Development and manufacture of an investigational human living dermal equivalent (ICX-SKN)

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Aim: To design and manufacture an investigational living skin graft replacement (ICX-SKN) that is able to incorporate into the host, providing healing by primary intent without the need for a second intervention. **Materials & Methods:** The ICX-SKN skin graft replacement has been designed as an allogeneic dermal substitute comprising an extracellular matrix composed largely of human collagen and human dermal fibroblast cells (HDFs). ICX-SKN is first formed by casting a provisional matrix of fibrin, into which HDFs are seeded. Through a process of maturation, HDFs are induced to lay down collagen and other extracellular matrix materials and, as the construct matures, the original fibrin is largely replaced by collagen, which provides tensile strength and flexibility to the construct. In order to design a product and manufacturing system that lends itself to large-scale production the process was developed as a discontinuous process consisting of four stages: 1. batch casting and maturation of the initial construct (pSKN), 2. freeze-drying of pSKN to produce a second intermediate (dSKN), 3. sterilization by γ -irradiation of dSKN to produce a third intermediate (sSKN), and finally, 4. repopulation of sSKN by fresh HDFs to produce the final product, ICX-SKN skin graft replacement. Preliminary characterization of ICX-SKN and its application in a preclinical model are described. **Results:** The 7-week maturation period resulted in a construct (pSKN) with robust handling properties, which was composed mainly of human collagen I. Following development of a process for freeze-drying and subsequent sterilization, the matrix was successfully repopulated with fresh HDFs. In addition, it was demonstrated that human keratinocytes attached and differentiated on the matrix. Application of human keratinocytes to the repopulated constructs (ICX-SKN) resulted in expression of markers of basement membranes that was largely dependent on the presence of living HDFs on the constructs. ICX-SKN graft replacements applied to excision wounds in mice healed and were rapidly re-epithelialized. **Conclusions:** ICX-SKN has been developed as a platform product that can be used as a skin graft replacement and the process by which it is manufactured has been designed for the product to be available to the end-user off-the-shelf and for ease-of-use in practice.

The aim of this study was to develop and manufacture a dermal equivalent suitable to replace autologous skin grafts for wound closure. Several living skin equivalents (LSEs) and living dermal equivalents (LDEs) have been reported previously [1–4], and although many of the currently available products were initially designed to work as skin grafts, in reality no current living product meets the rigorous requirements necessary to accomplish this function [4–6]. Rather, the dermal component of these products rapidly degrades in the wound, releasing the cells, which then contribute to wound healing by secondary intention. In contrast with earlier LSEs and LDEs that have used either a preformed collagen matrix or biodegradable synthetic mesh as the initial support system, the intent from the start of this program therefore was to develop a more

robust extracellular matrix and to investigate the ability of human dermal fibroblast cells (HDFs) themselves to produce this matrix *in vitro*. It had been demonstrated previously [4,5] that HDFs cast into a provisional matrix of fibrin were induced to lay down collagen, predominantly collagen I, and other extracellular matrix materials [7,8]. ICX-SKN skin graft replacement is based on the concept that the fibrin matrix provides a 3D fibrillar network that can be aligned by the cells in the construct and serves as a template for remodeling with the addition of collagen auto-synthesized by the HDFs. In addition, the provisional matrix provides mechanical constraint that provides the stimulus for developing structure as collagen is laid down [8] and probably also provides biochemical signals to upregulate collagen synthesis [7].

Keywords: autossynthesized human matrix, epithelialization, living skin equivalent, skin graft, tissue engineering and manufacture, wound healing

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A preliminary design specification for ICX-SKN skin graft replacement was developed which included: efficacy, convenient size and shape, ease of use, off-the-shelf availability, and permanent healing effect. In order to realize these aims, the emphasis was placed on maximizing deposition of insoluble human collagen, minimizing matrix contraction, maximizing the functional properties of the constructs (handling, rigidity/flexibility/strength, biological compatibility), and establishing a preliminary product stability profile. The studies described in this report were intended to provide a means for producing ICX-SKN skin graft replacement dermal constructs to these specifications that could be directly translated to clinical manufacture and ultimately be scaled-up both in size and number. Thus, manufacture of ICX-SKN skin graft replacement consists of four stages:

- Constructs were batch-cast by embedding HDFs into a provisional fibrin matrix and matured for 7 weeks in collagen-boosting medium to produce a first intermediate, pSKN;
- pSKN was freeze-dried to produce the second intermediate, dSKN;
- dSKN was sterilized by γ -irradiation to produce the third intermediate, sSKN;
- Living HDFs were reintroduced to the sSKN matrix to yield the final product, ICX-SKN skin graft replacement, which was then packaged ready for application.

Once placed *in situ*, it is intended that ICX-SKN skin graft replacement will maintain a biochemically balanced and moist wound environment and provide structural support for tissue regeneration and beneficial cytokines and growth factors to the wound bed. Future iterations may also be provided preseeded with keratinocytes for the treatment of large area defects; in much of the work described here, the dermal portion alone was studied as it was felt that this was the key area requiring attention in order to provide a construct that could withstand the harsh environment of a healing wound.

We describe a series of processes through which the research concept was translated to ICX-SKN skin graft replacement constructs produced aseptically in a manufacturing facility suitable for application in Phase I clinical trials in human volunteers [9]. In this report we describe the design, development and manufacture of these constructs, and preclinical data for the use of this matrix in wound closure.

Materials & methods

Manufacture of ICX-SKN skin graft replacement

All products used in clinical trials were manufactured as a batch of 48 individual constructs in accordance with EU standards of good manufacturing practice (GMP) in a licensed GMP manufacturing facility at Intercytex Ltd, UK [10].

Cells & cell growth conditions

HDFs were obtained from neonatal foreskin (donated under informed consent) essentially as described previously [11], expanded in culture to passage six at which point they were cryopreserved to produce a working cell bank (WCB). HDF cells were grown in Dulbecco's modified eagle's medium (DMEM) (Lonza, Basel, Switzerland) supplemented (DMEM-10) with γ -irradiated 10% foetal bovine serum (FBS; JRH Corp. MA, USA) and L-glutamine (0.29 mg/ml; Molekula) and were used at passages 7–9 to cast into a human plasma protein, Tisseel™ VH (Baxter AG, IL, USA) as described below. HDF cells sufficient to yield a batch of 48 constructs were thawed from cryopreserved stock or trypsinized from growing cultures, washed in DMEM-10 and finally resuspended in DMEM supplemented with 2% FBS and L-glutamine (0.29 mg/ml) at 3×10^6 HDFs/ml.

Production of pSKN

The human fibrinogen sealant powder (Tisseel™ VH, Baxter AG) was reconstituted 1:1 in aprotinin solution as supplied by the manufacturer, resulting in a 'working fibrinogen sealant.' Thrombin powder (Baxter AG) was reconstituted 1:300 in phosphate buffered saline (PBS) ($-\text{Mg}^{2+}$, $-\text{Ca}^{2+}$), resulting in a 'working thrombin solution.' For each construct of ICX-SKN to be cast, working cell suspension (1.8×10^6 cells/construct), working fibrinogen sealant and working thrombin solution were mixed (3.7 ml total) as indicated (see Results) and immediately dispensed into the centre of a single (3.5×2.5 cm) well of an eight-well plastic culture plate (Nunc Inc., NY, USA). The constructs were left to gel for 30 min at room temperature, then were covered with DMEM-10 before being placed into a humidified incubator (37°C , 5% CO_2) for approximately 2 days. This size of construct proved suitable for all current preclinical and clinical studies; however, larger constructs up to 10×10 cm have been produced in other custom-modified casting chambers.

Constructs were maintained and matured by changing the medium every 2–3 days for up to 7 weeks. In order to mature the constructs and encourage the deposition of collagen, DMEM 10 medium was replaced with total medium (TM-3:1 DMEM and Ham's-F12 [Cambrex Inc., MD, USA], 10% FBS, 0.29 mg/ml L-glutamine, 100 μ M ethanolamine, 6.78 ng/ml selenious acid, 50 ng/ml ascorbate, 0.2 μ g/ml L-proline, 0.1 μ g/ml glycine, 50 μ g/ml gentamycin [Molekula, MO, USA], 0.4 μ g/ml hydrocortisone [Spectrum Lab Products Inc., CA, USA], 100 μ M *O*-phosphoryl-ethanolamine [Merck, NJ, USA], 5 μ g/ml apo-transferin, 1.35 ng/ml 3,3',5-tri-iodo-L-thyronine, 0.05% polyethylene glycol MW 3400–3700 [SigmaCorp., NJ, USA], 20 pM/ml transferrin, 5 μ g/ml insulin [Serologicals, MA, USA], 250 ng/ml amphotericin B [BufaB.V.], 62 ng/ml EGF, 5 ng/ml TGF- β [R & D Systems, MN, USA], 0.01 unit/ml plasmin [Calbiochem, Merck KGaA, Darmstadt, Germany]) starting with the first feed 2–3 days post-casting. Matured constructs were collected from their casting wells for freeze-drying.

Production of dSKN

Each matured pSKN construct was removed from the casting chamber and placed into a 60 mm Petri dish (Corning Inc., NY, USA), which was heat-sealed into a Tyvek/film pouch (Oliver Medical Products BV, Netherlands). The packaged pSKN units were placed at -20°C for a minimum 12 h prior to freeze-drying. Packaged and frozen pSKN were then placed into the vacuum chamber of a Modulyo 4K freeze-drying unit with attached RV8 vacuum pump (Edwards) that had been pre-cooled to -40°C . In order to maintain the pSKN units frozen, each frozen construct was placed onto a brass disk chilled at -80°C for at least 4 h and the vacuum was applied. The freeze-drying cycle was conducted at 2×10^{-1} to 8×10^{-2} mBar for a minimum of 16 h. Each dried construct (dSKN) was then placed into a plastic roto-seal bag and was subjected to terminal sterilization.

Production of sSKN

Packaged dSKN constructs were subjected to a minimum dose of 25 kGy γ -irradiation (Swann-Morton, Sheffield, UK). The resulting sSKN was stored excluding light at ambient temperature and constituted a raw material for the final manufacture of the ICX-SKN skin graft replacement.

Manufacture of ICX-SKN skin graft replacement

In order to manufacture the final product, ICX-SKN skin graft replacement, sSKN constructs were first removed aseptically from their packaging, visually inspected to check their integrity, rehydrated with water for injection (WFI) (JRH Inc., UK) for 10 min at room temperature, with two subsequent changes of water. Constructs were then washed with DMEM-10 for 10 min. After removal of as much of the DMEM-10 as possible, HDFs (0.2 ml at 9×10^6 cells/ml in DMEM-10) were applied directly to the top surface of each construct. The constructs were placed in an incubator (humidified, 37°C , 5% CO_2) for a minimum of 30 min to allow cells to adhere to the construct, at which point DMEM-10 was added to each construct and then returned to the incubator for 48 h prior to assessment of cell viability via reduction of Alamar Blue™ (SeroTec) and packaging for storage and/or shipping to clinic.

Constructs for cold storage or shipping were packaged into sterile polyester/poly/foil/poly pouches (Oliver Medical Products BV, Netherlands) containing 5 ml of CO_2 -independent transport medium (L-15 medium [Cambrex] supplemented with 202.5 μ g/ml sodium bicarbonate, 4.5 mg/ml dextrose, 24.4 μ g/ml adenine, 0.29 mg/ml L-glutamine, 100 μ M ethanolamine, 100 μ M *O*-phosphoryl-ethanolamine, 0.4 μ g/ml hydrocortisone, 5 μ g/ml human recombinant insulin, 6.78 ng/ml selenious acid, 1.35 ng/ml of 3,3',5-tri-iodo-L-thyronine, 5 μ g/ml apo-transferrin, and 2% FBS). The pouches were heat sealed and visually assessed for leakage. All units were stored at $2\text{--}8^{\circ}\text{C}$ during shipping and before use.

Organotypic culture of ICX-SKN with keratinocytes

Human keratinocytes (HKs) cells were obtained from neonatal foreskin (donated under informed consent), and expanded in culture. Cells were used at passage 3–4 and were expanded in EpiLife® medium supplemented with EpiLife defined growth supplement (EDGS; Cascade Biologicals). HKs (5×10^6 cells/construct) were placed onto the surface of rehydrated sSKN constructs or onto the top surface (the surface already populated with HDFs) of ICX-SKN. HKs were made to differentiate according to established procedures [12]. Briefly, constructs were incubated at 37°C , 5% CO_2 in a humidified incubator for 2 days in epidermal

medium (EM – 1:3 mixture of DMEM (Lonza) and Hams-F12 supplemented with 0.3% FBS, 0.29 mg/ml L-glutamine, 6.78 ng/ml selenious acid, 50 ng/ml ascorbate, 24.4 µg/ml adenine, 50 µg/ml gentamycin, 100 µM ethanolamine, 0.4 µg/ml hydrocortisone [Spectrum Lab. Products Inc], 100 µM *O*-phosphoryl-ethanolamine, 1.35 ng/ml of 3,3',5-tri-iodo-L-thyronine, 5 µg/ml insulin, 5 µg/ml apo-transferrin, 10 ng/ml EGF and 0.628 ng/ml progesterone [Amersham]). After 2 days this medium was replaced with extracellular matrix containing 265 µg/ml CaCl₂ (Sigma Corp.), but omitting EGF and constructs were incubated for a further 2 days. To allow the stratification of the epithelial layer the constructs were aseptically transferred onto the polycarbonate membrane of transwell dishes (Corning Inc.) and maintained in medium omitting both progesterone and CaCl₂, achieving a fluid level just to the surface of the carrier membrane to maintain a liquid–air interface. Constructs were maintained in a humidified incubator at 37°C, 5% CO₂ for 7 days with changes of the media every 2–3 days. Finally, constructs were maintained in medium omitting ascorbate for a further 10 days with medium changes every 2–3 days.

Analyses of ICX-SKN skin graft substitute

Histology

Samples for histological analysis were prepared by embedding in paraffin wax and sections (5 µm) were cut using a rotary microtome (Leica, IL, USA), placed onto microscope slides, then de-waxed and stained with hematoxylin and eosin (H&E) stain. Following staining slides were mounted in DPX mounting media (BDH, UK) and coverslips applied. Digital images were captured using a Nikon 4500 CoolPix digital camera under light microscopy.

Immunocytochemistry

Samples were embedded in optimal cutting temperature (OCT) embedding medium (RA Lamb, UK) and sections (10 µm) were obtained using rotary microtome (Leica). Sections were thawed at room temperature for 10 min, fixed in ice-cold acetone for 5 min and air-dried. Air-dried sections were rinsed in three changes of a wash buffer containing 1% normal goat serum (NGS) and 0.1% TritonX100 in PBS for 10 min. Sections were blocked for 30 min at room temperature using 20% NGS blocking solution. The slides were drained to remove the blocking

solution and the sections were incubated for 1 h at room temperature in the primary antibody. Primary antibodies (AbCam, UK) were: antihuman collagen 1 (1:200), antihuman fibrin (1:100), antihuman laminin-5 (1:250), antihuman keratin (1:250), and antihuman involucrin (1:200). Sections were rinsed in three changes of PBS for 10 min each, and incubated for a minimum 60 min at room temperature in the appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch) at 1:200 dilution. Finally, sections were rinsed in three changes of PBS for 10 min each, mounted in diamidinophenylindole (DAPI)-containing mounting solution (Vectashield, CA, USA) and coverslipped. Images were captured using a Nikon Coolpix 4500 digital camera mounted onto a fluorescence microscope (Leica) using an appropriate fluorescence filter.

Phalloidin staining of ICX-SKN constructs

Repopulated constructs were washed in citrate buffer solution (CBS) buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.1; 138 mM KCl; 3 mM MgCl₂; 2 mM ethylene glycol tetraacetic acid (EGTA); 11% sucrose), then fixed in 4% paraformaldehyde in CBS buffer for 1 h at 2–8°C. Samples were then frozen in OCT embedding medium and 8 µm sections were cut and placed onto microscope slides. Samples were then rinsed in TBS buffer (0.02 M Tris (pH 7.4–7.5); 0.15 M NaCl), permeabilized in TBS 0.5% TritonX100 for 10 min and rinsed three times in TBS 0.1% TritonX100. Sections were pretreated in block buffer (TBS 0.1% TritonX100, 2% BSA) for 10 min then incubated with fluorescence-phalloidin (10 µg/ml in block buffer) for 20 min. Samples were rinsed again in TBS 0.1% TritonX100, drained well, mounted with DAPI-containing mounting solution (Vectashield) and viewed directly by fluorescence microscopy. Images were stored as electronic files.

Live cell labeling with Cell Tracker™

CM-Dil dye

HDFs used for repopulation of sSKN constructs were stained with Vybrant CM-Dil (Invitrogen, CA, USA) dye according to the manufacturer's instructions. Briefly, cells were collected from growing cultures or were recovered from cryopreservation and washed twice in DMEM and then resuspended in DMEM (10 ml). CM-Dil dye (10 µl of 10 mM in Dimethyl sulfoxide [DMSO; Sigma Corp.]) was added to the cell

suspension and the cells were incubated at 37°C for 30 min. The cell suspension was then pelleted by centrifugation (5 min at 500 g) and cells were washed twice in DMEM-10 to remove all traces of the dye. Cells were finally resuspended in DMEM-10 (9×10^6 cells/ml) and were applied to the rehydrated sSKN matrix as described previously.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) analyses were performed on DSCQ100 machine (TA Instruments, DE, USA). Briefly, a sample (5 mg) collected on day 0 postcasting or fully matured sSKN, or as a control, porcine dermis, was placed into the aluminum pan (TA Instruments). Prior to analysis samples were fully rehydrated with a drop of PBS (Sigma Corp.) for at least 5 min at room temperature. Pans were hermetically sealed and inserted into the instrument. The samples were heated from 0–120°C at 5°C/min. The results were analyzed using TA Instruments software and denaturation temperature (Td) of the major component of the matrix was established. Porcine dermis, used as a control, was prepared by removal of the epidermis using dispase solution (Sigma Corp.) for 12 h at 4°C. The epidermis was scraped from the dermis, the dermis washed several times in PBS, and finally freeze-dried and sterilized.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed by the electron microscopy unit at Manchester University, Faculty of Life Sciences using a SEM (Cambridge Instruments, UK). The freeze-dried (dSKN) samples were broken into small pieces using tweezers and were attached onto double-sided adhesive tape and placed on the top of carbon coated tabs. The samples were then coated with gold using argon gas to disperse the gold molecules onto the samples. Samples were then inserted into the chamber of the SEM and the chamber evacuated to obtain vacuum for a minimum of 30 min. Images were then obtained using scanning microscopy image software (Cambridge Instruments, UK).

Resistance test

Repopulated constructs were tested to breaking point using a Multitest force gauge (Mecmesin) equipped with a 10 Newton load cell. The constructs were gripped with clamps attached to the force gauge in such a way that the force was applied along the longer side of the construct.

The constructs were first ‘exercised’ at 2 mm/min three times to a displacement of 1 mm each cycle, and allowed to relax to their original position between displacements, then finally displaced to breaking point.

Determination of cell viability

Alamar Blue™ (Serotec, NC, USA) assay for metabolic activity of cells was essentially performed according to manufacturers’ instructions on ICX-SKN constructs. Briefly, a 10% Alamar Blue™ solution was prepared in CO₂-independent transport medium. The construct was placed into a container to which the 10% Alamar Blue™ solution (6 ml) was added, then placed at 37°C. Alamar Blue™ reduction was measured in duplicate samples at 30-min intervals at 570 and 600 nm wavelengths. The reduction of Alamar Blue was calculated according to manufacturers’ instructions.

Open wound assay

Full-thickness excisions (0.8×1.8 cm²) were made to the dorsal skin of anaesthetized male CD-1 nu/nu mice (Charles River Laboratory, MA, USA). A piece of rehydrated sSKN or ICX-SKN skin graft replacement, cut to match the size and shape of the excision, was placed onto the wound. Four sutures were made on the four corners to fix the graft in place and a piece of adaptic nonadhering dressing gauze (Johnson and Johnson) was placed onto the graft before the wound was bandaged. Wounds were observed at 7-day intervals and the graft tissue was harvested from sacrificed animals for histological analysis

Results

Development of pSKN matrix

Once placed *in situ*, it is intended that ICX-SKN skin graft replacement will maintain a biochemically balanced and moist wound environment and provide robust structural support for tissue regeneration as well as beneficial cytokines and growth factors to the wound bed. During manufacture of pSKN matrix, fibroblasts initially embedded in fibrin *in vitro* begin the process of remodeling the provisional matrix by destruction of fibrin whilst incorporating insoluble collagen into the matrix [7]. A presumed threshold exists above which the two processes occur at a rate which means that fibroblasts are continually embedded in a dynamic, insoluble matrix composed variably of fibrin and/or collagen [9]. Below this threshold, destruction of fibrin runs

ahead of incorporation of collagen and the fibroblasts are released into the liquid medium. In order to embed HDF cells in a stable 3D scaffold variously composed of human fibrin and/or human collagen, different concentrations of fibrinogen and thrombin were tested. The aim was to obtain constructs that were easy and reliable to cast and that did not dissolve or contract during 7 weeks of maturation in collagen-boosting medium (TM). Several formulations were tested, including combinations of the precursor to human fibrin, fibrinogen, at initial concentrations of 7 mg and 1 mg and human thrombin at 83 or 8.3 units, respectively, per construct (Table 1). In order to slow the process of cleavage of fibrinogen by thrombin to produce fibrin, and allow sufficient time for casting the mixture, all reagents were chilled to 2–8°C. Each construct was cast individually into a 3.5 × 2.5 cm casting chamber, taking care that the surface tension of the casting mix was maintained once cast.

It was difficult to effectively cast constructs using formulations of high thrombin (Formulations 1 and 3, Table 1) as the fibrinogen/cell mix tended to set immediately on contact with thrombin in the casting pipette. Those that could be cast resulted in uneven constructs or constructs that contracted during maturation (Figure 1A). Conversely, constructs cast with high fibrinogen and low thrombin (Formulation 2, Table 1) dissolved before sufficient collagen was incorporated (data not shown). The most consistent results were obtained using low fibrinogen and low thrombin (Formulation 4, Table 1) and the precooled casting mix allowed a reasonable ‘casting window’ of 30–60 s, which resulted in even and well-formed constructs (Figure 1B). In addition, after maturation in TM,

the constructs obtained from the latter formulation had not contracted or dissolved and were easily excised from the casting dishes (Figure 1C) without being damaged.

In addition to entrapment of HDFs into a provisional matrix, the development of a prototype suitable for use as a dermal substitute in clinical applications required that HDFs synthesize and deposit substantial amounts of collagen and other components of extracellular matrix into robust insoluble structures in order to resist the physicochemical forces at work on human skin. It has been shown that supplementing media with factors such as TGF-β, insulin, ascorbate and plasmin can induce collagen deposition [7,8]. We developed TM by combining components already described [7,8,201], in addition to other cell-sustaining components. In order to evaluate the effect on collagen and ECM components synthesis by HDFs, constructs (formulated with low fibrinogen and thrombin as described above) were subjected to three-times per week feeding [8] with TM or with TM omitting EGF, TGF-β, insulin and plasmin (MPM) and sample constructs were collected at 7, 14, 21 and 52 days of maturation. Constructs fed in TM medium over the period of maturation became increasingly opaque relative to those constructs fed with MPM (Figure 2A), suggesting that ECM proteins were being laid down in the constructs during maturation. By contrast, constructs maintained in MPM retained a transparent, gel-like appearance. In addition, compared with MPM, constructs fed with TM were more robust in their handling properties; whereas MPM-fed constructs were easily torn upon removal from the casting dish, TM constructs remained intact upon manipulation.

TM- and MPM-treated constructs were immunostained using antihuman-collagen I antibody (Figure 2B) and revealed that collagen I was present in constructs maintained in TM to a greater extent than in constructs maintained in MPM. Furthermore, collagen I was deposited as early as 10–14 days of maturation (Figure 2B iv) in those constructs maintained in TM.

Comparative analysis by DSC of constructs matured in TM (pSKN), porcine dermis and constructs composed largely of fibrin (i.e., day 0 (d0) of maturation) was performed (Table 2). One major component from each matrix was detected; however, the Td of these major components differed substantially between the two constructs, showing that during maturation the initial fibrin matrix was converted.

Table 1. Formulations for pSKN.			
Formulation	Fibrinogen mg/construct	Thrombin units/construct	Final volume (μl)
1. High fibrinogen/high thrombin	7	83	3670
2. High fibrinogen/low thrombin	7	8.3	3670
3. Low fibrinogen/high thrombin	1	83	3700
4. Low fibrinogen/low thrombin	1	8.3	3700

Figure 1. Constructs cast with different formulations of fibrinogen and thrombin.

Demonstrated by comparison with the Td of porcine dermis, the fibrin matrix was converted to collagen during the 49-day maturation period.

Development of dSKN, sSKN & ICX-SKN skin graft replacement

pSKN was produced in 7 weeks by intensive feeding with medium supplemented with a cohort of growth factors designed to boost the synthesis and deposition of collagen. At this point, pSKN could be used directly in clinical application. However, the ability to preserve the matrix at this stage would add value to pSKN by both allowing for an increased shelf-life of the product and also enabling material to be stockpiled for use. One approach for increasing shelf-life of similar products has been cryopreservation [101]. However, the need for specialized storage both at manufacture site and clinic, and the requirement for complex recovery methods from cryopreservation before application rendered this method of preservation for pSKN unsuitable in the manufacture of ICX-SKN skin graft replacement.

Freeze-drying was chosen as the method for preservation of the matrix since it could provide a means of storing the pSKN product (known as dSKN once dried) at ambient temperature for long periods. pSKN constructs were subjected to washing in different solutions prior to freeze-drying with the aim of obtaining constructs that maintained their 3D structure and rectangular shape, without becoming brittle. In addition, since the process of freeze-drying destroyed cells within the pSKN construct, the freeze-dried constructs had to maintain the ability to sustain cells once repopulated with fresh HDFs (i.e., remain 'cell friendly'). Constructs were

freeze dried in Soltran, 0.1 M Tris pH 7.0 and TM medium and were subsequently rehydrated and repopulated with fresh HDFs (see Materials and Methods) then assessed for viability of the fresh HDFs.

Constructs dried in osmotically balanced biological sample preservation solution (Soltran), lost integrity upon freeze-drying (Figure 3A). However, pSKN matrices freeze-dried in Tris or TM remained intact, maintaining their thickness and flexibility even when dry (Figure 3A iv). These constructs were readily rehydrated and, in contrast to constructs freeze-dried in other formulations (data not shown), both were able to support attachment and sustained cell viability once repopulated with fresh HDFs (Figure 3B). Despite comparable results freeze-drying constructs in Tris, freeze-drying directly from TM was chosen since it removed the need for a further manipulation (i.e., washing pSKN constructs in Tris), an important consideration when designing a manufacturing process for cell-therapies.

To view the 3D structure of freeze-dried constructs in more detail, constructs were analyzed by SEM. Constructs freeze-dried from TM media showed a relatively well-preserved 3D structure of the collagen matrix, with evident pores of variable size (Figure 3C).

The dSKN matrix was sterilized by γ -irradiation (resulting in sSKN) to render it suitable for final product assembly and application in clinical trials. γ -irradiation with a terminal minimal dose of 25 kGy is an approved method of sterilization of medical products and devices [13]. It is also a nonproduct-contact sterilization method that does not leave residues on the product or product packaging. It has been reported previously that γ -irradiation has a detrimental effect on integrity of collagen matrix [14,15]. Sample pSKN, dSKN and sSKN constructs were subjected to cyclic stress and the breaking stress determined. In contrast to pSKN, whose strength was not measurable with the test method utilized (data not shown), rehydrated and repopulated dSKN and sSKN constructs demonstrated resistance to cyclic stress (Figure 4A) and had comparable ultimate tensile strength (Table 3), indicating the matrix was not compromised by the applied dose of γ -irradiation. Furthermore, sSKN had a demonstrable capacity to sustain viable cells on the matrix once rehydrated and repopulated as described above (Figure 4B) to yield the final product, ICX-SKN skin graft replacement.

Stress fibers are maintained in living cells and staining with fluorescent-phalloidin allowed viewing of the live cells on or within the matrix

(Figure 4C) without interference from cells that had been within the construct at the time of freeze-drying (i.e., dead cells). That the matrix

Table 2. Comparative differential scanning calorimetry analysis.

Sample	Td (mean $\pm$ SD)
Fibrin construct (d0)	55.6 $\pm$ 3.0
pSKN (d49)	43.4 $\pm$ 1.3
Porcine dermis	46.4 $\pm$ 1.6

SD: Standard deviation; Td: Denaturation temperature.

was able to support attachment of living HDFs was supported by detection on repopulated ICX-SKN constructs of actin-stress fibers 2 days after reintroduction of live HDFs, which were present mostly on the top surface of the construct to which they were applied. The nuclei of cells present when pSKN was freeze dried were also evident.

When stored at 2–8°C in CO₂-independent transport media (see Materials and Methods), ICX-SKN skin graft replacement was stable for at least 5 days (Figure 4B) maintaining approximately 75% of initial cellular activity.

Addition of keratinocytes to ICX-SKN skin graft replacement

ICX-SKN skin graft replacement is a platform that has the potential to be developed as a fully differentiated bilayered skin substitute. In order to investigate the potential of constructs to support attachment and differentiation of keratinocytes, human neonatal keratinocytes were seeded onto ICX-SKN skin graft replacement constructs and onto rehydrated sSKN constructs. Keratinocytes were incubated on the constructs for 2 days before being exposed to EM for a further 2 days and finally brought to the air–liquid interface to induce keratinocyte differentiation and cornification (see Materials & methods). Constructs repopulated with HDFs (ICX-SKN) and/or HKs (sSKN) both expressed keratin (Figure 5A). Involucrin, a specific marker of keratinocyte differentiation, was detected on ICX-SKN constructs after exposure to differentiation induction but was not detected in any constructs before differentiation induction. A low level of involucrin expression was observed on rehydrated sSKN constructs exposed to differentiation conditions onto which only keratinocytes had been seeded. ICX-SKN constructs (previously populated with HDFs) onto which keratinocytes were seeded displayed clear evidence of involucrin expression, stratification and cornification (Figure 5B). Constructs were examined for expression of laminin-5, a basement membrane

marker expressed by HKs in the presence of HDFs; only ICX-SKN constructs repopulated with HKs and HDFs demonstrated robust laminin-5 expression but stratification was observed in both types of construct (Figure 5C).

Preclinical study of ICX-SKN skin graft replacement

ICX-SKN skin graft replacement and rehydrated sSKN constructs were grafted onto full thickness excisions made on the dorsal surface in male nu/nu mice. In total eight of each graft type were applied and no adverse effects of the grafts to animals were observed over 28 days. All animals recovered well and integration of the grafts was noted in all cases (Figure 6A) after application of both graft types, and all wounds were completely healed by 28 days. Epidermis formed over the graft site. Although there was extensive contraction to the wound sites, no scarring or blistering was observed. It was felt that contraction was due to dehydration of the grafts during the remodeling period, resulting from the difficulty of maintaining hydration in the animal model. Care was taken during the clinical study on human volunteers to ensure that secondary dressings maintained graft hydration and wound contraction was not observed [9].

At each time-point, two animals from each experimental group were sacrificed and the graft and surrounding tissue was excised and analyzed by H&E tissue stain. ICX-SKN skin graft replacement constructs appeared to induce accelerated healing relative to rehydrated sSKN matrix as samples taken at 7 days postgrafting revealed that, whereas host epithelium had begun to migrate over the ICX-SKN skin graft replacement, there was no evidence of migration over the sSKN graft from the same time-point (Figure 6B, compare top right to top left). However, both types of graft were completely covered by epidermis after 28 days (Figure 6B bottom panels). In addition, the newly formed epidermis appeared to be bonded well with underlying ICX-SKN skin graft replacement matrix, without any gaps or blistering, suggesting that proper attachment of both layers was achieved.

The expression of laminin-5 and collagen-IV, both protein markers of basement membrane, was investigated in ICX-SKN grafts excised at day 28. In order to distinguish expression of these proteins in basement membranes present in the host tissue and expression over the grafted

material, HDFs used to repopulate ICX-SKN constructs were first labeled with Vybrant CM-Dil dye. Collagen-IV and laminin-5 were present in the membrane directly overlying the grafts at construct–host epithelia boundary (Figure 6C) and staining for these proteins was contiguous between ICX-SKN skin graft replacement matrix and host epithelium. In

addition, HDFs were detected at 28 days after graft application, suggesting persistence of applied cells in the healed wound (Figure 6C iii). Hairs or hair follicles around the graft on one of the subject animals (Figure 6A & C middle panel) were observed. However, their presence was most likely owing to variability in phenotypic expression in this strain and not as a result of the graft.

Figure 4. Characterization of ICX-SKN skin graft replacement.

(A) Stretch and strength characteristics of an example (freeze-dried and sterilized) sSKN constructs (N/seconds) on day 0 post repopulation with HDFs ($n = 51$). **(B)** Metabolic activity of example (i) dSKN ($n = 2$) and ICX-SKN constructs (ii) 0 days ($n = 51$) and (iii) 5 days ($n = 18$) in cold storage as measured by reduction of Alamar Blue™ (% reduction/hour) **(C)** Stress fibers in fresh HDFs (left) stained with phalloidin and nuclei (right) from fresh HDFs (large arrows) and original HDFs (small arrows) stained with DAPI in a cross-section of ICX-SKN skin graft replacement. Scale bar: 250 μm .
DAPI: Diamidinophenylindole; HDF: Human dermal fibroblast cells.

Discussion

ICX-SKN skin graft replacement can be regarded as a platform product: a matrix consisting largely of human collagen autotransformed by HDFs over a 7-week maturation period. In order to maximize the utility of this matrix, it is freeze-dried and

sterilized so that it can be stored under ambient conditions and can be regarded, essentially, as a raw material in the manufacturing process of the final product. The platform can be repopulated with living cells and used in clinical applications, one of which we have already described [9].

Table 3. Comparative analysis of strength and stretch characteristics of dSKN and repopulated sSKN constructs.

Formulation	Peak load (N)	Displacement at peak load breaking point (mm)
dSKN (n = 5)	0.60 ± 0.18	2.37 ± 0.44
sSKN (n = 13)	0.66 ± 0.11	3.83 ± 0.98

Data shown are the mean ± standard deviation.

A number of consultant surgeons in dermatology, plastic and reconstructive, and burns surgery were contacted in order to garner their expert opinion in concrete terms on the desirable characteristics of a skin graft replacement. Among the key desirable characteristics for such a product was persistent healing effect such that, although over time physiologic processes should remodel the application, the wound would be permanently closed. In addition, efficacy, ease-of-use and off-the-shelf availability were felt to be essential for the success of such an application. From the original research concept the product was designed and developed with this utility in mind.

Although many of the currently available cell therapy-based products were initially designed to work as skin grafts, in reality no current product meets the rigorous requirements necessary to accomplish this function [16,17]. Unlike some of these earlier products that were based on cells embedded in purified bovine collagen [17], ICX-SKN skin graft replacement is based on synthesis of collagen by HDFs embedded in a provisional matrix of human fibrin over a period of maturation. Earlier work by Tuan, Neidert and others demonstrated that in similar compositions, HDFs in bovine fibrin were induced to lay down collagen and other extracellular matrix materials [7,8]. As the construct matured, the original fibrin was augmented or replaced by collagen that became, or could be induced to become, cross-linked. We would distinguish ICX-SKN on the basis that the collagen produced by allogeneic HDFs embedded initially in the human fibrin gel is autotranscribed and thus more likely to resemble collagen laid down in the authentic wound-healing process. Ultimately, the extracellular matrix manufactured by HDFs as the construct matures lends the functional properties of tensile strength, elasticity and flexibility to the ICX-SKN constructs.

In order to maximize the clinical and commercial potential of the extensive maturation period, the manufacturing process that results in

the final product, ICX-SKN skin graft replacement, encompasses several processes in a discontinuous manufacturing cycle (Figure 7). The construct is cast and matured to produce pSKN, the first intermediate. The 7-week aseptic process during which HDFs were stimulated to lay down collagen and other extracellular matrix proteins posed several logistical hurdles:

- Extensive manipulation (feeding, etc.) exposed the nascent constructs to the possibility of contamination, so antibiotics were included in the feeding medium
- Since pSKN was produced by living cells the shelf-life would be limited if pSKN were the final product.

Thus, a process was designed in which pSKN was instead an intermediate in manufacture of ICX-SKN, whose final manufacture can be scheduled according to customer demand. The second intermediate, dSKN, is the result of freeze drying pSKN, allowing preservation of the matured matrix which could, upon γ -irradiation, be stored at ambient temperature as a raw material. Irradiation, to produce the third intermediate, sSKN, was made necessary by the presence in the pSKN manufacturing process of antibiotic, which is bacteriostatic rather than bacteriocidal, and together with aseptic processing in a qualified clean-room environment, ensured sterility of the entire batch. Together the processes of freeze-drying and irradiation consume 1 week. Finally, sSKN was rehydrated, washed and repopulated with fresh HDFs to produce ICX-SKN skin graft replacement, which was packaged and shipped to the clinic. Relative to the manufacture of pSKN, ICX-SKN is ready for shipment after 2 days of repopulation. The capacity to hold sSKN under ambient conditions together with accessibility to a fully qualified bank of HDFs results in product that can be quickly assembled when needed and can be available off-the-shelf to the end user. In addition, at several points, processes were rationalized to reduce manipulation of the product, for

Figure 5. Addition of keratinocytes to ICX-SKN skin graft replacement.

example, the choice of freeze-drying medium, TM, which requires no washing of the pSKN constructs prior to freeze drying. The conditions of packaging and shipping mean that the product can be applied directly from the package with no further preparation by the end-user.

We have demonstrated here in preclinical models and in first-in-man use [9] that the platform product is biologically acceptable and integrates well with host tissues and that host epidermis readily colonized grafts in both cases.

Contraction in the animal model was observed and contrasts with lack of contraction observed in the Phase I clinical trial [9]; although the relative size of wound created in the preclinical study was larger and hydration of wounds in animal models is technically difficult, perhaps this outcome also underlines the paucity of adequate animal models for the study of wound healing. The development of epidermis over the graft was apparently accelerated by the application of keratinocytes and HDFs to the graft, although grafts repopulated with keratinocytes alone eventually developed epidermis as well. In addition, we have demonstrated that keratinocytes applied *in vitro* colonize the platform and that specific protein markers of basement membranes, collagen IV and laminin-5, were expressed. Furthermore, expression of both proteins was observed at the site of graft in pre-clinical models. Although delamination was not observed in the mouse wound treated with ICX-SKN matrix at 28 days, delamination upon histological processing was evident in some of the clinical subjects [9], suggesting that the basement membranes were not fully formed at the time (1 month after grafting) that the grafts were excised. Therefore, further investigation will be required to determine if basement membranes truly develop *in vivo*. Furthermore, although we have described some of the physical properties of ICX-SKN and its intermediates, ICX-SKN is not fully characterized as to its physiological, biochemical and physical features. The degree of characterization presented here is, however, exactly consistent with the requirements expected of a product at this stage of its clinical development.

The uses of such a product include skin applications such as skin graft following excision of skin cancers (mostly basal and squamous cell carcinomas), following loss of skin volume due to trauma or disease, burns and scar revision. Applying epidermal keratinocytes to the collagen construct and allowing cornification to occur *in situ* could provide further strength, rigidity, and function, particularly in large-area defects.

Conclusion

The current ICX-SKN application has been designed as a platform product suitable for use in human clinical trials as a dermal replacement. A four-stage discontinuous manufacturing process provides convenience to both the manufacturer and the end-user and results in a product that is intended to be available off-the-shelf and

Figure 6. Preclinical study of ICX-SKN skin graft replacement.

Rehydrated sSKN or ICX-SKN constructs grafted onto full thickness excisions made on the dorsum of nu/nu mice were observed weekly over 28 days. **(A)** Gross appearance of sSKN (left panels) and ICX-SKN (right panels) grafts at 7 days (upper) and 28 days (lower). Scale bar: 1 mm. **(B)** H&E sections from excised sSKN (left panels) or ICX-SKN (right panels) grafts at 7 (top panels) and 28 (bottom panels) days; Scale bar: 500 μ m. **(C)** ICX-SKN constructs repopulated with Di-I-labelled HDFs (left panels, red), were excised 28 days after grafting and stained for expression of collagen-IV (top panels) or laminin-5 (bottom panels). Arrows indicate the epidermis overlying the Di-I-labelled HDFs of the graft. Control sections were stained with secondary antibody only (right panels). Scale bar: 250 μ m.

d: Mouse dermis; e: mouse epidermis; HDF: Human dermal fibroblast cells; H&E: hematoxylin and eosin stain; SK: SKN graft matrix.

to provide ease-of-use to the clinician. At each stage of its development and manufacture, ICX-SKN and its intermediates have been assessed for characteristics that include functional assays indicating its suitability as a clinical trial material. This first iteration of the product lacks barrier function and is intended to be used as a dermal template under conditions where the patient's own epidermis would grow over the dermal graft, such as small excision wounds [9], and to provide contour where tissue has been lost due to trauma or disease. However, we have shown in the current study through the application of keratinocytes and using preclinical models, that this product may eventually be applied as a fully bilayered skin substitute. In addition, the current study suggests the potential for this platform to be used in further indications by the application of different functional cell types.

Future perspective

ICX-SKN skin graft replacement has been designed with the end-user in mind: the end-user expects an efficacious product but also desires a product that will be easy to use, without complicated preparative steps prior to its application, and off-the-shelf availability. Even in its current iteration, ICX-SKN is a complex product with a long, multistep manufacturing cycle. Marrying the expectations of the customer with the practicalities of manufacturing a living product is one of the continuing challenges for regenerative medicine.

Further development of ICX-SKN and products like it will require input of expertise from many disciplines, particularly engineering, to sustain the scale-up and scale-out that will be necessary to commercialize cell-based therapeutic products. However, engineering must understand and accommodate the particular demands of making, storing and shipping a living product such that the essential characteristics that provide the therapeutic benefit are maintained. In addition, the manufacturer of these living products will need to negotiate, and perhaps drive, the complex regulations that govern the use in humans of these therapies and applications.

We have described a manufacturing cycle that is very much based in developments made in the R&D environment. One of the hallmarks of regenerative medicine is that throughout the investigational use of this platform, and its derivatives, the product and the process will be subject to iterative cycles of development. With each iteration, manufacture must be made more cost-effective and streamlined without compromising

Figure 7. Process flow of ICX-SKN production.

Stages of production are marked by numbers and points of exit/entry to GMP are indicated by dashed lines. Casting of the constructs within GMP facility occurs at stage 1: Reagents are chilled, mixed sequentially and cast immediately upon the addition of thrombin as described (Materials and Methods). Maturation occurs at stage 2, where constructs are fed three times per week with TM for 7 weeks. Matured constructs (pSKN) are freeze dried to preserve the matrix (stage 3 – dSKN), sterilized (stage 4 – sSKN) within 1 week postcollection of pSKN, and finally reintroduced to GMP to be repopulated with HDF (stage 5 – ICX-SKN) over 2 days and packaged, producing the final product for use in the clinic.

GMP: Good manufacturing practice; HDF: Human dermal fibroblast cells; TM: Collagen-boosting medium.

on safety or value to the customer. Effective research translation depends on the realization that a research concept has a long journey before it is suitable for clinical application.

Finally, for a product to be successful will require solution of the three-way equation – what science can achieve, what the regulations allow and what the market will bear.

In memoriam

David Shering passed away on October 27th 2007 from a sudden illness. He was 29. He made a major contribution to the development of ICX-SKN and the rest of the team would like to dedicate this paper to his memory. He will be sorely missed.

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All authors apart from D Marshall are current employees of Intercytex and as such hold either stock or stock options in the Company. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Executive summary

ICX-SKN is a platform human dermal matrix autosynthesized by human dermal fibroblasts initially embedded in a provisional fibrin matrix.

Discontinuous manufacturing process was designed to provide ready inventory for off-the-shelf availability and ease-of-use for the end user.

Dermal matrix platform remained cell friendly through several intermediate processes in the manufacturing process and was well integrated in host models *in vivo*.

Potential for this application to be further developed with the addition of keratinocytes to form a fully bilayered product skin equivalent.

Ethical conduct of research

The author states that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all

human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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