Regenerating human epithelia with cultured stem cells: feeder cells, organoids and beyond

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Abstract

More than 40 years ago, Howard Green’s laboratory developed a method for long-term expansion of primary human epidermal keratinocytes by co-culture with 3T3 mouse embryonic fibroblasts. This was a breakthrough for in vitro cultivation of cells from human skin and later for other epithelia: it led to the first stem cell therapy using cultured cells and has vastly increased our understanding of epithelial stem cell biology. In recent years, new methods to expand epithelial cells as three-dimensional organoids have provided novel means to investigate the functions of these cells in health and disease. Here, we outline the history of stratified epithelial stem cell culture and the application of cultured epithelial cells in clinical therapies. We further discuss the derivation of organoids from other types of epithelia and the challenges that remain for the translation of novel stem cell therapies toward clinical use.

Keywords 3T3 cells; adult stem cells; cell culture; epithelial cells; organoids

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See the Glossary for abbreviations used in this article.

Introduction

Primary cell culture of human epithelial cells has been possible since the mid-1970s, but the ability to establish long-term cultures has varied depending on which organ cells are isolated from. Nonetheless, research has made considerable progress in understanding the mechanisms by which stem and progenitor cells orchestrate the homeostatic turnover and regenerative potential of adult epithelia. These cells reside within complex niches throughout the body that are composed of differentiated epithelial cells, diverse mesenchymal cells, vasculature, neuronal cells, and surrounding extracellular matrix (ECM).

Cell culture imposes a very different, harsh environment to which epithelial cells must adapt and proliferate extensively without losing their functional potential or entering a senescent state. Defining conditions for expanding primary epithelial cells without immortalization has been a challenge, but, under the correct conditions, cells can undergo more population doublings than they might in vivo. Lately, improvements in culture protocols to minimize the loss of clonogenic cells by maintaining a balance between cell proliferation and differentiation have allowed the expansion of sufficient numbers of primary human epithelial cells for diverse applications, including regenerative medicine (De Luca et al., 2006), disease modeling (Schweiger & Jensen, 2016), toxicology (Hynds & Giangreco, 2013), and drug discovery (Ranga et al., 2014).

In this Review, we focus on the application of cultured epithelial cells in regenerative medicine. In particular, we discuss the 3T3 mouse embryonic fibroblast (MEF) co-culture system that has enabled stratified epithelial stem cell therapy for clinical use. We further discuss recent advances in 3D organoid culture that allow non-stratified epithelial cell culture and consider the challenges that these methods face to emulate the translational successes that have used cultured stratified epithelial cells.

A history of epithelial cell expansion using 3T3 cells

3T3 cells were first isolated in Howard Green’s laboratory in the early 1960s as part of his experiments to better control the process of cell line derivation (Fig 1). Mouse embryonic cells were plated to select for adherent cells after embryo disaggregation and then expanded for 2–3 days until confluence. Following this transfer or “passage”, culture conditions—inoculation densities and transfer frequency—were systematically varied to study the growth characteristics of murine fibroblasts (Todaro & Green, 1963). Higher inoculation densities increased the possibility of routine culture establishment, but one cell line was established from a low-density culture. “3T3” is thus an abbreviation of this culture protocol—a 3-day transfer period with $3 \times 10^5$ cells plated at each transfer. In common with MEF cultures established using
other protocols, 3T3 cells developed altered chromosome number and gross karyotypic abnormalities, but were contact-inhibited upon cell confluence. Initially, the cells were used in studies of viral transformation, as loss of contact inhibition can be easily identified in a confluent monolayer (Todaro & Green, 1966).

Reproduction of the 3T3 protocol in other laboratories led to isolation of independent 3T3 cell lines from BALB/C and NIH Swiss mouse strains, giving rise to BALB/3T3 and NIH/3T3 lines, respectively (Aaronson & Todaro, 1968; Jainchill et al, 1969). These lines differ from the original 3T3 line as they are less contact-inhibited and more susceptible to viral transformation. Other subclones of Green’s 3T3 cells were selected for the presence of lipid accumulation. Upon cell confluence, these cells accumulate triglyceride lipids in cytoplasmic droplets and generate mature fat pads after injection into athymic mice (Green & Kehinde, 1974, 1979; Green & Meuth, 1974). These cells, known as 3T3-L1, are now widely used to model adipogenesis.

With regard to epithelial cell expansion in vitro, a key breakthrough was made during attempts to derive primary cell cultures from murine teratomas. When cultured on plastic, these tissues gave rise to fibroblasts with few, poorly proliferative epithelial cells. However, co-culture with mitotically inactivated 3T3 cells reduced fibroblast contamination and enabled serial expansion of epithelial cells resembling keratinocytes (Rheinwald & Green, 1975a). Application of this protocol to human epidermal keratinocytes, which had until then proven impossible to expand substantially in culture, created progressively growing, strongly adhesive colonies of epithelial cells that pushed away the surrounding feeder cells (Rheinwald & Green, 1975b). These colonies are genuine 3D structures that can display partial stratification, with cells resembling basal cells in contact with the culture dish.

Both the feeder cells and the culture medium were further optimized for human keratinocyte culture, which now uses 3T3-J2 cells, a subclone of Green’s 3T3 line that are more supportive of keratinocyte cultures (Rheinwald, 1989). Initially, co-cultures were performed in medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 in a 3:1 ratio with added fetal bovine serum (FBS) and hydrocortisone. Later refinements saw the addition of adenine (Peehl & Ham, 1980), cholera toxin (Green, 1978; Okada et al, 1982), insulin (Tsoa et al, 1982), triiodothyronine (Maciag et al, 1981), and epidermal growth factor (Rheinwald & Green, 1977). 3T3 co-culture in supplemented FAD (F12, adenine, and DMEM) medium allowed the long-term expansion of human epithelial cells for the first time.

Importantly, long-term expansion of epidermal cells in culture is enabled by the maintenance of epidermal stem cells; this was shown by seminal experiments that allowed the retrospective identification of stem cells by analyzing the differential growth capacity of colony-forming human epithelial cells in vitro (Barrandon & Green, 1987). When individual colonies formed from a single cell are re-plated in secondary cultures, they can be classified into three different clonal types: the “holoclone” has the greatest expansion capacity as at least 95% of the colonies in secondary cultures are large and contain small, highly proliferative cells; the “paraclone” gives rise only to small colonies of cells that undergo terminal differentiation within a few doublings (<15); finally, the “meroclone” represents an intermediate stage between holoclones and paraclones that contains both types of colonies (Barrandon & Green, 1987). Cells that form holoclones are the epidermal stem cells that are able to reconstitute a functional epidermis lasting for a lifetime in the treatment of full-thickness burns (Pellegrini et al, 1999). The number of holoclones in vivo is affected by aging, whereas loss of stemness in culture may occur by clonal conversion—from holoclones, through meroclones to paraclones—during which growth potential progressively decreases and telomere-independent senescence takes hold (Barrandon et al, 2012).

Clinical translation of epidermal and corneal stem cells

Endogenous regeneration mediated by adult stem cells is not always capable to completely repair damaged tissue. For example,
full-thickness epidermal wounds larger than 4 cm in diameter do not heal appropriately without medical intervention (MacNeil, 2007). Current therapies involve repair with split-thickness autografts of epidermis and a thin layer of dermis from a non-affected body site. However, this approach is poorly suited for extensive burns and increases the risk of sepsis. The clinical application of autologous stem cells expanded \textit{ex vivo} has addressed this problem.

By the early 1980s, pre-clinical work demonstrated that epithelial sheets could be generated by culturing keratinocyte colonies to confluence and detaching them using enzymes that target cell–substrate but not cell–cell junctions, such as dispase (Banks-Schlegel & Green, 1980) or thermolysin (Germain \textit{et al}, 1993). Engraftment of these cultured epidermal autografts (CEAs) on wound beds in immune-compromised mice was robust, and the histological morphology of cultured human epidermis remained intact for several months after transplantation (Banks-Schlegel & Green, 1980).

Initially, two patients received CEAs in combination with conventional autografts to treat extensive third-degree burns (O’Connor \textit{et al}, 1981). Large-scale production followed and two patients with third-degree burns of more than 80% of the total skin surface also received CEAs (Gallico \textit{et al}, 1984). Engraftment was successful, and around 50% of the life-saving epithelial regeneration was mediated by cultured cells. Overall, 60–100% of grafts take (Gallico \textit{et al}, 1984; Pellegrini \textit{et al}, 1999), but the success rate is highly variable between individual patients (De Luca \textit{et al}, 2006) and grafts can fail owing to suboptimal wound bed preparation and infection.

An improvement to the CEA protocol has involved culturing keratinocytes on alternative substrates as dispase-mediated detachment shrinks the epithelial surface area by 50% and generates fragile epithelial sheets that can only be used during a short window around the time of cell confluence. These alternative substrates include fibrin (Pellegrini \textit{et al}, 1999; Ronfard \textit{et al}, 2000), an insoluble protein.
mesh that is formed during normal wound healing, ECM proteins (Myers et al, 1997; Horch et al, 2000), and chemically defined surfaces (Zhu et al, 2005), but preservation of epidermal stem cells has only been demonstrated on tissue-culture plastic and fibrin substrates. These substrates have multiple advantages as they remove the need for enzymatic dissociation, retain ECM proteins produced by epithelial cells, and are more easily manipulated during surgery. Crucially, substrate-enabled CEAs can be transplanted subconfluent, which permits a more flexible time window for transplantation. The performance of CEAs was also appreciably improved by the use of cadaveric skin allografts, which adhere to the wound bed; the epidermis can be mechanically removed later for CEA engraftment (Cuono et al, 1986). This reduces the risk of infection and therefore promotes successful engraftment by maintaining a skin barrier prior to CEA transplantation and also provides a surgically prepared wound bed of alloderm.

Clinical experience and post-treatment analysis showed that CEAs are safe and that the morphological features of epidermis return, albeit over variable timeframes: scar tissue in the dermal layer takes up to 5 years to resolve, whereas Langerhans cells are present within 6 months and melanocytes within a year. Normal interfollicular epidermal histological appearance is maintained for many years post-transplantation (Compton et al, 1989; Odessey, 1992). In this way, 3T3 co-culture has benefitted thousands of patients worldwide (Watt, 2014). However, epidermal appendages such as hair follicles, sebaceous glands, and sweat glands are not currently regenerated in CEA skin, although progress has been made toward understanding the molecular mechanisms that govern their development (Lu et al, 2016; Chacon-Martinez et al, 2017; Lei et al, 2017) and designing methods to incorporate these into clinical transplants (Higgins et al, 2013).

Notably, CEA transplantation has revealed novel insights into the extent of epidermal cell plasticity. Palm and sole epidermis for instance is characterized by a thickened stratum corneum and cyto-keratin-9 (KRT9) expression, which are not found in epidermis from other anatomical sites. When palm-derived CEAs were grafted onto a patient’s upper leg, they retained memory of their site of origin (Mavilio et al, 2006). Similarly, cultured oral keratinocytes from the palate preserve donor site characteristics after transplantation (De Luca et al, 1990). It appears that keratinocyte location specificity is cell intrinsic and not lost during culture or re-wired by the microenvironment following transplantation. The epigenetic mechanisms underlying this phenomenon are not currently understood.

More recently, CEAs have enabled epithelial gene therapy in patients with functional epidermolysis bullosa (JEB), a blistering disease in which epithelia are inadequately anchored to the basement membrane owing to mutations in genes encoding laminin 332 (LAMA3, LAMB3, and LAMC2). Autologous epidermal keratinocytes expanded in culture and retrovirally transduced with full-length LAMB3 have been successfully engrafted as sheets onto surgically prepared wound beds (Mavilio et al, 2006; Hirsch et al, 2017). LAMB3 mRNA was detectable in grafts and expression of the transgene allowed proper keratinocyte-basement membrane interaction via integrin γ6β4. Healthy epithelium containing a normal number of ΔNP63 isoform α-positive keratinocyte basal cells was regenerated in transplanted areas (De Rosa et al, 2014). In a very recent report, more than 80% of a patient’s total epidermal surface (Hirsch et al, 2017) was transplanted after in vitro gene correction. This is a landmark successful in vitro gene therapy for a genetic disease of the epithelium. Nevertheless, these gene therapy studies face the risk that more than one-third of retroviral integration sites can fall within transcriptionally active genes; however, since long-term regeneration depends only on a small number of stem cells, the significance of deleterious insertion sites might be overstated, especially when balanced with absence of treatment that, in cases like this, invariably results in the patient’s death. Together, these studies demonstrate that cultured epithelial cells can engraft and contribute to long-term regeneration providing that they are given the correct cues for self-renewal in vitro. Future application of gene editing technologies, such as adeno-associated viral vectors (AAVs; Melo et al, 2014) or high-fidelity CRISPR-Cas9 systems (Kleinstiver et al, 2016), could be safe and effective means to generate gene-corrected, clonally derived CEAs for transplantation but are not yet in clinical use (Droz-Georget Lathion et al, 2015).

3T3 co-culture is effective for culturing other stratified squamous epithelia (Romagnoli et al, 1990), most notably for the generation of corneal epithelium to treat severe ocular burns (Atallah et al, 2016). Owing to the anatomical location of the stem cells responsible for maintaining the clear corneal epithelium in the limbus (Cotsarelis et al, 1989)—the border area between cornea and conjunctiva—ocular burns can deplete the stem/progenitor population. In their absence, conjunctival cells migrate across the cornea causing a connective coverage that leads to complete loss of vision. Limbal restoration is therefore essential for successful therapy. For patients with minimal residual limbal tissue or an uninjured eye, limbal stem cells expanded in culture restore vision upon transplantation (Pellegrini et al, 1997; Rama et al, 2010). To date, several hundred patients have been treated and, in an Italian cohort, vision was successfully restored in 78% of cases (De Luca et al, 2006).

The most important lesson from these epithelial therapies is that of stem cell retention during culture. In CEAs and limbal transplants alike, the retention of stem cells, rather than the histological appearance of the epithelium in vitro, is the critical indicator for graft success (Pellegrini et al, 1999). If culture is suboptimal, and stem cells lost by clonal conversion, grafts generate an atrophic, fragile epithelium that eventually fails due to the ever-decreasing ability of transplanted cells to maintain epithelial integrity (Pellegrini et al, 2013). Importantly, a retroviral integration study of genetically defined clones in engraved epidermis in the recent clinical application of CEA technology for JEB clearly demonstrates that only a small number of transplanted stem cells maintain long-term regeneration in vivo (Hirsch et al, 2017).

Yet, the retrospective method for identifying stem cells in vitro by clonal culture is not well suited for monitoring stem cell maintenance in cultures intended for clinical application. As an alternative, expression levels of the transcription factor P63 are a more direct method to assess stem cell content, at least in limbal epithelial cell cultures (Pellegrini et al, 2001; Di Iorio et al, 2005). In mice, P63-knockouts are born with stratified epithelia consisting of differentiated cell types, but their maintenance of basal epithelial stem cells is deficient (Yang et al, 1999). There are at least 10 P63 isoforms classified as either transactivating (TP63) or N-terminal truncated isoforms (ΔNP63) each with different C-termini (α, β, γ, δ, and ε; Mangiulli et al, 2009). The ΔNP63 isoform α is enriched in stem cells and is essential for their retention (Blanpain & Fuchs, 2007; Senno et al, 2007). Holoclone formation and ΔNP63 expression
levels could both be used for clinical quality control (Pellegrini et al., 2013). Recently, a non-invasive, time-lapse imaging method was developed which determines epidermal stemness during colony growth through analysis of cell movement; colonies with collective motion are more likely to be holoclones (Nanba et al., 2015).

Recreating a niche in vitro

3T3 cells: a mechanistic enigma

3T3 co-culture remains the gold standard for stem cell expansion in many settings as feeder- and serum-free media, which often replace these with crude bovine pituitary extract (Peehl & Ham, 1980), stimulate proliferation for only short periods. 3T3 co-culture starts with producing feeder layers by inactivating 3T3 cells with ~60 gray (Gy) ionizing (gamma) radiation, which causes direct DNA damage through double-stranded breaks and the generation of free radicals that interact with DNA, or by treating 3T3 cells with mitomycin C, which covalently cross-links double-stranded DNA and prevents the separation of DNA strands during replication. Both methods cause irreversible cell cycle arrest and altered cellular metabolism; the inactivated state might be required in its own right for epithelial cell support (Palechor-Ceron et al., 2013).

Nonetheless, the contribution of 3T3 cells in co-culture is poorly understood, which explains the difficulties in producing defined media that fully recapitulate their growth-supportive effects. This effect is not simply caused by an increase in the overall density of cells and is fibroblast-specific. Indeed, other strains of MEFs or even virally transformed 3T3 cells are not supportive of keratinocyte expansion to the same extent (Green et al., 1977). Although conditioned medium from 3T3 cells also cannot fully reproduce the effect of co-culture, diffusible factors likely mediate some of their key effects as separation of epithelial and feeder cells using transwell membranes allows colony formation (Palechor-Ceron et al., 2013). Instead, a constant supply of feeder cell factors, which cannot be recreated using conditioned medium, might be necessary. Further work is required to assess the effect of cell–cell contact on clonogenicity.

Previous work has also stressed the importance of reciprocal signaling between epithelial and feeder cells for optimal culture conditions. The receptor tyrosine kinase response of epithelial cells treated with 3T3 fibroblast-conditioned medium is surprisingly modest (R.E. Hynds, P. Bonfanti, S.M. Janes, unpublished data), but cultured keratinocytes constitutively produce interleukin-1 (IL-1), which causes c-JUN-dependent production of proliferation-enhancing growth factors by 3T3 cells, including keratinocyte growth factor (KGF), granulocyte–macrophage colony-stimulating factor (GM-CSF), and hepatocyte growth factor (HGF; Szabowski et al., 2000; Schnickmann et al., 2009). Insulin-like growth factors (IGFs) have also been identified as relevant 3T3 cell factors since IGF inhibition in keratinocytes or replacing 3T3-J2 cells with BALB/3T3 cells, which do not produce IGFs, reduces epithelial proliferation (Barreca et al., 1992). The secretion of IGF2 in 3T3-J2 cells appears to be due to their higher expression of the Wnt pathway antagonist Dact1, which suppresses transforming growth factor-β2 (TGFβ2) production in response to epithelial-derived Wnt signals (Suzuki & Senoo, 2015). This synergy between epithelial cells and fibroblasts, along with the fact that xenogeneic proteins bind to and activate receptors on human epithelial cells with different efficiency to species-relevant proteins, has complicated studies to unravel the mechanistic basis of 3T3 co-culture.

A recent protocol to improve multiple feeder-free stratified epithelial cell culture systems uses A83-01 and DMH-1, inhibitors that target activin-like kinase (ALK) receptors in the TGFβ and BMP signaling cascades, respectively (Mou et al., 2016). Interestingly, this raises the possibility that the role of feeder cells might be the elimination of factors that are detrimental for epithelial cell propagation, such as TGFβ in serum or produced by epithelial cells, rather than to provide feeder factors. Nevertheless, the ability of feeder-free systems to preserve stem cells has not been demonstrated and so, while our understanding of how 3T3-J2 cells support diverse stratified epithelial cells remains limited, this system remains the gold standard for epidermal and limbal regenerative medicine.

Development of organoid cultures

The term organoid has been applied to organotypic systems that more closely represent in vivo tissues (Fig 2) than 2D cultures which often lose key aspects of tissue physiology (Sasai, 2013). The loss of the complex microenvironment ex vivo leads to less representative cellular organization or even an inability to initiate cell cultures. By seeding cells into a 3D matrix in the presence of mesenchyme (Ootani et al., 2009) or in medium containing epidermal growth factor (EGF), Noggin and a Wnt pathway potentiator, R-spondin 1 (Sato et al., 2009), epithelial cells can be expanded long term even from simple epithelia that were traditionally hard to maintain in cell culture. By way of example, the dependence of LGR5+ gastrointestinal stem cells on Wnt pathway activation has allowed the formulation of media to expand organoids from the human gastrointestinal tract that retain the crypt–villus architecture of the native intestine (Sato et al., 2011a). Subsequently, self-renewing organoids have been derived from organs, including the prostate, liver, and pancreas, expanding the repertoire of human epithelial tissues from which primary culture is possible (Clevers, 2016; Kretzschmar & Clevers, 2016).

There are differences between epithelia that can be cultured as 2D 3T3 co-culture and as 3D organoids. 3T3 cells support long-term P63+ stratified epithelial stem cell expansion, whereas there are currently no reports of organoid culture methods to expand stratified epithelia long-term, suggesting that these cells might have different self-renewal requirements. Wnt signaling, an essential requirement for organoid propagation, is unlikely to be the basis of 3T3 cell keratinocyte support as 3T3-J2 cells express comparable levels of Wnt proteins to other MEFs that do not support expansion (Suzuki & Senoo, 2015). Furthermore, Green’s protocol has remained relatively unchanged since in vivo tissues (Fig 2) than 2D cultures which often lose key aspects of tissue physiology (Sasai, 2013). The loss of the complex microenvironment ex vivo leads to less representative cellular organization or even an inability to initiate cell cultures. By seeding cells into a 3D matrix in the presence of mesenchyme (Ootani et al., 2009) or in medium containing epidermal growth factor (EGF), Noggin and a Wnt pathway potentiator, R-spondin 1 (Sato et al., 2009), epithelial cells can be expanded long term even from simple epithelia that were traditionally hard to maintain in cell culture. By way of example, the dependence of LGR5+ gastrointestinal stem cells on Wnt pathway activation has allowed the formulation of media to expand organoids from the human gastrointestinal tract that retain the crypt–villus architecture of the native intestine (Sato et al., 2011a). Subsequently, self-renewing organoids have been derived from organs, including the prostate, liver, and pancreas, expanding the repertoire of human epithelial tissues from which primary culture is possible (Clevers, 2016; Kretzschmar & Clevers, 2016).

Organoid cultures have yielded insight into the cellular mechanisms of epithelial homeostasis and helped to elucidate specific niche factors. For example, small intestinal Paneth cells, an antimicrobial-secreting cell population adjacent to LGR5+ stem cells, produce EGF receptor ligands, Notch ligands, and WNT3, all of which are essential for stem cell maintenance in small intestinal organoids (Sato et al., 2011b). c-KIT+/REG4-expressing deep crypt secretory cells function in a similar manner in the colonic epithelium (Rothenberg et al., 2012; Sasaki et al., 2016). This highlights the
importance of both secreted and membrane-bound intraepithelial signals as niche components, in addition to more commonly studied mesenchymal–epithelial pathways (Farin et al., 2016).

Furthermore, the location specificity of LGR5+ gastrointestinal tract stem cells is retained during organoid culture (Middendorp et al., 2014). Consistent with the finding that epidermal stem cells retain memory of their donor site in clinical studies (Mavilio et al., 2006), murine small intestinal organoids transplanted into the murine colon retain transcriptional hallmarks of small intestinal epithelium 4 weeks after engraftment (Fukuda et al., 2014). Expression of colonic epithelium-specific markers after transplantation of fetal small intestinal organoids suggests that these cells might be more plastic (Fordham et al., 2013). Overall, however, existing data do not support the notion that adult stem cell expansion protocols “reprogram” cells to greater potency but rather maintain their native identity and functionality.

Despite the rapid progress in organoid technologies, the field is at a relatively early stage of development in terms of clinical use (Fig 3); in particular, questions remain as to whether these cells can maintain tissue homeostasis after transplantation. Experiments involving transplantation of murine colonic organoids in an acute colitis model show promising results: organoids derived from a single stem cell were capable of engrafting, surviving, and contributing to histologically normal epithelium for more than 25 weeks (Yui et al., 2012). Data demonstrating engraftment of human organoids in orthotopic murine transplantation experiments are currently limited to human hepatic organoids in acute liver injury (Huch et al., 2015). Transplanted cells differentiated to express hepatocyte markers and

Figure 2. Strategies of adult stem cell-based epithelial cell culture.
(A) Pathway for isolating human epithelial cells in 3T3 co-culture and methods for their “re-differentiation” in organotypic assays. (B) Pathway for isolating human epithelial cells as 3D organoids.
human albumin and alpha-1-antitrypsin could be detected in the blood of successfully transplanted animals after 120 days. However, the functionality of the engrafted cells, including their contribution to the hepatic stem cell pool, was not analyzed.

These initial transplantation experiments have also revealed practical hurdles for regenerative applications as vast numbers of organoid-derived cells have enabled only very modest engraftment rates. Experimentally, this might be addressed by improving the reproducibility between and within organoid cultures, by enhancing delivery methods, and by upscaling organoid cultures for medical applications, particularly within clinically useful timeframes. Overall, the evidence for using organoids as a cell source in translational applications falls short of demonstrating fully functional epithelia and further pre-clinical animal models should be advanced.

**Dependence of cultured epithelial cells on xenogeneic factors**

A commonality between 3T3 co-culture and organoid culture systems is that the gold standard protocols for stem cell culture depend on murine-derived products. Attempts to replace 3T3 cells with human feeder cells and/or defined media have been largely unsuccessful in terms of stem cell maintenance, while the regulatory environment for cell therapies has changed substantially. Early grafts from 3T3 co-culture were sanctioned through collective agreement about the scientific rationale by a small number of clinicians (Green, 2008). More recently, regulatory bodies in the USA and Europe have classified tissues based on 3T3 co-culture as xenografts. The use of murine cells and bovine serum raises concerns about the transmission of infectious agents. It was also suggested that co-culture of human embryonic stem cells (hESCs) with murine feeder cells leads the former to incorporate a potentially immunogenic non-human sialic acid, Neu5Gc (Martin et al., 2005). However, it was later shown that human sera do not reduce hESC viability substantially and that minor effects are independent of cellular Neu5Gc content (Cerdan et al., 2006). Notably, these concerns are unsupported by the long-term engraftment and survival of transplanted epithelial cells in patients. While adverse events are reported, these primarily relate to the preparation of the graft site and the quality of epithelial cells transplanted, rather than the presence of 3T3 cells, which only account for 0–2% of cells in the final CEA product (Ronfard et al., 2000). Importantly, no tumor development has been reported in any patients even after follow-up over several decades. CEAs have received regulatory approval in a number of countries including the USA, Japan, and Korea. In Europe, limbal stem cell therapy was recently approved contingent upon the generation of a well-characterized 3T3-J2 master cell bank in which all feeder cells are infection-free and cultured in accordance with Good Manufacturing Practice (Pellegrini et al., 2014).

To make matters more complicated, the interactions between epithelial cells and the ECM secreted by murine feeder cells in 3T3 co-culture are poorly understood. The complex ECM is known to contain fibronectin, laminin, and collagen types I, IV, and V (Alitalo et al., 1982), but their relative importance for epithelial stem cell retention is not known. Laminin is an interesting candidate: a recent feeder-free protocol employs laminin-rich conditioned medium from 804G rat bladder carcinoma cells to deposit a substrate for epithelial cell expansion (Mou et al., 2016) and the common organoid ECM from murine sarcoma-derived Matrigel contains predominantly laminin, collagen IV, and nidogen-1, but it also includes more than

![Figure 3. Milestones in regenerative medicine using cultured epithelial stem cells.](Image)
1,000 other proteins with variability between batches (Hughes et al., 2010). Laminin 511 has been used as a matrix for feeder-free growth of pluripotent (Nakagawa et al., 2014) and epithelial cells (Polisetti et al., 2017) but, overall, the physiological presentation of ECM secreted by cells seems to be more efficient compared with systems that use recombinant ECM proteins.

Replacing Matrigel with a better-defined product will be critical for future translation of human organoids for clinical applications. The potential use of bovine collagen I as a substitute (Jabaji et al., 2014) might address some concerns, but the conditions for complete organoid differentiation in collagen gels have not been described and their effects on the long-term maintenance of stem cells are unclear. Recent studies have begun to pave the way for Matrigel replacement with synthetic polymers: one elegant study sets out to determine minimal in vitro ECM niche requirements using a bottom-up bioengineering approach (Gjorevski et al., 2016). LGR5+ stem cells could be maintained in a synthetic RGD-functionalized polyethylene glycol (PEG) hydrogel, but these organoids lacked differentiated cell types. Hydrogel stiffness, communicated through YAP signaling, was the key determinant of stem cell retention and differentiation: an initially stiff matrix was required for stem cell expansion and subsequent matrix softening allowed differentiated cell types to emerge. The dynamic biomechanical properties of Matrigel therefore likely support both stem cell maintenance and organoid differentiation. A second study, investigating an alternative synthetic approach based on PEG-4MAL macromer-based hydrogels, also emphasized the importance of polymer mechanical properties and showed that these could expand pluripotent cell-derived intestinal organoids that could engraft in vivo (Cruz-Acuna et al., 2017). In such studies, full biocompatibility and rate of polymer degradation should be carefully analyzed before clinical translation.

Maintaining stem cells in culture

As discussed above, the abundance of stem cells, or holoclones, in transplanted skin and corneal grafts determines the long-term survival of the graft. It might therefore be beneficial to use products with a high proportion of stem cells. In epidermal 3T3 co-cultures, partial differentiation of colonies occurs during their expansion, while the stem cell population is retained by balanced self-renewal of stem cells. Isolation of these cells in the presence of Rho-associated protein kinase inhibition (ROCKi) can improve the number of cells expanded in culture, potentially including a greater number of stem cells (Chapman et al., 2010; Terunuma et al., 2010), likely by decreasing the extent of anoikis in the initial cell suspensions (Watanabe et al., 2007). Continuous ROCKi or mammalian target of rapamycin (mTOR) inhibition using rapamycin (Iglesias-Bartolome et al., 2012) also increase epithelial cell proliferation and reduce terminal differentiation (McMullan et al., 2003; Nanba et al., 2013) but, despite evidence that cells expanded with ROCKi can differentiate appropriately in vivo (Butler et al., 2016), the capacity of cells grown in these conditions to contribute to long-term organ homeostasis after transplantation has not yet been shown. This stands in contrast to Green’s protocol, which has clearly demonstrated this ability in multiple epithelial cell types.

ROCKi is also used during 3D organoid protocols to improve cell viability during isolation and transfer but does not significantly alter the cellular composition of organoids. Multiple groups have explored strategies to preferentially expand LGR5+ stem cells, rather than their differentiated progeny, in 3D organoid cultures. Addition of the Wnt pathway activator CHIR99021 and the histone deacetylase inhibitor valproic acid to culture medium favors expansion of murine Lgr5+ intestinal stem cells over differentiated cell types in organoid culture (Yin et al., 2014). Interestingly, the combination of 3T3 co-culture and medium with niche factors used in organoid culture allows long-term, pure expansion of genome-stable human gastrointestinal stem cells in 2D culture (Wang et al., 2015), suggesting that apico-basal polarity generated in 3D organoid cultures is key to the emergence of differentiated cell types. It will be important to establish in pre-clinical transplantation models whether stem cell-enriched transplants outperform those that contain differentiated cell types.

Future perspectives

Highly expandable cultures containing epithelial stem cells from a variety of organs can be established in co-culture with 3T3 cells. These co-cultures, in combination with organotypic differentiation assays, already offer a great tool for pre-clinical research, but the use of murine feeder cells is a limitation for their routine use in therapy. However, since stem cell retention is essential for long-term integration of cultured epithelia, the premature translation of therapies that attempt to replace components of the culture system without clear evidence that they avoid the loss of stem cells should be cautioned against. Alternatively, trials should have a clear rationale for the use of cells that will not support long-term tissue homeostasis. Unraveling the mechanisms involved in 3T3 support of stratified epithelial stem cells has proved difficult, but it is yielding more knowledge about basic stem cell biology. The determination of essential feeder factors and the production of defined growth conditions for pluripotent stem cells give hope that it will be possible to replace murine feeder cells for epithelial stem cell culture in the future (Nakagawa et al., 2014).

There is much excitement in regenerative medicine about the potential clinical use of iP5 cells and much progress has been made toward differentiating these into multiple organ-specific lineages, including epithelium. Yet, there remain greater hurdles to the use of iP5-derived organoids compared to tissue-specific, stem cell-derived cultures, including mutational burden, culture duration,
differentiation efficacy, epigenetic changes, and ultimate functionality. The impact of culture on the genome of cells is a particular concern. While the absence of any clinically apparent mutations in patients receiving cultured epithelial cell grafts to date is encouraging, rare genomic alterations could still occur during expansion. Initial data suggest that small numbers of de novo mutations are introduced during organoid culture, but this nevertheless compares favorably to the number of mutations that are introduced during induced pluripotent stem (iPS) cell culture (Huch et al., 2015). For these reasons, adult epithelial cells remain the first choice for clinical application, but iPS options should be explored if the use of adult stem cells is not feasible (Bilousova & Roop, 2014). This might include simple epithelia, cases where adult stem cells have been destroyed by injury—such as severe bilateral burns of the cornea—or if whole tissue substitutes (e.g., full-thickness skin) can be developed (Workman et al., 2017). Overall, the cell culture advances described here raise the prospect that the translational success achieved for skin and cornea could be repeated in further epithelial tissues.

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Conflict of interest
The authors declare that they have no conflict of interest.

References
Aaronson SA, Todaro GJ (1968) Development of 3T3-like lines from Balb/C mouse embryo cultures – transformation susceptibility to Sv40. J Cell Physiol 72: 141 – 148
Altalal K, Kuismanen E, Myllyla R, Kiistala U, Asko-Seljamaa S, Vaheeri A (1982) Extracellular matrix proteins of human epidermal keratinocytes and feeder 3T3 cells. J Cell Biol 94: 497 – 505
Atallah MR, Palioura S, Perez VL, Amescua G (2016) Limbal stem cell transplantation: current perspectives. Clin Ophthalmol 10: 593 – 602
Banks-Schlegel S, Green H (1980) Formation of epidermis by serially cultivated human epidermal cells transplanted as an epithelium to athymic mice. Transplantation 29: 308 – 313
Barrandon Y, Green H (1987) Three clonal types of keratinocyte with different capacities for multiplication. Proc Natl Acad Sci USA 84: 2302 – 2306
Barrandon Y, Grasset N, Zaffalon A, Gorostidi F, Claudinot S, Droz-Georget SL, Nanba D, Rochat A (2012) Capturing epidermal stemness for regenerative medicine. Semin Cell Dev Biol 23: 957 – 944
Barreca A, De Luca M, Del Monte P, Bondanza S, Damonte G, Cariola G, Di Marco E, Giordano G, Cancetta R, Minuto F (1992) in utero paracrine regulation of human keratinocyte growth by fibroblast-derived insulin-like growth factors. J Cell Physiol 151: 262 – 268
Bilousova G, Roop DR (2014) Induced pluripotent stem cells in dermatology: potentials, advances, and limitations. Cold Spring Harb Perspect Med 4: a015164
Blanpain C, Fuchs E (2007) p63: reving up epithelial stem-cell potential. Nat Cell Biol 9: 731 – 733
Butler CR, Hynds RE, Gowers KH, Lee D, Brown JM, Crowley C, Teixeira VH, Smith CM, Urbanl I, Hamilton NJ et al (2016) Rapid expansion of human epithelial stem cells suitable for airway tissue engineering. Am J Respir Crit Care Med 194: 156 – 168
Cerdan C, Bendall SC, Wang L, Stewart M, Werbowski T, Bhatia M (2006) Complement targeting of nonhuman siolic acid does not mediate cell death of human embryonic stem cells. Nat Med 12: 1113 – 1114; author reply 1115
Chacon-Martinez CA, Klose M, Niemann C, Glauche I, Wickstrom SA (2017) Hair follicle stem cell cultures reveal self-organizing plasticity of stem cells and their progeny. EMBO J 36: 151 – 164
Chapman S, Liu X, Meyers C, Schlegel R, McBride AA (2010) Human keratinocytes are efficiently immortalized by a Rho kinase inhibitor. J Clin Invest 120: 2619 – 2626
Clevens H (2016) Modeling development and disease with organoids. Cell 165: 1586 – 1597
Compton CC, Gill JM, Bradford DA, Regauer S, Gallico GG, O'Connor NE (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
Costarelis G, Cheng SZ, Dong C, Sun TT, Lavker RM (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 57: 201 – 209
Cruz-Acuña R, Quiros M, Farkas AE, Dedhia PH, Huang S, Siuda D, Garcia-Hernandez V, Miller AJ, Spence JR, Nusrat A et al (2017) Synthetic hydrogels for human intestinal organoid generation and colonic wound repair. Nat Cell Biol 19: 1326 – 1335
Cuono C, Langdon R, McGuire J (1986) Use of cultured epidermal autografts and dermal allografts as skin replacement after burn injury. Lancet 1: 1123 – 1124
De Luca M, Albanese E, Megna M, Cancetta R, Mangiante PE, Cadoni A, Franzì AT (1990) Evidence that human oral epithelium reconstituted in vitro and transplanted onto patients with defects in the oral mucosa retains properties of the original donor site. Transplantation 50: 454 – 459
De Luca M, Pellegrini G, Green H (2006) Regeneration of squamous epidermis from stem cells of cultured grafts. Regen Med 1: 45 – 57
De Rosa L, Carulli S, Cocchiarella F, Quaglinò D, Enzo E, Franchini E, Giannetti A, De Santis G, Recchia A, Pellegrini G et al (2014) Long-term stability and safety of transgenic cultured epidermal stem cells in gene therapy of junctional epidermolysis bullosa. Stem Cell Reports 2: 1 – 8
Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M (2005) Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. Proc Natl Acad Sci USA 102: 9523 – 9528
Droz-Georget Lathion S, Rochat A, Knott G, Recchia A, Martinet D, Benmohamed S, Grasset N, Zaffalon A, Besuchet Schmutz N, Savioz-Dayer E et al (2015) A single epidermal stem cell strategy for safe ex vivo gene therapy. EMBO Mol Med 7: 380 – 393
Hynds RE, Ianniello DV, de Punder K, Angers S, Peters PJ, Maurice MM et al (2016) Visualization of a short-range Wnt gradient in the intestinal stem-cell niche. *Nature* 530: 340–343
Fordham RP, Yui S, Hannan NR, Soenenda C, Madwick A, Schweiger PJ, Nielsen OH, Vallier L, Pedersen RA, Nakamura T et al (2013) Transplantation of expanded fetal intestinal progenitors contributes to colon regeneration after injury. *Cell Stem Cell* 13: 734–744
Fukuda M, Mizutani T, Mochizuki W, Matsumoto T, Nozaki K, Sakamaki Y, Ichinose S, Okada Y, Tanaka T, Watanabe M et al (2014) Small intestinal stem cell identity is maintained with functional Paneth cells in heterotopically grafted epithelium onto the colon. *Genes Dev* 28: 1752–1757
Gallico GG III, O'Connor NE, Compton CC, Kehinde O, Green H (1984) Permanent coverage of large burn wounds with autologous cultured human epithelium. *N Engl J Med* 311: 448–451
Germain L, Rouabha M, Guignard SF, Carrier L, Bouvard V, Auger FA (1993) Improvement of human keratinocyte isolation and culture using thermolysin. *Burns* 19: 99–104
Gjorevski N, Sachs N, Manfrin A, Giger S, Bragina ME, Ordonez-Moran P, Germain L, Rouabhia M, Guignard R, Carrier L, Bouvard V, Auger FA (1993) Concise review: the relevance of human stem cell-derived organoid models for epithelial translational medicine. *EMBO Molecular Medicine* 5: 903–910
Huch M, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MM, Ellis E, van Gjorevski N, Sachs N, Manfrin A, Giger S, Bragina ME, Ordonez-Moran P, Germain L, Rouabhia M, Guignard R, Carrier L, Bouvard V, Auger FA (1993) Concise review: the relevance of human stem cell-derived organoid models for epithelial translational medicine. *EMBO Molecular Medicine* 5: 903–910
Hadjivassiliou M, Williams DR (1996) Large bowel disease in the elderly: role of polyp surgery. *N Engl J Med* 335: 277–283
Jabaji Z, Brinkley GJ, Khalil HA, Sears CM, Lei NY, Lewis M, Stelzer M, Martin MG, Dunn JC (2014) Type I collagen as an extracellular matrix for the *in vitro* growth of human small intestinal epithelium. *PLoS One* 9: e107814
Kainth J, Aarons S, Gajic B (1983) Minor sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. *J Virol* 4: 549–553
Klein stiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK (2016) High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 529: 490–495
Kretzschmar K, Clevers H (2016) Organoids: modeling development and the stem cell niche in a dish. *Deu Cell* 38: 590–600
Lei M, Schumacher LJ, Lai YC, Yuan WT, Yeh CY, Wu P, Jiang TX, Baker RE, Widelitz RB, Yang L et al (2017) Self-organization process in newborn skin organoid formation inspires strategy to restore hair regeneration of adult cells. *Proc Natl Acad Sci USA* 114: 67101–67110
Lu CP, Polak L, Keyses BE, Fuchs E (2016) Spatiotemporal antagonism in mesenchymal-epithelial signaling in sweat versus hair fate decision. *Science* 354: aa6102
Maciag T, Nemos RE, Weinstein R, Gilchrest BA (1981) An endocrine approach to the control of epidermal growth: serum-free cultivation of human keratinocytes. *Science* 211: 1452–1454
MacNeil S (2007) Progress and opportunities for tissue-engineered skin. *Nature* 445: 874–880
Mangiulli M, Valletti A, Caratozzolo MF, Tullo A, Sbisa E, Pesole G, D’Erchia AM (2009) Identification and functional characterization of two new transcriptional variants of the human p53 gene. *Nucleic Acids Res* 37: 6092–6104
Martin MJ, Muotri A, Gage F, Arkai V (2005) Human embryonic stem cells express an immunogenic nonhuman salic acid. *Nat Med* 11: 228–232
Mavilio F, Pellegrini G, Ferrari S, Di Nunzio F, Di Iorio E, Recchia A, Maruggi G, Ferrari G, Provasi E, Bonini C et al (2006) Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. *Nat Med* 12: 1397–1402
McMullen R, Snoxall J, Robertson VH, Radford DJ, Broad S, Watt FM, Rowles A, Croft DR, Olson MF, Hotchin NA (2003) Keratinocyte differentiation is regulated by the Rho and ROCK signaling pathway. *Curr Biol* 13: 2185–2189
Melo SP, Lisowski L, Bashkirova E, Zhen HH, Chu K, Keene DR, Marinovich MP, Kay MA, Ora AE (2014) Somatic correction of junctional epidermolysis bullosa by a highly recombinogenic AAV variant. *Mol Ther* 22: 725–733
Middendorp S, Schneebberger K, Wiegerinck CL, Mokry M, Akkerman RD, van Wijngaarden S, Clevers H, Nieuwenhuis EE (2014) Adult stem cells in the small intestine are intrinsically programmed with their location-specific function. *Stem Cells* 32: 1083–1091
Mou H, Vinarsky V, Tata PR, Brazauskas K, Choi SH, Crooke AK, Zhang B, Solomon GM, Turner B, Bihler H et al (2016) Dual SMAD signaling inhibition enables long-term expansion of diverse epithelial basal cells. *Cell Stem Cell* 19: 217–231
Myers SR, Grady J, Soranzo C, Sanders R, Green C, Leigh IM, Navsaria HA (1997) A hyaluronic acid membrane delivery system for cultured keratinocytes: clinical “take” rates in the porcine kerato-dermal model. *J Burn Care Rehabil* 18: 214–222
Nakagawa M, Taniguchi Y, Senda S, Takizawa N, Ichisaka T, Asano K, Morizane A, Doi D, Takahashi J, Nishizawa M et al (2014) A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. *Sci Rep* 4: 3594

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Nanba D, Toki F, Matsushita N, Matsushita S, Higashiyama S, Barrandon Y (2013) Actin filament dynamics impacts keratinocyte stem cell maintenance. *EMBO Mol Med* 5: 640 – 653

Nanba D, Toki F, Tate S, Imai M, Matsushita N, Shiraishi K, Sayama K, Toki H, Higashiyama S, Barrandon Y (2015) Cell motion predicts human epidermal stemness. *J Cell Biol* 209: 305 – 315

O’Connor NE, Mulliken JB, Banks-Schlegel S, Kehinde O, Green H (1981) Grafting of burns with cultured epithelium prepared from autologous epidermal cells. *Lancet* 1: 75 – 78

Odyssey R (1992) Addendum: multicenter experience with cultured epithelial autograft for treatment of burns. *J Burn Care Rehabil* 13: 174 – 180

Okada N, Kitano Y, Ichihara K (1982) Effects of cholera toxin on proliferation of cultured human keratinocytes in relation to intracellular cyclic AMP levels. *J Invest Dermatol* 79: 42 – 47

Ootani A, Li X, Sangiorgi E, Ho QT, Ueno H, Toda S, Sugihara H, Fujimoto K, Okada N, Kitano Y, Ichihara K (2012) Identification of a cKit(+ ) colonic crypt base secretory cell that supports Lgr5( + ) stem cells in mice. *Gastroenterology* 142: 1195 – 1205 e1196

Ottaviani A, Li X, Sangiorgi E, Ho QT, Ueno H, Toda S, Sugihara H, Fujimoto K, Okada N, Kitano Y, Ichihara K (2012) Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5(+ ) stem cells in mice. *Gastroenterology* 142: 1195 – 1205 e1196

Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M (1990) Suppression of malignant transformation by keratinocyte growth factor activation promotes keratinocyte migration via CEACAM1. *Cell Death Differ* 7: 5399 – 5407

Polisetti N, Sorokin L, Kalisky T, Lee JJ, Dalerba P, Scheeren F, Lobo N, Kulkarni S, Sim S, Qian D et al. (2012) Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5( + ) stem cells in mice. *Gastroenterology* 142: 1195 – 1205 e1196

Polisetti N, Sorokin L, Kalisky T, Lee JJ, Dalerba P, Scheeren F, Lobo N, Kulkarni S, Sim S, Qian D et al. (2012) Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5( + ) stem cells in mice. *Gastroenterology* 142: 1195 – 1205 e1196

Peehl DM, Ham RG (1980) Clonal growth of human keratinocytes with small amounts of dialyzed serum. *In Vitro* 16: 526 – 540

Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M (1997) Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 347: 990 – 993

Pellegrini G, Ranno R, Stracuzzi G, Bondanza S, Guerra L, Zambruno G, Micali G, De Luca M (1999) The control of epidermal stem cells (holoclones) in the treatment of massive full-thickness burns with autologous keratinocytes cultured on fibrin. *Transplantation* 68: 868 – 879

Pellegrini G, Dellambra E, Colisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin D, McKeon F, De Luca M (2001) p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA* 98: 3156 – 3161

Pellegrini G, Rama P, Matuska S, Lambiase A, Bonini S, Pocobelli A, Colabelli RG, Spadae L, Fasciani R, Balestrazzi E et al. (2013) Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells. *Regen Med* 8: 553 – 567

Pellegrini G, Rama P, Di Rocco A, Panaras A, De Luca M (2014) Concise review: hurdles in a successful example of limbal stem cell-based regenerative medicine. *Stem Cells* 32: 26 – 34

Polisetti N, Sorokin I, Okumura N, Koizumi N, Kinosita S, Kruse FE, Schlotzer-Schrehardt U (2017) Laminin-511 and -521-based matrices for efficient ex vivo expansion of human limbal epithelial progenitor cells. *Sci Rep* 7: 5152

Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G (2010) Limbal stem-cell therapy and long-term corneal regeneration. *N Engl J Med* 363: 147 – 155

Ranga A, Gjorevski N, Lutolf MP (2014) Drug discovery through stem cell-based organoid models. *Adv Drug Deliv Rev* 69–70: 19 – 28

Rheinwald JG, Green H (1975a) Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. *Cell* 6: 317 – 330

Rheinwald JG, Green H (1975b) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6: 331 – 343

Rheinwald JG, Green H (1977) Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature* 265: 421 – 424

Rheinwald JG (1989) Methods for clonal growth and serial cultivation of normal human epidermal keratinocytes and mesothelial cells. In *Cell growth and division: a practical approach*, Baserga R (ed.), pp 81 – 94. Oxford: IRL Press

Romagnoli G, De Luca M, Faranda F, Bandelloni R, Franzì AT, Caliotti F, Cancedda R (1990) Treatment of posterior hypospadias by the autologous graft of cultured urethral epithelium. *N Engl J Med* 323: 527 – 530

Ronfard V, Rives JM, Neveux Y, Carsin H, Barrandon Y (2000) Long-term regeneration of human epidermis on third degree burns transplanted with autologous cultured epithelial grown on a fibrin matrix. *Transplantation* 70: 1588 – 1598

Rothenberg ME, Nusse Y, Kalisky T, Lee JJ, Dalerba P, Scheeren F, Lobo N, Kulkarni S, Sim S, Qian D et al. (2012) Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5( + ) stem cells in mice. *Gastroenterology* 142: 1195 – 1205 e1196

Sasi Y (2013) Next-generation regenerative medicine: organogenesis from stem cells in 3D culture. *Cell Stem Cell* 12: 520 – 530

Sasaki N, Sachs N, Wiebrands K, Ellenbroek SI, Fumagalli A, Lyubimova A, Begthel H, van den Born M, van Es JH, Karthaus WR et al. (2014) Reg4+ deep crypt secretory cells function as epithelial niche for Lgr5+ stem cells in colon. *Proc Natl Acad Sci USA* 113: E5399 – E5407

Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ et al. (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459: 262 – 265

Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, Van Houdt WJ, Pronk A, Van Gorp J, Siersma PD et al. (2011a) Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett’s epithelium. *Gastroenterology* 141: 1762 – 1772

Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, Barker N, Shroyer NF, van de Wetering M, Clevers H (2011b) Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* 469: 415 – 418

Schnickmann S, Camacho-Trullio D, Bissinger M, Eils R, Angel P, Schirmacher V, Terunuma A, Limgala RP, Park CJ, Choudhary I, Vogel JC et al. (2012) Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett’s epithelium. *Gastroenterology* 141: 1762 – 1772

Schweiger PJ, Jensen KB (2016) Modeling human disease using organotypic cultures. *Curr Opin Cell Biol* 43: 22 – 29

Senoo M, Pinto F, Crum CP, McKeon F (2007) p63 is essential for the proliferative potential of stem cells in stratified epithelium. *Cell* 129: 523 – 536

Suzuki D, Senoo M (2015) Dact1 regulates the ability of 3T3-J2 cells to support proliferation of human epidermal keratinocytes. *J Invest Dermatol* 135: 2894 – 2897

Szaboowski A, Maas-Szaboowski N, Andrecht S, Kolbus A, Schopp-Kistner M, Fusenig NE, Angel P (2000) c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. *Cell* 103: 745 – 755

Terunuma A, Limgala RP, Park CJ, Choudhary I, Vogel JC (2010) Efficient procurement of epithelial stem cells from human tissue specimens using a Rho-associated protein kinase inhibitor Y-27632. *Tissue Eng Part A* 16: 1363 – 1368
Todaro GJ, Green H (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 17: 299–313

Todaro GJ, Green H (1966) Cell growth and the initiation of transformation by SV40. Proc Natl Acad Sci USA 55: 302–308

Tsao MC, Walthall BJ, Ham RG (1982) Clonal growth of normal human epidermal keratinocytes in a defined medium. J Cell Physiol 110: 219–229

Wang X, Yamamoto Y, Wilson LH, Zhang T, Howitt BE, Farrow MA, Kern F, Ning G, Hong Y, Khor CC et al (2015) Cloning and variation of ground state intestinal stem cells. Nature 522: 173–178

Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K et al (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25: 681–686

Watt FM (2014) Mammalian skin cell biology: at the interface between laboratory and clinic. Science 346: 937–940

Workman MJ, Mahe MM, Trisno S, Poling HM, Watson CL, Sundaram N, Chang CF, Schiesser J, Aubert P, Stanley EG et al (2017) Engineered human pluripotent-stem-cell-derived intestinal tissues with a functional enteric nervous system. Nat Med 23: 49–59

Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C et al (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398: 714–718

Yin X, Farin HF, van Es JH, Clevers H, Langer R, Karp JM (2014) Niche-independent high-purity cultures of Lgr5+ intestinal stem cells and their progeny. Nat Methods 11: 106–112

Yui S, Nakamura T, Sato T, Nemoto Y, Mizutani T, Zheng X, Ichinose S, Nagaishi T, Okamoto R, Tsuchiya K et al (2012) Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5(+) stem cell. Nat Med 18: 618–623

Zhu N, Warner RM, Simpson C, Glover M, Hernon CA, Kelly J, Fraser S, Brotherston TM, Ralston DR, MacNeil S (2005) Treatment of burns and chronic wounds using a new cell transfer dressing for delivery of autologous keratinocytes. Eur J Plast Surg 28: 319–330

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