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14. ABSTRACT This project aims to identify adult prostate stem cells, using tissue recombination technology. To date, we have successfully directed the differentiation of mouse and human embryonic stem (ES) cells as outlined in the original statement of work. We have shown pathologically and histologically that the resultant tissue recombinants are highly characteristic of mouse and human prostate. We have refined the technology so that we reliably get prostate-like tissues in the absence of spontaneous differentiation of ES cells into non-prostate like tissues. We are currently restrained by the technology associated with the transfection of human embryonic stem cells, but hope to complete all the tasks of the project during 2005. This work has been presented at a total of 6 meetings including both national meetings within Australia internationally in the USA. We plan to publish the initial findings early in 2005. Work is ongoing to tag and isolate epithelial cells from these tissues in order to identify the adult prostate stem cell.					
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INTRODUCTION

This project set out to use human embryonic stem cells as a source of normal human prostate epithelial cells. Normal human prostate tissue from adult men in the prime of this life is difficult to obtain, and human fetal tissue is of limited availability. We successfully achieved this goal and recently published the findings in Nature Methods (Taylor et al., 2006).

The intended use for this model was to identify the adult stem cell population within the prostate gland. It is well accepted that stem cells reside within the prostate epithelium and that these cells have a major role to play in the initiation and progression of prostate carcinogenesis; therefore identifying these cells will have major implications in treating prostate cancer.

BODY

Task 1: To generate prostatic ductal structures from mouse ES cells using tissue recombination techniques (months 1-6).

When the project was initiated, mouse ES cell lines were the most well characterized and easily accessible. Therefore we established the protocols for tissue recombination with ES cells using mouse ES cells.

We were able to establish protocols for pre-inducing mouse ES cells to form endoderm in a hanging drop culture system, generating embryoid bodies (EBs). In addition, we were able to utilize these EBs in tissue recombination experiments with both rat and mouse urogenital mesenchyme (UGM) and seminal vesicle mesenchyme (SVM). These tissues proved to be a suitable source of inductive instructive mesenchyme in that they directed differentiation of mouse ES cells into prostate-like tissue structures. This result was consistent and reproducible over multiple experiments. The tissues were analyzed by pathological and histological techniques, and revealed appropriate cellular structures and biological markers as one would expect of mouse prostate.

One difficulty we faced was finding a definitive marker for mouse prostate tissue. There are several markers that are characteristic of prostate tissue, but very few that are prostate-specific. Prostate specific antigen (PSA) is the gold standard in the field, but the antibody that is available does not

recognize mouse prostate epithelial cells. Alternatively, Donjacour and Cunha (1993) developed an antibody against mouse prostatic protein secretion, named mDLP, that recognizes some mouse prostate lobes, but we could not get positive immunolabeling on our ES cell recombinants. Since we are using SVM and UGM to differentiate mouse ES cells, we were not certain of the analogous mouse prostate lobe that was being created and perhaps that was the problem with the staining.

Since there was no easy way around this problem (short of raising a new panel of antibodies to mouse prostate), we decided to move directly onto task 4, using human ES cells, since the PSA antibody was available and would definitely identify human prostate epithelium. Currently the results from Task 1 are unpublished, but were a critical in establishing the model. These experiments provided 'proof of concept' that rodent prostatic mesenchyme was capable of inducing prostatic differentiation in ES cells.

Task 2: To use these tissues for the isolation of sufficient numbers of basal and intermediate cell types from the epithelia, that can be tagged with appropriate markers prior to differentiation (months 6 – 18).

During the course of this project we worked towards this aim, but failed to complete this task. As mentioned, we concentrated on establishing the differentiation model using human ES cells (task 4), rather than mouse ES cells as originally planned. The technology for transfecting human ES cells has been troublesome to date, but recent developments mean that this task is now possible and we hope to complete this in the future.

Nonetheless, we successfully generated two constructs that can be used to transfect ES cells prior to recombination in preparation for cell sorting following the grafting period (task 3). The first construct is the human keratin 18 gene promoter driving DsRed expression and the second is the human keratin 5 gene promoter driving GFP expression. These constructs are available for use as soon as the appropriate transfection technology can be employed.

Task 3: To determine which cell type is the prostatic stem cell of the epithelia (months 12-24)

We did not make any progress on this task since we were unable to successfully transfect ES cells with our tagging constructs. Once we achieve that aim, we will embark on tracking prostate epithelial differentiation using the methods described in this task.

Several recent publications described the isolation of normal prostate stem cells (Collins et al., 2001; Richardson et al 2004) and prostate cancer stem cells (Collins et al., 2006) from primary tissue samples. These papers describe a number of cell surface markers that are useful in isolating prostate stem cells including CD133, CD44 and $\alpha 2\beta 1$ integrin. These cell surface markers were not available when this project was initiated and these recent reports may in fact alleviate the need to transfect the hESCs with our tagging constructs long term.

Task 4: To adopt the same strategies and techniques to prove the identity of human prostate stem cells using human ES cells (months 24-36).

Although we originally stated that we would not undertake these experiments until months 24-36 of the funding, we initiated these studies within the first year. Human and mouse embryonic stem cells grow under distinctly different culture conditions and therefore we needed time to adapt the recombination technique for hESCs.

In summary, we successfully established the tissue recombination technique to direct the differentiation of human ES cells into prostate tissues and these findings were recently published in Nature Methods (Taylor et al., 2006). We described fetal and mature prostate tissues derived from hESCs in terms of a number of markers for epithelial cells (CK8, 18, p63, chromogranin A) and stromal cells (smooth muscle actin) as well as prostate-specific proteins including Nkx3.1 and prostate-specific antigen (PSA), and hormone receptors including androgen receptor, estrogen receptor α (ER α) and estrogen receptor β (ER β) [please see published results in appendix 1; Figure 1 and Figure 2].

The final year of this funding has concentrated on 1) exploring the relative efficiencies of SVM versus UGM in directing the differentiation and 2) demonstrating biological functional responses in the hESC-derived prostate grafts.

1) We originally hypothesized that UGM and or SVM could direct the differentiation of hESCs into the endodermally derived prostatic lineage. We found that all SVM+hESC and UGM+hESC recombinants directed hESCs to differentiate into glandular structures with 27-47% of each recombinant graft identifiable as glandular tissue after 8-12 weeks of growth. We found that only some of these glands were immunopositive for PSA (definitive prostate marker). As shown in the table 1, there was a significant difference in the efficiency of each mesenchyme; rat SVM (~81%) was a more powerful inducer of prostate than rat UGM (~11%).

Recombinant Type	% of Recombinant that is Glandular Tissue	% of Glandular Tissue that is PSA Positive
hESC alone	38.49 $\pm$ 5.41	0
UGM alone ND	ND	ND
SVM alone	ND	ND
UGM + hESC	47.13 $\pm$ 4.77	11.56 $\pm$ 4.37
SVM + hESC	27.01 $\pm$ 3.24	81.17 $\pm$ 3.78

Mean $\pm$ SEM; $n = 4$ per recombinant type ($n = 2$ at 8 and 12 week time points per recombinant type)
 ND: Non-Detectable

Table 1: The proportion of glandular tissue expressing PSA in tissue recombinant following 8-12 weeks growth *in vivo*. Using a systematic uniform random sampling procedure, the entire tissue recombinant was examined to determine the percent of glandular tissue expressing PSA ($n = 4$ for each group). This table shows no PSA was expressed in the teratomas formed from hESC alone and in UGM and SVM controls due to a failure to develop glandular structures. Although the percentage of glandular tissue in UGM + hESC recombinants compared to SVM + hESC recombinants is higher, the conversion to glandular tissue expressing PSA is greater in SVM + hESC recombinants.

2) Androgen withdrawal by surgical or chemical castration is a classic technique used to study the biological function of prostate. Therefore we tested the effects of androgen withdrawal in SVM + hESC tissue recombinants. Tissue recombinants were grown for 8 weeks in intact male hosts prior to surgical castration and removal of testosterone implant in host SCID mice; grafts were harvested 4 days later. Upon castration of host mice, androgen deprivation led to regression and atrophy in SVM + hESC recombinants, as shown in Figure 1.

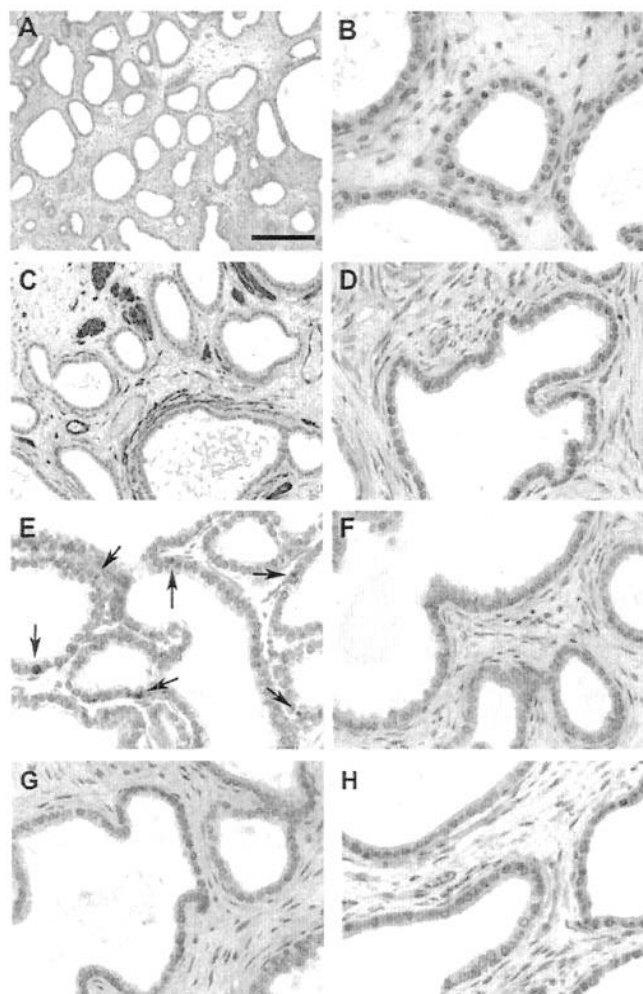


Figure 1: *Glandular regression of prostate tissues derived from SVM + hESC following androgen withdrawal by castration. A, B.* Androgen deprivation led to regression and atrophy in SVM + hESC tissue recombinants, 4 days after castration. **C.** Immunolocalisation of smooth muscle α -actin revealed stromal de-differentiation. **D.** Atrophy of the secretory epithelium was associated with an apparent increase in the proportion of p63-positive cells lining the glands. **E.** Apoptosis was detected in epithelial cells post-castration and labeled by Apoptag (marked by $\rightarrow$). **F-H.** Androgen receptor (**F**) and ER β (**G**) localization were significantly decreased following androgen withdrawal, whilst epithelial ER α was up-regulated (**H**). Bar = 125 μ m (A, C), 25 μ m (B, D-H)

In summary, we have established a model of normal human prostate tissue derived from human ES cells. Glandular epithelial cells derived from hESCs show normal epithelial differentiation and therefore future work will use this model further understand the stem cell hierarchy in human prostate.

KEY RESEARCH ACCOMPLISHMENTS

List of key research accomplishments emanating from this research:

- Proved concept of controlled differentiation of ES cells using tissue recombination.
- Directed differentiated mouse embryonic stem cells into prostate-like tissues (unpublished).
- Directed differentiated human embryonic stem cells into prostate-like tissues (published).
 - Generated human prostate tissue expressing prostate-specific antigen (PSA) within 8-12 weeks in vivo.
 - This tissue was functionally responsive to androgen withdrawal, resulting in glandular regression and atrophy.
- Generated constructs for CK18 and CK 5 for future transfections.

REPORTABLE OUTCOMES

	<i>Reportable outcomes that have resulted from this research:</i>
Manuscripts	• Taylor RA, Cowin PA, Cunha GR, Pera M, Trounson AO, Pedersen J, Risbridger GP (2006) Formation of human prostate tissue from embryonic stem cells. <i>Nature Methods</i> 3(3):179-181.
Abstracts Presentations	1. Risbridger GP (2005) Stromal influences on prostate pathology. <i>2nd Pacific Rim Breast and Prostate Cancer Meeting</i>, Palm Springs, CA, USA 2. Taylor RA, McPherson SJ, Ellem SJ, Frydenberg M, Risbridger GP (2005) All power to the prostatic stroma. <i>Annual Scientific Meeting of the Urological Society of Australasia</i>, Melbourne, Australia. (Poster presentation) 3. Taylor RA, Cunha GR, Trounson AO, Risbridger GP (2005) Stromal microenvironment influences stem cell differentiation in normal and malignant prostate. <i>17th Lorne Cancer Conference</i>, Phillip Island, Australia. (Oral presentation) 4. Jarred RA, Wang H, Trounson AO, Risbridger GP (2004) Directed differentiation of human embryonic stem cells into prostate using tissue recombination. <i>The 86th Annual Meeting of the Endocrine Society</i>, New Orleans, USA. (#This abstract was selected for inclusion in Endo Newslite television program and was awarded travel grant from the Endocrine Society as well as the Australian Women in Endocrinology DSL New Investigator Travel Award; poster presentation) 5. Jarred RA, Wang H, Trounson AO, Risbridger GP (2004) Directed differentiation of human embryonic stem cells into prostate using tissue recombination. <i>The 2nd International Society for Stem Cell Research Conference</i>, Boston, USA. (Poster presentation) 6. Jarred RA, Wang H, Trounson AO, Risbridger GP (2003) Directed differentiation of human embryonic stem cells into prostate using tissue recombination. <i>The 1st National Stem Cell Centre Scientific Conference</i>, Melbourne, Australia. 7. Risbridger GP, Jarred RA, Wang H, Trounson AO (2003) All power to the prostatic stroma. <i>The 3rd National Prostate Cancer Symposium</i>, Melbourne, Australia.

Patents and licences	- Provisional patent application filed March 2002 (663129) - Full patent application filed March 2003 (663129)
Degrees obtained	Nil.
Development of cell lines	Nil.
Tissue or serum repositories	Nil.
Informatics such as databases and animal models	Nil.
Funding applied for based on this work supported by this award	Dr. Renea Taylor (nee Jarred) was awarded a Postdoctoral Traineeship Award from the Prostate Cancer Research Program, Department of Defense in 2004. <i>Title:</i> Role of tumor stroma in prostate carcinogenesis <i>Award number:</i> W81XWH-04-1-0047.
Employment or research opportunities applied for and/or received based on experience/training supported by this award	Dr. Renea Taylor's (nee Jarred) Postdoctoral Traineeship Award as described above. This position was awarded based on preliminary observations from this work to continue her studies in the laboratory of Professor Risbridger.

CONCLUSIONS

In summary, we have shown that normal prostate differentiation and development can be induced using chimeric rodent-human recombinations. Rodent prostatic mesenchyme was demonstrated to be a powerful inducer of prostate differentiation in human ES cells. The resultant tissues were morphological and functionally identical to human fetal and adult prostate. This is a reliable and reproducible model system that can be used to study human prostate development and maturation over 8-12 weeks and is comparable to the process that takes decades in the human male. This model can now be used to identify the adult prostate stem cell.

This finding will have major implications to our understanding of prostate biology. In the same way that differentiation of embryonic stem cells into neurons has advanced the neurological field, these findings will have a major impact on urology. This research will further our understanding of the factors that induce prostate differentiation, that have eluded us until now.

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Formation of human prostate tissue from embryonic stem cells

Renea A Taylor^{1,2,6}, Prue A Cowin^{1,6},
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John Pedersen⁵ & Gail P Risbridger¹

Rodent models and immortalized or genetically modified cell lines are frequently used—but have limited utility—for studying human prostate development and maturation. Using rodent mesenchyme to establish reciprocal mesenchymal-epithelial cell interactions with human embryonic stem cells (hESCs), we generated human prostate tissue expressing prostate-specific antigen (PSA) within 8–12 weeks. This human prostate model shows species-conserved signalling mechanisms that could extend to integumental, gastrointestinal and genital tissues.

Rodent models provide controversial evidence of early origins of adult disease, and it is imperative that we establish a model of normal human prostate that can be studied from development to maturation¹. The human prostate gland is a slow-growing organ that reaches functional maturity at puberty, and maturation is

marked by PSA secretion from glandular epithelial cells. The normal adult prostate is relatively growth-quiescent, but after a considerably long period of latency, growth resumes in aged men leading to benign enlargement or neoplastic disease. Normal human prostate tissue from adult men in the prime of their life is difficult to obtain, and human fetal tissue is of limited availability.

Our approach of generating human prostate fetal tissue that can mature to normal adult glands was based on the method of tissue

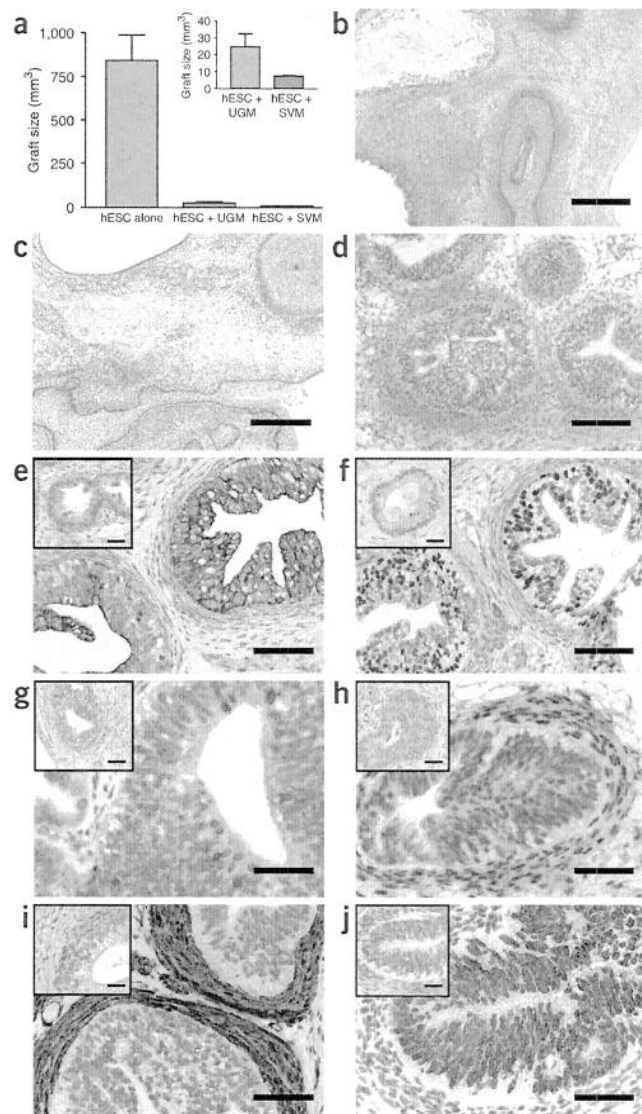


Figure 1 | Formation of prostate tissue from hESCs by tissue recombination with UGM or SVM. (a) Graft size measured after grafting of hESCs alone or in combination with UGM or SVM. Data are plotted as mean $\pm$ s.e.m. ($n = 5$). (b,c) hESCs grafted alone result in formation of teratoma (H&E-stained teratoma tissue, b). Immunohistochemical analysis of PSA expression in these teratomas indicates the absence of mature PSA-positive human prostate tissue (c). (d) H&E-stained SVM + hESC recombination tissue demonstrating presence of glandular structures. (e–j) Immunohistochemical analysis of these glandular structures in SVM + hESC recombination tissue was done; immunopositive cells were detected using the chromagen diaminobenzidine (brown stain). Localization of CK8 and CK18 identified epithelial cells in glandular structures (e). Presence of p63-positive cells within epithelium of glandular structures demonstrated stratification of epithelium and identified basal cells (f). Nkx3.1 expression analysis demonstrated the commitment to the endoderm prostate lineage (g). AR protein expression was evident in undifferentiated epithelial cells and surrounding mesenchyme, characteristic of immature human fetal prostate (h). Epithelial cords surrounded by thick sheaths of smooth muscle marked by smooth muscle α -actin (i). Epithelial cells were identified as human by immunolocalization of anti-human Lamin B1 (j). Insets, negative controls. Analysis was done 4 weeks after grafting (a–g,i) or 2 weeks after grafting (h,j). Bar, 125 μ m (b), 50 μ m (c–f), 25 μ m (g–j).

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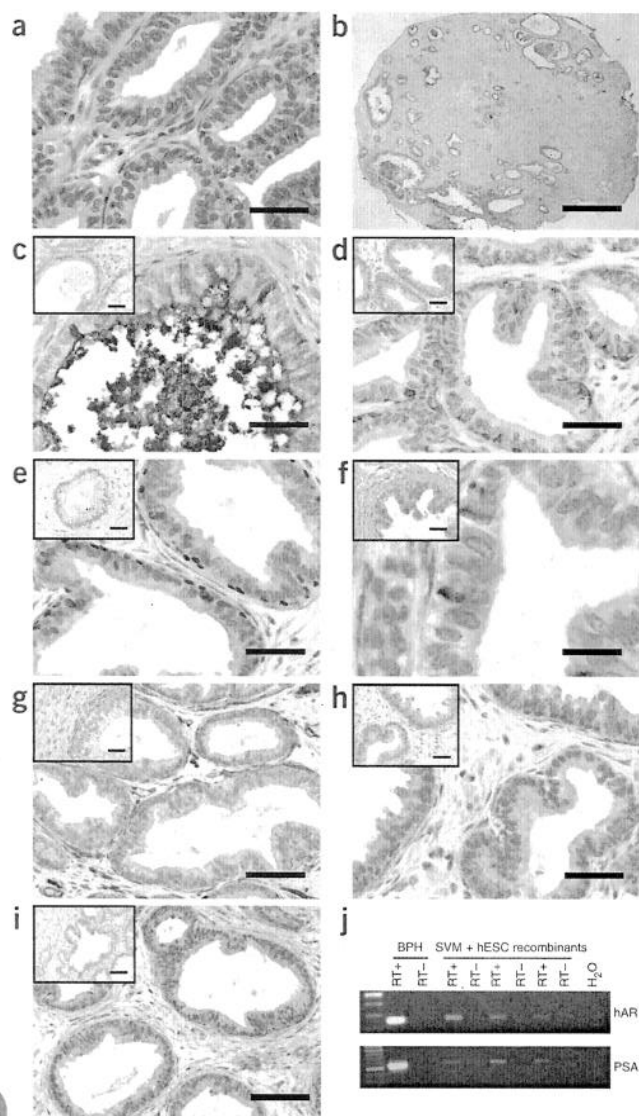


Figure 2 | Maturation of prostate derived from SVM + hESC after 8–12 weeks growth in intact male hosts. (a) H&E staining of normal benign human prostate tissue derived from SVM + hESC recombinants. (b,c) Immunolocalization of PSA in secretory epithelial cells. (d,e) Basal epithelial cells were detected by immunolocalization of high-molecular-weight cytokeratins (d) and p63 (e). (f) Neuroendocrine cells were rarely observed as evident by Chromagranin A expression. (g) Stromal differentiation resulted in organization of smooth muscle layers around the ductal structures marked by α -smooth muscle actin. (h,i) Immunolocalization of ER β (h) and AR (i) in both stromal and epithelial cells. (j) Analysis of expression of mRNA encoding human AR and PSA in three separate SVM + hESC recombinants using reverse transcriptase PCR. Benign prostatic hyperplasia (BPH)-positive control. Insets, negative controls. Analysis was done 12 weeks after grafting (a–g, i–j); or 8 weeks after grafting (h). Bar, 50 μ m (a,d,e,g–i), 125 μ m (b), 25 μ m (c) and 10 μ m (f).

and data presented here are derived from the directed differentiation of hES2 cells. We generated heterospecific tissue recombinants composed of mouse UGM or rat SVM and hESCs that were grown for up to 12 weeks ($n = 5$ per time point) under the renal capsule of intact adult male severe combined immunodeficiency (SCID) mice containing testosterone implants to augment androgen levels^{9,10}. (See **Supplementary Methods** online for descriptions of experimental procedures.) All animal handling and procedures were carried out in accordance with Australian National Health and Medical Research Council (NHMRC) guidelines for the Care and Use of Laboratory Animal Act and according to the Animal Experimentation and Ethics Committee at Monash Medical Centre, Clayton, Australia.

There was a reduction in graft size when SVM or UGM was combined with hESCs and grafted, compared to hESCs grafted alone (Fig. 1a). Sub-renal capsule grafting of undifferentiated hESCs resulted in the formation of large teratomas (Fig. 1b) confirming pluripotentiality of these cells as previously described¹¹, but the grafting did not result in formation of glandular tissue expressing PSA (Fig. 1c). UGM and SVM grafted alone did not develop into glands, as previously described¹². All SVM + hESC and UGM + hESC recombinants directed hESCs to differentiate into glandular structures with 27–47% of each recombinant graft identifiable as glandular tissue after 8–12 weeks of growth. PSA, a product of the secretory epithelial cells, is universally used as a marker of mature prostate function. Using PSA as the ‘gold standard’ of differentiation to human prostate, we showed the percentage of PSA-positive glandular tissue was 12% and 82% in UGM + hESC and SVM + hESC grafts, respectively. PSA was not expressed in mouse or rat mesenchyme or in hESC alone (see **Supplementary Table 1** online).

We observed immature glandular structures 2–4 weeks after grafting and expressed known markers of developing prostate epithelia including CK8 and CK18, p63, Nkx3.1 and androgen receptor (AR; Fig. 1d–h). We observed immature glands surrounded by α -smooth muscle actin–positive stroma (Fig. 1i). We confirmed that epithelial cells were human by immunolocalization of anti-human Lamin B1 (Fig. 1j).

At 8–12 weeks, we observed mature human prostate tissues derived from hESCs (Fig. 2) that were evident by hematoxylin and eosin (H&E) staining (Fig. 2a,b). Maturation was evident based on expression of PSA (confirmed by detection of mRNA that encodes PSA; Fig. 2j) by secretory epithelial cells associated with a basal cell layer expressing p63 and high-molecular-weight cytokeratin together with sporadic neuroendocrine cells identified by

recombination. Tissue recombination is a common and reliable method of inducing prostatic differentiation that has led to important advances in our understanding of prostate biology during development and disease^{2–5}—in particular, the concept that the mesenchyme is a key determinant, rather than a passive player, in the differentiation process⁴. We hypothesized that urogenital sinus mesenchyme (UGM) and/or seminal vesicle mesenchyme (SVM) could direct the differentiation of hESCs into the endodermally derived prostatic lineage. The rationale for using SVM is based on its ability to induce and support differentiation of seminal vesicle and anterior prostate, the near equivalent ability of SVM and UGM to induce prostatic differentiation from endodermally derived epithelia and the preservation of valuable breeding females when using neonatal SVM rather than embryonic UGM^{6,7}, as previously discussed⁵. The developmental outcome is determined by the germ-layer origin of the epithelia.

We obtained two hESC lines, hES2 (karyotype 46,XX; genetically female) and hES4 (karyotype 46,XY; genetically male) cells from ES Cell International Pte Ltd. and cultured them as previously described⁸. Differentiation was not influenced by stem cell gender,

chromogranin A (Fig. 2c–f). Patterning of the surrounding mesenchyme (marked by α -actin) was evidence of reciprocal interactions between rodent and hESCs (Fig. 2g). mRNA encoding AR as well as AR and estrogen receptor β (ER β) were expressed in the induced prostate epithelia indicating hormone-responsive tissue (Fig. 2h–j). Upon castration of host mice, androgen deprivation led to regression and atrophy in SVM + hESC recombinants (Supplementary Fig. 1 online).

This study is further evidence that signaling mechanisms are well conserved throughout species. We have shown that normal prostate differentiation and development can be induced using chimeric rodent-human recombinants. We report mesenchymal induction of immature and mature human prostate that is morphologically and functionally identical to human fetal and adult prostate from hESCs. This is a reliable and reproducible model system that can be used to study human prostate development and maturation over 8–12 weeks and is comparable to the process that takes decades in the human male. Furthermore, because the *in vivo* system uses rat mesenchyme and the grafts are hosted in mice, both the mesenchyme and host environment can be manipulated to identify critical systemic or local (stromal-epithelial) factors that influence prostate development and maturation.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

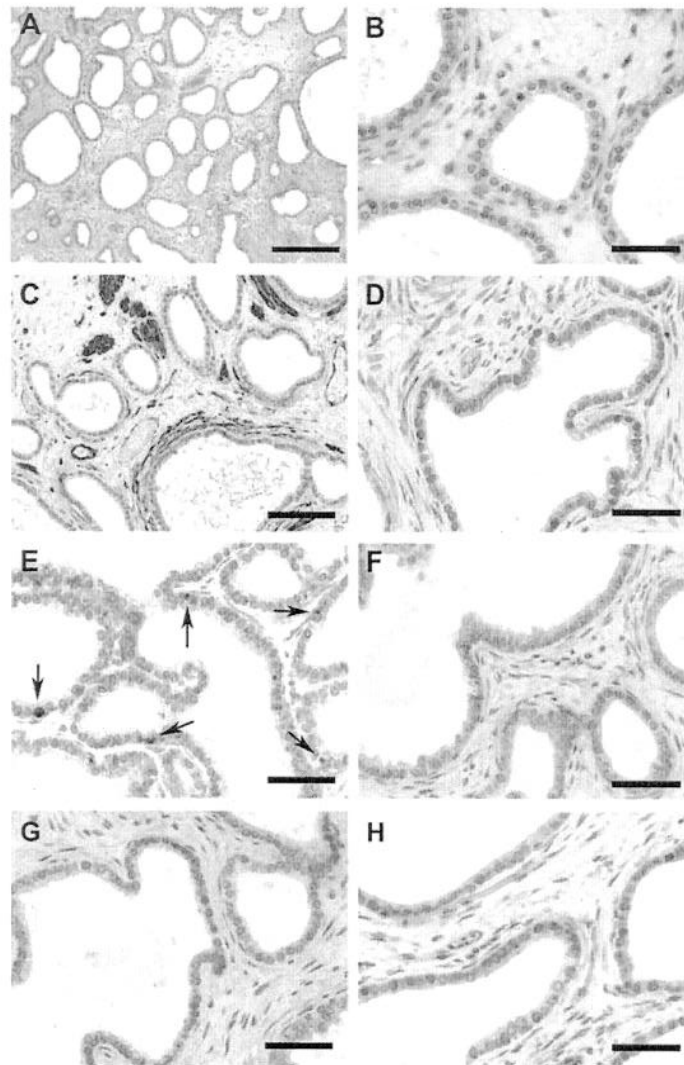
The authors declare that they have no competing financial interests.

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SUPPLEMENTARY FIGURE 1

Glandular regression of prostate tissues derived from SVM + hESC following androgen withdrawal by castration.



A, B. Androgen deprivation led to regression and atrophy in SVM + hESC tissue recombinants, 4 days after castration. **C.** Immunolocalisation of smooth muscle α -actin revealed stromal de-differentiation. **D.** Atrophy of the secretory epithelium was associated with an apparent increase in the proportion of p63-positive cells lining the glands. **E.** Apoptosis was detected in epithelial cells post-castration and labelled by Apoptag (marked by $\rightarrow$). **F-H.** Androgen receptor (**F**) and ER β (**G**) localization were significantly decreased following androgen withdrawal, whilst epithelial ER α was up-regulated (**H**).
Bar = 125 μ m (A, C), 25 μ m (B, D-H)

SUPPLEMENTARY TABLE 1

The proportion of glandular tissue expressing PSA in tissue recombinants following 8-12 weeks growth *in vivo*

Recombinant Type	% of Recombinant that is Glandular Tissue	% of Glandular Tissue that is PSA Positive
hESC alone	38.49 ± 5.41	0
UGM alone	ND	ND
SVM alone	ND	ND
UGM + hESC	47.13 ± 4.77	11.56 ± 4.37
SVM + hESC	27.01 ± 3.24	81.17 ± 3.78

Mean ± SEM

n = 4 per recombinant type (*n* = 2 at 8 and 12 week time points per recombinant type)

ND: Non-Detectable

The proportion of glandular tissue expressing PSA in tissue recombinant following 8-12 weeks growth in vivo. Using a systematic uniform random sampling procedure, the entire tissue recombinant was examined to determine the percent of glandular tissue expressing PSA (*n* = 4 for each group). This table shows no PSA was expressed in the teratomas formed from hESC alone and in UGM and SVM controls due to a failure to develop glandular structures. Although the percentage of glandular tissue in UGM + hESC recombinants compared to SVM + hESC recombinants is higher, the conversion to glandular tissue expressing PSA is greater in SVM + hESC recombinants.

SUPPLEMENTARY METHODS

Animals

Timed pregnant Balb/C mice were obtained from Monash University central Animal Services, killed at 16 days gestation (plug day = day 0) and urogenital sinuses obtained from male embryos as previously described [1]. Postnatal day 0 (day 0 = day of birth) male rats were obtained from Monash University Central Animal Services and seminal vesicles dissected. All animal handling and procedures were carried out in accordance with National Health and Medical Research Council (NHMRC) guidelines for the Care and Use of Laboratory Animal Act and according to the Animal Experimentation and Ethics Committee at Monash Medical Centre, Clayton, Australia.

Human Embryonic Stem Cells

Human embryonic stem cell lines, hES2 (karyotype: 46XX; genetically female) and hES4 (karyotype: 46XY; genetically male) cells were obtained from ES Cell International Pte Ltd and cultured as previously described [2]. Briefly, stem cell lines were cultured on mitomycin C mitotically inactivated mouse embryonic fibroblast feeder cells (isolated from day 13.5 post-coitum fetuses of 129/SV strain) in gelatin-coated tissue culture dishes.

Tissue Separation, Recombination and Grafting

Using a dissecting microscope (SZX12, Olympus Corporation, Tokyo, Japan) and dissecting tools, urogenital tracts and seminal vesicles were removed from male mice and rat pups respectively. Tissue collection involved microdissection in a modified watch glass (maximov depression slide; San Francisco, CA), in the presence of dissecting media (basal medium of Dulbecco's Modified Eagles Media (DMEM) and Hams F-12 (1:1 vol/vol) supplemented with penicillin and streptomycin (5mls/ltr) and fungizone (20µg/ml) at pH 7.3). Following digestion in 1% trypsin (Difco, Detroit, MI) in Hank's calcium and magnesium free Balanced Salt Solution (HBSS; Gibco,

Invitrogen, Vic, Australia) for 60 minutes, urogenital mesenchyme (UGM) and seminal vesicle mesenchyme (SVM) were obtained by mechanical separation and contained approximately 5×10^4 and 1×10^5 cells respectively. hESC colonies were manually propagated and in order to obtain standard sized pieces of hESC a colony was placed on a cutting grid and divided into pieces containing approximately 1×10^3 cells per piece using a glass pipette (cell number was obtained by counting the number of cells following dispersion). Commonly, ~9 pieces were obtained from one colony. The justification for using a piece of hESC rather than dispersed cells was based on the observation that hESC cells are more viable as aggregates rather than single cell suspensions and $\sim 1 \times 10^3$ cells is the lowest number of cells favourable for aggregation and formation of embryoid bodies [3]. Tissue recombinants were generated by combining a hESC piece with the UGM or SVM. Tissue recombinants were grown for 48 hours at 37°C on a solidified agar medium consisting of 1% agar (Oxoid Ltd, Hampshire, England) in 2x Dulbecco's Modified Eagle's Medium (DMEM; Gibco, NY, USA) with 10% (v/v) heat-inactivated foetal calf serum (FCS) (PA Biologicals Co. Pty Ltd, NSW, Australia) and antibiotics (100UI/ml penicillin and 10g/ml streptomycin; CSL Ltd, Parkville, Vic, Australia).

The procedures of tissue recombination and sub-renal grafting were performed as previously described [4, 5]. Briefly, heterospecific tissue recombinants were grafted under the kidney capsule of adult male immune-deficient SCID mice bearing subcutaneous 5mm testosterone (Sigma Aldrich, USA) implants to augment androgen levels (silastic implants were prepared using medical-grade polydimethylsiloxane tubing (Dow Corning, Corp., Midland, MO, USA; inner diameter, 1.98 mm; outer diameter, 3.18 mm), and medical adhesive silicone type A) [6, 7]. Recombinants were grown in host mice for 2, 4, 8 or 12 weeks. UGM and SVM were grafted alone as a method of detecting contamination by UGE or SVE. hESC were grafted alone to confirm pluripotency.

Quantitation of Conversion to Prostate Tissue

A gross examination of the grafts was performed by measuring the width, length and the depth of the grafts to provide a rough estimate of graft volume. Further image analysis was performed to estimate the conversion to PSA expressing glandular tissue. Entire grafts were sectioned and beginning from a randomly selected tissue section, a systematic uniform sampling procedure was applied to select 10% of the tissue throughout the explant. Using a computer assisted stereological analysis program (C.A.S.T V1.10 software (Computer Assisted Stereological Toolbox) (Olympus Danmark A/S, Denmark), the total area of each section and the area within each section that was glandular/PSA positive was measured. The area of glands and PSA positive glands within each section was expressed as a percentage of the total area. The mean for each graft was derived from the mean values of at least 10 sections. The mean value of glandular tissue (PSA positive) for the different graft types, was obtained from not less than 4 grafts per group. The numbers in **Supplementary Table 1** are Mean +/- SE of the percent of glandular and PSA positive glandular tissue.

Development and Maturation of SVM + hESC-derived prostate tissue

Recombinants were grown in host mice for a period of 2, 4, 8 or 12 weeks before analysis. At least $n=5$ grafts were analysed for each time point.

Hormonal Withdrawal

To study the effects of androgen withdrawal, recombinants were grown for 8 weeks in intact male hosts prior to surgical castration and removal of testosterone implant in host SCID mice; grafts were harvested 4 days later.

mRNA

Total RNA was extracted from prostate tissues (human BPH tissue and LNCaP cells) and SVM + hESC recombinant tissues using TRIzol Reagent (Invitrogen Life Technologies, Rockville, MD) according to manufacturer directions. RNA concentration was determined by PCR using the following specific primers to human AR and PSA: PSA 5'-GATGACTCCAGCCACGACCT-3' (sense) and 5'-CACAGACACCCCATCCTATC-3' (antisense) generating a 710bp product; AR 5'-GCCTTGCTCTCTAGCCTCAA-3' (sense) and 5'-GCTGTACATCCGGGACTTGT-3' (antisense) generating a 252bp product. All primers were designed from published mRNA sequences using PRIMER3, and none of the primer pairs showed significant cross-over binding as determined by a BLAST search (NCBI). The PCR products were subjected to gel electrophoresis on 1% agarose gels containing 0.5 µg/ml of ethidium bromide. DNA sequence analysis was performed with primers to confirm the identity of the PCR products.

Immunohistochemistry

Immunohistochemistry was performed using the DAKO Autostainer Universal Staining System (DAKO A/S, Denmark) to identify prostatic tissues [8]. Both negative and positive controls were performed for each of the antibodies. Negative and positive controls were concentration matched IgG. Positive control tissue specimens were obtained from men with benign prostate hyperplasia (*data not shown*). Antibodies to AR, Nkx 3.1 and p63 were purchased from Santa Cruz (CA, USA); to ER α , CgA, CKH and PSA from DAKO (Denmark); to α -Actin from Sigma (MO, USA); to lactoferrin from Abcam (Cambridge, UK) and used as previously described [9-11] or according to company specifications. Lamin B was purchased from Oncogene (Boston, MA, USA) and detected using frozen tissue sections.

Detection of Apoptosis

Apoptosis was analysed by ApopTag *In Situ* Apoptosis Detection Kit (Integren, Purchase, NY, USA) as previously described [9].

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