Production of Transgenic Cloned Miniature Pigs with Membrane-bound Human Fas Ligand (FasL) by Somatic Cell Nuclear Transfer

Ki Myung Choi[1,2], Dong Il Jin[2], Seung Pyo Hong[1], Ji Yeon Yoo[1], Soo Hyun Kim[1], Young Chul Park[1], Yun Jin Yun[1], Kwang Wook Park[3], Jae Young Heo[1], Jae Goo Seol[1]

1. MGEN, Inc., #1101 World Meridian Venture Center, 60-24 Gasan-Dong, Guemchun-Gu, Seoul 153-781, Korea.
2. Division of Animal Science & Resources, Research Center for Transgenic Cloned Pigs, Chungnam National University, 220 Gung-Dong, Yuseong-Gu, Daejeon 305-764, Korea.
3. Sunchon National University, 413 Jungangro, Sunchon 540-742, Korea.

Correspondence to: Jae Goo Seol[1] e-mail:seolmgen@hanmail.net

ABSTRACT

Cell-mediated xenograft rejection, including NK cells and $CD8^+$ CTL, is a major obstacle in successful pig-to-human xenotransplantation. Human $CD8^+$ CTL and NK cells display high cytotoxicity for pig cells, mediated at least in part by the Fas/FasL pathway. To prevent cell-mediated xenocytotoxicity, a membrane-bound form of human FasL (mFasL) was generated as an inhibitor for CTL and NK cell cytotoxicity that could not be cleaved by metalloproteinase to produce putative soluble FasL. We produced two healthy transgenic pigs harboring the mFasL gene via somatic cell nuclear transfer (SCNT). In a cytotoxicity assay using transgenic clonal cell lines and transgenic pig ear cells, the rate of $CD8^+$ CTL-mediated cytotoxicity was significantly reduced in transgenic pig's ear cells compared with that in normal minipig fetal fibroblasts. Our data indicate that grafts of transgenic pigs expressing membrane-bound human FasL control the cellular immune response to xenografts, creating a window of opportunity to facilitate xenograft survival.

Introduction

A major well-known immunological barrier to xenotransplantation of pig organs into humans is the binding of human natural anti-Gal antibody to α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R) abundantly expressed on pig cells, inducing complement activation, hyperacute rejection, and acute vascular rejection of the xenograft.[1] Generation of an α-1,3-galactosyltransferase gene-knockout pigs may aid in overcoming this antibody-mediated hyperacute rejection.[2] Once hyperacute rejection is overcome, xenografts are rejected directly due to immunity mediated by cells including NK cells and $CD8^+$ CTL.[3,4,5,6] Previous studies have demonstrated that human $CD8^+$ CTL directly recognizes MHC classl (SLA-l) on pig endothelial cells, which have the ability to directly kill xenograft cells in xenograft recipients.[5,6] These findings indicate that human $CD8^+$ CTL-mediated cytotoxicity in xenograft recipients is a new immunologic barrier for successful pig-to-human xenotransplantation. In the present study, we aimed to produce transgenic pigs in which this CTL-mediated response against xenograft cells is blocked, and assess the mechanism underlying direct destruction of xenograft cells by human $CD8^+$ CTL.

Studies with mice deficient in perforin/granzyme or FasL have indicated that the perforin/granzyme and FasL systems are the major pathways for CTL-mediated cytotoxicity.[7,8,9] $CD8^+$ CTL predominantly uses the Fas/FasL pathway to kill xenografted cells.[6]

Fas (CD95), a type I transmembrane protein and a member of the tumor necrosis factor (TNF) receptor family, is expressed on a variety of hematopoietic cells, including activated NK and T cells, monocytes and polymorphonuclear neutrophilic leukocytes (PMN), as well as hepatocytes and

certain tumor cells.[10] On the other hand, the expression of FasL (CD178), a type II membrane protein, is more restricted and involves activated cytotoxic T lymphocytes (CTL),[11] NK cells,[12] and cells in 'immune privileged' sites.[10,13] Fas-expressing cells undergo apoptosis upon interactions with FasL.[10]

One valuable strategy for inhibiting the xenocytotoxic activity of human CTL involves the expression of human FasL on pig xenograft cells to kill human CTL via activation of human Fas.[14] However, this technique fails to protect xenograft cells because strong chemotaxis of human polymorphonuclear neutrophils is induced by soluble FasL released from cells expressing ligand.[15,16] In the present study, membrane-bound human FasL, which is not cleaved to the soluble form of FasL by metalloproteinase, was used to inhibit human CTL cytotoxicity against xenograft cells.

Pigs are useful in biomedical research due to their anatomic and physiologic similarities to humans.[17] In particular, miniature pigs are extensively used owing to their smaller body size[18] and well-defined genetic background,[19] compared with domestic pigs, including Landrace, Yorkshire, Duroc, and their hybrids. However, domestic pigs have the advantage of lower cost, higher prolificacy and wide availability.[20]

One of the most remarkable research fields using miniature pigs is xenotransplantation. Transgenic pigs have been produced for this purpose using the somatic cell nuclear transfer (SCNT) technique.[21] Recent advances in genetic engineering technology, particularly using SCNT, has allowed the generation of genetically modified miniature pigs for the development of xenotransplantation.[22,23] Limited information is available on SCNT clones using donor cells from miniature pigs.[23,24] Numerous factors affect the efficiency of SCNT in the pig, including SCNT

procedure,[25,26] artificial activation conditions,[27,28,29] stages of donor cells.[30]

In the case of SCNT miniature pig embryos, it is reasonable to use IVM oocytes derived from domestic pig ovaries, which are easily available from the slaughterhouse. Recent studies have shown that nuclear-cytoplasmic compatibility between different species or strains exerts a profound effect on the development of SCNT embryos,[31,32] indicating that hybrid embryos reconstructed from the donor nuclei of miniature pigs with domestic pig cytoplasts display different developmental outcomes in a cell source-specific manner.

A previous investigation on *in vivo*-generated embryos reports that both placenta development and maintenance of pregnancy are influenced by fetal and maternal breed.[33] Here, we hypothesize that breed differences between cell donors and recipients is an important factor affecting the success of cloning of miniature pigs.

RESULTS

Establishment of cells expressing human FasL

Miniature pig fetal fibroblasts were transfected with pcDNA/FasL vector. Transfected cells were selected using hygromycin (200 μg/Mℓ) for 2 weeks, and selected colonies were screened for the presence of the FasL gene using PCR. A stable cell line positive for wild-type FasL (W1-1) and two clones positive for mFasL (F3-140 and F4-41) were established for the transgenic clonal cell lines. Transgene expression was analyzed using FACS and immunocytochemistry analyses. A normal porcine cell line and three FasL-transfected clonal cell lines were analyzed by FACS with mouse anti-human Fas ligand to determine the surface expression profiles of wild-type and mutant FasL. FACS analysis revealed high FasL expression in transgenic clonal cells (Fig. 1a-c). FasL expression in transgenic clonal cell lines was increased, relative to that in normal fetal cells. Immunocytochemistry experiments indicated strong FasL staining in all transgenic clonal cell lines (Fig. 2). The presence of the human FasL gene in genomic DNA of the clonal cell lines was confirmed using FISH. F4-41 cells were identified as stable transgenic cells, while others were mixed with non-transgenic cells. The F4-41 cell line displayed a normal chromosome number (36,XX). The integration site on the chromosome in F4-41 was 3p17. Accordingly, nuclear transfer was performed using F4-41 transgenic cells as the nuclear donor.

Production of transgenic cloned pigs

Cloned miniature piglets can be produced using both miniature and domestic pig recipients (Table 1). Pregnancy rates after transferring cloned miniature pig embryos into recipient pigs were higher in miniature than domestic recipients. However, delivery rates of miniature pig embryos transferred to domestic pig recipients were significantly higher than the rates of those transferred to miniature pig recipients. Domestic recipient pigs delivered one healthy female piglet and two stillborn piglets. The miniature recipient also delivered one healthy female piglet. No abnormalities were observed upon initial physical examination of the piglets. The presence of the mFasL gene in individual piglets was confirmed by PCR using DNA from the umbilical cord (Fig. 3a). Analysis of ear cells using FISH probes for the membrane-bound FasL gene revealed transgene integration in all piglets identical to that observed in metaphase spreads derived from the F4-41 cell line (Fig. 3b,c). Transgene expression was analyzed using FACS. Transgenic pig ear fibroblasts were prepared from transgenic piglets at 30 days after birth. FACS analysis revealed a slightly lower mFasL level in transgenic piglet ear cells than in donor cells. However, we observed stable expression of mFasL (Fig. 1d).

The 11 microsatellite markers were used for differentiating between donor cell lines as well as recipients. As shown in Table 2, nuclear DNA genotypes were identical between the cell lines and SCNT clones, but differed from those of the surrogate recipients. Our data confirm that the piglets obtained are derived from the cell lines used for SCNT.

Phenotype analysis of cultured lymphocytes

Although all subpopulations were detected among fresh lymphocytes, the majority (>80%) of lymphocytes cultured for 14 days were $CD8^+$ T cells and small amounts (<20%) were positive for the anti-CD56 antibody. B cells and macrophages were not detected among the cultured lymphocytes (Fig. 4a). Furthermore, $CD8^+$ CTLs present in 14-day cultures were confirmed as T cells capable of killing porcine fibroblasts following isolation using magnetic beads (Fig. 4b). Our findings indicate that strong cytotoxicity of cultured lymphocytes is associated with the $CD8^+$ T population.

Overexpression of mFasL effectively prevents $CD8^+$ CTL-mediated cytotoxicity against ear fibroblasts from transgenic piglet

Freshly obtained lymphocytes from healthy donors displayed no cytotoxic activity towards normal mini-pig cells. However, cells cultured for 14 days had highly detrimental effects. Furthermore, strong cytotoxicity against mini-pig fibroblasts was associated with $CD8^+$ lymphocytes. Downregulation of CTL-mediated directed cell lysis by mFasL was observed in two clonal cell lines and transgenic piglet ear fibroblast cells. Overexpression of membrane-bound FasL in transgenic mini-pig ear fibroblasts resulted in marked cytoprotection from $CD8^+$ CTL activity. The rate of CTL cytotoxicity was reduced to 38.4±19.2% compared with the control (60.8 ± 11.3%) at a 5:1 E/T ratio, and reduced to 31.2±47.4% compared with the control (72.5±31.9%) at a 10:1 E/T ratio (Fig. 5). This finding indicates that beneficial effects of inhibition of $CD8^+$ CTL-mediated xenocytotoxicity and is elicited by expression of membrane-bound FasL in transgenic

mini-pigs.

DISCUSSION

In the present study, transgenic miniature pigs with membrane-bound human FasL gene were successfully produced. Moreover, overexpression of membrane-bound FasL on transgenic pig cells prevented human CTL-mediated cytotoxicity. In transgenic pig ear cells, cytotoxicity was significantly suppressed at effector:target ratios of 5:1 and 10:1. This finding indicates that expression of membrane-bound FasL molecules on the pig cell surface is required to bind the human Fas receptor, which is sufficient for protection against CTL killing.

Previous studies indicated that human $CD8^+$ CTL-mediated killing by xenograft recipients represents an immunological barrier because these are highly toxic to pig xenograft cells.[6] Our results additionally demonstrate activation of $CD8^+$ CTL that directly kill xenograft target cells *in vitro*. Incubation of lymphocytes with pig primary cells, followed by the addition of IL-2 after 3 days, resulted in the emergence of a $CD8^+$ T cell population comprising more than 80% of cultures incubated for 14 days (Fig. 4a). These $CD8^+$ CTL predominantly use the Fas/FasL pathway to kill xenografted cells.[6] Binding of FasL to Fas induces formation of the death-inducing signaling complex (DISC), which contains the Fas receptor as well as several signaling components, such as Fas-associated death domain (FADD) and caspase 8, which destroy the cells.[38] A membrane-bound form of human FasL with a serial deletion at the cytoplasmic region and the metalloproteinase cleavage site was constructed with the intention of inhibiting human CTL cytotoxicity. Human FasL is a type Ⅱ membrane protein with a proline-rich amino terminal cytoplasmic region.[39] Tanaka et al.[35] reported that the introduction of intact human FasL cDNA into various cell lines did not

generate stable transformants expressing high levels of FasL. This may be due to destabilization of FasL on the cell surface by the proline-rich sequence. Deletion of residues 8 to 69 induced overexpression of human FasL. FasL is cleaved by a metalloproteinase to produce the soluble form (sFasL), which is functional in inducing apoptosis. Tanaka and colleagues produced mFasL by deleting the cleavage site (residues 110 to 134).[36] Upon binding of TNF to its receptor, the complex is internalized and degraded, leading to receptor downregulation.[40] The sFasL/Fas complex may be easily internalized, whereas internalization of mFasL with Fas is likely to be retarded.

The mFasL gene was transfected into miniature pig fetal fibroblasts for producing transgenic clonal cells. The F4-41 clonal cell line was selected as a nuclear transfer donor cells using PCR, FACS, immunocytochemistry, and FISH analyses. Two healthy transgenic miniature pigs with mFasL were produced via SCNT. Expression of mFasL was low in transgenic pig ear cells compared with that in donor and other clonal types (Fig. 1). At present, the reason for the difference in expression levels in these cells is unknown, but may be due to the integration site or epigenetic modification of the transgene. Especially, Kang and colleagues reported that tissue-specific expression of the transgene correlates with DNA demethylation at specific CpG sites as well as significant changes in histone modification.[41] Although mFasL expression was low in transgenic ear cells, cytotoxicity was significantly reduced in transgenic pig cells compared with the control at effector:target ratios of 5:1 and 10:1 (Fig. 5). Our data are consistent with previous studies showing that mFasL overexpression on porcine endothelial cells and pig islet xenografts significantly suppressed human CTL cytotoxicity at low mFasL level.[42,43,44,45] CTL cytotoxicity was suppressed in the majority of transgenic clonal cell lines, but was still higher than that in transgenic pig ear cells. This may be attributed to senescence of the clonal cell line during transfection, drug selection

or other passing conditions for establishing transgenic clonal cell lines. These data indicate that xenograft cells isolated from transgenic pigs with mFasL control the cellular immune response to xenografts.

Our study demonstrates that transgenic cloned miniature pigs can be produced using both miniature and domestic pig recipients (Table 1). Using miniature pig cells as nuclear donors, we obtained pregnancy rates of 16% and 35% in miniature and domestic recipient animals, respectively. The production efficiency of cloned piglets using common domestic pigs as recipients tended to be higher, although no clear statistical differences were evident due to the small number of transfer experiments. In particular, miniature pig SCNT clones displayed considerably more fetal lethality at day 45 of gestation. Our findings were consistent with those of a previous study performed with Yucatan using SCNT embryos.[46] In view of resorbing around implantation periods, Yucatan SCNT clones have considerably more fetal lethality at day 30 of gestation. The placentas of Yucatan SCNT clones are smaller than those of occidental breed clones, which may contribute to the higher fetal mortality in Yucatan. Moreover, Ueno *et al*. suggested the possibility of embryonic loss in transferring cloned embryos with damaged zona pellucida to the oviduct.[47] Overall, reduced efficiencies in the miniature pig are unlikely to be an issue of technical variability or oocyte or recipient quality because higher efficiencies with domestic breeds using analogous oocyte sources as donors and the same recipient herd were obtained by our group.[48] Lower viability of the miniature pig is caused by factors other than experimental variability. These factors, including mitochondrial incompatibility and reduced compatibility between the miniature pig placenta and domestic uterine environment, remain to be fully characterized. According to previous studies performed with Meishan and Yorkshire pigs using *in vivo* fertilized embryos, pregnancy rates were

determined by the uterine environment up to day 90 of gestation, regardless of the fetal genotype.[33,49] Recently, domestic pigs were recommended as an embryo recipient for production of cloned miniature pigs due to their ability to accommodate more fetuses.[50] Compared with common domestic pigs, miniature pigs produce fewer piglets and production costs are higher. Consequently, establishing a mass production system for cloned miniature pigs using IVM oocytes derived from abattoir ovaries should further accelerate the usage of miniature pigs in this field.

To our knowledge, this is the first report to describe the generation of pigs containing transgenes of membrane-bound human FasL. Overexpression of mFasL on xenograft cells leads to strong protection against human CTL killing that may subsequently control the cellular response to xenografts and thus creates a window of opportunity to facilitate xenograft survival. In the present study, the SCNT technique originally developed for common domestic pigs was applied to miniature pigs, with efficient production of reconstructed embryos. Furthermore, our findings indicate both miniature and common domestic pig recipients can be effectively used for the production of transgenic cloned miniature pigs.

Acknowledgments

This work was supported by the BioGreen 21 Program (#PJ004200201003), Rural Development Administration, Republic of Korea.

Author Contributions

Ki Myung Choi and Jae Goo Seol designed and executed experiments, and wrote the manuscript. Dong Il Jin contributed to the manuscript and performed experiments. Seung Pyo Hong, Ji Yeon Yoo, Soo Hyun Kim, Young Chul Park, Yun Jin Yun, Kwang Wook Park and Jae Young Heo performed experiments.

REFERENCES

1. Galili, U. Interaction of the natural anti-Gal antibody with alpha-galactosyl epitopes: a major obstacle for xenotransplantation in humans. *Immunol. Today*. **14**, 480-482 (1993).

2. Phelps, C.J. et al. Production of α1,3-galactosyltransferase deficient pigs. *Science* **299**, 411-414 (2003).

3. Auchincloss, H., & Sachs, D.H. Xenogeneic transplantation. *Annu. Rev. Immunol.* **16,**433 (1998).

4. Itescu, S., Kwiatkowski, P., Artrip, J.H., Wang, S.F., Ankersmit, J., Minanov, O.P., & Michler, R.E. Role of natural killer cells, macrophages, and accessory molecule interactions in the rejection of pig-to-primate xenografts beyond the hyperacute period. *Hum. Immunol.* **59**, 275-286 (1998).

5. Shishido, S., Naziruddin, B., Howard, T., & Mohanakumar, T. Recognition of porcine major histocompatibility complex class I antigens by human CD8+ cytolytic T cell clones. *Transplantation* **64**, 340-346 (1997).

6. Tanemura, M., Chong, A.S., DiSesa, V.J., & Galili, U. Direct killing of xenograft cells by CD8+ T cells of discordant xenograft recipients. *Transplantation* **74**, 1587-1595 (2002).

7. Itoh, N. et al. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66**, 233-243 (1991).

8. Nagata, S., & Golstein, P. The Fas death factor. *Science* **267**, 1449-1456 (1995).

9. Nagata, S. Apoptosis by death factor. *Cell* **88**, 355-365 (1997).

10. Sharma, K., et al. Death the Fas way: regulation and pathophysiology of CD95 and its ligand. *Pharmacol. Ther*. **88**, 333-347 (2000).

11. Suda, T. et al. Expression of the Fas ligand in cells of T cell lineage. *J. Immunol*. **154**, 3806-3813 (1995).

12. Arase, H., Arase, N., & Saito, T. Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J. Exp. Med*. **181**, 1235-1238 (1995).

13. Green, D.R., & Ferguson, T.A. The role of Fas ligand in immune privilege. *Nat. Rev. Mol. Cell. Biol.* **2**, 917-924 (2001).

14. Rodríguez-Gago, M., de Heredia, A., Ramírez, P., Parrilla, P., Aparicio, P., & Yélamos, J. Human anti-porcine gammadelta T-cell xenoreactivity is inhibited by human FasL expression on porcine endothelial cells. *Transplantation* **72**, 503-509 (2001).

15. Kang, S.M., Schneider, D.B., Lin, Z., Hanahan, D., Dichek, D.A., Stock, P.G., & Baekkeskov, S. Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. *Nat. Med*. **3**, 738-743 (1997).

16. Seino, K. et al. Chemotactic activity of soluble Fas ligand against phagocytes. *J. Immunol*. **161**, 4484-4488 (1998).

17. Svendsen, O. The minipig in toxicology. *Exp. Toxicol. Pathol*. **57**, 335-339 (2006).

18. Wakai, T. et al. Production of viable cloned miniature pig embryos using oocytes derived from domestic pig ovaries. *Cloning. Stem . Cells*. **10**, 249–262 (2008).

19. Yao, S.K., Zhang, Q., Sun, F.Z., & Liu, P.Q. Genetic diversity of seven miniature pig breeds (strains) analyzed by using microsatellite markers. *Yi. Chuan*. **28**, 407–412 (2006).

20. Estrada, J.L. et al. Successful cloning of the Yucatan minipig using commercial/occidental breeds as oocyte donors and embryo recipients. *Cloning. Stem. Cells.* **10**, 287-296 (2008).

21. Sprangers, B., Waer, M., & Billiau, A.D. Xenotransplantation: where are we in 2008? *Kidney.*

Int. **74**, 14–21 (2008).

22. Dai, Y. et al. Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. *Nat. Biotechnol.* **20**, 251-255 (2002).

23. Lai, L. et al. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* **295**, 1089-1092 (2002).

24. Hoshino, Y. et al. Developmental competence of somatic cell nuclear transfer embryos reconstructed from oocytes matured in vitro with follicle shells in miniature pig. *Cloning. Stem. Cells.* **7**, 17-26 (2005).

25. Miyoshi, K., Saeki, K., & Sato, E. Improvement in development of porcine embryos reconstituted with cells from blastocyst-derived cell lines and enucleated oocytes by optimization of reconstruction methods. *Cloning* **2**, 175–184 (2000).

26. Du, Y. et al. Piglets born from handmade cloning, an innovative cloning method without micromanipulation. *Theriogenology* **68**, 1104-1110 (2007).

27. Cheong, H.T. et al. Effect of elevated Ca2+concentration in fusion/activation medium on the fusion and development of porcine fetal fibroblast nuclear transfer embryos. *Mol. Reprod. Dev*. **61**, 488–492 (2002).

28. Zhu, J. et al. Improvement of an electrical activation protocol for porcine oocytes. *Biol. Reprod*. **66**, 635–641 (2002).

29. Ziecik, A.J. et al. Influence of estrus synchronization of prepubertal gilts on embryo quality. *J. Reprod. Dev.* **51**, 379–384 (2005).

30. Prather, R.S., Boquest, A.C., & Day, B.N. Cell cycle analysis of cultured porcine mammary cells. *Cloning* **1**, 17–24 (1999).

31. Hiendleder, S. et al. Heteroplasmy in bovine fetuses produced by intra- and inter-subspecific somatic cell nuclear transfer: neutral segregation of nuclear donor mitochondrial DNA in various tissues and evidence for recipient cow mitochondria in fetal blood. *Biol. Reprod*. **68**, 159-166 (2003).

32. Hiendleder, S. et al. Nuclear-cytoplasmic interactions affect in utero developmental capacity, phenotype, and cellular metabolism of bovine nuclear transfer fetuses. *Biol. Reprod*. **70**, 1196-1205 (2004).

33. Biensen, N.J., Wilson, M.E., & Ford, S.P. The impact of either a Meishan or Yorkshire uterus on Meishan or Yorkshire fetal and placental development to days 70, 90, and 110 of gestation. *J. Anim. Sci.* **76**, 2169–2176 (1998).

34. Abeydeera, L.R. et al. Development and viability of pig oocytes matured in a protein-free medium containing epidermal growth factor. *Theriogenology* **54**, 787-797 (2000).

35. Tanaka, M. et al. Fas ligand in human serum. *Nat. Med*. **2**, 317-322 (1996).

36. Tanaka, M., Itai, T., Adachi, M., & Nagata, S. Downregulation of Fas ligand by shedding. *Nat. Med*. **4**, 31-36 (1998).

37. Lim, H.T. et al. Establishment of a microsatellite marker set for individual, pork brand and product origin identification in pigs. *J. Anim. Sci. & Technol. (Kor)* **51**, 201-206 (2009).

38. Kischkel, F.C. et al. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO. J*. **14**, 5579-5588 (1995).

39. Takahashi, T., Tanaka, M., Inazawa, J., Abe, T., Suda, T., & Nagata, S. Human Fas ligand: gene structure, chromosomal location and species specificity. *Int. Immunol*. **6**, 1567-1574 (1994).

40. Watanabe, N. et al. Continuous internalization of tumor necrosis factor receptors in a human

myosarcoma cell line. *J. Biol. Chem*. **263**, 10262-10266 (1998).

41. Kang, J.K. et al. Coordinated change of a ratio of methylated H3-lysine 4 or acetylated H3 to acetylated H4 and DNA methylation is associated with tissue-specific gene expression in cloned pig. *Exp. Mol. Med*. **39**, 84-96 (2007).

42. Kawamoto, K. et al. Preventing human CD8+ cytotoxic T lymphocyte-mediated cytotoxicity against swine endothelial cells by overexpression of human decoy Fas antigen. *Transplant. Proc*. **37**, 500-502 (2005).

43. Kawamoto, K. et al. In vivo controlling of cellular response to pig islet xenografts by adenovirus-mediated expression of either membrane-bound human FasL or human decoy Fas. *Transplant. Proc*. **41**, 331-333 (2009).

44. Tanemura, M. et al. Synergistic effects on the inhibition of human CD8+ cytotoxic T lymphocytes-mediated killing against xenograft cells by coexpression of membrane-bound Human FasL and decoy Fas antigen. *Transplant. Proc*. **37**, 4607-4609 (2005).

45. Tanemura, M. et al. Intracellular and extracellular remodeling effectively prevents human CD8(+)cytotoxic T lymphocyte-mediated xenocytotoxicity by coexpression of membrane-bound human FasL and pig c-FLIP(L) in pig endothelial cells. *Transplant. Proc*. **41**, 391-394 (2009).

46. Estrada, J.L. et al. Successful cloning of the Yucatan minipig using commercial/occidental breeds as oocyte donors and embryo recipients. *Cloning. Stem . Cells.* **10**, 287-296 (2008).

47. Ueno, S. et al. Association between embryonic loss and damage to the zona pellucida by invasive micromanipulation during oviductal transfer of early-stage embryos in pigs. *J. Reprod. Dev*. **53**, 1113-1118 (2007).

48. Park, K.W. et al. Production of transgenic recloned piglets harboring the human granulocyte-

macrophage colony stimulating factor (hGM-CSF) gene from porcine fetal fibroblasts by nuclear transfer. *Theriogenology* **70**, 1431-1438 (2008).

49. Wilson, M.E., Biensen, N.J., Youngs, C.R., & Ford, S.P. Development of Meishan and Yorkshire littermate conceptuses in either a Meishan or Yorkshire uterine environment to day 90 of gestation and to term. *Biol. Reprod*. **58**, 905–910 (1998).

50. Kurome, M. et al. Production of transgenic and nontransgenic clones in miniature pigs by somatic cell nuclear transfer. *J. Reprod. Dev*. **54**, 156–163 (2008).

FIGURE LEGENDS

Figure 1. Overexpression of wild-type FasL or mFasL on transgenic clonal cell lines. Normal minipig fetal fibroblasts (open histogram) and transgenic cells expressing wild-type FasL or mFasL (closed histogram) were stained with mouse anti-human FasL Ab and FITC-conjugated anti-mouse IgG Ab, and analyzed using flow cytometry, as described in the Methods section. Mean fluorescence intensities of stained cells are expressed in the upper right region of the histogram. a; transfected with wild type FasL (W1-1), b and c; transfected with membrane-bound FasL (F3-140 and F4-41), d; transgenic pig ear cells.

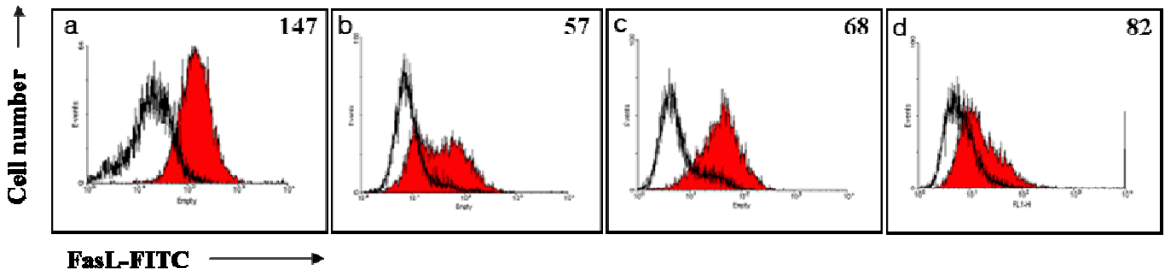
Figure 2. FasL expression on the clonal cell surface via immunocytochemistry. Images were obteined under bright fields (a,d,g,j), fluorescent fields (b,e,h,k), and merge (c,f,i,l). Normal cells (a-c); non-transfected fetal fibroblasts, W1-1 (d-f); transfected with wild type FasL , F3-140 (g-i) and F4-41 (j-l); transfected with membrane-bound FasL. Original magnification ×100.

Figure 3. PCR identification and fluorescence *in situ* hybridization (FISH) of the mFasL gene in transgenic piglets. Genomic DNA was subjected to PCR (a) using a specific primer set for mFasL. FISH analysis using a rhodamine-labeled probe containing the human FasL gene (b) and karyotyping revealed integration of cells obtained from transgenic cloned pigs (c) with normal karyotype (d).

Figure 4. Phenotype of human lymphocytes. Human lymphocytes were prepared from the peripheral blood of healthy volunteers by density centrifugation through Ficoll. Human lymphocytes were cultured at 37℃ for 14 days with IL-2. To determine the profile of human lymphocytes after culture, cells were stained with PE-conjugated anti-$CD8^+$, CD19, CD11 and CD56, respectively (a). To isolate $CD8^+$ lymphocytes, cells were incubated with $CD8^+$ DynaBeads for 20 min at 4℃. After isolation, cells were stained with PE-conjugated anti-$CD8^+$ (b).

Figure 5. The cytoprotective effect of FasL expression on miniature pig cells against human $CD8^+$ lymphocytes. The cytotoxicity assay was performed in cultured $CD8^+$ lymphocytes from healthy volunteers. Effector cell and target cell ratios are 5:1 (closed circles) and 10:1 (open circles), respectively. Negative control; non-transfected fetal fibroblasts, F4-41; donor cell, W3-104; transfected with wild-type FasL, Transgenic pig; transgenic cloned miniature pig ear cells.

Figure 1.



465

Figure 2.

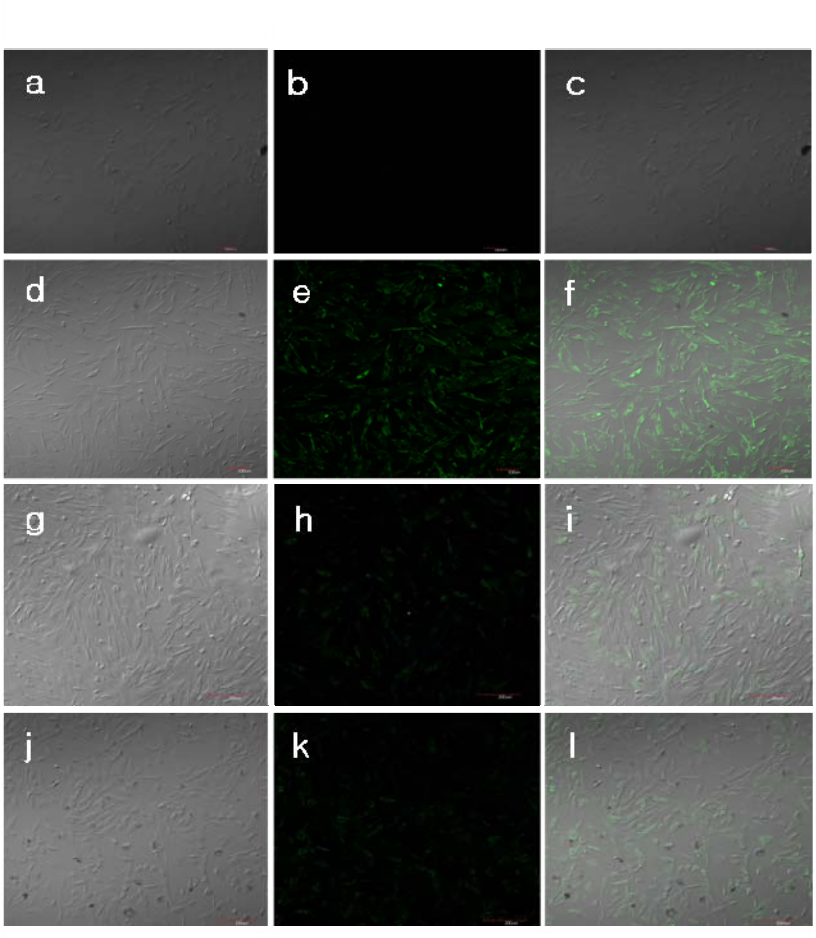
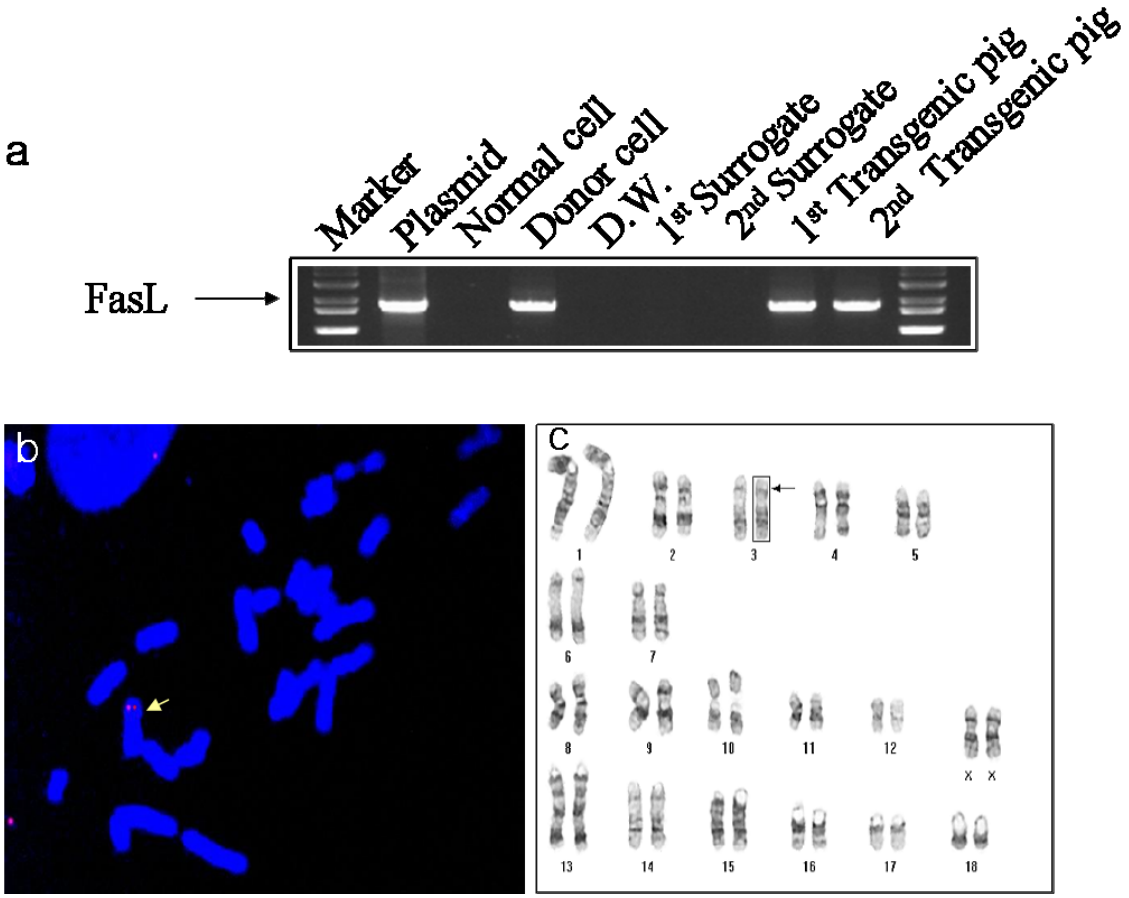


Figure 3.



480 **Figure 4.**

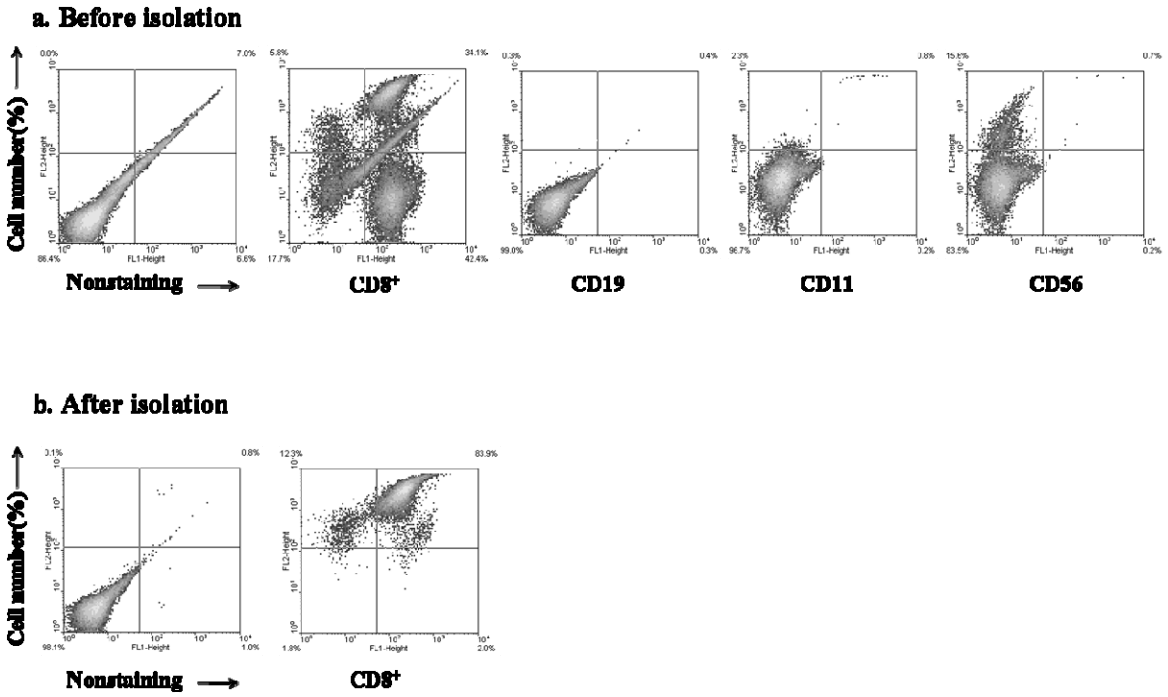


Figure 5.

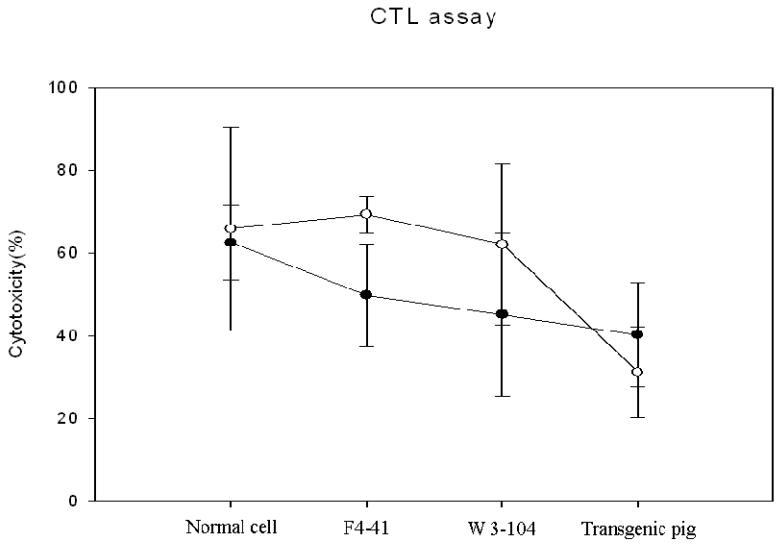


Table 1. *In vivo* development of cloned porcine embryos derived from fetal fibroblasts of miniature pig and transferred to miniature or domestic recipient pigs

Donor cell	Recipient Breed	No. of recipients	Pregnancy (%)	Delivery (%)	No. of piglets (Stillborn)
F4-41	Miniature	14	5 (35)	1 (7)	1
	Domestic	6	1 (16)	1 (16)	1 (2)

Table 2. Microsatellite (MS) analysis of clonal cell line, surrogates and piglets[a]

Markers	Donor cell	1st surrogate	1st piglet	2nd surrogate	2nd piglet
S0005	222/246	222/246	222/246	236/242	222/246
S0026	100/104	104/106	100/104	100/106	100/104
S0155	165/167	163/165	165/167	165/165	165/167
S0225	186/186	186/192	186/186	174/192	186/186
SW122	120/120	120/120	120/120	122/128	120/120
SW24	104/118	130/130	104/118	118/124	104/118
SW632	168/168	168/176	168/168	178/178	168/168

SW72	105/125	105/107	105/125	115/115	105/125
SW787	150/164	152/158	150/164	162/166	150/164
SW939	118/118	118/118	118/118	100/114	118/118
SW951	129/139	129/139	129/139	127/131	129/139

[a] **For each microsatellite marker, genotype was determined by size (base pairs). Litter 2 results from markers provide the strongest support for the genetic identity of fetal fibroblasts by donor cells (F4-41) and nuclear transfer piglets.**