The Quest to Derive Keratinocytes from Pluripotent Stem Cells

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Abstract The derivation of keratinocytes from pluripotent stem cells (PSCs) is a worthy goal. Epidermal and corneal keratinocytes cultured from normal human tissue have been very well-characterized for their growth and differentiation properties and their ability to regenerate permanent tissue as autologous transplants. PSC-derived keratinocytes would therefore be ideal for establishing proof-of-principle that normal, well-defined, and functional somatic cell types can be derived from PSCs and used successfully in tissue replacement therapies. Much progress has been made to identify experimental protocols for deriving cells that display some keratinocyte properties from ES and iPS cells. However, the proliferative potential of these cells typically is very limited and many features of these cells are common to all members of the large family of p63 epithelial cell types. Thus many lines of investigation remain to be examined more thoroughly in this important and exciting field.

Keywords Epithelial · p63 · Embryonic · Ectoderm · Cultured cells · Keratin

Introduction

The cultivation of pluripotent embryonic stem (ES) cell lines from mice [1, 2] became the basis for gene knockout technology, which supports much of current experimental

biology. Generation of similar cell lines from human embryos [3, 4] engendered interest in the idea of inducing these cells to differentiate in culture into clinically useful adult somatic cell types, especially those that are postmitotic in the adult and may deteriorate or become damaged, resulting in severe pathologies. With the discovery that transient expression of four factors can reprogram somatic cells such as skin fibroblasts to a pluripotent state similar to that of ES cells [5–8] (“induced pluripotent stem (iPS) cells”) came the prospect of producing autologous somatic cell types from iPS cells for therapeutic transplantation [9]. The subject of this review is research aimed at producing epidermal and corneal keratinocytes from ES and iPS cells. There is no pressing clinical need for PSC-derived keratinocytes, since autologous tissue-derived keratinocytes can be expanded rapidly in culture and transplanted to provide permanent epidermal coverage of full-thickness burn wounds [10, 11] and corneal restoration in cases of chemical damage to the eye surface [12]. However, more is known about the keratinocyte than any other cultivable cell type, with respect to growth regulation, protein expression, differentiation, and methods of transplantation to regenerate permanent tissue. It therefore is the ideal cell type for proof-of-principle that authentic, normal, and functional adult somatic cell types can be generated from pluripotent stem cells (PSCs).

More than a dozen research groups have reported generating keratinocytes from PSCs during the past several years, so this is an appropriate time for a comprehensive review. The author of this review devoted four years to this effort and appreciates the challenges of finding effective and reproducible protocols to generate keratinocytes from PSC and characterizing them appropriately. With the objective of encouraging research to advance the field, this is a critical review. As is true for all PSC research, scientists

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from many disciplines have been attracted to this particular endeavor. A consequence of this is that progress has been impeded by insufficient awareness of concepts from classical developmental biology and from contemporary comparative epithelial cell biology. A major problem is an unwarranted interpretation of marker expression to identify epithelial cells derived from PSC cells as “keratinocytes”. The modest proliferative potential and undetermined growth regulatory characteristics of PSC-derived epithelial cells raises questions about how normal they are and, therefore, their potential value for tissue replacement. The propensity of many cell types of the large family of epithelia that express p63 to undergo epidermoid histogenesis in the organotypic culture assay for tissue-forming potential also has led to premature conclusions about cell type identity. These issues will be discussed at the end of the article, with suggestions for future research.

Properties of Somatic Tissue-Derived Keratinocytes in Culture

A compelling rationale for generating keratinocytes from PSC cells is that so much is known about the behavior of human tissue-derived keratinocytes. Thus the properties of PSC-derived cells can be compared with those of primary keratinocyte cell lines. A culture system employing co-culture with lethally irradiated 3T3 fibroblast feeder cells and use of a mixed DMEM+F12 nutrient medium supplemented with serum, hydrocortisone, EGF, cholera toxin, insulin, thyroxine, and adenine was developed to promote clonal growth and long-term [25–80 population doublings (PDs)] growth of normal human epidermal keratinocytes [13–15]. This culture system supports equally well the growth of keratinocyte subtypes that form other stratified squamous epithelia (i.e., oral, esophageal, corneal, conjunctival, vaginal, and exocervical epithelium) as well as a diverse set of other epithelial cell types, including mammary, tracheo-bronchial, and bladder urothelial cells [15, 16, 17–20]. Semi-defined and defined media were developed subsequently that promote excellent clonal growth and serial propagation of keratinocytes without feeder cells, achieved by optimizing amino acid and vitamin concentrations and substituting bovine pituitary extract (BPE) for serum [21–23]. One of these that is widely used is K-sfm (GIBCO/Invitrogen/Life Technologies), a published modification [22] of an earlier Ham’s MCDB150 series medium [21, 23].

Normal human keratinocytes grow well in $[Ca^{2+}]$ as high as 1.0 mM, producing some stratifying, terminally differentiated cells while retaining a basal layer of proliferative cells [13, 21, 24, 25]. Primary murine epidermal keratinocytes require culture in media containing <0.1 mM Ca^{2+} to maintain basal layer-like, proliferation potential, since higher $[Ca^{2+}]$ triggers permanent growth arrest and

expression of terminal differentiation markers in most cells [26]. Murine keratinocytes can be serially propagated in low $[Ca^{2+}]$ versions of the FAD + 3T3 feeder system [27] or of K-sfm medium [28]. The FAD + fibroblast feeder system and K-sfm medium have been used in most protocols aimed at generating keratinocytes from PSCs.

Major markers of basal keratinocytes, which comprise the stem cell compartment of stratified squamous epithelia, are $\Delta Np63\alpha$, K5/K14 [29], $\alpha 6\beta 4$ integrin, and the Lam $\alpha 3$, Lam $\beta 3$, and Lam $\gamma 2$ subunits of the basal lamina protein Laminin-332. The many stratified squamous epithelia differ from one another with respect to additional keratins made by the basal cells and by the set of proteins involved in structural integrity expressed by the terminally differentiated, suprabasal stratified cells. These include the specialized keratin pairs K1/K10 by the epidermis and also expressed by exocervix and gingiva, K4/K13 by so-called non-keratinizing stratified squamous epithelia such as esophagus, oral buccal and floor of mouth mucosa, and conjunctiva, and K3/K12 by the cornea [30]. Additional suprabasal differentiation proteins are the cornified envelope proteins Involucrin (IVN) [24] and Loricrin (LOR) [31] and the K1/K10 filament aggregating protein Filaggrin (FIL) [32].

Serial culture and xenograft transplantation studies have demonstrated clearly that human keratinocyte subtypes are intrinsically distinct from one another and maintain their identity and preferred pattern of histogenesis in the absence of any specific signals from regional fibroblasts [18, 19, 33]. An “organotypic” culture system, developed to promote formation of an optimally differentiated epidermis from cultured human epidermal keratinocytes [34], instigates some K1/K10 expression and epidermal-like stratum corneum (sc) formation in all keratinocyte subtypes [33] as well as in other members of the p63+ family of epithelial cell types [16]. This generally unrecognized limitation of standard organotypic culture for determining epithelial cell identity is important in evaluating reports claiming epidermal keratinocyte identity of PSC-derived epithelial cells. Finally, gene expression profiling to compare PSC-derived epithelial cells with normal somatic keratinocytes and other epithelial cell types to determine cell type identity and normality generally has not been used.

Overview of the Literature

The literature on deriving cells exhibiting features of the keratinocyte lineage is summarized in Tables 1 and 2. The highlights are described in the following text.

Murine ES-Derived Epithelial Cells

The first report of possible conversion of murine ES (mES) cells to keratinocytes was by Bagutti, Watt, and colleagues

Table 1 Published reports of “keratinocytes” derived from murine PSCs in culture

Ref.	PSC line	Initiating conditions, factors	Culture conditions for derived epithelial cells	Markers	Organotypic or graft	Replicative potential	Authors' conclusions about cell type derived
[35, 36]	mES D3	EB, fibro CM, KGF, TGF α	EM, gelatin coat	K14, $\alpha 6\beta 4$ int, K10	nd	nd ^a	“Keratinocyte”
[38]	mES EB5	BMP4	EM, fixed PA6 fibro feeders	K14	nd	nd ^a	“Epidermal cell”
[39, 40]	mES CGR8	BMP4 or ascorbate	EM, Hu fibro matrix	K14 K10, Col 4, FLG, Lam $\alpha 3$, $\alpha 6\beta 4$ int, CDSN	Organo, strat., sc	nd ^a	“Epidermal keratinocyte”
[71]	mES R1	EB	EM, Matrigel coat	K14, K1, IVN, FLG	nd	nd ^a	“Epidermal keratinocyte”
[72]	mES CCE	EB	FAD	K14, IVN	nd	nd ^a	“Keratinocyte-like”
[44]	mES CGR8	BMP4	EM, fixed fibro feeders ^b	p63, K5, K14, K10, IVN	nd	nd ^a	“Epidermal keratinocyte”
[73]	mES		EM, MEF feeders 7 passages; then EM without feeders	K14	nd	10–30 PD	“Keratinocyte-like”
[28 ^c]	mIPS ^c	EB +RA, then COL4-coat dish +BMP4	ESCM-LJF, then DK-sfm; rapid attachment to COL4 coat	K5, K14, K1, LOR	If graft with fibro and dp cells, form epidermis, hair, and seb. glands	18 PD	“Multipotent epidermal and piloseb keratinocytes”

Note that “Markers” lists proteins detected by immunostaining or Western blot analysis but not gene transcripts detected by RT-PCR

3T3 the Todaro and Green 3T3 mouse embryonic fibroblast line, *CM* conditioned medium, *coat* precoated dish, *DK-sfm* fully defined version of K-sfm, *dp* dermal papilla, *EB* embryoid body, *EM* various versions of Eagle's medium supplemented with nonessential amino acids and serum, *ESCM* typical PSC culture medium, including EM, knockout serum replacement, and LIF, *FAD*: serum and growth factor supplemented Rheinwald/Green medium typically used for keratinocyte growth with fibroblast feeder cells, *feeders* monolayer of lethally irradiated or mitomycin-treated cells co-plated with the PSC or epithelial cells, *Fibro* fibroblast, *Hu* human, *immort* immortalized by transduction to express HPV16E6E7 or bml1 + hTERT, *int* integrin, *KGM2* a proprietary modification of MCDB154 [21, 23] keratinocyte medium (Lonza), *K-sfm* keratinocyte serum-free medium [22] (GIBCO/Invitrogen/Life Technologies), *Lam* laminin, *LIF* Leukemia Inhibiting Factor, *matrix* secreted products remaining on the dish after cells have been removed, *MEF* mouse embryo fibroblasts, *nd* not determined, *organo* organotypic culture, *piloseb* pilosebaceous unit, *PD* population doublings, *PSC* pluripotent stem cell, *RA* retinoic acid, *Ref.* reference, *sc* stratum corneum, *seb* sebaceous, *strat* stratification

^a Not a highly-enriched or pure cell population

^b If cultured without serum can isolate a stable K8/K18+ cell type identified by the authors as “embryonic ectoderm”. If add serum or engineer to express $\Delta Np63\alpha$, some of these cells convert to K14+ cells that can also express K10

^c Derived from skin fibroblasts

Table 2 Published reports of “keratinocytes” derived from human PSCs in culture

Ref.	PSC line	Initiating conditions, factors	Culture conditions for derived epithelial cells	Markers	Organotypic or graft	Replicative potential	Authors' conclusions about cell type derived
[41, 42]	hES H9	EB or teratoma	FAD + 3T3 feeders	p63, K14, BNC1, IVN	nd	<15 PD	“Abnormal keratinocytes”
[16 ^a]	H9	H9-derived epithelial lines from [42]	FAD + 3T3 feeders; K-sfm	p63, K14, IVN, K10, Lam γ 2	Organo. strat, no sc	Experimentally immortal	“Abnormal p63 + ep. cell, undetermined identity”
[45]	hES H1, H9	EB -bFGF	K-sfm or FAD, COL1 coat	K14, p63, IVN, FLG	nd	5 PD	“Epidermal keratinocyte”
[46 ^a , 47]	hES H1, H9	EB or direct plating, +RA	DK-sfm, gelatin coat	K14, p63, K10, IVN, FLG	Organo. strat, sc	~10 PD	“Epidermal keratinocyte progenitor” ^a
[54]	hES H1, NCLK	Corneal limbal fibro CM	FAD, COL4 coat	transient p63, K3/K12	nd	nd ^b	“Corneal keratinocyte”
[43 ^a]	hES H9	BMP4	ESCM -LIF on fixed fibro feeders; then FAD + 3T3 feeders ^c	p63, K14	nd	nd ^b	“Keratinocyte”
[57]	hES H9	BMP4	FAD + fixed MEF feeders; then FAD uncoat; then COL1 coat	K12	Organo. strat., no sc	nd ^b	“Corneal Keratinocyte”
[48 ^a , 49]	hES H9, SA01	BMP4 + Ascorbate	FAD + 3T3 feeders; then FAD + 3T3 feeders or KGM2	p63, K5, K14, K10, IVN, FLG, α 6 β 4int, Lam β 3, Col7	Organo. strat with sc-grafts form epidermis-like tissue	40–60 PD	“Epidermal keratinocyte”
[50 ^a]	hES H9	EB, RA + Ascorbate +Activin A	FAD + H9-derived fibro feeders or matrix; then DK-sfm, COL4 coat	p63, K14, α 6int, IVL-FIL	nd	Slower growth rate; “7–10 passages”	“Epidermal keratinocyte”
[51 ^a]	hiPS ^d	On Matrigel, RA + BMP4	DK-sfm; then DK-sfm, FN + collagen coat	K14, K5, p63, DSG3, K1, α 6 β 4int, Col7, Lam β 3, LOR	Organo. strat., no sc	up to “5 passages”	“Epidermal keratinocyte”
[51 ^a , 52, 55]	hiPS ^{d,e}	Corneal fibro CM, BMP4	COL4 coat; then FAD + 3T3 feeders	K14, p63, K3, PAX6	Organo. strat, no sc	nd ^b	“Corneal keratinocyte”
[52]	hiPS ^d	BMP4, Ascorbate, TGF β R1 inhibitor	FAD + 3T3 feeders 30d	K14, p63, K10	nd	nd ^b	“Epidermal keratinocyte”
[53]	hiPS ^d	EB, RA + BMP4	DK-sfm, gelatin coat	K5	nd	nd ^b	“Keratinocyte”

Note that “Markers” lists proteins detected by immunostaining or Western blot analysis but not gene transcripts detected by RT-PCR

3T3 the Todaro and Green 3T3 mouse embryonic fibroblast line, CM conditioned medium, coat precoated dish, DK-sfm fully defined version of K-sfm, dp dermal papilla, EB embryoid body, EM various versions of Eagle's medium supplemented with nonessential amino acids and serum, ESCM typical PSC culture medium, including EM, knockout serum replacement, and LIF, FAD serum and growth factor supplemented Rheinwald/Green medium typically used for keratinocyte growth with fibroblast feeder cells, feeders monolayer of lethally irradiated or mitomycin-treated cells co-plated with the PSC or epithelial cells, Fibro fibroblast, Hu human, immortalized by transduction to express HPV16E6E7 or bmi1 + hTERT, int integrin, KGM2 a proprietary modification of MCDB154 [21, 23] keratinocyte medium (Lonza), K-sfm keratinocyte serum-free medium [22] (GIBCO/Invitrogen/Life Technologies), Lam laminin, LIF Leukemia Inhibiting Factor, matrix secreted products remaining on the dish after cells have been removed, MEF mouse embryo fibroblasts, nd not determined, organo organotypic culture, piloseb pilosebaceous unit, PD population doublings, PSC pluripotent stem cell, RA retinoic acid, Ref. reference, sc stratum corneum, seb sebaceous, strat stratification

^a Oligonucleotide microarray analysis revealed many differences in gene expression compared to normal tissue-derived keratinocytes

^b Not a highly-enriched or pure cell population

^c If cultured without serum can isolate a stable K8/K18+ cell type identified by the authors as “embryonic ectoderm”. If add serum or engineer to express Δ Np63 α , some of these cells convert to K14+ cells that can also express K10

^d Derived from skin fibroblasts

^e Derived from hair follicle keratinocytes

[35, 36]. The basis for their approach was the prior observation [37] that a variety of developmental lineages form in culture inside aggregates of mES cells termed “embryoid bodies (EBs)”. ES cells are encouraged to aggregate into EBs by suspending them in a small hanging drop of culture medium. In the Bagutti studies, EBs allowed to attach and spread on gelatin-coated dishes yielded some K14+ and $\alpha 6 \beta 4$ int+ cells within several weeks. Kawasaki, Sasai, and colleagues [38] advanced this field as part of a study aimed at generating neural cells from mES. They found that BMP4 suppressed neurectoderm commitment of mES cells, thereby diverting more cells toward epithelial lineages including some K14+ cells. This study [38] also discovered that prior EB formation was not essential but that K14+ cell frequency was increased by plating mES cells on living or fixed fibroblast or bone marrow stromal cell feeder cells.

Coraux, Aberdam, and colleagues [39, 40] reported formation of both epidermal- and connective tissue-like cells by plating mES cells pretreated with BMP4 or ascorbate onto a dish coated with proteins secreted by fibroblasts. When plated at the air–liquid interface, the heterogenous mES-derived cell population sorted into a stratified epithelial layer expressing many markers of epidermal differentiation and an underlying connective tissue-like layer.

Human ES-Derived Epithelial Cells

Green, Iuchi, and colleagues published the first report that human ES (hES) cells can produce epithelial cells that express p63 and K14 and that can stratify and express IVN [41]. These cells were noted to have an unusually limited proliferative potential compared to somatic tissue-derived keratinocytes but they could be immortalized if engineered to evade both p16^{INK4A}- and telomere-related senescence barriers [16•, 42]. Their colony morphology in the FAD + 3T3 feeder system differed from that of somatic keratinocytes. The authors concluded that these cells were abnormal keratinocyte-like cells [42]. This was investigated more thoroughly by Dabelsteen, Rheinwald, and colleagues [16•], who determined that the cells had abnormal growth regulatory mechanisms and concluded that their identity could be any of the many p63+ cell types.

Medawar, Aberdam, and colleagues [43•, 44] identified a stable, potential developmental intermediate during conversion of ES cells to p63/K14+ cells. mES or hES cells plated on a fixed fibroblast layer and treated for several days with BMP4 in the absence of serum produced many K8/K18+ epithelial cells that lacked pluripotency markers and could be serially passaged. The authors proposed that these were embryonic ectodermal cells. If treated with serum or engineered to express Δ Np63 α , some

of these cells began expressing K14 and could stratify to form K10+ cells.

Metallo, Ji, Palacek, and colleagues [45–47] identified retinoic acid (RA) as an alternative to BMP4 as an agent to increase the frequency of hES-derived epithelial cells expressing p63, K14, IVN, K10, and $\text{Lam}\gamma 2$. In organotypic culture these cells formed a stratified, epidermal-like epithelium. Importantly, these cells were found to have a gene transcription pattern clearly different from, although sharing many key features with, normal somatic epidermal keratinocytes.

Guenou, Nissan, Baldeschi, and colleagues [48•, 49] plated clumps of hES cells into the FAD + 3T3 feeder system supplemented with BMP4+ ascorbate. After 40 days, 40–50 % of the cells had formed K14+ clusters with morphology reminiscent of keratinocytes. These were isolated and expanded by subculture either in the FAD + 3T3 feeder system or in semi-defined medium. They were found to express many markers of epidermal keratinocytes and to form an epidermal-like epithelium in organotypic culture and in grafts to immunodeficient mice. The authors stated, although without showing serial growth data, that these cells possessed a replicative lifespan of 40–60 PD, similar to that of somatic tissue-derived keratinocytes. This is notable since PSC-derived, keratinocyte-like cells produced by other labs consistently displayed a very short lifespan of 5–15 PD (Table 2).

Kidwai, Cao, and colleagues [50•] found that addition of Activin A for a specific 3 day window of time markedly increased the yield of p63+ cells from hES cells at the expense of cells that express the neurectoderm marker PAX6.

Derivation of Epidermal Keratinocyte-like Cells from iPS Cells

Recently several groups have derived epithelial cells displaying properties of keratinocytes from iPS cells generated from skin fibroblasts. Bilousova, Roop, and colleagues [28•] formed EBs from murine iPS (miPS) cells and treated them with RA. These were then plated on COL4-coated dishes, treated with BMP4 for 3 days, and the medium then changed to DK-sfm for 6 days. At this time many cells that had migrated out of the attached EBs were p63/K14+. EBs were selectively removed and the remaining cell population was progressively enriched for K14+ cells by serial culture in DK-sfm on COL4-coated dishes. The cells could express K1 and Loricrin (LOR) and had a replicative lifespan of ~18 PD, similar to that of newborn murine somatic epidermal keratinocytes cultured in the same conditions. This study was unique in demonstrating that miPS-derived p63/K14+ cells could form a stratified epidermis as well as hair follicles and sebaceous glands when

grafted onto the skin as a cell suspension mixed with normal skin fibroblast and dermal papilla cells.

Itoh, Christiano, and colleagues [51•] plated clumps of hiPS cells on Matrigel-coated dishes in DK-sfm supplemented with RA and BMP4 for the first 4 days. After 30 days, 45 % of the cells were K14+, increasing to 70 % after subculture to coated dishes in DK-sfm, and some cells expressed DSG3 and K1. In organotypic culture, the cells stratified and expressed suprabasal K1 and LOR. Subsequent studies by others [51•, 52, 53] have reported derivation of p63/K5/K14+ cells from hiPS cells bearing a variety of inherited mutations that have a severe epidermal phenotype.

Research Aimed at Generating Corneal Keratinocytes from PSC Cells

Several groups have sought to derive corneal keratinocytes from hES cells by attempting to direct lineage commitment by early exposure to products secreted by corneal fibroblasts. Ahmad, Lako, and colleagues [54] plated clumps of hES cells on COL4-coated dishes in medium conditioned by human corneal limbal fibroblasts. They observed a transient generation of p63+ and K3/K12+ cells peaking at 1 week but such cells were not sustained in the population. Shalom-Feuerstein, Aberdam, Petit, and colleagues [55] generated hiPS cells from hair follicle keratinocytes and skin fibroblasts and plated these in corneal fibroblast conditioned medium (CM) with a 4 day BMP4 treatment. 2 weeks later, many cells appeared by immunostaining and FACS analysis to coexpress p63, K14, K3, and PAX6. PAX6 was proposed by the authors as a marker of corneal epithelial identity but, in fact, is expressed by early neuroectoderm and many mature neurons as well as being essential for development of all structures of the eye [56]. These cells formed a bilayer in organotypic culture with suprabasal K3 expression. Hewitt, Aberdam, Garlick, and colleagues [57] used a complex multistep approach involving a possible ectoderm-like intermediate cell line, but without attempting to direct cells into a corneal lineage. They generated epithelial cells that stratified and produced some suprabasal K12+ cells. These studies are intriguing but none compared the effects of dermal versus corneal fibroblast CM or looked for expression of the epidermal keratin pair K1/K10, nor were homogenous and serially cultivable cell populations obtained.

Critique

The literature to date permits a number of important conclusions. The common feature of successful protocols is plating PSCs as aggregates into culture conditions permissive for growth of keratinocytes and other members of

the p63 family of epithelial cell types (e.g., the FAD + fibroblast feeder system or a semi-defined or defined keratinocyte medium). Then, provided that there is sufficient space on the dish to permit outward migration and further proliferation, during the following 1–4 weeks the PSCs lose pluripotency markers and some cells begin expressing p63 and K14. Prior EB formation by PSC does not enhance generation of p63/K14+ cells. No inducers of PSC commitment are essential as medium additives to generate such cells. However, RA, BMP4, Activin A, ascorbate, and TGF β receptor inhibitor, either individually or in various combinations, increase the frequency of p63/K14+ cells. An intermediate state, in which pluripotency markers no longer are expressed but K8/K18 expression is increased, may precede generation of p63/K14+ cells. As yet there is no evidence that conversion can be directed toward a specific keratinocyte subtype (e.g., cornea) by use of region-specific fibroblasts or their products. Derived p63/K14+ cells typically also express α 6 β 4 integrin, Laminin-332, and K5, the partner of K14 for filament formation, although none of these markers are specific to keratinocytes. The replicative lifespan of PSC-derived p63/K14+ cells typically is much shorter and the growth rate slower than that of tissue-derived somatic keratinocytes. However, two studies have reported replicative lifespans of miPS-derived and hES-derived p63/K14+ cells equivalent to that of normal keratinocytes [28•, 48•].

The stated objective of all the above studies was to derive keratinocytes from PSCs. Many studies using human PSCs claim to have achieved the goal of generating keratinocytes useful for clinical tissue replacement. Almost all of these studies mistakenly used expression of p63, K14, α 6 β 4 integrin, Laminin-332, and K5 as evidence that the proliferative cells they obtained were basal layer keratinocytes. The K5/K14 keratin pair (originally termed the 58kd and 50kd keratins for their electrophoretic mobility) have been known since the early 1980s to be expressed by the basal, proliferative cells of many epithelial tissues, including mammary gland, tracheobronchus, and urothelium in addition to the epidermis and other stratified squamous epithelia [15, 29, 58]. α 6 β 4 integrin and its cognate basal lamina protein ligand Laminin-332 also are expressed by the basal cells of these epithelia. The discovery of p63 in the late 1990s included the observation that this transcriptional regulator is expressed by the basal cell compartments of all of the above-mentioned epithelia and that knockout of p63 in mice results in failure to develop all of these epithelia [59–61].

Members of this large family of p63/K14+ cell types arise from all three embryonic germ layers (Fig. 1). The various keratinocyte subtypes that form different stratified squamous epithelia arise from all three embryonic germ layers. Thus, PSCs are not obliged to convert first to an

ectodermal precursor in order to undergo further commitment to a keratinocyte lineage. Interestingly, soon after the development of the FAD + 3T3 feeder system to culture epidermal keratinocytes it was found that this system also is ideal for clonal growth and serial propagation of all epithelial cell types now known to express p63 [15, 17, 20]. K-sfm and other semi-defined “keratinocyte” media also prove to promote excellent growth of all p63 epithelial cell types in addition to p63+ hES-derived epithelial cells (e.g., [16•, 25]). Thus, expression of p63 and markers of keratinocyte basal cells does not support the conclusion that PSC cells have converted to a keratinocyte lineage.

Many studies identified in their PSC-derived, p63/K14+ populations stratifying cells expressing IVL, K1/K10, and other epidermal terminal differentiation proteins. In normal epithelia *in vivo*, this would narrow down identification to epidermis, exocervix, and thymus, with occasional expression in gingiva and hard palate [62]. An organotypic culture system is widely used to assess the ability of cells to undergo epidermoid differentiation. This system was initially optimized to promote complete epidermoid differentiation by serially cultured primary human epidermal keratinocytes [34]. However, in this system other keratinocyte subtypes, such as the non-keratinizing buccal and floor of mouth mucosal keratinocytes, abnormally express suprabasal K1/K10 [33], despite the fact that keratinocytes retain their subtype identity during serial culture and ability to regenerate their appropriate pattern of suprabasal differentiation without specific stromal instruction in xenograft transplants [18, 19]. The study by Dabelsteen, Rheinwald, and colleagues [16•] found that somatic tissue-derived human urothelial and tracheobronchial epithelial cells stratify and form an epidermal-like stratum corneum with suprabasal K1/K10 and IVL in organotypic culture. Even human prostate epithelial cells undergo epidermoid differentiation in organotypic culture (W. Wei, P. Barron, and J. Rheinwald, unpublished). Mindful that chronic Vitamin A (RA) deprivation *in vivo* causes reversible epidermoid differentiation of many p63+ family epithelial tissues [63, 64], we tried adding RA to the medium but found that p63+ epithelial cell types continued to display features of epidermoid differentiation in organotypic culture [16•, 33]. This result precludes the use of standard organotypic assays, or of the occasional expression of IVL or K1/K10 by stratifying cells in conventional culture, as evidence of epidermal keratinocyte identity. This issue is especially relevant for evaluating studies in which an appropriate variety of other normal epithelial cell types is not compared to the PSC-derived cell for histogenesis and marker expression. The strongest evidence for epidermal identity has been obtained in several studies that demonstrated formation of an epidermal-like tissue by PSC-derived epithelial cells xenografted to the skin (Tables 1 and 2). However, the extent to which other p63+

epithelial cell types may change their differentiation to more epidermoid character in response to ectopic transplantation to the skin surface remains to be determined.

Most studies reporting PSC to keratinocyte-like cell conversion have focused on demonstrating expression of cell type and differentiation markers. However, this is only one of two essential criteria for generation of an authentic and useful somatic cell type. The other is whether the PSC-derived cell displays growth regulatory mechanisms characteristic of the normal somatic cell in question, which can help to both confirm identity or close similarity to the tissue-derived cell type and predict whether they would behave normally as tissue stem cells in transplants. Our study [16•] examining the growth properties of three clonally isolated, hES-derived p63/K14+ cell lines revealed that they grew less well in K-sfm and were less dependent on EGF and less sensitive to growth inhibition by serum than normal keratinocytes or urothelial cells. PSC-derived cells, even if able to undergo the desired differentiation, may not be suitable for transplantation if they lack normal growth regulatory systems.

With the exception of one report [48•], the p63/K14+ epithelial cells generated from human PSCs in all studies displayed a very short replicative lifespan, precluding isolation as pure populations as well as potential use for epidermal tissue regeneration. Differences in the starting PSC cells or in the protocols may yield cells that differ with

Fig. 1 Embryonic germ layer origin of p63-expressing epithelial cell types and sensitivity of their differentiation to retinoid deprivation *in vivo*. Keratinocyte subtypes are shown in blue font. Asterisks indicate those epithelia for which Vitamin A [retinoic acid (RA)] deprivation *in vivo* causes a reversible conversion to an epidermoid, stratified squamous epithelial form of differentiation. Panel shows an H9 hES-derived epithelial colony (hESderK clone 2A2/E6E7 [16•, 42]) immunostained for p63 (red) and K14 (green) (Color figure online)

respect to cell type or degree of “normality”. Protocols that do not include clonal isolation or other very rigorous purification of p63/K14+ cells may maintain some K8/K18+ ectoderm- or endoderm-like precursor cells in the population during serial passage. Such cells were noted to have a long lifespan and the potential to convert spontaneously to p63/K14+ cells [43•, 44], the result of which would be an apparently long lifespan of the p63/K14+ cells.

Future Directions

Many groups have established that it is possible, using a variety of protocols and facilitating factors, to generate p63/K14+ epithelial cells having the potential to undergo epidermoid differentiation. Effort now should be devoted to understanding the PSC-to-stratified epithelial cell process in more detail. The time course and quantitation of conversion under various conditions should be compared by disaggregating cultures to single cells and plating in the FAD + 3T3 feeder system to identify cells able to form epithelial colonies of at least a size of 1,000 cells (~10 PD). Such colonies can be immunostained for p63, K14, and other markers, or to look for loss of Oct4, expression of K8, etc. Some colonies can be isolated at different time points after beginning the conversion protocol to expand for further analysis, experimentally immortalizing them if necessary to permit this (e.g., [16•, 42]).

The stable, propagable K8/K18+ epithelial cells that can be derived from mES and hES cells [43•, 44] should be examined more thoroughly to determine their identity and similarity to early developmental intermediates *in vivo*. The conclusion that these cells are embryonic ectoderm is premature. K8/K18 were first discovered as “Endo A and Endo B”—cytokeratins of early parietal and visceral endoderm [65] and BMP4 not only suppresses neur ectoderm formation from ectoderm but also promotes formation of endoderm from ES cells [66]. Furthermore, although RA is used in some protocols aimed at generating epidermal keratinocytes, this agent was first noted to convert mouse embryonal carcinoma and ES cells toward an early endoderm phenotype [67]. It would be interesting to learn whether PSC-derived, embryonic endoderm-like cells preferentially produce p63/K14+ cell types that normally arise from endoderm during development (see Fig. 1). Related to this topic, whether and how PSC conversion can be directed toward a specific p63+ epithelial cell type remains a major question. Although postnatal somatic keratinocytes do not require signaling from their regional underlying connective tissue to differentiate appropriately in xenograft systems [18, 19], acquisition of specific cell type identity (e.g., corneal, tracheobronchial, urothelial)

during development may require exposure to regional connective tissue fibroblasts. Studies should compare dermal versus other connective tissue fibroblasts and embryonic versus adult for such inductive activity.

Accurate identification of PSC-derived, p63/K14+ cells and determination of how closely they resemble normal somatic epithelial cells will be aided by microarray analysis of gene expression on pure, homogenous cell populations. An initial study [46•] of an uncloned hES-derived population greatly enriched for K14+ cells revealed many differences between these cells and normal primary epidermal keratinocytes. PSC-derived populations may be mixtures of several p63+ cell types or otherwise heterogeneous with respect to gene expression. Clonally isolated populations should be compared to epidermal keratinocytes and a variety of normal tissue-derived p63+ cell types. Growth requirements in culture, including mitogen-dependence and inhibitor sensitivity, also are excellent bellwethers of normality [16•]. When cultured in semi-defined media, all normal p63+ epithelial cell types require either EGF, KGF, or HGF for growth [25] and are strongly growth inhibited by TGFβ [68, 69] and by serum [25]. Organotypic and transplantation assays used to determine cell type identity should be permissive for various types of epithelial differentiation and not push all cells toward epidermoid differentiation. The internal skin flap xenograft system [18, 19, 70] is one example of a permissive system.

Conclusions

There is a wonderful quote by the author Louis Lamour: “There will come a time when you believe that everything is finished. That will be the beginning.” As described in this review, despite much progress this field is still very much in an initial, discovery phase. Awareness that many p63+ cell types may be arising from PSC cells expands the opportunities for discovery immensely. PSC-derived, p63/K14+ cells now should be characterized more precisely and their normality examined more critically using better growth and differentiation assays and global gene expression profiling. Protocols should be compared quantitatively and qualitatively for frequency and identity of cells generated. Progress would be aided immensely by free exchange of PSCs and derived p63/K14+ cell lines among laboratories. Continued research on this subject will expand our understanding of the essential factors for embryonic development of the many keratinocyte subtypes and other p63+ epithelial cell types.

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References

Papers of particular interest, published recently, have been highlighted as:

- Of importance

- Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154–156
- Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 78:7634–7638
- Shamblott MJ, Axelman J, Wang S et al (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA* 95:13726–13731
- Thomson JA, Itskovitz-Eldor J, Shapiro SS et al (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147
- Lin SL (2011) Concise review: deciphering the mechanism behind induced pluripotent stem cell generation. *Stem Cells* 29:1645–1649
- Takahashi K, Tanabe K, Ohnuki M et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872
- Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676
- Yu J, Vodyanik MA, Smuga-Otto K et al (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318:1917–1920
- Robinton DA, Daley GQ (2012) The promise of induced pluripotent stem cells in research and therapy. *Nature* 481:295–305
- Compton CC, Gill JM, Bradford DA et al (1989) Skin regenerated from cultured epithelial autografts on full-thickness burn wounds from 6 days to 5 years after grafting. A light, electron microscopic and immunohistochemical study. *Lab Invest* 60:600–612
- Gallico GG 3rd, O'Connor NE, Compton CC et al (1984) Permanent coverage of large burn wounds with autologous cultured human epithelium. *N Engl J Med* 311:448–451
- Pellegrini G, Traverso CE, Franzi AT et al (1997) Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 349:990–993
- Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331–343
- Watt FM, Green H (1981) Involucrin synthesis is correlated with cell size in human epidermal cultures. *J Cell Biol* 90:738–742
- Wu YJ, Parker LM, Binder NE et al (1982) The mesothelial keratins: a new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia. *Cell* 31:693–703
- Dabelsteen S, Hercule P, Barron P, et al. (2009) Epithelial cells derived from human embryonic stem cells display p16^{INK4A} senescence, hypermotility, and differentiation properties shared by many p63+ somatic cell types. *Stem Cells* 27:1388–1399. *This paper identified growth control abnormalities in hES-derived, p63/K14+ cells and showed that the pattern of differentiation these cells display in organotypic culture is similar to that of many p63+ epithelial cell types and not only epidermal keratinocytes*
- Lechner JF, Haugen A, Autrup H et al (1981) Clonal growth of epithelial cells from normal adult human bronchus. *Cancer Res* 41:2294–2304
- Lindberg K, Brown ME, Chaves HV et al (1993) In vitro propagation of human ocular surface epithelial cells for transplantation. *Investig Ophthalmol Vis Sci* 34:2672–2679
- Lindberg K, Rheinwald JG (1990) Three distinct keratinocyte subtypes identified in human oral epithelium by their patterns of keratin expression in culture and in xenografts. *Differentiation* 45:230–241
- Taylor-Papadimitriou J, Shearer M, Stoker MG (1977) Growth requirements of human mammary epithelial cells in culture. *Int J Cancer* 20:903–908
- Boyce ST, Ham RG (1983) Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J Invest Dermatol* 81:33s–40s
- Pirisi L, Yasumoto S, Feller M et al (1987) Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *J Virol* 61:1061–1066
- Shipley GD, Pittelkow MR (1987) Control of growth and differentiation in vitro of human keratinocytes cultured in serum-free medium. *Arch Dermatol* 123:1541a–1544a
- Rice RH, Green H (1979) 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
- Wei W, Barron PD, Rheinwald JG (2010) Modulation of TGF-beta-inducible hypermotility by EGF and other factors in human prostate epithelial cells and keratinocytes. *In vitro Cell Dev Biol Animal* 46:841–855
- Hennings H, Michael D, Cheng C et al (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* 19:245–254
- Nowak JA, Fuchs E (2009) Isolation and culture of epithelial stem cells. *Methods Mol Biol* 482:215–232
- Bilousova G, Chen J, Roop DR (2011) Differentiation of mouse induced pluripotent stem cells into a multipotent keratinocyte lineage. *J Invest Dermatol* 131:857–864. *This paper describes a protocol for routine generation from miPS cells of p63/K14+ cells possessing a replicative lifespan similar to that of normal primary murine keratinocytes and the ability to form epidermis, hair follicles, and sebaceous glands in vivo when transplanted admixed with skin fibroblasts and dermal papilla cells*
- Sun TT, Eichner R, Nelson WG et al (1983) Keratin classes: molecular markers for different types of epithelial differentiation. *J Invest Dermatol* 81:109s–115s
- O'Guinn WM, Galvin S, Schermer A et al (1987) Patterns of keratin expression define distinct pathways of epithelial development and differentiation. *Curr Top Dev Biol* 22:97–125
- Mehrel T, Hohl D, Rothnagel JA et al (1990) Identification of a major keratinocyte cell envelope protein, loricrin. *Cell* 61:1103–1112
- Lynley AM, Dale BA (1983) The characterization of human epidermal filaggrin. A histidine-rich, keratin filament-aggregating protein. *Biochim Biophys Acta* 744:28–35
- Schön M, Rheinwald JG (1996) A limited role for retinoic acid and retinoic acid receptors RAR α and RAR β in regulating keratin 19 expression and keratinization in oral and epidermal keratinocytes. *J Invest Dermatol* 107:428–438
- Parenteau NL, Nolte CM, Bilbo P et al (1991) Epidermis generated in vitro: practical considerations and applications. *J Cell Biochem* 45:245–251
- Bagutti C, Hutter C, Chiquet-Ehrismann R et al (2001) Dermal fibroblast-derived growth factors restore the ability of β 1 integrin-deficient embryonic stem cells to differentiate into keratinocytes. *Dev Biol* 231:321–333
- Bagutti C, Wobus AM, Fassler R et al (1996) Differentiation of embryonic stem cells into keratinocytes: comparison of wild-type and β 1 integrin-deficient cells. *Dev Biol* 179:184–196
- Doetschman TC, Eistetter H, Katz M et al (1985) The in vitro development of blastocyst-derived embryonic stem cell lines:

- formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol* 87:27–45
38. Kawasaki H, Mizuseki K, Nishikawa S et al (2000) Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* 28:31–40
 39. Aberdam D (2004) Derivation of keratinocyte progenitor cells and skin formation from embryonic stem cells. *Int J Dev Biol* 48:203–206
 40. Coraux C, Hilmi C, Rouleau M et al (2003) Reconstituted skin from murine embryonic stem cells. *Curr Biol* 13:849–853
 41. Green H, Easley K, Iuchi S (2003) Marker succession during the development of keratinocytes from cultured human embryonic stem cells. *Proc Natl Acad Sci USA* 100:15625–15630
 42. Iuchi S, Dabelsteen S, Easley K et al (2006) Immortalized keratinocyte lines derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 103:1792–1797
 43. • Aberdam E, Barak E, Rouleau M, et al. (2008) A pure population of ectodermal cells derived from human embryonic stem cells. *Stem Cells* 26:440–444. *This paper identified a stable K8/K18 + epithelial cell type derived from hES cells that has the potential to spontaneously, or if engineered to express ΔNp63α, express K14*
 44. Medawar A, Virolle T, Rostagno P et al (2008) ΔNp63 is essential for epidermal commitment of embryonic stem cells. *PLoS ONE* 3:e3441
 45. Ji L, Allen-Hoffmann BL, de Pablo JJ et al (2006) Generation and differentiation of human embryonic stem cell-derived keratinocyte precursors. *Tissue Eng* 12:665–679
 46. • Metallo CM, Azarin SM, Moses LE, et al. (2010) Human embryonic stem cell-derived keratinocytes exhibit an epidermal transcription program and undergo epithelial morphogenesis in engineered tissue constructs. *Tissue Eng Part A* 16:213–223. *This paper was the first to identify substantial differences in global gene transcription between hES-derived p63/K14 + cells and normal primary human epidermal keratinocytes*
 47. Metallo CM, Ji L, de Pablo JJ et al (2008) Retinoic acid and bone morphogenetic protein signaling synergize to efficiently direct epithelial differentiation of human embryonic stem cells. *Stem Cells* 26:372–380
 48. • Guenou H, Nissan X, Larcher F, et al. (2009) Human embryonic stem-cell derivatives for full reconstruction of the pluristratified epidermis: a preclinical study. *Lancet* 374:1745–1753. *This paper is the only report to date of generating long replicative lifespan p63/K14 + cells from hES cells in culture*
 49. Nissan X, Denis JA, Saidani M et al (2011) miR-203 modulates epithelial differentiation of human embryonic stem cells towards epidermal stratification. *Dev Biol* 356:506–515
 50. • Kidwai FK, Liu H, Toh WS, et al. (2013) Differentiation of human embryonic stem cells into clinically amenable keratinocytes in an autogenic environment. *J Invest Dermatol* 133:618–628. *This paper describes a thorough comparison of several protocols for producing p63/K14+ cells from hES cells and identifies strong positive effects of Activin A*
 51. • Itoh M, Kiuru M, Cairo MS, et al. (2011) Generation of keratinocytes from normal and recessive dystrophic epidermolysis bullosa-induced pluripotent stem cells. *Proc Natl Acad Sci USA* 108:8797–8802. *This paper is the first report of routine generation of p63/K14 + cells from human iPS cells*
 52. Shalom-Feuerstein R, Serron L, Aberdam E et al (2013) Impaired epithelial differentiation of induced pluripotent stem cells from ectodermal dysplasia-related patients is rescued by the small compound APR-246/PRIMA-1MET. *Proc Natl Acad Sci USA* 110:2152–2156
 53. Tolar J, Xia L, Lees CJ et al (2013) Keratinocytes from induced pluripotent stem cells in junctional epidermolysis bullosa. *J Invest Dermatol* 133:562–565
 54. Ahmad S, Stewart R, Yung S et al (2007) Differentiation of human embryonic stem cells into corneal epithelial-like cells by in vitro replication of the corneal epithelial stem cell niche. *Stem Cells* 25:1145–1155
 55. Shalom-Feuerstein R, Serron L, De La Forest Divonne S et al (2012) Pluripotent stem cell model reveals essential roles for miR-450b-5p and miR-184 in embryonic corneal lineage specification. *Stem Cells* 30:898–909
 56. Simpson TI, Price DJ (2002) Pax6; a pleiotropic player in development. *BioEssays* 24:1041–1051
 57. Hewitt KJ, Shamis Y, Carlson MW et al (2009) Three-dimensional epithelial tissues generated from human embryonic stem cells. *Tissue Eng Part A* 15:3417–3426
 58. Moll R, Franke WW, Schiller DL et al (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 31:11–24
 59. Mills AA, Zheng B, Wang XJ et al (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 398:708–713
 60. Yang A, Kaghad M, Wang Y et al (1998) p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 2:305–316
 61. Yang A, Schweitzer R, Sun D et al (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398:714–718
 62. Moll R, Divo M, Langbein L (2008) The human keratins: biology and pathology. *Histochem Cell Biol* 129:705–733
 63. Wolbach SB, Howe PR (1925) Tissue changes following deprivation of fat-soluble A vitamin. *J Exp Med* 42:753–777
 64. Wolbach SB, Howe PR (1933) Epithelial repair in recovery from vitamin A deficiency: an experimental study. *J Exp Med* 57:511–526
 65. Howe WE, Klier FG, Oshima RG (1986) Murine endodermal cytokeratins Endo A and Endo B are localized in the same intermediate filament. *J Histochem Cytochem* 34:785–793
 66. Conley BJ, Ellis S, Gulluyan L et al (2007) BMPs regulate differentiation of a putative visceral endoderm layer within human embryonic stem-cell-derived embryoid bodies. *Biochimie Biologie Cellulaire* 85:121–132
 67. Soprano DR, Teets BW, Soprano KJ (2007) Role of retinoic acid in the differentiation of embryonal carcinoma and embryonic stem cells. *Vitam Horm* 75:69–95
 68. Rollins BJ, O'Connell TM, Bennett G et al (1989) Environment-dependent growth inhibition of human epidermal keratinocytes by recombinant human TGFβ. *J Cell Physiol* 139:455–462
 69. Shipley GD, Pittelkow MR, Wille JJ Jr et al (1986) Reversible inhibition of normal human prokeratinocyte proliferation by type β transforming growth factor-growth inhibitor in serum-free medium. *Cancer Res* 46:2068–2071
 70. Barrandon Y, Li V, Green H (1988) New techniques for the grafting of cultured human epidermal cells onto athymic animals. *J Invest Dermatol* 91:315–318
 71. Troy TC, Turksen K (2005) Commitment of embryonic stem cells to an epidermal cell fate and differentiation in vitro. *Dev Dyn* 232:293–300
 72. Haase I, Knaup R, Wartenberg M et al (2007) In vitro differentiation of murine embryonic stem cells into keratinocyte-like cells. *Eur J Cell Biol* 86:801–805
 73. Huang HJ, Gao QS, Tao BF et al (2008) Long-term culture of keratinocyte-like cells derived from mouse embryonic stem cells. *In Vitro Cell Dev Biol* 44:193–203