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Engineering epithelial-stromal interactions *in vitro* for toxicology assessment

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Abstract

Crosstalk between epithelial and stromal cells drives the morphogenesis of ectodermal organs during development and promotes normal mature adult epithelial tissue homeostasis. Epithelial-stromal interactions (ESIs) have historically been examined using mammalian models and *ex vivo* tissue recombination. Although these approaches have elucidated signaling mechanisms underlying embryonic morphogenesis processes and adult mammalian epithelial tissue function, they are limited by the availability of tissue, low throughput, and human developmental or physiological relevance. In this review, we describe how bioengineered ESIs, using either human stem cells or co-cultures of human primary epithelial and stromal cells, have enabled the development of human *in vitro* epithelial tissue models that recapitulate the architecture, phenotype, and function of adult human epithelial tissues. We discuss how the strategies used to engineer mature epithelial tissue models *in vitro* could be extrapolated to instruct the design of organotypic culture models that can recapitulate the structure of embryonic ectodermal tissues and enable the *in vitro* assessment of events critical to organ/tissue morphogenesis. Given the importance of ESIs towards normal epithelial tissue development and function, such models present a unique opportunity for toxicological screening assays to incorporate ESIs to assess the impact of chemicals on mature and developing epidermal tissues.

Keywords

Epithelial cells; Epithelial-stromal interactions; Stromal cells; Multipotent stromal cells; Bioengineering; Organotypic; Morphogenesis; Development; Palate fusion

1. Introduction

Epithelial cells actively promote tissue function by serving as a protective and responsive barrier that regulates molecular transport or by secreting specialized macromolecules into blood stream or ducts. Epithelial cells (ECs) exhibit a range of different morphologies (*e.g.* squamous, cuboidal, columnar) characteristic of their phenotype and function in the tissue

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Competing interests

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milieu. For example, squamous alveolar ECs are specialized to secrete surfactants and provide a thin, protective, and selectively permeable barrier between atmospheric air and the pulmonary circulation (Ward and Nicholas, 1984). Cuboidal ECs can form hollow lumens and are specialized to secrete endocrine hormones into the blood stream or exocrine products into ducts (Hassiotou and Geddes, 2013; Holmberg and Hoffman, 2014). A critical aspect of epithelial tissue function is active crosstalk between ECs, the cell microenvironment, and the tissue stroma.

Reciprocal epithelial-stromal interactions (ESIs) direct tissue morphogenesis during development and regulate homeostasis in adults (Cunha et al., 1985, 2004). ESIs broadly consist of biochemical and biophysical crosstalk between stromal cells (*e.g.* fibroblasts, endothelial cells, smooth muscle cells, adipocytes), the extracellular matrix (ECM), and ECs, and promote normal epidermal (*e.g.* skin, cornea, tooth, mucosa) and glandular (*e.g.* mammary, pancreas, salivary, lacrimal) tissue development and function. ESIs can also contribute to tumor growth and metastasis (Parrinello et al., 2005) and disease progression (Strand and Hayward, 2010) as discussed in other reviews. During development, fusion of the neural tube, cardiac endocardial cushions, palatal shelves, and urethral folds are all critically dependent on ESIs (Ray and Niswander, 2012). These developmental processes are sensitive to perturbation with environmental contaminants that result in birth defects, and this motivates the need for robust models of developmental morphogenesis *in vitro*.

Culture models of developmental processes currently lack the throughput and human developmental relevance needed for high throughput toxicity assessment. Historically, tissue recombination, trans-well, and conditioned medium assays have all been used to study ESIs *in vitro*. For example, recombination of mammalian epithelial and stromal tissues has contributed to understanding the importance of fibroblast growth factor-10 (FGF-10)/sonic hedgehog (SHH) crosstalk (Rice et al., 2004) and epidermal growth factor (EGF) signaling (Tyler and Pratt, 1980) during embryonic palate fusion. Tissue explant studies are inherently low throughput and cannot fully recapitulate the relative contribution of signaling pathways that underlie human developmental processes (Fougerousse et al., 2000). In comparison, trans-well and conditioned medium assays can be used to interrogate paracrine signaling between epithelial and stromal cells *in vitro* (Miki et al., 2012). However, these assays are fundamentally limited by dilution of paracrine factors and depletion of nutrients in the conditioning phase that are needed by the secondary cell type. Trans-well and conditioned medium assays also cannot recapitulate epithelial tissue architecture, wherein stroma and epithelium are separated only by a basement membrane (BM) that promotes cell adhesion and regulate ESIs (Yurchenco, 2011). In contrast, three-dimensional (3D) culture of stromal and ECs (Fig. 1) can support tissue morphogenesis by recapitulating the tissue architecture and mass transport properties essential to tissue morphogenesis (Shamir and Ewald, 2014). The purpose of this review is to introduce the reader to bioengineering strategies that have been used to incorporate ESIs into models of epidermal and glandular tissues using primary cells and stem cells and to emphasize the importance of incorporating ESIs into toxicological screening assays. Finally, we derive insights into how engineered ESIs could enable the design of embryonic tissues *in vitro* that would enable the assessment of developmental morphogenesis for toxicology applications.

2. Layer-by-layer assembly to engineer tissues that promote ESIs

2.1. Skin tissue engineering

Skin is the foremost example of an adult tissue with which a bottom-up approach has been taken to promote ESIs and functional tissue formation *in vitro*. Skin tissue function is dependent on ESIs between epidermal keratinocytes, squamous epithelia, and BM separating the epithelium and underlying dermis containing ECM proteins (primarily collagens) and dermal fibroblasts (Fig. 1B) (Werner et al., 2007). Skin tissue engineering approaches can provide important insights into the materials, cell types, and methods (Table 1) that promote ESIs and normal epithelial tissue phenotype and function.

Human skin equivalents are commonly produced using layer-by-layer assembly (Fig. 1A) of encapsulated or monolayer stromal cells with a juxtaposed monolayer of keratinocytes to recapitulate the stratified nature of mature skin tissue. Typical 3D culture approaches involve encapsulating cells by mixing them in a soluble ECM that can solidify upon a stimulus to form a hydrated matrix with embedded cells. For example, skin equivalents comprising human epidermal keratinocytes (HEKs) seeded on gelatin-chondroitin-6-sulfate-hyaluronic acid hydrogels (Wang et al., 2006), fibrin (Flasza et al., 2007) or BioReagent-coated collagen (Kinsner et al., 2001) with encapsulated human dermal fibroblasts (HDFs) maintained epithelial and stromal phenotype and skin-like tissue stratification *in vitro* (Table 1). Similar skin equivalents using collagen I (Casasco et al., 2001; Eaglstein et al., 1995; Mirastschijski et al., 2006), collagen-glycosaminoglycan (Cooper et al., 1993), plastic compressed collagen (Hartmann-Fritsch et al., 2013), or decellularized amniotic membrane (Sanluis-Verdes et al., 2015; Yang et al., 2009) successfully engrafted to immune-compromised mice, demonstrating their biocompatibility. This approach is contrasted by traditional 2D cell culture techniques wherein cells are attached to a substrate on only one side. For example, HEKs and HDFs seeded on opposite sides of decellularized dermis (Ojeh and Navsaria, 2014) or collagen-glycosaminoglycan-chitosan (Black et al., 2005) exhibited skin-like stratification and BM formation and supported both epithelial and stromal phenotypes.

Alternative sources of cells, including multipotent stromal cells and pluripotent stem cells, have been used to recapitulate skin *in vitro*. For example, skin tissue equivalents constructed of amniotic ECs seeded on the apical side of collagen gels with encapsulated amniotic stromal cells exhibited engineered tissue stratification and promoted cell proliferation and engineered skin engraftment to immune-compromised mice (Li et al., 2012). Human Wharton's jelly stromal cells (HWJSCs) are a multipotent cell population, derived from the Wharton's jelly of the umbilical cord, that have been used both as a stromal and an epithelial component of engineered skin tissue equivalents. A model consisting of collagen-encapsulated stromal cells (HDFs, human bone marrow stromal cells (BMSCs), or HWJSCs) with HaCaT cells seeded on the apical side of the gel promoted epithelial polarization and stable expression of BM proteins relative to the same model substituting collagen for cell-secreted matrix (Schneider et al., 2010). The authors demonstrated that HDF-produced, but not BMSC- or HWJSC-produced, matrix was instructive for epithelial polarization, stratification, and differentiation (Schneider et al., 2010). Keratinocytes alone

can exhibit stratification upon stimulation with EGF (Riva et al., 2007), insulin-like growth factor-1 (IGF-1) (Eming et al., 1996), or SHH (Kameda et al., 2001), and these key signaling pathways may suggest a mechanism whereby HDFs may be more instructive for skin-like tissue formation relative to MSCs. An alternative approach using HWJSCs seeded on the apical side of fibrin-agarose gels with encapsulated HDFs elicited stratified epithelium *in vitro*, though *in vivo* grafting was required to differentiate the engineered HWJSC-derived epithelium, as indicated by a time-dependent increase in expression of cytokeratin (CK1/4/13), plakoglobin, filaggrin, and involucrin upon engraftment *in vivo* (Garzon et al., 2013). These studies highlight the ability of HWJSCs to exhibit either stromal or epithelial phenotype based on their context and culture conditions, which agrees with data demonstrating their expression of ectoderm, endoderm, and mesoderm markers (Fong et al., 2011) and ability to undergo ectodermal differentiation (Jadalannagari and Aljitawi, 2015). HWJSCs are an attractive cell source for tissue engineering due to their ease of isolation (relative to other multipotent stromal cells) (Kalaszczynska and Ferdyn 2015) and their multipotency. Both BMSCs (Via et al., 2012) and HWJSCs (Troyer and Weiss, 2008) can be induced to differentiate to bone, cartilage, adipose, muscle, neural, and other tissue types, but caution should be exercised when culturing either BMSCs or HWJSCs for tissue engineering applications, as spontaneous differentiation in culture can compromise reproducibility and engineered tissue phenotype and function. Finally, Hewitt et al. developed a procedure to generate epidermal-like tissue (Fig. 2A) from human embryonic stem cells (hESCs). Serum-containing medium with EGF/BMP4 promoted hESC differentiation to primitive ectodermal cells expressing typical stromal markers (vimentin, CD73, CD105, CD106, CD44, CD10, CD13). Subsequent culture of hESC-derived primitive ectoderm on keratinocyte-secreted ECM/collagen 1 promoted CK12+/CK18- phenotype and reduced the expression of CD73, CD106, and CD10, consistent with an epithelial-like phenotype. hESC-derived epithelial-like cells exhibited BM (collagen IV) deposition when cultured on collagen I gels containing hESC-derived primitive ectodermal cells, and only in co-culture did the authors observe BM formation (Hewitt et al., 2009).

Skin bioengineering approaches employing more than two cell types have been hypothesized to better recapitulate the complexity of ESIs in mature human skin (Fig. 1B). Martins-Green et al. developed a multi-layered skin model consisting of juxtaposed layers of encapsulated HDFs, human primary microvascular endothelial cells, and HDFs with HEKs seeded on the apical surface. The multilayered skin model supported microvasculature formation, epithelial stratification, BM deposition, and angiogenic factor production *in vitro* (Martins-Green et al., 2004). A similar multi-layered approach using fibrin-embedded human adipose-derived stromal cells (ADSCs) and HDFs with HEKs seeded on the apical surface exhibited cellular architecture similar to native skin, supported ADSC and HDF proliferation, and promoted glycerol production (indicating adipogenesis) (Kober et al., 2015). Finally, co-culture models of HEKs, HDFs, and human primary melanocytes can control engineered skin pigmentation. Skin equivalents composed of collagen-encapsulated HDFs and seeded primary human melanocytes and HEKs exhibited stratification, engraftment, and endogenous melanin production (Biedermann et al., 2015; Bottcher-Haberzeth et al., 2014, 2013). Validated non-animal methods for skin irritation and sensitization currently use either epidermis-only or full-thickness keratinocyte/fibroblast co-cultures (Ward, 2016), but there

exist opportunities to increase the complexity and physiological relevance of engineered skin models (such as incorporating sweat glands and hair follicles) and apply the same bioengineering principles to the design of other epithelial tissues with relevance to toxicology screening.

2.2. Corneal tissue engineering

The structure of the cornea consists of stratified layers of cells and ECM that together contribute to the transparent and diffusible properties of the cornea. Corneal ECs are attached to Bowman's layer (collagen I and heparan sulfate proteoglycans) (Torricelli et al., 2013), and the stroma between Bowman's layer and Descemet's membrane (collagen IV) consists of corneal fibroblasts and collagen I. Corneal endothelial cells underlie Descemet's membrane and serve as a barrier to the aqueous humor (Thomas, 1955). The sclera surrounds the cornea, and the limbus, forming the border between the cornea and sclera, contains limbal ECs, limbal fibroblasts (Van Buskirk, 1989), and a corneal epithelial stem cell niche (Li et al., 2007). Corneal tissue is stratified and highly dependent on ESIs similarly to skin tissue (Wilson et al., 1999), but engineered corneas must be uniquely diffusible and transparent.

Layer-by-layer assembly of epithelial and stromal cells has been used for corneal tissue engineering to recapitulate the stratified architecture of the cornea. For example, silk dual-layer fibroin mats seeded on opposing sides with limbal epithelial and limbal stromal cells supported BM formation and both epithelial (CK3/12+, Np63+) and stromal (CD73+/CD90+/CD105+/α-smooth muscle actin+) phenotypes while maintaining transparency (Bray et al., 2012). Corneal fibroblasts encapsulated in fibrin/agarose and seeded with limbal epithelial cells or HWJSCs exhibited similar stratification, phenotype, and proteoglycan/crystallin expression relative to native corneal tissue, which suggests that HWJSCs could exhibit an epithelial (CK+, connexin 43+) or stromal (collagen+, fibronectin+) phenotype (Garzon et al., 2014) in agreement with skin tissue engineering approaches using HWJSCs. Corneal fibroblasts can produce ECM rich in collagen I, VI, XII, and fibronectin (consistent with the cornea) after 35 days in culture, which provides understanding of their role in promoting corneal epithelial attachment (Lake et al., 2013). Plastic compressed collagen I with or without encapsulated human limbal fibroblasts supported human limbal EC attachment, phenotype (CK3/15+, β1-integrin+), and microvilli formation and promoted BM formation (collagen IV+, laminin+) while maintaining transparency (Levis et al., 2010). Finally, layer-by-layer assembly of corneal endothelial cells, fibroblasts, and ECs elicited tissue architecture that was consistent with porcine cornea and exhibited drug permeability that was similar to that of porcine corneal explants (Reichl et al., 2004). This study demonstrates a potential application of organotypic culture for assessing drug and toxin permeability through a model cornea that is relevant to ocular drug delivery assessment and toxicology assessment applications. Although non-animal tests have been validated for eye irritation, currently no *in vitro* alternative method uses complex corneal co-cultures (Ward, 2016), which are likely important to faithfully recapitulate the response of engineered corneal tissue to chemical challenge. Future work will require that additional features of ocular physiology are incorporated *in vitro* such as tear fluid flow and mechanical forces generated by a representative aqueous humor (Shafaie et al., 2016). Corneal equivalents for

toxicology applications must also undergo validation and scale-up in order to fully replace the *in vivo* and *ex vivo* ocular sensitivity tests currently used for toxicology applications (Wilson et al., 2015).

2.3. Mucosal tissue engineering

ECs lining the airways and in the alveoli of the lungs serve as both a barrier and a source of lubrication. Airway ECs are arranged in a pseudostratified architecture and exhibit distinct phenotypes – ciliated (columnar) ECs, Goblet (cuboidal) ECs that secrete mucus, and basal (cuboidal) ECs that can differentiate and replace damaged epithelium (Ward and Nicholas, 1984). In the respiratory airways, the pseudostratified epithelium is separated from the lamina propria, containing capillaries, connective tissue (*e.g.* elastin, collagen), fibroblasts, smooth muscle cells, and glands, by a thin BM (Liu et al., 2011). To date, there are no validated non-animal models for assessing toxicity in mucosal tissues (Ward, 2016), and thus there is a need to develop and validate mucosal barrier tissues for toxicity testing.

The co-culture strategy of layering stroma and epithelium as described for skin and corneal tissue engineering have also been used for bronchial and oral mucosal engineering. For example, bronchial equivalents consisting of human bronchial fibroblasts encapsulated in collagen with monolayer human bronchial ECs exhibited pseudostratified architecture and contained a BM (collagen IV+, laminin+) and rudimentary cilia (Chakir et al., 2001). Human BMSCs (basal side) and human bronchial ECs (apical side) seeded on opposite sides of a polyvinylidene difluoride membrane maintained epithelial (CK+) and stromal (vimentin+) phenotype, promoted epithelial microvilli formation, and accelerated mucin production relative to ECs alone (Le Visage et al., 2004). A tri-culture microfluidic model of layered lung microvascular endothelial cells, lung fibroblasts, and bronchial ECs separated by nanoporous membranes formed a semi-permeable barrier consistent with lung epithelium and supported mucin production and cilia formation in ECs cultured at an air-liquid interface (Sellgren et al., 2014). The importance of the stromal compartment for governing epithelial phenotype is exemplified by several studies showing that stromal cells from a diseased donor could alter the phenotype of the resulting engineered oral or bronchial mucosa tissue (Gaballah et al., 2008; Massie et al., 2015; Paquette et al., 2004). For example, asthmatic human bronchial fibroblasts within bronchial equivalents or conditioned medium from asthmatic fibroblasts elicited human bronchial EC detachment with fewer cilia compared to bronchial equivalents with non-asthmatic fibroblasts (Paquette et al., 2004). These studies highlight the ability to test for the effect of chemicals on cell-based models from patients susceptible to inflammation.

Oral mucosa exhibits the same stratified nature of the bronchial mucosa with stratified mucus-producing epithelium, BM, and underlying lamina propria. A bioengineered oral mucosa model generated using layer-by-layer assembly (Fig. 1A) of human oral fibroblasts encapsulated in fibrin/agarose with monolayer human oral keratinocytes exhibited similar stromal-epithelial phenotype and stratification as native oral mucosa (Vinuela-Prieto et al., 2014). Layer-by-layer assembly of gingival fibroblasts and gingival keratinocytes on gelatin-polycaprolactone electrospun scaffolds supported epithelial (CK1/10/14+, involucrin+) and stromal (vimentin+) phenotype and promoted re-epithelialization of a porcine oral gingival

wound upon engraftment relative to sham, suggesting the biocompatibility of engineered mucosa (Schulz et al., 2014). Oral mucosa models have also been used to study dysplasia, as a dysplastic epithelial morphology could be elicited by seeding normal oral keratinocytes on collagen-embedded dysplastic buccal fibroblasts (exhibiting high MMP9 and hepatocyte growth factor expression) or by seeding dysplastic keratinocytes on embedded normal buccal fibroblasts (Gaballah et al., 2008). Oral mucosal models have also been used for proof-of-principle toxicity assessment *in vitro*. Co-culture models of human oral keratinocytes and human oral fibroblasts on decellularized dermis or encapsulated within nylon mesh enabled assessment of the epithelial interface with titanium implants (Chai et al., 2010) and enabled the identification of toxic (nickel, zinc, copper, indium, and cobalt) or pro-inflammatory (zinc, copper, indium, and cobalt) effects of common dental implant materials (Schmalz et al., 1997), respectively. Finally, pluripotent stem cells have been explored for reconstituting mucosal tissue *in vitro*. Engineered mucosa with hESC-derived ECs (CK18+/p63+) seeded on encapsulated vocal fold fibroblasts exhibited stratification, cilia formation, differential expression of CK14 (basal) and CK13 (suprabasal) markers, and exhibited formation of BM (laminin-5), tight junctions, gap junctions, adherens junctions, and desmosomes (Leydon et al., 2013). These studies together highlight the importance of ESIs for recapitulating oral mucosa for the study of disease phenotypes, toxicology, host-pathogen interactions, and drug permeability.

2.4. Urinary system tissue engineering

Urinary tract tissues contain a stratified transitional epithelium (urothelium), which is composed of a basal layer of ECs, an intermediate layer of highly proliferative ECs, and a superficial layer of fully differentiated ECs that contain microvilli and form an impenetrable barrier (Arrighi 2015). *In vitro* tissues to model the urinary bladder, or vesical equivalents, composed of vimentin+ canine oral fibroblasts (basal side) and canine oral keratinocytes (luminal side) seeded on electrospun silk-fibroin scaffolds exhibited stratified epithelial architecture and promoted urothelial repopulation and wound healing in a urethral defect model, whereas the control cell-free implants elicited no urothelial regeneration at the defect site (Xie et al., 2014). Vesical equivalents, consisting of layered bladder fibroblasts, bladder endothelial cells, and urothelial cells exhibited epithelial stratification, microvasculature, and microvilli and exhibited lower permeability to urea than fibroblast sheets alone (Bouhout et al., 2010), suggesting barrier function of vesical equivalents. These studies highlight an opportunity to examine *in vitro* the toxicity of chemicals in urinary epithelium, which currently requires animal testing to evaluate.

2.5. Summary: layer-by-layer assembly promotes ESIs and epithelial tissue stratification

The tissue engineered skin, cornea, mucosa, and urinary tissue models described here used either collagen, fibrin, glycosaminoglycans, ECM-coated electrospun fibers, or decellularized matrices to promote attachment of epithelial and stromal cells in culture. Matrices rich in collagen I can support the attachment and stratification of epithelial-fibroblast co-cultures (Table 1), and collagen III may prevent epithelial invasion into the stromal layer (Merne and Syrjanen, 2003). Epithelial culture on BM mimics (*e.g.* Matrigel) may prevent their differentiation in co-culture (Merne and Syrjanen, 2003) and has been used to promote epithelial morphogenesis to complex tissue types including hair follicle,

tooth, and glands as discussed in the next section. Though the ECM composition of the engineered tissue must promote initial epithelial attachment, literature supports the ability of ECs to secrete their own BM proteins in culture only in the presence of stromal cells (Hahn et al., 1987; Smola et al., 1998), which is supported by the studies discussed here. BM formation and epithelial stratification may also require seeding heterogeneity of stromal and epithelial cell types, wherein epithelial cells and stromal cells are seeded at different times or in distinct locations within an engineered construct. Seeding epithelial and stromal cells together on a provisional matrix resulted in a stratified epithelium in only one out of three studies discussed herein. Finally, while most of the studies described in this section cultured the epithelial cells at an air-liquid interface, it was not required to form a stratified epithelium (Table 1), though air-liquid interfaces can promote the phenotype of airway epithelial cells (Pezzulo et al., 2011) and the differentiation of epidermal keratinocytes (Fuchs, 1990). The studies described in this section highlight similarities between the development of engineered skin, cornea, mucosa, and urinary tissues, wherein the major differences were in the origin of the starting primary EC and stromal cell types (Table 1). The strategies employed in this section to generate stratified epithelial tissues could be generalized to instruct the design of simple embryonic epithelial tissues for developmental toxicology applications, as discussed in Future Directions.

3. Bioengineering approaches to promote epithelial morphogenesis and formation of complex 3D epithelial tissues

Complex tissues such as hair follicle, tooth, pulmonary, and glandular tissue require distinct approaches to promote ESIs and morphogenesis to generate functional tissue (Table 2). Novel epithelial, stromal, and stem cell types can exhibit biologically-driven self-assembly into complex structures without the use of provisional matrices given the appropriate microenvironment and culture conditions. Alternatively, 3D co-encapsulation within an instructive provisional matrix followed by *in vitro* culture or organ germ culture can promote 3D morphogenesis and differentiation into functional tissues. The methods discussed here may translate to more complex screening tools for toxicity assessment applications.

3.1. Biologically-drive assembly of engineered tissues

3.1.1. Hair follicle—Hair follicle engineering remains a critical obstacle in skin tissue engineering. The hair follicle consists of a dermal papilla (ECM, microvasculature), hair matrix with embedded ECs and melanocytes, a root sheath with cuboid ECs and the cuticle, a bulge region with stem cells, sebaceous and apocrine sweat glands, and a network of capillaries, muscles, and sensory receptors (Fig. 1D) (Paus and Cotsarelis, 1999). ESIs between ECs and stromal cell types (melanocytes, dermal papilla cells, bulge cells, myocytes) can regulate the function of the hair follicle (Paus and Cotsarelis, 1999) and serve as putative targets for hair follicle engineering approaches. For example, intracutaneous co-injection of dissociated mouse epidermal cells and mouse dermal stromal cell aggregates at approximately equal cell numbers yielded cycling hair follicles with stem cells, stromal cells, and immature and keratinized ECs, whereas dermal stromal cell or epidermal cell injections alone yielded fibrosis or cysts, respectively (Zheng et al., 2005). Alternatively, ectodermal precursor cells (CK14/18+, TP63+), derived from human induced pluripotent

stem cells (hiPSCs), exhibited Wnt/SHH crosstalk with dermal papilla cells (DPCs) *in vitro* and formed primitive hair follicle-containing cysts upon subcutaneous injection (Veraitch et al., 2013).

Hair follicle formation depends on direct coordination between stromal cells and ECs in tissue-like 3D contexts. Yen et al. demonstrated that mouse foot pad keratinocytes and mouse primary DPCs self-assembled into stratified microtissues *in vitro* (CK+ keratinocytes on periphery, vimentin+ DPCs on interior), consistent with native hair follicles, when co-cultured on poly (ethylene-co-vinyl alcohol) surfaces (Fig. 1C). This suggests that DPC-secreted ECM can sufficiently drive stratification and phenotypic specification of hair follicles *in vitro*. Upon intracutaneous injection to mouse hypodermis, only injected microtissues formed primitive hair follicles while co-injected suspensions of keratinocytes/DPCs failed to produce hair follicle-like structures (Yen et al., 2010). This observation disagrees with literature showing that injected dermal cells together with stem cell-derived ectodermal cells, HEKs, or epidermal cells robustly formed hair follicle-like structures *in vivo* (Veraitch et al., 2013; Zheng et al., 2005) and motivates future studies to clarify the nature of ESIs required for hair follicle formation. The importance of ESIs for hair follicle formation is exemplified by a study wherein co-encapsulation of outer root sheath keratinocytes (ORSKs) and DPCs was required to elicit CK6/14+ proliferative hair follicle-like organoids *in vitro* when co-cultured with HDFs. DPCs and ORSKs co-encapsulated in collagen-Matrigel beads exhibited ORSK clustering, epithelial (CK6+) and stromal (versican+) phenotype, and responsiveness to hair growth inhibitors (*e.g.* tretinoin) and hair growth stimulators (*e.g.* hepatocyte growth factor) (Havlickova et al., 2009).

3.1.2. Kidney—Kidney organoids derived from pluripotent stem cell-derived metanephric mesenchyme provide another example of the utility of biologically-driven assembly to generate complex epithelial tissue morphologies *in vitro*. mESC aggregates that were first differentiated to metanephric mesenchyme (Osr1+, Wt1+, Pax2+, Sall1+, Six2+) exhibited renal tubulogenesis, vascularized glomerulus formation, and podocyte clustering after implantation with embryonic spinal cords to the subrenal capsule *in vivo*. Similarly, hiPSC-derived metanephric mesenchyme aggregates exhibited renal tubulogenesis and glomerulus formation upon co-culture with mouse embryonic spinal cords *in vitro* (Taguchi et al., 2014). Takasato et al. developed a distinct approach wherein 2D cultures of hESCs were differentiated to metanephric mesenchyme (Wt1+, Six2+, and Pax2+). Pellet culture of metanephric mesenchymal cells for 4 days in serum-containing medium promoted structures consistent with ureteric epithelium and renal tubulogenesis (Takasato et al., 2014). A modified differentiation protocol and 3D pellet culture of hESC- or hiPSC-derived metanephric mesenchyme (Table 3) resulted in renal tubulogenesis (microvilli on collecting duct structures, brush border and tight junctions on proximal tubule structures), glomeruli formation, podocyte clustering, tubular endothelial networks surrounded by perivascular cells, and renal interstitial cells (Fig. 2B). Renal organoids exhibited acute apoptosis in the presence of a nephrotoxicant, cisplatin, which indicates an application of engineered kidney organoids for renal toxicology applications (Takasato et al., 2015). This study suggests that metanephric mesenchyme pellet culture in serum-containing or serum replacement conditions can sufficiently promote vascularized renal organoid formation *in vitro* that may

obviate the need for organ germ or spinal cord co-culture. Engineered renal organoids could be suitable alternatives to animal studies for testing renal toxicity, as currently there are no OECD validated *in vitro* renal toxicity models (Ward, 2016).

3.2. Three-dimensional encapsulation

3.2.1. Lung—Pulmonary alveoli consist of branched epithelial structures that can only be adequately modeled using 3D culture. Two independent research groups engineered lung alveolus-like tissues *in vitro* using 3D culture of fetal pulmonary cells (FPCs). A 3D culture model of FPCs (mixed isolectin+ cells, alveolar type II cells (SpC+), and CK+ cells) encapsulated in Matrigel elicited alveolar ring-like structures (no growth factors) or branched structures (with exogenous FGF-2, -7, and -10) lined with CK+ and SpC+ ECs similar to fetal lung tissue. Importantly, FGFR2 expression was only found in 3D cultures of FPCs relative to 2D Matrigel culture (Mondrinos et al., 2006), which suggests that 3D culture promoted FGF-dependent morphogenesis of FPCs consistent with FGF-dependent epithelial branching morphogenesis (Varner and Nelson, 2014). A distinct 3D culture model of FPCs (mixed CK+, SpC+ cells, and vimentin+ cells), suspended in alginate beads and encapsulated within collagen I, formed sheets of branched alveolus-like structures that exhibited lamellar body formation (indicative of secretory granules) and higher SpC expression than culture without alginate beads (Zhang et al., 2011b). Future work will be necessary to incorporate further components of alveolar physiology including vascular networks and alveolar macrophages. Nonetheless, engineered embryonic pulmonary tissues constitute a valuable *in vitro* model system for assessing developmental toxicity that is specific to lung development (Miller and Marty, 2010).

3.2.2. Mammary gland—Endocrine and exocrine glands form through the invagination of ECs to tubular (simple lumen) or alveolar (complex branched lumen) structures (Fig. 1F) (Conrady et al., 2016; Hassiotou and Geddes, 2013; Holmberg and Hoffman, 2014), and 3D encapsulation of glandular epithelial and stromal cells provides a microenvironment to drive glandular morphogenesis. For example, Wang et al. developed a co-culture system of MCF10A (human fibrocystic mammary epithelial cell line) and human ADSCs encapsulated within an ECM containing silk fibroin, collagen I, and Matrigel. The presence of ADSCs reduced MCF10A proliferation and stimulated casein expression and the formation of polarized acinar and ductal morphologies, likely through hepatocyte growth factor (HGF) signaling (Fig. 1E) (Wang et al., 2009) consistent with HGF-dependent epithelial morphogenesis (Bowes III et al., 1999; Johnson et al., 1993). Several studies support the ability of MCF10A to form polarized acinar morphologies in 3D culture (Debnath et al., 2003; Wang et al., 2013) that is context-dependent (Plachot et al., 2009), but caution should be exercised with their use in modeling mammary morphogenesis as MCF10A represent a cancerous phenotype (Keller et al., 2010; Qu et al., 2015). Murine amniotic fluid-derived stem cells constitute an alternative stromal cell source for promoting ESIs and mammary morphogenesis, as they directly associate with mammary acini *in vitro* and can reconstitute the stroma of newly formed mammary ducts in mice (Klemmt et al., 2010).

3.2.3. Tooth—Human tooth development is a complex 3D process involving morphogenesis of dental ECs, which are separated from ectomesenchymal cells (of neural

crest cell origin) by a BM. Dental ECs form buds that become surrounded by ectomesenchymal cell aggregates that constitute the dental papilla. ESIs promote epithelial differentiation and stratification to enamel-secreting ameloblasts and dental papilla differentiation to predentin-secreting odontoblasts (Tucker and Sharpe, 2004) and further promote tooth crown formation and maturation (Fig. 1H) (Gritli-Linde, 2002). Tooth engineering requires cell contact-mediated ESIs, as organotypic cultures comprising a dental EC monolayer seeded on encapsulated dental pulp stromal cells exhibited higher alkaline phosphatase activity in culture than either encapsulated cell type alone or both cell types segregated in trans-well culture. Only heterotypic organotypic cultures exhibited tooth bud formation upon implantation *in vivo*, characterized by ameloblast differentiation (amelogenin+), dentin production, and odontoblast differentiation (bone sialoprotein+) (Honda et al., 2007). Finally, tooth-like organoids of collagen-embedded human dental pulp stromal cells juxtaposed to Matrigel-embedded porcine dental ECs exhibited epithelial rosette formation, differentiation to ameloblasts and odontoblasts, and BM formation *in vitro* and exhibited more abundant mineralization upon subcutaneous implantation than constructs containing dental epithelial or dental pulp stromal cells alone (Zhang et al., 2010).

3.3. Organ germ method

Tsuji and colleagues developed a novel bioengineering approach to recapitulate complex 3D tissue types by promoting ESIs. The organ germ method (Fig. 1G) consists of isolating epithelial and mesenchymal cells from embryonic tissues, encapsulating epithelial and mesenchymal cells in a provisional collagen matrix, and implanting organ germs in sub-renal capsules in mice. Implanted organ germs can be explanted and cultured *in vitro* or implanted *in situ* to examine engineered tissue function. Such an approach was used to demonstrate hair follicle formation *in vitro*, as explanted organ germs composed of mouse embryonic dermal ECs and mouse embryonic dermal mesenchymal cells exhibited hair-like epithelial and mesenchymal expression patterns and formed innervated cycling hair follicles upon *in situ* engraftment to mice (Toyoshima et al., 2012). Similar organ germs contained dermal papilla and inner and outer root sheaths *in vitro* and, upon *in situ* implantation, produced innervated cycling hair follicles (with bulge, bulb, and sebaceous gland) (Asakawa et al., 2012). Although the organ germ method has limited throughput and thus limited relevance to toxicology, it provides evidence of the critical need for ESIs in the development of complex epithelial tissue models.

3.3.1. Tooth—In their initial report on the organ germ method, Tsuji and coworkers demonstrated that organ germs of mouse embryonic dental ECs and mouse embryonic dental pulp mesenchymal cells promoted an architecture and dentin deposition reminiscent of tooth bells. The origin of the cell types used in the organ germ determined the ultimate identity of the engineered tooth, and tooth germs exhibited vascular and neuronal ingrowth *in situ* (Nakao et al., 2007). Embryonic molar ECs have been shown to differentiate to matrix-producing ameloblasts upon organ germ culture with embryonic molar mesenchymal cells (Takahashi et al., 2010). Molar organ germs formed occluding tooth structures upon implantation to an empty tooth socket in mice, and organ germs exhibited similar mechanical properties to teeth and exhibited neuronal response (gelatinin+, c-Fos+) to forceful stimulus (Ikeda et al., 2009). A modified organ germ method using CellMatrix

supported FGF/SHH-mediated crosstalk between embryonic dental ECs and dental pulp cells that elicited tooth formation wherein the cusp number of engineered teeth positively correlated with the size of the epithelial-stromal tissue interface (Ishida et al., 2011). Tooth organ germs with a 1:1 ratio of rat apical bud cells and dental pulp stromal cells exhibited the highest alkaline phosphatase activity and elicited tooth-like structures containing dentin, differentiated ameloblasts and odontoblasts, and enamel, whereas germs with a higher proportion of bud cells or pulp cells lacked odontoblasts or ameloblasts, respectively (Yu et al., 2008). Finally, hESC-derived ECs (CK14+) expressed amelogenin and formed primordial tooth-like structures when co-encapsulated with fetal dental pulp stromal cells in culture and after implantation to sub-renal capsules (Zheng et al., 2013).

3.3.2. Glands—The organ germ method provides an appropriate 3D context to promote glandular morphogenesis (Fig. 1G) as previously discussed in the context of 3D encapsulation. Organ germs composed of embryonic mouse glandular epithelial and mesenchymal cells self-assembled into acinar ducts and upon *in situ* engraftment exhibited a phenotype (E-cadherin+, aquaporin 5+, calponin+, lactoferrin+) and inducible tear production consistent with innervated glandular epithelium. Importantly, engineered lacrimal glands restored corneal epithelium thickness in mice with deficient lacrimal gland, which suggests a functional recovery of tear formation (Hirayama et al., 2013, 2015). Tsuji and colleagues used a similar organ germ method with embryonic mouse submandibular gland (SMG) epithelial and mesenchymal cells and demonstrated epithelial phenotype (aquaporin-5+, calponin+, and α -smooth muscle actin+) and inducible saliva production after *in situ* implantation consistent with innervated glandular epithelium (Ogawa et al., 2013). Matrigel and *in vitro* culture may provide an alternative to organ germ culture, as pellet culture of mouse embryonic SMG epithelial and mesenchymal cells on Matrigel elicited ductal morphogenesis and epithelial phenotype (E-cadherin+, β -catenin+). Co-culture promoted aquaporin-5 and SMG protein C expression relative to pellet culture of ECs alone, which suggests that SMG mesenchymal cells promoted glandular function of cultured SMG-ECs (Wei et al., 2007). Tissues engineered with the organ germ method demonstrate remarkable complexity that is able to reconstitute both the structure and function of particular target tissues. However, ongoing research is needed to unravel the microenvironment required for complex 3D epithelial tissue morphogenesis, as subrenal capsule engraftment is a low-throughput and invasive method to generate organoids for screening applications and may present hurdles to clinical translation.

4. Future directions: engineered ESIs to mimic developmental biology

The epithelial tissue engineering approaches described here each constitute state-of-the-art *in vitro* tools amenable to toxicity assessment, although each must undergo throughput enhancement and validation to achieve approval as an alternative to animal testing. Nevertheless, the lack of approved *in vitro* methods for testing renal toxicity, pulmonary toxicity, and mucosal barrier toxicity motivates approaches to harness ESIs and increase the complexity and physiological and developmental relevance of toxicity testing methods. ESI-promoting strategies also may broadly be generalized to instruct the design of assays of developmental processes that are similarly dependent on ESIs and currently can only be

studied using animal models. Tissue morphogenesis and fusion events during development are highly dependent on ESIs and EC polarity, and organotypic models that mimic the architecture of the target embryonic tissue are necessary to study morphogenesis *in vitro*. For example, fusion of the endocardial cushions during heart development is a critical step in the formation of the separated chambers of the heart and the separation of the aortic and pulmonary arches and is dependent on endocardial-epicardial-myocardial cell crosstalk (Ray and Niswander, 2012). Urethral tube fusion during genital tubercle formation involves crosstalk between cloacal mesoderm, hindgut endoderm, and surface ectoderm (Blaschko et al., 2012). Similarly, morphogenetic fusion of the palate during early human development is dependent on signaling crosstalk, mediated by FGF-10/SHH (Rice et al., 2004), Wnt/ β -catenin/TGF β 3 (He et al., 2011), and EGF (Tyler and Pratt, 1980), between the embryonic palatal mesenchyme and epithelium (Abbott, 2010). Palatal fusion in particular has largely been studied *in vivo* with mice or rats or *ex vivo* using explanted mouse, rat, or human embryonic palatal tissue in a dish. Animal models are inherent low throughput, and further, mouse and human palates *ex vivo* exhibit differential sensitivity to and transcriptional response to a model cleft palate teratogen, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Abbott, 2002), which together highlight the need to develop human organotypic models of embryonic fusion events *in vitro* for developmental toxicity assessment. In this section, we comment on the methods used to generate epithelial tissues *in vitro* and their potential utility in modeling embryonic tissues *in vitro*.

The methods of layer-by-layer assembly, biologically-driven self-assembly, 3D encapsulation, and organ germ can instruct the design of embryonic epithelial tissues *in vitro*. Layer-by-layer assembly is useful for generating a keratinized epithelium or stratified mucosal, corneal, or urinary tissues, but these models are inherently planar and elicit a stratified, mature epithelium that may not adequately represent ectodermal embryonic tissues. Layer-by-layer assembly and self-assembly methods may be required to mimic primitive ectodermal tissue, which often consists of a stroma surrounded by a thin layer of immature epithelium. Spheroid culture is a well-established technique for self-assembly of adhesion-dependent stromal cell types (Baraniak and McDevitt, 2012; Fennema et al., 2013), and spheroids can be generated *via* forced aggregation in non-adherent round-bottom plates, rotary culture in non-adherent flasks, orbital shaker culture in non-adherent plates, or hanging drop culture (Fennema et al., 2013). Previous evidence of engineered skin, cornea, and mucosal tissue demonstrate that ECs can attach to and deposit BM at the interface with a stromal compartment composed of various provisional ECMs (Table 1), thus we posit that stromal cell spheroids with an outer surface presenting ECM motifs (*e.g.* collagen, fibrin, glycosaminoglycans) would promote epithelial attachment, BM deposition, and barrier formation by cultured ECs. However, one important caveat of this approach is that the identity and multipotency of the stromal cell population can cause disorganized ECM deposition (Merne and Syrjanen, 2003). This observation suggests that the stromal compartment should be selected or engineered to secrete an ECM (*e.g.* collagen I) that is known to support epithelial attachment, polarization, and BM deposition. Alternatively, the epithelial-stromal interface can be engineered to exploit known intermolecular interactions such as that between avidin and biotin, as established to promote the attachment of streptavidin-coated NIH3T3 fibroblasts to the surface of aggregated biotinylated myoblast

cells in a proof-of-concept model (De Bank et al., 2007). Spheroids of stromal cells could thus be generated and the surface stroma engineered to promote epithelial cell adhesion through coating with provisional ECM or through biotinylation of the surface-exposed stromal cells. ECs representing a naïve or immature ectodermal phenotype (*e.g.* primitive non-neural ectoderm derived through prolonged treatment of PSCs with BMP4 (Kawasaki et al., 2000)) could be seeded on stromal spheroids and cultured over time to generate heterotypic spheroids that would theoretically mimic the architecture of a primitive embryonic ectodermal tissue.

Alternatively, cell aggregates can be differentiated to elicit self-assembly into an architecture that mimics embryonic ectodermal tissue. Biologically driven assembly of a heterogeneous population into an organized, tissue-like architecture *in vitro* has been demonstrated with engineered hair follicles, lung organoids, and renal organoids (Table 3). Renal organoid self-assembly in particular was driven by mesenchymal-epithelial transition, or MET, to form stromal, epithelial, and vascular structures consistent with renal tissue. A similar method could be used to generate stromal aggregates with an outermost layer of ectodermal cells *via* biologically driven assembly of aggregated stromal cells, which can undergo MET through BMP signaling (as demonstrated with renal fibroblasts) (Zeisberg et al., 2005) or FGF signaling (as demonstrated with metanephric mesenchymal cells) (Taguchi et al., 2014; Takasato et al., 2014, 2015). Epithelial-mesenchymal transition has also been proposed to regulate the formation of renal tissue (Zhang et al., 2011a) and corneal tissue (Gourdie et al., 2012) *in vitro*, but future studies will be needed to rule out the possibility of cell contamination in these primary cell cultures. Biologically-driven assembly of co-cultured stromal and epithelial cell types, such as that described between ORSKs and DPCs for hair follicle engineering (Yen et al., 2010), could also be employed with embryonic mesenchyme and ectodermal cells to drive the formation of an embryonic epithelial tissue without the need for a differentiation step.

Emerging bioengineering techniques can also be used to promote ESIs and ectodermal tissue architecture through encapsulation of the stromal compartment in a provisional ECM. Stromal cells can secrete a variety of ECM proteins that may or may not resemble the tissue ECM, thus a provisional ECM can be designed to specifically recapitulate the ECM of the target tissue. Encapsulated stromal cells within natural ECMs including collagen and fibrin can support epithelial attachment as previously described (Table 1), and synthetic ECMs present an alternative strategy to promote ESIs in a modular chemically-defined format. For example, hyaluronic acid (HA) is abundant in embryonic palate (Gritli-Linde, 2007), and HA hydrogels can be crosslinked and functionalized to provide a 3D provisional matrix capable of promoting stromal cell and epithelial cell attachment and remodeling *in vitro* (Nicodemus and Bryant, 2008). Alternatively, poly(ethylene glycol) hydrogels can be readily crosslinked and functionalized to generate a provisional matrix able to promote stromal and epithelial cell attachment (Pierschbacher et al., 1983) and remodeling (Nagase and Fields, 1996). Additional functionalities can be incorporated to PEG or HA hydrogels to study the influence of morphogen sequestering that would be hypothesized to promote morphogenesis through gradient generation (Belair et al., 2014) (*e.g.* FGF, HGF). Encapsulating stromal cells in natural or synthetic matrices in cell-dense hydrogel spheroids followed by

attachment of naïve EC cells to the outer spheroid surface may provide a chemically-defined method to generate stromal-epithelial co-cultures competent of fusion.

Blood vessels are a critical component of developing epithelial tissues. There is evidence of vascular network formation in engineered tissues upon implantation of tissue organoids and vessel ingrowth, upon co-culture with endothelial cells, or upon stromal cell differentiation to endothelial lineage. The latter evidence is substantiated by the mesodermal origin of vascular networks during embryonic vasculogenesis (Goldie et al., 2008) and tooth development in particular (Rothova et al., 2011). Literature also suggests that BMSCs can differentiate to an endothelial-like lineage upon culture in vascular endothelial growth factor (Janeczek Portalska et al., 2012; Oswald et al., 2004). Developmental processes require signaling crosstalk between endothelial cells and developing tissues and organs (Cleaver and Melton, 2003). Therefore, engineered vascular networks using the methods described here may be required to characterize response to teratogens within *in vitro* models of developmental processes.

5. Conclusion

Epithelial-stromal interactions drive the morphogenesis of ectodermal organs during development and regulate homeostasis in adult epithelial tissues. The importance of ESIs for tissue development and function highlights the need to incorporate ESIs into the next generation of toxicological screening assays. Bioengineering strategies to leverage ESIs using stem cells, epithelial cells, and stromal cells have enabled the generation of human *in vitro* models of mature epidermal tissues and glands, and these engineered tissues present an opportunity to replace animal testing in toxicology applications including skin sensitization and irritation, eye irritation, mucosal tissue irritation, renal toxicity, and lung developmental toxicity. Developmental processes are currently modeled almost exclusively using animal studies, animal tissue explants, or rudimentary 2D cultures that potentially lack human physiological relevance needed for screening applications. Emerging bioengineering strategies to culture cells in 3D and harness ESIs have enabled the development of complex epithelial tissue structures *in vitro* that can instruct the design of embryonic ectodermal tissues and enable for the first time the study of ectodermal tissue morphogenesis processes in a dish. The availability of biomimetic matrices, cell types from a variety of pluripotent and primary sources, and advanced cell culture techniques will enable toxicological screening assays to achieve the complexity and throughput needed to eventually replace animal models as the gold standard for toxicity assessment.

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Abbreviations

2D/3D	two-dimensional/three-dimensional
ADSC	adipose-derived stromal cell
BM	basement membrane
BMP	bone morphogenetic protein
CK	cytokeratin
DPC	dermal papilla cell
EC	epithelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
ESI	epithelial-stromal interaction
FGF	fibroblast growth factor
FPC	fetal pulmonary cell
HDF	human dermal fibroblast
HESC	human embryonic stem cell
HEK	human epidermal keratinocyte
HGF	hepatocyte growth factor
HIPSC	human induced pluripotent stem cell
HMVEC	human microvascular endothelial cell
HWJSC	human Wharton's jelly stromal cell
IGF-1	insulin-like growth factor-1
MESC	mouse embryonic stem cell
MMP	matrix metalloproteinase
MSC	multipotent stromal cell
ORSK	outer root sheath keratinocyte
SHH	sonic hedgehog
SMG	submandibular gland

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Fig. 1. Methods to Generate 3D Organotypic Culture Models (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) Schematic demonstration of organotypic culture of epithelial (green) and stromal (blue) cells derived from primary sources or from immortalized human cell lines. A: Recombination of epithelial and stromal cells using in a layer-by-layer assembly approach has been used to generate engineered skin, cornea, mucosa, or vesicals either in submerged or air-liquid interface culture. B: Schematic of primitive skin tissue comprising keratinocytes (white), epithelium (light green), melanocytes (pink), basement membrane (BM; purple), blood vessels (BV; dark blue, red), adipocytes (yellow), ECM, and fibroblasts (blue). C: Recombination and self-assembly of epithelial and stromal cells to hair follicle-like structure

after seeding on poly (ethylene-co-vinyl alcohol) (pEVAL) substrates. D: Schematic of primitive hair follicle, comprising hair shaft, inner root sheath (IRS; light green), outer root sheath (white), BM (purple), dermal papilla (DP; blue), BV (dark blue/red), sebaceous glands, and bulge region (stem cell niche). E: 3D encapsulation of epithelial and stromal cells has previously been used to generate mammary gland or pulmonary tissue through culture either in survival factors or morphogens (*e.g.* HGF, FGF). F: Schematic of primitive mammary gland comprising adipocytes (yellow), epithelial alveolus/acinus (light green), BM (purple), fibroblasts (blue), and BV (dark blue/red). G: The organ germ method encompasses 1. dissociation of embryonic tissues and segregation of epithelial and mesenchymal tissues, 2. encapsulation of epithelial and mesenchymal cells, 3. implantation of ‘organ germs’ to mouse sub-renal capsule, and 4. transplantation of engineered organ germs *in situ* to assess their function. The organ germ method has been used to generate salivary and lacrimal gland, tooth, hair follicle, and renal tissue. H: Schematic of primitive tooth crown at the late bell stage of development, showing the outer epithelial sheath (dark green), stellate reticulum (purple shading), ameloblasts (light green), enamel (white), dentin (pink shading), and a dental papilla (light blue shading) consisting of odontoblasts (dark blue), neural crest (NC) mesenchyme (light blue), and BV (blue/red).

A. Engineered Epidermal Tissue**B. Engineered Kidney Organoids** **Fig. 2. Leveraging Mesenchymal-Epithelial Transition in Bioengineered Tissues**

Schematic representation of the generation of stratified epidermal tissue (A) and kidney organoids (B) using pluripotent stem cells. A: Organotypic layer-by-layer assembly of encapsulated hESC-derived stromal cells (CD73+/CD105+/CD106+/CD44+/CD10+/CD13+) and monolayer epithelial-like (CD73-/CD105+/CD106-/CD44-/CD10-/CD13+) cells gives rise to a stratified epidermal-like tissue with CK12+ keratinized epithelial layer and CK18+ stromal layer separated by a collagen IV basement membrane B: Pellet culture of metanephric mesenchyme cells generates kidney organoids with renal tubular structures (ECAD-/LTL+ or ECAD+/LTL-), collecting duct structures (ECAD+/GATA3+),

glomerulus structures (WT1+), and stromal tissue comprising CD31+/KDR+/Sox17+ primitive vascular networks associated with PDGFRA+ stromal pericyte-like cells.

Table 1

Bioengineering Approaches to Generate Mature Epidermal Tissues *via* ESIs.

Tissue	EC Type (s)	Stromal Cell Type (s)	Extracellular Matrix (ECM)	Culture Method	Tissue Characteristics	Ref
Skin	HaCaT keratinocytes	HFF-1 fibroblasts	Collagen I	Bioprinted (ALI)	Stratified	(Lee et al., 2014)
	HEKs ^a	HDFs ^a	Decellularized dermis	Bi-layered (ALI)	Stratified, BM+	(Ojeh and Navsaria, 2014)
	HEKs ^a	HDFs ^a	Collagen-GAG-Chitosan	Bi-layered (ALI)	Stratified, BM+	(Black et al., 2005)
	HEKs ^a	HDFs ^a	Gelatin-C6S-HA	Bi-layered (ALI)	Stratified, p63+	(Wang et al., 2006)
	HEKs ^a	HDFs ^a	Fibrin	Bi-layered (ALI)	Stratified, BM+, engrafted	(Fiasza et al., 2007)
	HEKs ^a	HDFs ^a	BioReagent-coated Collagen	Bi-layered (ALI)	Stratified	(Kinsner et al., 2001)
	HEKs ^a	HDFs ^a	Collagen-coated PCL	Co-seeding	Cell attachment	(Palamaro et al., 2013)
	HEKs ^a	HDFs ^a	Decell. amniotic membrane	Bi-layered (ALI)	Stratified, BM+, engrafted	(Yang et al., 2009)
	HEKs ^a	HDFs ^a	Decell. amniotic membrane	Bi-layered	Stratified, BM+	(Sanluis-Verdes et al., 2015)
	HEKs ^a	HDFs ^a	Compressed Collagen I	Bi-layered	Stratified, BM+, engrafted	(Hartmann-Fritsch et al., 2013)
	HEKs ^a	HDFs ^a	Collagen I-GAG	Bi-layered	Stratified, BM+, engrafted	(Cooper et al., 1993)
	HEKs ^a	HDFs ^a	Collagen I	Bi-layered (ALI)	Stratified, engrafted	(Casasco et al., 2001; Mirastschijski et al., 2006)
	HWJSCs ^{a,b}	Fibroblasts ^a	Fibrin-agarose	Bi-layered (ALI)	Stratified, keratinized, engrafted	(Garzon et al., 2013)
Cornea	Amniotic ECs ^a	Amniotic stromal cells ^a	Collagen I	Bi-layered (ALI)	Stratified, keratinized, engrafted	(Li et al., 2012)
	HaCaT keratinocytes	HDFs ^a or BMSC ^a or HWJSC ^a	Collagen G	Bi-layered	Stratified, BM+	(Schneider et al., 2010)
	HEKs ^a	HDFs ^a , HMVECs ^a	Vitrogen, Collagen	Tri-layered (ALI)	Stratified, BM+, engrafted	(Martins-Green et al., 2004)
	HEKs ^a	HDFs ^a , ADSC ^a	Fibrin	Tri-layered (ALI)	Stratified, glycerol produced	(Kober et al., 2015)
	HEKs ^a	HDFs ^a or tonsil-derived stromal cells ^a , dermal melanocytes ^a	Collagen I	Bi-layered	Stratified, BM+, pigmented, engrafted	(Botcher-Haberzeth et al., 2014, 2013)
	Keratinocytes ^a	Fibroblasts ^a , melanocytes ^a	Collagen I	Bi-layered	Stratified, pigmented, engrafted	(Biedermann et al., 2015)
	Limbal ECs ^d	Limbal fibroblasts ^d	Electrospun PLGA	Co-seeded (ALI)	Cell attachment, outgrowth	(Ortega et al., 2014)
	Limbal ECs ^a	Limbal stromal cells ^a	Silk fibroin mat	Bi-layered	Stratified, semitransparent	(Bray et al., 2012)
	Comeal ECs ^a , keratinocytes ^a	HWJSCs ^a	Fibrin-agarose	Bi-layered (ALI)	Stratified, crystallin+	(Garzon et al., 2014)

Tissue	EC Type (s)	Stromal Cell Type (s)	Extracellular Matrix (ECM)	Culture Method	Tissue Characteristics	Ref
Mucosa	Corneal ECs ^a or ^d	Corneal fibroblasts ^a	Fibroblast-secreted ECM	Bi-layered	Cell attachment, BM+	(Lake et al., 2013)
	Limbic ECs ^a	Limbic fibroblasts ^a	Compressed Collagen I	Bi-layered (ALI)	Stratified, BM+	(Levis et al., 2010; Massie et al., 2015, 2014)
	Immortalized corneal ECs ^a	Corneal fibroblasts ^a	Collagen I	Tri-layered (ALI)	Stratified, drug-permeable	(Reichl et al., 2004)
	Bronchial ECs ^a	Bronchial fibroblasts ^a	Bovine Collagen I	Bi-layered (ALI)	Stratified, BM+, ciliated	(Chakir et al., 2001; Paquette et al., 2004)
	Bronchial ECs ^a	HDFs ^a	BioVaSC (SIS-)	Bi-layered (ALI)	Stratified, keratinized, ciliated	(Steinke et al., 2014)
	Bronchial ECs ^a	BMSCs ^a	Transwell membrane	Bi-layered (ALI)	Stratified, ciliated, mucin +	(Le Visage et al., 2004)
	Bronchial ECs ^a	Lung fibroblasts ^a , lung HMVEC ^a	Collagen I, III, or IV coatings	Tri-layered (flow)	Ciliated, mucin+, barrier function	(Sellgren et al., 2014)
	Oral keratinocytes ^a	Oral fibroblasts ^a	Cadaver decellularized	Co-seeded (ALI)	Stratified	(Chai et al., 2010)
	Oral keratinocytes ^a	Oral or buccal fibroblasts ^a	Collagen	Bi-layered (ALI)	Stratified, keratinized	(Gaballah et al., 2008)
	Oral keratinocytes ^a	Oral fibroblasts ^a	Fibrin-agarose	Bi-layered (ALI)	Stratified, BM+, keratinized	(Vinuela-Prieto et al., 2014)
Urethra	Gingival keratinocytes ^a	Gingival fibroblasts ^a	Electrospun Gelatin-PCL	Bi-layered	Stratified, engrafted	(Schulz et al., 2014)
Bladder	Oral keratinocytes ^e	Oral fibroblasts ^e	Electrospun silk-fibroin	Bi-layered (ALI)	Stratified, engrafted	(Xie et al., 2014)
	Uroepithelial cells ^c	Dermal fibroblasts ^c , Uroendothelial cells ^c	Fibroblast-secreted ECM	Tri-layered (ALI)	Stratified, BM+, ciliated, barrier	(Bouhout et al., 2010)

Abbreviations: ALI = Air-Liquid Interface; ADSC = Adipose-Derived Stem Cell; BM+ = BM positive; BMSC = Bone Marrow Stromal Cell; C6S = Chondroitin-6 Sulfate; EC = Epithelial Cell; ECM = Extracellular Matrix; GAG = Glycosaminoglycan; HA = Hyaluronic Acid; HDF = Human Dermal Fibroblast; HEK = Human Epidermal Keratinocyte; HMVEC = Human Microvascular Endothelial Cell; HWJSC = Human Wharton's Jelly Stromal Cell; PCL = Polycaprolactone; PLGA = Polylactide-co-glycolide.

^aHuman primary.

^bDifferentiated to epithelial lineage.

^cPorcine primary.

^dRabbit primary.

^eCanine primary.

Table 2
 Bioengineering Approaches to Promote Complex Epidermal Tissue Formation and Gland Formation *via* ESIs.

Target Tissue	EC Type (s)	Stromal Cell Type (s)	Extracellular Matrix (ECM)	Culture Method	Tissue Characteristics	Ref.
Hair Follicle	Epidermal cells ^c	Inductive dermal cells ^c	–	Co-injection <i>in vivo</i>	Engrafted, cycling hair follicles	(Zheng et al., 2005)
	HEKs ^a , hiPSC-EPCs ^b	Dermal papilla fibroblasts ^a	–	2D co-culture <i>in vitro</i> , co-injected <i>in vivo</i>	Engrafted, cycling hair follicles	(Veratch et al., 2013)
	Keratinocytes ^d	Dermal papilla cells ^d	–	2D self-assembly on pEVAL	Stratified <i>in vitro</i> , engrafted, hair+	(Yen et al., 2010)
	ORSK ^a	Dermal papilla fibroblasts ^a	Collagen I, Matrigel	3D encapsulation, self-assembly	Stratified <i>in vitro</i> , inductive prolif.	(Havlickova et al., 2009)
	ORSK ^a	HDFs ^a , dermal papilla fibroblasts ^a	Collagen I, Matrigel	Tri-layered assembly	Stratified <i>in vitro</i> , versican+/CK+	(Havlickova et al., 2004)
	EDEC ^c	EDMC ^c	Collagen	3D organ germ method	Stratified, engrafted, cycling HFfs	(Asakawa et al., 2012)
Tooth	EDEC ^c or adult bulge ECs ^c	EDMC or adult vibrissae dermal papilla ^c	Collagen	3D organ germ method	Stratified, engrafted, cycling HFfs, neural innervation <i>in vivo</i>	(Toyoshima et al., 2012)
	Dental ECs ^e	Dental pulp stromal cells ^e	Porcine Collagen I, III	Bi-layered assembly	Stratified <i>in vitro</i> , engrafted, dentin +/- enamel+	(Honda et al., 2007)
	Dental ECs ^e	Dental pulp stromal cells ^a	Collagen, Matrigel	Bi-layered assembly	Stratified, BM+, engrafted, calcified	(Zhang et al., 2010)
	Embryonic dental ECs ^c	Embryonic dental pulp mesenchymal cells ^c	Collagen	3D organ germ method	Stratified, engrafted, structural tooth formation (vascularized, innervated)	(Ikeda et al., 2009; Nakao et al., 2007; Takahashi et al., 2010)
	Embryonic dental ECs ^c	Embryonic dental pulp mesenchymal cells ^c	CellMatrix type I-A	3D organ germ method	Stratified, controlled cusp formation	(Ishida et al., 2011)
	Apical bud cells ^d	Dental pulp stromal cells ^d	–	2D or 3D co-culture (subrenal graft)	Stratified, engrafted, dentin+/enamel+	(Yu et al., 2008)
Mammary Gland	MCF10A	ADSC ^a	Silk fibroin (+Collagen/Matrigel)	3D encapsulation	Alveolar/branched morphogenesis, polarized, BM+, differentiated	(Wang et al., 2009)
Lacrimal Gland	Mammary ECs ^c	Amniotic fluid stem cells ^c	Matrigel	2D culture (Matrigel), co-injected <i>in vivo</i>	Ductal morphogenesis <i>in vitro</i>	(Klemmt et al., 2010)
	Embryonic glandular ECs ^c	Embryonic glandular mesenchymal cells ^c	Collagen	3D organ germ method	Ductal morphogenesis, engrafted, innervated, inducible tear production	(Hirayama et al., 2013, 2015)

Target Tissue	EC Type (s)	Stromal Cell Type (s)	Extracellular Matrix (ECM)	Culture Method	Tissue Characteristics	Ref.
Salivary Gland	Embryonic SMG ECs ^c	Embryonic SMG mesenchymal cells ^c	Collagen	3D organ germ method	Ductal morphogenesis, engrafted innervated, inducible saliva production	(Ogawa et al., 2013)
	Embryonic SMG ECs ^c	Embryonic SMG mesenchymal cells ^c	-	Cell pellet culture	Branched structures, SpC+, AQP5+	(Wei et al., 2007)

Abbreviations: ADSC = Adipose-Derived Stromal Cell; BM = BM; CK = Cytokeratin; EC = Epithelial Cell; EDEC = Embryonic Dermal EC; EDMC = Embryonic Dermal Mesenchymal Cell; HDF = Human Dermal Fibroblast; HEK = Human Epidermal Keratinocyte; HF = Hair Follicle; hiPSC-EPCs = Human Induced Pluripotent Stem Cell-derived Ectodermal Precursor Cells; MCF10A = Human Fibrocystic Mammary Epithelial Cell Line; ORSK = Oral Root Sheath Keratinocyte; pEVAL = Poly (ethylene-co-vinyl alcohol); SMG = Submandibular Gland.

^aHuman primary.
^bDifferentiated to epithelial lineage.
^cMouse primary.
^dRat primary.
^ePorcine primary.

Table 3
Bioengineering Approaches to Generate Epithelial Tissues using Pluripotent Stem Cells or Fetal Cells.

Target Tissue	Stem Cell	Stromal Cell	Differentiation Protocol	ECM	Culture Method	Ref.
Skin	hESC	–	Keratinocyte (EGF, BMP)	Collagen I	Bi-layered	(Hewitt et al., 2009)
Mucosa	hESC	VFF ^a	Epithelial (BMP, RA, Noggin)	Collagen I	Bi-layered	(Leydon et al., 2013)
Tooth	hESC	FDPSC ^a	Epithelial (RA, BMP4)	Matrigel	3D co-culture	(Zheng et al., 2013)
Alveolus	FPC ^b	–	Epithelial (FGF)	Matrigel, Col I, PDL	3D encapsulation	(Mondrinos et al., 2006)
	FPC ^b	–	–	Matrigel, Collagen I	3D encapsulation	(Zhang et al., 2011b)
Kidney	1. hESC	–	1. BMP4/CHIR (2d), FGF9 + Hep (4d, 6d), No GFs (6d)	–	1. 2D diff. → pellet	1. (Takasato et al., 2014)
	2. hESC or hiPSC	–	2. CHIR (4d), FGF9 + Hep (3d), pellet formation, CHIR pulse, FGF9 + Hep (5d), No GFs (6d)	–	2. 2D diff. → pellet	2. (Takasato et al., 2015)
	1. mESC	–	1. Act (1d), BMP4/CHIR (4.5d), Act/BMP4/CHIR/RA/ROCKi (1d), CHIR/FGF9/ROCKi (2d)	–	1. EB differentiation	(Taguchi et al., 2014)
	2. hiPSC	–	2. BMP4/ROCKi (1d), Act/FGF2 (2d), BMP4/CHIR (6d), Act/BMP4/CHIR/RA/ROCKi (2d), CHIR/FGF9/ROCKi (3d)	–	2. EB differentiation	

Abbreviations: Act = Activin A; BMP = Bone Morphogenetic Protein; CHIR = CHIR99021 (Wnt agonist); EB = Embryoid Body; EGF = Epidermal Growth Factor; FDPSC = Fetal Dental Pulp Stromal Cells; FGF = Fibroblast Growth Factor; FPC = Fetal Pulmonary Cell; Hep = Heparin; hESC = Human Embryonic Stem Cell; hiPSC = Human Induced Pluripotent Stem Cell; mESC = Mouse Embryonic Stem Cell; PDL = Poly(D-Lysine); RA = Retinoic Acid; ROCKi = Rho-associated Coiled-coil Containing Protein Kinase Inhibitor; VFF = Vocal Fold Fibroblast.

^aHuman primary.

^bMouse primary.