/ml) was not significantly altered from untreated controls.

3.8.5.2 Effect of Permixon® on detected levels of PSA expression in androgen stimulated co-cultured epithelial cells

Androgen pulsed co-cultured epithelial cells were treated with 10µg/ml Permixon® and the effect on increased production of PSA was investigated. Conditioned medium collected from the cells was analysed using the same method as described previously. The results show no inhibition of androgen induced increase in PSA production by Permixon® (fig 3.36). The measured levels of PSA in the conditioned medium from treated cells was 0.43±0.003 ng/ml which was not significantly different from the concentration measured in androgen pulsed controls. These results suggest that Permixon® at a concentration of 10µg/ml does not affect androgen binding.
3.9 Effect of Permixon® on Intracellular Membranes of Cultured Cells from Prostate, Skin and Breast.

3.9.1 Effect of Permixon® on prostate fibroblast and epithelial cell membranes

3.9.1.1 Effect of Permixon® on co-cultured prostate epithelial cell membranes

The physical effects of Permixon® (10µg/ml) on treated cells were investigated by employing electron microscopy. The results shown in fig 3.37 are representative of the disruption of the intracellular membranes by Permixon® (10µg/ml) in epithelial cells. The electron micrographs show accumulation of lipid droplets within the cytoplasm of the cells (C). At a higher magnification, it becomes easier to see the damage to mitochondrial membranes (Mi) and the nuclear membrane (M). There is also evidence of condensation of the chromatin within the nucleus, which may be indicative of apoptosis. Untreated cells show no such intracellular damage as caused by Permixon® (10µg/ml) treatment (Fig 3.27).

3.9.1.2 Effect of Permixon® on co-cultured prostate fibroblast cell membranes

The intracellular damage to fibroblast cells caused by Permixon® (10µg/ml) was similar to the changes seen in epithelial cells (fig 3.38). Electronmicrographs show disruption of mitochondrial membranes (Mi), nuclear membrane (M) and accumulation of lipid droplets in the cytoplasm of the cell (C). Untreated cells appeared normal (fig 3.28) in comparison showing none of the signs of membrane disruption seen in the Permixon® treated samples.
Fig. 3.37 Electronmicroscopy of Permixon® (10µg/ml) treated co-cultured prostate epithelial cells.

Permixon® treated co-cultured epithelial cells show many physical changes (plates A (mag x5292), B (mag x56160), C (mag x138240). There is an accumulation of lipids in the cytoplasm (C) of the cells and vacuolation (V) of the cytoplasm. The mitochondria (Mi) show membrane damage resulting in rupture of the organelle. The chromatin appears polarised in the nucleus and the nuclear membrane (M) demonstrates signs of physical damage. Tonofibrils (T) are still evident in the cytoplasm of the cells.
Fig. 3.38 Electronmicroscopy of Permixon® treated (10μg/ml) co-cultured prostate fibroblast cells.

Permixon® treated fibroblast cells show many of the changes seen in the epithelial cells. Plates A (mag x15984), B (mag x103680). The appearance of lipid droplets (F) in the cytoplasm (C), damage to the nuclear membrane (M) and disruption of organelle membranes (mitochondria; (Mi)).
3.9.2 Effect of Permixon® on skin fibroblast cell membranes

The effects of Permixon® on none prostate derived cells was investigated by investigating the effect of Permixon® on skin fibroblasts. The skin fibroblasts were treated with Permixon® at a concentration of 10μg/ml in accordance with the protocol employed with the prostate derived cells. The results from investigation by electron microscopy are shown in figs 3.39.. There is no detectable physical effect of Permixon® (10μg/ml) on these cells derived from skin when compared to controls (fig 3.40). All intracellular membranes appear normal and there is no visible disruption.

3.9.3 Effects of Permixon® on breast fibroblast cell membranes

The physical effects of Permixon® were also investigated in fibroblast cells derived from breast tissue. The breast fibroblast cells were treated with Permixon® (10μg/ml) in the same manner as the previous studies. The cells were analysed by electron microscopy and are displayed in fig 3.41.. The results are similar to those provided by skin fibroblasts with no detectable physical changes to the treated cells in comparison to controls (fig 3.42).
Electronmicrographs of Skin derived fibroblast cells treated with Permixon®. Cells demonstrate large numbers of cilia (Ci), convoluted nucleus (N) and large amounts of endoplasmic reticulum (E) in the cytoplasm (C). In contrast to prostate fibroblasts treated with Permixon®, skin fibroblasts do not show any damage of the nuclear membrane (M). There is also minimal demonstrable lipid accumulation in the cytoplasm (F).

Plate A (mag x28080), plate B (mag x3888), plate C (mag x9720).
Fig 3.40 Electronmicrographs of skin derived fibroblast cells. Untreated skin fibroblast cells demonstrate large convoluted nuclei (N) and large amounts of endoplasmic reticulum (E) in the cytoplasm (C). There is no demonstrable difference between Permixon® treated and untreated cells. Plate A (mag x20520), plate B (mag x5292).
Fig 3.41 Electronmicrographs of breast derived primary cultured fibroblast cells treated with Permixon®. Cells demonstrate a characteristic convoluted nucleus (N) and cytoplasm (C) abundant with endoplasmic reticulum. There is no evidence of membrane damage and the nuclear membrane (M) appears intact. A small amount of lipid accumulation in the cytoplasm is evident (F). Plate A (mag x5292), plate B (mag x15984)
Fig 3.42 Electronmicrographs of breast derived primary cultured fibroblast (negative control). Cells demonstrate a characteristic convoluted nucleus (N) and cytoplasm (C) abundant with endoplasmic reticulum. There is no demonstrable difference between the treated and untreated cells. Plate A (mag x5292), plate B (mag x7854)
3.10 Summary of Results

The results presented in this chapter can be summarised as follows:

1. The co-culture model is a better in-vitro model of BPH than any of the other models currently available as it expresses both isoenzymes of 5α-reductase, androgen receptors and PSA which is not the case with primary cultures or with the prostate cancer cell lines.

2. The factor which is responsible for inducing the expression of the 5α-reductase type II isoenzyme in prostatic epithelial cells is secreted only by prostatic fibroblast cells. It is approximately 10kD in size, is anionic and may exist as a dimer.

3. 5α-reductase types I and II show different testosterone metabolising profiles in the human prostate.

4. Permixon® is an effective inhibitor of 5α-reductase activity in prostate cells and does not interfere with androgen receptor binding at pharmacological dosages.

5. Permixon® only affects the intracellular structures of prostate derived cells and does not appear to have any physically damaging effects on none prostate derived cells.
4 DISCUSSION

This study was set up primarily to identify the factor(s) responsible for controlling the expression of 5α-reductase in the human prostate and to establish whether the action of Permixon® in BPH is mediated through these new pathways. In order to investigate the control of 5α-reductase in the prostate, it was necessary to use a model, which expressed both isoenzymes of 5α-reductase as is seen in the in-vivo prostate. At present there are a rather limited number of models available for the in-vitro study of prostate biology. The models, which are available, provide a somewhat less than ideal platform to investigate this increasingly studied area. As a result, it became necessary to develop a new model of BPH, which provided a suitable alternative to the current models. The results presented in this thesis provide evidence for a novel in-vitro model of BPH, which maintains many of the characteristics of the in-vivo prostate. The data also demonstrates the need for a better understanding of epithelial-fibroblast interactions in the adult prostate and their role in health and disease.

4.1 In-vitro models of BPH

The models currently available for the investigation of processes involved in the development of BPH are the cell lines (DU145, PC3, LNCaP etc) and primary cultures of epithelial and fibroblast cells from hyperplastic tissue obtained from TURP [169, 171]. The cells lines are all derived from prostate cancer metastasis and although offering certain advantages do not give an ideal representation of the in-vivo prostate [168, 169]. The three cell lines utilised in this study were all immortalised cells, a process which tends to alter the phenotype of the cell, the cell lines are all clones which results in a completely homogenous population of cells, a situation which is not indicative of the heterogenous population found in the prostate, all three cell lines were obtained from metastases from patients who had undergone oestrogen treatment, chemotherapy and orchidectomy [151, 162, 164, 165] obviously, cells obtained from metastasised prostate cancer are not directly comparable to benign hyperplastic cells.
The cell lines also reflect none of the stromal-epithelial interactions, which are possible in the prostate gland itself. All these conditions result in cells, which are altered in nature compared to benign hyperplastic cells. The alterations in the phenotype of the cells are exemplified by comparing the basic characteristics of the cells with normal prostate tissue. Androgen receptors, an integral part of BPH development and research, are only expressed by the LNCaP cells and not by the other two cell lines. Work on the androgen receptor expression in LNCaP cells has shown that the androgen receptor in the LNCaP cell line is mutated resulting in an altered response to certain antagonists [157, 158]. The data presented in this thesis also demonstrated that all three cell lines utilised only express 5α-reductase type I with no detectable expression of 5α-reductase type II either functionally or by RT-PCR. As it has been shown in prostate tissue that both isoforms are expressed and active [128], this represents a serious shortcoming for the investigation of processes underlying the control of these enzymes.

The next in-vitro model to be considered is the primary culture model. In this model, cells are obtained from TURP tissue, which is treated with collagenase and the cell types separated by centrifugation [169, 171]. The separated cells are then cultured in appropriate medium. This offers the obvious advantage over the cell lines of being derived from BPH tissue, which has not undergone any form of endocrine or chemo therapy. These cells in theory should be representative of the originating tissue as they are not clonal in origin and have not been immortalised. They should therefore express all the same characteristics as the tissue of origin. Unfortunately, the process of culturing causes changes in the cell characteristics due to the change in environment brought on with culturing. These changes can be quite marked and it is clear from the observed effect of androgens on these cultured cells that like the cell lines, they do not express functional androgen receptors. This finding is in agreement with the findings of McKeelhan et al (1984) [179], Merchant (1990) [180] and Grant et al (1996) [158]. Data is also presented which demonstrates that these cells only express the one isoenzyme of 5α-reductase (type I) after a short period in culture. There is also no expression of PSA by primary cultured epithelial cells, which once again is unlike the situation seen in the intact organ. It has been speculated that these cells are undifferentiated basal cells or dedifferentiated secretory epithelial cells. This is also in agreement with the observations of Montpetit et al (1988) [181] on epithelial cultures derived from the rat
ventral prostate which lacked the expression of prostatic binding protein genes which are markers of secretory epithelium in this tissue. The hypothesis that these primary cultured epithelial cells are derived from basal cells is further supported by data presented in this thesis which demonstrates the detection of cytokeratins 1,5,10 and 14, which are markers in basal epithelial cells and are not detectable in luminal secretory epithelial cells. There are three cell types in the prostate, which can be described as epithelial. These are secretory, basal and endocrine-paracrine. All three cell types differ in their marker expression and hormone regulation. Of these three cell types, we shall consider only the basal and luminal epithelial cells. Secretory cells express PSA and cytokeratins 8 and 18 [182, 183, 184], require continuous support from androgens for maintenance and express androgen receptors. Basal cells are an androgen independent population but can proliferate under the influence of oestrogens due to the expression of oestrogen receptors [185, 186, 187, 188]. The marker profile of basal cells is significantly different from that of the secretory cells. Basal cells lack PSA expression and exclusively express high molecular weight cytokeratins [182, 183, 184, 189, 190, 191].

In terms of studying BPH and prostate cancer, the ability to culture secretory, androgen responsive epithelial cells would be of the greatest benefit. But as can be seen from the results presented in this thesis, epithelial cells grown in culture using the conditions of Chaproniere and M'Keehan [171, 179] are not representative of secretory cells and instead express the cytokeratin markers of basal epithelial cells. The primary cultured cells lack 5α-reductase type I expression, androgen receptors and PSA. In comparison to other models, the primary cultured cells appear more like the cell lines than tissue. Some of these changes in phenotype can be accounted for by the lack of cell interaction between epithelial and fibroblast cells, which is a result of the culturing process. The role of these cell interactions will be further discussed when considering the co-culture model in which soluble factors are able to pass between epithelial and fibroblast cells and thereby influence the expression of certain characteristics by these cells, which are otherwise suppressed by primary culturing.

The final model presented is the novel co-culture model, the development of which is one of the main subjects of this thesis. This model offers several advantages over the other two cell models detailed so far. Unlike the cell lines, but similar to the
primary cultures, it is derived from tissue obtained from TURP operations of patients suffering from BPH but who have received no treatment in the form of endocrine or chemotherapy. This offers the same advantages as the primary cultured cells in that the cells are not clonal in origin, are derived from the original tissue and not from metastasis and have not been immortalised. Unlike primary cultured cells, by employing these cells in a co-culture system, it has been possible to maintain or re-induce many of the important characteristics of the host organ. This is achieved through the maintenance of cellular interaction between the epithelial and fibroblast cells by culturing the two cell types in a system, which although preventing any direct cell contact, allows diffusion of soluble factors between the two cell types. Data presented here demonstrates that co-cultured epithelial and fibroblast cells derived from the prostate will continue to express androgen receptors and both isoenzymes of 5α-reductase (types I & II). Also, co-cultured epithelial cells secrete PSA and are androgen responsive.

Characterisation of the epithelial cells after growth in co-culture would seem to indicate that they are derived from basal epithelial cells, as is the case with the primary cultured epithelial cells. In the prostate, basal epithelial cells do not express androgen receptors or PSA. This would imply that these basal cells are being stimulated to express these characteristics by secretory products from the prostatic fibroblast cells. Indeed, there is evidence from the immunohistochemistry using high molecular weight cytokeratins that a second population of cells which do not stain for the high molecular weight cytokeratins is differentiating from the basal epithelial cells under the influence of soluble factors secreted by the fibroblast cells. If, as the evidence suggests, these are basal epithelial cells, which are being stimulated into expressing the characteristics of secretory epithelial cells or are differentiating under the influence of the fibroblast cells, it offers an explanation for the characteristics of the primary cultured epithelial cells. As the primary cultured cells are no longer influenced by the fibroblast cells, they are unable to differentiate and therefore maintain their basal nature. There is further evidence in the form of electronmicroscopy which shows a distinct difference in appearance between the primary cultured and the co-cultured epithelial cells. The primary cultured cells are noted to have large nuclei with a small amount of cytoplasm, which contains disorganised organelles. The co-cultured epithelial cells when viewed
by electron microscopy have a smaller nucleus with a relatively larger amount of cytoplasm, which appears to be highly organised. The results also add more weight to the theory of basal epithelial being progenitors of secretory epithelial cells. This theory was propounded by Trump et al (1981) [192]. Basically, it states that basal epithelial cells act as a "reserve" for secretory cells and can replace the luminal cells via one or more differentiation stages. Growing epithelial cells on basement membrane has been shown to have an effect on differentiation of the basal epithelial cells in-vitro (Fong et al, 1991) [193]. It is worth considering that the combination of fibroblast conditioned medium and basement membrane may result in complete differentiation of the basal cells into luminal cells. Although there is no evidence of a stem cell model in the prostate as yet, the evidence pointing to such as system being in operation is mounting.

4.2 5α-reductase Isoenzyme Expression and Activity in Cultured Human Prostate Cells – Different Roles for Different Isoenzymes.

Several groups have demonstrated the presence of both isoenzymes of 5α-reductase in prostatic tissue and there has been a great deal of debate over the possible roles of these isoenzymes in-vivo. This is in comparison to primary cultured cells and cell lines which only express 5α-reductase type I. The primary cultured cells lose the ability to express 5α-reductase type II after a short period in culture. This loss of 5α-reductase type II expression can be prevented or re-induced by co-culturing the epithelial cells with fibroblast cells or by growing the epithelial cells in fibroblast conditioned medium. The fact that these cells continue to express 5α-reductase type I and stop expressing 5α-reductase type II unless stimulated to do so by some as yet unidentified secretory product of fibroblast cells suggests that the 5α-reductase type II isoenzyme is the more important of the two in androgen sensitive tissues and that the 5α-reductase type I enzyme is not under the control of external influences. Other tissues such as the skin and liver which are androgen sensitive to a degree, have been shown to express only 5α-reductase type I, which brings into question the role of the two isoenzymes. If both are equally active with the same affinity for substrate, then why are two active isoenzymes expressed in the prostate and male genital tract and nowhere else? This would appear to suggest that the enzyme 5α-reductase type I, is
ubiquitous to androgen sensitive tissues and has a distinct and separate role from that of 5α-reductase type II. It also suggests that in terms of growth and function of these androgen sensitive organs, 5α-reductase type II is the enzyme of import. The separate roles of the isoenzymes is more clearly demonstrated by diseases such as pseudohermaphroditism which is a condition exemplifying the role of 5α-reductase type II and DHT in the normal development of the male genitalia. Pseudohermaphroditism arises as a result of the lack of 5α-reductase type II activity in the male genital tract during development [194]. This results in a condition characterised by female phenotype at birth with a clitoris like phallus, a blind ending vaginal pouch and pronounced hypospadias. The patient also exhibits a XY chromosomal genotype, functioning but undescended testes and a rudimentary prostate, which never develops BPH. When the child reaches puberty, the normal male phenotype develops under the influence of androgens. When investigated, serum testosterone levels are normal, as are androgen receptor levels and function. The condition is a result of low levels of DHT. Administration of DHT results in growth of the prostate. Further molecular investigation of this condition leads to the realisation that it was a result of the lack of 5α-reductase type II expression in the affected organs [6, 7, 106, 195, 196, 197]. This alone would seem to indicate that there are distinct roles for both isozymes of 5α-reductase in the metabolism of androgens. If 5α-reductase type I is as effective at metabolising testosterone to DHT then the condition of pseudohermaphroditism would not exist as 5α-reductase type I continues to be expressed in the affected organs of these individuals. As this is clearly not the case, a role for each isozyme must be found in order to understand better the development of BPH.

When the pattern of metabolism of cultured cells expressing only 5α-reductase type I is investigated, using cell lines and primary cultured epithelial and fibroblast cells derived from the prostate, it has been shown that 5α-reductase type I appears to have a higher specificity for androstenedione than for testosterone [111]. In these cells, there appears to be a high level of 17β hydroxysteroid dehydrogenase activity, which metabolises testosterone into androstenedione. It is only after the metabolism of testosterone into androstenedione that there are any 5α reduced metabolites become detectable. Work presented in this thesis appears to be in agreement with this hypothesis in that primary cultured epithelial and fibroblast cells demonstrate
production of large amounts of androstenedione and androstanedione before any DHT becomes detectable. This is in comparison to co-cultured cells, which express both isoenzymes. In the co-cultured cells, DHT is readily detectable after only a short period of incubation with testosterone and becomes detectable before any androstanedione can be observed. There is still a large production of androstenedione and androstanedione but the levels of DHT produced are much higher than in the primary cultured cells. This indicates that 5α-reductase type I has a substrate preference for androstenedione. In support of this hypothesis, work by Levy et al (1996) [111] has shown that the human 5α-reductase isoenzymes have different substrate specificities with 5α-reductase type II having a greater affinity for testosterone than for androstendione. The presence of two isoenzymes could possibly be because of dual roles in the metabolism of testosterone. 5α-reductase type II converts T to DHT, a more active compound, to gain maximum benefit from its effects, while 5α-reductase type I may be expressed as an attempt to prevent the conversion of testosterone to oestrogen following metabolism to androstenedione due to the action of 17β hydroxysteroid dehydrogenase. As 5α reduced androgens are not aromatase substrates this metabolic pathway would maximise the amount of testosterone converted into DHT and prevent the aromatisation of testosterone to oestrogen. Tissues, which only express 5α-reductase type I, are not as dependent upon the action of DHT as the prostate and the accessory sex organs and therefore do not require the relatively high levels of DHT the action of 5α-reductase type II would produce. By expressing 5α-reductase type I only, these tissues prevent the production of high levels of oestrogen via aromatisation of testosterone by metabolising testosterone to androgens which cannot undergo aromatisation to oestrogen. This theory is supported by the observations of Mahendroo et al (1997) [100] in foetal mice. In this study, it was noted that excess production of oestrogen resulting from the lack of 5α-reductase type I expression precipitated the death of the foetal mice. The excess levels of oestrogen were attributed to a failure to 5α reduce testosterone to metabolites which could not undergo aromatisation, leading to conversion of testosterone to oestrogen instead. By administering an aromatase inhibitor or an oestrogen antagonist, the high levels of foetal death could be reduced. Although these observations were made in the female mouse, they provide an intriguing insight into the possible actions of 5α-reductase type I in the human male.
When these observations made in the mouse are taken into consideration alongside the observations noted in published papers [101] and this thesis, it is reasonable to suggest that this may have great significance for the treatment of BPH in the human. The implication of this hypothesis for the treatment of BPH, is that there is a need to block not only the 5α-reductase type II, which is clearly the most active enzyme, but also to inhibit the action of 5α-reductase type I. Blocking both isoenzymes will not however prevent BPH, as there is a great deal of evidence to demonstrate that oestrogen will cause fibromuscular hypertrophy of the prostate through the activation of oestrogen receptors in the stromal compartment of the gland [198, 199]. This would make it necessary to not only block the activity of 5α-reductase types I & II, but also to block the action of oestrogen on the prostate through either oestrogen receptor antagonists or through administration of aromatase inhibitors.

4.3 Epithelial – Fibroblast Cell Interactions.

The role of cell interaction in the prostate and the relevance it has for the development of BPH and the treatment of the disease has largely been ignored. Cell interactions during development of the prostate have been clearly detailed and provide a possible point of reference for investigations into cellular interactions in the mature gland. The importance of embryonic mesenchyme in the prostatic ductal morphogenesis and epithelial growth and differentiation has been demonstrated by tissue recombinant experiments carried out by Cunha et al [8, 9, 200, 201]. Other observations have indicated that these interactions may retain an integral role in the adult gland as well. Tenniswood [202] has also postulated over the role of an epithelial-fibroblast interaction in the adult prostate. The theory proposed by Tenniswood suggested that the androgenic influence on the gland is mediated by growth factors, which stimulate cell growth and differentiation. From the studies detailed in this thesis, it has become evident that cellular interactions play a significant role in the growth and differentiation of epithelial and fibroblast cells. Under the influence of fibroblast cells, epithelial cells begin to express androgen receptors and 5α-reductase type II. In a similar manner, under the influence of epithelial cells, fibroblast cells also express androgen receptors and express 5α-reductase type II. As 5α-reductase is more active in
the epithelial cells than it is in the fibroblast cells, it is possible that the fibroblast cells rely on the epithelial cells to provide DHT and through the actions of this hormone, the fibroblast cells secrete growth factors which in turn effect the epithelial cells thereby forming a paracrine system.

Androgen receptors have been described in the stromal/fibroblast component of the in-vivo prostate gland but have not been described in cultured fibroblast cells. The possibility exists that under the influence of androgens, the fibroblast cells may differentiate into smooth muscle cells thereby leading to an increase in the mass of the gland. If this was the indeed the case, it would provide a possible system for the control of 5α-reductase function and fibroblast growth through the actions of androgens. By influencing the expression of 5α-reductase type II in the epithelial cells, the fibroblast cells could in theory affect their own growth by altering the concentration of DHT produced through the actions of 5α-reductase type II.

The work presented in this thesis, on the co-culture model, demonstrates that there is a need to investigate further the interaction of the different cell types in the prostate and the paracrine effects of cellular products on the other compartments of the gland. Indeed, work by Hyvtinen et al [203] on the development of a tumour model in mice using the LNCaP cell line showed that in order for the LNCaP cells to grow in the host animal, they had to be implanted along with normal fibroblast cells. If the LNCaP were implanted on their own, there was no demonstrable tumour growth. If the LNCaP cells were implanted with fibroblast cells derived from tumours, the LNCaP cells did not grow either. In order for the LNCaP cells to adhere and grow, it was necessary to associate them with normal fibroblast cells which suggests that the fibroblast cells are secreting some factor which is necessary for the LNCaP cells to grow and adhere. Although associated with tumour growth, this work demonstrates a role for prostatic fibroblasts in the growth of prostatic epithelial cells and clearly demonstrates cellular interaction between prostate cells.

The data gathered from the effect of fibroblast conditioned medium on epithelial cells demonstrates that an anionic protein of approximately 10kD secreted by fibroblast cells can induce the expression of 5α-reductase type II in primary cultured epithelial cells as detected by RT-PCR and protein functional assays. The role of this protein and its effects on the levels of DHT production in the prostate gland require to be
investigated further. The possibility exists that the expression of this as yet uncharacterised protein may be androgen sensitive and that by altering the DHT concentration within the prostate gland, it may be possible to effect the expression of this protein by the fibroblast cells. When primary cultured fibroblasts from skin and breast tissue were investigated, they were shown to have no effect on the expression of 5α-reductase type II in primary cultured, prostate derived epithelial cells. Unlike primary cultures of prostate derived fibroblast cells, which induced the expression of 5α-reductase type II in primary cultured prostate epithelial cells.

This observation is in keeping with other work carried out in this laboratory which shows that following metastasis, prostate cancer (CaP) cells lose the ability to express 5α-reductase type II RNA [Habib et al, in Press]. When considered along side the data presented here, there is a strong suggestion that the expression of 5α-reductase type II is dependent upon factors secreted by prostatic fibroblasts and that these factors are not secreted by fibroblasts originating from other organs. Expression of PSA in prostate epithelial cells is associated with differentiation of the cells into luminal cells. Other authors have shown that by growing epithelial cells on collagen, epithelial cells can be induced to differentiate into luminal cells and thereby begin to express PSA [193]. Evidence presented in this thesis demonstrates that secreted products from the fibroblast cells can also cause differentiation in basal epithelial cells and thereby induce expression of PSA. By utilising a monoclonal anti-body against high molecular weight cytokeratins, it is possible to detect two distinct cell populations in the co-cultured epithelial cells. One of these populations demonstrates positive staining for the high molecular weight cytokeratins whereas the second population does not. This suggests that the basal cell population has been induced to differentiate into another type of cell. The expression of PSA in these co-cultured cells would suggest that this second cell type is luminal in nature as only luminal epithelial cells secrete PSA. Data presented here suggests that the differentiation of basal epithelial cells can be regulated by products secreted by prostate fibroblast cells.

Taking this data and the role of cell interactions in the developing prostate into consideration, the suggestion is that differentiation of basal epithelial cells into secretory luminal cells may be due to paracrine factors secreted by fibroblast cells in the stromal compartment of the prostate. These results suggest that epithelial cells are
responsible for the metabolism of T to DHT, which in turn has a mitogenic effect on the fibroblast cells causing the secretion of growth factors, which regulate the growth and differentiation of the prostatic epithelial cells [204].

This model would also account for the presence of a second 5α-reductase isoenzyme in the prostate. As previously discussed, the role of 5α-reductase type I may be to prevent the build up of aromatisable androgens in the prostate, which would result in an increased local concentration of oestrogen [101]. With 5α-reductase type II being regulated by a cellular interaction system between the fibroblast and epithelial cells, any reduction in the expression of 5α-reductase type II would result in an increased level of aromatisable androgens in the prostate. By expressing 5α-reductase type I, these androgens can be metabolised to compounds which cannot undergo aromatisation to oestrogen thereby maintaining a constant oestrogen concentration in the tissue.

This model of cellular interaction would also account for the need for androgen receptor expression in the mesenchyme of the prostate in order to induce prostatic growth and development. And for the markedly different levels of 5α-reductase activity in the two cell types, which is discussed in the previous section. The growth response of co-cultured epithelial and fibroblast cells to androgens, may not be as a direct effect of the androgens on the cells, but through a more indirect effect involving the secretion of growth factors from fibroblast cells under the influence of testosterone metabolised to DHT by the epithelial cells [205]. This also offers a possible explanation for the time difference seen in the effects of T and DHT on the growth of the co-cultured cells. Cells pulsed with DHT demonstrate a quicker response than those pulsed with T. As DHT is more biologically active than T, the lag phase in the cell growth may be due to the conversion of T to DHT to a high enough concentration to induce mitogenesis. Also the secretion of growth factors from the fibroblast cells may be dependent upon the action of DHT rather than T. Considering the condition of pseudohermaphroditism, it seems evident that this is the case, as the pseudohermaphrodite does not develop a normal prostate unless treated with DHT.

By utilising cellular interaction between fibroblast and epithelial cells, it is possible to closely control the expression of 5α-reductase type II and thereby control the growth of the stromal compartment of the prostate through the levels of DHT produced. With increasing size of the prostate with age, there would be an increase in the levels of
5α-reductase type II expressed which would lead to an increase in the local levels of DHT produced. This in turn would result in increased growth of the stromal compartment of the prostate thereby instigating a vicious cycle resulting in the development of BPH. Therefore, by limiting the expression of the growth factor responsible for the control of 5α-reductase type II in the prostate epithelial cells, it may be possible to prevent the onset of BPH.

4.4 The Mechanism of Action of Permixon® and its Role in the Treatment of BPH.

Permixon® is an orally administered phytotherapeutic drug available in many European countries for the medical treatment of BPH. It consists of free and esterified long chain fatty acids, several sterols including β-sitosterol, campesterol, stigmasterol and cycloartenol. It also contains flavonoids and various polyprenic compounds [206]. It is the possible actions of the fatty acids, the sterols and the flavonoids, which are thought to be responsible for the effects of Permixon® on the prostate [207, 208, 209, 210]. Permixon® is one of the few phytotherapeutic agents, which have been studied in any detail [211]. Other phytotherapeutic agents are detailed in table 1.1. When compared to other phytotherapeutic drugs, Permixon® is shown to be one of the most effective treatments for BPH. Many of the other compounds have been shown to improve symptoms of BPH but the studies were flawed in one form or another by either having inadequate controls, low patient numbers or by inappropriate study design. Permixon® is the only agent to have undergone appropriate placebo controlled trials, which showed a significant improvement in symptom relief compared to placebo treated groups [209, 210]. Permixon® was also the subject of a multi-centre, randomised, double-blind trial, which compared Permixon® (320mg per day) with Finasteride (5mg per day) in 1098 patients [212].

In this direct comparison, both drugs were administered over a 6 month period and effects on International Prostate Symptom Score (I-PSS), urinary flow rate, residual volume and prostate volume were measured. The patients also filled out a questionnaire on quality of life and sexual function. Of the 951 patients who completed the trial, 467 received Permixon® and 484 received Finasteride. Both groups of patients recorded a decrease in symptom scores of 38%, which was significant in comparison to baseline.
There was no difference between the two groups. When comparing the improvement in quality of life, 70% of patients in both groups registered an improvement. The urinary flow rate was increased by >3ml/sec in 36% of the Permixon® group and 39% of the Finasteride group. The difference was not significant. Sexual function was unchanged in the Permixon® treated group but the Finasteride treated group registered a significant decrease in sexual function. The prostate volume was reduced by 16% in Finasteride treated patients and by 7% in Permixon® treated patients after 3 months. There was no further change in the prostate volume in either group in the following 3 months. The other parameter, which was measured was PSA. Finasteride caused a significant reduction in PSA whereas Permixon® showed no effect on PSA levels. This study demonstrated that Permixon® was as effective as Finasteride at relieving symptoms of BPH but did not have the undesirable side effects of Finasteride. As it has no effect on PSA, there is no risk in a developing cancer being undetected by medical staff.

The only other phytotherapeutic agent which has undergone any scientific evaluation is Cernilton, an extract from rye grass pollen, which demonstrated an improvement in overall symptom scores compared to placebo but had no effect on either prostate volume or urinary flow rate [213]. Cernilton has not been compared in a trial to any other commercially available drug either physiotherapeutic or synthetic.

Permixon® appears to have multiple mechanisms of action as demonstrated by in-vitro studies. These actions include inhibition of both 5α-reductase type I and type II [178, 214, 215, 216], prolactin receptor signal transduction [217], anti-oestrogenic properties [218], anti-androgenic properties [219], inhibition of arachidonic acid metabolism and reduction in oedema [220]. Most of these actions have been attributed to the flavonoid constituents of the extract, however the exact mechanism by which these effects are achieved is as yet unknown. The effect of lignans and isoflavonoids on 5α-reductase and 17β hydroxysteroid oxidoreductase (17β HSOR) in genital skin fibroblast and prostate tissue was investigated by Evans et al (1995) [221]. The results show that certain isoflavonoids and lignans are effective at inhibiting 5α-reductase and 17β HSOR. In the prostate, it was noted that these compounds were more effective at inhibiting both isoenzymes of 5α-reductase than they were at inhibiting 17β HSOR. Lignans and flavonoids have also been shown to inhibit the action of the enzyme aromatase, which metabolises testosterone to oestrogen. Other effects of Permixon®
have also been emulated using individual flavones or fatty acids [222]. It is reasonable to assume that the overall effect of Permixon® is due to the synergistic action of these compounds and is not due to one individual compound alone.

The data presented in this thesis on the effects of Permixon® in a novel co-culture model of the prostate is in agreement with previous studies, which have shown that Permixon® inhibits both isoenzymes of 5α-reductase [215]. However, most of the previous studies were carried out in insect expression systems or in cell lines or primary cultured cells which were not representative of the hyperlastic prostate to the degree that the co-culture model has been shown to be. These results detailed here were obtained from a system, which more closely emulates the prostate and demonstrates that Permixon® is an effective inhibitor of 5α-reductase types I & II. The data presented is also in agreement with that of Carraro et al (1996) [212] which demonstrated that Permixon® at therapeutic dosages had no effect on PSA expression in the prostate.

By examining Permixon® treated cells by electronmicroscopy we have been able to show that human prostate cells demonstrate large amounts of intracellular damage. This is in keeping with results obtained from Permixon® treated prostate cancer cell lines which demonstrated comparable cellular damage [223]. A possible explanation for this observation is the presence of fatty acids in the plant extract. These fatty acids may be inserting into the membrane and thereby disrupting the structure of the membrane. This would also explain the inhibition of both isoenzymes of 5α-reductase. These enzymes require to be membrane bound in order to function [224]. By disrupting the membrane environment, Permixon® is inhibiting the action of the enzymes. This data implies that the fatty acid component of Permixon® is of more importance than previously thought.

Previous studies have reported an inhibition of androgen and oestrogen receptor binding by Permixon® [218, 219]. The androgen receptor binding data appears to be in contradiction to the work presented here. This can be explained by the different concentrations of Permixon® used in these studies. The concentrations used to inhibit androgen binding were much higher than those utilised in the co-culture model. The lower concentration of Permixon® was utilised in the co-culture model to mimic the concentrations, which would be expected in a human patient. However, DiSilverio et al (1992) [218] have shown that tissue obtained from patients who had been receiving
Permixon® for 3 months there was a decrease in nuclear oestrogen and androgen receptors compared to control but no effect on cytosolic receptor numbers. This was explained by the blocking of translocation of the receptors across the nuclear membrane by Permixon®. This leads us once again to the theory that a constituent of the Permixon® is producing effects which are consistent with membrane destruction. It would also explain the lack of effect of Permixon® on androgen regulated processes such as growth and PSA secretion which have been shown both in-vivo and in the in-vitro co-culture model not to be affected by treatment with Permixon®.

Other effects of Permixon® may be attributable to the flavonoid component of the extract. Flavonoids have been demonstrated to influence the metabolism of arachidonic acid to a considerable extent [225], to inhibit the actions of cyclo-oxygenase (CO) [226, 227] and also to be potent inhibitors of lipoxygenase (LO). These actions are attributed to the anti-oxidant properties of flavonoids [228]. The inhibition of CO and LO by flavonoids endows them with anti-inflammatory properties with potencies intermediate to indomethacin and aspirin. Permixon® has been shown to have anti-inflammatory properties in cultures of rat prostate tissue [229]. Comparison of Permixon® with α adrenergic antagonists has shown that Permixon® is also capable of increasing urinary flow rate, reducing residual volume and reducing urinary frequency.

When all these possible actions are taken into consideration, it appears that Permixon® is useful drug in the first line treatment not only of BPH but also of prostatitis. By having such widespread effects, Permixon® treats many of the symptoms of prostate disease without the need to administer several drugs and thereby reducing the number of possible side effects and interactions this would involve.

The next consideration has then to be the tissue specificity of Permixon®. As it is a mixture of several compounds, there is a strong possibility that these compounds will damage and affect other cells in the same manner as it does prostate cells. The data obtained from electromicroscopic investigation of the structure of Permixon® treated primary cultured of breast and skin demonstrated no disruption of the intra-cellular membranes. There were no detectable changes in the treated cells compared to the controls. This suggests that the compounds, which comprise Permixon®, are in some way selective to the prostate and have no physical effects on non-prostate derived cells.
Although this would appear to be a strange result, it is in keeping with the data published by [222] in which they demonstrated that radiolabelled fatty acids accumulated in the prostate in much higher concentrations than in any other gland. This selectivity of Permixon® for the prostate also opens the possibility of using Permixon® as a carrier drug for drugs used in the treatment of CaP. By accumulating the carrier drug in the gland, it becomes possible to deliver a toxic drug to the required area without risking damage to other unaffected but susceptible tissues. Thereby the side effects of many drugs could be reduced and treatment achieved more effectively.

4.5 Future Studies.

As the data presented in this thesis demonstrates, there is still a great deal of research to be done on the role of epithelial-fibroblast interactions in the prostate and the effect of these interactions on prostate disease. There is also much to learn about Permixon® and its mode of action in the prostate. With further investigation, it may be possible to answer many of the questions, which remain. Studies, which could be carried out to further the work presented here, are:

1. The co-culture model has been partially characterised in terms of the cell types utilised and the effect of co-culturing on some of these factors. It would however be useful to further investigate the interaction of these cells in the co-culture model and to look at the effect of epithelial secreted factors on fibroblast cells. Further characterisation of the cell types is required in terms of the expression of differentiation markers by both fibroblast and epithelial cells. Although we were unable to detect the presence of any smooth muscles cells in the cultures by electron microscopy, it is feasible that these cells are present and may be differentiating from the fibroblast cells under the influence of epithelial cells. The detection of smooth muscle cells by immunohistochemistry was, until recently, unreliable due to the anti-bodies available cross reacting with fibroblast antigens. However, new anti-bodies have now appeared on the market which are specific for smooth muscle cell antigens and would allow the easy detection of this cell type in the co-culture model. It would also be useful to investigate the expression of PMA in the co-cultured epithelial cells. The co-culture
model also provides an opportunity to further investigate possible factors, which may affect the differentiation of basal epithelial cells into luminal epithelial cells.

2. The elucidation of the exact nature and identity of the protein factor secreted by fibroblast cells which stimulates the expression of 5α-reductase type II in epithelial cells and the factors which affect it's secretion from the fibroblast cells is an area which should be more fully investigated. Possible areas for investigation include the effect of a variety of pH's and different temperatures on the activity of the protein factor. Another area of further study is the effect of trypsin over time periods and the effect on the activity of the secreted factor. The effect of Permixon® on the expression of this protein by fibroblast cells also requires further investigation but this is dependent upon the further characterisation and identification of the secreted factor. Should Permixon® affect the expression of the secreted factor it would be a novel mode of action for the inhibition of 5α-reductase type II expression. By investigating the nature of the protein and possible factors, which control the expression, it may be possible through drug intervention to alter the secretion of this growth factor, which may offer a new treatment regime for the control of BPH.

3. Although there is evidence for biological functions for both 5α-reductase isoenzymes, it is necessary to determine the exact role they play in prostate biology and their link with prostate disease. Possible mechanisms of action and hypotheses have been propounded which offer explanations in keeping with presently available data. It is however necessary to determine which if any of these possible roles is the correct one. As 5α-reductase has been implicated rightly in the onset of BPH there has been much interest in developing effective drugs to inhibit the action of this enzyme. Many of these attempts have only been partially successful mainly due to our lack of understanding of prostate biology in health and disease. If 5α-reductase was the only cause of these disease states, then there would be no progression disease in patients receiving 5α-reductase inhibitors. As this is not the case, it becomes necessary to look towards other possible causative agents. To this end, it is necessary to investigate the role of growth factors in the prostate and their impact on cellular interactions with a view to better understanding the control of growth of stromal and epithelial
compartments of the gland. It is also necessary to investigate the role of 5α-reductase in the aromatisation of androgens to oestrogens. As detailed in the discussion, there exists the possibility that the inhibition of both isoforms of 5α-reductase may result in higher concentrations of oestrogens in the prostate which, along with increased concentrations of testosterone, may predispose to the development of BPH. Should this be proved to be the case in the human prostate, the use of 5α-reductase inhibitors in the treatment of BPH would become contra-indicated and another avenue for therapeutic intervention would be required.

4. Permixon® as has already been detailed is a complex mixture of plant sterols, fatty acids and flavonoids. The role of each of these compounds in the prostate needs to be investigated and also the factor(s) which result in the cell-damaging effects of Permixon®. Work presented here has shown that Permixon® appears to be selective for prostate cells when compared to the cell types we have so far investigated. This work needs to be expanded to include more cells types from other organs such as liver, kidney and seminal vesicle. Also, many of the changes seen in Permixon® treated cells indicate the induction of apoptosis. Whether this is indeed the case needs to be ascertained not only in-vitro but in-vivo too. This could be achieved fairly readily in-vitro through the use of one of several readily available commercial kits for the detection of apoptosis in cells. The possibility also exists that the apparent apoptotic events are in fact a result of terminal differentiation of the cell or indeed due to interruption of the cell cycle. Once again, these are areas, which require greater investigation. The fact that Permixon® appears to only target prostate cells would seem to indicate that these cells are different from other body cells in some way. Investigations into the lipid composition of the cell membranes and their interaction with some of the compounds found in Permixon® may provide a valuable insight into the mechanism by which Permixon® targets these cells. It may also allow the development of a more refined and effective treatment for the management of BPH.
REFERENCES CITED


2 Lowsley OS. The development of the human prostate gland with reference to the development of other structures at the neck of the urinary bladder. American Journal of Anatomy, 1912; 13: 299-349

3 Timms BG, Mohs TJ, Didio LJA. Ductal budding and branching patterns in the developing prostate. Journal of Urology, 1994; 151: 1427-1432


8 Cunha GR. Epithelio-mesenchymal interactions in primordial gland structures which become responsive to androgenic stimuli. Anatomical Record. 1972; 172: 179-196

9 Cunha GR. Tissue interactions between epithelium and mesenchyme of urogenital and integumental origin. Anatomical Record, 1972; 172: 529-536


30b Tut TG, Ghadessy FJ, Tifiro MA, Pinsky L, Yong EL. Long polyglutamine tracts in the androgen receptor are associated with reduced transactivation, impaired sperm production and male infertility. *Journal of Endocrinology & Metabolism*. 1997; 82: 3777-3782


58 Tenniswood M. Role of epithelial-stromal interactions in the control of gene expression in the prostate: an hypothesis. *The Prostate*, 1986; **9**: 375-385


80 Kyprianou N, Isaacs JT. Activation of programmed cell death in the rat ventral prostate after castration. *Endocrinology*, 1989a; **122**: 552-562


82 Chang C, Saltzman AG, Sorensen NS, Hiipaka RA, Liao S. Identification of Glutathione S-transferase Yb1 mRNA as the androgen repressed mRNA by cDNA
cloning and sequence analysis. *Journal of Biological Chemistry*, 1987; **262**: 11901-11903


84 Hsieh JT, Lin SH. Androgen regulation of cell adhesion molecule gene expression in rat ventral prostate during organ degeneration. C-CAM belongs to a class of androgen-repressed genes associated with enriched stem/amplifying cell population after prolonged castration. *Journal of Biological Chemistry*, 1994; **269**: 3711-3716


86 Franks LM, Barton AA. The effects of testosterone on the ultrastructure of the mouse prostate *in-vivo* and in organ culture. *Experimental Cell Research*, 1960; **19**: 35-50

87 Cunha GR. The role of androgens in the epithelio-mesenchymal interactions involved in prostatic morphogenesis in embryonic mice. *Anatomical Record*, 1973; **175**: 87-96


90 Beato M. Gene regulation by steroid hormones. 1989; **56**, 3: 335-344


100 Mahendroo MS, Cala KM, Landrum CP, Russell DW. Fetal death in mice lacking 5α-reductase type I caused by estrogen excess. Molecular Endocrinology. 1997; 11, 7: 917-927.


114 Berman DM, Russell DW. Cell-type specific expression of rat steroid 5α-reductase isozymes. *Proceedings of the National Academy of Science USA*, 1993; 90: 9359-9363


120 Blandy JP. The indications for prostatectomy. *Urologia Internationalis*, 1978; 33: 159-170


127 Habib FK, Ross M, Bayne CW, Grigor K, Buck AC, Bollina P, Chapman K. The localisation of 5α-reductase type I and II mRNAs in human hyperplastic prostate and in prostate primary cultures. *Journal of Endocrinology*, 1997; accepted for publication

128 Cabot AT The question of castration for enlarged prostate. *Annals of Surgery*, 1896; **24**: 265-309


133 Bosch RJ, Griffiths DJ, Blom J, Schroeder FH. Treatment of benign prostatic hyperplasia by androgen deprivation: effects on prostate size and urodynamic parameters. *Journal of Urology*, 1989; 141: 68-72


151 Wilding G, Chen M, Gelmann EP. Aberrant response in-vitro of hormone-responsive prostate cancer cells to antiandrogens. The Prostate, 1989; 14: 103-115

152 Schuurmans ALG, Bolt J, Mulder E. Androgens stimulate both growth rate and epidermal growth factor receptor activity of the human prostate tumour cell LNCaP. The Prostate, 1988; 12: 55-63


156 Veldscholte J, Ris-Stalpers C, Kuiper GGJM, Jenster G, Berrevoets C, Claassen E, van Rooij HCJ, Trapman J, Brinkmann AO. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and
response to ant-androgens. *Biochemical and Biophysical Research Communications*, 1990; **17**: 534-540

157 Grant ES, Batchelor KW, Habib FK. Androgen independence of primary epithelial cultures of the prostate is associated with a down-regulation of androgen receptor gene expression. *The Prostate*, 1996; **29**: 339-349

158 Gibas Z, Becher R, Kawinski E, Horoszewicz J, Sandberg AA. A high-resolution study of chromosome changes in a human prostatic carcinoma cell line (LNCaP). *Cancer Genetics and Cytogenics*, 1984; **11**: 399-404


160 Pousette A, Carlström K, Henriksson P, Grande M, Stege R. Use of a hormone-sensitive (LNCaP) and a hormone-resistant (LNCaP-r) cell line in prostate cancer research. *The Prostate*, 1997; **3**: 198-203


165 Burrows MT, Burns JE, Suzuki Y. The culture of bladder and prostate tumours outside the body. Journal of Urology, 1917; 1: 3-15

166 Webber MM. Growth and maintenance of normal prostatic epithelium in-vitro - a human cell model. Progress in Clinical and Biological Research, 1980; 37: 181-216


170 Buck AC. Phytotherapy for the prostate. *British Journal of Urology*, 1996; **78**: 325-336


180 Merchant DJ. Terminally differentiating epithelial tissues in primary explant culture: a model of growth and development. *In Vitro*, 1990; 26: 543-553


182 Wernert N, Seitz G, Achtstätter T. Immunohistochemical investigations of different cytokeratins and vimentin in the prostate from fetal period up to adulthood and in prostate carcinoma. *Pathological Research and Practice*, 1987; 182: 617-626


194 Ellsworth K, Harris G. Expression of the type 1 and 2 steroid 5α-reductases in human fetal tissues. *Biochemical and Biophysical Research Communications*, 1995; **215**: 774-780


202 Hyvtinen ER, Thalmann GN, Zhau HE, Karku R, Kallioniemi OP, Chung LW, Visakorpi T. Genetic changes associated with the acquisition of androgen-independent


204 Chang SM, Chung LWK. Interaction between prostatic fibroblast and epithelial cells in culture: Role of androgen. *Endocrinology*, 1989; **125**: 2719-2727


216 Iehle C, Delos S, Martin P-M, Raynaud J-P. Comparative inhibition of type 1 and type 2 5 alpha-reductase activity by the n-hexane lipid/sterol extract of *Serenoa repens* (Permixon®) and by finasteride. In *BPH: from Molecular biology to Patient Relief*. Eds. Dimopoulos CA and Di Silverio, Monduzzi Editore, Bologna, Italy. 1996: 22-33


221 Evans BAJ, Griffiths K, Morton MS. Inhibition of 5α-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavonoids. *Journal of Endocrinology*, 1995; 147: 295-302


PAPERS PUBLISHED
