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In Vivo

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13. ABSTRACT (Maximum 200 Words) Our objective is to develop a realistic preclinical model of prostate cancer by developing methodology that supports the survival, growth and differentiation of primary cultures of prostate cells in mice. We hypothesize that implantation under the highly vascularized renal capsule of cells cultured on gas permeable OptiCell membranes will achieve this purpose. In year one, we accomplished our goal of determining the optimal conditions for growing prostatic epithelial and stromal cells, individually or in co-culture, on OptiCell membranes in vitro. We also accomplished our goal of characterizing the phenotypes of cells cultured in this manner. Finally, we implanted cells grown on OptiCell membranes under the renal capsule of mice and recovered and characterized the cells grown in vivo. We concluded that OptiCell membranes induce an inflammatory reaction and scar formation and will not provide a suitable platform for implantation of primary cultures. Next year, we will investigate other means of implantation, and in particular will explore novel materials being developed for tissue bioengineering. Our hypothesis remains viable and, although OptiCell membranes did not perform as anticipated, there are other alternate approaches to achieving our original aims.				
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INTRODUCTION

Currently available models of prostate cancer do not realistically predict activity of experimental therapeutic agents in clinical trials. The objective of our proposed research is to develop a model system that will allow the translation of *in vitro* results to an *in vivo* environment and provide a more realistic preclinical model of prostate cancer than currently exists. Primary cultures, which provide a key *in vitro* model of normal and malignant prostate biology, could fulfill this objective if we can devise a means by which they can be maintained *in vivo* and express appropriate structural and functional differentiation. Our preliminary studies show that primary cultures transplanted into *nude* mice via standard subcutaneous injection methods rapidly become squamous. We hypothesize that hypoxia is the factor that triggers inappropriate squamous formation that prevents appropriate growth and prostate-specific differentiation of primary cultures *in vivo*. Our experimental plan is to circumvent hypoxia by transplanting cells on a unique gas permeable membrane under the highly vascularized subrenal capsule of the mouse. Our aims are (1) To transplant primary epithelial cell cultures grown on OptiCell™ membranes under the renal capsule of *nude* mice, (2) to transplant primary stromal cell cultures grown on OptiCell™ membranes under the renal capsule of *nude* mice, and (3) to transplant co-cultures of epithelial and stromal cells on OptiCell™ membranes under the renal capsule of *nude* mice.

BODY

Our first designated task was to transplant primary epithelial cell cultures grown on OptiCell membranes under the renal capsule of nude mice (months 1-12). Our specific goals were to (a) prepare epithelial cell cultures on OptiCell membranes *in vitro*, (b) characterize epithelial cells grown on OptiCell membranes *in vitro*, (c) transplant epithelial cells grown on OptiCell membranes *in vivo*, and (d) characterize epithelial cells grown *in vivo*. We have accomplished all components of this aim.

(a) First, we examined several protocols for growing primary cultures of prostatic epithelial cells on gas permeable OptiCell membranes. The variables we explored were cell density, attachment surface, and culture medium. Regarding cell density, we found that if we inoculated cells onto the membranes at low density, the cells did not proliferate well and did not expand to cover the membrane. Alternatively, if we inoculated at too high density, the cells tended to deteriorate and slough off. We concluded that an inoculation of about one million cells per 100 cm² membrane was optimal. At this density, the cells formed a confluent monolayer upon attachment and the cells maintained a healthy appearance for at least several days.

Concurrently, we also tested several media formulations, including "Complete 105", a serum-free medium that optimally supports clonal growth of prostatic epithelial cells (1); "Complete PFMR-4A", another serum-free medium that optimally supports high density growth (1); KSFM, a serum-free medium often used for transformed prostatic epithelial cells (2); and MCDB 105 supplemented with serum. The latter medium is used for culture of prostatic stromal cells (1), and we wanted to evaluate the health of the epithelial cells in this medium in preparation for co-culture experiments in Aim 3. In conjunction with an inoculum of one million cells per membrane, Complete PFMR-4A demonstrated the best ability to maintain the monolayer.

However, cellular appearance was quite good in the stromal medium as well, paving the way for potential use in co-culture experiments in Aim 3.

The third variable that we explored was the substrate. Our primary cultures are routinely grown on dishes coated with collagen (1), so we tested uncoated OptiCell membranes or membranes coated with collagen. The collagen-coated OptiCell membranes proved superior. In summary, we concluded from this set of experiments that an inoculum of one million epithelial cells into Complete PFMR-4A medium onto a membrane coated with collagen was the optimum protocol for preparing epithelial cell cultures on OptiCell membranes *in vitro*.

In preparation for *in vivo* implantation studies in part (d) of this aim, we also evaluated the growth and maintainance of several prostate cell lines on OptiCell membranes. We chose DU 145, an established line of prostate cancer cells, and pRNS-1-1, an immortal line of SV40-transformed prostatic epithelial cells (3). DU 145 cells were selected to use as a positive control in part (d) because of their known tumorigenic potential, and pRNS-1-1 cells were of interest because, like primary cultures of prostate cancer cells, pRNS-1-1 cells do not form tumors when implanted subcutaneously into nude mice. pRNS-1-1 cells in fact form squamous, keratinized cysts *in vivo* (3), and we were curious to determine whether implantation under the highly vascularized renal capsule might also prevent squamous differentiation and promote tumor formation as hypothesized for primary cultures. As for the primary cultures, we determined the optimal cell number for inoculation and medium for optimal maintainance of each of these cell lines on OptiCell membranes.

(b) In addition to evaluating the morphology and overall appearance of epithelial cells cultured on OptiCell membranes in (a), we also used immunocytochemistry to evaluate cellular differentiation. For this purpose, we used antibodies specific for basal epithelial cells (keratin 5, p63), for secretory epithelial cells [keratin 18, androgen receptor and prostate-specific antigen (PSA)], neuroendocrine cells (chromogranin A), and squamous cells (keratins 10 and 16). The first sets of antibodies were of interest to determine whether the phenotype differed between cells grown on OptiCell membranes versus on dishes in standard monolayer culture. On dishes, the cells predominantly are basal, with expression of keratin 5 and p63 in the majority of cells. A subset of cells on dishes also typically expresses secretory keratin 18, but the markers characteristic of fully differentiated secretory cells, androgen receptor and PSA, are not present. In general, the antigenic phenotype of cells cultured on OptiCell membranes was similar to that of cells in dishes, and there was no evidence of the induction of a fully differentiated phenotype or of a neuroendocrine phenotype. However, we did note that certain media conditions caused a few changes. For example, cells grown in the stromal medium, MCDB 105 with serum, completely lacked expression of p63, which is present in ~100% of cells cultured in Complete PFMR-4A. Also, keratin 18 was expressed in a smaller percentage of cells grown on OptiCells compared to cells grown in dishes. We do not know the basis for these differences but they are probably not of major significance for our study. The most critical finding was that few cells grown on OptiCell membranes were positive for the squamous keratins 10 and 16. This was important because our hypothesis is that hypoxia prevents the survival, growth and appropriate differentiation of prostatic epithelial cells implanted into mice by making them squamous, so therefore the cells cannot be squamous prior to implantation.

(c) After verifying the status of epithelial cells cultured on OptiCell membranes, we initiated transplantation studies. To date, we have carried out 3 experiments. In the first experiment, we successfully performed surgery and implanted prostatic epithelial cells grown on OptiCell membranes under the renal capsules of 3 nude mice. One day later, we sacrificed one mouse,

recovered the kidney, and fixed the kidney containing the OptiCell membrane in formalin. Another mouse was sacrificed on day 8, and the final mouse was taken on day 16. The fixed kidneys were embedded in paraffin and 5 μ m sections were cut for histologic analysis of H & E-stained tissues. In the second experiment, we implanted mice with pRNS-1-1 cells on OptiCell membranes. In the final experiment, we implanted DU 145 cells on OptiCell membranes, and in the contralateral kidney, also implanted OptiCell membranes alone with no cells.

(d) In the final part of this Aim, we characterized the epithelial cells implanted in vivo. In the first experiment, we could see the site where the OptiCell membrane had been implanted, although the membrane itself dissolves in xylene during the processing of the fixed tissues. We could not definitively identify the single layer of epithelial cells. However, the most obvious finding in this experiment was that there was a significant inflammatory response at the site of the implanted membrane. By day 16, the implantation site was overtaken by substantial scar formation.

In the second experiment, in which we had implanted mice with pRNS-1-1 cells on OptiCell membranes, we again observed an inflammatory response and scar formation, and no epithelial cells were clearly seen. In the final experiment in which we implanted DU 145 cells on OptiCell membranes and also OptiCell membranes alone with no cells, we saw an inflammatory response with scar formation even with the OptiCell membranes alone. We concluded that the membranes will not be suitable as an implantation platform because they clearly cause an undesirable inflammatory response. The membranes, composed of a proprietary plastic, are rather rigid and presumably are causing physical trauma to the kidney, hence the inflammatory reaction and scar formation.

Our second aim was to transplant primary stromal cell cultures grown on OptiCell™ membranes under the renal capsule of nude mice (months 13-20). Our specific goals were to **(a)** prepare stromal cell cultures on OptiCell membranes in vitro, **(b)** characterize stromal cells grown on OptiCell membranes in vitro, **(c)** transplant stromal cells grown on OptiCell membranes in vivo, and **(d)** characterize stromal cells grown in vivo.

(a) Although our original intent had been to not initiate studies with stromal cells until year two, we decided to do a few experiments in conjunction with the epithelial cells in year one. As for the epithelial cells, we first wanted to evaluate the ability of stromal cells to attach and grow or survive on OptiCell membranes. We tested similar conditions to those that we routinely use to culture stromal cells on dishes, and the cells looked fine. We concluded that using uncoated OptiCell membranes, inoculating ~ one million stromal cells per membrane, and using either MCDB 105 supplemented with 10% serum or SCGM with 5% serum and FGF-2 and insulin was satisfactory.

(b) As for the epithelial cells, we used immunocytochemistry to evaluate the phenotype of stromal cells grown on OptiCell membranes. Prostatic stromal cells can exist as undifferentiated fibroblasts or as differentiated smooth muscle cells. Treatment with transforming growth factor (TGF)- β typically induces smooth muscle differentiation in prostatic stromal cells (4), so we evaluated stromal cells cultured on OptiCell membranes in media with or without TGF β . In both conditions, ~100% of the cells were positive for vimentin and fibronectin, as is characteristic of stromal cells. In medium without TGF β , ~50% of the cells were positive for smooth muscle- α -actin, a marker of smooth muscle differentiation. Since high density also causes smooth muscle differentiation of prostatic stromal cells, this degree of staining was not unanticipated for cells at

this density. However, we did not observe an increase in smooth muscle- α -actin in cells treated with TGF β , which was not as anticipated. Since growth factors such as FGF-2 can block induction of smooth muscle differentiation (5), it is possible that the presence of serum containing growth factors such as FGF-2 was responsible for lack of response of the stromal cells. Alternatively, there may be something different about the conditions provided by the OptiCell membrane; this remains to be clarified in additional experiments.

(c) Since we had initiated transplantation studies with epithelial cells and had noted a problematic inflammatory response, we decided to transplant stromal cells on OptiCell membranes to see if a similar inflammatory response would occur. The kidneys of 4 mice were implanted with either OptiCell membranes alone (no cells) or with stromal cells grown on OptiCell membranes. Mice were sacrificed at several time points up to three months following implantation and kidneys were fixed, embedded and sectioned.

(d) Histologic analysis of the implanted membranes revealed the same phenomenon as noted in the experiments with epithelial cells. Even the OptiCell membrane itself with no cells caused inflammation and extensive scar formation, as was also seen with the membranes carrying cells. This validated our conclusion from the previous studies that the membranes acted as an irritant in the kidney and this property precludes their utility as a platform for implantation of cell cultures.

Our third aim was to transplant co-cultures of epithelial and stromal cells on OptiCell™ membranes under the renal capsule of nude mice (months 21-36). Our specific goals were to (a) co-culture epithelial and stromal cells on OptiCell membranes in vitro, (b) characterize co-cultures of epithelial and stromal cells grown on OptiCell membranes in vitro, (c) transplant co-cultures of epithelial and stromal cells on OptiCell membranes in vivo, and (d) characterize co-cultures of epithelial and stromal cells in vivo.

(a) Although we had not planned to initiate co-culture experiments until year 3, we decided to start some of these experiments since we were already working with epithelial and stromal cells in Aims 1 and 2. An important consideration in creating co-cultures is the optimal medium. Since the optimal media conditions for epithelial versus stromal cells differ significantly, we evaluated co-cultures in various media formulations. We also evaluated varying densities of epithelial and stromal cells. In the first two experiments, we first created a dense monolayer of stromal cells on the OptiCell membrane, then the next day inoculated a large number of epithelial cells. One co-culture was maintained in stromal medium (MCDB 105 with 10% serum), and the other was maintained in epithelial medium (Complete PFMR-4A). Either medium appeared to support survival of both cell types, and in both conditions, we observed that epithelial cells pushed stromal cells aside so that they could reach and attach to the substratum. In a third experiment, we used the epithelial medium (Complete PFMR-4A) supplemented with retinoic acid and androgen (10 nM R1881), factors associated with induction of epithelial differentiation. In this medium, epithelial cells formed "domes" in between the stromal cells. In a fourth experiment, stromal cells were first inoculated onto an OptiCell membrane in MCDB 105 with 10% serum, then were treated with TGF β for several days in an attempt to increase smooth muscle differentiation. Epithelial cells were then inoculated onto the TGF β -treated stromal monolayer. We perceived no obvious morphological differences between co-cultures maintained +/- TGF β .

(b) The OptiCell membranes carrying the co-cultures described in (a) were fixed and evaluated by immunocytochemistry. In the first two co-cultures with epithelial (Complete PFMR-4A) medium versus stromal (MCDB 105 with 10% serum) medium, we observed that the stromal cells did not express smooth muscle actin (hence were fibroblasts and not differentiated smooth muscle cells), and p63 (a basal epithelial cell marker) was not expressed by the epithelial cells. Chromogranin A was absent, indicating lack of neuroendocrine differentiation, as was PSA, suggesting lack of full differentiation of the epithelial cells. However, keratin 18 (a secretory cell marker) was expressed in some epithelial cells, and was especially prominent in the stromal medium. Keratin 10 was not present in either of the co-cultures, showing that squamous differentiation was absent, as desired.

In the third experiment, in which the co-cultures were maintained in medium with factors associated with epithelial differentiation (retinoic acid and R1881), we noted that basal epithelial (keratin 14-positive) cells were present, as were secretory (keratin 18-positive) cells. However, neither androgen receptor nor PSA were expressed, showing lack of full differentiation.

In the fourth experiment, we did not observe expression of smooth muscle α -actin in the stromal cells, despite our attempts to induce smooth muscle differentiation with TGF β . As noted in Aim 2, lack of effect of TGF β may be due to inhibition by serum, inhibition by epithelial cells, or different conditions imposed by growth on OptiCell membranes. In this co-culture, epithelial cells expressed basal (keratin 14) and secretory (keratin 18) markers, but not PSA.

(c) and (d) We did not attempt to implant any co-cultures on OptiCell membranes into nude mice given the problem with inflammation that we encountered in Aims 1 and 2.

KEY RESEARCH ACCOMPLISHMENTS

- Identified optimal media and cell density for maintenance of primary cultures of prostatic epithelial cells, primary cultures of prostatic stromal cells, co-cultures of epithelial and stromal cells, and several immortal prostate cell lines on the unique gas permeable cell culture membrane OptiCell
- Developed protocol for fixation and immunocytochemical labeling of cells cultured on OptiCell membranes and used this protocol to evaluate phenotype of epithelial and stromal cells cultured on OptiCell membranes
- Determined that differentiation status of stromal and epithelial cells varied depending on culture conditions, but most importantly, found that inappropriate squamous differentiation of epithelial cells did not occur regardless of conditions, as desired for implantation studies
- Mastered technique of implantation of OptiCell membranes under the renal capsule of nude mice and implanted and recovered OptiCell membranes carrying various cell configurations
- By histologic examination of OptiCell membranes recovered from mice, concluded that the membranes themselves elicit an inflammatory response and scar formation that precludes their use as a platform for implantation of cell cultures under the renal capsule of mice

REPORTABLE OUTCOMES

None.

CONCLUSIONS

We achieved all elements of our first aim, and several components of Aims 2 and 3. For Aim 1, we met our goals of preparing epithelial cell cultures on OptiCell membranes in vitro and characterizing the phenotype of cells cultured in this manner. We successfully transplanted epithelial cells on OptiCell membranes under the renal capsule of nude mice, and we were able to recover and examine these membranes at various times subsequent to transplantation. We also accomplished several components of Aims 2 and 3 by preparing stromal cell cultures on OptiCell membranes, characterizing these cultures, implanting into mice, and recovering and examining the membranes. Co-cultures of epithelial and stromal cells on OptiCell membranes were also created and analyzed.

From our studies so far, we conclude that OptiCell membranes provide a suitable substrate for in vitro culture of prostatic epithelial and stromal cells, both individually or in co-culture, although the phenotype of the cells is not identical to that in standard culture dishes and culture conditions must be somewhat modified to maintain healthy cells. Most importantly, the epithelial cells do not undergo inappropriate squamous differentiation when cultured on OptiCell membranes, a prerequisite for implantation into mice.

However, we conclude that OptiCell membranes do not provide a suitable platform for implantation of cultured cells under the renal capsule of nude mice. Although the membranes are readily cut into small pieces suitable for implantation, the membranes themselves, even those without cells, evoke a strong inflammatory response that leads to extensive scar formation. We surmise that this is due to the rigidity of the membrane, which facilitates implantation but must act as an irritant, thus provoking the inflammatory response.

While the failure of OptiCell membranes to perform as desired is a disappointment, this does not negate our ability to continue to test our hypothesis. The gas permeable nature of OptiCell membranes made them especially attractive for our project, but alternative approaches are feasible. We will return to an old technique of culturing cells on nitrocellulose filters, which still allows the cultured cells to be implanted in situ on the filters. These filters are rigid enough to be transplanted, but we know from previous experience that they do not evoke an inflammatory reaction. In addition, we will investigate hydrogels and other bioengineering materials that are being developed at Stanford for tissue regeneration projects. There is a great deal of activity in this area and we believe that we can take advantage of innovations in this field to develop novel methods of implantation that will achieve our purpose.

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APPENDICES

None.