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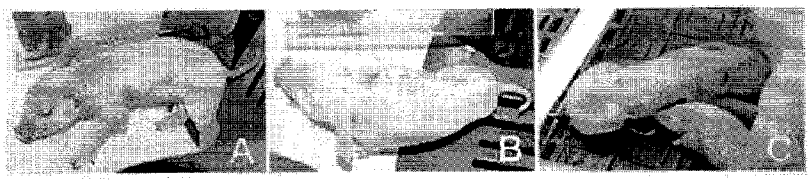
(54) Title: PIGLET PRODUCTION METHOD BY SIMULTANEOUS TRANSFER OF EMBRYOS FORMED BY IN VITRO FERTILIZATION AND NUCLEAR TRANSFER

【Figure 2】



(57) Abstract: Disclosed herein is a piglet production method comprising the steps of : (A) collecting and culturing immature oocytes from a butchered pig: (B) preparing a) embryos by in vitro fertilization of the mature oocytes prepared in the step (A) with liquid semen and b) somatic cell-cloned embryos by enucleation of the mature embryos prepared in the step (A) and transfer of donor somatic cells into the enucleated oocytes, and culturing the prepared embryos (a) and (b) separately; and (C) transferring the in vitro fertilized embryos and the somatic cell-transferred embryos, prepared in the step (B), simultaneously into one surrogate. The method facilitates the implantation of somatic cell-cloned embryos, which are generally rarely implanted, so as to increase the production of cloned piglets. Also, according to the method, the in vitro culture period of in vitro fertilized embryos can be greatly reduced, normal delivery is possible even from a small number of embryos, and piglets derived from in vitro fertilized embryos and piglets derived from somatic cell-cloned embryos can be simultaneously produced.

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【DESCRIPTION】

【Invention Title】

PIGLET PRODUCTION METHOD BY SIMULTANEOUS TRANSFER OF EMBRYOS FORMED BY IN VITRO FERTILIZATION AND NUCLEAR TRANSFER

【Technical Field】

The present invention relates to the production of piglets by embryo transfer, and more particularly to a method of producing piglets by *in vitro* maturation of immature oocytes and then simultaneous transfer of *in vitro* fertilized embryos and somatic cell-cloned embryos.

【Background Art】

Until recently, the improvement of pigs in reproductive terms has relied mainly on artificial insemination with superior boars, but there is a limitation in the improvement of pigs only by artificial insemination. Thus, pig improvement using superior sows was paid to attention, and the technology developed due to this need is embryo transfer. Embryo transfer is important because it can greatly contribute to studies not only on the genetic improvement of pigs, but also on sex ratio adjustment, monozygous twin production, nuclear transfer, foreign gene introduction, the identification of mechanisms of early embryonic development and gene expression, the control of diseases, etc.

A person who first succeeded in the transfer of mammalian embryos is Heape (1890, Proc. Roy. Soc. 48:457). He succeeded in producing a litter rabbit by transferring Angora rabbit embryos into a Belgian doe. A person who first succeeded in the transfer of pig embryos is Kvansnickii (1951, Interbreed ova transplantation. Sovetsk, Zootech. 1:36). Since embryo collection and transfer methods, which are currently used in most laboratories, were developed in the 1960s by Hancock and Hovell (1962, J. Reprod. Fert. 4: 195), Dziuk et al. (1964, J. Anim. Sci. 23:37), and Vincent et al. (1964, J. Anim. Sci. 23: 1084), they have been limited mainly to studies in laboratories.

However, recently, the production of normal piglets by *in vitro* fertilization of oocytes matured *in vivo* was reported by Cheng (1985, *In vitro* fertilization of farm animal oocytes. Ph. D. Thesis. Council for Nation Academic Awards), Yoshida (1987, Jpn. Vet. Sci. 49: 711; 1989, Jpn. J. Anim. Reprod. 35:34) and Nagai et al. (1988, J. Reprod. Fert. 84:585), the production of normal piglets by *in vitro* fertilization of oocytes matured *in vitro* was reported by Mattioli et al. (1989, Theriogenology 31: 1201), the possibility of practical use of embryo transfer technology appeared. In Korea, since Lee et al. (1988, Korean Journal of Animal Sciences, 30(7):405) produced 18 piglets by collecting 67 embryos from 5 donor pigs and

transferring the embryos into 5 recipient pigs, the history of pig embryo transfer has started.

Kikuchi et al. (2002, Biology of Reproduction 66:1033) produced a total of 19 piglets by subjecting pig oocytes matured in vitro to *in vitro* fertilization, incubating the fertilized oocytes *in vitro* for 5 and 6 days, selecting only good-quality blastocysts from the fertilized embryos and transferring 50 blastocysts into each of 3 surrogates. Also, Suzuki et al. (2006, Theriogenology 65: 374) produced 7 piglets by maturing oocytes *in vitro* in a bovine fetal serum-containing medium, incubating the oocytes for 6 days and transferring the resulting blastocysts into two surrogates. However, the prior reports have shortcomings in that many oocytes should be prepared to obtain a large number of blastocysts, and fertilized embryos should be incubated for 5-6 days before transfer.

Nuclear transfer is a method of producing a genetically equivalent individual by transferring the cell nuclei of a desired transgenic individual or a good genetic character into enucleated oocytes and is also called "somatic cell nuclear transfer". Recently, studies on nuclear transfer have been actively conducted for the mass production of superior domestic animals and the use thereof in the medical and pharmacological fields on the basis of fundamental studies using experimental animals. As a result of such efforts,

since the cloning of sheep was successful for the first time (Wilmut et al., 1997, Nature 385:810), examples of success in the cloning of mice and cattle have occurred in succession, and recently, the production of somatic cell clone animals in pigs known to be most difficult to clone was successful (PPL Therapeutics, 2000).

With the recent development of transplantation surgery, the prolongation of life by the transplantation of organs, including hearts, kidneys and liver, has spread. However, because the source of organs is limited to human beings, the supply of organs is absolutely inadequate compared to the demand thereof. Thus, as the possibility of use of animal organs for securing the source of transplant organs is suggested, the importance thereof has been highlighted. Particularly, it has been suggested that pigs that are most similar to human beings in terms of physiological structures and morphology can be used as organ transplant substitutes. Thus, together with the production of animals for providing organs, cell therapy of transplanting a specific cell of the relevant organ or tissue to treat chronic diseases, such as Alzheimer's disease, Parkinson's disease and diabetes, receives attention. In addition, when cloned embryos are prepared using as recipient embryos, the oocytes of domestic animals, such as easily available pigs, according to cross-species nuclear transplantation of

transplanting human somatic cells, are allowed to grow up to a stage before transplantation, and then are allowed to differentiate into stem cells such as embryonic cells, the desired cells can be directly produced in large amounts.

There is a report that the above-described nuclear transfer technique, which is highly useful in terms of domestic animal reproduction or medical application, was used to produce cloned pigs (Park et al., 2001, Animal Biotechnology 12: 173; Lai et al., 2002, Science 295:1089). The pregnancy of pigs is sensed when four or more embryos become implanted on the uterine wall at 12 days of pregnancy (Polge et al., 1966, Reproduction & Fertility 12:395), and nuclear transfer-derived embryos have a problem in that the percentage of development to normal blastocysts thereof is low, and thus a plurality of embryos should be transplanted. Also, after nuclear transfer, the percentage of development to normal blastocysts is low, and thus pregnancy rate is very low, leading to a great decrease in piglet production (Lai et al., 2003, Reproductive Biology and Endocrinology 1:82).

【Disclosure】

【Technical Problem】

The present invention has been made in order to solve the above-described problems occurring in the prior art, and it is an object of the

present invention to a piglet production method, which can increase the normal delivery rate of cloned pigs by simultaneous transfer of *in vitro* fertilized embryos and somatic cell-cloned embryos.

Another object of the present invention is to provide a method of producing piglets by *in vitro* fertilization, in which the culture period of embryos is greatly reduced and the delivery rate from a small number of oocytes is increased.

【Technical Solution】

To achieve the above objects, the present invention provides a piglet production method comprising the steps of: (A) collecting and culturing immature oocytes from a butchered pig: (B) preparing a) embryos by *in vitro* fertilization of the mature oocytes prepared in the step (A) with liquid semen and b) somatic cell-cloned embryos by enucleation of the mature embryos prepared in the step (A) and transfer of donor somatic cells into the enucleated oocytes, and culturing the prepared embryos (a) and (b) separately; and (C) transferring the *in vitro* fertilized embryos and the somatic cell-cloned embryos, prepared in the step (B), simultaneously into one surrogate.

The *in vitro* culture of the immature oocytes is the most fundamental step for the success of *in vitro* fertilization and somatic cell cloning and

can be carried out according to any method known in the art. However, it is preferably carried out in a medium containing follicle shells for 36-48 hours in order to the efficiency of *in vitro* maturation. In the case of immature pig oocytes, nuclear maturation is usually achieved 36 hours after *in vitro* culture, and the nuclei of the oocytes become arrested at the metaphase II stage up to before sperm stimulation (*in vitro* fertilization). For this reason, the oocytes should be cultured for at least 36 hours. However, because cytoplasm maturation is also required for complete maturation of the oocytes, it is most preferable that the *in vitro* culture be carried out for 44-48 hours. The mature oocytes at the interphase stage after 48 hours of culture are degraded by themselves in the absence of sperm stimulation, such that they are not normally fertilized.

It is known that follicle shells enhance the cytoplasm maturation of porcine immature oocytes (Mattioli et al., 1989, Theriogenology 31:1201) and increase the concentrations of cyclic adenosine monophosphate (cAMP) and glutathione in immature oocytes to stimulate early embryonic development (Abeydeera et al., 1998, Biol Reprod 58:213). Thus, Abeydeera et al. (Bio Reprod 58:213, 1998) collected follicular fluid from follicles with a syringe, subjectively selected follicle shell pieces from follicular fluid under a microscope, and then washed the selected follicle shell pieces for

use in the *in vitro* culture of immature oocytes. However, this was not applied in practice due to a complicated treatment process. In the present invention, follicle shells having a diameter of 4-6 mm were cut off with surgical scissors from ovaries collected in butcheries and were cultured in maturation medium with porcine immature oocytes. Although specific data are not shown in Examples of the present invention, oocytes maturated *in vitro* in a mature medium containing follicle shells were fertilized *in vitro*, cultured in a CO_2 incubator for 7 days and then examined for their development rate, and as a result, they showed an increase rate of a maximum of about 175% compared to when follicle shells were not added. This suggests that the addition of follicle shells has a significant effect on the development of embryos to blastocysts.

The oocytes maturated *in vitro* in the step (A) were used to prepare each of *in vitro* fertilized embryos and somatic cell-transferred embryos.

In vitro fertilization can be performed using liquid semen according to a conventional method, and it is preferable to use liquid semen stored at 4℃ as used in Examples of the present invention. The 4℃-stored liquid semen in the present invention is prepared using a porcine sperm dilution for artificial insemination, comprising, based on 100 ml of the dilution, 10-15 g

of lactose hydrate, 15-25 ml of egg yolk and 0.01-0.1 g of N-acetyl-D-glucosamine. Although specific data are not described in Examples of the present invention, the liquid semen was excellent in the motility, acrosome and viability of sperms even after it was stored at 4℃ for 5 days, and thus it is expected that the liquid semen can increase the fertility rate and offspring number of pigs.

The production of embryos by somatic cell transfer can also be easily performed using any technique known in the art, and the technique is not limited to a specific method.

The *in vitro* fertilized embryos and the somatic cell-transferred embryos are separately cultured *in vitro* for 16-36 hours, and then are transferred into a surrogate. In a conventional method of producing piglets by *in vitro* fertilization, *in vitro* fertilized embryos are cultured *in vitro* for 5-6 days, and then only good-quality blastocysts are selected from the cultured embryos and transferred, whereas the present invention has an advantage in that *in vitro* fertilized embryos may be transferred directly after they are cultured only for 16-36 hours.

The *in vitro* fertilized embryos and the somatic cell-transferred embryos, each cultured *in vitro*, are simultaneously transferred into one surrogate by laparotomy. The total number of the embryos for use in transfer

is preferably 150-250.

Generally, when oocytes matured *in vitro* are subjected to *in vitro* fertilization and somatic cell cloning, and then the embryos are cultured for 7 days, the blastocyst development rate thereof is about 20%. However, considering that the implantation rate of blastocysts in the practical transfer of embryos is as low as about 2%, when embryos are transferred after one-day culture, a minimum of 250 embryos theoretically should be transferred for successful impregnation. Meanwhile, when blastocysts are transferred, embryos should be cultured for 5-7 days, and then only good-quality blastocysts should be selected therefrom and 50 or more blastocysts among them should be injected.

When only the somatic cell-cloned embryos produced in Examples of the present invention were transferred into a surrogate according to the same surgical method as in Examples to attempt the production of cloned piglets, the surrogate was not pregnant in spite of 11 attempts as shown in Table 1 below. This test was conducted over a period ranging from July 15, 2005 to June 2, 2006. This result coincides with the fact that the pregnancy rate of somatic cell-cloned embryos is very low. However, 100 somatic cell-cloned embryos were transferred into a surrogate simultaneously with 100 one-day-cultured *in vitro* fertilized embryos, and as a result, the surrogate was

pregnant in only one attempt, and thus three piglets were normally produced.

【Table 1】

Surrogate pigs (Landrace gilt)	Number of Transferred embryos		Pregnancy	Pregnancy Period	Number of born piglets
	In vitro Fertilized	Somatic cell cloned			
1	-	200	-	-	-
2	-	189	-	-	-
3	-	240	-	-	-
4	-	164	-	-	-
5	-	246	-	-	-
6	-	250	-	-	-
7	-	220	-	-	-
8	-	255	-	-	-
9	-	243	-	-	-
10	-	217	-	-	-
11	-	144	-	-	-
12	100	100	+	115 days	3

The three piglets were compared with the DNAs of the somatic cells used in somatic cell cloning and the semen used in *in vitro* fertilization and, as a result, it could be seen that two piglets were derived from the *in vitro* fertilized embryos, and one piglet was derived from the somatic cell-cloned embryos. The birth weights of the piglets were normal weights of 1.2 kg and 2.0 kg for the piglets derived from *in vitro* fertilized embryos), and 1.2 kg for the piglet derived from somatic cell-cloned embryos.

【Advantageous Effects】

Even though the delivery rate of piglets by somatic cell cloning is

very low, the normal delivery of two *in vitro* fertilized embryo-derived piglets and one somatic cell-cloned embryo-derived piglet by only one attempt suggests that *in vitro* fertilized embryos have an advantage effect on the implantation of somatic cell-cloned embryos, which are generally rarely implanted. Also, the fact that two piglets were produced even though only 100 *in vitro* fertilized embryos were transferred before blastocyst formation suggests that the transfer of somatic cell-cloned embryos simultaneously with the *in vitro* fertilized embryos had a synergy effect on increases in fertility rate and normal delivery rate.

【Description of Drawings】

FIG. 1 is a photograph showing follicle shells used in the *in vitro* maturation of immature oocytes in the porcine ovary.

FIG. 2 is a photograph showing *in vitro* fertilized embryo-derived piglets (A and B) and a somatic cell-cloned embryo-derived piglet (C).

【Best Mode】

Hereinafter, the present invention will be described in further detail with reference to examples. It is to be understood, however, that these examples are illustrative only and the scope of the present invention is not limited thereto.

Examples

1. Collection and *in vitro* maturation of porcine oocytes

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to the laboratory in 0.9% saline at 30~35℃. Follicular fluid and cumulus-oocyte complexes (COCs) were aspirated from follicles of 2~6 mm in diameter using an 18-gauge needle fixed to a 10 ml disposable syringe. The follicular contents were pooled into 50 ml tube and allowed to sediment, and the sediment was placed into HEPES buffered Tyrode-Lactate medium (TL-HEPES-PVA) containing 0.1% polyvinyl alcohol (PVA). Oocytes with uniform ooplasm surrounded by a compact cumulus cell mass were selected and washed with TL-HEPES-PVA and then washed twice with the maturation medium. The basic media used for *in vitro* maturation with modified tissue culture medium (TCM)199 supplemented with 26.19 mM sodium bicarbonate, 3.05 mM glucose, 0.91 mM sodium pyruvate, 75 μg/ml sodium penicillin G, 50 μg/ml streptomycin sulfate and 0.1% PVA. COCs were cultured in maturation medium containing the 0.5 μg/ml luteinizing hormone (LH), 0.5 μg/ml follicle stimulating hormone (FSH), 10 ng/ml epidermal growth factor (EGF), 10% porcine follicular fluids (pFF) and 0.57 mM cysteine in the presence of follicle shells. As shown in FIG 1, whole antral follicle with 4~6 mm in diameter ("→" in FIG 1) for obtaining follicle shells was cut from ovaries using a fine scissors. The follicle shells were then inverted using scissors

and forceps and washed with the TL-HEPES-PVA medium.

After 22 hr of culture, oocytes were cultured without hormones for 22 hr at 38.5℃, 5% CO_2 in air.

2. Semen collection and liquid semen processing

Semen was collected from Duroc boars once weekly. The sperm-rich portion of ejaculates with greater than 85% motile sperm and normal apical ridge (NAR) acrosomes were used in the experiments. Semen was slowly cooled to room temperature by 2 h after collection. Semen was transferred into 15 ml tubes, centrifuged at room temperature for 10 min at 800×*g*, and supernatant solution was poured off. The concentrated sperm was resuspended with 5 ml LEN (11.0 g lactose hydrate, 20 ml egg yolk and 0.05 g N-acetyl-D-glucosamine and 100 ml distilled water) diluent to provide 1.0×10^9 sperm/ml at room temperature. The resuspended semen was cooled in a refrigerator to 4℃ and preserved for 5 days.

3. *In vitro* fertilization (IVF) and culture of oocytes

After 44 h of maturation, cumulus cells of oocyte were removed by 0.1% hyaluronidase in TL-HEPES-PVA and washed twice with TL-HEPES-PVA and modified

Tris-buffered media (mTBM; 113.1 mM NaCl, 3.0 mM KCl, 10.0 mM $CaCl_2$, 20.0 mM Tris(hydroxymethyl aminomethane), 11.0 mM glucose, 5.0 mM sodium pyruvate, 1.0 mM caffeine, 75 mg/ml penicillin, 50 mg/ml streptomycin, 0.57 mM cystein and 1 mg/ml bovine serum albumin (BSA)), respectively. Thereafter, 30~40 oocytes were transferred into each well of a 4-well multidish (Nunc, Denmark) containing mTBM that had been covered with mineral oil and equilibrated at 38.5℃, 5% CO_2 in air for 4 h. The dishes were kept in a CO_2 incubator until spermatozoa were added for insemination. For IVF, 0.5 ml liquid semen was washed twice in TL-HEPES-PVA and then resuspended with mTBM to adjust a sperm concentration to 1×10^6 sperm/ml. Oocytes and sperm were co-incubated in 500 μl mTBM for 6 h. After insemination, oocytes were transferred into 500 μl NCSU-23 containing 50 μM 2-mercaptoethanol, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate and 0.4% BSA. After 52 h of culture, cleaved oocytes were transferred into 500 μl NCSU-23 (108.73 mM NaCl, 4.78 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4 \cdot 7H_2O$, 5.55 mM D-glucose, 1.00 mM glutamine, 7.00 mM taurine, 5.00 mM hypotaurine, 25.07 mM $NaHCO_3$, 1.70 mM $CaCl_2 \cdot 2H_2O$, 75 mg/ml penicillin G, 50 mg/ml streptomycin) containing 50 mM 2-mercaptoethanol, 0.17 mM sodium pyruvate and 2.73 mM sodium lactate, and cultured for 16-36 hours, followed

by embryo transfer.

4. Nuclear transfer of somatic cell

The oocytes maturated for 44 hours in the section 1 were transferred into TL-HEPES-PVA containing 0.1% hyaluronidase and pipetted to remove expanded cumulus cells. The porcine oocytes were enucleated by micromanipulation under an inverted microscope (Nikon, Japan) equipped with a micromanipulator (Narishige, Japan), and fetal fibroblasts isolated and cultured from a fetus extracted from a pig 35 days pregnant were injected into the oocytes.

For fusion to other cells, the oocytes were given electrical stimulation with an ECM 2001 electrocell manipulator (BTX Inc., San Diego, CA, USA) in conditions of 1.2 kV/cm, 30 ms and 1 DC pulse. The fused oocytes were cultured in TCM-199 medium for 3 hours, and then cultured in NCSU-23 for 16-36 hours. Then, the oocytes were used in embryo transfer.

5. Embryo transfer and piglet production

The estrous cycle of pigs is usually 20-22 days (21 days in average), and the estrus duration is 48 hours in average. Thus, porcine estrus was checked two times a day, and at 12-24 hours after the detection of estrus,

embryo transfer was performed.

For embryo transfer, a surrogate gilt was allowed to be quiet by injecting 35 mg of acepromazine maleate and 2.5 mg of atropine sulfate subcutaneously into the neck muscle behind the ear. After 30 minutes, the surrogate mother was generally anesthetized by injecting 20 mg of acepromazine maleate and 1,200 mg of ketamine hydrochloride into the ear vein. The anesthetized pig was placed on an operating table in a supine position, a portion to be operated was washed, shaved, sterilized, and then incised 15 cm along the median line between the final nipple and the next nipple. The subcutaneous fat was separated from the incised portion to the left and right sides, and then the muscle and peritoneum were excised and the uterus, fallopian tube and ovary were exposed, followed by embryo transfer.

1-4 cell stage embryos (100 *in vitro* fertilized embryos and 100 somatic cell-cloned embryos, that is, 200 embryos for transfer into one surrogate) were transferred deep into the fallopian tube of one surrogate using a nontoxic surgical polyethylene tube (Intramedic, Clay Adams, USA) having an inner diameter of 0.86 mm and an outer diameter of 1.27 mm. After transfer, the uterine, fallopian tube and ovary were restored to the original positions, and suture was conducted in the order of the peritoneum, muscle and skin.

On September 25, 2006, 115 days after the embryo transfer, three normal piglets were produced.

6. DNA paternity testing

The DNAs of the piglets, the surrogate gilt and the donor cells were analyzed to test whether the piglets were derived from the *in vitro* fertilized embryos or derived from the somatic cell-cloned embryos. The DNA paternity testing was carried out in National Livestock Research Institute (564 Omokshun-dong, Gwonsun-Gu, Suwon, Korea), in which the DNA sample of the porcine liquid semen, the DNA sample of each of the three piglets, and the DNA sample of the donor cells, were analyzed for 13 microsatellite markers (MS) in the following manner.

1) DNA extraction

The ear of each of the three piglets and one surrogate gilt was cut to a size of 1-2 cm and placed in a 1.5 ml tube. The sample of the semen used in the *in vitro* fertilization was prepared by placing 1 ml of the semen in a 1.5 ml tube, centrifuging the semen in a microcentrifuger (Bio-spin, Hanil, Korea) at 10,000 rpm for 20 minutes and collecting the remaining pellets. The sample of the somatic cells used in the *in vitro* cloning was prepared by isolating the somatic cells with 0.05% trypsin-EDTA (ethylenediamine tetra

acetic acid) and washing the isolated cells two times with phosphate buffered saline (PBS).

To each of the prepared samples, 500 ml of DNA extraction buffer (50 nM Tris, 100 mM EDTA, 10% sodium dodecyl sulfate (SDS)) and 10 mg/ml of proteinase K (U302B, Promega, Madison, WI, USA) were added, and the mixture was vertically shaken. Each of the samples was incubated in a water bath at 55 ℃ for about 8-24 hours to confirm whether the samples became hazy, and 500 ml PCI (phenol: chloroform: isoamylalcohol= 25:24:1, Cat. C9017, Bioneer, Korea) was added thereto and the mixtures were vertically shaken and then centrifuged at 10,000 rpm for 10 minutes. Only the centrifuged supernatant was taken and placed in a tube, the same amount of CI (chloroform: isoamylalcohol= 24:1 (v/v)) was added thereto and vertically shaken, the mixture was centrifuged at 10,000 rpm for 10 minutes, and only the supernatant was placed in a fresh tube. 3 M sodium acetate (pH 6.0) was added in an amount of 1/10 of the supernatant, and 100% ethanol was added thereto in an amount corresponding to two times the mixture and well shaken. The mixture was left to stand at room temperature for at least 5 hours to form a precipitate, and then centrifuged at 10,000 rpm for 10 minutes. In a clean bench, the supernatant was discarded and only pellets were collected. To remove salts from the pellets, the pellets were added to 100 ml of 70%

ethanol, well shaken, discarded two times, added to 100 ml of 70% ethanol, well shaken and then discarded one time. The washed pellets were completely dried at room temperature for 20 minutes, and the solid pellets were dissolved in 20-50 ml of distilled water. The UV absorbance of the aqueous solution was measured at a 260-nm wavelength with a UV/visible spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, Cambridge, UK) to measure the optical density (OD) thereof. Then, the concentration of DNA in the aqueous solution was calculated to adjust final DNA concentration to 1 mg/ml. The DNA samples were stored at -20℃ before use.

2) Isolation and concentration measurement of genomic DNA

For the extraction of genomic DNA, from the F_2 354 reference group, 10 ml of whole blood was collected in a tube containing 1 ml of 0.5 M EDTA (pH 8.0), and genomic DNA was extracted from the whole blood using the Wizard genomic DNA purification kit (Promega Co., USA). 30 ml of a cell lysis solution was added to 10 ml of the collected whole blood, and red blood cells were lyzed at room temperature for 10 minutes. After lysis, the blood solution was centrifuged at 2,000×g for 10 minutes to collect only white blood cells, and then the pellets were dissolved in 10 ml of nuclei lysis solution. The resulting solution was treated with RNase A (20 mG/ML) in a

shaking incubator at 37℃ for 15 minutes, and then strongly suspended in 3.3 ml of protein precipitation solution for 20 seconds. The suspension was purified by centrifugation at 2,000×*g* for 10 minutes, and the DNA of the suspension was precipitated with isopropanol. Then, the DNA was washed with 70% ethanol, dried and dissolved in 50 ml if Tris-EDTA buffer (pH 8.0) for use in PCR.

3) Microsatellite markers

The Microsatellite markers present in LEPR (leptin receptor) used for genotype analysis were selected on the basis of base sequence information (Accession No. AF184172) and were in the form of a nucleotide repeat sequence of (CA)n. As primers for PCR, the following primers were used: Forward primer: 5'-aatggaaactcttcccagct-3' (SEQ ID NO: 1); and reverse primer: 5'-cattcgaactgttcattgccat-3'(SEQ ID NO: 2).

4) DNA amplification by PCR

A PCR reaction mixture contained PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_3$), 2.5 mM dNTPs, 3 pmol fluorescent dye labeling primer pairs, 10 ng template DNA, 0.5 U Tag DNA polymerase (Takara Shuzo Co., Shiga, Japan) and ddH_2O and had a total reaction volume of 10 ml. The PCR reaction was performed using the GeneAmp PCR System 9600 (Perkin-

Elmer Co., USA) in the following conditions: pre-denaturation at 94℃ for 5 min, and then 35 cycles of denaturation at 94℃ for 30 sec, annealing at 60℃ for 40 sec and extension at 72℃ for 1 min, followed by final extension at 72 ℃ for 10 min. The PCR amplification product was electrophoresed on 2% agarose gel containing EtBr (ethidium bromide) and observed in the presence of UV, in order to confirm whether the size of the amplified fragment was in the expected allele size range and whether the PCR conditions were suitable.

5) Genotype analysis

The PCR product was diluted in a suitable amount of DI (deionized) water, and DNA: formamide: size standard (Genescan-350 TAMRA) were mixed at a ratio of 1 ml: 12 ml: 0.5 ml. The mixture was denatured at 95℃ for 3 minutes, and then analyzed using the ABI310 Genetic Analyzer (Perkin-Elmer Co., USA). Data on the amount and size of the DNA fragment as the PCR product were collected using the GeneScan software version 2.1 (Perkin-Elmer Co., USA). In electrophoresis, performance optimized polymer (POP4) (PE Applied Biosystems) and 10x buffer (with EDTA) were used as a 1x dilution, and the run time was 22 minutes. The genotype was analyzed using the Genotype software version 2.5 ((Perkin-Elmer Co., USA).

6) DNA analysis results

The DNAs of the three piglets, the sperms used in *in vitro*

fertilization and the somatic cells used as donor cells in somatic cell cloning were comparatively analyzed. As a result, as shown in Table 2 below, in the case of two piglets, the sperms used in fertilization and 12 microsatellites were almost consistent, and in one piglet, all of 12 microsatellites were consistent.

【Table 2】

ID	sw240		s0090		s0155		s0228		s0301		s0355	
Sperm	91	95	248	248	152	152	225	225	261	261	246	246
Piglet 1	95	109	248	248	152	158	225	225	257	261	246	250
Piglet 2	91	95	248	248	158	158	225	225	251	261	250	258
Piglet 3	95	107	244	248	156	160	225	225	257	261	246	250
somatic cell	95	107	244	248	156	160	225	225	257	261	246	250
Surrogate	107	111	248	252	156	162	223	225	257	261	246	272

ID	s0378		s0386		sw857		sw911		sw787		sw24	
Sperm	103	105	163	163	150	150	155	159	154	158	102	108
Piglet 1	103	109	163	163	150	152	159	159	152	154	102	110
Piglet 2	105	109	161	161	140	152	155	159	152	152	102	110
Piglet 3	105	105	153	161	140	148	159	159	154	158	102	102
somatic cell	105	105	153	161	140	148	159	159	154	158	102	102
Surrogate	105	109	153	163	144	156	155	159	156	162	110	114

Thus, it could be confirmed that the piglets 1 and 2 (A and B in FIG. 2) were piglets derived from *in vitro* fertilized embryos and the piglet 3 (C in FIG. 2) was a piglet derived from somatic cell-cloned embryos.

【Industrial Applicability】

As described above, according to the present invention, simultaneous

transfer of *in vitro* fertilized embryos and somatic cell-cloned embryos allows the normal delivery of cloned pigs having a very low normal delivery rate. In addition, the *in vitro* culture period of *in vitro* fertilized embryos can be greatly reduced, normal delivery is possible even from a small number of embryos, and piglets derived from *in vitro* fertilized embryos and piglets derived from somatic cell-cloned embryos can be simultaneously produced.

【CLAIMS】

【Claim 1】

A piglet production method comprising the steps of:

(A) collecting and culturing immature oocytes from a butchered pig:

(B) preparing a) embryos by *in vitro* fertilization of the mature oocytes prepared in the step (A) with liquid semen and b) somatic cell-cloned embryos by enucleation of the mature embryos prepared in the step (A) and transfer of donor somatic cells into the enucleated oocytes, and culturing the prepared embryos (a) and (b) separately; and

(C) transferring the *in vitro* fertilized embryos and the somatic cell-transferred embryos, prepared in the step (B), simultaneously into one surrogate.

【Claim 2】

The method of Claim 1, wherein a medium for culturing the immature oocytes in the step (A) includes 2~6 follicle shell having a size of 4-6 mm.

【Claim 3】

The method of Claim 1, wherein the *in vitro* fertilized embryos in the step (B) are prepared using a porcine semen dilution for artificial fertilization for storage at 4℃, the semen dilution comprising, based on 100 ml of the dilution, 10-15 g of lactose hydrate, 15-25 ml of egg yolk and

0.01-0.1 g of N-acetyl-D-glucosamine.

【Claim 4】

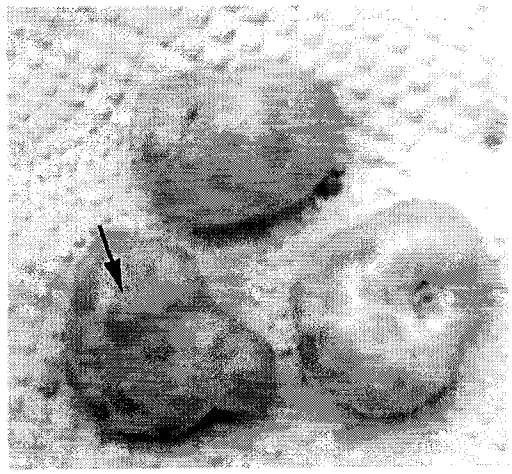
The method of Claim 1, wherein the *in vitro* fertilized embryos and the somatic cell-cloned embryos are transferred into the surrogate after they are separately cultured for 16~36 hours.

【Claim 5】

The method of Claim 1, wherein the total number of embryos used in the step (C) is 150-250.

【DRAWINGS】

【Figure 1】



【Figure 2】

VIII-5-1	**Declaration: Non-prejudicial disclosure or exceptions to lack of novelty** Declaration as to non-prejudicial disclosures or exceptions to lack of novelty (Rules 4.17(v) and 51bis.1(a)(v)): Name (LAST, First)	in relation to this international application THE INDUSTRY AND ACADEMIC COOPERATION IN CHUNGNAM NATIONAL UNIVERSITY (IAC) declares that the subject matter claimed in this international application was disclosed as follows:
VIII-5-1(i)	Kind of disclosure:	publication
VIII-5-1(ii)	Date of disclosure:	12 January 2007 (12.01.2007)
VIII-5-1(iii)	Title of disclosure:	The dong-a ilbo etc.
VIII-5-1(iv)	Place of disclosure:	Republic of Korea

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/002428

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/16(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 C12N 5/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Utility models and applications for Utility Models since 1975

Japanese Utility models and applications for Utility Models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS(KIPO internal), PubMed, DELPHION, "porcine, pig*, fertiliz*, nuclear transf*, embryo, co-transf*, and similar terms"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KING T.J. et al., Embryo development and establishment of pregnancy after embryo transfer in pigs: coping with limitations in the availablility of viable embryos, Reproduction, 2002, Vol. 123, pages 507-515. See the whole document.	1-5
A	SATOSHI WATANABE et al., A novel method for the production of transgenic cloned pigs: electroporation-mediated gene transfer to non-cultured cells and subsquent selection with puromycin, Biology of Reproduction, 2005, Vol.72, pages 309-315. See the whole document.	1-5
A	KAZUHIRO KIKUCHI, Developmental competence of porcine blastocysts produced in vitro, Journal of Reproduction and Development, 2004, Vol.50(1), pages 21-28. See the whole document.	1-5
A	KAZUHIRO KIKUCHI et al., Successful piglet production after transfer of blastocysts produced by a modified in vitro system, Biology of Reproduction, 2002, Vol. 66, pages 1033-1041. See the whole document.	1-5

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 DECEMBER 2007 (14.12.2007)	**14 DECEMBER 2007 (14.12.2007)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer KIM Jung Tae Telephone No. 82-42-481-8385

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/002428

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

   a. type of material
      - [x] a sequence listing
      - [ ] table(s) related to the sequence listing

   b. format of material
      - [ ] on paper
      - [x] in electronic form

   c. time of filing/furnishing
      - [x] contained in the international application as filed
      - [ ] filed together with the international application in electronic form
      - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: