data demonstrate that compound **4** is a potent LFA-1 antagonist, which binds LFA-1, blocks the binding of ICAM-1, and inhibits LFA-1 mediated lymphocyte proliferation and adhesion in vitro. It achieves this with a potency significantly greater than cyclosporine A, and demonstrates its equivalence to anti-CD11a in the level of its inhibition of the immune system's response in vivo.

Compounds **1** through **4** emerged from considerations of the ICAM-1 epitope via kistrin, the RGDMP peptides and H_2N-CGY$^{(m)}$DMPC-COOH. Each of these LFA-1 ligands was able to compete with a fluorescein-conjugated analog of compound **3** for LFA-1 binding. SAR similarities suggest a common presention of a carboxylic acid moiety to a binding site on LFA-1 as the basis of this competition. A comparison of the structures and molecular functionality of compound **4** and ICAM-1 responsible for their LFA-1 binding reveals that the carboxylic acid, sulfide, phenol, and carboxamide groups of the ICAM-1 epitope are embodied in compound **4** (Fig. 3) (*14*). This allows us to propose that compounds **2** through **4** are mimics of ICAM-1 resulting from the transfer of the ICAM epitope to a small molecule. A definitive proof of this mimicry will require the determination of the structures of LFA-1 and its complexes with ICAM-1 and compounds **2** through **4**.

We believe the work presented here (*23, 24*) represents the first reduction of a nonlinear, discontinuous but contiguous protein epitope (encompassing five residues spanning three different β strands across the face of a protein surface) from a protein to a small molecule. In contrast to more traditional approaches, this rational, structurally directed hypothesis and information driven lead discovery process utilized molecular modeling and structure activity relationships to identify pharmacophoric similarities within ICAM-1, kistrin, the peptides, and ultimately compounds **1** through **4**. This provided the perspective to recognize compound **1** as a viable lead and rapidly elaborate it into compounds **2** through **4**, and demonstrates the value of antibodies, protein mutagenesis, and structural (SAR) data for native protein ligands as leads in the identification of pharmaceutical agents, which block large protein-protein interactions. What remains to be seen with these small-molecule LFA-1 antagonists is a clinical evaluation of their safety and effectiveness in the control of human diseases relative to humanized anti-CD11a and other small-molecule agents discovered by other means (*25–28*).

References and Notes

1. R. W. McMurray, *Semin. Arthritis Rheumatism* **25**, 215 (1996).
2. J. H. Spragg, in *Molecular Biology of Cell Adhesion Molecules*, M. A. Horton, Ed. (Wiley, New York, 1996), chap. 8, pp. 131–154.
3. N. Oppenheimer-Marks, P. E. Lipsky, *Clin. Immunol. Immunopathol.* **79**, 203 (1996).
4. M. K. Connolly, E. A. Kitchens, B. Chan, P. Jardieu, D. Wofsy, *Clin. Immunol. Immunopathol.* **72**, 198 (1994).
5. K. Kakimoto *et al.*, *Cell. Immunol.* **142**, 326 (1992).
6. E. K. Nakakura *et al.*, *Transplantation* **55**, 412 (1993).
7. W. A. Werther *et al.*, *J. Immunol.* **157**, 4986 (1996).
8. A. Gottlieb *et al.*, *J. Am. Acad. Dermatol.* **42**, 428 (2000).
9. H. Granlund, in *Curr. Opin. Anti-Inflammatory Immunomodulatory Invest. Drugs* **2**, 332 (2000).
10. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. X indicates any residue.
11. K. L. Fisher *et al.*, *Mol. Biol. Cell* **8**, 501 (1997).
12. J. M. Casasnovas, T. Stehle, J.-h. Liu, J.-h. Wang, T. A. Springer, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4134 (1998).
13. D. E. Staunton, M. L. Dustin, H. P. Erickson, T. A. Springer, *Cell* **61**, 243 (1990).
14. Supplementary material is available at *Science* Online at www.sciencemag.org/cgi/content/full/295/5557/1086/DC1
15. M. S. Dennis *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2471 (1989).
16. M. Adler, P. Carter, R. A. Lazarus, G. Wagner, *Biochemistry* **32**, 282 (1993).
17. S. C. Bodary, P. Gribling, W. P. Lee, unpublished data.
18. M. S. Dennis, P. Carter, R. A. Lazarus, *Proteins Struct. Funct. Genet.* **15**, 312 (1993).
19. R. S. McDowell *et al.*, *J. Am. Chem. Soc.* **116**, 5069 (1994).
20. R. S. McDowell, T. R. Gadek, *J. Am. Chem. Soc.* **114**, 9245 (1992).
21. R. S. McDowell *et al.*, *J. Am. Chem. Soc.* **116**, 5077 (1994).
22. D. J. Burdick, in *PCT Int. Appl.* (Genentech, USA). WO 9949856 (1999), p. 9.
23. Experimental methods for an LFA-1 ELISA assay (p. 65), the mixed lymphocyte reaction (MLR) (p. 67) and the synthesis of compounds **2** through **4** (p. 68) are found in D. J. Burdick, *PCT Int. Appl.* (Genentech, USA). WO 9949856 (1999).
24. In the course of the studies presented here, several reports appeared describing small molecule antagonists of LFA-1/ICAM (*25–27*). Two have identified compounds which interact with LFA-1's allosteric I domain site (*26, 27*). Preliminary studies with compound **4** indicate that it binds at a different site on LFA-1, and that the allosteric site antagonist(s) do not inhibit the binding of compound **4** to LFA-1.
25. T. A. Kelly *et al.*, *J. Immunol.* **163**, 5173 (1999).
26. J. Kallen *et al.*, *J. Mol. Biol.* **292**, 1 (1999).
27. G. Liu *et al.*, *J. Med. Chem.* **44**, 1202 (2001).
28. G. Liu, *Expert Opin. Ther. Patents* **11**, 1383 (2001).
29. J. Bella, P. R. Kolatkar, C. W. Marlor, J. M. Greve, M. G. Rossman, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4140 (1998).

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Production of α-1,3-Galactosyltransferase Knockout Pigs by Nuclear Transfer Cloning

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The presence of galactose α-1,3-galactose residues on the surface of pig cells is a major obstacle to successful xenotransplantation. Here, we report the production of four live pigs in which one allele of the α-1,3-galactosyltransferase locus has been knocked out. These pigs were produced by nuclear transfer technology; clonal fetal fibroblast cell lines were used as nuclear donors for embryos reconstructed with enucleated pig oocytes.

Clinical transplantation has become one of the preferred treatments for end-stage organ failure since the introduction of chronic immunosuppressive drugs in the mid-1980s. One of the novel approaches to dealing with the limited supply of human organs is the use of alternative species as a source of organs (xenotransplantation). The pig is considered the primary alternative species because of ethical considerations, breeding characteristics, infectious disease concerns, and its compatible size and physiology (*1*).

A major barrier to progress in pig-to-primate organ transplantation is the presence of terminal α-1,3-galactosyl (Gal) epitopes on the surface of pig cells. Humans and Old World monkeys have lost the corresponding galactosyltransferase activity in the course of

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evolution and therefore produce preformed natural antibodies to the epitope, which are responsible for hyperacute rejection of porcine organs. The temporary removal of recipient antibodies to Gal through affinity adsorption and expression of complement regulators in transgenic pigs has allowed survival of pig organs beyond the hyperacute stage. However, returning antibody and residual complement activity are believed to be responsible for the acute and delayed damage that severely limit organ survival even in the presence of high levels of immunosuppressive drugs and other clinical intervention (*2*). Competitive inhibition of galactosyltransferase in α-1,2-fucosyl-transferase transgenic pigs has resulted in only partial reduction in epitope numbers (*3*). Similarly, attempts to block expression of Gal epitopes in *N*-acetylglucosaminyltransferase III transgenic pigs also resulted in partial reduction of Gal epitope number but failed to substantially extend graft survival in primate recipients (*4*). Given the large number of Gal epitopes present on pig cells (*5*), it seems unlikely that any dominant transgenic approach of this nature can provide sufficient protection from damage mediated by antibodies to Gal. In contrast, a genetic knockout of the α-1,3-galactosyltransferase (GGTA1) locus in pigs would provide permanent and complete protection.

Viable α-1,3-galactosyltransferase knockout mice have been produced by embryonic stem cell technology (*6*). The development of nuclear transfer technology has provided a means for locus-specific modification of large animals, as demonstrated by the production of viable sheep by means of in vitro targeted somatic cells (*7*). Successful cloning (*8–11*) and production of transgenic pigs by nuclear transfer of genetically modified somatic cells (*12*) have been reported. Attempts at targeting the GGTA1 locus in pigs (*11*) and sheep (*13*) have also been reported, but these failed to result in live birth of animals with the desired modification. In both cases, difficulties in obtaining viable targeted donor cell clones were encountered.

We chose to knock out the GGTA1 locus in a highly inbred, major histocompatibility complex–defined miniature pig line. Descendant from lines long used for xenotransplantation studies (*14, 15*), this line is an ideal size match for eventual use in clinical transplantation and has animals that consistently test negative for transmission of porcine endogenous retrovirus (PERV) to human cells in vitro (*16*). Cells were isolated from one male (F9) and three female (F3, F6, and F7) fetuses at day 37 of gestation for production of donor cell lines (*17*). A gene trap targeting vector, pGalGT, was used for homologous replacement of an endogenous GGTA1 allele (Fig. 1). The vector contains about 21 kb of homology to the GGTA1 locus, with the coding region upstream of the catalytic domain disrupted by insertion of a selection cassette consisting of a Bip internal ribosome entry site followed by sequences encoding G418 resistance. After transfection and 14 days of G418 selection, viable cell clones (*18*) were passaged in triplicate for further analysis and cryopreservation (*19*).

A reverse transcription polymerase chain reaction (RT-PCR) was performed on crude cell lysates the day after passage with a forward primer from exon 7 (upstream of the 5′ end of the targeting vector) and a reverse primer from the selection cassette (*20*). Dot blot hybridization of the RT-PCR products with an exon 8 probe detected targeting in 22 of 159 clones analyzed.

The structure of the GGTA1 locus was analyzed in two overlapping PCR reactions (*21*). Clones with a targeted insertion of the cassette relative to vector external primer sites both upstream and downstream of the cassette, indicative of a replacement-type targeting event, were considered candidates for use in nuclear transfer. Of 17 clones analyzed, 8 were found to have undergone the desired recombination event, and one from each fetus (F3-C5, F6-C3, F7-H6, and F9-J7) was used for nuclear transfer.

Nuclear transfer was performed with the use of in vitro matured oocytes and, except for 4 of 28 embryo transfers, cryopreserved donor cells without further culture (*22*). Asynchronous embryo transfer—that is, transfer to a surrogate at an earlier stage of the estrus cycle than the embryos themselves—had previously been used with minimally manipulated (*23*), pronuclear microinjected (*24*), and nuclear transfer (NT)–derived embryos (*10–12*). The observed benefit of asynchronous transfer suggests that any manipulation may result in a delay in early embryonic development. Because the manipulations required for nuclear transfer are quite extensive, and because previous reports suggest that miniature swine embryos of the NIH strain used here may normally develop at a relatively slower rate (*25*), naturally cycling large white gilts that had displayed standing estrus but had not yet completed ovulation were used as surrogates (*26*). [For detailed information on all 28 embryo transfers, see (*18*).]

A minimum of four viable embryos is required for establishment of pregnancy in pigs (*27*). Thus, we used two methods to increase the likelihood of establishing pregnancies with NT-derived embryos. Although pregnancy was established in five of seven surrogates receiving parthenogenetic "carrier" embryos, no live births resulted. Therefore, we transferred reconstructed embryos to a mated surrogate. In the one embryo transfer performed in this group, the surrogate (O212) was mated on the first day of standing estrus and received NT-derived embryos the same day. Although any fertilized embryos would theoretically be 43 to 55 hours behind development of the transferred NT-derived embryos, the actual in vivo development rate for NT-derived embryos is unknown. Early pig embryos have a lower rate of survival when present in a surrogate along with embryos at a slightly more advanced stage (*25*). Thus, an apparent embryonic asynchrony may be advantageous should NT-derived embryos develop at a slower rate than naturally fertilized embryos.

Seven piglets, four females and three males (Table 1), were delivered by cesarean section at term (*28*). Microsatellite analysis (*29*) revealed that six of six haplotypes for one female piglet (O212-2) were iden-

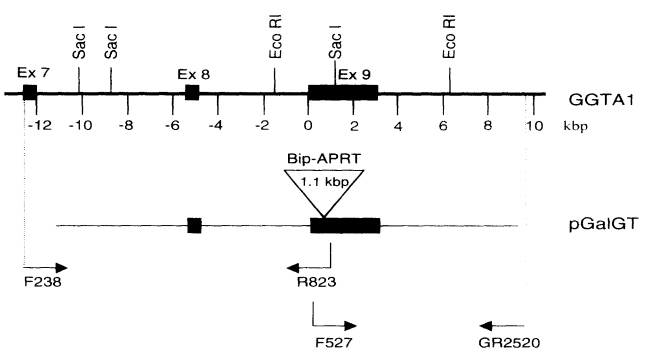

Fig. 1. pGalGT targeting vector and genomic PCR assays for targeting (35).

Table 1. Pregnancies carried to term after transfer of embryos reconstructed with GGTA1 knockout cell lines.

Surrogate (estrus day)	Donor line	NT embryos	Outcome
O212 (0)	F7-H6	116	Mated surrogate Seven born 9/21/01 One NT-derived female piglet
O226 (1)	F3-C5	92	Four NT-derived female piglets born 10/19/01
O230 (1)	F7-H6 cultured	130	Two NT-derived female piglets born 10/15/01

tical to that of the F7 fetal cell line from which knockout donor line F7-H6 was derived (*18*). Furthermore, three of six haplotypes of O212-2 were not compatible with mating of the surrogate. All other piglets had at least four haplotype mismatches with the F7 line and were compatible with mating of the surrogate.

We performed 20 additional transfers of only NT-derived embryos to unmated surrogates. Pregnancy was confirmed by ultrasound in six of these surrogates, with two continuing to cesarean section at term. Two live piglets were delivered from surrogate O230 (Table 1), one of which (O230-2) died from respiratory distress syndrome shortly after delivery. Four live piglets were delivered from surrogate O226 (Table 1), again with one (O226-4) dying shortly after birth from respiratory distress syndrome. Microsatellite haplotypes of all six piglets from the two litters were identical to the F7 and F3 donor parental cell lines, respectively.

Genomic targeting analysis was performed on DNA samples from all NT-derived piglets, the untransfected F3 and F7 donor parental cell lines, and surrogates (Fig. 2). For all piglets except O230-2, analysis of both ends of the GGTA1 locus revealed the presence of one replacement-type targeted allele. Whether O230-2 was derived from an untargeted miniature swine cell in the F7-H6 donor line or had a GGTA1 rearrangement incompatible with detection by the targeting assays performed has yet to be determined.

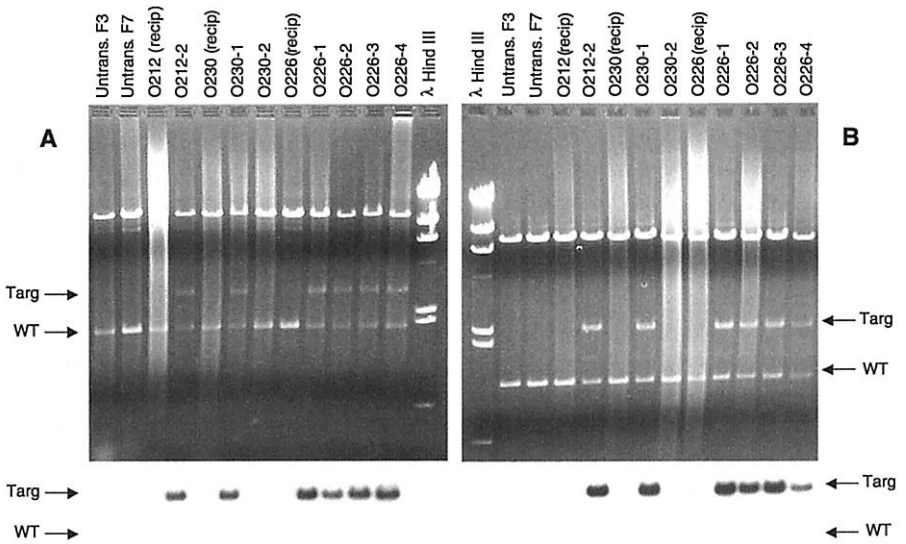

Fig. 2. Targeting analysis of NT-derived piglets, parental miniature swine fetal cell lines F3 and F7, and surrogate sows. See Fig. 1 for a description of the assays. (**A**) Upstream genomic PCR analysis with primers F238 and R823. (**B**) Downstream genomic PCR analysis with primers F527 and GR2520. After transfer, digested reactions were probed with an oligonucleotide (Bip419) from the IRES portion of the selection cassette. The analysis of all offspring, with the exception of O230-2, is consistent with a replacement-type targeting event at one GGTA1 allele.

Table 2 presents a health summary for the seven NT-derived piglets. Four of the five piglets surviving beyond the immediate postpartum period remain healthy, with a normal growth rate for miniature swine. The fifth, O226-3, died suddenly at 17 days of age during a routine blood draw. Necropsy revealed a dilated right ventricle and thickening of the heart wall. Another animal, O230-1, has shown cardiac defects similar to those reported in NT-derived animals of other species (*7, 12, 30, 31*).

A number of other abnormalities were noted at birth among surviving piglets, none of which appear to affect their overall health and well-being. Flexure tendon deformities similar to those reported here have been observed in previous NT-derived commercial strain pigs (*32*). It is unlikely that the abnormalities we have seen are related to the genetic modification, as there is not a consistent phenotype and only one allele has been targeted, but rather are the result of improperly reprogrammed epigenetic factors. With the exception of O212-2, the four surviving piglets were somewhat undersized, with birth weights of 450 to 650 g (strain average 860 g).

Under our growth and selection conditions, miniature swine fetal fibroblasts maintain a steady doubling time of about 24 hours. Clonal lines senesce after 30 to 32 days of culture on average. The ability to quickly select clonal lines for nuclear transfer is likely to be a requirement for introduction of other complex genetic alterations into the pig genome. The ability to use cryopreserved donor cells without further culture, demonstrated by two of our litters, is also advantageous, as it extends the number of potential donor lines available for use in nuclear transfer. Our efficiencies in producing NT-derived GGTA1 knockout animals are similar to those previously reported in which extensively cultured primary fetal cells were used as

Table 2. Health summary of NT-derived miniature swine piglets. H, healthy; NG, normal growth; D, dead.

Piglet	Physical findings	Birth weight (g)	Clinical symptoms/cardiac exam findings
O212-2 (H, NG)	Ocular defect, small ear flaps with no patent ear canals	1100	No clinical symptoms; no significant echocardiogram findings
O230-1 (H, NG)	Flexure deformity of distal interphalangeal joint at birth*	450	Mild abdominal ascites; right ventricular enlargement and pulmonary hypertension
O230-2 (D)	Flexure deformity of distal interphalangeal joint at birth; dysmaturity at birth	115	Died shortly after delivery of respiratory distress syndrome; no gross lesions observed at necropsy
O226-1 (H, NG)	Normal	600	No clinical symptoms; no significant echocardiogram findings
O226-2 (H, NG)	Flexure deformity of distal interphalangeal joint at birth†	650	No clinical symptoms; low-velocity regurgitation at center of tricuspid valve
O226-3 (D)	Flexure deformity of distal interphalangeal joint at birth*	550	Death during routine blood draw 17 days after birth; dilated right ventricle with thickening of heart wall observed at necropsy
O226-4 (D)	Cleft palate; dysmaturity at birth	250	Died shortly after delivery of respiratory distress syndrome; no gross lesions observed at necropsy

*Responded to physical therapy. †Responded to physical therapy plus splinting.

nuclear donors (*10, 11*), despite the nearly fourfold difference in adult size between the miniature swine strain modified here and the commercial oocyte donor and surrogate strains used. The ability to use readily available oocyte donors and surrogates in a nuclear transfer program is essential when modification of less commonly available animals is required.

The next step will be to create α-1,3-galactosyltransferase–null (homozygous knockout) pigs, either by breeding to a heterozygous male produced by nuclear transfer or by sequential nuclear transfer modification of cell lines produced from the four female pigs reported here. Because α-1,3-galactosyltransferase–null mice have already been produced (*6*), it is not anticipated that this genetic modification will be lethal in the null animals. We hope that α-1,3-galactosyltransferase–null pigs will not only eliminate hyperacute rejection but also ameliorate later rejection processes, and (in conjunction with clinically relevant immunosuppressive therapy) will permit long-term survival of transplanted porcine organs. At a minimum, the availability of galactosyltransferase-null pigs will allow a clearer evaluation of approaches currently in development aimed at overcoming potential delayed and chronic rejection mechanisms in porcine xenotransplantation.

References and Notes

1. R. W. Evans, in *Xenotransplantation*, J. L. Platt, Ed. (ASM Press, Washington, DC, 2001), pp. 29–51.
2. D. Lambrigts, D. H. Sachs, D. K. S. Cooper, *Transplantation* **66**, 547 (1998).
3. C. Costa *et al.*, *FASEB J.* **13**, 1762 (1999).
4. S. Miyagawa *et al.*, *J. Biol. Chem.* **276**, 39310 (2001).
5. U. Galili, *Curr. Opin. Organ Trans.* **6**, 30 (2001).
6. A. D. Thall, P. Maly, J. B. Lowe, *J. Biol. Chem.* **270**, 21437 (1995).
7. K. J. McCreath *et al.*, *Nature* **405**, 1066 (2000).
8. I. A. Polejaeva *et al.*, *Nature* **407**, 86 (2000).
9. A. Onishi *et al.*, *Science* **289**, 1188 (2000).
10. J. Betthauser *et al.*, *Nature Biotechnol.* **18**, 1055 (2000).
11. K. Bondioli, J. Ramsoondar, B. Williams, C. Costa, W. Fodor, *Mol. Reprod. Dev.* **60**, 189 (2001).
12. K. W. Park *et al.*, *Anim. Biotech.*, **12**, 173 (2001).
13. C. Denning *et al.*, *Nature Biotechnol.* **19**, 559 (2001).
14. D. H. Sachs *et al.*, *Transplantation* **22**, 559 (1976).
15. Fetuses for derivation of cell lines used here were the product of six generations of brother-sister mating and have an estimated inbreeding coefficient of 0.86. Two additional sib-mated generations have been produced from this inbred line, with no apparent decrease in fitness.
16. B. A. Oldmixon *et al.*, in preparation.
17. Primary fibroblasts were isolated from miniature swine fetuses by collagenase-trypsin digestion of minced tissue. Dissociated cells were plated at 2×10^5 cells/cm^2 on collagen-coated plates in Ham's F10 medium containing 20% fetal bovine serum (FBS) and antibiotics. Adherent cells were frozen the following day.
18. For supplementary data, see *Science* Online (www.sciencemag.org/cgi/content/full/1068228/DC1).
19. Fetal fibroblasts were thawed and cultured for 3 days to subconfluence before transfection. About 2×10^7 fibroblasts were electroporated at 260 V, 960 μFD in 0.8 ml of Hepes-buffered saline containing pGalGT (0.5 pmol/ml). The vector was restriction-digested at both ends of the GATA1 homologous sequences before use. Transfected cells were cultured in bulk for 2 days without selection, then plated in collagen-coated 96-well plates at 2×10^4 cells per well in Ham's nutrient mixture F10 containing 20% FBS and G418 (100 μg/ml). The low selection concentration was made possible by the absence of cells transiently expressing G418 resistance; untransfected cells were uniformly killed by G418 (50 to 75 μg/ml) after 5 to 7 days. After 14 days of selection in G418, growing cultures were passaged in triplicate for cryopreservation of donor cells, RT-PCR screening for targeting, and DNA isolation. Subconfluent donor cell cultures were trypsinized and frozen in 20-μl aliquots containing 1000 to 2000 cells each.
20. Lysates were prepared from 96-well cultures of selected clones the day after passage by three rounds of freezing and thawing in 10 μl of 2 mM dithiothreitol containing placental ribonuclease inhibitor (1 U/μl). The lysate was amplified in a one-tube amplification reaction using rTth polymerase, exon 7 forward primer F291 (5′-ACAACAGAGGAGAGCTTCCG) and reverse primer Bip419 (5′-CTCTCACACTCGCGAAACAC). PCR products were alkaline-denatured and transferred to nylon membranes before hybridization with an exon 8 oligonucleotide probe (5′-GGTCGTGACCATAACCAGATG).
21. Clones identified as putatively targeted in the RT-PCR screening assay were expanded into 24-well plates, and DNA was isolated for genomic analysis. About 250 ng of DNA was amplified in reactions with LA Taq DNA polymerase (Panvera, Madison, WI). Targeting was assessed by two PCR assays, each incorporating a primer outside of the vector homologous region. Upstream analysis used exon 7 forward primer F238 (5′-TTACCACGAAGAAGAAGACGC) and exon 9 reverse primer R823 (5′-AGGATGTGCCTTGTACCACC). Upon digestion with Eco RI, fragments of 2.0 kb (WT locus), 3.1 kb (targeted locus), and 10.4 kb (either locus) are produced. Downstream analysis used exon 9 forward primer F527 (5′-GGTTGGCCACAAAGTCATC) and reverse primer GR2520 (5′-CACTATTTGGAGGACAGGGTC). Upon digestion with Sac I, fragments of 1.2 kb (WT locus), 2.3 kb (targeted locus), and 8.1 kb (either locus) are produced. Southern blots of digested reactions were hybridized to internal ribosome entry site (IRES) region probe Bip419.
22. Oocytes derived from slaughtered gilts were matured in defined protein medium [TCM 199 supplemented with 0.1% polyvinyl alcohol, cysteine (0.1 mg/ml), epidermal growth factor (10 ng/ml), 0.91 mM Na-pyruvate, 3.05 mM D-glucose, follicle-stimulating hormone (0.5 μg/ml), luteinizing hormone (0.5 μg/ml), penicillin (75 μg/ml), and streptomycin (50 μg/ml)]. Oocytes from sow ovaries were purchased from BoMed Inc. (Madison, WI) and shipped overnight in their commercial maturation medium. After maturation, oocytes were freed of cumulus cells and were kept in TCM/BSA [TCM 199 supplemented with bovine serum albumin (BSA, 4 mg/ml)] until use. Enucleation of metaphase II oocytes was performed in medium supplemented with cytochalasin B (7.5 μg/ml), without staining the chromatin (as this may be detrimental to subsequent development) (*33*). Cryopreserved donor cells were thawed at 37°C and 10 volumes of FBS were added. The suspension was kept at room temperature for 30 min, and four volumes of TCM/BSA were added and the cells pelleted. Fibroblast cells were resuspended and directly used for NT. For NT-derived embryos transferred to four surrogates (O230, O203, O291, and O221), the cells were cultured for 1 week as above, then overnight in medium containing 0.5% serum before use in NT. All cells with an intact membrane were used, as the limited number of targeted cells did not permit selection. Nuclear transfer, fusion, and activation were performed as in Park *et al.* (*12*). Embryos were kept in TCM/BSA for another 30 to 60 min before the fusion rate was evaluated. Fused embryos were cultured in NCSU 23 supplemented with BSA (4 mg/ml) overlaid with mineral oil. The surviving embryos (intact plasma membrane) were selected for transfer into surrogates.
23. C. Polge, *Control of Pig Reproduction* (Butterworth, London, 1981), pp. 283–285.
24. M. J. Martin, C. A. Pinkert, *Transgenic Animal Technology* (Academic Press, New York, 1994), pp. 315–333.
25. W. F. Pope, S. Xie, D. M. Broermann, K. P. Nephew, *J. Reprod. Fertil. Suppl.* **40**, 251 (1990).
26. Potential surrogates were checked for estrus twice a day. Depending on the exact time of estrus, NT-derived embryo transfers were performed 5 to 17 hours or 20 to 36 hours after the actual onset of estrus for day 0 and day 1 surrogates, respectively. In prior control experiments using in vitro produced embryos cultured for 22 hours after fertilization and then transferred to a day 1 surrogate, 19 of 100 embryos recovered on day 6 were at blastocyst stage, with an average nuclear number of 65. For surgery, gilts were induced with Pentothal (Abbott Laboratories) and anesthesia was maintained with 2% Halothane (Halocarbon Laboratories, River Edge, NJ). A midventral laparotomy was performed, and embryos were loaded into a 3½ Fr. tomcat catheter and deposited into the oviduct. Examination of the ovaries during embryo transfer confirmed that none of the surrogates had completed ovulation.
27. C. Polge, L. E. A. Rowson, M. C. Chang, *J. Reprod. Fertil.* **12**, 395 (1966).
28. Surrogates displayed few if any signs of impending parturition, similar to findings reported in other species (*31, 34*). They also lacked mammary gland development and colostrum production. The surrogates all displayed mild signs of vulvar enlargement and elongation, but much less than would normally be expected. This would appear to indicate a defect in the signaling mechanism between the fetuses and dam, and would most likely result from an abnormality in placental function. These observations were made even in the mated recipient.
29. Microsatellite reactions were amplified with fluorescent primers kindly provided by the USDA-supported U.S. Pig Genome Coordination Project and analyzed by Lark Technologies Inc. (Houston, TX).
30. J. B. Cibelli *et al.*, *Science* **280**, 1256 (1998).
31. J. R. Hill *et al.*, *Theriogenology* **51**, 1451 (1999).
32. L. Lai *et al.*, unpublished data.
33. T. Tao, Z. Machaty, L. R. Abeydeera, B. N. Day, R. S. Prather, *Zygote* **8**, 69 (2000).
34. D. N. Wells, P. M. Misica, H. R. Tervit, *Biol. Reprod.* **60**, 996 (1999).
35. GGTA1 homologous sequences in the pGalGT vector begin ~0.8 kb downstream of exon 7 and continue to ~6.8 kb downstream of the end of exon 9. A selection cassette—consisting of a Bip internal ribosome entry site, APRT coding sequences (encoding G418 resistance), and flanking stop codons—is inserted into an Eco RV site upstream of the GGTA1 catalytic domain in exon 9. Targeting is assessed with two PCR assays, each incorporating a primer outside of the vector homologous region. Upstream genomic structure is assessed with primers F238 (exon 7, upstream of the 5′ end of the pGalGT vector) and R823 (exon 9 downstream of the selection cassette insertion site). Upon digestion with Eco RI, fragments of 2.0 kb (WT locus), 3.1 kb (targeted locus), and 10.4 kb (either locus) are produced. Downstream genomic structure is assessed with primers F527 (exon 9 upstream of the selection cassette insertion site) and GR2520 (downstream of the 3′ end of the pGalGT vector). Upon digestion with Sac I, fragments of 1.2 kb (WT locus), 2.3 kb (targeted locus), and 8.1 kb (either locus) are produced.

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