Development of a Genetically Modified Human Dermal Fibroblast for the Treatment of Recessive Dystrophic Epidermolysis Bullosa

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ABSTRACT and INTRODUCTION
Recessive dystrophic epidermolysis bullosa (RDEB) is an autosomal recessive, inherited skin disease caused by null mutations within the type VII collagen gene (COL7A1). The mutations cause an absence or reduction of functional collagen VII, which makes up anchoring fibrils that maintain binding of the epidermis to the dermis (Figure 2). The disease is characterized by a mechanical fragility and repeated blister formation in the sub-lamina densa, at the level of the structurally defective anchoring fibrils. Currently, there is no effective therapy for this disease, and death is usually the result of aggressive squamous cell carcinoma, sepsis, or malnutrition.

We are developing an autologous, genetically-modified fibroblast cell therapy that is anticipated to improve skin function in RDEB patients through restoration of collagen VII. A patient's fibroblasts will be harvested, genetically modified ex vivo with a functional COL7A1 gene, and expanded in culture (GM-COL7). Ex vivo transduction will occur through the use of a replication-defective, self-inactivating (SIN) lentiviral vector (Figure 2). After expansion, the fibroblasts are administered back to the patient as a local intradermal injection into target wound margins. The resulting increase in anchoring fibrils is anticipated to stabilize the connection between skin layers and reduce blistering tendency.

In vitro product development indicates that GM-COL7 can GM-HDF-COL7 cells express full-length type VII collagen exhibiting the proper trimeric structure, size, and binding functionality (Figures 3-7). A hybrid in which user pharmacology/immunoassay study using an organ culture/SCI/Mouse model is underway at Stanford University to confirm type VII collagen persistence, distribution, localization and bioactivity.

SUMMARY
• Production of UV-COL7 was successful resulting in an infectious titer of 9E+06 IU/mL
• GM-HDF cells were successfully produced by expanding LV-COL7-transduced RDEB patient fibroblasts from a biopsy sample
• The integrated transgene copy number per cell was dependent on the virus dose averaging 0.38 and 0.18 transgene copies per cell for high and low dose, respectively
• The COL7 expression from the GM-HDF cells was confirmed by RT-qPCR, immunofluorescence staining, and EUSA
• The COL7 expression from the GM-HDF cells was confirmed to be predominantly trimeric with LV-COL7-transduced cells expressing more COL7 than mock-transduced cells (Arms 1-2).

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Potential Conflicts
VAD, MIC, AE, and SK have financial interest in Intrexon stock. JM is the Vice President of Scientific Affairs has financial interest in Fibrocell Science and owns Xion stock. SK is Senior Vice President with Intrexon. MPM is a paid consultant for Intrexon and Fibrocell Science.

Figure 2
UV-COL7 transduction process: A COL7 expression cassette was cloned into a self-inactivating lentiviral backbone. A pilot scale production of UV-COL7 with a titer of ~1E+06 PAE/mL was generated for use in the UV-COL7 production process. Fibroblasts were isolated from RDEB patient biopsies, grown, then split into three arms for mock, high-, and low-dose UV-COL7 transduction. Each arm of fibroblasts was grown to 2 x 10^5 cells in tetracycline-processed (Drug Substance). For patients’ use, Drug Substance vials are harvested and formulated (Drug Product); then shipped back to the clinic for wound-stitch injection of the originating patient.

Figure 3
A. UV-COL7 copy number per cell: Drug Substance vials were thawed and assayed for UV-COL7 copies per cell using qPCR. Primers were specific for the UV shuttle vector. Results demonstrate dose-dependent levels of copies per cell with an average of 0.16 and 0.18 copies from the High and Low Dose arms (GM-COL7), respectively. B. Cytoplasmic UV-COL7 mRNAs levels compared to mock-transduced controls. Results demonstrate dose-dependent mRNA levels of UV-COL7 mRNA with ~3000 and ~7000 copies per cell using Quant-IT. C. Cytoplasmic UV-COL7 protein levels compared to mock-transduced controls. Results demonstrate dose-dependent increase in COL7 protein expression compared to mock-transduced cells.

Figure 4
A. Immunofluorescence detection of COL7 expression - Drug Substance (UV)-COL7 cells were thawed and cultured. Normal human dermal fibroblasts (NHDF) were also cultured. NHDF and RDEB cells from each UV transduction group were fixed, permeabilized, and stained with DAPI to visualize nuclei (blue) and COL7 antibody to COL7 (brown). 1.25 ug/ml AlexaFluor 555-conjugated goat anti-rabbit IgG to visualize COL7 expression (red). Images were acquired at 20X magnification using an exposure time of 250 ms. COL7 is depicted on NHDF, and High and Low Dose UV-COL7-transduced RDEB cell groups, but not mock-transduced RDEB cells.

Figure 5
A. COL7 expression levels produced by RDEB patient fibroblasts transduced with UV-COL7 - Drug Substance vials were thawed and cultured for 5 days. Conditioned cell supernatants were collected and assayed for COL7 levels by EUSA. Results show virus dose-dependent protein expression which ranges from 60 to 120 ng/mL COL7 in UV-COL7-transduced cells. B. Trimeric form of COL7 produced by RDEB patient fibroblasts transduced with UV-COL7 - Conditioned cell supernatants were also used in an immunoprecipitation assay. Immunoprecipitated COL7 was separated on an SDS gel and visualized by western blot. The COL7 produced by RDEB fibroblasts was predominantly trimeric with UV-COL7-transduced cells expressing more COL7 than mock-transduced cells (Arms 1-2). Some lower molecular weight species showing immunoreactivity were also observed.

Figure 6
A. Binding of purified COL7 dimer to Lamin312: COL7 was expressed in CHO-DG44 cells, purified by size exclusion chromatography, and was assayed for preferential binding to Lamin312 compared to BSA to establish the assay (increase in OD450 corresponds to increase in COL7 binding to Lamin312). B. Lamin312 binding of COL7 in UV-COL7-transduced RDEB fibroblasts culture supernatant: Drug Substance samples were thawed and cultured for 2 days. Conditioned cell culture supernatants were collected and assayed for binding to Lamin312 compared with BSA control. Results show virus dose-dependent binding to Lamin312.

Figure 7
Migration of UV-COL7-transduced RDEB Patient Fibroblasts: NHDFs and patient fibroblasts were assayed for COL7 and cultured and cells were seeded into culture dishes with an inset to prevent cell adhesion in a small area of the culture dish. The dish was then removed and the rate at which the cells migrated into the open area was monitored by microscopy and quantified using the irregular shape delineation plugin for ImageJ software. Percent migration (A) and images of migration (B) are shown. The results show that the mock-transduced RDEB patient fibroblasts migrate into the open area faster than MHDF, and transduction with UV-COL7 reverses the patient cells to a rate of migration similar to NHDFs. These results are consistent with those described by Chen, M. et al. J. Biol. Chem. (2000) 275:24293-25.