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Invited Review

Altered phenotype of cultured urothelial and other stratified epithelial cells: implications for wound healing

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Sun, Tung-Tien. Altered phenotype of cultured urothelial and other stratified epithelial cells: implications for wound healing. *Am J Physiol Renal Physiol* 291: F9–F21, 2006; doi:10.1152/ajprenal.00035.2006.—The differentiation of cultured stratified epithelial cells can deviate significantly from that of normal epithelium, leading to suggestions that cultured cells undergo abnormal differentiation, or a truncated differentiation. Thus cultured epidermal and corneal epithelial cells stop synthesizing their tissue-specific keratin pair K1/K10 and K3/K12, respectively. The replacement of these keratins in the suprabasal compartment by K6/K16 keratins that are made by all stratified squamous epithelia during hyperplasia rules out a truncated differentiation. Importantly, the keratin pattern of in vivo corneal epithelium undergoing wound repair mimics that of cultured rabbit corneal epithelial cells. Although cultured urothelial cells continue to synthesize uroplakins, which normally form two-dimensional crystalline urothelial plaques covering almost the entire apical urothelial surface, these proteins do not assemble into crystals in cultured cells. Cultured epithelial cells can, however, rapidly regain normal differentiation on the removal of mitogenic stimuli, the use of a suitable extracellular matrix, or the transplantation of the cells to an in vivo, nonmitogenic environment. These data suggest that cultured epithelial cells adopt altered differentiation patterns mimicking in vivo regenerating or hyperplastic epithelia. Blocking the synthesis of tissue-specific differentiation products, such as the K1 and K10 keratins designed to form extensive disulfide cross-links in cornified cells, or the assembly of uroplakin plaques allows epithelial cells to better migrate and proliferate, activities that are of overriding importance during wound repair. Cultured urothelial and other stratified epithelial cells provide excellent models for studying the regulation of the synthesis and assembly of differentiation products, a key cellular process during epithelial wound repair.

bladder epithelium; uroplakin; urothelial plaque; permeability barrier

THE ABILITY TO GROW CELLS of stratified squamous epithelia and to reproduce their in vivo differentiation program under well-defined in vitro conditions can greatly facilitate the study of the growth and differentiation of these cell types. Thus the in vitro cultivation of human epidermal cells using lethally irradiated or mitomycin-treated 3T3 feeder cells (80) made possible early breakthroughs in studying the synthesis of keratins (23, 24, 97, 98) and the formation of cornified envelope (83, 84, 95), and for using in vitro expanded epidermal cells to treat burn patients (21, 25). Studies of the differentiation of cultured corneal epithelial cells led to the discovery that corneal epithelial stem cells reside in the basal cell layer of peripheral corneal epithelium in a previously ignored area called the limbus (87) and to the use of cultured corneal epithelial cells in restoring patients' eyesight (73, 74). It is therefore not surprising that, in the area of urothelial biology, many efforts have been made to cultivate and characterize mammalian urothelial cells (see, e.g., Refs. 4, 56, 75, 86, 90, 98, 112). Using cell culture techniques, a number of investigators have demonstrated that mammalian urothelial cells can be expanded in vitro, forming urothelial epithelia that mimic to some degree the phenotype of in vivo urothelium (reviewed in Ref. 71). The interpretation of the data obtained from these cultured urothelial cells, which have been maintained under a wide range of conditions, remains somewhat problematic, however, with several important issues yet to be resolved. We will review here the differentiation of normal urothelium and cultured urothelial cells, paying special attention to a group of integral membrane proteins called uroplakins, which are synthesized as major urothelial differentiation products. We will also discuss the differentiation of cultured skin and corneal epithelial cells using the expression of specific keratins as markers, to derive the conclusion that cultured stratified epithelial cells undergo an alternative pathway of differentiation, rather than a truncated (normal) differentiation. The functional significance of these findings in terms of epithelial wound healing will be discussed.

TWO UNRESOLVED ISSUES REGARDING CULTURED UROTHELIAL CELLS

The urothelium is a stratified squamous epithelium (Fig. 1*A*) that covers much of the urinary tract, including the renal pelvis, ureter, bladder, and proximal urethra. As the progeny of

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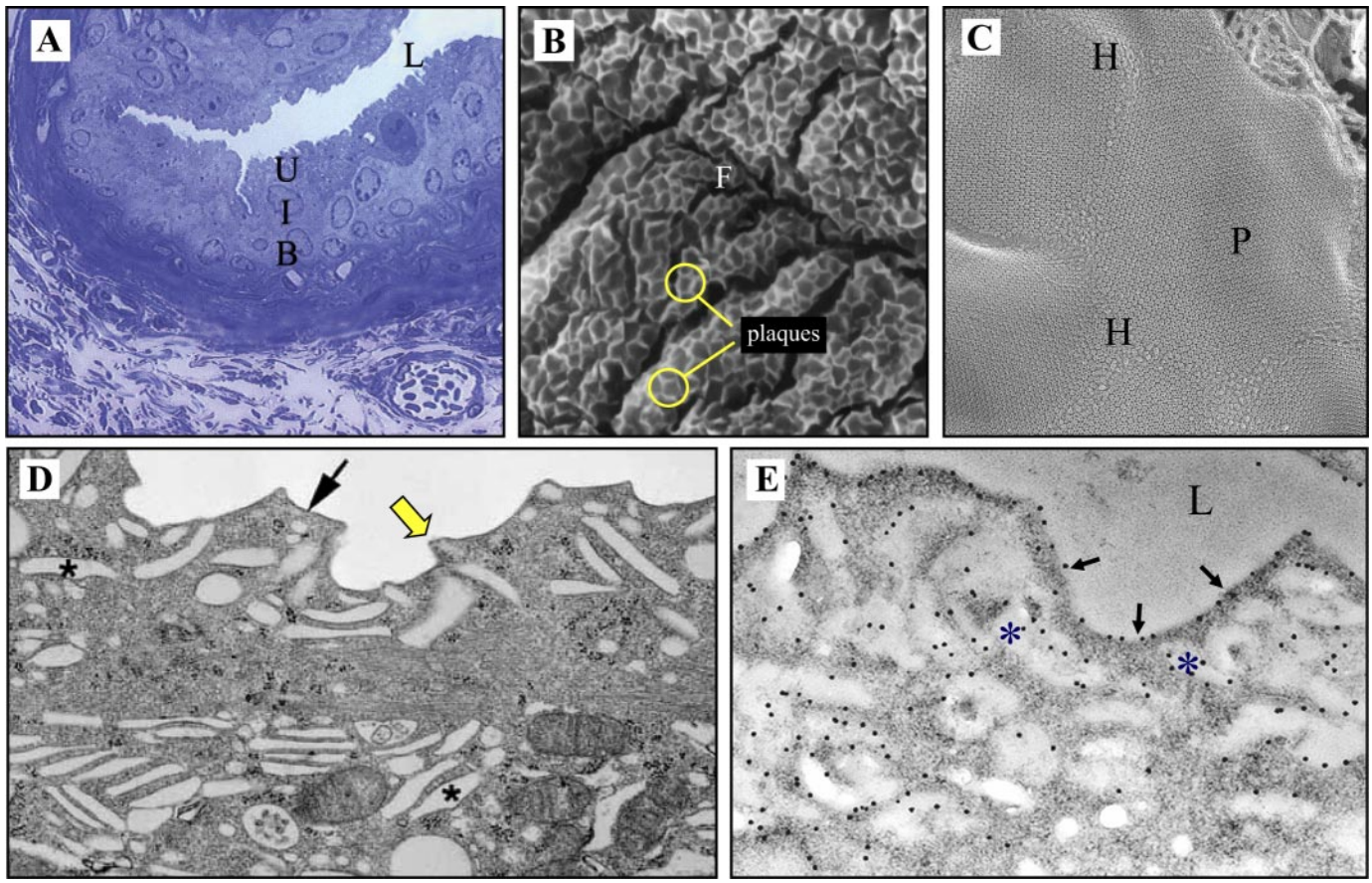

Fig. 1. Membrane specialization of mammalian urothelium. *A*: semithin section of mouse bladder (Toluidine blue-stained) showing the basal (B), intermediate (I), and superficial umbrella (U) cells that face the lumen (L). *B*: apical surface of mouse urothelial umbrella cells visualized by scanning EM showing prominent foldings (F) between the neighboring umbrella cells and urothelial plaques (courtesy of Edith Robbins, NYU Medical School). *C*: apical surface of mouse urothelial umbrella cells as seen by quick-freeze deep-etch showing that the urothelial plaques consists of 16-nm particles packed hexagonally forming two-dimensional (2D) crystals known as urothelial plaques (P) interconnected by hinges (H) that contain partially deformed 16-nm particles (45). The *top right* corner shows an area where the apical cellular membrane is broken, exposing submembranous cytoplasmic filaments and subcellular organelles. Reproduced with permission from Ref. 45. *D*: apical surface of mouse urothelial umbrella cell as seen by transmission electron microscopy showing the rigid-looking urothelial plaques (solid arrow) interconnected by hinges (open arrow), and the numerous cytoplasmic fusiform vesicles (*). *E*: ultrastructural immunogold localization of uroplakins on a section of mouse urothelium using a rabbit antiserum against total uroplakins. Note the labeling of fusiform vesicles (*) and apical plaques (arrows).

urothelial basal cells leave the basal layer, they undergo a highly specialized form of differentiation (Fig. 1, *B–E*), culminating in the formation of superficial umbrella cells containing a large amount of cytoplasmic vesicles involved in delivering uroplakin proteins to the apical surface (Fig. 1, *D* and *E*). As a result, the urothelial apical surface is almost completely covered by hexagonally packed 16-nm uroplakin particles, forming two-dimensional (2D) crystals also known as "urothelial plaques" (Fig. 1, *B* and *C*). These plaques are thought to play an important role in reversibly adjusting the apical surface area, stabilizing and preventing the apical urothelial surface from rupturing during bladder distention, and forming one of the most effective permeability barriers existing in nature (2, 38, 53, 66, 91, 96).

A key issue regarding cultured urothelial cells is potential urothelial heterogeneity, i.e., whether epithelial cells of various zones of the urinary tract are identical to each other. Because the epithelial lining of much of the urinary tract including the renal pelvis, ureter, bladder, and proximal urethra are all known simply as the "urothelium," earlier investigators have sometimes used the epithelial cells from these various zones interchangeably. Thus rat ureteral urothelium was suggested to be a model for studying urothelial cells in general (37). In human studies, the ureter has been used extensively as a source for cultured urothelial cells because it is much more readily available (from kidney donors) than the bladder epithelium. It has been said, e.g., that human urothelial cells from ureter and bladder behave similarly in culture, implying that what is learned from studying ureteral urothelial cells can be applied to bladder urothelium. Is it really true, then, that human ureteral and bladder urothelial cells are identical to each other and that whatever minor phenotypical differences may exist between these two urothelia in vivo are simply due to different mesenchymal signals?

Another important issue has to do with the interpretation of the differentiation state of cultured urothelial cells. It has been reported that cultured urothelial cells form stratified colonies that retain the ability to synthesize and assemble 2D crystals of urothelial plaques at the apical cell surface (102); however, others reported that cultured urothelial cells do not assemble these crystalline plaques (72, 78, 99). Is it therefore possible that cultured urothelial cells can exhibit a wide spectrum of

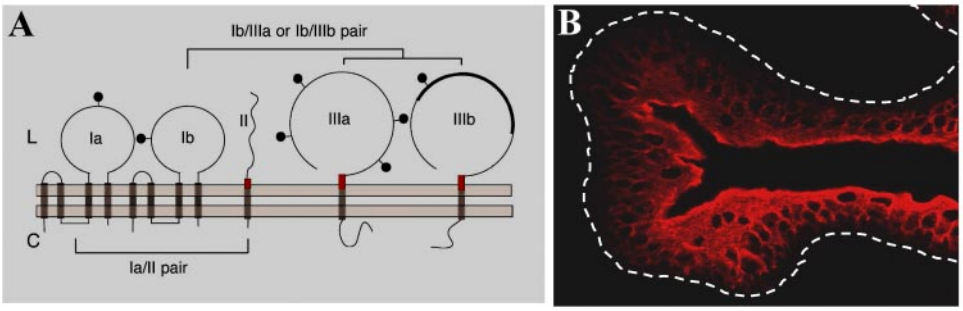

Fig. 2. Uroplakins (UPs) as major urothelial differentiation products. *A*: 4 major uroplakins (UPIa, 27-kDa; UPIb, 28-kDa; UPII, 15-kDa; and UPIIIa, 47-kDa) form 2 heterodimer pairs (Ia/II) and (Ib/IIIa). As a minor isoform of UPIIIa, UPIIIb also interacts with UPIb forming UPIb/IIIb heterodimer (16). C and L denote cytoplasm and lumen, respectively. *B*: immunohistochemical staining of mouse urothelium with an antiserum to uroplakin IIIa showing preferential staining of superficial umbrella cells, thus demonstrating the differentiation-dependent expression of uroplakins.

phenotypes ranging from the faithful reproduction of the in vivo urothelial phenotype to an apparently "defective" state? If the phenotype of cultured urothelial cells is this sensitive to the culture condition presented in different laboratory protocols, how can one compare the results obtained from these different culture systems, and what is the significance of the altered urothelial phenotype in vitro? Does it reflect a truncated normal urothelial differentiation, adopting a highly proliferative basal/intermediate cell phenotype without expressing markers associated with later/terminal urothelial differentiation (59, 90), or does it correspond to specific physiological or pathological states of the urothelium? It would be of interest to address the latter possibility because, should it be true, one might be able to take advantage of cultured urothelial cells as a model for studying such physiological or pathological conditions.

CLONAL AND SERIAL CULTURE OF MAMMALIAN UROTHELIAL CELLS

Although it has been relatively easy to grow epidermal keratinocytes using organ or explant culture, cultivating dissociated, single epidermal cells for clonal growth was not accomplished until Rheinwald and Green (80) introduced the use of lethally irradiated or mitomycin-treated 3T3 cells as a feeder layer. This culture system offers major advantages over other conventional culture systems in two important aspects. First, it enables the cultivation of epidermal keratinocytes at clonal densities, forming stratified colonies that can be serially subcultured (80). Second, it can achieve an expansion of the epidermal cells by a factor of 2^{30}-2^{60} (18, 81), making it possible to use a small piece of normal skin as the starting material for growing a sufficient number of cells to reconstitute the epidermis of the entire body of a burn patient (25). This culture system also works well for growing other stratified epithelial cells including those of the cornea, esophagus, thymus, as well as the bladder (20, 58, 75, 82, 92, 94). Because we have been using bovine urothelium, which is readily available fresh and in large quantities, as our starting material for the biochemical studies of uroplakins, I will use cultured bovine urothelial cells as an example in our discussion; I will also comment, however, on cultured urothelial cells of other species.

UROPLAKINS AS MARKERS OF UROTHELIAL DIFFERENTIATION

The identification of a panel of differentiation markers enables one to define, much more precisely, the differentiation state of an epithelium both in vivo and in culture. Mammalian urothelia synthesize, as their major differentiation products, a group of integral membrane proteins called uroplakins (Fig. 2*A*), which thus provide excellent markers for studying urothelial differentiation (Fig. 2*B*). Highly purified urothelial plaques from a number of mammalian species contain four major uroplakins, i.e., UPIa (~27 kDa), UPIb (~28 kDa), UPII (~15 kDa), and UPIIIa (~47 kDa), and a minor UPIIIb (~35 kDa; Fig. 2*A*) (16, 57, 108, 110, 113). UPIa and UPIb have four transmembrane domains (TMDs), a small (first) and a large (second) extracellular domain, the latter containing a highly conserved CCG signature motif. UPIa and UPIb share ~40% identical amino acid residues and belong to the tetraspanin family that include CD9, CD63, CD81, CD82, and CD151 (113). These tetraspanins play important roles in cell migration, immunological signaling, viral infection, and membrane architecture (6, 9, 10, 35, 52, 60). UPII and UPIIIa have only a single transmembrane domain and share a stretch of ~12 amino acid residues located on the extracellular side of the single transmembrane domain (Fig. 2*A*). Of these four major uroplakins, UPIIIa is the only one that has a relatively large cytoplasmic domain of ~50 amino acid residues, which has been postulated to play a role in interacting with the cytoskeleton (57, 110). Recent studies indicate that UPIa and UPIb bind to UPII and UPIIIa, respectively, forming heterodimers Ia/II and Ib/IIIa before they can exit from the endoplasmic reticulum (ER) (39, 103, 109). UPIb is exceptional as it can exit, by itself, from the ER (103); this finding, plus its presence in ocular surface and respiratory epithelia, suggests that UPIb may play a unique biological role (1, 59, 70). The sequence of the minor UPIIIb is related to that of UPIIIa, although the extracellular domain of the former contains a motif of ~85 amino acids that are related to a homolog of a DNA mismatch repair enzyme (16). Given the structural relatedness between UPIIIa and UPIIIb, it is not surprising that they both interact with UPIb (Fig. 2*A*) (16). Although the biological function of UPIIIb is not yet clear, recent phylogenetic studies indicate that it is encoded by a gene that is at least as ancient as that of UPIIIa (28). Because the amino acid sequences of all the mammalian

uroplakins, including bovine, human, and mouse, are highly conserved, these proteins must play important biological functions (28). The accumulation of these uroplakins in the terminally differentiated urothelial umbrella cells indicates that uroplakins can serve as excellent markers for urothelial differentiation (Fig. 2*B*) (55, 57, 109, 112). In addition, recent studies indicate that uroplakin Ia may serve as the urothelial receptor for type 1-fimbriated *Escherichia coli* that causes >90% of urinary tract infections (111, 117) and that the use of a urothelium-specific uroplakin II promoter made possible detailed analyses of the roles of specific oncogenes and tumor suppressor genes in bladder tumorigenesis (27, 29, 65, 116).

Ablation of mouse UPIIIa and UPII genes abolishes the formation of the uroplakin-delivering fusiform vesicles, greatly reduces or completely abolishes the apical accumulation of urothelial plaques, and leads to a compromised urothelial permeability barrier function. These findings indicate that uroplakins are integral protein subunits of urothelial plaques that contribute to the formation of the urothelial barrier (40, 41, 49). In addition, there is a great reduction of umbrella cell size, suggesting that continued insertion of urothelial plaques onto the apical surface may play a role in increasing and stabilizing the apical urothelial surface of the umbrella cells (49). Another important phenotype of both UPIIIa- and UPII-null mice is that a significant number of the mice suffer from vesicoureteral reflux (VUR) and severe hydronephrosis (40, 49). Litters from certain breeding pairs reproducibly die neonatally around *day 12*, indicating that uroplakin deficiency can have serious and even lethal consequences (40, 49). The possibility that major uroplakin mutations, such as deletion or truncation, can be lethal may explain why no such mutations have yet been found in human VUR patients (31, 44, 46). This may also explain why all the point mutations that have been detected so far in VUR patients do not seem to play a major role in this disease (31, 44, 46). Recent data indicate, however, that uroplakin defects may play a role in a rare congenital urinary tract problem, i.e., severe renal adysplasia (43).

UROTHELIAL HETEROGENEITY AND ITS IMPLICATIONS FOR CULTURED UROTHELIAL CELLS

Using uroplakins and specific keratins that are expressed differentially in various urothelial layers as markers, we compared the differentiation of urothelia in different zones of the bovine urinary tract (55). The umbrella cells of the ureteral urothelium show a much weaker cytoplasmic uroplakin staining than those of bladder, corresponding to a smaller number of fusiform vesicles, as seen by electron microscopy (55, 85). Immunoblotting confirmed that the in vivo ureteral urothelium contains much fewer uroplakins (~10-fold lower on a per milligram cellular protein basis) than bladder urothelium. To see whether such phenotypic differences can be explained by different mesenchymal signals (extrinsic modulation of epithelial differentiation) (14, 91), we compared the in vitro growth and differentiation of cultured bovine ureter, bladder, and bladder neck/proximal urethral urothelial cells after they had been serially expanded in an identical environment (with 3T3 feeder cells) to "dilute out" any residual mesenchymal signals that might have been present in the in vivo urothelium. The observation that cultured ureteral and bladder urothelial cells remain distinct in their morphology (Fig. 3), their uroplakin content, and their in vitro growth characteristics after serial expansion rules out a mesenchymal signal as a continuously required factor for the observed in vivo phenotypic differences (55). Rather, the data strongly support the idea that these two urothelia belong to two separate cell lineages that have become intrinsically divergent during development. Additional data indicate that the urothelium of the trigone (area defined by the urethra and 2 ureteral openings), which is said to be mesoderm derived and is thus distinct from the endoderm-derived bladder epithelium (22, 32), gives rise to cultures indistinguishable from those of bladder urothelium. This finding supports the idea that the urothelia of trigone and bladder actually belong to the same lineage (5, 101). Finally, we demonstrated that mouse bladder neck/proximal urethral urothelium preferentially responds to vitamin A deficiency, giving rise to foci of keratinization that expand to cover the rest of the bladder (Figs. 4 and 5). These results are consistent with the earlier finding that the mesoderm-derived ureteral urothelium and the endoderm-derived bladder urothelium respond differently to the same embryonic seminal vesicle mesenchymal instructions to form seminal vesicle and prostate epithelium, respectively (15, 19). Taken together, these data strongly suggest that there are at least three urothelial lineages, i.e., those of the ureter/renal pelvis, bladder/trigone, and bladder neck/proximal urethra (55), and that bladder urothelial metaplasia involves the replacement of one (bladder) urothelial lineage by cells from another (bladder neck/proximal urethral) lineage (55) (Figs. 4 and 5).

Although the above studies were done using bovine urothelia, consistent data exist for other mammalian species. An important difference in various urothelia is their in vitro growth property. In several culture media containing 0.1–1 mM calcium, urothelial cells of monkey bladder consistently grow faster and have a higher in vitro growth potential than those of the ureter (F. X. Liang, unpublished observations). The reverse is true, however, for humans, as cultured human ureteral urothelial cells grow much better than bladder urothelial cells, which are particularly difficult to cultivate and subculture, possibly reflecting an insufficiency, or the presence of a growth-inhibitory activity, in the tested culture media (F. X. Liang, unpublished observations). Although the significance of this species difference is unclear, these data clearly show that bladder and ureteral urothelial cells cultured under an identical in vitro environment remain phenotypically distinct. In addition, in vivo studies of mouse urothelium indicate that vitamin A deficiency induces preferentially the proliferation and keratinization of a subpopulation of bladder neck/proximal urethral urothelial cells (55). Together, these data indicate that the concept of urothelial heterogeneity may be generally applicable to all the mammalian species that have been studied so far (55). It would therefore be important to distinguish the urothelia of various regions in the urinary tract, e.g., bladder urothelium vs. ureteral urothelium, instead of using the term "urothelium" as if it is a homogeneous entity.

ALTERED UROPLAKIN AND KERATIN EXPRESSION IN CULTURED UROTHELIAL CELLS

Cultured bovine bladder urothelial cells form stratified colonies with large superficial cells that are frequently binucleated (99). Moreover, these cells continue to synthe-

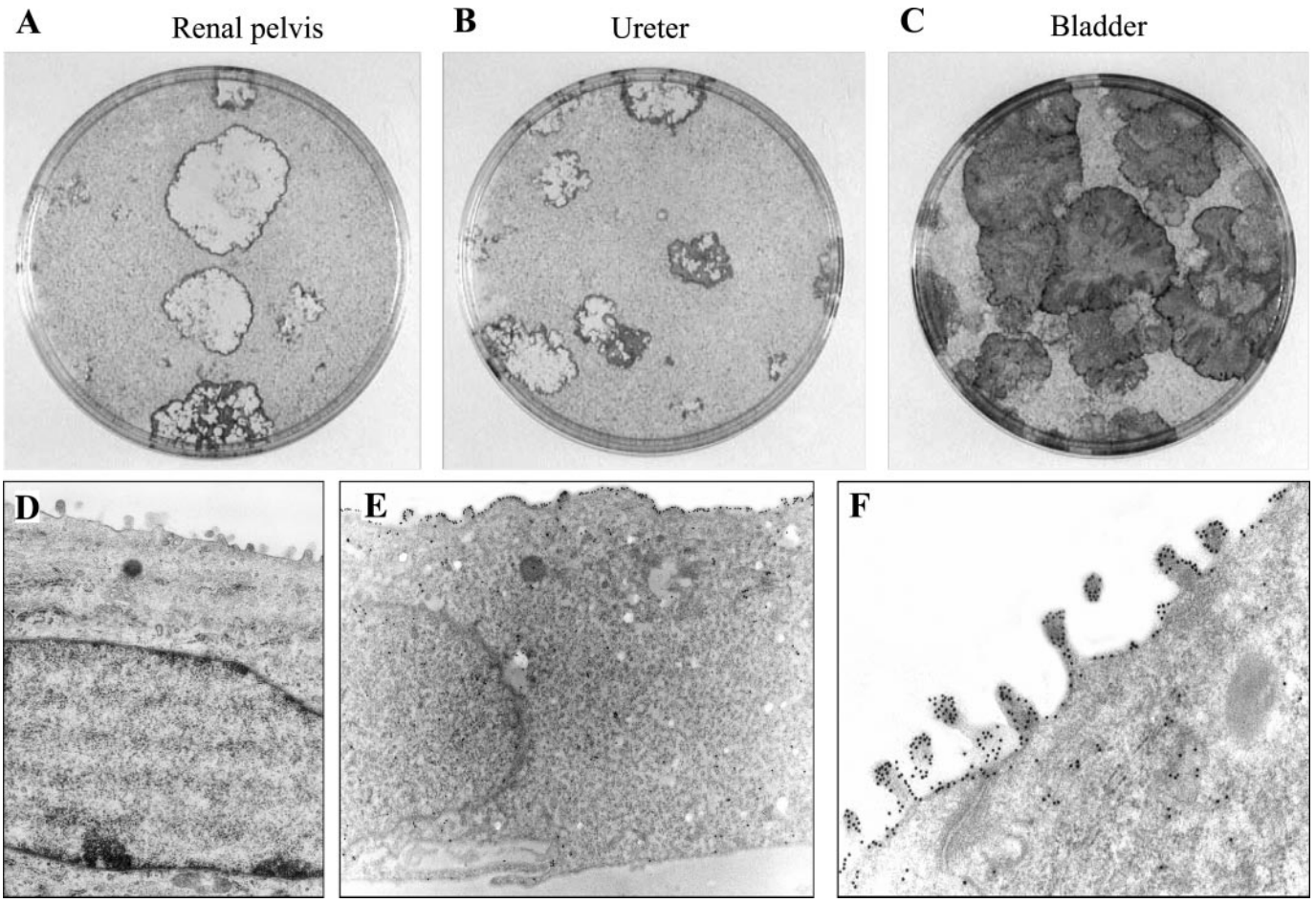

Fig. 3. Cultured bovine renal pelvis (*A*), ureteral (*B*), and bladder (*C*) urothelial cells. Cultured pelvis and ureteral urothelial cells form colonies that are much thinner (*A* and *B*) and, consistent with the in vivo data, synthesize ~10-fold fewer uroplakins than the cultured bladder urothelial cells (*C*). *D*: transmission electron microscopy of cultured bovine urothelial cells showing that their apical surface is covered by microvilli without any plaques. *E* and *F*: ultrastructural immunolocalization of uroplakins (using a rabbit anti-total uroplakins) on sections of cultured bovine urothelial cells showing the association of uroplakins with the apical surface including the tips of the microvilli. *A–C* are adapted from Ref. 55.

size uroplakins, albeit at a significantly lower level than normal urothelium (55). Both the mRNAs and protein complexes of uroplakins are remarkably stable in cultured urothelial cells (T.-T. Chen and A. Hu, unpublished observations). Immunofluorescent staining of nonpermeabilized, cultured urothelial cells using antibodies to individual uroplakins results in the surface labeling of some of the cells, indicating that the uroplakins can be targeted to the apical surface (114, 115). Electron microscopy revealed that the apical surface of such cultured urothelial cells harbors numerous microvilli with no detectable uroplakin crystals, indicating that the final steps of uroplakin assembly do not occur in serially cultured urothelial cells (Fig. 3, *D–F*) (34, 72, 99).

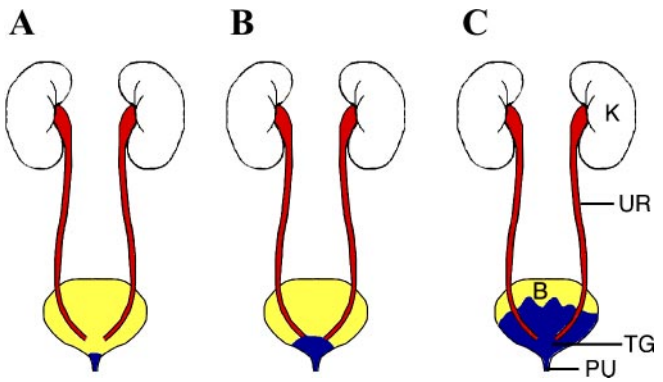

Fig. 4. Diagram showing that the urothelium can be divided into at least 3 lineages of ureter/renal pelvis (red), bladder (B)/trigone (TG; yellow), and bladder neck/proximal urethra (PU; blue; *A*). The diagram also shows that the vitamin A deficiency-induced urothelial metaplastic keratinization initiates in the proximal urethra and trigone area (*B*) and expands to cover the rest of the bladder (*C*). K, kidney; UR, ureter.

Another feature of cultured urothelial cells is altered keratin synthesis. Normal bovine urothelial cells synthesize mainly simple epithelial keratins, i.e., K8, K18, and K19, with trace amounts of keratinocyte keratins K5 and K6 (75, 99, 112). Cultured bovine bladder urothelial cells also express K8, K18, and K19 but in addition greatly upregulate the synthesis of keratinocyte keratins K5/K14 (markers for keratinocyte basal cells) (68, 93) and the K6/K16 (markers for hyperproliferation) (106). These observations raise the possibility that the primordial urothelium was a keratinocyte-type stratified squamous epithelium that later evolved to express only simple epithelial keratins and that, during hyperplastic conditions, the urothe-

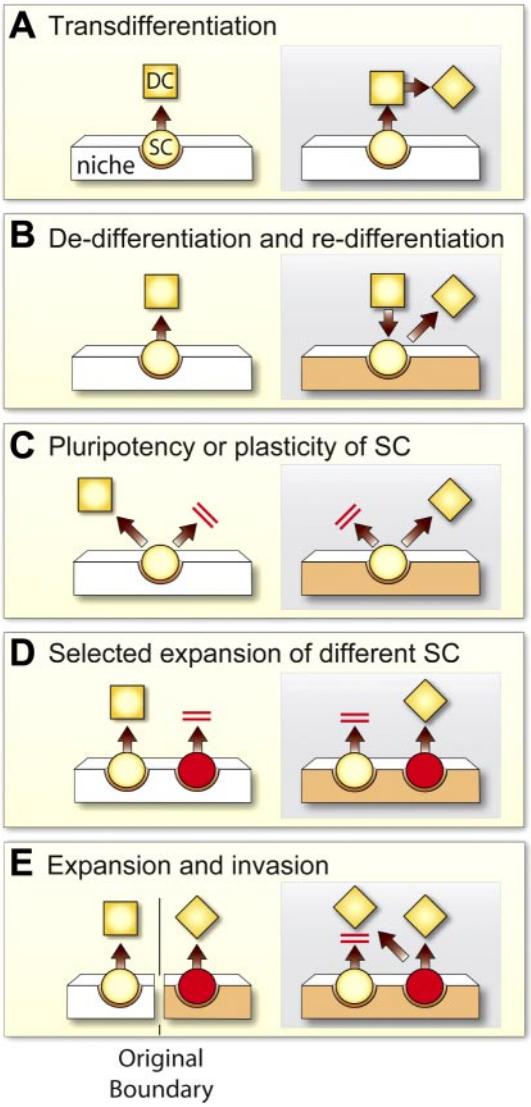

Fig. 5. Possible mechanisms of urothelial metaplasia. *A*: "transdifferentiation" model. A terminally differentiated cell (DC) can convert directly into another cell type in response to a change in environment (gray). *B*: "dedifferentiation and redifferentiation" model. A terminally differentiated cell can "dedifferentiate" to revert back to a stem cell (SC), which then gives rise to another cell type. *C*: "multipotent stem cell" model. A multipotent stem cell gives rise to different cell types in response to different environmental cues. *D*: "selective activation of stem cells" model. Two populations of stem cells are differentially activated depending on the environment. *E*: "expansion and replacement/invasion" model. Two separate populations of stem cells occupy 2 adjacent zones. Changes in environmental cues allow the progeny of 1 stem cell population to overgrow the other, resulting in a change in the apparent cellular phenotype. This last model appears to be able to best explain the vitamin A deficiency-induced urothelial keratinization (55).

lium reverts to a more "primitive" state of differentiation that is more keratinocyte-like (99).

FACTORS INFLUENCING THE GROWTH AND DIFFERENTIATION OF CULTURED UROTHELIAL CELLS

The culture environment can greatly influence the differentiation of all cultured stratified epithelial cells. In general, these cells stratify better in high-calcium (~1 mM) medium (75, 99, 112). Stratification of cultured urothelial cells is significantly suppressed in low-calcium medium, such as the keratinocyte growth medium (~0.1 mM calcium) designed for keeping the epithelial cells from stratification and hence in a relatively undifferentiated state (36, 78, 90). Various mitogenic factors, including protein factors such as EGF and PDGF, and small molecules such as retinoic acid that are present in calf or fetal calf serum employed in high concentrations in most culture media, tend to stimulate cell proliferation and suppress normal differentiation. Growing cells on a plastic surface stretches the basal cells flat, resulting in poor differentiation, whereas growing cells on a collagen or other extracellular matrix, preferably detached from the dish so that it can contract in response to cellular remodeling, allows more advanced epithelial differentiation. The mesenchymal tissues can clearly exert a major influence on urothelial differentiation (3, 14, 54, 71, 89, 91). Transmission of the nutrients from the epithelial basal cell surface, similar to the in vivo situation, offers an obvious advantage if the apical surface of neighboring cultured epithelial cells is sealed off with tight junctions. In addition, cultured normal human urothelial cells, probably ureter derived, can be stimulated by prostaglandin, which is present in abundance in the urine, achieving a higher degree of cytodifferentiation, as indicated by the upregulation of the synthesis of certain uroplakins (104).

Because most cell culture system are designed, fundamentally, to encourage cell proliferation, such systems utilize serum, which contains large amounts of blood clotting-released growth factors such as PDGF, which cells rarely encounter under normal circumstances, except during wound healing. When cells receive these potent wound-healing signals, they respond by proliferating and migrating to repair a wound, at the expense of normal differentiation. Such a transition from normal differentiation to wound repair is, by necessity, rapid and reversible. This is indeed the case for epidermal cells, which when placed in culture, can quickly turn off the synthesis of K1/K10 keratins. These suprabasally expressed keratins contain large amounts of cysteine residues designed to form extensive cross-linking to make the highly stable and physically tough cornified cells. Instead, the cultured epidermal cells turn on the synthesis in suprabasal cells of the more "generic" K6/K16 keratins that are made in all stratified squamous epithelia during hyperplasia (93, 106). When such cultured epidermal cells are trypsinized and placed subcutaneously in athymic mice, away from the mitogenic cell culture environment, they regain in a matter of hours a fully keratinized phenotype (20, 51). Similarly, cultured urothelial cells can reacquire a normal phenotype when transplanted to an appropriate in vivo environment (65, 69). Such a "Ying-Yang" relationship between growth and differentiation seems to hold true for all epithelia. In the case of urothelial cells, wounding can stimulate the normally slow-growing urothelial cells (with a labeling index of $<0.01\%$, which is among the lowest in stratified epithelia) to proliferate and can in the meantime downregulate the synthesis of uroplakins and other differentiation products (55). It is also conceivable that such proliferative stimuli can rapidly disassemble the 2D crystals of urothelial plaques (of the urothelial apical surface) that may interfere with urothelial cell proliferation and migration.

ALTERNATIVE PATHWAYS OF STRATIFIED EPITHELIAL DIFFERENTIATION

Given the large number of factors that can influence the differentiation state of cultured epithelial cells, one might presume that it would be difficult to compare the results obtained from cells cultured under different culture conditions. The results obtained from analyzing cultured skin epidermal epithelial cells using specific keratins as markers indicate, however, that such cells undergo two alternative pathways of differentiation. These two pathways are characterized by the suprabasal expression of either the normal basic K1 and acidic K10 keratins (markers for keratinization) or alternative basic K6 and acidic K16 keratins (markers for hyperproliferation) (Fig. 6*A*) (11, 93). Thus the suprabasal cells of normal, fully keratinized epidermis expresses exclusively K1/K10, whereas those of cultured epidermal cells maintained in mitogenic conditions express only K6/K16. The keratin patterns of partially keratinized epidermis, such as cultured epidermal cells maintained in vitamin A-deficient or other keratinization-inducing media, form a continuous spectrum between these two extremes (106). The ratio of the keratinization markers to hyperproliferation markers varies and is roughly proportional to the degree of morphological keratinization of the cultured epithelium. Moreover, the keratin patterns of a wide range of epidermal diseases, including psoriasis, actinic keratosis, basal cell carcinoma, and squamous cell carcinoma, also follow exactly the same rule: the more keratinized lesions express relatively more K1/K10, whereas the less keratinized ones

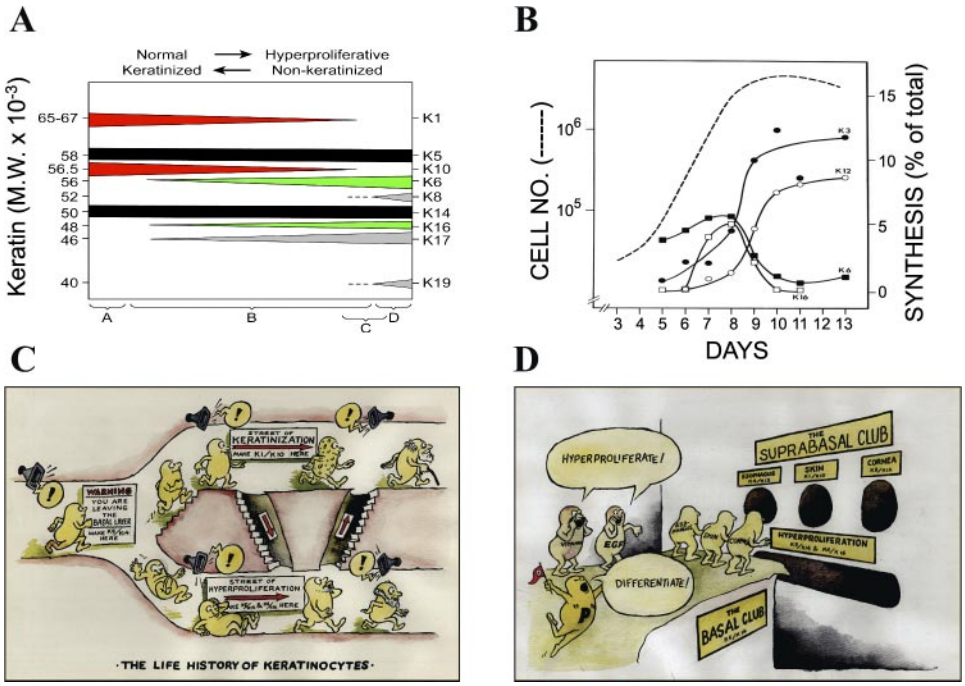

Fig. 6. Mutually exclusive expression of tissue-specific keratin markers and hyperproliferation keratin markers in cultured keratinocytes. *A*: schematic diagram summarizing the keratin patterns of human epidermal cells in normal epidermis (far *left*; *A*; expressing mainly K5/K14 markers for keratinocyte basal cells plus K1/K10 markers for keratinization or skin type differentiation), epidermal cells maintained under mitogenic conditions (far *right*; *D*; expressing mainly K5/K14 plus K6/K16 markers for hyperproliferative keratinocytes). Keratins of a wide range of epidermal diseases including psoriasis, basal cell and squamous cell carcinoma, and other epidermal diseases (*B*), and cultured epidermal cells in conditions favoring keratinization (vitamin A deficiency, air-liquid interface, etc; *C*) form a continuous spectrum in between the normal epidermis (*A*) and cultured epidermal cells (*D*). Note the amount of K1/K10 keratins (markers for keratinization) is proportional, whereas that of the K6/K16 keratins (markers for hyperproliferation) is inversely proportional, to the degree of epithelial morphological keratinization. Modified from Refs. 11 and 93. *B*: mutually exclusive synthesis of K6/K16 keratins and tissue-specific K3/K12 keratins (markers for corneal type differentiation) in cultured rabbit corneal keratinocytes. Reproduced from Ref. 88. *C*: highly simplified and dramatized cartoon based purely on keratin expression data, illustrating the alternative pathways of epidermal keratinocytes and their regulation by the environmental factors. The figure to the *left* represents an epidermal basal cell that, while still in the basal compartment, makes K5/K14 keratins (that are made by basal cells of all stratified squamous epithelia). Once leaving the basal compartment, the cell adopts 1 of the 2 alternative pathways of differentiation, labeled "Street of Keratinization" and marked by K1/K10 expression, or "Street of Hyperproliferation" and marked by K6/K16 expression, depending on the environmental factors/instructions ("!" from the loudspeakers). The total replacement of K1/K10 keratins by K6/K16 in suprabasal cells of cultured cells rules out the notion of cultured epidermal cells undergoing "incomplete" or "truncated" (normal) epidermal differentiation. Suprabasal cells can adopt one of, or may oscillate between, the 2 pathways of differentiation in response to environmental factors/instructions. Reproduced from Ref. 26 with permission. *D*: highly simplified and dramatized cartoon illustrating the importance of intrinsic and extrinsic factors in regulating the differentiation of skin, esophageal, and corneal epithelial cells. The 3 figures in the center represent a skin, esophageal, and corneal epithelial basal cell, as labeled. Like epidermal basal cells illustrated in (*A*), corneal and esophageal basal cells also faced a binary decision of whether to jump upward to undergo their normal in vivo differentiation pathways marked by the expression of tissue-specific keratin pairs, i.e., K3/K12 (maker for corneal-type differentiation) and K4/K13 (esophageal type differentiation), respectively, or jump downward to undergo the hyperproliferation pathway marked by a common K6/K16 keratin pair. This decision is made in response to the sum of opposing environmental factors, some mitogenic (figures labeling EGF, vitamin A), thus favoring hyperproliferation, whereas some others favor differentiation (cheering figure label "P" to denote a "permissive" in vivo environment that is devoid of the mitogenic factors). The 3 basal cell figures are intrinsically divergent from one another. Note that one to the *left* has a stronger right leg (esophageal basal cell), the one to the *right* has a stronger left leg (cornea), and the one in the *middle* has 2 balanced legs (skin), so that in response to the same permissive environment these 3 figures will jump to the *left*, *right*, or *middle* tunnels labeled esophageal, corneal, and skin type of differentiation, respectively. Reproduced from Ref. 26 with permission.

express more K6/K16 (106). The replacement of the K1/K10 keratins by the less tissue-specific K6/K16 keratins in cultured epidermal and other stratified squamous epithelial cells may be functionally important in terms of the physical as well as the signaling properties of the cytoskeleton (17, 107). The fact that the K1/K10 of suprabasal cells in cultured epidermal colonies maintained under mitogenic conditions are completely replaced by K6/K16 conclusively rules out the notion that such cultured cells undergo only the early stages of normal keratinization (equivalent to the basal and intermediate cell layers of normal epidermis). Rather, cultured epidermal cells undergo an alternative pathway of differentiation that is similar to that of hyperplastic epidermal lesions such as psoriasis. This idea is strongly supported by data on cultured corneal epithelial cells. In this case, the suprabasal cells of corneal epithelium (in the limbal stem cell zone) express basic K3 and acidic K12 (markers for corneal epithelial type differentiation), whereas those of cultured corneal epithelial cells initially express additional K6/K16 (markers for hyperproliferation) (Fig. 6*B*) (87, 88). Introducing a wound in rabbit corneal epithelium in vivo leads to the transient expression of K6/K16 keratins similar to cultured corneal epithelial cells; the keratin pattern returns to normal, however, when the wound is repaired (88). Together, these data indicate that cultured keratinocytes undergo an alternative pathway of differentiation that operates during hyperplasia as a result of wound repair or of a disease state (Fig. 6, *C* and *D*). As mentioned earlier, the differentiation of cultured urothelial cells also deviates from that of normal urothelium in that the former synthesize ~10 times less uroplakins, which, moreover, usually do not usually assemble into 2D crystals (72, 78, 99). Together with what we have learned about the differentiation of cultured keratinocytes, these data suggest that cultured urothelial cells also undergo an alternative pathway of differentiation.

ASSEMBLY OF UROPLAKIN PLAQUES AND ITS POSSIBLE REGULATION

As integral membrane proteins, uroplakins first form heterodimers (UPIa/II and UPIb/III) in the ER before they can be assembled into 16-nm particles that later form 2D crystals in post-Golgi vesicles (Fig. 7*A*). The structure of the urothelial plaque has been studied using a number of ultrastructural techniques including negative staining coupled with image

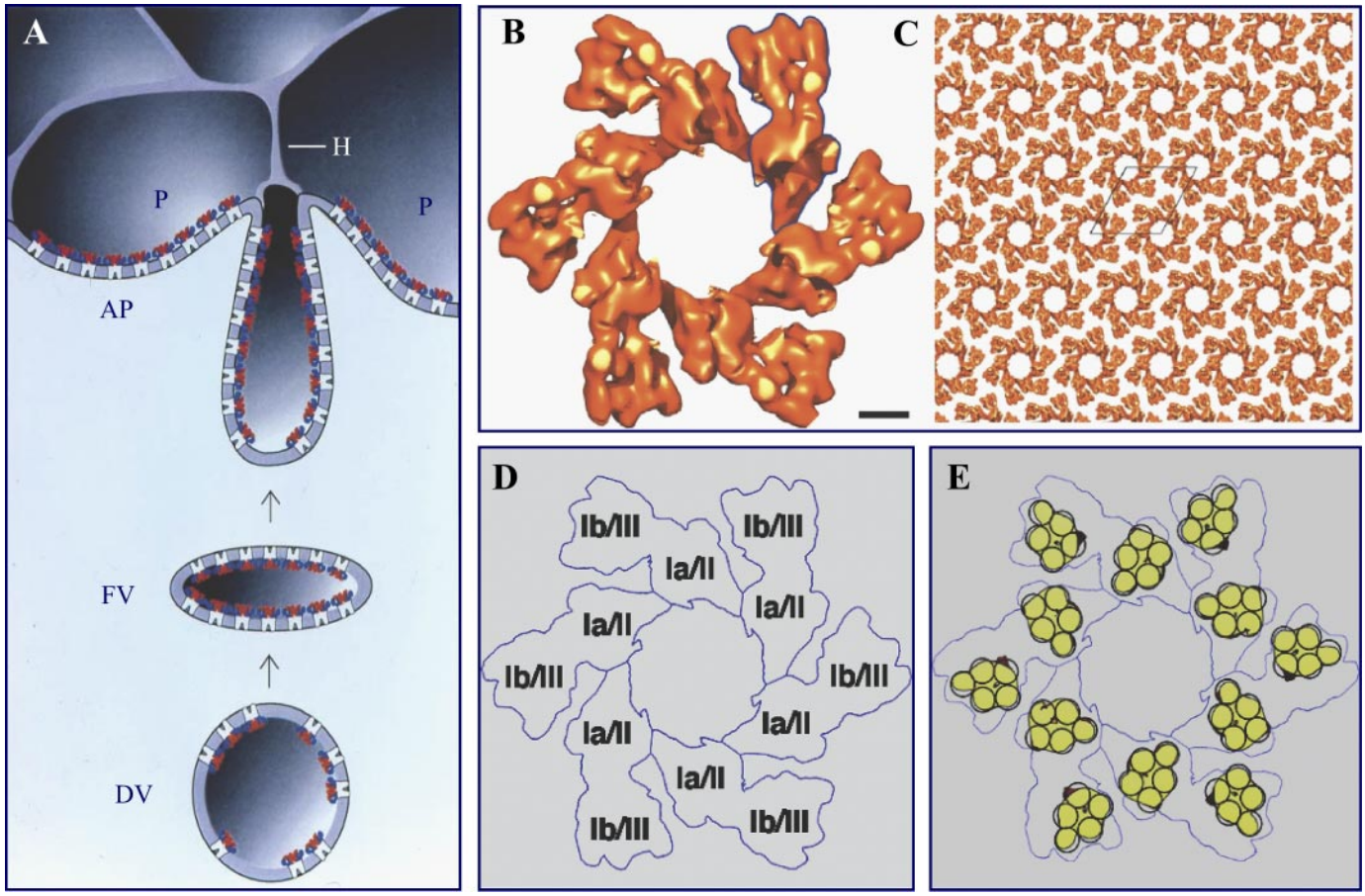

Fig. 7. Biosynthesis and assembly of uroplakins. *A*: schematic diagram showing that uroplakins, after being synthesized and forming appropriate heterodimers in the endoplasmic reticulum (ER), are assembled into 16-nm particles in post-Golgi vesicles [first in discoidal vesicles (DV) and later in more mature, fusiform (FV) vesicles], which eventually fuse with the apical urothelial surface (AP) covered with plaques (P) interconnected by hinges (H). *B* and *C*: top view of a single 16-nm particle (*B*) and of a crystalline array of the particles (*C*) as seen by the cryo-EM technique at ~10-Å resolution. *D*: hypothetical schematic diagram showing that the UPIa/II and UPIb/IIIA heterodimers are associated with the inner and outer subdomains, respectively, of the 16-nm particle, based on the immunolocalization of UPIa, the calculation of the mass of proteins that can be accommodated by the individual subdomains, and the visualization of 5 transmembrane domains (*E*) in each subdomain. *B–E* reproduced from Ref. 63 with permission.

processing, atomic force microscopy, quick-freeze deep-etch, and cryo-electron microscopy (45, 62, 63, 105). Such data revealed that each 16-nm particle can be resolved into six dumbbell-shaped subunits, each consisting of an inner and an outer subdomain (Fig. 7, *B* and *C*). Available data suggest that the UPIa/II and UPIb/IIIa heterodimers are associated with the inner and outer subdomain, respectively (Fig. 7*D*), based on the localization of UPIa using recombinant FimH (62, 117), the visualization of five transmembrane domains in all the subdomains (Fig. 7*E*) (62), and the scanning transmission EM-calculated mass that can be accommodated by each subdomain (63, 105).

Data from transfection and other studies have recently made clear that the "uroplakin I" recognized by AE31, a mouse monoclonal antibody first described in 1990 (115), is actually the 32-kDa pro-UPII, which assumes a hairpin-like configuration (39). Although pro-UPII by itself cannot exit from the ER and is quite unstable, once it binds to its partner, UPIa, the heterodimer becomes stabilized and acquires the ability to exit from the ER (Fig. 8) (39, 103). The removal of the prosequence of pro-UPII by furin, a *trans*-Golgi network-associated protease, leads to global conformational changes in mature UPII, as demonstrated by the use of a panel of conformation-dependent antibodies to a number of UPII epitopes (39). The data indicate that UPIa can stabilize and facilitate the maturation of pro-UPII and that UPIb can induce conformational changes in its partner UPIIIa and facilitate its maturation (39). These results indicate that tetraspanin uroplakins can induce conformational changes leading to the ER exit, stabilization, and cell surface expression of their associated, single-TMD partner proteins; these tetraspanin proteins can thus function as "maturation-facilitators" (Fig. 8) (39).

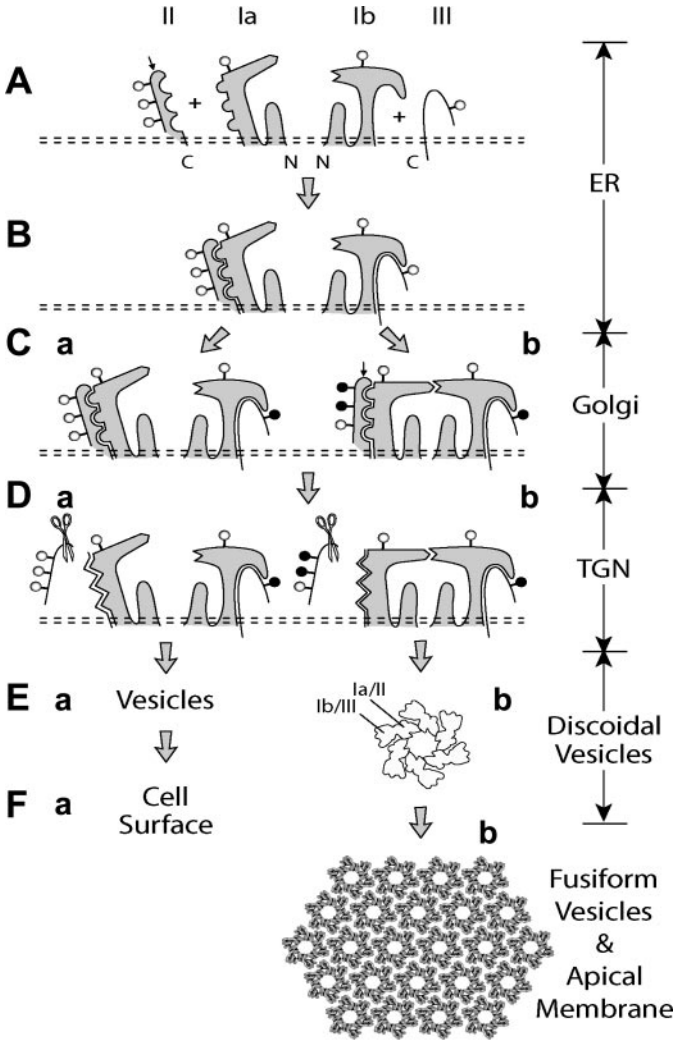

Fig. 8. Assembly of uroplakins in normal urothelium and in cultured urothelial cells. *A* and *B* show the assembly of UPIa/II and UPIb/IIIA heterodimers in the ER; *C* shows a differentiation-dependent glycosylation step, resulting in the formation of complex glycans on the prosequence of UPII in normal urothelial umbrella cells (*Cb*), but only high-mannose glycans in cultured urothelial cells (*Ca*), leading to different dimer conformations and assembly properties; *D* shows the removal of the UPII prosequence by the *trans*-Golgi network (TGN)-associated furin; *E* shows that only heterotetramers in normal urothelium can assemble into 16-nm particles (*Eb*) and later into 2D crystals (*Fb*), whereas those of cultured cells (*Ea* and *Fa*) cannot. Reproduced from Ref. 39 with permission.

POSSIBLE SIGNIFICANCE OF CULTURED UROTHELIAL CELLS: A MODEL FOR REGENERATIVE UROTHELIUM

What is the biological significance of these altered patterns of differentiation associated with cultured skin and urothelial cells? One possibility is that the accumulation of the highly cross-linkable K1/K10 filaments or the 2D crystals of uroplakins in their superficial cells can impair the ability of such cells and their underlying cells (through cell-cell interactions) to undergo migration and proliferation, which are, of course, of overriding importance during wound repair. This concept can easily explain why during wound healing the synthesis and assembly of some of the major epithelial specialization products are suppressed. Relatively little is known, however, about the mechanisms that link hyperplasia and the suppression of the synthesis and assembly of specific keratins and uroplakins. Cultured stratified epithelial cells provide excellent opportunities for studying how these processes may be regulated and for analyzing the roles of various soluble factors and EM in epithelial repair.

CULTURED UROTHELIAL CELLS AND TISSUE ENGINEERING

The fact that stratified squamous epithelial cells, including those of the bladder, can be greatly expanded in culture has made it possible to use such cells to reconstitute the epithelia in vivo (21, 25, 73, 74). There are several issues that are relevant when one uses cultured urothelial and other epithelial cells for tissue engineering. First, it would be useful to know which areas of the tissue are enriched in stem cells, e.g., the limbal region of the corneal epithelium (73) and the bulge area of the hair follicle (12). The importance of this point is underscored by the fact that, before our proposal in 1986 that corneal epithelial stem cells reside in the limbus (87), it was thought that conjunctival and corneal epithelia were equipotent and that the former can convert into the latter through "transdifferentiation" (30) and that limbal and corneal epithelia represented distinct entities (48). Without the realization that corneal epithelial stem cells reside in the limbus, it would not have been imaginable that limbal epithelium can be used as a source for corneal epithelial culture and tissue reconstruction (47, 74). Similar considerations apply to the source of urothelial cells, in terms of their lineage. Thus one should not assume

that the use of cultured human ureteral urothelial cells would necessarily have the same long-term effects as cultured human bladder urothelial cells. Second, the in vitro growth of epithelial stem cells, away from their normal in vivo niche which keeps the stem cells in a slow-cycling state, can rapidly exhaust the cells' proliferative potential (61, 79). The in vitro expansion of epithelial stem cells should therefore be kept to a minimum, before they are returned to an in vivo environment where the transplanted stem cells can interact with mesenchymal components to reconstitute a niche and can return to a slow-cycling state (61). Alternatively, stem cells can be passaged and expanded in an environment mimicking the in vivo stem cell niche; a prerequisite of this is a better understanding of the molecular and cellular basis of the niche. Third, cultured epithelial cells, even with a somewhat "abnormal" morphology, can be quite adequate for transplantation, because these cells can rapidly acquire a normal phenotype within days, if not hours, when they are placed in an appropriate in vivo environment (20, 51). Therefore, it may not be unnecessary to reconstitute in vitro a perfectly "normal" epithelium before it can be used for transplantation. Fourth, the mesenchymal tissue can exert a major influence on the differentiation phenotype of its overlying epithelium. A good example is provided by urothelium which, when transplanted onto an embryonic urogenital or adult intestinal mesenchyme, can be converted into a prostate-like or a mucin-secreting epithelium, respectively (3, 14, 54, 71, 89, 91). Finally, cultured epithelial cells can be used as a temporary "dressing" that can rapidly cover the wound, allowing the remaining epithelial stem cells located at the edge of the wound to eventually replace the transplanted cells. For this purpose, prefabricated allografts can prove to be convenient and versatile (8, 76, 100).

SUMMARY AND CONCLUSION

Significant progress has been made in growing stratified squamous epithelial cells under well-defined in vitro conditions. A remaining hurdle is that many such cultured epithelial cells, perhaps as a consequence of being exposed to a mitogenic environment, do not replicate their in vivo normal differentiation. However, this may be viewed as a virtue, instead of a handicap, of the cultured cells, if one recognizes that the altered differentiation state of such cultured cells mimics that of the in vivo wound-healing epithelium, as this provides unique opportunities to study mechanisms of epithelial wound repair. Moreover, conditions have been found to allow cultured epidermal keratinocytes (that are maintained on a collagen gel and exposed to an air-liquid interface) to achieve an advanced stage of keratinization with the formation of enucleated cornified cells (7, 50). Lessons can also be learned from the systematic studies by Nelson and Bissell (67), who have illustrated the critical importance of the extracellular matrix that, in three-dimensional cultures, allows an impressive degree of cytodifferentiation in normal and cancerous mammary epithelial cells. Further optimization of the stratified squamous epithelial culture should therefore pay attention to many factors including the mitogenic and differentiation-suppressing factors in the serum, the effects of extracellular matrix and three dimensional model, and the further development of synthetic, serum-free media allowing individual stratified squamous epithelial cell types to differentiate. A good strategy, which has not been adequately explored, is to grow epithelial cells in two stages: to first expand the cells in a mitogenic tissue culture medium, followed by a shift to a differentiation-favoring condition (13, 77). Although it has been reported that adult bone marrow stem cells have some degree of plasticity and can give rise to certain stratified squamous epithelia (33, 42), most adult stem cells work best within their lineages. Studies of urothelial cells should therefore pay attention to the urothelial lineage. Additional work should lead to refined cell culture conditions that will allow stratified epithelial cells to reproduce their in vivo differentiation patterns and will make possible the manipulation of the growth and differentiation state of stratified epithelial cells. Such culture systems will provide invaluable tools for studying the molecular mechanism of normal and wound repair-associated epithelial differentiation.

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REFERENCES

1. **Adachi W, Okubo K, and Kinoshita S.** Human uroplakin Ib in ocular surface epithelium. *Invest Ophthalmol Vis Sci* 41: 2900–2905, 2000.
2. **Apodaca G.** The uroepithelium: not just a passive barrier. *Traffic* 5: 117–128, 2004.
3. **Atala A.** Future trends in bladder reconstructive surgery. *Semin Pediatr Surg* 11: 134–142, 2002.
4. **Autrup H, Grafstrom RC, Christensen B, and Kieler J.** Metabolism of chemical carcinogens by cultured human and rat bladder epithelial cells. *Carcinogenesis* 2: 763–768, 1981.
5. **Batourina E, Tsai S, Lambert S, Sprenkle P, Viana R, Dutta S, Hensle T, Wang F, Niederreither K, McMahon AP, Carroll TJ, and Mendelsohn CL.** Apoptosis induced by vitamin A signaling is crucial for connecting the ureters to the bladder. *Nat Genet* 37: 1082–1089, 2005.
6. **Berditchevski F.** Complexes of tetraspanins with integrins: more than meets the eye. *J Cell Sci* 114: 4143–4151, 2001.
7. **Bernstam LI, Vaughan FL, and Bernstein IA.** Keratinocytes grown at the air-liquid interface. *In Vitro Cell Dev Biol* 22: 695–705, 1986.
8. **Bolivar-Flores J, Poumian E, Marsch-Moreno M, Montes de Oca G, and Kuri-Harcuch W.** Use of cultured human epidermal keratinocytes for allografting burns and conditions for temporary banking of the cultured allografts. *Burns* 16: 3–8, 1990.
9. **Boucheix C and Rubinstein E.** Tetraspanins. *Cell Mol Life Sci* 58: 1189–1205, 2001.
10. **Boucheix C, Silvie O, Rubinstein E, and Mazier D.** A tiny thread towards a tetraspanin function. *Pathol Biol (Paris)* 52: 55–57, 2004.
11. **Cooper D, Schermer A, and Sun TT.** Classification of human epithelia and their neoplasms using monoclonal antibodies to keratins: strategies, applications, and limitations. *Lab Invest* 52: 243–256, 1985.
12. **Cotsarelia G, Sun T-T, and Lavker RM.** Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogensis. *Cell* 61: 1329–1337, 1990.
13. **Cross WR, Eardley I, Leese HJ, and Southgate J.** A biomimetic tissue from cultured normal human urothelial cells: analysis of physiological function. *Am J Physiol Renal Physiol* 289: F459–F468, 2005.
14. **Cunha GR, Fujii H, Neubauer BL, Shannon JM, Sawyer L, and Reese BA.** Epithelial-mesenchymal interactions in prostatic development. I. Morphological observations of prostatic induction by urogenital

sinus mesenchyme in epithelium of the adult rodent urinary bladder. *J Cell Biol* 96: 1662–1670, 1983.

15. **Cunha GR, Young P, Higgins SJ, and Cooke PS.** Neonatal seminal vesicle mesenchyme induces a new morphological and functional phenotype in the epithelia of adult ureter and ductus deferens. *Development* 111: 145–158, 1991.
16. **Deng FM, Liang FX, Tu L, Resing KA, Hu P, Supino M, Hu CC, Zhou G, Ding M, Kreibich G, and Sun TT.** Uroplakin IIIb, a urothelial differentiation marker, dimerizes with uroplakin Ib as an early step of urothelial plaque assembly. *J Cell Biol* 159: 685–694, 2002.
17. **DePianto D and Coulombe PA.** Intermediate filaments and tissue repair. *Exp Cell Res* 301: 68–76, 2004.
18. **Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, Weinberg RA, Louis DN, Li FP, and Rheinwald JG.** Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol* 20: 1436–1447, 2000.
19. **Donjacour AA and Cunha GR.** Assessment of prostatic protein secretion in tissue recombinants made of urogenital sinus mesenchyme and urothelium from normal or androgen-insensitive mice. *Endocrinology* 132: 2342–2350, 1993.
20. **Doran TI, Vidrich A, and Sun TT.** Intrinsic and extrinsic regulation of the differentiation of skin, corneal and esophageal epithelial cells. *Cell* 22: 17–25, 1980.
21. **Ferrari S, Pellegrini G, Mavilio F, and De Luca M.** Gene therapy approaches for epidermolysis bullosa. *Clin Dermatol* 23: 430–436, 2005.
22. **Foster CS, Dodson A, Desmond AD, and Fordham M.** Embryology and anatomy of the urinary bladder. In: *Pathology of the Urinary Bladder*. Philadelphia, PA: Saunders, 2004, p. 1–14.
23. **Fuchs E and Green H.** Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell* 19: 1033–1042, 1980.
24. **Fuchs E and Green H.** The expression of keratin genes in epidermis and cultured epidermal cells. *Cell* 15: 887–897, 1978.
25. **Gallico GG III, O'Connor NE, Compton CC, Kehinde O, and Green H.** Permanent coverage of large burn wounds with autologous cultured human epithelium. *N Engl J Med* 311: 448–451, 1984.
26. **Galvin S, Loomis C, Manabe M, Dhouailly D, and Sun TT.** The major pathways of keratinocyte differentiation as defined by keratin expression: an overview. *Adv Dermatol* 4: 277–299, 1989.
27. **Gao J, Huang HY, Pak J, Cheng J, Zhang ZT, Shapiro E, Pellicer A, Sun TT, and Wu XR.** p53 Deficiency provokes urothelial proliferation and synergizes with activated Ha-ras in promoting urothelial tumorigenesis. *Oncogene* 23: 687–696, 2004.
28. **Garcia-España A, Chung PJ, Zhao XQ, Lee A, Pellicer A, Yu J, Sun TT, and DeSalle R.** Origin of the tetraspanin uroplakins and their co-evolution with associated proteins: implications for uroplakin structure and function. *Mol Phylogenet Evol.* In press.
29. **Garcia-Espana A, Salazar E, Sun TT, Wu XR, and Pellicer A.** Differential expression of cell cycle regulators in phenotypic variants of transgenically induced bladder tumors: implications for tumor behavior. *Cancer Res* 65: 1150–1157, 2005.
30. **Geggel HS, Thoft RA, and Friend J.** Histology of human conjunctival transplantation. *Cornea* 3: 11–15, 1984.
31. **Giltay JC, van de Meerakker J, van Amstel HK, and de Jong TP.** No pathogenic mutations in the uroplakin III gene of 25 patients with primary vesicoureteral reflux. *J Urol* 171: 931–932, 2004.
32. **Gosling JA and Dixon JS.** Embryology and anatomy. In: *The Bladder*, edited by Fitzpatrick JM and Krane RJ. Edinburgh: Churchill Livingstone, 1995, p. 1–16.
33. **Grove JE, Bruscia E, and Krause DS.** Plasticity of bone marrow-derived stem cells. *Stem Cells* 22: 487–500, 2004.
34. **Heatfield BM, Sanefuji H, El-Gerzawi S, Urso B, and Trump BF.** Surface alterations in urothelium of normal human bladder during long-term explant culture. *Scan Electron Microsc* 3: 61–70, 1980.
35. **Hemler ME.** Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. *Annu Rev Cell Dev Biol* 19: 397–422, 2003.
36. **Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, and Yuspa SH.** Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* 19: 245–254, 1980.
37. **Hicks RM.** The fine structure of the transitional epithelium of rat ureter. *J Cell Biol* 26: 25–48, 1965.
38. **Hicks RM.** The mammalian urinary bladder: an accommodating organ. *Biol Rev Cam Philos Soc* 50: 215–246, 1975.
39. **Hu CC, Liang FX, Zhou G, Tu L, Tang CH, Zhou J, Kreibich G, and Sun TT.** Assembly of urothelial plaques: tetraspanin function in membrane protein trafficking. *Mol Biol Cell* 16: 3937–3950, 2005.
40. **Hu P, Deng FM, Liang FX, Hu CM, Auerbach AB, Shapiro E, Wu XR, Kachar B, and Sun TT.** Ablation of uroplakin III gene results in small urothelial plaques, urothelial leakage, and vesicoureteral reflux. *J Cell Biol* 151: 961–972, 2000.
41. **Hu P, Meyers S, Liang FX, Deng FM, Kachar B, Zeidel ML, and Sun TT.** Role of membrane proteins in permeability barrier function: uroplakin ablation elevates urothelial permeability. *Am J Physiol Renal Physiol* 283: F1200–F1207, 2002.
42. **Huttmann A, Li S, and Dhursen U.** Bone marrow-derived stem cells and "plasticity." *Ann Hematol* 82: 599–604, 2003.
43. **Jenkins D, Bitner-Glindzicz M, Malcolm S, Hu CC, Allison J, Winyard PJ, Gullett AM, Thomas DF, Belk RA, Feather SA, Sun TT, and Woolf AS.** De novo uroplakin IIIa heterozygous mutations cause human renal adysplasia leading to severe kidney failure. *J Am Soc Nephrol* 16: 2141–2149, 2005.
44. **Jiang S, Gitlin J, Deng FM, Liang FX, Lee A, Atala A, Bauer SB, Ehrlich GD, Feather SA, Goldberg JD, Goodship JA, Goodship TH, Hermanns M, Hu FZ, Jones KE, Malcolm S, Mendelsohn C, Preston RA, Retik AB, Schneck FX, Wright V, Ye XY, Woolf AS, Wu XR, Ostrer H, Shapiro E, Yu J, and Sun TT.** Lack of major involvement of human uroplakin genes in vesicoureteral reflux: implications for disease heterogeneity. *Kidney Int* 66: 10–19, 2004.
45. **Kachar B, Liang F, Lins U, Ding M, Wu XR, Stoffler D, Aebi U, and Sun TT.** Three-dimensional analysis of the 16 nm urothelial plaque particle: luminal surface exposure, preferential head-to-head interaction, and hinge formation. *J Mol Biol* 285: 595–608, 1999.
46. **Kelly H, Ennis S, Yoneda A, Bermingham C, Shields DC, Molony C, Green AJ, Puri P, and Barton DE.** Uroplakin III is not a major candidate gene for primary vesicoureteral reflux. *Eur J Hum Genet* 13: 500–502, 2005.
47. **Kenyon KR and Tseng SC.** Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* 96: 709–722, 1989.
48. **Kinoshita S, Kiorpes TC, Friend J, and Thoft RA.** Limbal epithelium in ocular surface wound healing. *Invest Ophthalmol Vis Sci* 23: 73–80, 1982.
49. **Kong XT, Deng FM, Hu P, Liang FX, Zhou G, Auerbach AB, Genieser N, Nelson PK, Robbins ES, Shapiro E, Kachar B, and Sun TT.** Roles of uroplakins in plaque formation, umbrella cell enlargement, and urinary tract diseases. *J Cell Biol* 167: 1195–1204, 2004.
50. **Kopan R, Traska G, and Fuchs E.** Retinoids as important regulators of terminal differentiation: examining keratin expression in individual epidermal cells at various stages of keratinization. *J Cell Biol* 105: 427–440, 1987.
51. **Lavker RM and Sun TT.** Rapid modulation of keratinocyte differentiation by the external environment. *J Invest Dermatol* 80: 228–237, 1983.
52. **Levy S and Shoham T.** The tetraspanin web modulates immune-signalling complexes. *Nat Rev Immunol* 5: 136–148, 2005.
53. **Lewis SA.** Everything you wanted to know about the bladder epithelium but were afraid to ask. *Am J Physiol Renal Physiol* 278: F867–F874, 2000.
54. **Li Y, Liu W, Hayward SW, Cunha GR, and Baskin LS.** Plasticity of the urothelial phenotype: effects of gastrointestinal mesenchyme/stroma and implications for urinary tract reconstruction. *Differentiation* 66: 126–135, 2000.
55. **Liang FX, Bosland MC, Huang H, Romih R, Baptiste S, Deng FM, Wu XR, Shapiro E, and Sun TT.** Cellular basis of urothelial squamous metaplasia: roles of lineage heterogeneity and cell replacement. *J Cell Biol* 171: 835–844, 2005.
56. **Liebert M, Wedemeyer G, Chang JH, Stein JA, McKeever PE, Carey TE, Flint A, Steplewski Z, Buchsbaum DJ, Wahl RL, and Grossman HB.** Comparison of antigen expression on normal urothelial cells in tissue section and tissue culture. *J Urol* 144: 1288–1292, 1990.
57. **Lin JH, Wu XR, Kreibich G, and Sun TT.** Precursor sequence, processing, and urothelium-specific expression of a major 15-kDa protein subunit of asymmetric unit membrane. *J Biol Chem* 269: 1775–1784, 1994.
58. **Lindberg K, Brown ME, Chaves HV, Kenyon KR, and Rheinwald JG.** In vitro propagation of human ocular surface epithelial cells for transplantation. *Invest Ophthalmol Vis Sci* 34: 2672–2679, 1993.
59. **Lobban ED, Smith BA, Hall GD, Harnden P, Roberts P, Selby PJ, Trejdosiewicz LK, and Southgate J.** Uroplakin gene expression by

normal and neoplastic human urothelium. *Am J Pathol* 153: 1957–1967, 1998.

60. **Maecker HT, Todd SC, and Levy S.** The tetraspanin superfamily: molecular facilitators. *FASEB J* 11: 428–442, 1997.
61. **Miller SJ, Lavker RM, and Sun TT.** Interpreting epithelial cancer biology in the context of stem cells: tumor properties and therapeutic implications. *Biochim Biophys Acta* 1756: 25–52, 2005.
62. **Min G, Stolz M, Zhou G, Liang F, Sebbel P, Stoffler D, Glockshuber R, Sun TT, Aebi U, and Kong XP.** Localization of uroplakin Ia, the urothelial receptor for bacterial adhesin FimH, on the six inner domains of the 16 nm urothelial plaque particle. *J Mol Biol* 317: 697–706, 2002.
63. **Min G, Zhou G, Schapira M, Sun TT, and Kong XP.** Structural basis of urothelial permeability barrier function as revealed by cryo-EM studies of the 16 nm uroplakin particle. *J Cell Sci* 116: 4087–4094, 2003.
64. **Mo L, Cheng J, Lee EY, Sun TT, and Wu XR.** Gene deletion in urothelium by specific expression of Cre recombinase. *Am J Physiol Renal Physiol* 289: F562–F568, 2005.
65. **Moriya K, Kakizaki H, Murakumo M, Watanabe S, Chen Q, Nonomura K, and Koyanagi T.** Creation of luminal tissue covered with urothelium by implantation of cultured urothelial cells into the peritoneal cavity. *J Urol* 170: 2480–2485, 2003.
66. **Negrete HO, Lavelle JP, Berg J, Lewis SA, and Zeidel ML.** Permeability properties of the intact mammalian bladder epithelium. *Am J Physiol Renal Fluid Electrolyte Physiol* 271: F886–F894, 1996.
67. **Nelson CM and Bissell MJ.** Modeling dynamic reciprocity: engineering three-dimensional culture models of breast architecture, function, and neoplastic transformation. *Semin Cancer Biol* 15: 342–352, 2005.
68. **Nelson WG and Sun T-T.** The 50- and 58-kdalton keratin classes as molecular markers for stratified squamous epithelia: cell culture studies. *J Cell Biol* 97: 244–251, 1983.
69. **Oberpenning F, Meng J, Yoo JJ, and Atala A.** De novo reconstitution of a functional mammalian urinary bladder by tissue engineering. *Nat Biotechnol* 17: 149–155, 1999.
70. **Olsburgh J, Harnden P, Weeks R, Smith B, Joyce A, Hall G, Poulsom R, Selby P, and Southgate J.** Uroplakin gene expression in normal human tissues and locally advanced bladder cancer. *J Pathol* 199: 41–49, 2003.
71. **Pariente JL, Bordenave L, Bareille R, Baquey C, and Le Guillou M.** Cultured differentiated human urothelial cells in the biomaterials field. *Biomaterials* 21: 835–839, 2000.
72. **Pauli BU, Anderson SN, Memoli VA, and Kuettner KE.** The isolation and characterization in vitro of normal epithelial cells, endothelial cells and fibroblasts from rat urinary bladder. *Tissue Cell* 12: 419–436, 1980.
73. **Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, and De Luca M.** Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *J Cell Biol* 145: 769–782, 1999.
74. **Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, and De Luca M.** Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 349: 990–993, 1997.
75. **Phillips MA and Rice RH.** Convergent differentiation in cultured rat cells from nonkeratinized epithelia: keratinocyte character and intrinsic differences. *J Cell Biol* 97: 686–691, 1983.
76. **Phillips TJ, Kehinde O, Green H, and Gilchrest BA.** Treatment of skin ulcers with cultured epidermal allografts. *J Am Acad Dermatol* 21: 191–199, 1989.
77. **Ram-Liebig G, Meye A, Hakenberg OW, Haase M, Baretton G, and Wirth MP.** Induction of proliferation and differentiation of cultured urothelial cells on acellular biomaterials. *BJU Int* 94: 922–927, 2004.
78. **Reznikoff CA, Loretz LJ, Pesciotta DM, Oberley TD, and Ignjatovic MM.** Growth kinetics and differentiation in vitro of normal human uroepithelial cells on collagen gel substrates in defined medium. *J Cell Physiol* 131: 285–301, 1987.
79. **Rheinwald JG and Green H.** Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature* 265: 421–424, 1977.
80. **Rheinwald JG and Green H.** Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6: 331–343, 1975.
81. **Rheinwald JG, Hahn WC, Ramsey MR, Wu JY, Guo Z, Tsao H, De Luca M, Catricala C, and O'Toole KM.** A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. *Mol Cell Biol* 22: 5157–5172, 2002.
82. **Rheinwald JG and O'Connell TM.** Intermediate filament proteins as distinguishing markers of cell type and differentiated state in cultured human urinary tract epithelia. *Ann NY Acad Sci* 455: 259–267, 1985.
83. **Rice RH and Green H.** Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell* 18: 681–694, 1979.
84. **Rice RH and Green H.** The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. *Cell* 11: 417–422, 1977.
85. **Romih R, Korosec P, de Mello W Jr, and Jezernik K.** Differentiation of epithelial cells in the urinary tract. *Cell Tissue Res* 320: 259–268, 2005.
86. **Roszell JA, Douglas CJ, and Irving CC.** Polyamine-stimulated growth of cultured rat urinary bladder epithelial cells. *Cancer Res* 37: 239–243, 1977.
87. **Schermer A, Galvin S, and Sun TT.** Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 103: 49–62, 1986.
88. **Schermer A, Jester JV, Hardy C, Milano D, and Sun TT.** Transient synthesis of K6 and K16 keratins in regenerating rabbit corneal epithelium: keratin markers for an alternative pathway of keratinocyte differentiation. *Differentiation* 42: 103–110, 1989.
89. **Southgate J, Cross W, Eardley I, Thomas DF, and Trejdosiewicz LK.** Bladder reconstruction–from cells to materials. *Proc Inst Mech Eng [H]* 217: 311–316, 2003.
90. **Southgate J, Hutton KA, Thomas DF, and Trejdosiewicz LK.** Normal human urothelial cells in vitro: proliferation and induction of stratification. *Lab Invest* 71: 583–594, 1994.
91. **Staack A, Hayward SW, Baskin LS, and Cunha GR.** Molecular, cellular and developmental biology of urothelium as a basis of bladder regeneration. *Differentiation* 73: 121–133, 2005.
92. **Sun TT, Bonitz P, and Burns WH.** Cell culture of mammalian thymic epithelial cells: growth, structural, and antigenic properties. *Cell Immunol* 83: 1–13, 1984.
93. **Sun TT, Eichner R, Cooper D, Schermer A, Nelson WG, and Weiss RA.** Classification, expression, and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model. In: *The Cancer Cell: The Transformed Phenotype*, edited by Levine A, Topp W, Vande Woude G, and Watson JD. New York: Cold Spring Harbor Laboratory, 1984, p. 169–176.
94. **Sun TT and Green H.** Cultured epithelial cells of cornea, conjunctiva and skin: absence of marked intrinsic divergence of their differentiated states. *Nature* 269: 489–493, 1977.
95. **Sun TT and Green H.** Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. *Cell* 9: 511–521, 1976.
96. **Sun TT, Liang FX, and Wu XR.** Uroplakins as markers of urothelial differentiation. *Adv Exp Med Biol* 462: 7–18, 1999.
97. **Sun TT and Green H.** Keratin filaments of cultured human epidermal cells. Formation of intermolecular disulfide bonds during terminal differentiation. *J Biol Chem* 253: 2053–2060, 1978.
98. **Sun TT, Shih C, and Green H.** Keratin cytoskeletons in epithelial cells of internal organs. *Proc Natl Acad Sci USA* 76: 2813–2817, 1979.
99. **Surya B, Yu J, Manabe M, and Sun TT.** Assessing the differentiation state of cultured bovine urothelial cells: elevated synthesis of stratification-related K5 and K6 keratins and persistent expression of uroplakin I. *J Cell Sci* 97: 419–432, 1990.
100. **Thivolet J, Faure M, Demidem A, and Mauduit G.** Long-term survival and immunological tolerance of human epidermal allografts produced in culture. *Transplantation* 42: 274–280, 1986.
101. **Thomas JC, DeMarco RT, and Pope JC IV.** Molecular biology of ureteral bud and trigonal development. *Curr Urol Rep* 6: 146–151, 2005.
102. **Truschel ST, Ruiz WG, Shulman T, Pilewski J, Sun TT, Zeidel ML, and Apodaca G.** Primary uroepithelial cultures. A model system to analyze umbrella cell barrier function. *J Biol Chem* 274: 15020–15029, 1999.
103. **Tu L, Sun TT, and Kreibich G.** Specific heterodimer formation is a prerequisite for uroplakins to exit from the endoplasmic reticulum. *Mol Biol Cell* 13: 4221–4230, 2002.

104. **Varley CL, Stahlschmidt J, Lee WC, Holder J, Diggle C, Selby PJ, Trejdosiewicz LK, and Southgate J.** Role of PPARγ and EGFR signalling in the urothelial terminal differentiation programme. *J Cell Sci* 117: 2029–2036, 2004.
105. **Walz T, Haner M, Wu XR, Henn C, Engel A, Sun TT, and Aebi U.** Towards the molecular architecture of the asymmetric unit membrane of the mammalian urinary bladder epithelium: a closed "twisted ribbon" structure. *J Mol Biol* 248: 887–900, 1995.
106. **Weiss RA, Eichner R, and Sun TT.** Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. *J Cell Biol* 98: 1397–1406, 1984.
107. **Wong P and Coulombe PA.** Loss of keratin 6 (K6) proteins reveals a function for intermediate filaments during wound repair. *J Cell Biol* 163: 327–337, 2003.
108. **Wu XR, Manabe M, Yu J, and Sun TT.** Large scale purification and immunolocalization of bovine uroplakins I, II, and III. Molecular markers of urothelial differentiation. *J Biol Chem* 265: 19170–19179, 1990.
109. **Wu XR, Medina JJ, and Sun TT.** Selective interactions of UPIa and UPIb, two members of the transmembrane 4 superfamily, with distinct single transmembrane-domained proteins in differentiated urothelial cells. *J Biol Chem* 270: 29752–29759, 1995.
110. **Wu XR and Sun TT.** Molecular cloning of a 47 kDa tissue-specific and differentiation-dependent urothelial cell surface glycoprotein. *J Cell Sci* 106: 31–43, 1993.
111. **Wu XR, Sun TT, and Medina JJ.** In vitro binding of type 1-fimbriated *Escherichia coli* to uroplakins Ia and Ib: relation to urinary tract infections. *Proc Natl Acad Sci USA* 93: 9630–9635, 1996.
112. **Wu YJ, Parker LM, Binder NE, Beckett MA, Sinard JH, Griffiths CT, and Rheinwald JG.** The mesothelial keratins: a new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia. *Cell* 31: 693–703, 1982.
113. **Yu J, Lin JH, Wu XR, and Sun TT.** Uroplakins Ia and Ib, two major differentiation products of bladder epithelium, belong to a family of four transmembrane domain (4TM) proteins. *J Cell Biol* 125: 171–182, 1994.
114. **Yu J, Manabe M, and Sun TT.** Identification of an 85–100 kDa glycoprotein as a cell surface marker for an advanced stage of urothelial differentiation: association with the inter-plaque ("hinge") area. *Epithelial Cell Biol* 1: 4–12, 1992.
115. **Yu J, Manabe M, Wu XR, Xu C, Surya B, and Sun TT.** Uroplakin I: a 27-kD protein associated with the asymmetric unit membrane of mammalian urothelium. *J Cell Biol* 111: 1207–1216, 1990.
116. **Zhang ZT, Pak J, Huang HY, Shapiro E, Sun TT, Pellicer A, and Wu XR.** Role of Ha-ras activation in superficial papillary pathway of urothelial tumor formation. *Oncogene* 20: 1973–1980, 2001.
117. **Zhou G, Mo WJ, Sebbel P, Min G, Neubert TA, Glockshuber R, Wu XR, Sun TT, and Kong XP.** Uroplakin Ia is the urothelial receptor for uropathogenic *Escherichia coli*: evidence from in vitro FimH binding. *J Cell Sci* 114: 4095–4103, 2001.