Clinical Impact of Cryopreservation on Split Thickness Skin Grafts in the Porcine Model

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Vital, genetically engineered, porcine xenografts represent a promising alternative to human cadaveric allografts (HCA) in the treatment of severe burns. However, their clinical value would be significantly enhanced if preservation and long-term storage—without the loss of cellular viability—were feasible. The objective of this study was to examine the direct impact of cryopreservation and the length of storage on critical in vivo and in vitro parameters, necessary for a successful, potentially equivalent substitute to HCA. In this study, vital, porcine skin grafts, continuously cryopreserved for more than 7 years were compared side-by-side to otherwise identically prepared skin grafts stored for only 15 minutes. Two major histocompatibility complex (MHC)-controlled donor–recipient pairs received surgically created deep-partial wounds and subsequent grafting with split-thickness porcine skin grafts, differentiated only by the duration of storage. Clinical and histological outcomes, as well as quantification of cellular viability via a series of 3-4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assays, were assessed. No statistically significant differences were observed between skin grafts cryopreserved for 15 minutes vs 7 years. Parametric distinctions between xenografts stored for short- vs long-term durations could not be ascertained across independent clinical, histological, or in vitro evaluative methods. The results of this study validate the ability to reliably preserve, store, and retain the essential metabolic activity of porcine tissues after cryopreservation. Plentiful, safe, and readily accessible inventories of vital xenografts represent an advantageous solution to numerous limitations associated with HCA, in the treatment of severe burns.

Severe deep partial- (second degree) and full-thickness (third degree) burns are devastating, complex, and unforgiving injuries that widely affect civilians, first-responders, and military personnel. They occur unexpectedly, represent the majority of burn-related deaths,[1] and unfortunately remain far too prevalent today.[2–6]

It is widely accepted that the clinical treatment of severe burns requires early excision and subsequent grafting to provide the necessary wound closure.[7–10] Grafting to achieve temporary wound closure has several advantages: it prevents dehydration;[11] reduces loss of body heat, electrolytes, and pH homeostasis;[12] reduces infection;[13] and relieves pain by covering nerve ends.[7,14] Successful employment of this clinical modality is credited with remarkably increasing overall survivability of severe burns.[9,11,15–17]

The "gold standard" for temporary wound closure is human cadaver allograft (HCA), recognized to effectively reduce wound sepsis; reduce water, electrolyte, and protein losses; and simultaneously increase the comfort and well-being of the patient.[4,18–20] The fundamental characteristic that affords HCA such efficacy and permits such clinical impact is the inclusion of living cells and retention of overall tissue viability. Without intervention, skin loses up to 50% of its viability within the first 24 hours,[3,15,21] and as a result the American Association of Tissue Banks recommends "skin should be collected within 18 hours after death. ..."[22] The notion of "just-in-time" procurement of donor-derived, human skin is a logistical impossibility.[22] Thus, rapid preservation and efficient storage of HCA and equivalent materials are essential to adequately treat patients who endure severe and extensive burn wounds.

Fortunately, skin is unique among organs in its suitability for cryopreservation and extended storage. Preservation of skin by programmed freezing at a controlled rate (1°C/ min), followed by storage at a low temperatures (–80°C), and use of penetrative

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cryoprotectants such as dimethyl sulfoxide (DMSO) have been shown to protect morphology and tissue structure, and retain metabolic activity levels comparable to that of fresh skin.[4,23] Low mass and a naturally flat conformation result in permissive heat transfer mechanics; this allows accurate measurement of temperature, essential to successful processing. Thus, cryopreservation has emerged as an attractive method to achieve long-term storage for HCA,[24–26] and as a result, dedicated skin-banking facilities and techniques to preserve HCA have been established worldwide over the past several decades.[24,26–30]

Despite these advances to enhance HCA availability, many areas of the world still experience significant shortages.[31–35] Even countries with robust healthcare infrastructure remain vulnerable to large-scale, mass-casualty events which can rapidly overwhelm available supplies.[28,30,36,37] Resource and time intensive requirements for safe procurement, testing, and banking of human tissues, combined with limited numbers of medically suitable organ and tissue donors,[38,39] and global, cultural, and religious beliefs,[7,11] result in a deficit of available HCA around the world.

Patients with severe and extensive deep-partial and full-thickness burn wounds are in immediate need of alternative temporary treatment options with a similar mechanism of action that offer enhanced safety without sacrificing clinical efficacy. Potential biological or prosthetic interventions intended as life-saving alternatives to HCA must possess two critical characteristics. First, "adherence" to the wound bed is the most important factor to the survivability of the graft and is a pre-requisite for vascularization,[40] which permits functional wound closure and the necessary restoration of lost integumentary barrier function.[41] This, however, necessitates the inclusion of living viable cells.[20,25]

Cellular viability can be reliably measured via the enzymatic reduction of MTT ([3-4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) into a formazan metabolite.[42,43] This method is commonly used in industry to assess the viability of skin products and is widely cited in literature.[17,18,21,25,44,45] Additionally, a plausible alternative to HCA should also possess an adequate "shelf-life" that would allow for clinically meaningful durations of storage, affording the accumulation of sufficient inventories for both routine use and also disaster preparedness, with the ability for transport worldwide.

A promising candidate as an alternative to HCA is the use of skin from animal sources.[46–48] Morphological and physiological similarities to human skin at the microscopic level[46,49] cause vital swine skin to be especially promising, especially with the inclusion of immunologically favorable genetic alterations,[50] recently made more plausible and efficient with advanced technologies and techniques. Porcine xenografts have been reported to have similar qualitative properties to allografts,[7,46,48,51] and since the 1960s, have gained increasing acceptance,[7] becoming the most widely used source of xenografts today.[3]

However, the storage and cryopreservation of porcine skin have not been fully characterized, especially with regard to viability, as most porcine xenografts are intentionally devitalized, or "fixed" with glutaraldehydes or radiation treatment.[7,48,52] Such information is necessary to support the use of vital porcine skin grafts—or porcine skin transplants—as a temporary and clinically advantageous option in the treatment of severe burns.

Previously, we demonstrated that no distinguishable or statistical difference exists, in terms of overall outcome and time of graft adherence before immune-mediated rejection, between freshly procured (nonpreserved) and cryopreserved porcine grafts.[53] In this study, we specifically examine the isolated effects of long-term cryopreservation and storage on clinically useful metrics.

Absolute durations of graft survival are intentionally not the present focus. Instead, direct comparisons between otherwise equivalent materials are examined for meaningful, differential times of survival based solely on the duration of storage, holding all other factors constant. Side-by-side, in vivo evaluations are performed between equivalent grafts, preserved in identical fashion, and stored for periods of 15 minutes vs 7 years. Clinical gross assessments and photographs, paired with independent histological assessments, determine whether any appreciable differences in graft survival exist relative to the length of time in the frozen state. In tandem, separate in vitro assessments of graft viability, quantified by MTT-reduction assays, characterize the metabolic activity of cells post-cryopreservation and various storage terms. Furthermore, independent histomorphological analysis, using standard histological (H&E) staining, provides evidence as to whether these processes cause observable changes to the graft material at a structural level.

METHODS

Ethics Statement

This study was conducted in accordance with an Institutional Animal Care and Use Committee approved protocol (2005N000279, Amendment 69) at the Center for Transplantation Sciences at the Massachusetts General Hospital (Boston, MA), and in compliance with the United States Department of Agriculture's (USDA) Animal Welfare Act (9 CFR Parts 1, 2 and 3), the Guide for the Care and Use of Laboratory Animals, and all state and local laws and regulations. Study protocols, surgical procedures, and animal care guidelines were independently reviewed and monitored by a standing Institutional Animal Care and Use Committee at the Massachusetts General Hospital (Boston, MA).

Animals

A total of 13 swine were used in this study. Four served as donor-recipient subjects in the in vivo, surgical experimental series. Separately, four additional swine served solely as tissue source donors in 2009 and 2010. These eight swine were members of an inbred, MHC-swine leukocyte antigen (SLA) fixed, miniature swine colony[54] at the Massachusetts General Hospital (Boston, MA) (Table 1). At the time of surgery, the four swine that served as donor-recipient pairs were between 10 and 20 kg in total body weight and between 2 and 4 months of age. Immunosuppression regimen(s) were not administered at any time during this experiment. Subjects assigned to Cohort I represented a matched MHC SLA-Class I and SLA-Class II donor–recipient pair. Subjects assigned to Cohort 2 represented a matched MHC SLA Class I, but mismatched SLA Class II donor–recipient pair. Two of the four tissue source donors were

Table 1. Genotype, Swine Leukocyte Antigen (SLA) designations for MHC Class I and II, and date of surgery for all eight swine subjects used in the entire in vivo, surgical experimental series

Animal Number	Genotype	SLA	SLA MHC Class I (allele 1, allele 2)	SLA MHC Class II (allele 1, allele 2)	Date of Surgery
18671	GalT-KO	DD	d,d	d,d	September 2009
19189	GalT-KO	DD	d,d	d,d	November 2009
18811	Wild-Type	DD	d,d	d,d	September 2009
19198	Wild-Type	DD	d,d	d,d	February 2010
24074	Wild-Type	AD	a,d	a,d	March 2017
24075	Wild-Type	AD	a,d	a,d	March 2017
24043	Wild-Type	KK	d,d	c,c	March 2017
24070	Wild-Type	LL	d,d	a,a	March 2017

SLA designations are nomenclature developed by Dr. David Sachs, formerly of the Translational Biology Research Center (TBRC) of the Massachusetts General Hospital (MGH). GalT-KO indicates swine alpha 1,3-Galactosyltransferase knockout.

alpha-1,3 galactosyltransferase knockout (GalT-KO) genetically modified swine; the remaining animals used in the in vivo, surgical experimental series were wild-type swine from the same colony. These were included in the study design to observe any impacts attributed solely to the genetic modification.

Separately, for the in vitro, MTT series of analyses, five additional wild-type Göttingen miniature swine provided tissues for positive and negative controls.

Surgical Procedure—Split Thickness Skin Graft Procurement From Porcine Donors

Swine donors were anesthetized with intramuscular injection of 2 mg/kg telazol (tiletamine HCl and zolazepam HCl, Zoetis Inc., Kalamazoo, MI) and brought to the operating room for orotracheal intubation. Anesthesia was maintained using 2% isoflurane and oxygen. Skin surfaces were disinfected before surgery with chlorhexidine acetate (NolvasanR Surgical Scrub, Fort Dodge Animal Health, Fort Dodge, IA) and povidone-iodine, 10% (Betadine Solution, Purdue Products, L.P., Stamford, CT). The animals were then draped, leaving the right side of the dorsum exposed. Split-thickness skin grafts, measuring approximately 25 cm^2, were harvested between the scapula and inferior margin of the lowermost rib from each animal using an air-driven Zimmer dermatome (Medfix Solution, Inc., Tucson, AZ).

Surgical Procedure—Deep-Partial Wound Creation to Model Severe Burn Injury

Deep-partial wound defects were surgically introduced via additional passes with the dermatome after the initial split thickness graft harvest. Each subject received four deep-partial defects along the animal's right dorsum, in a linear (caudal to cranial) orientation, ordered from 1 to 4, respectively. The resulting 5×5 cm wound beds were uniform, free of visible debris, and intentionally interrupted, not made in a single continuous pass with the dermatome. Instead, care was given to create four, isolated but equivalent deep-partial wounds that would serve to model a severe burn injury.

Cryopreservation Procedure—Split-Thickness Skin Graft Storage

Following procurement, autografts intended as short-term storage controls were prepared via standardized institutional protocol, cryopreserved, and maintained at −80°C for a period of 15 minutes. Grafts used in the experimental groups were processed using the same institutional protocol and were stored at −80°C for a period of 15 minutes or continuously for more than 7 years. Freeze media was prepared by combining 15% DMSO cryoprotective media (Lonza BioWhittaker) with fetal porcine serum (FPS) or donor serum (if FPS is unavailable) in a 1:1 ratio, filtering (0.45 micron), and chilling to 4°C before use. Cryopreservation was achieved via a controlled rate, phase freezer at a rate of 1°C per minute to −40°C, and then rapidly cooled to a temperature of −80°C, before storage for the respective durations.

Surgical Preparation of Split-Thickness Skin Grafts Before Surgical Engraftment

To thaw grafts before surgery, sealed vials were placed in 37°C water baths for approximately 1 minute. The frozen graft was removed using sterile technique, and underwent three, 1-minute serial washes in normal saline with gentle agitation, in order to dilute and systematically remove ambient, residual DMSO and prevent loss of cell viability. Grafts were then taken to the surgical field in normal saline at ambient temperature.

Surgical Procedure and Experimental Design

Two separate, otherwise identical surgical events were performed in succession. The surgical plan included donor–recipient swine pairs with four wound beds each for a total of 12 experimental grafts and 4 technical controls. Animals were assigned to one of two experimental cohorts (Cohort 1 and Cohort 2) based on SLA-type as described previously. In total, sixteen (n = 16) grafts were placed. Four (n = 4) cryopreserved autografts served as internal, technical controls for the surgical cryopreservation, and thawing techniques (Wound Sites 1 most-caudal). Four (n = 4) allografts that had been cryopreserved and stored for 15 minutes (Wound Sites 2) and eight (n = 8) allografts that had been cryopreserved and stored for 7 years (Wound Sites 3 and 4) (Figure 1). Allografts at Wound Sites 3 and 4 were otherwise identical, except for the removal of the alpha-1,3-gal (Gal)-epitope via genetic knockout in the grafts located in each animal at Wound Site 4. Inclusion of GalT-KO

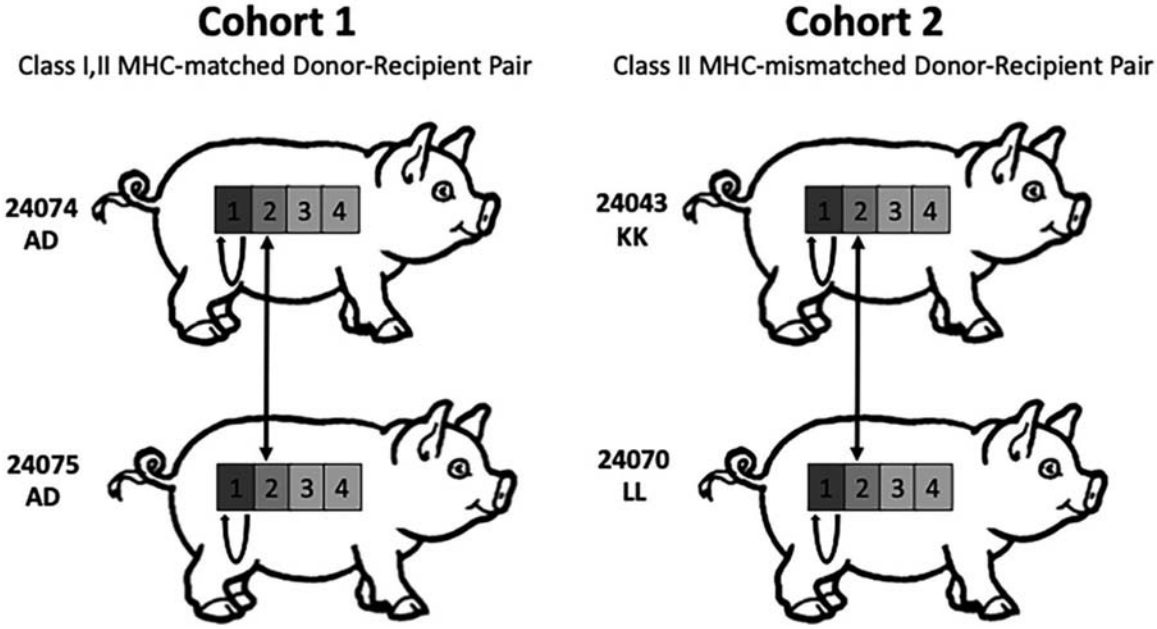

Figure 1. Overall experimental design and surgical schematic for donor–recipient surgical pairs in Cohort 1 and Cohort 2. Animals 24074 and 24075 were assigned to Cohort 1 and represented a MHC-matched donor–recipient pair. Animals 24043 and 24070 were assigned to Cohort 2 and represented a MHC Class I matched, MHC Class II mismatched donor–recipient pair. Each animal received four deep-partial wound defects along the animal's right dorsum, in a linear (caudal to cranial) orientation ordered from 1 to 4, respectively. At Wound Site 1 (most caudal), a split-thickness autograft was placed, serving as a technical control. At Wound Site 2, a split-thickness allograft from its respective cohort pair-mate was sutured into place, representing allografts exposed to cryopreservation for a limited duration (15 min at –80°C). At Wound Sites 3 and 4, split-thickness grafts, with identical SLA designation as those at Wound Site 2 were placed, representing allografts that had experienced "extended" storage in the cryopreserved state (more than 7 yr at –80°C). Wound Sites 3 were wild type grafts; Wound Sites 4 were GalT-KO grafts.

graft materials served two purposes. First, this both increased the number of experimental allografts stored for 7 years or greater, but also served to eliminate any possible differences existing as a result of the genetic modification in regards to cryopreservation.

Before engraftment, all split-thickness skin grafts were fenestrated to prevent seroma or hematoma formation. Graft test articles were independently placed on the prepared wound beds and uniformly sutured in place using simple interrupted, 3-0 nylon sutures, applied in a graft-to-wound bed manner. Approximately 16 points of fixation were introduced, spaced evenly around the graft, with the resulting knot located on the wound border, not the graft article. This technique ensured that adequate tension across the graft was present and uniform, necessary for optimal graft survivability.

Postoperative Surgical Care

Total postoperative observation was 20 days. Overlying pressure dressings, consisting of Xeroform petroleum gauze (Medtronic), Telfa nonadhesive dressing (Covidien, Minneapolis, MN), and sterile gauze were maintained in place and dry with multiple, overlapping sheets of Tegaderm (3M, St. Paul, MN). Recipients were then dressed with cotton jackets to reduce interference with the grafts. Graft dressings were removed on Post-Operative Day (POD) 2 and changed daily thereafter. All sutures were removed by POD-7. Animals were monitored for signs of pain including vocalization, tachypnea, loss of appetite, and changes in attitude, behavior, and mobility. Transdermal fentanyl patches were applied for postoperative analgesia.

Postoperative Assessment of Graft Survivability

At the time of each dressing change, all grafts were photographed, inspected, and characterized for signs of viability and integrity, such as graft adherence to the underlying wound bed (i.e., graft "take"). Adverse conditions such as hematoma, erythema, necrosis, or other visible evidence of immunologically mediated rejection were recorded.

The primary outcome measure for the in vivo evaluations was days of graft survival, assessed by both direct clinical observation and separately via blinded, photographic assessment by the Senior Author. Complete rejection of the graft was considered to occur when less than 10% of viable graft tissue covered the wound.[55–57]

Two 3-mm punch biopsies from each graft were obtained before redressing at each observation and were fixed in neutral buffered formalin and frozen in liquid nitrogen with optimum cutting temperature (OCT) gel. Care was taken to ensure all biopsy sites were sufficiently remote from border of the graft and surrounding wound bed, and that biopsy specimens included both graft and wound bed tissue layers. Hematoxylin and eosin (H&E) slides were evaluated for immunological graft rejection by a blinded, third-party pathologist, graded via the Banff 2007 Working Classification

of Skin-Containing Composite Tissue Allograft Pathology criteria and scale.[53,57,58]

Separately, comparative analysis of tissue sections that had been cryopreserved and stored for 15 minutes vs those stored for 7 years were evaluated for morphological or structural changes caused by the freeze-thaw process and varying durations of storage in the cryopreserved state.

Assessment of Graft Viability Via MTT-Reduction Assay

To complement the clinical, histological, and morphological analyses, a series of in vitro MTT-reduction assays were performed to evaluate the residual viability of porcine grafts after cryopreservation and long-term storage (Charles River Laboratories Edinburgh LTD, Tranent, East Lothian, UK). Mitochondria reduce MTT into a formazan metabolite which can be observed as a purple hue. Harnessing this phenomenon, an analysis of changes in optical density values measured by a spectrophotometer, can provide differential assessments of cellular viability, between experimental samples and positive and negative controls.

Four additional split-thickness grafts, procured from the same source animals and identically processed and stored alongside those used in the in vivo surgical experiments, were evaluated for mitochondrial activity. Positive control values were established using five samples of fresh skin tissue, procured from five wild-type Göttingen miniature swine, obtained at the time the assay was performed. Assay results from these tissues would represent levels corresponding to 100% cellular viability. Similarly, negative control values were established using five separate skin tissue samples, procured from the same source animals as the positive controls, but were then heat deactivated via boiling in distilled water for a minimum of 30 minutes. Assay results from these tissues would represent levels corresponding to 0% cellular viability. This entire testing schema was performed twice, yielding positive and negative controls for Series 1 and Series 2.

All samples measured approximately 1 cm^2 and were approximately 0.022 inch (0.55 mm) in thickness. Area and thickness of each sample (experimental and controls) were verified by micrometer for consistency and weights of each sample were recorded.

A solution of MTT was created by combining 0.3 mg/ml of MTT ([3-4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, Sigma–Aldrich Company Ltd., Gillingham SP8 4XT, United Kingdom), with 0.5 mg/ml Dulbecco's modified Eagle Medium (DMEM) (Life Technologies Ltd, Paisley PA4 9RF, United Kingdom). Incubations were performed in amber microcentrifuge tubes containing MTT solution (0.3 mg/ml in DMEM; 0.5 ml). Reactions were initiated by the addition of the test (or control) samples and continued for 180 ± 15 minutes, at 37°C, in an atmosphere containing 5% carbon dioxide. Tissues were then dried on absorbent paper and transferred to fresh microcentrifuge tubes with acidified isopropanol (0.8 ml), where formazan was extracted for 24 hours under controlled (limited) light conditions. 200-µL aliquots were transferred to 96-well plates for optical absorbance analysis via spectrophotometer at 550-nm, with 630-nm serving as the optical reference standard. Each measurement was performed in duplicate.

Statistical Analysis

All data are expressed as means +/– the standard deviation (geometric mean was calculated, where indicated). In assessment of graft survival between the allografts of a limited storage duration (15 minutes) and allografts of an extended storage duration (7 years), we estimated the mean difference in the number of days of survival between the groups, and the effect of histological vs clinical assessment using a linear mixed effect model with a random intercept for each animal.

For the reduction assay, statistical significance between comparative sample test groups of independent conditions was assessed via use of the Mann–Whitney *U* test. Statistical significance was considered for $\alpha = .05$, two-tailed test, ($P < .05$). Statistical analysis was performed using Microsoft EXCEL (2011) software.

Reported analytical values reflect the arithmetic average of the replicates for each test sample, for parameters of weight (mg) and formazan production (mg/ml). Formazan production was determined as a function of absorbance at 550-nm measured via spectrophotometer and then interpolated from individual calibration curves established before sample measurement. Fresh samples, test articles, and heat denatured, negative controls were analyzed immediately after harvest (or thaw).

RESULTS

Gross Clinical Assessment

Both donor-recipient pairs tolerated the surgical procedure and recovered fully without incident. All sixteen (n = 16) grafts revascularized without evidence of technical complication and uniformly exhibited adherence to the underlying wound bed (i.e., "good take") (Table 2). Over the course of the postoperative observational period, no grafts were affected by mechanical disturbance or infection.

All four (n = 4) autografts at Wound Site 1, in Cohorts 1 and 2, healed permanently and were indistinguishable from surrounding tissues at the respective study end point.

In Cohort 1, all six (n = 6) allogeneic grafts demonstrated equivalent adherence to the underlying wound bed and exhibited clinical signs of vascularization and perfusion. However, grafts that had been stored for 7 years appeared comparatively paler than allograft comparators stored for 15 minutes. This initial presentation resolved fully, in all grafts and both subjects, by POD-6.

Mild sloughing of the superficial epidermis was exhibited by all allografts on POD-8, but remained otherwise adherent and appeared healthy at inspection on POD-12. In one subject, all grafts were equally rejected on POD-14, regardless of duration of prior storage. In the second subject, beginning on POD-14, grafts at Wound Sites 2 and 3 showed initial signs of necrosis, progressive erythema, and loss of adherence, until final rejection on POD-18 (Figure 2). This subject not only showed prolonged survival of two grafts, but was also the only instance of demonstrated intrasubject variation

Table 2. Postoperative days of graft survival, as determined by surgeon's clinical gross assessment, for both Cohort 1 (MHC Class I and Class II Matched, Donor–Recipient Pair) and Cohort 2 (MHC Class I Matched, MHC Class II Mismatched, Donor–Recipient Pair)

Cohort 1 MHC Class I & Class II, Matched Donor-Recipient Paired Swine	**Clinical Assessment of Postoperative Graft Survival**			
Wound Site	1	2	3	4
Duration of Cryopreservation	15 min	15 min	>7 yr	>7 yr
Graft Type	Autograft	Allograft	Allograft	Allograft
Subject 24074 Graft Survival (in Days)	End of Study	18	18	14
Subject 24075 Graft Survival (In Days)	End of Study	14	14	14
Cohort 2 MHC Class I & Class II, Matched Donor-Recipient Pair MHC Class-I & Class II Mismatched Donor-Recipient Paired Swine	**Clinical Assessment of Postoperative Graft Survival**			
Wound Site	1	2	3	4
Duration of Cryopreservation	15 min	15 min	>7 yr	>7 yr
Graft Type	Autograft	Allograft	Allograft	Allograft
Subject 24043 Graft Survival (in Days)	End of Study	8	8	8
Subject 24070 Graft Survival (in Days)	End of Study	10	10	10

Graft survival ended on the date of observation when less than 10% of the original graft showed signs of adherence and vascularity.

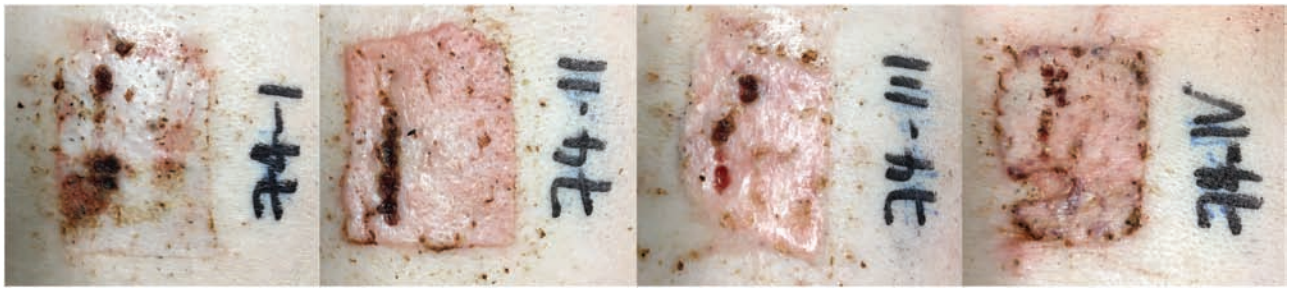

Figure 2. Subject 24074, Cohort 1, at POD-12. All grafts, irrespective of duration of storage, demonstrate equivalent vascularity, adherence, and survival at POD-12. From Left to Right: Side-by-side porcine split-thickness skin grafts at Wound sites 1, 2, 3, and 4, respectively.

in graft survival times. In this same subject, on POD-14, the graft located at Wound Site 4 (most-cranial) was conspicuously darker than those at Wound Sites 2 and 3, and ultimately survived until POD-18, when it exhibited signs of complete necrosis and was assessed to be clinically rejected (Figure 3).

In Cohort 2, all grafts presented similar clinical signs and uniformly displayed signs of rejection as those in Cohort 1, but at an accelerated pace. The initial pale appearance of the grafts cryopreserved for 7 years was also observed, resolving by POD-6. All grafts, in all subjects, demonstrated evidence of perfusion and adherence on POD-6. In one subject, all three allogeneic grafts were rejected by POD-8, and by POD-10 the remaining three grafts in the second subject were also necrotic (Figure 4). All grafts in Cohort 2, on an intrasubject basis, survived for the same duration, irrespective of the genetics or length of storage.

Quantities of Formazan Produced From Fresh (Non-Cryopreserved Samples) Were Statistically Greater Than Quantities of Formazan Produced From Heat-Denatured (Boiled) Samples

In the in vitro experiments, Series 1, the average formazan produced from fresh samples was 0.221 ± 0.022 mg/ml and the average of formazan produced by heat-denatured samples was 0.094 ± 0.020 mg/ml. In Series 2, the average formazan produced from fresh samples was 0.300 ± 0.035 mg/ml and the average formazan produced by heat-denatured samples was 0.105 ± 0.009 mg/ml. In both Series 1 and Series 2, there was a statistically greater amount of formazan produced in fresh vs heat-denatured samples ($P < .05$) (Figure 5a).

Quantities of Formazan Produced From Tissues Cryopreserved and Stored for 7 Years Were Statistically Greater Than Quantities of Formazan Produced From Heat-Denatured (Boiled) Samples

Using identical testing methods as the positive and negative controls, the average formazan produced from the four experimental samples (n = 4) was 0.281 ± 0.068 mg/ml (Figure 5b). When compared using a nonparametric statistical method (Mann–Whitney *U* test), no statistically significant difference was found between the fresh samples (n = 10) and those tissues that had been cryopreserved for more than seven years (n = 4) (Figure 5c). However, when applying the same methodology to compare the heat-deactivated samples (n = 10) and those tissues that had been cryopreserved for more than 7 years (n = 4), there was a statistically significant difference in

levels of formazan production ($P < .05$) (Figure 5d).

Lastly, comparing the formazan produced by the tissues that had been cryopreserved for more than 7 years, no meaningful statistical difference between the metabolic activity demonstrated by wild-type tissues (n = 2) vs GalT-KO tissues (n = 2) could be determined.

Histological Assessment

Overall, histological assessments closely mirrored the clinical findings (Table 3). Following surgery, all sixteen grafts exhibited early signs of acute inflammation during initial observations on POD-2, four of which later resolved with time. Similar to the clinical presentation, allografts in Cohort 2 uniformly exhibited histological evidence of an accelerated immune-mediated rejection process as compared to those in Cohort 1 (Figure 6). Histologically, all grafts, in Cohort 1 and 2, on an intrasubject basis, survived for the same duration irrespective of the genetics or length of storage. The six (n = 6) allogeneic grafts in Cohort 1 and three allogeneic grafts (n = 3) in Cohort 2 demonstrated histological evidence and microscopic signs of rejection coterminous with the clinical observations (Figure 7). The three allografts from Cohort 2 that clinically survived until POD-10 each received Banff scores of 4 but were not histologically assessed to be rejected until POD-12. The allograft at Wound Site 4, in Cohort 1, consistent with the clinical evidence of rejection 4 days before its counterparts, demonstrated concurrent histological evidence of rejection at POD-14 (Figure 8).

Independent pathologist assessments of tissues of short- vs long-storage duration demonstrated no observable morphological changes or structural disruption of the cellular organization, or appreciable histomorphological differences between the two groups. Routine H&E stained sections from the cryopreserved allografts that had been stored for 7 years showed viable normal skin with intact epidermis. The blood vessels and adnexal structures were also normal (Figure 9).

Overall, using a linear, mixed effect model with random intercept, the mean survival of grafts at Wound Site 3 was 0.00 (95% CI: −1.10, 1.10 days) less than allografts at Wound Site 2. The mean survival of grafts at Wound Site 4 was 2.00 (95% CI: 1.10, 3.10 days) less than allografts at Wound Site 2. Histological assessment found, on average 0.5 days more survival than grafts assessed grossly, but this was not statistically distinguishable ($P = .28$).

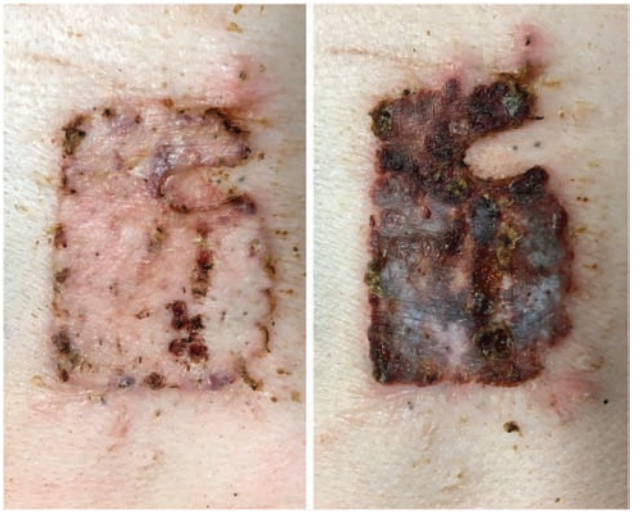

Figure 3. Subject 24074, Wound Site 4. Left: POD-12, Right: POD-14. Cryopreserved, split thickness porcine skin graft on partial thickness wound defect, demonstrating rapid progression of immune-mediated rejection and dramatic presentation of necrosis within 48 hours, assessed to be fully rejected at POD-14.

DISCUSSION

Protocols and techniques of cryopreservation are designed and continually optimized to minimize disruption to the structure, cell morphology, organization, and extracellular matrices. Despite such efforts, disruption cannot be completely eliminated or avoided. The aim of this series of experiments was to investigate whether, following cryopreservation, the duration of storage in the frozen state would affect the outcome of surgically ingrafted split-thickness grafts, specifically those obtained from porcine donors.

We were advantaged by the availability of materials that had been stored, uninterrupted, for extended time, along with the associated surgical records and existing institutional protocols. Furthermore, processing methods and protocols between the comparative groups were standardized, and identically applied with respect to cryopreservation and thawing protocols, reagents, and preparation methods employed. Combined, this allowed for isolated side-by-side evaluation of the effects due to the duration of storage, and alleviated the need to model or extrapolate findings, or otherwise use predictive methods.

Figure 4. Subject 24070, Cohort 2, at POD-10. All allografts are considered to be fully immunologically rejected, as assessed by gross clinical observation and independent, histological evaluation. From Left to Right: Side-by-side porcine split-thickness skin grafts at Wound sites 1, 2, 3, and 4, respectively. Cryopreserved autograft (wound site 1/technical control) healed fully without complications. Equivalent adherence, vascularity, and survival rates observed for all three allografts, indistinguishable between those cryopreserved for limited duration (15 min) or those stored for an extended duration (>7 yr). (Note: Skin pigmentation of porcine recipient 24070 was naturally, phenotypically dark.)

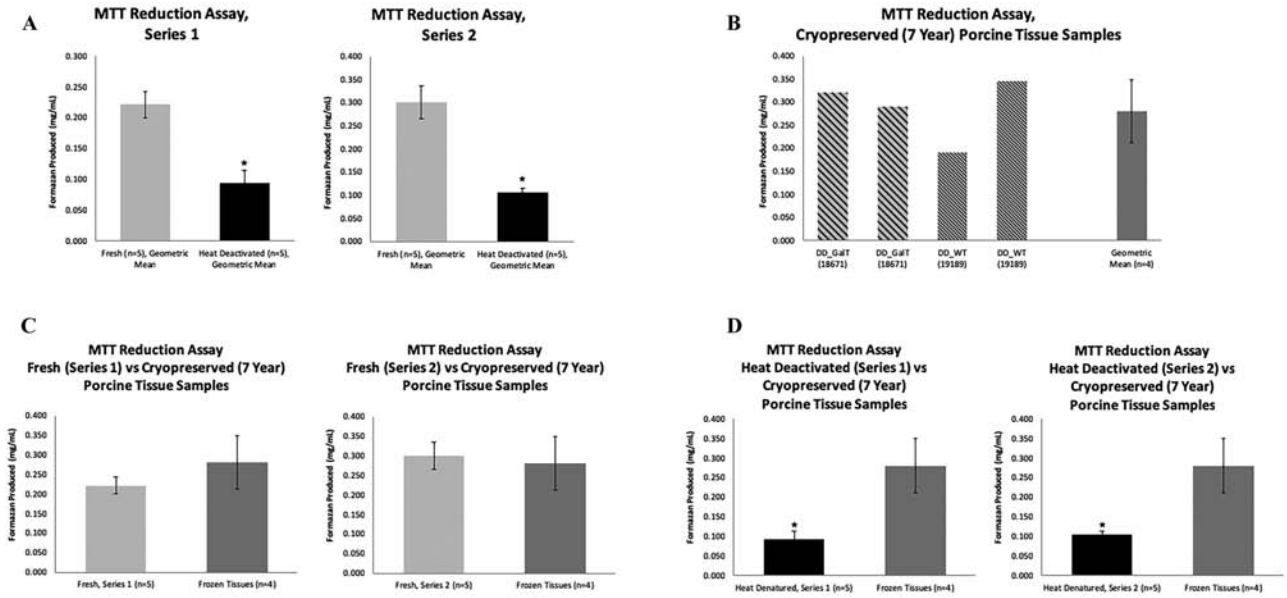

Figure 5. (a) Quantities of formazan metabolite produced independently during MTT-reduction assays, representing mitochondrial metabolic activity as a surrogate indicator of cellular viability of porcine skin tissues. During two separate evaluations, fresh tissues (positive controls) and heat-denatured tissues (negative controls) were compared. Statistically significant differences in formazan production were observed in both independent groups, Series 1 and 2. (b) Quantities of formazan metabolite produced independently during MTT-reduction assays from split-thickness experimental grafts, cryopreserved via standard protocol, and stored without interruption for >7 years. (c) Fresh tissues from Series 1 and Series 2 were compared against the four experimental grafts that had been cryopreserved and stored for more than 7 years. No statistical difference in the quantity of formazan produced during MTT-reduction assays was observed. (d) Heat-denatured tissues from Series 1 and Series 2 were compared against the same four experimental grafts that had been cryopreserved and stored for more than 7 years. A statistically significant difference in quantity of formazan produced was observed.

Table 3. Postoperative days of graft survival, as determined by pathologist's morphohistological assessment, for both Cohort 1 (MHC Class I and Class II Matched, Donor–Recipient Paired Swine) and Cohort 2 (MHC Class I Matched, MHC Class II Mismatched, Donor–Recipient Paired, Swine)

Cohort 1 MHC Class I & Class II Matched Donor-Recipient Paired Swine	**Histological Assessment of Postoperative Graft Survival**			
Wound Site	1	2	3	4
Duration of Cryopreservation	15 min	15 min	>7 yr	>7 yr
Graft Type	Autograft	Allograft	Allograft	Allograft
Subject 24074 (SLA-AD) Graft Survival (in Days)	End of Study	18	18	14
Subject 24075 (SLA-AD) Graft Survival (in Days)	End of Study	14	14	14
Cohort 2 MHC Class I Matched, Class II Mismatched Donor-Recipient Paired Swine	**Histological Assessment of Postoperative Graft Survival**			
Wound Site	1	2	3	4
Duration of Cryopreservation	15 min	15 min	>7 yr	>7 yr
Graft Type	Autograft	Allograft	Allograft	Allograft
Subject 24043 (SLA-KK) Graft Survival (in Days)	End of Study	8	8	8
Subject 24070 (SLA-LL) Graft Survival (in Days)	End of Study	12	12	12

Graft survival ended on the date of observation when BANFF criteria indicated Class IV necrosis.

The use of MHC-matched and Class II mismatched donor–recipient pairs in this model of allogeneic skin transplantation served as internal controls to both confirm the identity of the tissues obtained 7 years earlier, and thus, the veracity of the surgical notes and documentation. Equivalent behavior exhibited by the allografts also demonstrates that the antigenicity of the grafts was not altered as a result of the duration of storage. Lastly, the immunological impact as a result of the use of the GalT-KO material, was not expected or observed in the current model; porcine subjects do not elicit antibody mediated rejection due to the Gal-epitope.

Graft Survivability Was Indistinguishable, Regardless of Duration of Storage

Seven of the eight experimental grafts demonstrated identical characteristics as their intrasubject comparators. With the exception of the graft at Wound Site 4 that rejected 4 days earlier than its comparators for idiopathic reasons, the in vivo

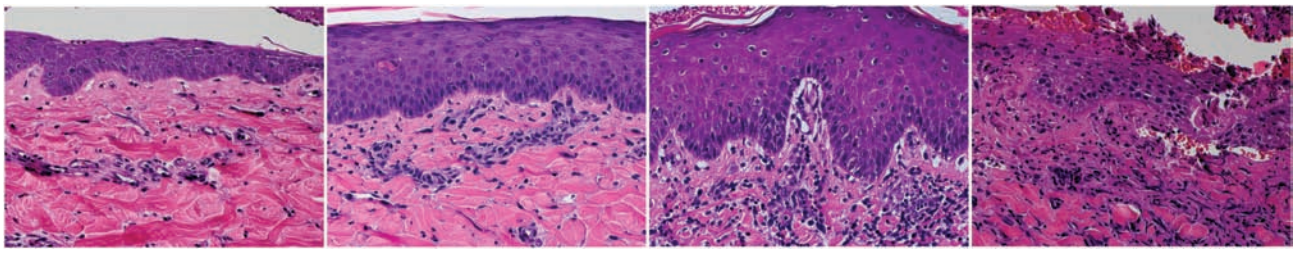

Figure 6. H&E slide images (40× magnification) of sequential biopsies obtained from porcine split thickness skin allografts, at various times during the course of the study, post-operatively, in chronological order. Increasing cellular infiltrates and other histological evidence of progressive signs of immune-mediated rejection are shown, also scored by Banff Rejection Scale. From Left to Right (Banff Score, Subject, POD): Grade 1, 24043, POD-4; Grade 1–2, 24075, POD-6; Grade 3, 26160, POD-10; Grade 4, 24074, POD-16.

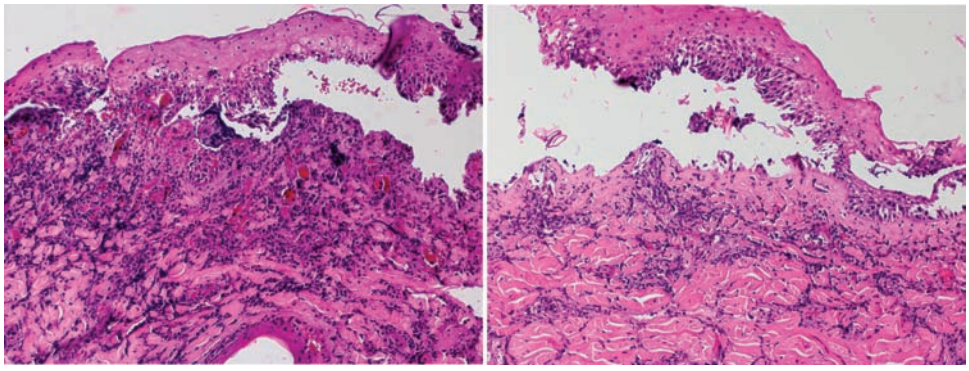

Figure 7. H&E images (20× magnification) of biopsy sections obtained from porcine split thickness skin grafts. Subject 24043, at Wound Sites 2 and 3, at time of histological graft rejection on POD-8. Left: Allograft cryopreserved and stored for a limited duration (15 min). Right: Allograft cryopreserved and stored for an extended duration (>7 yr).

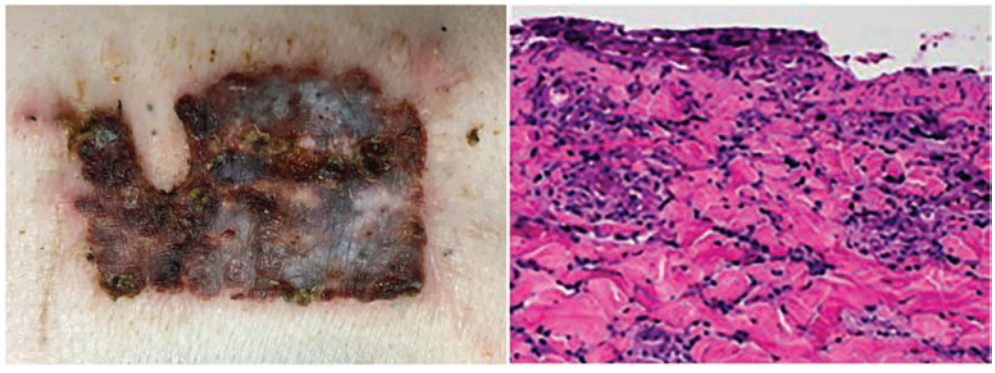

Figure 8. Subject 24074, Wound Site 4, on POD-14. Photograph and correlated H&E slide image (40× magnification) of porcine split thickness skin graft that had been cryopreserved for >7 years before engraftment. Gross clinical signs of rejection (left) coincide with histological assessment of immune-mediated rejection, Banff Rejection Scale, Grade 4.

experiments showed no difference in graft performance and survivability between allografts that had been preserved for short-or-long durations.

Graft Viability Demonstrated Via Independent Analytical Methods

Viability was evidenced uniformly in all grafts, across three independent evaluation methods. The statistical analysis of the MTT-reduction assay showed no statistical difference between cryopreserved and fresh specimens, but significant differences were noted between cryopreserved and heat-denatured tissues. This demonstrates the metabolic activity of the cryopreserved materials and this finding was empirically witnessed in vivo, as all 7-year grafts demonstrated adherence to the wound bed and prolonged survivability. Such survivability would not have been exhibited by nonvital allografts.

Absolute Quantities of Formazan Represent a Relative, Comparative Index of Viability

Regarding the MTT-reduction assays, substantial variability existed between the absolute values resulting from such assays, from specimen-to-specimen and from cohort-to-cohort.

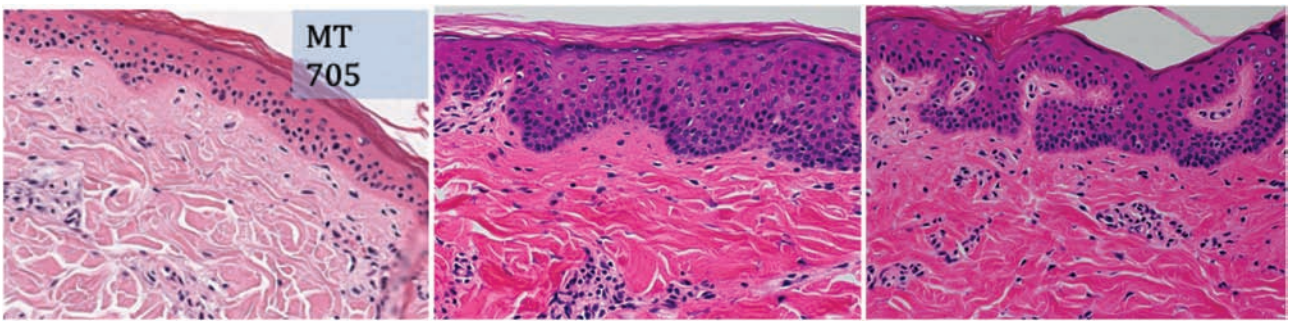

Figure 9. Histology demonstrates morphological equivalence regardless of duration of storage. Routine H&E stained sections from cryopreserved grafts stored for 7 years show viable normal skin with intact epidermis. The blood vessels and adnexal structures were also normal. No distinguishable differences could be seen at a histological level based on duration of storage. From Left to Right (Duration of Storage): 15 minutes, 7 years, 7 years.

Indeed, absolute values of formazan production among cryopreserved samples were actually higher than those obtained from noncryopreserved samples.

As has been suggested in the literature,[59] values should only be considered relative to samples that are assessed contemporaneously and via identical methods. Even subtle variations (exposure to light, length of incubation, absorbance wavelength, interpretation from the calibration curves, for example) affect assay absolute values. Skin composition also presents a tremendous potential for variability. Cell type and number will vary widely with anatomic location. For example, lipid structures have a negative influence on MTT assay outcomes, whereas low-metabolism thymocytes or splenocytes have relatively lower levels of enzymatic activity as opposed to rapidly dividing cells. Other factors, such as varying levels of NADH, environmental pH, glucose concentrations, and other nutrient disparities can affect absorbance values.

As a consequence, it is advisable to obtain and process positive and negative controls concomitantly with each test sample and use the results as relative comparisons. Furthermore, use of optical absorbance values from the spectrophotometer in calculations is preferred, in lieu of extrapolation of quantities of formazan production from the calibration curves.

GalT-KO Porcine Skin Provides Functional Temporary Wound Coverage After 7 Years of Cryopreservation

We initially hypothesized that graft take and overall survival, would be inversely proportional to the length of storage duration. That is, the longer the graft had been frozen, the less likely it would survive and mimic the comparator grafts preserved for shorter durations. It appears, based on these findings not to be the case, if preserved continuously at the appropriate temperature.

It is clear from these data that porcine skin can be cryopreserved for extended durations, 7 years in this case, and retain significant cell viability. Moreover, we observed that the genetic modification (GalT-KO) did not affect metabolic activity, when compared with wild-type skin processed identically. Lastly, our study demonstrates that the MTT-reduction assay can reliably provide an accurate, useful diagnostic method, applicable to the assessment of porcine skin graft viability.

The promising results of this study indicate that it may be feasible to cryopreserve and store porcine skin for logistically relevant durations, and our findings are consistent with current industry practices and the multiyear "shelf life" guidance that the American Association for Tissue Banks has suggested for human cadaveric tissues.[60]

Most importantly, these data suggest that scalable, clinically useful methods of preserving and storing porcine skin with adequate viability are possible. Vital porcine skin grafts that can be effectively stored and distributed would be beneficial in the treatment of severe and extensive, deep partial- and full-thickness burn wounds. Widespread availability of such grafts would improve the outcomes for those patients unable to access HCA due to lack of supply or other limitations. Thus, the results presented here offer great promise for patients in need.

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