EpiX™ expansion technology enables *ex vivo* tissue engineering of skin using autologous patient-derived cells for regenerative medicine applications

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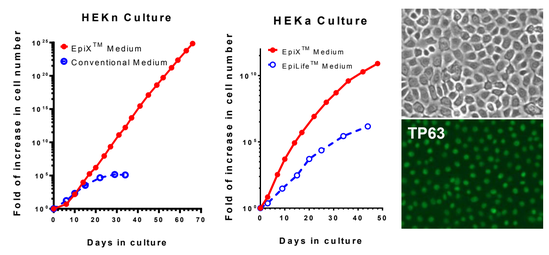
Abstract

It remains a challenge to preserve stem and progenitor cells during *ex vivo* expansion of epidermal keratinocytes under serum-free and feeder-cell-free culture condition. This limitation greatly hinders the development of advanced autologous cell and gene therapeutics for inherited skin diseases such as epidermolysis bullosa and injuries such as severe burns. We have developed a serum-free and feeder-cell-free culture technology (EpiX™) that allows rapid generation of more than one-trillion epidermal keratinocytes while retaining the stem and progenitor cell population. In-depth whole genome sequencing and *in vivo* tumorigenicity studies demonstrated that the EpiX™-expanded cells maintain genetic stability and do not form tumors. The preservation of stem cell character is evidenced by repeated single cell cloning capability to enrich genetic engineered cells via CRISPR/Cas9-mediated gene knock-in into the AAVS1 safe harbor locus.

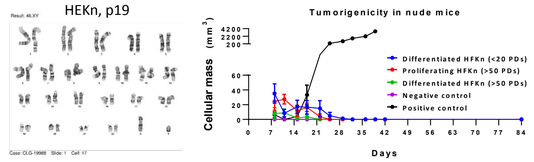
EpiX™-expanded keratinocytes maintain a basal cell phenotype during *ex vivo* expansion and readily differentiate into stratified epidermis in organotypic culture on the air-liquid interface. When grafted into immunocompromised mice, human keratinocytes survived over several months *in vivo* and seamlessly integrated with wounded mouse skin. An improved manufacturing process allows us to make suturable clinical-sized (75 cm^2) skin graft sheets with mesenchymal cell-populated dermis and stratified epidermis layers, thereby enabling the development of a range of gene-engineered cellular therapeutics for diseases and injuries of the skin.

Quick *ex vivo* expansion to generate trillions of primary keratinocytes using the EpiX™ medium

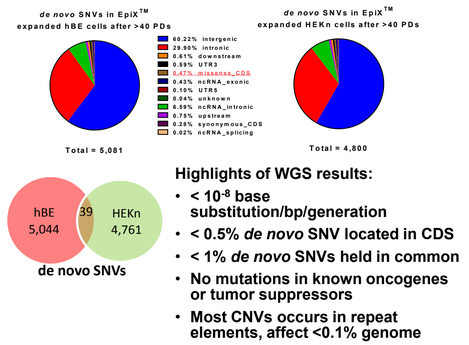


(Left) Human epidermal keratinocytes-neonatal (HEKn) achieved over 10^{25}-fold expansion in less than 70 days using the EpiX™ medium, while quickly stopped growth after merely 10^6-fold expansion in a conventional medium. **(Middle)** Human epidermal keratinocytes-adult (HEKa) achieved 10^{11}-fold expansion in the EpiX™ medium but stopped proliferation in the EpiLife medium after 10^5-fold expansion. **(Right)** The keratinocytes maintained ubiquitous TP63 expression in EpiX™ medium.

EpiX™-expanded cells maintain genetic stability and are not tumorigenic

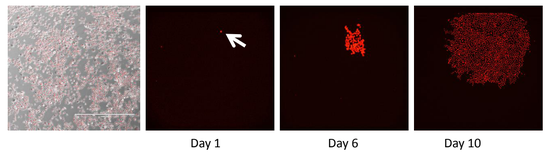


HEKn expanded in EpiX™ medium for 19 passages remained diploid. Nude mice that received 10 x 10^6 keratinocytes subcutaneously showed no evidence of forming a tumor mass (3 month trial).

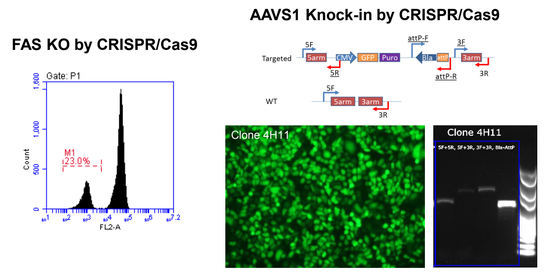


Whole genome sequencing results showed that the cells retain genome integrity after extensive *in vitro* expansion, did not exhibit a heightened mutational load, nor had experience any accumulation of known tumor-driving gene mutations.

EpiX™ medium enables genetic engineering and single cell cloning of human primary keratinocytes

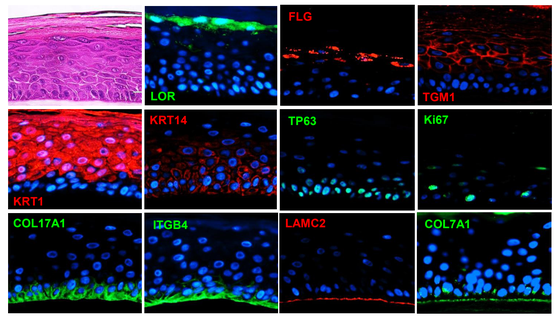


Stable RFP-expressing transgenic cell lines are derived by lentivirus transduction and used for single cell cloning in EpiX™ medium



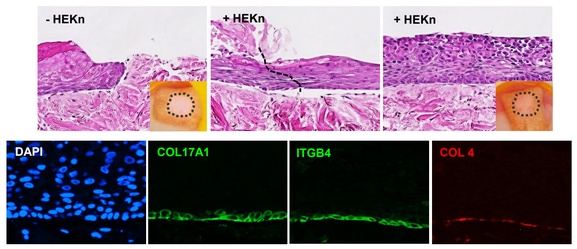
Keratinocytes expanded in the EpiX™ medium are used for genetic engineering using CRISPR/Cas9, retrovirus, etc. Keratinocytes were transduced with lentiviral particles expressing Cas9 and gRNA to target human FAS/CD95 (Left). Keratinocytes were transfected with CRISPR/Cas9 RNP, which led to targeted gene knock-in into the AAVS1 site (right). Clonal cell populations were subsequently isolated from the transduced cellular pool.

EpiX™-expanded keratinocytes differentiate into stratified epithelium



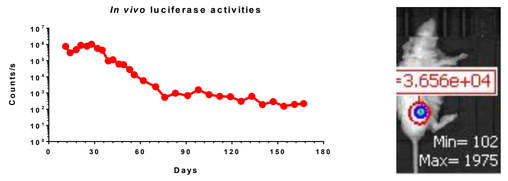
EpiX™-expanded HEKn were lifted to air-liquid-interface (ALI) culture condition for 7 or 14 days. H&E and IHC-P staining showed stratification. There were stem cells resident in basal layer at day 14 of ALI culture as $TP63^+$, and some of them were actively proliferating as $Ki67^+$.

EpiX™-expanded keratinocytes integrate into wounds in human skin model

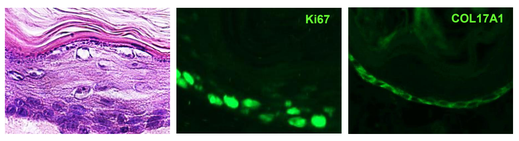


In an *ex vivo* wound-healing model using human skin, EpiX™-expanded keratinocytes integrated into wounds seamlessly and differentiated into multi-layer epithelium. The cells in the basal layer expressed high levels of Integrin β4, Collagen XVII and Collagen IV.

Differentiation and long-term self-renewal of EpiX™-expanded keratinocyte *in vivo*

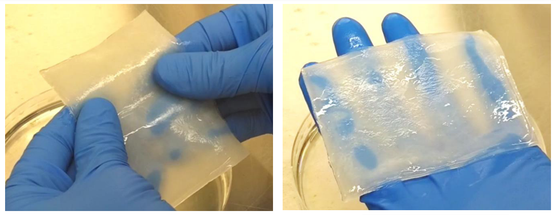


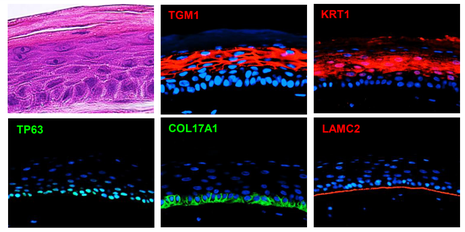
EpiX™-expanded keratinocytes survived for over 5 months when they were implanted subcutaneously in immune-compromised NSG mice, as monitored by the expression of luciferase transgene.



Subcutaneously implanted keratinocytes recapitulated differentiation in immunocompromised mice *in vivo*. All cells in the basal layer expressed Collagen XVII and many cells were positive for Ki67.

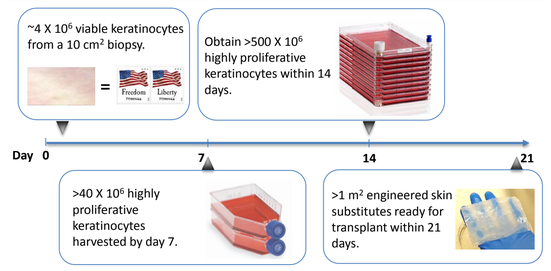
Development of 75 cm^2 skin substitute sheets using EpiX™-expanded keratinocytes





EpiX™-expanded keratinocytes were used in manufacturing suturable engineered skin substitutes in a clinically-relevant size, i.e. ~75 cm^2. The engineered skin substitutes had enough tensile strength to be handled easily.

Workflow of producing skin substitutes for severe burn injuries



Summary

EpiX™ stem cell expansion technology allows for a trillion-fold expansion of primary epidermal keratinocytes in a short timeframe. During this rapid expansion phase, the cells remain genetically stable and do not become transformed *in vitro* or form tumors *in vivo*. EpiX™ technology enables genetic engineering and clone selection. After expansion, if the cells are placed into air-liquid interface culture conditions, they rapidly differentiate into a stratified epidermis structure *in vitro* that resembles the architecture of normal healthy skin. The cells can also generate a well-differentiated multilayer epithelium in a mouse wound healing model *in vivo*. Using keratinocytes expanded with EpiX™ technology, we have developed a process for making an engineered skin construct of sufficient size and mechanical strength to enable the manufacture of suturable skin substitutes. We believe these advances in creating an tissue engineered skin substitute will address multiple unmet medical needs including wound healing and curative treatments for inherited skin diseases such as Epidermolysis Bullosa.

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