

Production of Transgenic Miniature Piglets by Transfer of *ifn-γ* Gene Transfected Somatic Cells into Enucleated Oocytes

Suo Li*, Jin-Dan Kang*, Jun-Xue Jin, Cheng-Du Cui, Wen-Xue Li, Qing-Shan Gao, Yu Hong, Hai-Ying Zhu, Long Jin and Xi-Jun Yin

Department of Animal Science, Agricultural College of Yanbian University

Corresponding author: Xi-Jun Yin

Agricultural College of Yanbian University

Yanji 133002, P. R. China

Tel: +86-0433-2435623; Fax: +86-0433-2435622

E-mail: yinxj33@msn.com

Received: January 10, 2014, Accepted: January 20, 2014, Published: January 22, 2014.

*These authors contributed to the work equally and should be regarded as co-first authors.

ABSTRACT

Generation of transgenic pigs by somatic cell nuclear transfer (SCNT) has provided a new avenue to modify disease resistance or susceptibility in pigs. In this study, we evaluated the efficiency of producing cloned transgenic piglets by SCNT. Donor cells were isolated from a 2-year-old female Wuzhishan miniature piglet. Cells transfected with either the swine broad-spectrum anti-viral interferon- γ gene (IFN- γ) and non-transfected cells between passages 3 and 5, were used for cloning. We examined the developmental potential of porcine nuclear transfer (NT) embryos when IFN- γ and non-transfected cells were used as donor cells. There was no significant difference in cleavage and development rates to the blastocyst stage for IFN- γ NT embryos compared with non-transgenic donor cells (78.4% and 10.8% vs. 80.6% and 16.2%, respectively). Cloned embryos reconstructed with IFN- γ gene were introduced into 4 surrogate mothers and 1 of these gave birth to 7 cloned piglets. Genomic DNA from cloned piglets was screened, and 5 PCR-positive founders were identified. The control group were introduced into 8 surrogate mothers. Of these mothers, 5 became pregnant as detected by ultrasonography on days 24–26 after embryo transfer. 3 surrogate mothers gave birth to 18 cloned piglets by eutocia after 114–117 days of gestation. While the number of piglets surviving to adulthood was not significantly different between transgenic and non-transgenic donor cells (57.1% vs. 66.6%). We succeeded to product transfer of IFN- γ gene Wuzhishan inbred miniature pigs by somatic cell nuclear transfer, to provide a suitable approach to produce resistant transgenic pigs. The disease resistance of IFN- γ transgenic pigs has yet to be further study.

Key words: transgenic pig, somatic cell nuclear transfer, IFN- γ

INTRODUCTION

Somatic cell nuclear transfer (SCNT) using oocytes matured in vitro has been successfully employed to produce clones of a wide range of farm animals, including sheep [1], cattle [2], pigs [3]. The first successful gene transfers into farm animals by microinjection were reported in 1985 [4]. This approach has the potential of developing completely new breeding strategies and novel applications. Transgenic pigs with random insertions are very valuable in biomedical research, as well as in agriculture. As reviewed over a decade ago [5], genetic modification can create animals that are resistant to specific diseases. Westhusin et al. have reported an example involving cattle and the rescue of a genome affording natural disease resistance, and genetic basis of natural disease resistance against bovine brucellosis [6]. they decided to utilize these cells for somatic cell nuclear transfer to attempt the production of a cloned bull and salvage the valuable

genotype. To prove the existence of some valuable genes will have a certain ability to resist disease.

Interferons (IFNs) are the first line of innate defense against viral infection [7]. They confer resistance against many different viruses, inhibit proliferation of normal and malignant cells, impede multiplication of intracellular parasites, enhance macrophage and granulocyte phagocytosis, augment natural killer cell activity, and show several other immunomodulatory functions. Most species have three IFNs: IFN- α , IFN- β and IFN- γ . IFN- γ , a type II interferon, is an important immunoregulatory molecule whose initial biological properties were first reported in 1965. Although the effects of IFN γ in vitro and in vivo and at the molecular level have been widely investigated.

Since 1993, transgenic mice carrying a hybrid gene consisting of ovine I-lactoglobulin gene sequences and human γ -interferon (hIFN- γ) cDNA have been produced [8]. To this day, it has not

been reported Production of transfer of IFN- γ gene farm animals by somatic cell nuclear transfer, such as pigs. Miniature pigs are considered to represent an attractive species for use as experimental models for biomedical research, due to the many characteristics including anatomy and physiology that are similar to humans, and that their small body size is easier to handle, and cheaper to maintain compared with larger domestic pigs [9]. The Wuzhishan inbred miniature pig is a Chinese inbred mini pig with the highest inbreeding coefficient and one of the candidates of experimental models.

We succeeded to product 7 cloned piglets transfer of IFN- γ gene Wuzhishan inbred miniature pigs by somatic cell nuclear transfer, 5 PCR-positive founders were identified. To provide a suitable approach to produce resistant transgenic pigs.

MATERIALS AND METHODS

Plasmids, chemicals and animals

Swine broad-spectrum anti-viral interferon- γ (IFN- γ) plasmids were provided by Professor Jin Ning-yi (Laboratory of Genetic Engineering of PLA, Academy of Military Medical Sciences, China). All chemicals used in this study were purchased from Sigma Chemical Company (Sigma-Aldrich, China), unless otherwise indicated. Laboratory animals were purchased from the Agricultural Science and Technology Institute of Yanbian, China. This research was carried out in accordance with the Ethics Committee of Yanbian University.

Donor cell culture

Ear fibroblasts were isolated from a 2-year-old female Wuzhishan miniature piglet and cultured in a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ in air at 38°C. The cells were used as donors for nuclear transfer (NT) between passages 3 and 5. A single cell suspension was prepared by standard trypsinization immediately before NT.

Transfection of resistance gene into ear fibroblasts

The day before transfection, confluent ear fibroblasts at passages 3 to 5 were trypsinized, counted, plated onto 35mm culture dishes, and cultured to 80% confluency. Two μ L of the IFN- γ plasmid and 8 μ L of FuGENE® HD (Roche Diagnostics, Indianapolis, IN, USA) were added to 100 μ L of serum-free DMEM. After incubating for 15 min at room temperature, the transfection mixture was added dropwise to the culture dish containing 800 μ L medium. After 5–6 h, the transfection mixture was replaced with lab stock medium. Approximately 24 h thereafter, cells were cultured in media supplemented with 200 μ g/mL G418 for 15 days. The surviving colonies were picked up with 5mm cloning dishes and transferred into 96-well plates to achieve stable integration of the gene into the genome before SCNT.

Retrieval and in vitro maturation of oocytes

Ovaries were obtained from pigs at a local slaughterhouse and transported to the laboratory within 2 h of collection in 0.9% NaCl containing 75 μ g/mL penicillin G and 50 μ g/mL streptomycin sulfate at 30–39°C. Cumulus-oocyte complexes (COCs) were aspirated from 2–5 mm diameter follicles with a 20 gauge needle attached to a 10 mL disposable syringe and washed three times in handling medium. Good-quality COCs were selected on the basis of size (~120 μ m) and the presence of a homogeneous granulated cytoplasm and at least three uniform

layers of compact cumulus cells. COCs were washed three times in TL-HEPES containing 0.1% polyvinyl alcohol (PVA) and cultured in four-well plates (Nunc), with each well containing 500 μ L NCSU-37 medium supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP), 0.1 IU/mL human chorionic gonadotropin (hCG) and 0.1 IU/mL pregnant mare serum gonadotropin (PMSG) for 20 h. Thereafter, COCs were cultured for an additional 18–24 h in the absence of dbcAMP and hCG, as previously reported [10], followed by incubation in in vitro maturation (IVM) medium under mineral oil at 37°C in a humidified atmosphere of 5% CO₂ in air. Mature eggs with the first polar body were collected.

Nuclear transfer

NT was performed as described by Yin et al. [11]. In brief, mature eggs with the first polar body were cultured in media supplemented with 0.4 μ g/mL demecolcine and 0.05 M sucrose for 1 h. Eggs with a protruding membrane were then transferred to media containing 5 μ g/mL cytochalasin B (CB) and 0.4 μ g/mL demecolcine. The protrusion was removed with a beveled pipette, and the first polar body and a portion of cytoplasm directly beneath it were aspirated.

A single donor cell was injected into the perivitelline space of each egg and electrically fused using two direct current pulses of 150 V/mm for 50 μ sec in 0.28 M mannitol supplemented with 0.1 mM MgSO₄ and 0.01% PVA. Fused eggs were cultured in NCSU-37 medium for 1 h before electro activation and then cultured in medium supplemented with 5 mg/mL of CB for 4 h. The reconstructed oocytes were activated by two direct current pulses of 100 V/mm for 20 msec in 0.28 M mannitol supplemented with 0.1 mM MgSO₄ and 0.05 mM CaCl₂. Activated eggs were cultured in medium for 6 days in a humidified atmosphere of 5% CO₂ and 95% air at 38.5°C. At the end of the culture period, blastocysts were mounted onto slides with a mixture of glycerol and PBS (9:1) containing 10 μ g/mL Hoechst 33342. After coverslipping and sealing the edges with nail polish, the number of nuclei was counted under ultraviolet light.

Embryo transfer

Cloned embryos after SCNT and overnight culture were surgically transferred to the oviducts of surrogate mothers on the day of, or 1 day after, the onset of estrus. Pregnancy was diagnosed by ultrasonography on day 25 (day 0 was the day of SCNT) and monitored weekly. Cloned piglets were delivered naturally or induced by an intramuscular injection of prostaglandin F_{2 α} (Ningbo Second Hormone Factory, Zhejiang, China) on day 118 of gestation, if farrowing did not start spontaneously.

Quality evaluation of embryos cultured in vitro

To monitor in vitro development, NT embryos were cultured in culture medium for 7 days. Cleavage and blastocyst rates were determined 2 and 7 days after activation. To determine the total number of cells, blastocysts were stained with 10 μ g/mL Hoechst 33342 for 5 min, mounted onto slides with 100% glycerol, and visualized under a fluorescence microscope.

Identification of positive clones

PCR analysis was performed on total genomic DNA isolated from piglet fibroblasts. DNA from each clone was adjusted to the same concentration. The primers used to amplify IFN- γ (501 bp PCR transcript) were 5' -ACA ACTTATTTCTTGCTTTTCAG-3' (forward) and 5' -CTTGG AACATAGTCTGACTTCTC-3' (reverse). The PCR reaction mixture contained 2 μ L of genomic DNA, 25 mM

MgCl₂, 1 μ L of 10 mM deoxynucleoside triphosphates, 1 μ L of each primer at 20 pM, and 5U of Taq DNA polymerase (Takara Bio Inc., Shiga, Japan) in a final volume of 25 μ L. The PCR reaction was performed using the following conditions: 30 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 30 s, and synthesis at 72°C for 30 s. The PCR products were fractionated on a 1% agarose gel.

Statistical analysis

There were at least three replicates for each treatment. The nuclear maturation of oocytes was analyzed by one-way analysis of variance (ANOVA). Differences were identified by Duncan multiple comparison test using SPSS 17.0 software (SPSS Inc, IL, USA). Data were expressed as mean \pm SEM and $p < 0.05$ was considered significant.

RESULTS

Embryo development following SCNT with nuclei obtained from IFN- γ transfected or non-transfected fibroblasts

The present study examined the effects of different types of transgenic donor cells on the developmental potential of porcine NT embryos. There was no significant difference in the developmental rate of IFN- γ NT embryos to the 2–4 cell stage and non-transgenic donor cells (78.4% vs. 80.6%, respectively, $p < 0.05$) (Table 1). Furthermore, there was no significant difference in the developmental rate of NT embryos to the blastocyst stage and transgenic or non-transgenic donor cells (10.8% vs. 16.2%, respectively, $p < 0.05$). The average number of cells in blastocysts following transgenic SCNT was 36.0 \pm 6.0.

Generation of transgenic Wuzhishan miniature pigs

We used IFN- γ transgenic cells as donor cells to reconstruct NT embryos by SCNT. Approximately 200 NT blastocysts were transferred to a surrogate mother, while cloned embryos reconstructed with IFN- γ or control group were introduced into 12 surrogate mothers. Of these mothers, 7 became pregnant as detected by ultrasonography on days 24–26 after embryo transfer. 4 surrogate mothers gave birth to 25 cloned piglets by eutocia after 114–117 days of gestation, respectively 7 (IFN- γ group) and 18 (control group) piglets. These transgenic Wuzhishan miniature pigs were called Wmp-IFN. While the number of piglets surviving to adulthood was not significantly different between transgenic and non-transgenic donor cells (57.1% vs. 66.6%) (Table 2).

Genomic DNA from offspring was screened by PCR, and six PCR-positive founders were identified (Fig.1). While five out of seven SCNT-wmp offspring carried the IFN- γ transgene. Total three IFN- γ transgene positive piglets survived to adulthood (Fig.2).

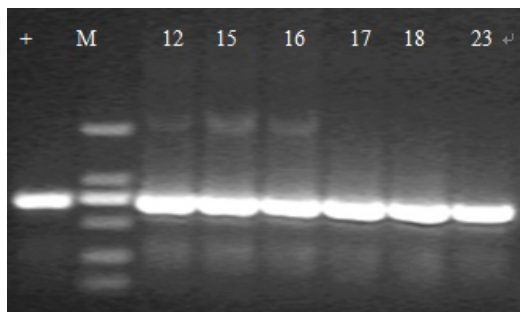


Fig.1. PCR of genomic DNA from transgenic pigs. lane +: positive control, lane M: 100 bp ladder marker, lanes 12,15,16,17,18, and 23: genomic DNA from different transgenic pigs.

DISCUSSION

Genetic modifications in swine are useful in both agriculture and human medicine. Current research focuses on improving productivity traits, 'gene farming', the establishment of animal models for human diseases, and the provision of organs for xenotransplantation [12]. Another important aspect is the improvement of animal health by transgenic means [13]. Approaches to reduce disease susceptibility are not only important for animal welfare but also to the economy. Genetic improvement of livestock achieved by both conventional breeding techniques and recently developed techniques in molecular biology may reduce expenses associated with the treatment of disease that have been estimated to account for 10–20% of total production costs.

Gene transfer into farm animals by microinjection offers the prospect of developing completely new breeding strategies and novel applications. For example, Clements et al.[14] constructed transgenic sheep that expressed an envelope gene of the visna virus under the control of the visna long terminal repeat to investigate the role of the env gene in the pathogenesis of lentiviral disease in its natural host. Disease resistance was significantly improved in these transgenic sheep. Bleck et al. [15] generated sows that produced bovine α -lactalbumin in their milk, while Golovan et al.[16] introduced a phytase gene that is expressed in the salivary glands of pigs. In both cases, animals produced manure with less phosphorous. Another area that might be important is to create animals that produce antibodies in their milk. This would protect nursing animals from diseases such as transmissible gastroenteritis [17].Golding MC et al. [18] have explored the utility of RNA interference (RNAi) in silencing the expression of genes in livestock. SCNT using oocytes matured in vitro has been successfully employed to produce clones of goat fetus by shRNA targeting caprine and bovine prion protein (PrP).Richt et al. [19] have reported the generation and characterization of PrPC-deficient cattle produced by a sequential gene-targeting system. At over 20 months of age, brain tissue homogenates are resistant to prion propagation in vitro as assessed by protein misfolding cyclic amplification. Those results provide strong evidence that the approach described here will be useful in producing transgenic livestock conferring potential disease resistance and provide an effective strategy for suppressing gene expression in a variety of large-animal models.

Previous studies have demonstrated that IFN- α and IFN- β can rapidly induce a nonspecific protective response against all FMDV serotypes in vitro [20]. Beneficial effects of interferon therapy in FMDV infections have been observed; porcine IFN- α in combination with adenovirus has been used to protect swine from FMDV[21]. IFN- γ , the only type II interferon, can also inhibit FMDV in vitro [22], but the in vivo effect is not well established. Yao et al. have demonstrated that porcine IFN- γ was useful as an anti-FMD agent to more rapidly induce protection in susceptible animals such as swine. And porcine IFN- γ protects swine from foot-and-mouth disease virus (FMDV)[23].

In the research field of transgenic animal the combination of cloning and somatic cell transgenic technology was the essential trend of development. It was because that the technology could greatly decline the production cost. In this search, we used the gained the positive cell which was transplanted IFN- γ gene as the donor and constructed the transgenic reconstructed embryo which was trans ferred into the denudeated oocyte via the nuclear transplantation and electrofusion. The transgenic blastula was

successfully gained , and the development situation in vitro of clone embryo was contrasted . The result showed that there was no significant difference in the developmental rate of IFN- γ NT embryos to the 2–4 cell stage and non-transgenic donor cells. The blastocyst ratio in vitro of IFN- γ transgenic reconstructed embryo was 10.8 % and had no obvious difference ($P > 0.05$) with the untransformed one (16.2 %). It corresponded with the research

conclusion of Hitomi Matsunari et al . [24]. The literature reported that the long tmie drug screening on the transgenic cell could decline the cloning efficiency of somatic cell [25-26] . But in the research , the situation didn't appear , and the developmental capability in vitro of transgenic cell had no significant difference with the gene clone embryo . It might relate to the used cell line .



Fig.2. Three MMLV-IFN transgene positive Wuzhishan miniature cloned piglets survived to adulthood.

Table 1. Development ability of transgenic SCNT embryos and non-transgenic SCNT embryos.

Transgenic donor cells	No. of transferred embryos	No. of 2–4 cell(%)	No. of blastocysts (%)
IFN- γ	74	58(78.4) ^a	8(10.8) ^a
Non-transgenic	93	75 (80.6) ^a	15(16.2) ^a

Table 2. The pregnancy of recipients of tansgenic SCNT embryos and non-transgenic SCNT embryos

Transgenic donor cells	No. of recipient s	No. of pregnant recipients (%)	No. of parturition recipients (%)	No. of cloned piglets	Piglets surviving to adulthood, No. (%)	PCR-positive (%)	No. of positive piglets surviving to adulthood
IFN- γ	4	2 (50.0)	1 (25.0)	7	4(57.1)	5(71.4)	3
Non-transgeni	8	5(62.5)	3(37.5)	18	12(66.6)	-	-

Cloned embryos reconstructed with IFN- γ gene were introduced into 4 surrogate mothers and 1 of these gave birth to 7 cloned piglets. Genomic DNA from cloned piglets was screened, and 5 PCR-positive founders were identified. The control group were introduced into 8 surrogate mothers. Of these mothers, 5 became pregnant as detected by ultrasonography on days 24–26 after embryo transfer. 3 surrogate mothers gave birth to 18 cloned piglets by eutocia after 114–117 days of gestation. While the number of piglets surviving to adulthood was not significantly

different between transgenic and non-transgenic donor cells (57.1% vs. 66.6%).

At present there were few about the effect of exogenous gene on the pregnancy of recipients. There were many reasons , such as a mount of the transplantation, embryo quality,surrogate sows pecies , mimune system and so on .Besides the above reasons , there were other elements that affected the transplantation efficiency of pig embryo , such as the surrogate mother , anesthesia , operation , transplanted site ,nursing after the

operation and so on . Every link all decided the success and efficiency, increased the difficulty of the research.

We succeeded to product transfer of IFN- γ gene Wuzhishan inbred miniature pigs by somatic cell nuclear transfer, to provide a suitable approach to produce resistant transgenic pigs. The disease resistance of IFN- γ transgenic pigs has yet to be further study.

Acknowledgements

Supported in part by the Genetically Modified Organisms Breeding Major Project (2009ZX08006-002B) and the State Key Development Program for Basic Research of China (Grant No. 2012CB526710).

REFERENCES

- Campbell KH, McWhir J, Ritchie WA, Wilmut I, Sheep cloned by nuclear transfer from a cultured cell line, *Nature* . 380(1996)64–66.
- Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM, Cloned transgenic calves produced from nonquiescent fetal fibroblasts, *Science*. 280(1998) 1256–1258.
- Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry AC. Pig cloning by microinjection of fetal fibroblast nuclei. *Science*. 289(2000)1188–1190.
- Hammer, R.E., Pursel, V.G. Rexroad Jr., C.E., Wall, R.J., Palmiter, R.D. and Brinster, R.L. Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* . 315(1985)680–683.
- Wheeler M.B. Transgenic technology and applications in swine. *Theriogenology*. 56(2001)1345–1369.
- M. E. Westhusin, T. Shin, J. W. Templeton, R. C. Burghardt and L. G. Adams .Rescuing valuable genomes by animal cloning: A case for natural disease resistance in cattle. *J ANIM SCI*. 2007; 85:138–142.
- Samuel, C.E. Antiviral actions of interferons. *Clin. Microbiol. Rev.* 14(2001) 778–809.
- V.N. Dobrovolsky, O.V. Lagutin, T.V. Vinogradova, I.S. Frolova, V.P. Kuznetsov, O.A. Larionov . Human γ -interferon expression in the mammary gland of transgenic mice. *FEBS Letters*. 1993; 319(1993): 181–184.
- Bollen P., Ellegaard L., *Pharmacol Toxicol.* [J], 80(1997) 3.
- Funahashi H, Cantley TC, Day BN. Synchronization of meiosis in porcine oocytes by exposure to dibutyl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. *Biol Reprod* . 57(1997)49–53.
- Yin XJ, Tani T, Yonemura I, Kawakami M, Miyamoto K, Hasegawa R, et al. Production of cloned pigs from adult somatic cells by chemically assisted removal of maternal chromosomes. *Biology of reproduction*. 67(2002)442–446.
- Müller, M. and Brem, G.. Transgenic strategies to increase disease resistance in livestock. *Reprod. Fert.* 6(1994)605–613.
- Brem, G. and Müller, M. Large transgenic animals. In: Maclean, N. (Ed.) *Animals with Novel Genes*, Cambridge University Press, Cambridge, UK.(1994)179–244.
- Clements J.E., Wall R.J., Narayan O., Hauer D., Schoborg R., Sheffer D., Powell A., Carruth L.M., Zink M.C., Rexroad C.E..Development of transgenic sheep that express the visna virus envelope gene. *Virology*. 200(1994) 370–380.
- Bleck G.T., White B.R., Miller D.J., Wheeler M.B..Production of bovine alpha-lactalbumin in the milk of transgenic pigs *J. Anim.* 76(1998)3072–3078.
- Golovan S.P., Meidinger R.G., Ajakaiye A., Cottrill M., Wiederkehr M.Z. r, Barney D.J.. Pigs expressing salivary phytase produce low-phosphorus manure. *Nat. Biotechnol* . 19(2001)741–745.
- Castilla J..Engineering passive immunity in transgenic mice secreting virus-neutralizing antibodies in milk. *Nat. Biotechnol.* 16(1998)349–354.
- Golding MC, Long CR, Carmell MA, Hannon GJ, Westhusin ME. Suppression of prion protein in livestock by RNA interference. *Proc Natl Acad Sci U S A*. 103(2006)5285–90.
- Richt J A, Kasinathan P, Hamir A N, et al. Production of cattle lacking prion protein[J]. *Nature biotechnology*. 25(2006)132–138.
- Sevilla, N., Domingo, E.. Evolution of a persistent aphthovirus in cytolytic infections: partial reversion of phenotypic traits accompanied by genetic diversification. *J. Virol.* 70(1996) 6617–6624.
- Chinsangaram, J., Moraes, M.P., Koster, M., Grubman, M.J.. A novel viral disease control strategy: adenovirus expressing interferon alpha rapidly protects swine from foot-and-mouth disease. *J. Virol.* 77 (2003)1621–1625.
- Zhang, Z.D., Hutching, G., Kitching, P., Alexandersen, S.. The effects of gamma interferon on replication of foot-and-mouth disease virus in persistently infected bovine cells. *Arch. Virol.* 147(2002)2157–2167.
- Qingxia Yao, Qinfeng Huang, Yi Cao, Ping Qian, Huanchun Chen. Porcine interferon-gamma protects swine from foot-and-mouth disease virus (FMDV). *Veterinary Immunology and Immunopathology*. 122(2008)309–311.
- Matsunari H , Onodera M , Tada N , Mochizuki H , Karasawa S , Haruyama E , Nakayama N , Saito H , Ueno S , Kurome M , Miyawaki A , Nagashima H .Transgenic-cloned pigs systemically expressing red fluorescent protein, Kusabira-Orange. *Cloning Stem Cells*.10 (2008) 313–23.
- Jeff Betthausen, Erik Forsberg, Monica Augenstein, et al. Production of cloned pigs from in vitro systems. *Nature Biotechnology*. 18(2000)1055 – 1059.
- Atsuo Ogura, Kimiko Inoue, Narumi Ogonuki, et al. Production of Male Cloned Mice from Fresh, Cultured, and Cryopreserved Immature Sertoli Cells. *Biology of Reproduction*, 62(2000) 1579–1584 .