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Prostate epithelial stem cell culture

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Abstract

The prostate gland is the site of the second most common cancer in men in the UK, with 9,280 deaths recorded in 2000. Another common disease of the prostate is benign prostatic hyperplasia and both conditions are believed to arise as a result of changes in the balance between cell proliferation and differentiation. There are three types of prostatic epithelial cell, proliferative basal, secretory luminal, and neuroendocrine. All three are believed to be derived from a common stem cell through differentiation along different pathways but the mechanisms behind these processes is poorly understood. In particular, there has until recently been very little information about prostate stem cell growth and differentiation. This review will discuss ways of distinguishing these prostate cell types using markers, such as keratins. Methods available for the culture of prostate epithelial cells and for the characterisation of stem cells both in monolayer and three-dimensional models are examined.

Abbreviations: AR, Androgen receptor; BPH, Benign prostatic hyperplasia; DHT, Dihydrotestosterone; K, Keratin; NE, Neuroendocrine; PSA, Prostate specific antigen; PAP, Prostatic acid phosphatase

Introduction

The prostate is part of the male reproductive system and is a small gland surrounding the urethra at the base of the bladder. The functions of the prostate are unclear although prostatic secretions make up around 30% of seminal fluid (Cunha et al., 1987) and may provide nutrients for sperm. One component, prostate specific antigen (PSA), a form of chymotrypsin, functions as an anticoagulant maintaining the fluidity of semen. Unfortunately the prostate gland has a high tendency to develop diseases later in life and prostate cancer is the second most common cancer in men in the UK, with 21,770 cases recorded in 1997 alone (Office of National Disease Statistics, UK data) and 9,280 deaths in 2000. Even more prevalent is benign prostatic hyperplasia (BPH) and most men will experience BPH symptoms in old age with around 10% of men over 50 requiring surgical intervention to relieve symptoms of obstruction. In middle age, the prostate begins to enlarge in the transition zone, an area that surrounds the urethra close to where it leaves the bladder. As BPH develops it compresses the urethra, restricting outflow and causing symptoms relating to bladder irritability and obstructed voiding. If left untreated BPH can result in more severe conditions including hydroureter and hydronephrosis (leading to kidney damage) and uninhibited detrusor contractions (resulting in incontinence).

Changes in the balance between proliferation and differentiation of epithelial cells are implicated in the aetiology of both BPH and prostate cancer (Isaacs and Coffey 1989; Bonkhoff and Remberger 1996). The elucidation of the control mechanisms of epithelial differentiation is therefore vital in understanding the development of prostatic disease. Until recently, however, there was very little information about these processes. Our understanding of prostate cell differentiation has been advanced by recent reports presenting evidence for the existence of epithelial stem cells in

this tissue (Hudson et al., 2000, Collins et al., 2001) and we are now in a better position to investigate the process of stem cell proliferation and differentiation.

The purpose of this review is to summarise the progress made to date in the identification and localisation of prostate epithelial stem cells and to describe methods for their *in vitro* culture.

Epithelial stem cells

Although it is believed that stem cells are involved in the development of both prostate cancer and BPH there has been little direct evidence for their existence or information on their characteristics. Stem cells are well characterised in other epithelial tissues, such as the gut (Potten and Loeffler 1990) and the skin epidermis (Jones and Watt 1993, Jones et al., 1995). In these tissues a small population of multipotent stem cells is responsible for maintaining the full repertoire of differentiated epithelial cell types and the stem cells have the capacity to self-renew throughout life. Under normal conditions a stem cell divides rarely, undergoing asymmetric division to give rise to a new stem cell along with a more differentiated transit amplifying daughter cell. Transit amplifying cells can divide rapidly but have a limited proliferative capacity and will ultimately leave the proliferative compartment to produce the various differentiated cell lineages. *In vitro*, however, stem cells have high clonogenicity and undergo rapid cell division (Jones and Watt 1993).

Epithelial cell types within the prostate

Prostate tissue consists of a complex system of branching epithelial ducts surrounded by a stromal matrix containing smooth muscle and fibroblast cells together with neurovascular components. The epithelium is arranged in two cell layers surrounding a central lumen. The innermost cells are the secretory 'luminal' cells and these sit on a continuous layer of smaller basal cells. As most proliferation occurs in the basal layer (Bonkhoff et al., 1994; Hudson et al., 2001) this is where the stem cells are believed to reside.

Different epithelial cell types in the prostate can be distinguished by the expression of a number of marker proteins. Cells in the luminal layer express the androgen receptor (AR), keratins (K) 8 and 18 and secrete prostate specific antigen (PSA) and prostatic acid phosphatase (PAP). Conversely, basal cells are non-secretory, have no androgen receptor expression and express CD44, K5 and K14. Interspersed amongst the basal and luminal cells is the largest number of neuroendocrine (NE) cells in any organ of the male or female urogenital tract. These are non-proliferative and express a range of endocrine specific markers such as chromogranin A, serotonin and neuron-specific enolase (Abrahamsson 1999) but do not express AR or Bcl-2. NE cells also stain positively for some exocrine markers such as PSA and keratins, indicating that they are of epithelial origin (Bonkhoff et al. 1994; Xue et al., 1997). It is believed that the three epithelial cell types, basal, luminal and neuroendocrine, are the progeny of a common stem cell produced via alternative differentiation pathways (Bonkhoff and Remberger 1996).

Keratins as markers of epithelial differentiation

Keratins are cytoskeletal proteins that perform structural functions within epithelial tissues. Expression patterns of members of the keratin family are distinctive for different epithelial tissues and cell types (Moll et al., 1982). The family members are divided into two major subgroups, basic, type I keratins (numbered 1–9) and acidic, type II keratins (numbered 10–20). Keratins are expressed in cells as heterodimers consisting of one polypeptide of each type. Basal layer cells in all multilayered epithelia express K5 with K14 (Purkis et al., 1990) but this expression is downregulated during differentiation and switched to alternative tissue specific keratin dimers as cells move into suprabasal layers. These newly expressed keratins include K1/K10 in cornified tissues, such as epidermis, K4/K13 in non-cornifying squamous epithelia, and K3/K12 in cornea. In glandular tissues, such as breast and prostate, the basal layer also expresses K5/K14, while luminal secretory cells express K8/K18.

Keratin expression patterns in the prostate have provided evidence for other epithelial cells with phenotypes intermediate between those of basal and luminal cells. Occasional cells in the luminal layer express both K5 and K18 while a sub-population of basal cells lacks K14 but expresses low levels of K18 with K5 (Verhagen et al., 1992; Xue et al., 1998; van Leenders et al., 2000). We have also shown that the intermediate basal K14-negative populations additionally express keratins 15, 17 and 19. We found K19 to be expressed by some basal and some luminal cells but also in a subset of cells that appear to be located midway between the two. This led us to propose K19 as a marker of cells undergoing the transition between the basal and luminal cell layers (Hudson

et al., 2001). This differentiation pathway is shown diagrammatically in Figure 1. The stem cells reside within the K5/14 expressing population and as cells begin to differentiate K14 expression is lost, to be replaced by other keratins including K15, K17 and K19. K15 and K17 expression is limited to basally located intermediate cells and, as the cells move up out of the basal layer only K19 expression is maintained in the luminal layer. As the luminal cells differentiate further K19 expression is lost and these cells only express K8/18. K19 has been implicated in the differentiation of other epithelial cell types, such as oral epithelium, mammary gland ducts and the hair follicle (Stasiak et al., 1989). It is postulated to act as a switch keratin that permits the changeover from one cytoskeleton type to another, in the case of prostate from K5/14 to K8/18.

The epithelial stem cell origin of neuroendocrine (NE) cells is still somewhat controversial. One alternative school of thought believes that, in common with other neuroendocrine cells, they are derived from the neural crest during embryogenesis. (Aumuller et al. 1999, 2001). Recently, however, Rumpold and colleagues (Rumpold et al. 2002) demonstrated that the differentiated cells that emerge from previously basal primary cultures included some cells that expressed chromogranin A, together with K5, K14 and K8. This supports the stem cell theory that NE cells are produced by the differentiation of basal epithelial cells along an alternative pathway to that producing the luminal cells.

The idea that the stem cells are in the basal layer has been reinforced by experiments carried out in a rat androgen ablation model. Castration of male rats to eliminate testosterone production results in rapid involution of the prostate. However, while there is an almost total loss of androgen dependent luminal cells, the basal layer remains relatively intact. Upon exogenous re-administration of androgen, the luminal epithelium regenerates through rapid expansion of a cell population with keratin expression patterns intermediate between that of basal and luminal cells as described above.

Recent advances in prostate cell culture systems have led to an increasing number of studies aiming to identify and characterise prostate stem cells. Some of these are described below.

Prostate epithelial cell culture

Prostate epithelial cells can be cultured in two ways, either as mixed cultures from tissue explants or clonally from a single cell suspension, depending on the requirements of the study. In explant or epithelial organoid culture, cells grow out from minced or partially digested tissue pieces. This method is widely used as it is relatively easy to carry out and large cell numbers can be obtained for study. However, the starting material is fairly heterogeneous and a mixture of cell types, including migrating differentiated epithelial cells and fibroblasts, can emerge from the organoids. Since early studies describing the growth of cells from organoids and the optimisation of serum free media (Lechner et al., 1980; Peehl and Stamey, 1986) there have been many papers published that use this method. The techniques for the isolation of cells used in these studies are basically the same. Prostate tissue obtained from radical prostatectomies or transurethral resection of the prostate (TURP) is minced into 1mm pieces and either plated directly into flasks with medium (van Leenders et al., 2000; Rumpold et al., 2002) or digested overnight in collagenase solution to separate the stromal material and cells from the epithelia acini (Robinson et al., 1998; Fry et al., 2000). Initial outgrowths from organoids produced either way are of predominantly basal cells, expressing K5/K14. With time, another basal phenotype emerges that contains cells that express K5/K8 and not K14 (van Leenders et al., 2000) and this has been classified as an intermediate population. As these cultures reach confluence more differentiated cell types appear and gland-like buds can be seen that have basal cells surrounding luminal type cells in the centre.

A major disadvantage of the explant growth method is that it is not possible to follow the lineage of cells in the cultures. Therefore, in order to study clonogenicity within the tissue, it became necessary to develop methods of growing cells from a single cell suspension. Peehl and colleagues (Peehl et al., 1988) demonstrated that it is possible to grow cells at very low densities (as low as 100 cells per 6cm dish) in serum free medium containing bovine pituitary extract. Our group and others have proceeded to study the different types of colonies formed in such cultures to look for evidence of proliferative heterogeneity, consistent with the presence of stem cells and transit amplifying populations.

Prostate stem cell cultures

In order to identify and characterise stem cells in culture we used a clonal growth assay with freshly isolated prostate epithelial cells plated at low dens-

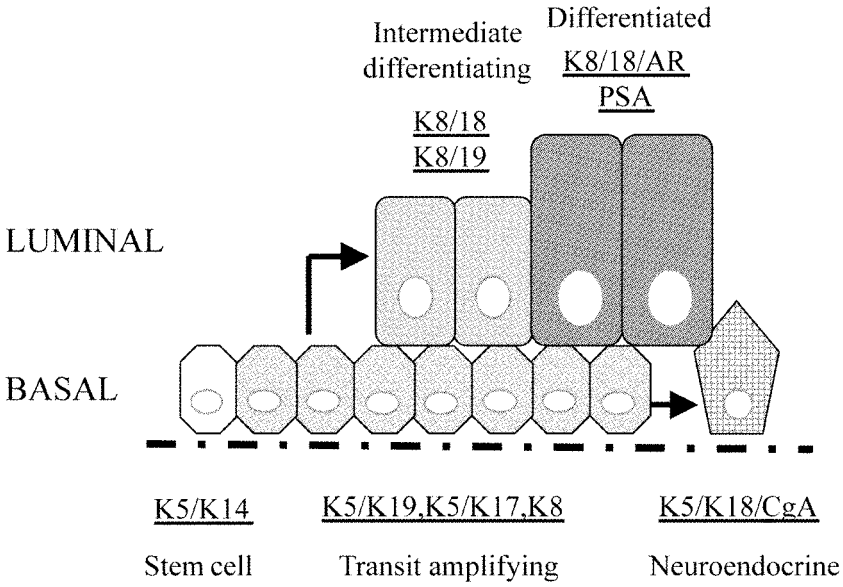

Figure 1. Hypothetical differentiation pathway for human prostate epithelial cells based on patterns of keratin staining. Basal stem cells (K5/14 only) give rise to an intermediate population expressing K19 along with K15 and K17. These cells differentiate into luminal cells with transient expression of K19 together with K8/18 before loss of K19 expression and onset of secretion of PSA and PAP signals complete differentiation. In an alternative pathway intermediate cells begin to express chromogranin A (CgA) and differentiate into neuroendocrine cells. (Redrawn from Hudson et al 2001 with permission of the Histochemical Society, 2002).

ity (1000 cells per 6cm dish) on a feeder layer of irradiated mouse 3T6 cells. We showed that primary epithelial cells had a colony forming efficiency of around 6% of plated cells (Hudson et al., 2000). Colonies containing 32 or more cells after 14 days growth were classified into two types, termed types I and II, based on their morphology and size (Figure 2). The majority of colonies formed were irregular in shape and contained between 32 and 8,500 cells at a density of around 30 cells per linear mm. These were termed type I. Type II colonies were larger that type I, containing between 8,000 and 40,000 cells after 14 days, with a high cell density of 30 to 50 cells per linear mm. Immunostaining for keratins showed most cells in type II colonies to be positive for K14, with an absence of K8. Conversely type I colonies contained a mixture of small K14 and larger K8 positive cells with many intermediate cells expressing both keratins. We postulated that type II colonies are established from stem cells with the more differentiating type I colonies being the progeny of transit amplifying cells. Type II colonies represented approximately 10% of total colonies formed, giving a stem cell frequency of around 0.5% of plated cells. As there are three times as many cells in the luminal layer of prostate epithelium as in the basal layer (Hudson et al., 2001) the stem cell frequency in basal cells is around 2%.

Keratinocyte stem cells have been shown to have high cell surface levels of functional α 2 and β 1 integrins and these has been used to isolate these cells using their rapid adhesion to extracellular matrix (ECM) proteins, such as collagen 1 (Jones and Watt, 1993). We analysed adhesion of primary prostate cells to type I collagen and showed that we too could enrich for a proliferative population that included the stem cells although it did not separate type I and type II colony forming cells. Subsequently Collins and co-workers (Collins et al., 2001) also described the selection of colony forming cells by rapid attachment to ECM and additionally used flow cytometry to show that the most rapidly adherent population had the highest levels of cell surface α 2 integrin. This population had the highest colony forming ability and the rapidly attaching cells mostly expressed K5/14.

Although monolayer cell cultures are useful for the study of stem cell clonogenicity and proliferation, the differentiation of their progeny is fairly limited and the expression of functional markers such as the secretion of PSA and PAP are rarely seen. To grow prostate cells under more physiological conditions several

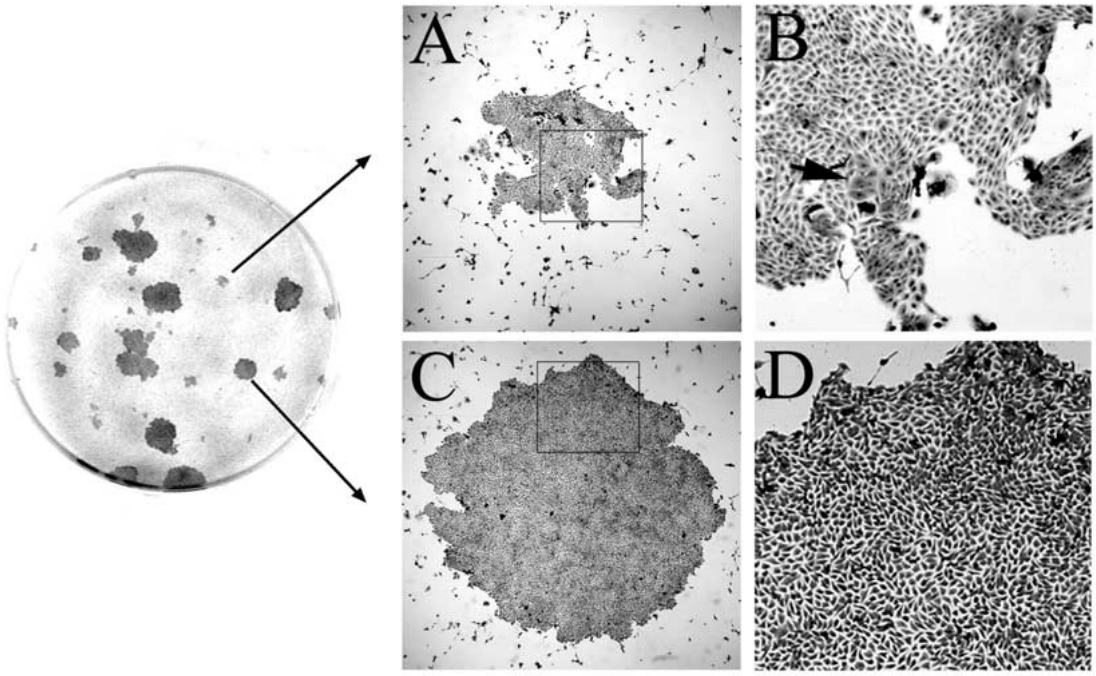

Figure 2. Colony types formed in primary cultures of prostate epithelial cells. Freshly isolated epithelial cells (10^3) were plated onto a 6 cm dish with a feeder layer of irradiated 3T6 cells and grown for 14 days. The cells were fixed and stained with 1% Nile blue/rhodamine. Two types of colony were formed: Type 1 (A and B) are relatively small with a mixture of small proliferative and larger differentiated cells (arrow in B). These colonies are irregular in shape, have a cell density below 30 cells per linear mm and contain between 32 and 8500 cells. Type II colonies (C and D) and are large and regular in shape. They consist mainly of small cells and have a high density of between 30 and 50 cells per mm. Type II colonies contain between 8,000 and 40,000 cells after 14 days of culture.

groups, including us, have used three dimensional model systems.

Three dimensional stem cell culture

When cells are grown in monolayer culture they quickly lose their more differentiated phenotypes. This is largely as a result of differentiating cells being shed into the medium and a failure to develop the intercellular interactions between cells that occur in three-dimensional tissues. One culture method that more closely mimics the in-vivo situation is the growth of cells suspended in a semi-solid gel. The most effective medium used to date is Matrigel, a reconstituted extracellular membrane produced by Engelbroth-Holm-Swarm (EHS) mouse sarcoma cells. It consists mainly of laminin with type IV collagen, heparan sulphate proteoglycans and nidogen (Kleinman et al., 1982). It additionally contains a number of growth factors including FGF, TGF β and tissue plasminogen activator. At room temperature and above Matrigel polymerises into a biologically active matrix that resembles *in vivo* mammalian basement membrane.

Experiments comparing culture methods carried out by Fong and colleagues (Fong et al., 1991) showed that when grown on plastic, epithelial cells grew in a monolayer with a flattened spread morphology. In contrast, cells seeded onto Matrigel assumed a rounded morphology with cells aggregating into organoid-like clusters that could be maintained for up to three weeks. Analysis by electron microscope of these clusters showed multiple cell layers connected by desmosomal structures. Cells containing secretory vesicles surrounded a central lumen that accumulated secreted material. While the cells on plastic grew rapidly those on Matrigel maintained a steady cell number. There were also striking differences in functional differentiation of the cells with a more than 8-fold increase in PSA and a 5-fold increase in PAP secretion in Matrigel compared to that on plastic. This could be further enhanced by the addition of stromal conditioned medium and di-hydrotestosterone (DHT) to the growth medium.

Since one of the characteristics of a stem cell is pluripotency, the ability to regenerate several different

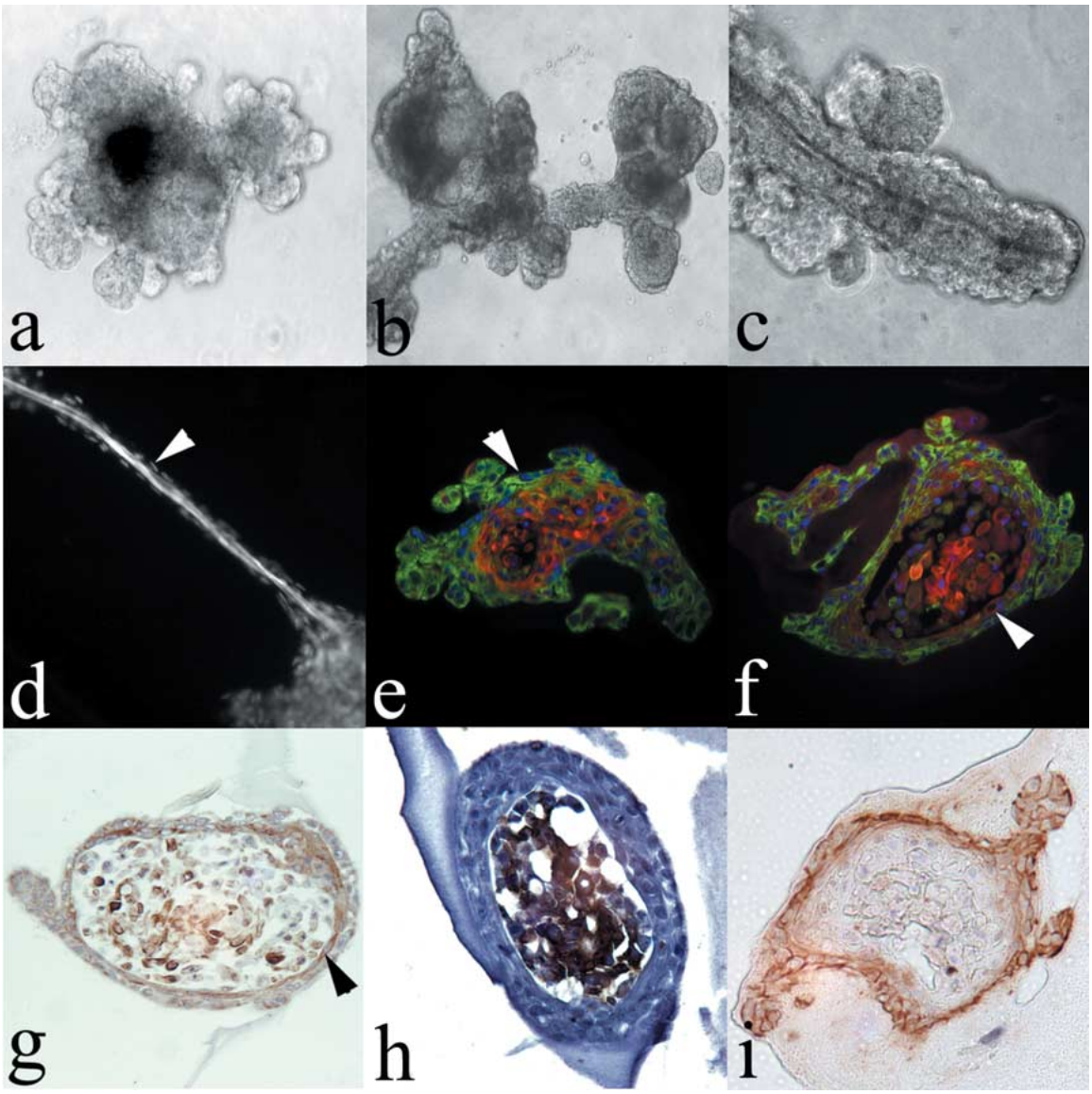

Figure 3. Analysis of three-dimensional epithelial cell cultures. Type II colonies were ring cloned and transferred to individual wells of a 24-well plate containing matrigel. Cells were grown for 17 days in the presence of 3T6 cell conditioned medium with 10^{-9} M mibolerone and photographed. Cultures were then fixed, paraffin embedded, and 5 μm sections cut. a-c) Phase contrast images of unfixed structures showing acini budding and duct formation. d) Hoechst stain showing detail of connecting ductal structure. e-f) Sections through spheroid double stained for; e) K14 (green) and K17 (red). f) K14 (green) and K19 (red). Antibodies detected with IgG isotype specific conjugates. Note basal K14 staining (arrow in e) and luminal staining for K19 (arrow in f). g-i) Sections through spheroid stained with anti K8 (g), anti-androgen receptor (h) and anti-CD44 (i) detected with DAB using the Vector Elite kit. Figures 2 and 3 reproduced and modified from Hudson et al. (2000) with kind permission of the publishers, Lippincott Williams and Wilkins 2002.

cells types from a common progenitor, we analysed the ability of our putative stem cell populations to differentiate fully in this more physiological three-dimensional environment. (Hudson et al., 2000). Cells were prepared as before and grown at clonal density for 14 days. Type II colonies were individually harvested using cloning rings and all the cells from individual colonies transferred separately to wells containing Matrigel. Cells were cultured for up to 17 days in the presence of 3T6 conditioned medium and DHT. After as little as 3 days the cells formed aggregates that developed, by 7 days, into three-dimensional spherical

structures with side branches (Figure 3a,b). As these elongated there was the appearance of thin tubular structures running through the centre (Figure 3c). Occasionally these spheres appeared to be connected by ductal structures and Hoechst staining showed these to consist of a monolayer of cells surrounding a tube (Figure 3d).

The cells within these spheroid structures were well organised into two or more cell layers surrounding a central lumen containing shed differentiating cells. Keratin staining revealed that the basal layer stained strongly for basal markers CD44 and keratins K5 and K14 while the inner layers stained more strongly for K17 and K19, putative transit amplifying cell markers (Hudson et al., 2001) together with luminal marker K8 (Figure 3 e-i). Cells that had accumulated in the lumen stained positively for androgen receptor but failed to express either PSA or PAP. Thus cells from the type II colonies are capable of maintaining prostate gland-like structures with both a proliferative basal layer and differentiating luminal cells, consistent with them being progeny of stem cells. It appears therefore that, although the cells are capable of undergoing differentiation along the previously described keratin pathway, secretory functions are still lacking in this model. This may be due to the fact that the cells have been cultured on plastic for 2 weeks before transfer to Matrigel and all studies where PSA secretion has been reported, either in coculture or in three-dimensional systems, have involved the direct use of freshly isolated cells (Bayne et al., 1998; Collins et al., 2001; Lang et al., 2001). It is possible that some irreversible loss of androgen response occurs or that there is a signal required to reactivate PSA secretion in a culture once it is lost. Recently Lang et al (Lang et al., 2001) further optimised this system and showed that the addition of 2% serum to the growth medium, together with oestrogen produced the most complete differentiation of primary cells in Matrigel and this may help clonal three-dimensional cell cultures too.

Conclusions

Great advances have been made in recent years in prostate epithelial cell culture and this has allowed more studies into the stem cell characteristics of the tissue. It is clear that there are a small number (1–3%) of basal epithelial cells that have both proliferative and pluripotent characteristics consistent with those of stem cells. Whilst we can produce cultures containing colonies we believe to be the progeny of stem cells our ability to purify these cells is still limited. It is likely that the type II colonies produced by stem cells contain a mixture of both stem and transit amplifying cells since sub-culture of these colonies produces cultures with a mixture of colony types, similar to those produced by keratinocyte stem cells (Barrandon and Green, 1987). We have, however, yet to identify a marker for stem cells, although appear to be a subset of cells within the high β 1 expressing population. Advances in cDNA microarray technology (Richardson et al., 2002) and flow sorting techniques (Bhatt et al., 2002) over recent years are now beginning to have an impact on prostate research and it is only a matter of time before definitive markers for the stem cell population are available.

Recently the idea that tumours also have a stem cell hierarchy has regained acceptance (Reya et al., 2001). Since tumours often contain both differentiated and undifferentiated cells it is possible that these represent stem and transit amplifying populations. An important aspect of prostate cancer treatment is that therapies, such as androgen ablation, often alleviate symptoms by reducing tumour load but ultimately hormone refractory secondary tumours return. It is possible that the androgen independent cells are a stem cell population that turns over very slowly but whose transit amplifying progeny fail to survive until further mutations allow them to grow in the absence of hormones. It may therefore be advantageous to target this population. It is not at present, however, known if cancer stem cells are similar to normal stem cells (Bonkhoff, 1996) or if they more closely resemble transit amplifying cells (van Leenders and Schalken, 2001; Verhagen et al., 1992) or even if they arise through the de-differentiation of luminal cells (Nagle et al., 1987; Liu et al., 1999). The identification of a definitive normal prostate epithelial stem cell population would solve this question and have a potentially enormous influence on the development of future prostate cancer therapies.

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