Technical Summary on the EpiX™ Technology – A Unique Solution that Unleashes the Potential of Tissue-Resident Epithelial Stem and Progenitor Cells for Regenerative Cell Therapy

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Background

The epithelial stem and progenitor cells residing in diverse human epithelia have lifetime self-renewal capability *in vivo* to ensure tissue homeostasis and wound repair processes, however they can be expanded for only a few passages *in vitro* due to a multitude of disparate stress factors (Figure 1). This greatly limits their utility in drug discovery and has stalled advances in regenerative cell therapy exploiting their potential. Pluripotent stem cells, especially induced pluripotent stem cells (iPSCs), have been the subject of intense research in the hope that they will offer representative models and may one day provide solutions for cell replacement therapy. However, this approach faces multiple challenges including donor variability, high cost, laborious procedures, acquired oncogenic mutations, and imperfect differentiation efficiency towards mature cell types[1,2].

Feeder cells can assist the continuous *in vitro* propagation of keratinocytes from the skin and the cornea, and this method has garnered interest due to its use in developing curative therapy for burn patients[3] (e.g., EpiCel©) and in expanding patient-derived keratinocytes to remedy inherited skin diseases (e.g., epidermolysis bullosa)[4,5]. However, the use of feeder cells presents challenges to meet regulatory expectations for manufacturing cell therapy products[6]. Recently, sophisticated feeder-free 3D organoid culture methods have been developed to expand epithelial stem cells from colon, liver, pancreas, and stomach[7–9]. Still, these methods are limited to small-scale *in vitro* research due to the prohibitive cost to produce critical reagents, scale-up challenges, and its dependency on murine-derived extracellular matrix protein mixtures.

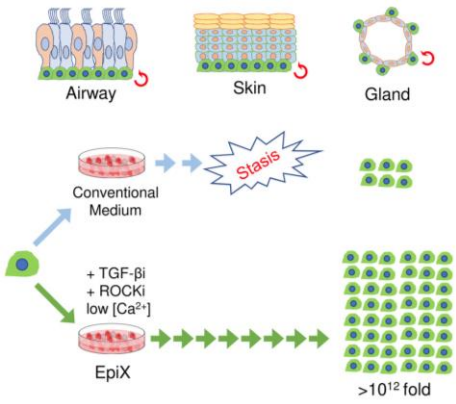

Figure 1. By pharmacologically modulating the PAK1-ROCK-Myosin II and TGF-β signaling, the EpiX medium supports over one trillion-fold ex vivo expansion of epithelial stem and progenitor cells from various human epithelia in the absence of feeder cells.

To address the aforementioned issues, including cost, reproducibility, scale-up compatibility and regulatory concerns[6], we commenced to develop cell culture media formulations that support long-

term expansion of epithelial stem and progenitor cells in the absence of any material derived from an animal source. As cell behaviors are influenced by diverse external signals, we screened a knowledge-based collection of small molecules modulating diverse biological pathways governing stem cell self-renewal and differentiation processes, and successfully identified several families of small molecules that sustain the continuous *in vitro* proliferation of epithelial stem and progenitor cells without the use of feeder cells or animal serum (Figure 1)[10]. Strategically evaluating the permutations of combinatory use of these small molecules allowed us to develop a patented ex vivo epithelial cell expansion technology without animal serum and feeder cells, and ultimately accomplish the development of chemically-defined and animal-origin-free epithelial cell expansion medium formulations to unleash the potential of tissue-resident epithelial stem and progenitor cells for regenerative cell therapy (EpiX™; US patents 9,790,471, 9,963,680, 10,066,201, 10,100,285 and 10,119,121).

EpiX Technology

As summarized in Figure 2 and detailed in our recent publication in *Cell Reports*[10], the EpiX technology enables the expansion of epithelial cells from the skin, airway, mammary, prostate and other epithelial tissues to more than one trillion fold, far beyond the meager 10,000-fold expansion supported by conventional commercial media. Importantly, whole genome sequencing study revealed that the EpiX-expanded epithelial cells maintain remarkable genome integrity. The cells retain normal diploidy and have an extremely low natural genetic drift comparable to what is observed in stem cells in vivo[11]. Importantly, no genetic change occurs in oncogenic or tumor suppressor genes. Keratinocytes that have been expanded with the EpiX medium for more than 10^{15}-fold did not develop tumors in immune-deficient mice, confirming that EpiX-expanded epithelial cells are not tumorigenic. Of note, it has been reported that human ESC and iPSC lines acquire genomic structural variations including TP53 mutations with successive passages even under cGMP-guided expansion procedures and raise the concern for its safety[1].

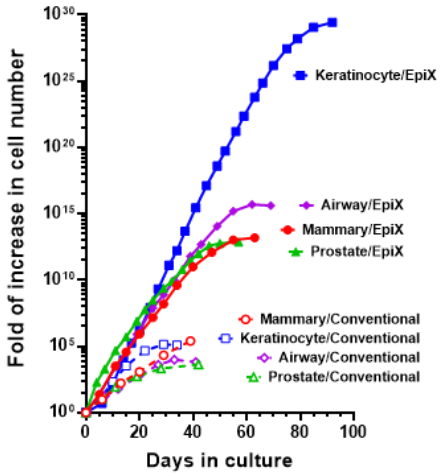

Figure 2. EpiX™ technology supports ex vivo expansion of epithelial cells from various tissue for over one trillion-fold more than conventional methods.

Epithelial cells expanded with the EpiX medium faithfully retain their lineage-restricted differentiation capacity even after long-term *in vitro* expansion (Figure 3). For example, EpiX-

expanded dermal keratinocytes fully matured into a stratified epidermis-like epithelium consisting of the regenerative basal layer, the spinous layer, the granulated layer and the cornified layers after 2–3 weeks of culture at the air-liquid interface (Figure 3 A-B). Similarly, bronchial epithelial cells expanded using the EpiX medium mature into a mucociliary epithelium consisting of multi-ciliated cells that spontaneously beat as well as goblet cells that produce mucin (Figure 3C). The mucociliary epithelium also maintain physiological functions, showing a significant increase in goblet cells in response to IL-13 stimulation (Figure 3D). Additionally, nasal epithelial cells derived from Cystic Fibrosis patients using the EpiX medium maintain the ion channel physiology expected for their CFTR genotypes and respond to therapeutic CFTR modulators in test tube, paving the way to develop precision medicine strategy using patient-derived cells.

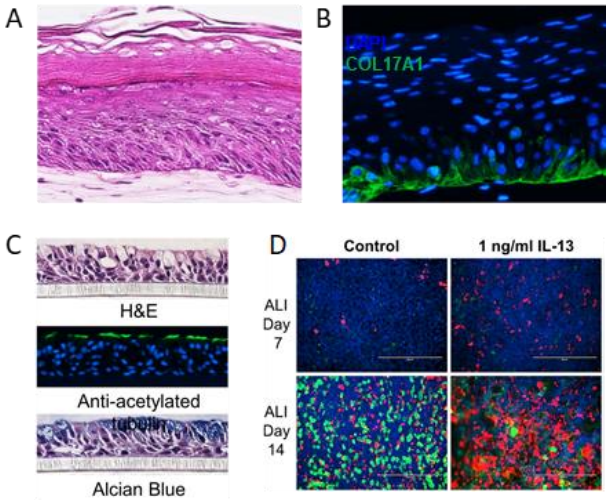

Figure 3. Epithelial cells readily differentiate mature epithelium after withdrawn from the EpiX expansion condition. **A-B**. EpiX-expanded dermal keratinocytes form multilayer epidermis after 2 weeks under ALI condition. **C**. EpiX-expanded bronchial epithelial cells form mucociliary epithelium. **D.** IL-13 induces more mucin-producing goblet cells (stained in red with anti-MUC5AC) at the expense of multiciliated cells (stained in green with anti-acetylated tubulin).

Since the EpiX medium provides a favorable *in vitro* environment for the epithelial stem and progenitor cells to expand, it supports multi-round single-cell cloning with high efficiency (Figure 4). The extended proliferation runway in EpiX medium enables sophisticated genetic engineering in human epithelial cells. As shown in Figure 4, we successfully used the CRISPR/Cas9 technology to insert the GFP gene into the AAVS1 locus (a safe harbor in the human genome) in human keratinocytes so that all cells express the transgene.

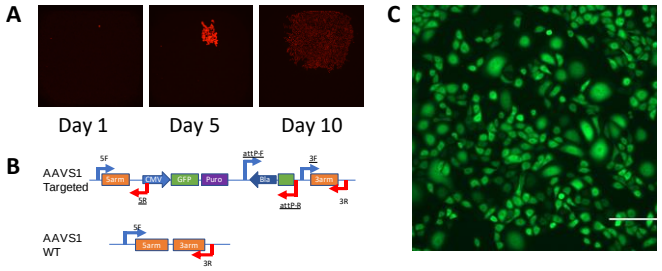

Figure 4. EpiX medium supports single cell cloning and genetic engineering by CRISRP/Cas9. **A**. One single keratinocyte (expressing RFP) grew into a colony in less than 2 weeks in 384-well plate. **B**. Introducing a GFP-expression cassette into the AAVS1 locus of human keratinocytes by CRISRP/Cas9. **C**. A clonal human keratinocyte cell line is established and all cells express GFP from the AAVS1 safe harbor.

This ability to consistently create differentiated tissue models from individual human biopsy sources Is enabling to a broad range of basic and industrial research applications. Having tissue models that represent natural and specific genotypes – without having to genetically engineer the cells – is highly desired in drug discovery and toxicology research. To facilitate this field, we have recently invented

scalable methods for the creation of apical side-outward (ASO) airway spheroids that are stable in culture and can be transported (Figure 5). In vitro-generated spheroids are typically apical side-inwards such that the environmentally exposed surface is facing inwards to the spheroid lumen. In our ASO spheroids however, the epithelial cells are in the natural configuration with the apical surface being the first point of contact with aerosolized drugs, pollutants, xenobiotics and toxins, and infectious agents.

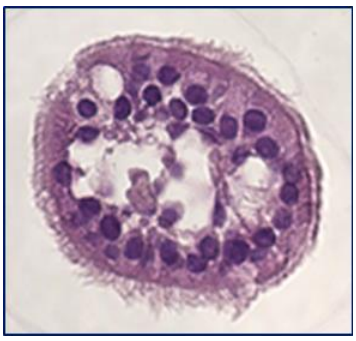

Figure 5. EpiX-expanded human airway epithelial cells can be organized using a novel process to expose their apical side outwards to the surrounding environment, mimicking the normal structure of the airway lining. Note the cilia on the surface of the spheroid; these beat at normal frequency to native airway cells. Mucin secretion by goblet cells within the ASO spheroid also occurs on the apical surface.

Summary

In summary, we developed the EpiX medium which supports over one trillion-fold expansion of human epithelial stem and precursor cells from diverse tissues. The EpiX medium presents unique solutions to unleash the potential of tissue-resident adult stem cells for regenerative medicine applications, with the following unmatched innovative advantages:

- Patented animal origin-free and chemically-defined formulation allowing for over one trillion-fold expansion of primary human epithelial stem and progenitor cells *ex vitro*
- Epithelial stem/progenitor cells expanded using the EpiX medium retain genome integrity and are non-tumorigenic
- Epithelial cells that are propagated using the EpiX technology retain their lineage-committed differentiation capacity
- EpiX enables the production of clinical-scale tissue biomaterial in a short time frame (2-3 weeks) from small biopsy samples
- Extended proliferation runway empowers advanced genetic engineering (e.g., CRISPR/Cas) to develop regenerative cell therapy

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