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REGENERATIVE MEDICINE (A ATALA, SECTION EDITOR)

Production of Urothelium from Pluripotent Stem Cells for Regenerative Applications

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Abstract As bladder reconstruction strategies evolve, a feasible and safe source of transplantable urothelium becomes a major consideration for patients with advanced bladder disease, particularly cancer. Pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are attractive candidates from which to derive urothelium as they renew and proliferate indefinitely in vitro and fulfill the non-autologous and/or non-urologic criteria, respectively, that is required for many patients. This review presents the latest advancements in differentiating urothelium from pluripotent stem cells in vitro in the context of current bladder tissue engineering strategies.

Keywords Bladder · Urothelium · Differentiation · Human embryonic stem cells · Induced pluripotent stem cells · Grafts

Introduction

The urinary bladder is a reservoir that stores and voids urine from the body by means of complex biological function and coordination. The bladder is made up of three tissue layers, the luminal urothelium, the lamina propria, and the muscle, which are dispersed with complex nerve and vascular networks.

The urothelium is the specialized and essential epithelial lining of the bladder, which provides the impenetrable barrier that keeps urine and pathogens from being reabsorbed into the bloodstream. From the basement membrane to the lumen, the urothelium is made up of three cell types: basal, intermediate, and superficial (umbrella) cells [1]. Basal cells are the most progenitor of the urothelial layers and are proposed to harbor the bladder stem cells that provide lifelong renewal of the urothelial layer. Intermediate cells are highly proliferative and responsible for rapid regeneration of the urothelium in times of injury or infection. The umbrella cells are the fully differentiated luminal cells that are responsible for maintaining the high-resistance barrier function of the urothelium. Specialized tight junctions between the umbrella cells, as well as the assembly of urothelial cell-specific uroplakin proteins into plaques, create this necessary, near impermeable barrier [2, 3].

Patients with neuropathic bladder disorders, trauma, or bladder cancer often require augmentation or reconstruction of the urinary bladder. The current protocol for bladder tissue replacement calls for the use of gastrointestinal tissue. While this approach does suffice for improved bladder function, the diametrically opposed functions of bladder and intestinal tissues often lead to significant complications including electrolyte imbalances, urinary stones, chronic infections, and increased cancer risk [4]. Thus, tissue engineering has become a field of intense focus for many urology clinicians and researchers, as a need for improved replacement bladder tissue prevails.

Current Tissue Engineering Strategies

To improve upon the current use of gastrointestinal tissue for cystoplasty, strategies to bioengineer bladder grafts have taken center stage. However, the complex functions of the bladder pose significant challenges to the engineering of tissue. Neobladder tissue is being bioengineered using scaffolds,

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synthetic or natural, reconstituted with or without the various tissue layers of the bladder wall [5].

While natural scaffolds are considered the best choice with regard to biocompatibility, they also succumb to graft contraction upon transplant. Synthetic scaffolds are of great interest due to their defined and reproducible characteristics but are hard-pressed to exhibit the mechanical qualities that are imperative for proper bladder filling and voiding.

Regardless of the material, acellular scaffolds that have been transplanted with cells function better post-transplant than do scaffolds that have not been seeded [6–9, 10, 11]. Non-seeded scaffolds rely on native cell in-growth for tissue regeneration and thus are limited to use in small reconstructions. In-growth of muscle, nerve, and vasculature is a rate-limiting event to the use of acellular scaffolds for larger bladder reconstructions. Furthermore, it was recently reported that bladder grafts, prepared from synthetic scaffolds of PGA reconstituted with smooth muscle and urothelial cell layers, were unsuccessful in providing functional augments to pediatric patients in a clinical trial [12]. This clinical trial highlights the challenge of replicating functional bladder tissue, specifically with regard to blood supply.

The co-transplantation of stem cells to aid in bladder repair and/or regeneration is also under significant investigation. The contribution of transplanted stem cells to tissue regeneration is not only through differentiation and cell replacement but also via paracrine effects [13]. Accordingly, various sources of stem cells (embryonic stem cells (ESCs), BM-derived SCs, tissue-specific SCs) have been shown to enhance tissue regeneration upon bladder injury or reconstruction [14–20]. In particular, Sharma et al. demonstrated the signaling effects of bone marrow-derived mesenchymal stem cells (MSCs) and hematopoietic stem cells in inducing regeneration of vasculature, nerves, muscle, and urothelium upon bladder augmentation with a synthetic scaffold in a murine model [21]. While stem cells can induce native urothelial regeneration over a graft, they do not regenerate urothelium de novo.

The urothelium is highly regenerative and capable of growing over bioengineered scaffolds, but the urothelium from patients with advanced bladder disease may not be so capable or desirable [22]. This is particularly true with regard to bladder cancer, which is primarily of urothelial origin. Bladder cancer is believed to follow the two-hit hypothesis of neoplasia, such that known genetic predispositions, like mutations in DNA repair or tumor suppressor genes, are compounded by a secondary hit from long-term exposure of urothelium to urine carcinogens [23]. After endoscopic resection, 70 % of patients have tumor recurrence in a different part of the bladder, classifying bladder cancer as a field defect with premalignant urothelium scattered throughout the bladder. Two theories of bladder cancer cell origin have been proposed to account for this field defect (reviewed in [24]). One theory postulates bladder cancer to be monoclonal, developing from a single transformed cell that gives rise to cancerous progeny, which spreads throughout the bladder by various mechanisms [25]. An alternate theory proposes that many transformed cells arise independently and are genetically distinct as a result of long-term insult to the entire urothelium [26]. Whether the etiology of bladder cancer recurrence is a result of either one or both of these mechanisms, the potential for malignancy in all urothelial cells of a bladder cancer patient precludes the use of autologous cells for regenerative purposes. Therefore, there is a multifaceted need for alternative sources of urothelium that can meet patient-specific needs for reconstruction. In such cases of advanced disease, alternative non-urologic or non-autologous sources of urothelium are a necessity for bladder reconstruction via tissue engineering. Thus, whether it is for reconstruction of a bladder graft de novo or be for mere improvements to current cystoplasty techniques via replacement of enteric epithelium with urothelium, many patients would benefit from having a readily available and healthy source of urothelium.

Potential Sources of Urothelium for Bladder Reconstruction

The use of autologous cells for transplantation is ideal, as adverse immunological responses and graft rejection are negated. However, since most patients in need of bladder reconstruction have advanced bladder disease or bladder cancer, the use of autologous urothelium for seeding bladder grafts is not ideal or safe. Urothelium from patients with benign bladder diseases has been shown to have reduced proliferative abilities in vitro [22]. Thus, urothelium from these patients may not be capable of being produced to the quantities needed for engineering bladder tissue, while it may also have impaired function when returned in vivo. Particularly in the case of cancer, patients would require non-urologic, genetically healthy tissue for a source of regenerated urothelium. Thus, alternate sources for transplantable, non-pathogenic urothelium are focused on that derived from either adult stem cells or pluripotent stem cells, including human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs).

Adult stem cells are fairly easy to obtain and culture, are autologous, and are therefore an attractive source for urothelium in bioengineering. Urothelium has been derived from multiple adult stem cells, including adipose-derived stem cells, urine-derived stem cells, and bone marrow-derived MSCs [27–29]. Fetal or post-natal stem cells, which are not autologous but do have relatively low immunogenicity properties, have also been differentiated into urothelium [30–32]. The primary limitation of using adult and fetal stem cell-derived urothelium hinges on a poorly understood differentiation process that typically occurs through either transdifferentiation or cell fusion. For instance, the long-term function and safety of mesenchymal lineage cells becoming

epithelial has not been sufficiently investigated. Furthermore, adult cells have limited proliferation potential in vitro and fetal cells have potential immunological repercussions.

However, the pluripotent characteristics of hESCs and hiPSCs make them attractive candidates for cell therapy, as they have the potential to renew and proliferate indefinitely and differentiate into any cell type. The concept of using urothelium derived from autologous hiPSCs for patients with a history of bladder cancer is predicated on current fast developments in genomic mapping that may show that urothelium, derived from somatic cells outside the bladder, is free of secondary mutations associated with bladder cancer. Like adult stem cells, pluripotent stem cells for use in clinical application are not without issue. hESCs are non-autologous and derived from human embryos, which is of significant worldwide ethical debate, while hiPSCs are derived from somatic cells that have been reprogrammed with known oncogenes, potentially posing a safety risk.

All points considered, human ESCs and hiPSCs may still provide efficacious sources from which to derive urothelium for use in future bladder tissue engineering, particularly for bladder cancer patients.

Differentiation of Urothelium from Pluripotent Stem Cell Sources

Early investigations proved the ability to differentiate urothelium from pluripotent stem cells using mouse models. Oottamasathien et al. used murine embryonic bladder mesenchyme in tissue recombination experiments to differentiate urothelium from mouse ESC [33], while murine embryoid bodies have also been directed toward the urothelial lineage upon in vivo transplantation into mice [34]. More recently, it has been shown that retinoic acid (RA), via GATA-4/6 signaling, induces efficient differentiation of murine ESCs and iPSCs to urothelium in vitro [35••, 36].

While the murine model provided evidence of feasibility, only recently has the differentiation of human urothelium from pluripotent stem cells been reported. As such, hESCs and hiPSCs have now been efficiently induced into urothelium in vitro, providing significant progress toward urothelial cell transplant for clinical applications [37••, 38••, 39•]. These in vitro models of differentiation also provide a useful and needed study model for human urothelial development.

In Vitro Induction of Urothelium Through a Developmental Stage Process

During embryogenesis, differentiation of endodermal lineages (epithelial cells, including urothelium) from ESCs follows a specific progression of development through the definitive endoderm (DE) stage. Presumably, the most efficient differentiation to mature endodermal tissues in vitro would therefore follow this in vivo developmental progression. To that end, d'Amour et al. developed a protocol to induce DE from hESCs in vitro, which appropriately mimics the known gene expression transitions of gastrulation [40•]. With mouse embryonic fibroblast (MEF) feeders, low serum supplementation, and activin A stimulation, DE production was highly efficient, approximating 80 %. The induced DE was also functional, as DE could be differentiated into various endodermal lineages in in vivo transplantation experiments.

Using this DE induction protocol, human ESCs and hiPSCs were recently induced to urothelium [37••]. Although multiple culture conditions for urothelial induction from DE were tested, the most efficient induction proved to be culturing under standard conditions for human urothelium (keratinocyte basal medium with epidermal growth factor (EGF) and bovine pituitary extract (BPE)), supplemented with low levels of serum (Table 1). Expression of uroplakins, specific markers of urothelial cell lineage, showed the urothelial cell yield to be up to 60 % for both hESCs and hiPSCs. Upon subsequent passages and selective expansion in urothelial cell growth medium, culture purity reached 90 % urothelium.

In addition to this in vitro culture system being effective at producing urothelium, it also appeared to mimic what is known about the developmental stages of urothelial cell differentiation during embryogenesis. The expression of various stage-specific transcription factors followed a directed process from hESC to DE to urothelium, as depicted in Fig. 1. Furthermore, co-expression of uroplakins with known urothelial fate specification mediators in a temporal fashion suggested the appropriate development to urothelium. FoxA2 is a marker of DE that is downregulated as urothelium matures. Accordingly, the association of FoxA2 with uroplakins decreased over the urothelial induction period. The transcription factors IRF-1, Get1, and GATA-4 have been shown to be involved in urothelial fate specification and were appropriately associated with uroplakin-expressing cells within the first week of DE induction to urothelium [35••, 37••, 41, 42].

More recently, Kang et al. altered this development-directed induction approach to minimize the use of xenogeneic products with the intent to derive human urothelium that could be used in clinical applications (Table 1) [38••]. The urothelium expressed appropriate lineage markers (UPs, cytokeratins) and tight junction proteins, while exhibiting physiologically appropriate low permeability in a functional assay.

Human iPSCs, generated from urinary tract stromal tissue, were also previously induced to urothelium (Table 1) [39•]. However, the hiPSCs were directly differentiated to urothelium using a conditioned medium culture approach, rather than a development-directed approach. The resulting cells displayed a mixed stromal and urothelial phenotype, which may suggest incomplete or inappropriate differentiation that may, in part, be due to an undefined developmental path.

Table 1 Comparison of in vitro models of urothelial cell induction from human pluripotent stem cells

	Pluripotent stem cells		Definitive endoderm			Urothelium			
Pluripotent stem cells	Feeder cells	Medium and maintenance factors	Induction medium	Induction factors	Matrix	Induction medium	Induction factors	Matrix	Reference
hESC (H9, HuES8) hiPSC (IMR-90, CD34+ HSC-derived)	MEFs	DMEM:F12 20 % knockout serum replacement FGF2 (4 ng/ml)	RPMI	2 % FBS Act A (100 ng/ml)	None	RPMI	2 % FBS BPE hEGF	None	Osborn et al. [37••]
hESC (H9) hiPSC (CRL-2097 fibroblast-derived)	None (matrigel)	mTeSR-1 FGF2 (10 ng/ml)	RPMI	2 % B27 serum replacement Act A (100 ng/ml) Wnt3a (50 ng/ml)	Matrigel	K-SFM	10 uM RA BPE hEGF	Matrigel	Kang et al. [38••]
hiPSC (urinary tract-derived)	MEFs	Knockout DMEM 20 % serum replacement FGF2 (8 ng/ml)					Conditioned medium (bladder urothelial cells; ureter stromal cells)	None	Moad et al. [39•]

The derivation of urothelium in vitro through an intermediary DE step is efficient. The rendered urothelium expresses the appropriate urothelial-specific markers and functions as an impermeable barrier in vitro. While the data suggests that urothelium derived from pluripotent stem cells in this manner has high likelihood of being able to be used for cell replacement in vivo, more intense studies are necessary to show safety and efficacy of the induced urothelium. Nonetheless, this directed culture system also provides a useful in vitro model for studying human bladder development.

The Role of Retinoic Acid in Urothelial Development

Retinoic acid is a primary vitamin A derivative that functions in a wide array of cellular processes, including fate specification of various endodermal tissues. It has been shown to be important for bladder development from the urogenital sinus and for differentiation of mouse urothelial cells from pluripotent stem cells [35••, 43, 44]. While RA was shown to act through GATA-4/6 in differentiating urothelium from mouse ESCs, the role of RA and GATA-4/6 signaling in human urothelial cell fate has not been definitively determined. Induction of urothelium in the presence of serum was not enhanced by the addition of exogenous RA [37••]. However, RA significantly enhanced the differentiation of urothelium in a serum-free system [38••]. Thus, as serum contains RA, it likely contributes to specification of urothelium in vitro. In serum-free systems, it appears necessary to supplement exogenous RA for induction of urothelium. Thus, while it is likely that RA is important for urothelial differentiation, it is unclear whether its action is through GATA-4/6 in humans, as only a nominal association of GATA-4 was found with UP-expressing cells of hESC-derived urothelium [37••].

Xenogeneic-Free Strategies for Clinical Applications

It is widely accepted that animal and human products, particularly feeder cells and serum (FBS), need to be avoided in culture systems that will produce cells for human clinical applications. The significant challenge to these in vitro differentiation systems is in maintaining the pluripotency of stem cells, the unique property that allows them to be differentiated into nearly any cell type. Although not without flaws, current strategies utilize serum replacers in lieu of FBS and various extracellular matrices (ECM), such as matrigel, collagen, and fibronectin, instead of feeder cells [45, 46].

Serum replacers are often proprietary and even if the constituents are known, the concentrations are typically not. Like serum replacers, the exact composition of matrigel is unknown. Importantly, matrigel also contains growth factors that can contribute to differentiation and proliferation. Accordingly,

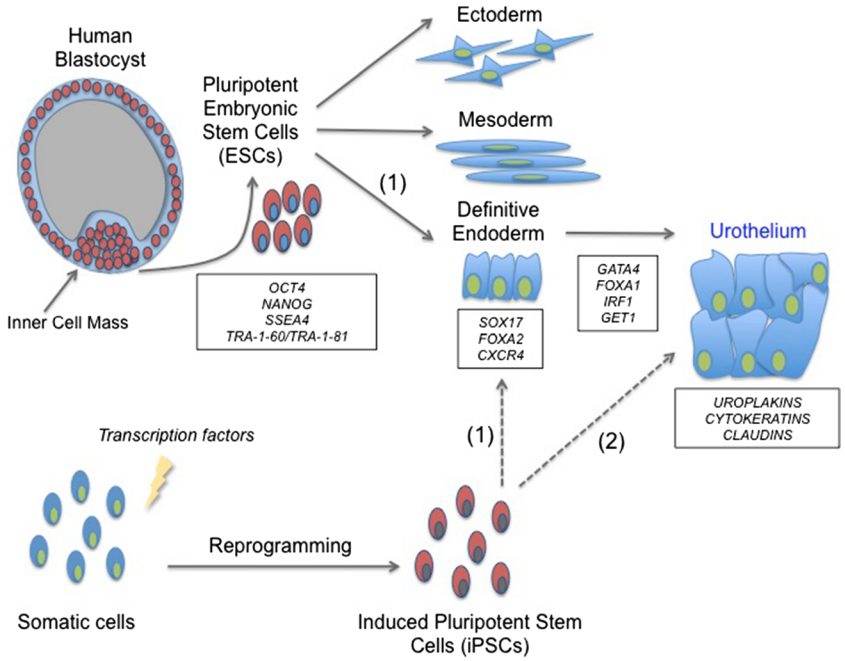

Fig. 1 The induction of urothelium from human pluripotent stem cells. The pluripotency of two classes of stem cells can be harnessed to produce various differentiated cell types in vitro. Embryonic stem cells (ESCs) are the pluripotent stem cells that make up the inner cell mass of the human blastocyst during embryogenesis and that give eventual rise to all three germ layers: endoderm, mesoderm, and ectoderm. Induced pluripotent stem cells (iPSCs) are differentiated somatic cells that have been reprogrammed to a pluripotent state using forced expression of distinct sets of transcription factors via retroviral or lentiviral infection. From the pluripotent state, ESCs and iPSCs can be induced to the urothelium either through a developmental stage (*1*) or direct induction (*2*) approach. The developmental stage approach follows the specification of the urothelium during embryogenesis, such that ESCs and iPSCs are differentiated to definitive endoderm and subsequently to endodermal cell lineages such as the urothelium (*1*). The direct induction approach drives pluripotent stem cells directly to the urothelium, using conditioned medium to mimic the epithelial-mesenchymal interactions of the bladder (*2*). Transcription factors, known to associate with the different cell types at various stages of endodermal development, are indicated in the boxes below the phase at which they are expressed

hESCs and hiPSCs were capable of being induced to DE and subsequently urothelium using a culture system that substituted matrigel for MEF feeder cells and a serum replacer for FBS [38••]. Although the production of DE and urothelium in this culture system was less efficient than the previously published system, the derived cells nonetheless fulfilled phenotypic and functional expectations of urothelium in vitro [37••]. While a truly chemically defined system for culturing pluripotent stem cells has yet to evolve, these modifications in serum and feeders do limit the xenogeneic material in the culture system.

If progress can be made in the xenogeneic-free culturing of pluripotent stem cells and their induction to DE, there is a significant potential for the induction step to urothelium to be achieved in such a manner. Human urothelial cells are typically grown in vitro using a keratinocyte basal medium supplemented with EGF and bovine pituitary extract [47–51]. Induction of urothelium from DE was achieved using these standard urothelial cell growth conditions supplemented with RA, either exogenous or in the form of serum. Thus, exogenous RA could replace serum in the induction medium. Previous studies in both mice and human relied heavily on cell co-culture or conditioned medium environments to induce urothelium from various stem cell sources [29–31, 33, 34, 39•, 52–54]. However, differentiation of human urothelium from hESCs and hiPSCs in vitro was efficiently achieved in their absence [37••, 38••]. Interestingly, cell co-culture and conditioned medium methods produced urothelium less efficiently than standard culture conditions [37••].

Matrices were also not required for efficient induction to urothelium [37••]. Although feeders were used for support in the initial stem cell cultures, these feeder cells were typically dead and removed from culture by the end of DE induction (Osborn and Kurzrock, unpublished observations). Thus, urothelium was induced from DE in the absence of feeders and/or matrices. In contrast, Kang et al. maintained cell cultures on matrigel throughout all phases of differentiation to urothelium and noted that matrigel significantly increased the expression of urothelial-specific markers, as compared to other matrices [38••]. Considering the known potential for unwanted effects of matrigel on growth and differentiation of cells, this observation needs to be considered carefully and the quality of the induced urothelium investigated moving forward. As urothelium can be induced in the absence of a matrix, removal of matrigel from the urothelial induction phase might be a better option.

Urothelium can be efficiently cultured long-term and expanded in serum-free conditions and in the absence of a matrix for growth support in vitro [47, 48] (Osborn and Kurzrock,

unpublished observations). Therefore, the post-induction culturing of stem cell-derived urothelium can likely be achieved under xenogeneic conditions as well.

Practical and Future Applications of In Vitro-Derived Urothelium

Admittedly, more thorough study of hESC- and hiPSC-derived urothelium needs to be conducted, specifically with regard to in vivo function and safety, prior to clinical application. Induced urothelium phenotypically resembled normal urothelium and likewise performed well in in vivo functional tests of permeability. In a preliminary assay, hESC-derived urothelium did not form teratomas when orthotopically transplanted in vivo, suggesting biosafety [37••]. However, as hiPSCs are reprogrammed using known oncogenes, the current human application of these pluripotent stem cells and their derivatives needs further evaluation. Although far from ready for clinical use, the data supports continued validation studies.

It has also been reported that urinary tract fibroblasts can be reprogrammed to pluripotency (hiPSCs) and subsequently differentiated into urothelium [39•]. Although this provides an interesting model to study development and epigenetics, it most likely does not have utility in clinical practice. Most patients requiring bladder reconstruction have advanced bladder diseases and/or cancer. These patients would benefit most from urothelium derived from non-urologic yet autologous sources. As skin iPSCs can be efficiently driven to urothelium, this approach seems more relevant to the clinical application of bioengineered bladder tissue [37••, 38••].

Conclusions

The current protocols for bladder reconstruction are less than optimal, as use of gastrointestinal tissue leads to significant complications. The increased risk of cancer due to the juxtaposition of intestinal epithelium with urine is of particular concern as the life expectancy of patients receiving augments also increases. As there is a dire need for improvement in bladder grafts to be used for reconstruction, tissue engineering has become a significant focus of the urologic research community. Since the majority of patients who require cystoplasty suffer from advanced bladder disease, it is often not feasible, practical, or safe to utilize autologous urothelium for tissue engineering purposes. This is particularly true in the case of bladder cancer, which is deemed a field defect, rendering the entire urothelium to be considered premalignant. Thus, the ability to differentiate urothelium from pluripotent stem cells such as ESCs and iPSCs provides a potentially valuable non-urologic source of urothelium for tissue engineering. While urothelium can now be efficiently produced from pluripotent stem cells in vitro using developmentally directed culture methods, the translational application of utilizing pluripotent stem cells and the in vivo functional capacity of the induced urothelium require validation.

Compliance with Ethics Guidelines

Conflict of Interest Dr. Stephanie L. Osborn and Dr. Eric A. Kurzrock each declare no potential conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- •• Of major importance

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