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# Prolonged Skin Graft Survival and Histocompatibility in Highly Inbred Miniature Swine

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**Background.** Pigs are potential organ donors for humans. Some proposed xenotransplant tolerance regimens require genetically identical cells from different animals (eg, juvenile bone marrow and mature organs or tissues). We therefore sought to develop a highly inbred line of miniature swine for this purpose. The aim of this study was to test histocompatibility in a new subline of highly inbred miniature swine. **Methods.** Pigs from 2 generations with coefficient of inbreeding (COI) of 92% (n = 6) and 94% (n = 4) each received 2 split-thickness skin grafts (STSGs): an autograft and an allograft from the same generation. This was repeated in a group of 4 pigs from the generation with COI 92%. STSGs were followed for 28–35 d (COI 92%) or >380 d (COI 94%). **Results.** For the pigs with COI of 92%, 1 pig rejected the first allograft on day 9. All other pigs showed prolonged (>24 d) STSG survival. All subsequently rejected a second matched allograft in <14 d, indicating sensitization to minor histocompatibility antigens still segregating in the herd. For the pigs with COI of 94%, 1 pig rejected its allograft at day 9 while the other 3 accepted their allografts >386 d. **Conclusions.** At COI of 92%, highly inbred swine experienced prolonged STSG survival, but persistent minor histocompatibility antigen disparities caused delayed skin graft rejection. Most pigs with COI of 94% accepted reciprocal skin grafts long-term without immunosuppression, indicating homozygosity of the skin graft donors for all relevant histocompatibility loci. Organ transplants within this new inbred line are expected to be accepted indefinitely without a requirement for exogenous immunosuppression, facilitating experiments requiring genetically identical cells from different animals.

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## INTRODUCTION

Organ transplantation is a lifesaving procedure for patients affected by terminal organ failure. However, the number of people on waiting lists is far greater than the number of available organs.[1] Pigs are potential organ donors because of their many anatomical and physiological similarities to humans.[2,3] Sachs miniature swine[4] are a specific breed that are similar in size to humans and for which our laboratory has developed inbred sublines with fixed swine leukocyte antigen (SLA, the porcine equivalent of HLAs, HLA) by intentional, selective inbreeding. We further inbred certain sublines such that their tissues behaved as if genetically identical for the purposes of transplantation. The

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first subline of highly inbred pigs (denoted DD for SLA$^{dd}$ haplotype) enabled in vivo transplantation studies in the absence of concurrent immunosuppression, including the first studies of adoptive transfer of tolerance in a large animal model.[5,6] Since the availability of animals from this subline was compromised by loss of fertility of the latest generation, we are currently attempting to reestablish that line by cloning from fibroblasts. Considering our current need for inbred animals and the advantages of having more than 1 inbred subline, we decided that establishment of a second inbred subline with a different haplotype would be worthwhile. We therefore started with another partially inbred haplotype (denoted HH for SLA$^{hh}$), with a higher fertility ratio. We are in the process of carrying out sequential brother-sister matings to establish a highly inbred HH line, which, like the DD line, should be able to provide histocompatible animals for studies of transplantation and for use as xenograft donors. The aim of the present study was to test histocompatibility in the 2 latest generations of this new HH inbred line by split-thickness skin graft (STSG) transplantation, a very stringent test for histocompatibility.[7,8]

## MATERIALS AND METHODS

All procedures were approved by the local Institutional Animal Care and Use Committees, protocols AC-AABV1657 and Accuro 7, and performed in accordance with the Guide for the Care and Use of Laboratory Animals.[9]

### Animals and Housing

Ten Sachs miniature swine[4] (females n = 5, males n = 5) of the 2 latest generations of the SLA$^{hh}$ haplotype (the HH line) were chosen for this study. Two pigs were transported from Accuro Farms, Chazy, NY, to the Institute of Comparative Medicine, Columbia University, NY, where they were housed in single cages, measuring 1.5 m$^2$, within sight and sound of conspecifics. The pigs were fed Laboratory Mini-Pig Grower Diet 5081 (LabDiet, St Louis, MO) twice a day and had free access to water. The pigs also received daily edible enrichment and toys according to a schedule developed by the Institute of Comparative Medicine, Columbia University. A 12:12 h light schedule, lights on at 7 AM, was applied. Room temperature was kept at 72 ± 3°F and humidity at 50% ± 20%. Eight pigs were housed at Accuro Farms, Chazy, NY. The pigs were kept in single cages measuring 3.2 m$^2$, within sight and sound of conspecifics. The pigs were fed Blue Seal Home Fresh Sow Developer (KENT Nutrition Group, Muscatine, IA) twice a day and had free access to water. A 12:12 h light schedule, light on at 6 AM, was applied. Room temperature was kept at 71 ± 10°F and humidity at 50% ± 20%. Animal characteristics are presented in Table 1.

### Experimental Setup

Experiments 1 and 2 were carried out with pigs with a coefficient of inbreeding (COI) of 92%, calculated by Wright formula[10] using BreedMate Pedigree software (Wild Systems, Sydney, NSW, Australia). Experiment 1 was carried out as a pilot with 2 pigs (26430, 26433) that received 2 STSGs, 1 autologous and 1 sex-mismatched allogeneic from the other pig. This was followed by experiment 2 in which 4 pigs (26322, 26323, 26429, 26432) each received 2 STSGs in 2 rounds. First, they received 1 autologous STSG and 1 from a sex-matched allogeneic pig from the same generation of the highly inbred line. Seventy-six days later they received another autologous STSG and an allograft from a sex-mismatched allogeneic pig from the same generation of the highly inbred line. In experiment 3, 4 pigs (26746, 26748, 26750, 26751) from the next generation (COI of 94%) each received 2 STSGs, 1 autologous and 1 sex-mismatched allogeneic from the same generation. The experimental timelines are presented in Figure 1.

### Skin Transplantation and Rejection Monitoring

All skin transplants were carried out under general anesthesia. Hair was trimmed using a clipper and the skin was prepared for sterile surgery with povidone iodine. STSGs (0.6 mm) were harvested with a Zimmer dermatome (Zimmer Biomet, Warsaw, IN) and briefly maintained in sterile, cold saline before they were transplanted to the recipients. The grafts were fenestrated to allow for release of any serous accumulation during healing. To prepare the graft bed on the recipients, 2 passes with the dermatome at 0.6 mm were used to deepen the wound. The grafts were protected with an occlusive pressure dressing for the first 3–4 d postoperatively

**TABLE 1.**

**Animal characteristics, recipient-donor pairs, and day of rejection of split-thickness skin allografts**

| Pig Idenitfication (ID) recipient | Sex | Age at first transplant (mo) | Pig ID (sex) donor first allograft | Day of rejection first allograft | Pig ID (sex) donor second allograft | Day of rejection second allograft |
|---|---|---|---|---|---|---|
| Experiment 1 | | | | | | |
| 26430 | F | 4 | 26433 (M) | 25 | N/A | N/A |
| 26433 | M | 4 | 26430 (F) | >28 | N/A | N/A |
| Experiment 2 | | | | | | |
| 26322 | F | 14 | 26429 (F) | 9 | 26432 (M) | 9 |
| 26323 | M | 14 | 26432 (M) | 40 | 26429 (F) | 7 |
| 26429 | F | 9 | 26322 (F) | 29 | 26323 (M) | 13 |
| 26432 | M | 9 | 26323 (M) | 27 | 26322 (F) | 13 |
| Experiment 3 | | | | | | |
| 26746 | F | 2.5 | 26751 (M) | >386 | N/A | N/A |
| 26748 | F | 2.5 | 26750 (M) | 9 | N/A | N/A |
| 26750 | M | 2.5 | 26748 (F) | >386 | N/A | N/A |
| 26751 | M | 2.5 | 26746 (F) | >386 | N/A | N/A |

F, female; M, male; N/A, not applicable.

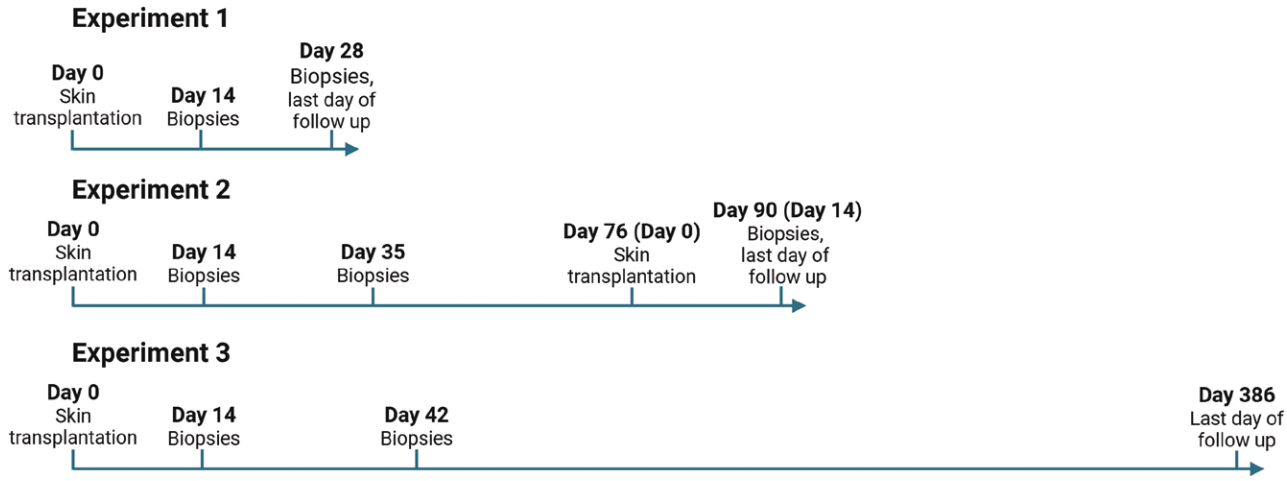


**FIGURE 1.** Timeline for experiment 1, 2, and 3. For experiment 2, days in brackets indicate days after the second round of skin transplantation.

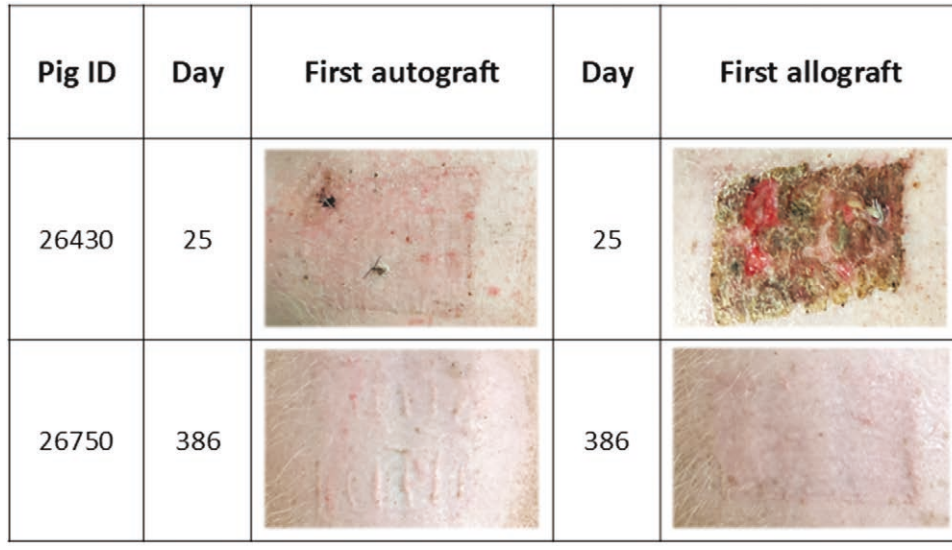


**FIGURE 2.** Representative photographs of auto- and allografts from animals with and without rejection. Pig ID 26430 (top) from experiment 1 rejected its allograft on day 25. Pig ID 26750 (bottom) from experiment 3 accepted its allograft long term, >386 d.

and then inspected at least every other day for evidence of healing or rejection. The day of rejection was defined as the day on which <10% of the skin graft appeared viable to gross inspection, as judged by color, texture, and warmth to touch. For grafts inspected every other day, it is possible that grafts classified as rejected would have been classified as rejected 1 d earlier if inspections had been made every day. Unless the STSGs were already rejected, biopsies were collected in formalin on postoperative day 0, 14, and 28 (experiment 1), 35 (experiment 2), or 42 (experiment 3). Fixed tissues were embedded in formalin, and 3 µm sections were stained with hematoxylin and eosin. Sections were scored grade 0–4 based on the Banff 2007 working classification of skin-containing composite tissue allograft pathology.[11]

## RESULTS

### Experiment 1

For both pigs, the self-grafts showed a normal appearance throughout the study. For the male pig (26433), the allograft demonstrated hyperemia on day 9 but remained warm and soft to touch. The hyperemia resolved spontaneously by day 15, and the graft showed normal appearance for the remainder of the study. Histology from allograft biopsy collected on day 28 showed grade 1 rejection. We did not rebiopsy, since the graft remained grossly normal after that point. For the female pig (26430), the allograft demonstrated more severe hyperemia starting on day 9. This continued until day 21, at which point it darkened to purple, progressing to full rejection by day 25. Histology from a biopsy collected on day 24 confirmed grade 4 rejection.

### Experiment 2

For all 4 pigs, the self-grafts from both the first and second transplants showed a normal appearance throughout the study. In the first round, 1 female (26322) rejected the allograft on day 9 based on visual appearance, and histology from a biopsy collected on day 14 confirmed grade 4 rejection. For 2 pigs (1 female, 1 male), the allografts both turned hyperemic on day 11, slowly progressing to full rejection on day 27 (26432) and 29 (26429), respectively. Histology from biopsies collected at day 35 confirmed grade 4 rejection. For 1

male pig, the allograft demonstrated hyperemia on day 16 but remained warm and soft to touch. The hyperemia resolved spontaneously by day 22 and the graft showed a normal appearance for the remainder of the day 35 follow-up period. However, histology from a biopsy collected on day 35 showed grade 3 rejection, which progressed to full rejection by day 40 as assessed by visual inspection. In the second round of transplants, 76 d after the first round, for which the same animals were used in different combinations, all 4 allografts became hyperemic on day 5 (first day of inspection) and progressed to full rejection at day 7 (26323), 9 (26322), and 13 (26429 and 26432). Histology from biopsies collected on day 14 confirmed grade 4 rejection for all pigs.

### Experiment 3

For all 4 pigs in the cohort with COI of 94%, the self-grafts showed a normal appearance throughout the study. One female (26748) rejected the sex-mismatched allograft on day 9 based on visual appearance, and histology from a biopsy collected on day 14 confirmed grade 4 rejection. For the other 3 pigs (26746, 26750, 26751), the allografts appeared grossly healthy throughout the study, surviving >386 d. Biopsies collected from 2 of the pigs (26746 and 26750) showed mild changes consistent with grade 1 rejection on day 14, while the biopsy collected from the third pig (26751) showed grade 3 rejection on day 14, which decreased to grade 1 at day 41. No further biopsies were performed because the appearance of the grafts returned to normal.

## DISCUSSION

Deriving a subline of swine that has been bred to the point of histocompatibility is important for both allogeneic and xenogeneic studies. For allotransplantation, one example of their utility is to facilitate our adoptive transfer studies.[5,6] Histocompatible swine also have major advantages for use in the field of xenotransplantation, where porcine xenografts could potentially bridge the gap between organ supply and demand. For example, one use of the highly inbred line is to construct composite "thymo-islet-kidney" grafts. Such an experiment requires islets from a very large and mature swine to be allowed to engraft under the kidney capsule of a juvenile swine together with thymus from a juvenile animal.[12,13] Bone marrow can also be added from a separate juvenile swine. The significance of this approach is that the use of concomitant donor thymus and/or bone marrow has facilitated the successful induction of tolerance in allogeneic transplant models.[14] The level of immunosuppression required for xenotransplantation remains higher, and its efficacy lower, than for allogeneic transplants. Therefore, the success of these tolerogenic strategies would make xenotransplantation more feasible, and these strategies are possible only by combining tissue from histocompatible donors.

The importance of having inbred strains of donor animals for use as xenotransplant donors may not be apparent in the current era of large animal cloning, but there are marked differences between inbred animals and cloned animals in this regard. Inbred animals, produced by sequential brother-sister matings, become homozygous for an increasing percentage of all genetic loci with each generation.[15] When homozygosity is achieved at all loci that produce allelic proteins, peptides of which can serve as minor transplantation antigens, then animals of the strain become histocompatible, meaning that they do not reject tissue transplants from each other.[15] Further breeding of these animals within the strain leads to similarly histocompatible offspring—essentially an unlimited number of identical twins, with regard to transplantation. In contrast, cloning is a much more costly process and is subject to cloning artifacts.[16] Furthermore, since the genetic loci are heterozygous, breeding produces reassortment and loss of any semblance of genetic identity.[15]

Skin grafts are used to assess histocompatibility because the skin is particularly immunogenic, requiring higher doses of immunosuppression to avoid rejection than are required for most solid organ transplants,[7,8] and making it a very stringent test for histocompatibility in this experiment. This procedure mandates homozygosity for the gene products capable of producing minor histocompatibility antigens (mHAs), rather than just assuring a level of overall homozygosity. In understanding our reporting of skin graft findings, it is important to note that, in our experience, the most accurate means of assessing long-term survival of experimental skin grafts is the gross appearance of the graft (rather than histology) since so many nonimmunologic variables (including local surface irritation, topographical differences, trauma, etc.) can affect the histologic appearance. Histological examination of some allografts showed mild changes consistent with grade 1 rejection; however, an active immune response may be expected during the development of regulatory T cells,[17,18] and the gross appearance of the grafts returned to normal. In view of these considerations, there is very little chance that rejection would not have been observed. Figure 2 demonstrates the difference in appearance of a graft that was rejected versus a graft that was accepted long term.

Our data demonstrate the progress that can be made toward achieving histocompatibility with just 1 additional generation of inbreeding. In the first generation for which histocompatibility was assessed, 5 of the 6 recipients rejected skin grafts. It is unlikely that this was caused by mHAs encoded on the Y chromosome in pigs that remain disparate to female recipients despite inbreeding since rejection occurred even in sex-matched pairs. More likely genes coding for mHA disparities strong enough to cause skin graft rejection remained heterozygous in the 92% COI HH subline, despite the high level of inbreeding. In the next generation (COI of 94%), 3 pigs accepted the allograft for >386 d, suggesting homozygosity of all histocompatibility loci in the donors of these grafts, with 1 pig still heterozygous for a minor antigen strong enough to cause early rejection. With elimination of that donor from further inbreeding of this line, we subsequently expect the line to be fully histocompatible. mHAs have been shown to consist of peptides of allelic proteins within a species that are presented to T cells by MHC antigens and can cause rejection even between MHC-identical donor-recipient pairs.[19] Therefore, essentially any of the enormous number of proteins that are allelic in a species can serve as mHAs. However, the strength of these antigens varies widely, and when individual mHAs have been isolated in congenic, inbred strains of mice, they lead to skin graft rejection times ranging from 10 d to >3 mo.[20] After rejecting a graft on the basis of an mHA, subsequent grafts from the same strain show accelerated rejection because of sensitization to that minor antigen.[20] Thus, the results presented here are entirely consistent with what is known about minor antigens in mice.

We further expect that tissue transplanted between our most recent highly inbred generation of pigs would be accepted indefinitely without immunosuppression, since skin is generally considered the most highly antigenic of transplantable tissues.[7,8] Our previous results in the DD line of highly inbred miniature swine (COI 91%), in which markedly prolonged skin and heart graft survival was observed,[5] are consistent with this expectation. Assuming that we are able to recover the DD line from our current cloning of frozen cells from a previous generation, we expect to have 2 highly inbred strains of miniature swine for our planned studies of transplantation of allogeneic organs without immunosuppression and of xenotransplantation of cells, tissues, and organs from inbred donors. Such strains open the door to both allogeneic tolerance studies and clinical xenotransplantation protocols that would not otherwise be possible by any other strategies, including cloning.

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