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(54) Title: PIG MODEL FOR ATHEROSCLEROSIS

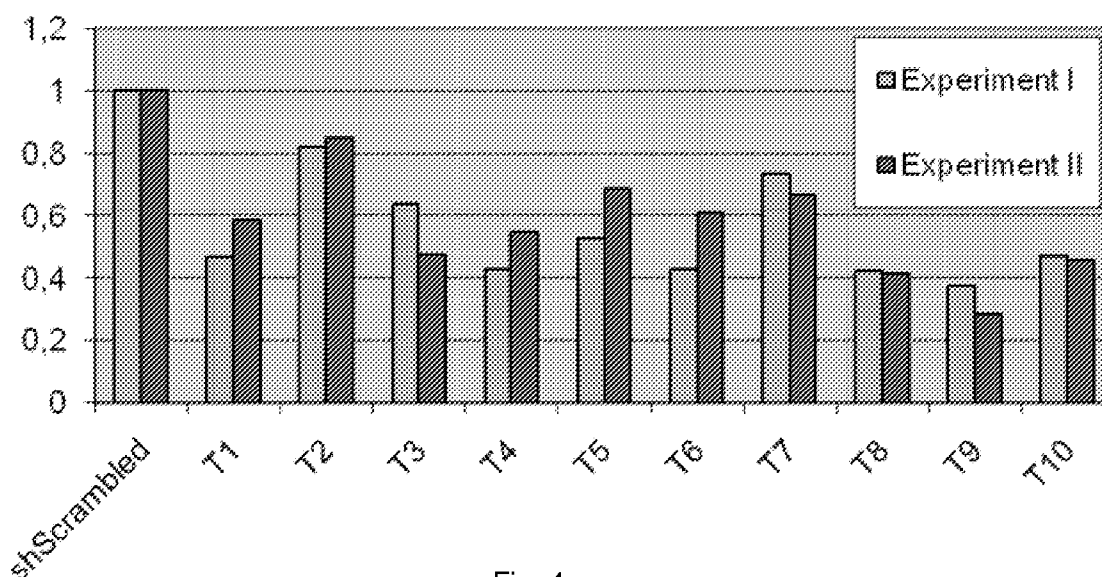


Fig. 4

(57) Abstract: The present invention relates to a genetically modified pig as a model for studying atherosclerosis. The modified pig model displays one or more phenotypes associated with atherosclerosis. Disclosed is also a modified pig comprising a mutation in the endogenous ApoE gene or part thereof and/or LDL gene or part thereof, and/or LDL receptor gene, and/or transcriptional and/or translational product or part thereof. The invention further relates to methods for producing the modified pig; and methods for evaluating the effect of a therapeutical treatment of atherosclerosis; methods for screening the efficacy of a pharmaceutical composition; and a method for treatment of a human being suffering from atherosclerosis are disclosed.

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Pig model for atherosclerosis

Field of invention

5 The present invention relates to a genetically modified pig as a model for studying atherosclerosis, wherein the pig model expresses at least one phenotype associated with said disease. The invention further relates to methods by which the genetically modified pig is produced. In addition, methods for evaluating the response of a
10 therapeutical treatment of atherosclerosis, for screening the efficacy of a pharmaceutical composition, and a method for treatment of human being suffering from atherosclerosis are disclosed.

Background of invention

Transgenic, non-human animals can be used to understand the action of a single gene or genes in the context of the whole animal and the interrelated phenomena of gene
15 activation, expression, and interaction. The technology has also led to the production of models for various diseases in humans and other animals which contributes significantly to an increased understanding of genetic mechanisms and of genes associated with specific diseases.

20 Traditionally, smaller animals such as mice have been used as disease models for human diseases and have been found to be suitable as models for certain diseases. However, their value as animal models for many human diseases is quite limited due to differences in mice compared to humans. Larger transgenic animals are much more suitable than mice for the study of many of the effects and treatments of most human
25 diseases because of their greater similarity to humans in many aspects. Particularly, pigs are believed to be valuable as disease models for human diseases.

Atherosclerosis is by far the most frequent cause of coronary artery disease (angina pectoris, myocardial infarction and sudden death), carotid artery disease (stroke), and
30 peripheral arterial disease. Atherosclerosis is referred to as 'hardening of the arteries' which is caused by the formation of numerous plaques within the arteries.

It is a chronic inflammatory disease, fueled by high plasma levels of cholesterol-rich lipoproteins, that leads to the development of atherosclerotic plaques of inflammatory cells, debris, and smooth muscle cells in large and medium-sized arteries¹. These
35 lesions by themselves rarely cause symptoms. The mechanical process wherein

plaques burst open, known as plaque ruptures, causes the devastating consequences of atherosclerosis. By this process the thrombogenic core of the plaque is exposed to the haemostatic system of the circulating blood and this may elicit an acute flow-limiting superimposed thrombus.

5 In most cases of atherosclerosis the genetic component is complex, but in some cases the inheritance of the disease is monogenic. These cases are mostly due to mutations in genes coding for proteins involved in lipoprotein trafficking, and the most severe in humans are caused by homozygous null mutations in the LDL receptor (homozygous familial hypercholesterolemia). Even though the disease process in homozygous LDL
10 receptor deficiency is immensely aggressive leading to severe coronary atherosclerosis in childhood, the disease is deemed qualitatively no different from that seen in more slowly developing atherosclerosis. Thus, monogenic causes of atherosclerosis can be used as tools to model atherosclerosis and atherosclerotic complications in genetically modified animal models.

15 Apolipoprotein E and LDL-receptor deficient mice with severe hypercholesterolemia and rapid development of atherosclerosis were created in the early 1990ies by homologous recombination in embryonic stem cells^{6,7}. These mouse models have been instrumental in understanding many aspects of plaque development, but they are limited as models for human atherosclerosis because they lack measurable coronary
20 atherosclerosis and do not develop the most feared complication of atherosclerosis, i.e. atherosclerotic plaque rupture and superimposed thrombosis. In addition – because of their small size - these animals have not aided research on bioimaging of atherosclerosis and percutaneous coronary intervention.

Similarities in cardiovascular structure and coronary artery distribution with humans
25 make swine an attractive species to explore cardiovascular function and diseases. On conventional diets (~3% fat, w/w), pigs have low plasma cholesterol levels (~2 mmol/l), but many strains of pigs are susceptible to hypercholesterolemia and moderate atherosclerosis when fed a diet high in saturated fat and cholesterol, including miniature Yucatan⁸ and Yorkshire farm pigs⁹. Yucatan minipigs are of particular interest
30 as models of human atherosclerosis because their adult weight compares well with that of humans (60-80 kg for males and 50-70 kg for females¹⁰) and thus equipment for imaging and percutaneous coronary intervention can be used directly.

The most pronounced lesions to date have been described in a progeny of large farm pigs identified in Wisconsin that harbor a single-nucleotide missense mutation in the

LDL receptor gene that reduces affinity of the receptor to its ligands¹¹. A colony of these pigs is now maintained in France by Professor Ludovic Drouet, INRA, Jouy en Josas. The pigs develop atherosclerosis in coronary arteries with many aspects of human atherosclerosis including plaque ruptures and superimposed thrombosis.

5 However, hypercholesterolemia is modest on a normal pig diet (total cholesterol 5-8 mM) and atherosclerosis develops only slowly over several years. By the time these pigs have developed atherosclerosis, they are by far too large for most scientific purposes.

10 Even though considerable advances in anti-atherosclerotic pharmacological therapy have been achieved in the past decades, atherosclerosis remains one of the leading causes of death and severe disability in Denmark and worldwide². There are at least three parts to an explanation for that.

First, the conventional population-based risk factor approach recommended in official guidelines is unable to identify those who need treatment on the level of the individual³.

15 Thus, even though we have access to effective preventive treatment we are unable to identify those to treat. This problem could be solved by diagnostic imaging of atherosclerosis, which is becoming theoretically possible with the advent of new high-resolution imaging technology^{4,5}. However, to develop tracers/contrast agents and imaging sequences that are able to visualize atherosclerotic plaques and
20 atherosclerotic disease activity, we need a human-sized animal model of the disease that can be examined in patient CT, MR and PET-scanners.

Second, anti-atherosclerotic therapy is effective in preventing atherosclerosis in the long-term, but there is a lack of medical therapy that is effective at rapidly decreasing the risk of thrombotic complications in persons that have established severe
25 atherosclerosis. E.g. in those persons that have identified themselves by presenting symptoms of atherosclerosis and in which maximal treatment is instigated, future events might still occur. Today, the major obstacle of developing such medicine is the lack of an animal model in which plaque rupture and arterial thrombosis occurs.

30 Third, the best treatment for coronary events today is primary percutaneous coronary intervention with placement of a stent, but these procedures are subject to complications including stent thrombosis and in-stent restenosis. Most research within this important area is carried out in non-diseased coronary pig arteries, but this approach has obvious limitations.

For these reasons, a human-sized pig model with severe human-like atherosclerosis, including plaque ruptures and thrombotic complications, is needed more than ever.

Even though the genes responsible for inherited atherosclerosis or involved in the development of disease have been identified in humans it does not follow that animals transgenic for such mutations display a phenotype comparable to that of the human disease. However, the present invention has surprisingly shown that the genetically modified pig models according of the present invention display the atherosclerosis phenotype.

Summary of invention

The present invention concerns a genetically modified pig model which allows for the study of atherosclerosis.

Thus, one aspect of the present invention relates to a genetically modified pig as a model for studying atherosclerosis, wherein the pig model expresses at least one phenotype associated with said disease and/or a modified pig comprising at least one mutation in the i) endogenous ApoE gene or part thereof and/or LDL gene or part thereof, and/or ii) endogenous ApoE gene or part thereof and/or iii) endogenous LDL receptor gene or part thereof, transcriptional and/or translational product or part thereof and/or a modified pig comprising at least one human and/or porcine PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.

Embodiments for the present invention comprises, mini-pigs for example selected from the group consisting of Goettingen, Yucatan, Bama Xiang Zhu, Wuzhishan and Xi Shuang Banna, including any combination thereof. However, another embodiment relates to pigs that are not a mini-pig, such as the species of *Sus domesticus*, for example where the pig is selected from the group consisting of Landrace, Yorkshire, Hampshire, Duroc, Chinese Meishan, Berkshire and Piétrain, including any combination thereof. In a preferred embodiment the pig is a Yucatan minipig.

Embodiments of the present invention comprise the modified pig, wherein the pig comprises at least one mutation in a ApoE and/or LDL gene or part thereof, transcriptional and/or translational product or part thereof. In another embodiment the modified pig comprises at least one mutation in an endogenous ApoE gene or part thereof, transcriptional and/or translational product or part thereof. In a further embodiment the modified pig comprises at least one mutation in an endogenous LDL

gene or part thereof, transcriptional and/or translational product or part thereof. In yet another embodiment the at least one mutation is introduced into the endogenous porcine ApoE or LDL gene by homologous recombination or alternatively by random integration. In yet a further embodiment the at least one mutation is introduced into the endogenous porcine ApoE or LDL gene, transcriptional and/or translational product or part thereof by siRNA. A further embodiment relates to the modified pig, wherein said pig comprises at least one human and/or porcine PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.

10 A second aspect of the present invention relates to a genetically modified porcine blastocyst derived from the genetically modified pig model as disclosed herein and/or a modified porcine blastocyst comprising at least one mutation in the i) endogenous ApoE gene or part thereof and/or LDL gene or part thereof, and/or ii) endogenous ApoE gene or part thereof and/or iii) endogenous LDL receptor gene or part thereof, transcriptional and/or translational product or part thereof and/or a modified blastocyst comprising at least one human and/or porcine PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.

20 A third aspect of the present invention relates to a genetically modified porcine embryo derived from the genetically modified pig model as disclosed herein and/or a modified porcine embryo comprising at least one mutation in the i) endogenous ApoE gene or part thereof and/or LDL gene or part thereof, and/or ii) endogenous ApoE gene or part thereof and/or iii) endogenous LDL receptor gene or part thereof, transcriptional and/or translational product or part thereof and/or a modified embryo comprising at least one human and/or porcine PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.

30 A fourth aspect of the present invention relates to a genetically modified porcine fetus derived from the genetically modified pig model as disclosed herein and/or a modified porcine fetus comprising at least one mutation in the i) endogenous ApoE gene or part thereof and/or LDL gene or part thereof, and/or ii) endogenous ApoE gene or part thereof and/or iii) endogenous LDL receptor gene or part thereof, transcriptional and/or translational product or part thereof and/or a modified fetus comprising at least one human and/or porcine PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.

A fifth aspect of the present invention relates to a genetically modified porcine donor cell derived from the genetically modified pig model as disclosed herein and/or a modified porcine donor cell comprising at least one mutation in the i) endogenous ApoE gene or part thereof and/or LDL gene or part thereof, and/or ii) endogenous ApoE gene or part thereof and/or iii) endogenous LDL receptor gene or part thereof, transcriptional and/or translational product or part thereof and/or a modified donor cell comprising at least one human and/or porcine PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.

In one embodiment of the present invention the at least one phenotype of the modified pig or pig derived from a modified porcine blastocyst, embryo, fetus and/or donor cell is hypercholesterolemia. The hypercholesterolemia is characterized by an at least 10% increase in total cholesterol level in the plasma as compared to a standard level of the pig. In another embodiment of the present invention the at least one phenotype of the modified pig or pig derived from a modified porcine blastocyst, embryo, fetus and/or donor cell is atherosclerosis. The atherosclerosis is for example determined by intravascular ultrasound, PET scanning, CT scanning and/or MR scanning.

A sixth aspect of the present invention relates to the genetically modified pig as described herein, porcine blastocyst, embryo, fetus and/or donor cell obtainable by nuclear transfer comprising the steps of

- i) establishing at least one oocyte having at least a part of a modified zona pellucida,
- ii) separating the oocyte into at least two parts obtaining an oocyte having a nucleus and at least one cytoplasm,
- iii) establishing a donor cell or cell nucleus with desired genetic properties,
- iv) fusing at least one cytoplasm with the donor cell or membrane surrounded cell nucleus,
- v) obtaining a reconstructed embryo,
- vi) activating the reconstructed embryo to form an embryo; culturing said embryo; and
- vii) transferring said cultured embryo to a host mammal such that the embryo develops into a genetically modified fetus.

wherein said genetically modified embryo obtainable by nuclear transfer comprises steps i) to v) and/or vi),

wherein said genetically modified blastocyst obtainable by nuclear transfer comprises steps i) to vi) and/or vii),

5 wherein said genetically modified fetus obtainable by nuclear transfer comprises steps i) to vii).

A seventh aspect of the present invention pertains to a method for producing a transgenic pig as a model for atherosclerosis, porcine blastocyst, embryo, fetus and/or
10 donor cell comprising:

- i) establishing at least one oocyte
- ii) separating the oocyte into at least three parts obtaining at least one cytoplasm,
- iii) establishing a donor cell or cell nucleus having desired genetic
15 properties,
- iv) fusing at least one cytoplasm with the donor cell or membrane surrounded cell nucleus,
- v) obtaining a reconstructed embryo,
- vi) activating the reconstructed embryo to form an embryo;
- 20 vii) culturing said embryo; and
- viii) transferring said cultured embryo to a host mammal such that the embryo develops into a genetically modified fetus,

wherein said genetically modified embryo obtainable by nuclear transfer comprises steps i) to v) and/or vi),

25 wherein said genetically modified blastocyst obtainable by nuclear transfer comprises steps i) to vi) and/or vii),

wherein said genetically modified fetus obtainable by nuclear transfer comprises steps i) to vii).

30 Embodiments of the sixth and seventh aspects comprise one or more of the features as defined in any of the preceding claims, wherein the method for activation of the reconstructed embryo is selected from the group of methods consisting of electric pulse, chemically induced shock, increasing intracellular levels of divalent cations and reducing phosphorylation. Further embodiments of the second and third aspects
35 comprise one or more of the features as defined above, wherein steps iv) and vi) are

performed sequentially or simultaneously, and embodiments comprising one or more of the features, wherein the embryo is cultured in vitro. Such embryo may be cultured in sequential culture. The embryo, for example at the blastocyst stage, is cryopreserved prior to transfer to a host mammal.

5

For the methods of the present invention embodiments cover pigs, mini-pigs for example selected from the group consisting of Goettingen, Yucatan, Bama Xiang Zhu, Wuzhishan and Xi Shuang Banna, including any combination thereof. However, another embodiment relates to pigs that are not a mini-pig, such as the species of *Sus scrofa domesticus*, for example where the pig is selected from the group consisting of Landrace, Yorkshire, Hampshire, Duroc, Chinese Meishan, Berkshire and Piêtrain, including any combination thereof. In a preferred embodiment is the Yucatan minipig.

15 A further aspect of the present invention pertains to method for evaluating the effect of a therapeutical treatment of atherosclerosis, said method comprising the steps of i) providing the pig model as disclosed herein, ii) treating said pig model with a pharmaceutical composition exerting an effect on said phenotype, and iii) evaluating the effect observed.

20 In one embodiment the method further comprises a step of advising on medical treatment based on the afore-mentioned observed effects.

Yet a further aspect of the present invention relates to a method for screening the efficacy of a pharmaceutical composition for atherosclerosis, said method comprising the steps of i) providing the pig model as disclosed herein, ii) expressing in said pig model said genetic determinant and exerting said phenotype for said disease, iii) administering to said pig model a pharmaceutical composition the efficacy of which is to be evaluated, and iv) evaluating the effect, if any, of the pharmaceutical composition on the phenotype exerted by the genetic determinant when expressed in the pig model.

30

Furthermore the present invention in another aspect relates to a method for treatment of a human being suffering from atherosclerosis, said method comprising the initial steps of i) providing the pig model as disclosed herein, ii) expressing in said pig model said genetic determinant and exerting said phenotype for said disease, iii) administering to said pig model a pharmaceutical composition the efficacy of which is

35

to be evaluated, and iv) evaluating the effect observed, and v) treating said human being suffering from atherosclerosis based on the effects observed in the pig model.

5 Description of drawings

Figure 1 shows the number of resistant clones after co-transfection of pSBT-HCRapoE-hAAT-PCSK9-bpA with pCMV-mSB (Left) and pCMV-HSB3 (Right).

Figure 2 shows transient expression of pSBT-HCRapoE-hAAT-PCSK9-BpA in HepG2 cells followed by immunocytochemical staining for PCSK9-FLAG using an anti-FLAG antibody and Alexa-594-conjugated streptavidin (red fluorescence). **A.** Non-transfected control HepG2 cells. **B.** pSBT-HCRapoE-hAAT-PCSK9-BpA transfected HepG2 cells. **C.** Transient transfection of the enhanced green fluorescent protein (eGFP)-expressing plasmid pEGFP-N1 (Invitrogen) to indicate transfection efficiency.

15

Figure 3 shows the Sleeping Beauty transposon-based pSBT/cHS4.H1p.PGK-puro.U6p.cHS4 plasmid for stable expression of shRNAs. Short hairpin RNAs can be expressed under the U6 or H1 promoter, or both. The expression cassettes are flanked by cHS4 insulator sequences (Chung JH et al. Proc Natl Acad Sci USA. 1997;94(2):575-580) to protect the expression cassettes from silencing position effects after genomic integration.

20

Figure 4 shows the knockdown efficiency of shRNA directed towards the 19-nucleotide targets T1 to T10 in Renilla luciferase-LDL receptor fusion RNAs. T8 and T9 perform best in the screening assay.

25

Figure 5 shows the bi-phased technology of the present invention in which an integrating SB vector, carrying a reporter gene and a selective marker gene, serves as a reporter for continuous gene expression and hence as a target for gene insertion. In a second modification step this vector may serve as a target for insertion of one or more gene expression cassettes in a well-characterized locus.

30

Figure 6 shows a schematic representation of pSBT/RSV-GFIP.

Figure 7 shows transposition of SB vectors in porcine fibroblasts. A standard transposon encoding a puromycin resistance gene (SBT/PGK-puro) was employed and varying levels of transposition were detected, resulting in about 75 drug-resistant colonies in cultures of fibroblasts co-transfected with pSBT/PGK-puro and pCMV-SB, less than 3 colonies appeared after transfection with pSBT/PGK-puro and pCMV-mSB, the latter which encodes an inactive version of the transposase. Interestingly, a mean of almost 140 colonies was obtained using the hyperactive transposase variant HSB3, indicating that HSB3 also in porcine cells mediates higher levels of transposition compared to the original SB transposase.

Figure 8 shows efficient insertion of a FRT-tagged SB vector in pig fibroblasts SB-tagged cell clones containing a Flp recombination target site for site-specific gene insertion were co-transfected the pSBT/loxP.SV40-loxP257 plasmid with pCMV-mSB, pCMV-SB, and pCMV-HSB3, respectively. HSB3 again showed the highest activity, resulting in about 30 drug-resistant colonies after transfection of 3×10^4 fibroblasts.

Figure 9 shows clone analysis by fluorescence microscopy of isolated and expanded puromycin-resistant colonies demonstrates efficient FRTeGFP expression

Figure 10. (a) Oocytes trisection; (b) couplets of fibroblast-oocyte fragment for the first fusion; (c) embryos reconstructed with triplets (note elongation under the AC currency); (d) triplets fusion. Scale bar = 50 μ m.

Figure 11. (a) In vitro matured oocytes after partial zona digestion. (b) Delipated oocytes after centrifugation. (c) Bisection of delipated oocytes. (d) Couplets of fibroblast-oocyte fragment for the first fusion. (e) Four-cell stage reconstructed embryos developed from delipated oocytes. (f) Four-cell stage reconstructed embryos developed from intact oocytes. (g) Re-expanded blastocysts from delipated embryos after warming. (h) Hoechst staining and UV illumination of re-expanded blastocysts from delipated embryos after warming. Bar represents 100 μ m.

Figure 12. Bisection at chemically assisted enucleation. Note the extrusion cone or polar body connected to the smaller part (putative karyoplast). Stereomicroscopic picture. Bar represents 50 μ m.

Figure 13. Hoechst staining and UV illumination of the absence and presence of chromatin. UV light, inverted fluorescent microscopic picture. Bar represents 50 μ m. (a) The absence of chromatin in putative cytoplasts (b) The presence of chromatin in putative karyoplasts.

5

Figure 14. Stereomicroscopic picture of Day 7 blastocysts produced with chemically assisted handmade enucleation (CAHE). Bar represents 50 μ m.

10

Figure 15. Hoechst staining and UV illumination of blastocyst developed after chemically assisted handmade enucleation (CAHE). Bar represents 50 μ m.

Detailed description of the invention

15

The present invention pertains to a genetically modified pig model, blastocysts, donor cells and/or fetuses for studying atherosclerosis wherein the pig model expresses at least one phenotype associated with atherosclerosis.

20

It will be appreciated that the invention does not comprise processes for modifying the genetic identity of pigs which are likely to cause them suffering without any substantial medical benefit to man or animal, or animals resulting from such processes.

25

The present invention also relates to genetically modified pig embryos obtainable by the methods described herein.

The methods for producing the pig model for studying atherosclerosis described herein do not encompass a surgical step performed on the pig.

30

The term 'endogenous' is used herein to specify a particular gene present naturally in the genome of of a particular target cell (for example cells of a pig).

35

The term "genetic determinant" is used herein to refer to a single-stranded or double-stranded "polynucleotide molecule" or "nucleic acid" comprising a structural gene of interest. The "genetic determinant" encodes a protein not ordinarily made in appreciable amounts in the target cells. Thus, "genetic determinants" include nucleic acids which are not ordinarily found in the genome of the target cell. "Genetic determinants" also include nucleic acids which are ordinarily found within the genome

of the target cell, but is in a form which allows for the expression of proteins which are not ordinarily expressed in the target cells in appreciable amounts. Alternatively, "genetic determinants" may encode a variant or mutant form of a naturally-occurring protein.

5

The terms "polynucleotide" and "nucleic acid" are used interchangeably, and, when used in singular or plural, generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as defined herein include, without limitation, single- and double-stranded DNA, DNA including single- and double-stranded regions, single- and double-stranded RNA, and RNA including single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or include single- and double-stranded regions. In addition, the term "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. The term "polynucleotide" specifically includes cDNAs. The term includes DNAs (including cDNAs) and RNAs that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritiated bases, are included within the term "polynucleotides" as defined herein. In general, the term "polynucleotide" embraces all chemically, enzymatically and/or metabolically modified forms of unmodified polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

The terms 'transgenic' pig and 'genetically modified' pig are used in identical meaning herein.

30

The present invention pertains to a modified pig model for studying atherosclerosis, wherein the pig model expresses at least one phenotype associated with atherosclerosis.

Pigs

The present invention relates to a modified pig as a model for studying atherosclerosis, wherein the pig model expresses at least one phenotype associated with atherosclerosis. The pig of the present invention may be any pig.

5

The pig is evolutionary close to humans as compared to for example rodentia. Furthermore, the pig has been widely used in bio-medical research because of the similarities between human and porcine physiology (Douglas, 1972; Book & Bustad, 1974).

10

In one embodiment the pig is a wild pig. In another embodiment the pig is the domestic pig, *Sus scrofa*, such as *S. domesticus*. In yet another embodiment the invention relates to mini pigs, as well as to inbred pigs. The pig can be selected e.g. from the group consisting of Landrace, Yorkshire, Hampshire, Duroc, Chinese Meishan, Berkshire and Piêtrain, such as the group consisting of Landrace, Yorkshire, Hampshire and Duroc, for example the group consisting of Landrace, Duroc and Chinese Meishan, such as the group consisting of Berkshire, Pietrain, Landrace and Chinese Meishan, for example the group consisting of Landrace and Chinese Meishan. In one embodiment, the pig is not a mini-pig. In another embodiment the pig of the present invention is an inbred pig.

20

In another embodiment of the present invention the pig is a mini-pig and the mini-pig is preferably selected from the group consisting of Goettingen, Yucatan, Bama Xiang Zhu, Wuzhishan and Xi Shuang Banna. Thus, the present invention relates to any of Goettingen, Yucatan, Bama Xiang Zhu, Wuzhishan and Xi Shuang Banna separately, or in any combination. In a preferred embodiment of the invention the Yucatan mini pig is used.

25

Due to its size and weight of about 200 kg the domestic pig is not easily handled in a laboratory setting. A preferred alternative to the domestic pig is the Goettingen (Göttingen) mini-pig that weighs about 30 kg. However, alternatives to the Goettingen minipig is the Yucatan minipig, Preferred embodiments of the present invention comprises Goettingen mini pig, or the Yucatan minipig.

30

Genetically modified

The genetic modifications are introduced in the somatic cell prior to cell nuclear transfer. However, the genetic modification may in another embodiment be introduced during the cell nuclear transfer process, for example by addition of transgenes at
5 different steps of the hand made cloning (HMC) procedure that will then find their way to the genome of the embryo.

The genetic modifications comprise random integration of a disease causing gene, mutated gene, into the genome of the somatic cell. It could also be random integration of a normal non-mutated gene that will cause a disease when expressed in a specific
10 tissue or at a specific expression level.

However, the invention also pertains to modified pigs, embryos, donor cells, blastocysts and/or fetuses obtained by transfer of mRNA and/or protein of the genes disclosed herein. Thus, the modification of the pig is in one embodiment does not lead to integration of a transgene into the genome of the pig, embryo, blastocyst and/or
15 fetus.

The introduced gene or transgene, transcriptional and/or translational product or part thereof may originate from any species, including bacteria, pig, human, mouse, rat, yeast, invertebrates, or plants. Regulatory sequences of the transgene may drive
20 ubiquitous or inducible or tissue- and/or time-specific expression and may also originate from any species including pig, human, mouse, rat, yeast, invertebrates, or plants.

Importantly, the genetic modification in the somatic cell may be targeted to a specific region in the porcine genome by homologous recombination of a targeting construct or
25 by gene editing procedures. This could be inactivation (e.g. knock-out) of specific genes that will cause a disease or phenotype.

Homologous recombination occurs between two homologous DNA molecules. It is also called DNA crossover. By homologous recombination, one DNA segment can
30 replace another DNA segment with a similar sequence. The process involves breakage and reunion between the homologous regions of DNA, which is mediated by specialized enzymes. The technique allows replacing one allele with an engineered construct without affecting any other locus in the genome. Using homologous recombination it is possible to direct the insertion of a transgene to a specific known
35 locus of the host cells genome. Knowing the DNA sequence of the target locus, it is

possible to replace any gene with a genetically modified DNA construct, thereby either replacing or deleting the target sequence. The technique comprises discovering and isolating the normal gene and then determining its function by replacing it in vivo with a defective copy. This procedure is known as 'gene knock-out', which allows for specific gene targeting by taking advantage of homologous recombination. Cloned copies of the target gene are altered to make them nonfunctional and are then introduced into ES cells where they recombine with the homologous gene in the cell's genome, replacing the normal gene with a nonfunctional copy.

Homologous recombination can similarly be exploited to generate fusion genes or insertion of point mutations in a 'knock-in' strategy, in which a targeting vector, comprising a relevant exon of the target locus fused with the cDNA sequence of chromosomal translocation-fusion partner, is transfected into embryonic stem cells, whereby the recombinant sequence is fused to an endogenous gene to generate fusion a gene.

Another applicable technique to exploits the phenomenon called RNA interference (RNAi), in which 21 nucleotide small interfering RNAs (siRNA) can elicit an effective degradation of specific mRNAs. RNA interference constitutes a new level of gene regulation in eukaryotic cells. It is based on the fact that presence of double stranded RNA in a cell eliminates the expression of a gene of the same sequence, whereas expression of other unrelated genes is left undisturbed. The siRNA stimulates the cellular machinery to cut up other single-stranded RNA having the same sequence as the siRNA. In a preferred embodiment of the present invention, siRNAs are directed towards porcine ApoE, and/or porcine LDL. In one embodiment, the siRNA may be selected from the siRNA sequences, described herein.

The genetic modifications introduced into the porcine genome prior or during the HMC procedure could also be epigenetic modifications (e.g. methylation of DNA or methylation or acetylation/deacetylation of histones) by incubating somatic cells, oocytes or reconstructed HMC embryos with chemical components such as trichostatin or compounds with similar effect.

The present invention relates to a modified pig, blastocyst, embryo, fetus and/or donor cell comprising a genetic determinant in the form of at least one mutation in an endogenous, and thus porcine, ApoE gene or part thereof, and/or at least one mutation

in an endogenous, and thus porcine, LDL gene or part thereof, and/or in the form of an overexpressed human and/or porcine PCSK9 gene or part thereof, separately or in combination as described in detail herein.

5 The present invention also relates to porcine embryos, blastocysts and/or fetuses derived from a modified pig expressing at least one phenotype associated with atherosclerosis.

10 It is within the scope of the present invention that the modified pig, blastocyst, embryo, fetus and/or donor cell comprises at least one mutation, such as 2, 3, 4, 5, 6, 7, 8, 9, 10 or 15 mutations in the ApoE gene and/or the LDL gene.

15 In one embodiment of the present invention the modified pig according to the present invention is mutated in the ApoE gene or part thereof, transcriptional and/or translational product or part thereof (SEQ ID NO: 1, SEQ ID NO:2) and/or the LDL gene or part thereof, transcriptional and/or translational product or part thereof (SEQ ID NO: 3, SEQ ID NO:4)) The genes are mutated so that the function of the gene is disrupted. It is appreciated that a mutation and/or disrupted function of the gene will result in a reduction in the amount of the transcriptional product or part thereof and/or translational product or part thereof of said gene compared to the amount of
20 transcriptional and/or translational product of the gene in question, here the ApoE gene and/or the LDL gene,

25 However, in another embodiment the transgenic pig is transgenic for a combination of mutations, for example at least one mutation in the porcine endogenous ApoE gene and/or at least one mutation in the porcine endogenous LDL gene.

The mutations introduced into the endogenous porcine ApoE gene and/or the endogenous porcine LDL gene or part thereof may be introduced by any method known to the person skilled in the art. In one embodiment the at least one mutation is introduced by knock out through homologous recombination. However, in another
30 preferred embodiment the at least one mutation is in the form of a reduction in the amount of transcriptional and/or translational product or part thereof of the endogenous ApoE and/or endogenous LDL gene compared to the amount of transcriptional and/or translational product or part thereof of a wild type ApoE gene and/or a LDL gene. A preferred method for reducing the amount of ApoE and/or LDL transcriptional and/or
35 translational product or part thereof is by use of small interfering RNAs (siRNA)

directed against the transcriptional products or part thereof of the ApoE gene and/or LDL gene. In a preferred embodiment the transcriptional product or part thereof of the LDL gene is targeted. Non-limiting targets for small interfering RNAs knock down of LDL receptors are shown in Table 1. Each of the targets may be chosen separately, or in any combination. In a preferred embodiment the target is T8. In another embodiment the preferred target is T9.

Table 1

Targets for shRNA directed LDL receptor knockdown

	First base in pig cDNA AF065990 sequence	Sequence
T1	763	tgtcaaagcggcgagtgca
T2	889	tcccatatctgcaatgacc
T3	1150	accctggaccgtagtgagt
T4	1308	tgacaccattattggcgaa
T5	1309	gacaccattattggcgaag
T6	1439	agactctctccaagagaa
T7	1553	tgaacggagtggacgtcta
T8	1814	tcacaggctcggacataca
T9	858	CCAACGAGTGTCTGGACAA
T10	1109	CCTACCTCTTCTTCACCAA

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One or more mutations of the ApoE and/or LDL gene may be in coding region of the ApoE and/or the LDL gene, however, one or more mutations of the ApoE and/or the LDL gene may also be determined in at least one regulatory sequence of the ApoE and/or LDL gene. By regulatory sequence is meant sequences that regulate the transcriptional and translational process, for example, promoters, enhancers, sequences that affect polyadenylation, translational or transcriptional start, splicing of transcriptional products. The promoters and enhancers that control the transcription of protein-encoding genes are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by each element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation. The ApoE and/or LDL gene may alternatively be mutated in one or more of its exons.

20

In one embodiment the endogenous porcine ApoE gene of the genetically modified pig, embryo, blastocyst, donor cell and/or fetus of the present invention is mutated in one or more of its exons, thus disrupting gene function of ApoE. Thus, any of exon 1, exon 2, exon 3, or exon 4 of the porcine ApoE gene may be mutated. Exon 1 is positioned at nucleotide 832-857 of the porcine ApoE gene, exon 2 at nucleotide 1663-1728, exon 3 at nucleotides 2473-2662, exon 4 at nucleotides 3037-3879 of the porcine ApoE gene. In a preferred embodiment the one or more exon of ApoE and/or LDL is mutated by disrupting the exon due to the insertion of a nucleotide construct by homologous recombination and knock-out technology.

Furthermore, it is appreciated the genetically modified pig, embryo, blastocyst, donor cell and/or fetus of the present invention comprises the transcriptional product or part thereof and/or the translational product or part thereof of the porcine ApoE and/or LDL genes as described above.

In most cases of atherosclerosis the genetic component is complex, but in some cases the inheritance of the disease is monogenic. These cases are mostly caused by mutations in genes coding for proteins involved in lipoprotein trafficking, and the most severe in humans are caused by mutations affecting LDL receptor-mediated lipoprotein uptake (recessive and autosomal dominant familial hypercholesterolemia). Recently, a gain-of-function mutation in the PCSK9 gene was described as the cause of autosomal dominant familial hypercholesterolemia (17) PCSK9 binds to the LDL receptor leading to its degradation (18). Therefore, gain-of-function mutations in humans and overexpression of PCSK9 transgenes in mice leads to functional LDL receptor deficiency (19). The modified pig, embryo, blastocyst, fetus and/or donor cell comprises at least one human and/or porcine PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.

In one embodiment the modified pig, embryo, blastocyst, fetus and/or donor cell comprises at least one porcine PCSK9 gene or part thereof, transcriptional and/or translational product thereof. In a preferred embodiment the modified pig, embryo, blastocyst, fetus and/or donor cell comprises at least one human PCSK9 gene or part thereof, transcriptional and/or translational product thereof.

Sequence identity

Functional equivalents and variants are used interchangeably herein. In one preferred embodiment of the invention there is also provided variants of the LDL receptor and/or the apolipoprotein A gene. When being polypeptides, variants are determined on the basis of their degree of identity or their homology with a predetermined amino acid sequence, said predetermined amino acid sequence being the LDL receptor and/or the apolipoprotein A gene products as described herein, or, when the variant is a fragment, a fragment of any of the aforementioned amino acid sequences, respectively.

Accordingly, variants preferably have at least 91 % sequence identity, for example at least 91% sequence identity, such as at least 92 % sequence identity, for example at least 93 % sequence identity, such as at least 94 % sequence identity, for example at least 95 % sequence identity, such as at least 96 % sequence identity, for example at least 97% sequence identity, such as at least 98 % sequence identity, for example 99% sequence identity with the predetermined sequence.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "predetermined sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity".

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A "predetermined sequence" is a defined sequence used as a basis for a sequence comparison; a predetermined sequence may be a subset of a larger sequence, for example, as a segment of a full-length DNA or gene sequence given in a sequence listing, such as a polynucleotide sequence of the LDL receptor and/or the apolipoprotein A genes as described herein or may comprise a complete DNA or gene sequence. Generally, a predetermined sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length.

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Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20

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contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a predetermined sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the
5 predetermined sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences.

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:
10 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or
15 by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term
20 "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and
25 multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a predetermined
30 sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the predetermined sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the predetermined sequence over the window of comparison. The predetermined
35 sequence may be a subset of a larger sequence, for example, as a segment of the full-

length LDL receptor and/or apolipoprotein A gene polynucleotide sequence illustrated herein.

Sequence identity is determined in one embodiment by utilising fragments of porcine or human ApoE sequence, or porcine or human LDL peptides, porcine or human PCSK9 peptides comprising at least 25 contiguous amino acids and having an amino acid sequence which is at least 80%, such as 85%, for example 90%, such as 95%, for example 96%, such as 97%, for example 98%, such as 99% identical to the amino acids as described herein, wherein the percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

Conservative amino acid substitutions:

Substitutions within the groups of amino acids, shown below, are considered conservative amino acid substitutions. Substitutions between the different groups of amino acids are considered non-conservative amino acid substitutions.

P, A, G, S, T (neutral, weakly hydrophobic)

Q, N, E, D, B, Z (hydrophilic, acid amine)

H, K, R (hydrophilic, basic)

F, Y, W (hydrophobic, aromatic)

L, I, V, M (hydrophobic)

C (cross-link forming)

By the term "transcriptional or translational products" is meant herein products of gene transcription, such as a RNA transcript, for example an unspliced RNA transcript, a mRNA transcript and said mRNA transcript splicing products, and products of gene translation, such as polypeptide(s) translated from any of the gene mRNA transcripts and various products of post-translational processing of said polypeptides, such as the products of post-translational proteolytic processing of the polypeptide(s) or products of various post-translational modifications of said polypeptide(s).

As used herein, the term "transcriptional product of the gene" refers to a pre-messenger RNA molecule, pre-mRNA, that contains the same sequence information (albeit that U nucleotides replace T nucleotides) as the gene, or mature messenger RNA molecule, mRNA, which was produced due to splicing of the pre-mRNA, and is a template for translation of genetic information of the gene into a protein.

Phenotypes

The phenotypes associated with atherosclerosis are many. It is appreciated that the pig model of the present invention expresses at least one phenotype associated with atherosclerosis, such as three, for example four, five, six, seven, eight, nine, ten, eleven, 12, 13, 14, 15, 16, 17, 18, 19 or 20 phenotypes associated with atherosclerosis. Non-limiting examples of said phenotypes are hypercholesterolemia, accumulation of fat, cholesterol and other substances in the walls of arteries, plaque formation, stenosis, blockage of blood flow, plaque rupture, infarction, and/or claudication.

At least one phenotype associated with atherosclerosis is hypercholesterolemia. Hypercholesterolemia is an increase in cholesterol amount as compared to a standard level in the pig observed before the onset of an increase in cholesterol amount or compared to a standard level determined from a population of pigs. The cholesterol amount is preferably measured in the plasma of pigs. An increase in plasma cholesterol is at least 10% compared to a standard level, such as at least 15%, for example 20%, such as 25%, for example 30%, such as at least 35%, for example at least 40%, such as at least 45%, for example at least 50%, such as at least 55%, for example at least 60%, such as at least 65%, for example at least 70%, such as at least 75%, for example at least 80%, such as at least 85%, for example at least 90%, such as at least 95%, for example at least 100%, such as at least 110%, for example at least 120%, such as at least 150%, for example at least 175%, such as at least 200%, for example at least 250%, such as at least 300%, for example at least 350%, such as at least 400%, for example at least 450%, such as at least 500 compared to a standard level.

Thus, a 10% increase in hypercholesterolemia in a pig having a standard level of 2 mmol/l corresponds to an amount of cholesterol of 2.2 mmol/l.

The phenotypes associated with atherosclerosis comprise the building up of fat, cholesterol and other substances in the walls of arteries and form plaques. Eventually, the plaque deposits can make the artery less flexible. The hardening of the artery may result in decreased blood flow (stenosis) and even blockage of blood flow.

Consequently, insufficient blood supply to the organs. The artery may compensate for narrowing of the artery by enlarging the artery which when excessive leads to the formation of an aneurysm. If blood flow in the arteries leading to the heart is reduced, chest pain can occur. Plaques can also break apart (plaque rupture), causing pieces of

material to move through the artery, causing the formation of a thrombus that will rapidly slow or stop blood flow, leading to death of the tissues affected by stop of blood flow, also known as infarction. This is a common cause of heart attack and stroke. Blood clots can also form around the plaque deposits. Clots block blood flow. If the clot moves into the heart, lungs, or brain, it can cause a stroke, heart attack, or pulmonary embolism.

Also claudication due to insufficient blood supply to the legs, typically due to a combination of both stenosis and aneurysmal segments narrowed by clots is an embodiment of the phenotypes associated with atherosclerosis. Another phenotype of the genetically modified pig is hypercholesterolemia in which consistently high levels of low-density lipoprotein (LDL) is observed compared to normal levels, leading to premature atherosclerosis of the coronary arteries. Typically in affected men, heart attacks occur in their 40s to 50s. In humans, hypercholesterolemia is often in the form of familial hypercholesterolemia which is mostly due to mutations in genes coding for proteins involved in lipoprotein trafficking, caused by homozygous null mutations in the LDL receptor.

A number of tests exist which aids in the diagnosis of atherosclerosis are known to the person skilled in the art such as a cardiac stress test which is a medical test performed to evaluate arterial blood flow to the myocardium (heart muscle) during physical exercise, compared to blood flow while at rest. Also low intensity ultrasound is used to detect blood flow velocity in arteries known as a Doppler study, magnetic resonance arteriography, CT scanning, arteriography using x-ray and special dye to see inside the arteries, intravascular ultrasound and/or ultrasonic duplex scanning are used to diagnose atherosclerosis.

Methods for producing pig model for studying atherosclerosis

The present invention provides improved procedures for cloning pigs by nuclear transfer which refers to the introduction of a full complement of nuclear DNA from one cell to an enucleated cell. The genetically modified pig of the present invention may be produced using any technique in which modified genetic material material, transcriptional product and/or translational product or part thereof is transferred from at donor cell to a host cell, such as an enucleated oocyte. A number of techniques exist such as introducing genetic material from a genetically modified somatic cell into an enucleated oocyte by for example microinjection or by nuclear transfer

In cloning, the transfer of the nucleus of a somatic (body) cell or somatic cell into an egg cell (oocyte) which has had its own nucleus removed (denucleated or enucleated) is called somatic cell nuclear transfer. The new individual will develop from this reconstructed embryo and be genetically identical to the donor of the somatic cell. In the present invention the modified pig model, porcine embryo, blastocyst and/or fetus is obtainable by somatic cell nuclear transfer comprising the steps of a) establishing at least one oocyte having at least a part of a modified zona pellucida, b) separating the oocyte into at least two parts obtaining at least one cytoplasm, c) establishing a donor cell or cell nucleus having desired genetic properties, d) fusing at least one cytoplasm with the donor cell or membrane surrounded cell nucleus, e) obtaining a reconstructed embryo, f) activating the reconstructed embryo to form an embryo; and g) transferring said cultured embryo to a host mammal such that the embryo develops into a genetically modified fetus, wherein said genetically modified embryo obtainable by nuclear transfer comprises steps a) to e) and/or f), wherein said genetically modified blastocyst obtainable by nuclear transfer comprises steps a) to e) and/or f), wherein said genetically modified fetus obtainable by nuclear transfer comprises steps a) to g).

The oocyte of b) may in another embodiment be separated into at least three parts obtaining at least two cytoplasts. It is appreciated that the donor cell or cell nucleus of c) harbours genetic determinants for atherosclerosis, for example in the form of modified human or porcine ApoE gene or part thereof and/or modified human or porcine LDL gene or part thereof. The host mammal of g) is in one embodiment a pig, preferably a Yucatan mini pig.

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However, the present invention also relates to a method for producing a transgenic pig, porcine blastocyst, embryo and/or fetus as a model for atherosclerosis comprising the steps of a) establishing at least one oocyte, b) separating the oocyte into at least three parts obtaining at least two cytoplasts, c) establishing a donor cell or cell nucleus having desired genetic properties, d) fusing at least one cytoplasm with the donor cell or membrane surrounded cell nucleus, e) obtaining a reconstructed embryo, f) activating the reconstructed embryo to form an embryo; and g) transferring said cultured embryo to a host mammal such that the embryo develops into a genetically modified fetus, wherein said genetically modified embryo obtainable by nuclear transfer comprises

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steps a) to e) and/or f), wherein said genetically modified blastocyst obtainable by nuclear transfer comprises steps a) to e) and/or f), wherein said genetically modified fetus obtainable by nuclear transfer comprises steps a) to g)

5 It is appreciated that the donor cell or cell nucleus of c) harbours genetic determinants for atherosclerosis, for example in the form of modified human or porcine ApoE gene or part thereof and/or modified human or porcine LDL gene or part thereof and/or transcriptional and/or translational products thereof, and/or the insertion of a porcine and/or human PCSK9 gene. The host mammal of g) is in one embodiment a pig, preferably a Yucatan mini pig.

10 The various parameters are described in detail below.

Oocyte

The term 'oocyte' according to the present invention means an immature female reproductive cell, one that has not completed the maturing process to form an ovum (gamete). In the present invention an enucleated oocyte is the recipient cell in the nuclear transfer process.

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The oocytes according to the present invention are isolated from oviducts and/or ovaries of a mammal. Normally, oocytes are retrieved from deceased pigs, although they may be isolated also from either oviducts and/or ovaries of live pigs. In one embodiment the oocytes are isolated by oviductal recovery procedures or transvaginal recovery methods. In a preferred embodiment the oocytes are isolated by aspiration. Oocytes are typically matured in a variety of media known to a person skilled in the art prior to enucleation. The oocytes can also be isolated from the ovaries of a recently sacrificed animal or when the ovary has been frozen and/or thawed. Preferably, the oocytes are freshly isolated from the oviducts.

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Oocytes or cytoplasts may also be cryopreserved before use. While it will be appreciated by those skilled in the art that freshly isolated and matured oocytes are preferred, it will also be appreciated that it is possible to cryopreserve the oocytes after harvesting or after maturation. If cryopreserved oocytes are utilised then these must be initially thawed before placing the oocytes in maturation medium. Methods of thawing cryopreserved materials such that they are active after the thawing process are well-known to those of ordinary skill in the art. However, in general, cryopreservation of oocytes and cytoplasts is a very demanding procedure, and it is especially difficult in

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pigs, because of the above mentioned general fragility of pig oocytes and cytoplasts, and because of the high lipid content that makes them very sensitive to chilling injury (i.e. injury that occurs between +15 and +5°C during the cooling and warming procedure).

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In another embodiment, mature (metaphase II) oocytes that have been matured in vivo, may be harvested and used in the nuclear transfer methods disclosed herein.

Essentially, mature metaphase II oocytes are collected surgically from either nonsuperovulated or superovulated pigs 35 to 48 hours past the onset of estrus or past the injection of human chorionic gonadotropin (hCG) or similar hormone.

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Where oocytes have been cultured in vitro, cumulus cells that are surrounding the oocytes in vivo may have accumulated may be removed to provide oocytes that are at a more suitable stage of maturation for enucleation. Cumulus cells may be removed by pipetting or vortexing, for example, in the presence of in the range of 0.1 to 5 % hyaluronidase, such as in the range of 0.2 to 5% hyaluronidase , for example in the range of 0.5 to 5 % hyaluronidase, such as in the range of 0.2 to 3% hyaluronidase , for example in the range of 0.5 to 3 % hyaluronidase, such as in the range of 0.5 to 2 % hyaluronidase , for example in the range of 0.5 to 1% hyaluronidase, such as 0.5% hyaluronidase.

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The first step in the preferred methods involves the isolation of a recipient oocyte from a suitable pig. In this regard, the oocyte may be obtained from any pig source and at any stage of maturation.

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The stage of maturation of the oocyte at enucleation and nuclear transfer has been reported to be of significance for the success of nuclear transfer methods. Immature (prophase I) oocytes from pig ovaries are often harvested by aspiration. In order to employ techniques such as genetic engineering, nuclear transfer and cloning, such harvested oocytes are preferably matured in vitro before the oocyte cells may be used as recipient cells for nuclear transfer.

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Preferably, successful pig embryo cloning uses the metaphase II stage oocyte as the recipient oocyte because it is believed that at this stage of maturation the oocyte can be or is sufficiently activated to treat the introduced nucleus as if it were a fertilising

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sperm. However, the present invention relates to any maturation stage of the oocyte which is suitable for carrying out somatic cell nuclear transfer, embryos, blastocysts, and/or transgenic pigs obtainable by the method of somatic cell nuclear transfer of the present invention.

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The in vitro maturation of oocytes usually takes place in a maturation medium until the oocyte has reached the metaphase II stage or has extruded the first polar body. The time it takes for an immature oocyte to reach maturation is called the maturation period.

10 In a preferred embodiment of the present invention the oocyte is from sow or gilt, preferably from a sow.

The donor (somatic cell or nucleus of somatic cell) and recipient (cytoplast) involved in the cell nuclear transfer method according to the present invention is a pig. Likewise, 15 reconstructed embryos may be implanted in a pig according to the present invention. The different pigs suitable as donor, recipient or foster mother are described elsewhere herein.

The donor pig according to the present invention may be female, or male. The age of the pig can be any age such as an adult, or for example a fetus.

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Embryo

According to the present invention a reconstructed embryo (i.e. single cell embryo) contains the genetic material of the donor cell. Subsequently, the reconstructed embryo divides progressively into a multi-cell embryo after the onset of mitosis. In vitro the 25 onset of mitosis is typically induced by activation as described herein.

In the present invention the term 'embryo' also refers to reconstructed embryos which are embryos formed after the process of nuclear transfer after the onset of mitosis by activation. Reconstructed embryos are cultured in vitro.

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When the embryo contains about 12-16 cells, it is called a "morula". Subsequently, the embryo divides further and many cells are formed, and a fluid-filled cystic cavity within its center, blastocoele cavity. At this stage, the embryo is called a "blastocyst". The developmental stage of the "fertilized" oocyte at the time it is ready to implant; formed 35 from the morula and consists of an inner cell mass, an internal cavity, and an outer

layer of cells called trophectodermal cells.

The blastocyst according to the present invention may be implanted into the uterus of a host mammal and continues to grow into a fetus and then an animal.

5 In the methods provided herein for producing genetically modified or transgenic non-human mammal, for cloning a non-human mammal, for culturing a reconstructed embryo, and /or for cryopreservation of a pig embryo, the embryo may be cultured in vitro. The embryo may for example be cultured in sequential culture. It will be appreciated that the embryo may be a normal embryo, or a reconstructed embryo as
10 defined elsewhere herein.

The present invention thus relates to a modified porcine embryo, blastocyst and/or fetus derived from the genetically modified pig model as disclosed herein and/or the modified porcine embryo comprises at least one modified human ApoE gene or part thereof and/or, human LDL gene or part thereof and/or, porcine APoE gene or part
15 thereof and/or, porcine LDL gene or part thereof, and/or comprises a human PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.

It is appreciated that the modified porcine embryo, blastocyst and/or fetus derivable from the modified pig model for studying atherosclerosis, expressing at least one
20 phenotype associated with atherosclerosis may have been the result of the crossing of for example a pig transgenic for at least one ApoE mutation and a pig transgenic for at least one LDL mutation, and/or a pig comprising a human PCSK9 gene.

Cytoplasm

25 An oocyte or a part of an oocyte from which the nucleus has been removed.

Donor Cell

By the term 'donor cell' of the present invention is meant somatic cell and/or cells derived from the germ line.

30 By the term 'somatic cell' of the present invention is meant any (body) cell from an animal at any stage of development. For example somatic cells may originate from fetal or adult tissue. Especially preferred somatic cells are those of foetal origin. However, cells from a germ line may also be used. According to the present invention a donor cell is a somatic cell. In another embodiment of the present invention the donor cell is a
35 cell derived from a germ cell line.

In a preferred embodiment of the present invention the donor cell harbours desired genetic properties. However, the donor cell may harbour desired genetic properties which have been gained by genetic manipulation as described elsewhere herein.

5 Somatic cells are selected from the group consisting of epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells.

10 These may be obtained from different organs, e. g., skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs.

The pigs from which the somatic cells may be derived are described elsewhere herein. A preferred embodiment of the invention is the use of somatic cells originating from the same species as the recipient oocyte (cytoplasm).

15 Preferably, the somatic cells are fibroblast cells as they can be obtained from both developing fetuses and adult animals in large quantities. Fibroblasts may furthermore be easily propagated in vitro. Most preferably, the somatic cells are in vitro cultured fibroblasts of foetal origin.

20 In a preferred embodiment the somatic cells are genetically modified. In yet a further preferred embodiment of the present invention the somatic cells are preferably of foetal origin, or for example from adults.

25 The present invention thus relates to a modified porcine donor cell derived from the genetically modified pig model as disclosed herein and/or the modified porcine embryo comprises at least one modified human ApoE gene or part thereof and/or, human LDL gene or part thereof and/or, porcine ApoE gene or part thereof and/or, porcine LDL gene or part thereof, and/or comprises a human PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.

30 It is appreciated that the modified porcine donor cell from the modified pig model for studying atherosclerosis, expressing at least one phenotype associated with atherosclerosis may have been the result of the crossing of for example a pig

transgenic for at least one ApoE mutation and a pig transgenic for at least one LDL mutation, and/or a pig comprising a human PCSK9 gene.

Type of genetic modification

- 5 The donor cells may be genetically modified by any of standard method known in the art. The genetic modification may be a modification of the genomic DNA by deletion, insertion, duplication and/or other forms of mutation, including point mutation. The modification may be made in coding sequences and/or non-coding sequences. DNA constructs for insertion may harbour a gene of interest and/or regulatory sequences
- 10 such as promoters, insulators, enhancers, repressors or ribosomal entry sites. In some embodiments, only one genetic modification is introduced in the genome. In other embodiments, however, the genome may be modified at more than one site. Suitable techniques for genetic modification of mammalian cells, such as fibroblasts, include techniques such as gene addition by nonhomologous recombination, gene
- 15 replacement by homologous recombination, and gene editing. This may include the use of retroviral insertion, transposon transfer and/or artificial chromosome techniques. Nonhomologous DNA recombination may e.g. be carried out as described in Kragh et al. (2004) *Reprod. Fert. Dev.* 16:290 or Kragh et al. (2004) *Reprod. Fert. Dev.* 16:315, Transposon-based gene transfer may be carried out as described in Izsvak et al.(1997)
- 20 *Cell* 91:501. Gene replacement by homologous recombination may e.g. involve the techniques described by Urnow et al. (2005) *Nature* 435:646. Techniques for gene editing have been described in Andersen et al. (2002) *J. Mol. Med.* 80:770, Liu et al (2002) *Gene Ther.* 9:118 and Sørensen et al.(2005) *J. Mol. Med.* 83:39. In a preferred embodiment the donor cell is genetically modified by random integration,
- 25 homologous recombination of the genes disclosed herein into the genome of the donor cell, or the transcriptional product or part of the human PCSK9 gene is modified by RNA interference..

- In a preferred embodiment of the present invention the donor cell is genetically
- 30 modified (as described in a copending application). The donor cell or nucleus carries a SB tagged genome containing a Flp recombination target site for site specific gene insertion or integration. The SB tagged genome result from the integration of a recombinant target vector comprising a DNA transposon construct and a bicistronic gene cassette comprising (i) a FRT recombination site and (ii) an IRES-driven selection
- 35 gene. The DNA transposon construct may be any construct in which any DNA

transposon is present. In the present invention the DNA transposon construct is the Sleeping Beauty (SB) DNA transposon vector. The FRT recombination site may be embedded in the coding sequence of a selection gene which allows for detecting whether a transposition has occurred. The selection gene of the present invention is not limited to any particular selection gene. In preferred embodiments the selection gene are genes conferring resistance to antibiotics or drugs, such as puromycin, tetracycline, streptomycin or hygromycin resistance genes, or the enhanced green fluorescent protein (eGFP) gene, red fluorescent protein genes or the like.

The FRT recombination site may thus be embedded in a SV40 promoter driven fusion variant of the selection gene. However, any promoter suitable for conferring expression of a selection gene may be used according to the present invention. Non-limiting examples of such promoters are CMV (cytomegalovirus) or PGK promoter.

The IRES-driven selection gene is similarly not limited to any particular selection gene. In preferred embodiments the selection gene are genes conferring resistance to antibiotics or drugs, such as puromycin, tetracycline, streptomycin or hygromycin resistance genes, or the enhanced green fluorescent protein (eGFP) gene, red fluorescent protein genes or the like.

The recombinant vector construct may also comprise at least one site for Cre recombinase. The at least one site for Cre recombinase may be located as disclosed in the examples herein.

The donor cell or nucleus may also originate from a genetically modified pig comprising at least one site for integration of at least one transgene. A preferred embodiment is a donor cell or nucleus in the form of a fibroblast, such as a primary fibroblast.

The present invention also relates to a method for producing a porcine cell comprising a SB tagged genome containing a Flp recombination target site for site-specific gene insertion. The method comprises the steps of

a) providing a mammalian cell, b) transfecting the cell of a) with a plasmid expressing a transposase and a recombinant target vector comprising a DNA transposon construct and a bicistronic gene cassette comprising (i) a FRT recombination site and ii) an IRES-driven selection gene, c) selecting SB tagged cells.

As described elsewhere herein the mammalian cell may be any cell. In one embodiment in which the porcine cell is subsequently to be used for producing a

genetically modified pig by nuclear transfer according to the hand-made protocol as described herein, the porcine cell is in a preferred embodiment a fibroblast and most preferred a porcine primary fibroblast.

5 It is appreciated that a desired transgene may be integrated directly into the at least one site for integration present in the genome of the cell. However, the cell in which the genome carries the at least one site for integration is in another embodiment used as a donor cell for the production of a genetically modified pig by for example microinjection of the donor cell or nucleus thereof into a oocyte or by for example somatic nuclear transfer. In a preferred embodiment the donor cell or the nucleus thereof is used for the
10 production of a genetically modified pig by somatic nuclear transfer using the procedure as described elsewhere herein.

The transgene or gene of interest to be integrated in the targeted cells of the present invention is not limited to any particular gene. In one embodiment the gene to be
15 integrated is a disease-causing gene which results in the formation of a genetically modified pig displaying a phenotype of interest. According to the present invention the gene of interest to be integrated into the porcine cell is Apolipoprotein-E (ApoE) and/or LDL receptor.

20 The integration of the transgene into the at least one site for integration present in the genome of the cell is employed by transfection into the cell of plasmid DNA containing the gene of interest and also FRT sites, and a plasmid expressing the Flp-recombinase used to support integration at the FRT sites.

25 Enucleation

The method of enucleation of an oocyte may be selected from the group of methods consisting of aspiration, physical removal, use of DNA-specific fluorochromes, exposure to ultraviolet light and/or chemically assisted enucleation. In one embodiment the present invention relates to the use of DNA-specific fluorochromes.

30 Enucleation may, however, be performed by exposure with ultraviolet light. In a particular embodiment enucleation is chemically assisted prior to physical removal of the nucleus. Chemically assisted enucleation using for example antineoplastic agents, such as demecolcine (N-deacetyl-N-methyl 1 colchicine), and/or for example etoposide or related agents may be performed prior to enzymatic modification of zona pellucida.

Chemically assisted enucleation comprises culturing matured COCs in maturation medium as described elsewhere herein supplemented with demecolcine for a particular period of time. In the range of 0.1 µg/ml to 10 µg/ml demecolcine, such as 0.2 µg/ml to 10 µg/ml, for example 0.3 µg/ml to 10 µg/ml, such as 0.25 µg/ml to 5 µg/ml, for example 0.3 µg/ml to 1 µg/ml, such as 0.25 µg/ml to 0.5 µg/ml, for example 0.4 µg/ml demecolcin may be supplemented to the maturation medium. Similarly, maturation medium may be supplemented with etoposide for example in the range of 0.1 µg/ml to 10 µg/ml etoposide, such as 0.2 µg/ml to 10 µg/ml, for example 0.3 µg/ml to 10 µg/ml, such as 0.25 µg/ml to 5 µg/ml, for example 0.3 µg/ml to 1 µg/ml, such as 0.25 µg/ml to 0.5 µg/ml, for example 0.4 µg/ml etoposide may be supplemented to the maturation medium. The time for culturing the COCs in the presence of antineoplastic agents ranges from 10 min to 5 hrs, such as 30 minutes to 5 hrs, for example 10 minutes to 2 hrs, such as 30 min to 2 hrs, for example 10 min to 1.5 hrs, such as 20 min to 3 hrs, for example 10 min to 3 hrs, such as 30 min to 1.5 hrs, for example 45 min.

In a particular embodiment chemically assisted enucleation is performed using 0.45 µg/ml demecolcine and/or etoposide added to the maturation medium for 45 min.

In a particular embodiment it is preferred that the enucleation is by physical removal of the nucleus. The physical removal may be by separation for example by bisection of the oocyte into two halves (two parts), one which contains the nucleus and the enucleated oocyte half, known as the cytoplast, removing the nucleated half of the oocyte and selecting the resulting cytoplast for further procedures of the invention. Alternatively the separation is by trisection, resulting in three parts of which two parts are cytoplasts. In another embodiment the oocyte may be separated into four parts, resulting in the production of three cytoplasts. The oocyte may even be separated into five parts by physical removal, resulting in four cytoplasts. Similarly, the oocyte may be separated into six parts, for example seven parts, such as eight parts, for example nine parts, such as ten or more parts.

The physical separation of the oocyte and subsequent removal of the nucleus-bearing part of the oocyte may be achieved by the use of a microsurgical blade. The oocytes may be screened to identify which oocytes have been successfully enucleated. Oocyte parts that harbour nuclear DNA may be identified by staining with Hoechst flourochrome, the staining procedure of which is known to a person skilled in the art. Oocyte parts harbouring nuclear DNA are discarded and the enucleated

oocytes (cytoplasts) are selected for further procedures.

Zona pellucida

5 Zona pellucida is a thick, transparent, noncellular layer or envelope of uniform thickness surrounding an oocyte

10 Generally, an intact zona pellucida is considered to be important in cell nuclear transfer due to a number of parameters. One parameter is to keep the polar body close to the metaphase plate of the oocyte in order to indicate the appropriate site for enucleation. Another parameter relates to the keeping of the donor cell close to the oocyte cytoplasm before and during fusion. The zona is also believed to confer protection for the donor cell and cytoplasm during fusion. Finally, embryo development after reconstitution and activation is believed to be supported by the zona pellucida.

15 Modification of at least a part of the zona pellucida can be performed by a number of methods. For example physical manipulation can be used to modify the zona. But also chemical treatment with agents such as acidic solutions (acidic Tyrode) can be employed. One example of chemical agents that can be employed in the present invention is acidic solutions, for example Tyrode. In a particular embodiment of the invention the zona pellucida is modified by enzymatic digestion. Such enzymatic digestion may be performed by enzymes comprising for example trypsin. Alternatively
20 a specific protease may be used, such as pronase.

25 In a preferred embodiment the enzymatic digestion results in at least a partial digestion of a part of zona pellucida which in a preferred embodiment of the present invention means that at least a part of the zona pellucida is being removed, or that the zona pellucida is partly removed. In the present context the zona pellucida is not completely removed.

According to an especially preferred embodiment of the present invention the partially digested part of zona pellucida is characterized by the zona pellucida still being visible and by the fact that the oocyte has not become misshaped.

30 The partial digestion may be achieved by exposure to a protease. In another embodiment of the present invention the partial digestion may be accomplished by the

use of a pronase. In yet another embodiment the partial digestion may be achieved by a combination of a protease and pronase.

In a preferred embodiment the concentration of pronase is in the range of 0.1 mg/ml to 10 mg/ml, such as 0.5 mg/ml to 10 mg/ml, for example 1 mg/ml to 10 mg/ml, such as
5 1.5 mg/ml to 10 mg/ml, for example 2 mg/ml to 10 mg/ml, such as 2.5 mg/ml to 10 mg/ml, for example 2.75 mg/ml to 10 mg/ml, such as 3 mg/ml to 10 mg/ml, for example 3.25 mg/ml to 10 mg/ml, such as 3.3 mg/ml to 10 mg/ml, for example 3.5 mg/ml to 10 mg/ml.

A preferred embodiment is a pronase concentration in the range of 2 mg/ml to 5 mg/ml,
10 such as 2.25 mg/ml to 5 mg/ml, for example 2.5 mg/ml to 5 mg/ml, such as 2.75 mg/ml to 5 mg/ml, for example 2.8 mg/ml to 5 mg/ml, such as 2.9 mg/ml to 5 mg/ml, for example 3 mg/ml to 5 mg/ml, such as 3.1 mg/ml to 5 mg/ml, for example 3.2 mg/ml to 5 mg/ml, such as 3.3 mg/ml to 5 mg/ml.

A particular embodiment of the present invention is a pronase concentration in the
15 range of 1 mg/ml to 4 mg/ml, for example 1 mg/ml to 3.9 mg/ml, such as 1 mg/ml to 3.8 mg/ml, for example 1 mg/ml to 3.7 mg/ml, such as 1 mg/ml to 3.6 mg/ml, for example 1 mg/ml to 3.5 mg/ml such as 1 mg/ml to 3.4 mg/ml, for example 1 mg/ml to 3.3 mg/ml.

In a preferred embodiment the pronase concentration is in the range of 2.5 mg/ml to 3.5 mg/ml, such as 2.75 mg/ml to 3.5 mg/ml, for example 3 mg/ml to 3.5 mg/ml. In a
20 special embodiment the pronase concentration is 3.3 mg/ml.

It is clear to the skilled person that the pronase should be dissolved in an appropriate medium, one preferred medium according to the present invention is T33 (Hepes buffered TCM 199 medium containing 33% cattle serum (*as described earlier - Vajta, et al., 2003*)).

25 The time of incubation of the oocyte in the pronase solution is in the range of 1 second to 30 seconds, such as 2 seconds to 30 seconds, for example 3 seconds to 30 seconds, such as 4 seconds to 30 seconds, such as 5 seconds to 30 seconds.

In another embodiment of the present invention the incubation time is in the range of 2 seconds to 15 seconds, such as 2 seconds to 14 seconds, for example 2 seconds to
30 13 seconds, such as 2 seconds to 12 seconds, for example 2 seconds to 11 seconds,

such as 2 seconds to 10 seconds, for example 2 seconds to 9 seconds, such as 2 seconds to 8 seconds, for example 2 seconds to 7 seconds, such as 2 seconds to 6 seconds, for example 2 seconds to 5 seconds.

5 In a particular embodiment of the present invention the incubation time is in the range of 3 seconds to 10 seconds, such as 3 seconds to 9 seconds, for example 4 seconds to 10 seconds, such as 3 seconds to 8 seconds, for example 4 seconds to 9 seconds, such as 3 seconds to 7 seconds, for example 4 seconds to 8 seconds, such as 3 seconds to 6 seconds, for example 4 seconds to 7 seconds, such as 3 seconds to 5 seconds, for example 4 seconds to 6 seconds, such as 4 seconds to 5 seconds. An
10 especially preferred incubation time is 5 seconds.

In a preferred embodiment of the present invention the oocyte is treated for 5 seconds in a 3.3 mg/ml pronase solution at 39°C.

Reconstructed embryo

15 By the term 'reconstructed embryo' is meant the cell which is formed by insertion of the donor cell or nucleus of the donor cell into the enucleated oocyte which corresponds to a zygote (during normal fertilisation). However, the term 'reconstructed embryo' is also referred to as the 'reconstituted cell'. In the present invention the donor cell is a somatic cell. However, the donor cell may also be derived from a germ line cell.

20 Fusion

The transfer of a donor cell or a membrane surrounded nucleus from a donor cell to at least cytoplasm is according to the present invention performed by fusion. In the scenarios described below the term 'donor cell' also refers to a membrane surrounded nucleus from a donor cell. Fusion may be achieved by a number of methods.

25 Fusion may be between a donor cell and at least one cytoplasm, such as between a donor cell and at least two cytoplasts, for example between a donor cell and at least two cytoplasts, such as between a donor cell and at least three cytoplasts, such as between a donor cell and at least four cytoplasts, for example between a donor cell and at least five cytoplasts, such as between a donor cell and at least six cytoplasts, for
30 example between a donor cell and at least seven cytoplasts, such as between a donor cell and at least eight cytoplasts.

Fusion may be performed according to the listed combinations above simultaneously or sequentially. In one embodiment of the present invention the fusion is performed simultaneously. In another embodiment fusion of the at least one cytoplasm and a donor cell is performed sequentially.

- 5 For example fusion may be achieved by chemical fusion, wherein a donor cell and the at least one cytoplasm are exposed to fusion promoting agents such as for example proteins, glycoproteins, or carbohydrates, or a combination thereof. A variety of fusion-promoting agents are known for example, polyethylene glycol (PEG), trypsin, dimethylsulfoxide (DMSO), lectins, agglutinin, viruses, and Sendai virus. Preferably
10 phytohemagglutinin (PHA) is used. However mannitol and, or polyvinylalcohol may be used.

- Alternatively, fusion may be accomplished by induction with a direct current (DC) across the fusion plane. Often an alternating current (AC) is employed to align the donor and recipient cell. Electrofusion produces a sufficiently high pulse of electricity
15 which is transiently able to break down the membranes of the cytoplasm and the donor cell and to reform the membranes subsequently. As a result small channels will open between the donor cell and the recipient cell. In cases where the membranes of the donor cell and the recipient cell connect the small channels will gradually increase and eventually the two cells will fuse to one cell.

- 20 Alignment of the at least one cytoplasm and the donor cell may be performed using alternating current in the range of 0.06 to 0.5 KV/cm, such as 0.1 to 0.4 KV/cm, for example 0.15 to 0.3 KV/cm. In a preferred embodiment alignment of the at least one cytoplasm and the donor cell may be performed using alternating current at 0.2 KV/cm.

- Fusion may be induced by the application of direct current across the fusion plane of
25 the at least one cytoplasm and the donor cell. Direct current in the range of 0.5 to 5 KV/cm, such as 0.75 to 5 KV/cm, for example 1 to 5 KV/cm, such as 1.5 to 5 KV/cm, for example 2 to 5 KV/cm. Another preferred embodiment of the present invention is the application of direct current in the range of 0.5 to 2 KV/cm. In a further preferred embodiment the direct current may be 2 KV/cm.

- 30 The direct current may preferably be applied for in the range of 1-15 micro seconds, such as 5 to 15 micro seconds, for example 5 to 10 micro seconds. A particular embodiment may be 9 micro seconds.

In an especially preferred embodiment fusion with direct current may be using a direct current of 2 KV/cm for 9 micro seconds.

Electrofusion and chemical fusion may however be also be combined.

Typically electrofusion is performed in fusion chambers as known to the skilled person.

- 5 Fusion may be performed in at least one step, such as in two steps, for example three steps, such as in four steps, for example in five steps, such as six steps, for example seven steps, such as in eight steps.

- 10 Fusion may be performed in for example a first step wherein the at least one cytoplasm is fused to the donor cell. A second step of fusion may comprise fusion of the fused pair (cytoplasm-donor cell, reconstructed embryo) with at least one cytoplasm, such as at least two cytoplasts, for example three cytoplasts, such as four cytoplasts, for example five cytoplasts, such as six cytoplasts, for example seven cytoplasts, such as eight cytoplasts. The second step of fusion with fusion of at least one cytoplasm and the fused pair may be performed sequentially or simultaneously. In one embodiment the at least
15 two cytoplasts are fused to the fused pair simultaneously. In another embodiment the at least two cytoplasts are fused to the fused pair sequentially.

In one embodiment of the invention the second step of fusion may also be an activation step wherein the reconstructed embryo is activated to enter mitosis. As described elsewhere herein.

20 Activation

In a preferred embodiment the reconstructed embryo may be allowed to rest prior to activation for a period of time in order to allow for the nucleus of the donor cell to reset its genome and gain totipotency in the novel surroundings of the enucleated cytoplasm. The reconstructed embryo may for example rest for one hour prior to activation.

25

Preferably, the reconstructed embryo may be activated in order to induce mitosis. Methods for activation may preferably be selected from the group of consisting of electric pulse, chemically induced shock, increasing intracellular levels of divalent cations or reducing phosphorylation. A combination of methods may be preferred for
30 activation.

In one particular embodiment of the invention the activation and the second step of fusion may be performed simultaneously. However, the activation of the reconstituted embryo and the at least one additional step of fusion between the reconstructed embryo and the at least one cytoplasm may be performed sequentially.

5

Reducing the phosphorylation of cellular proteins in the reconstructed embryo by known methods such as for example by the addition of kinase inhibitors may activate the reconstituted embryo. A preferred embodiment may involve the use of agents that inhibit protein synthesis, for example cycloheximide. A further preferred embodiment

10

may be using agents that inhibit spindle body formation, for example cytochalasin B.

In one embodiment of the invention the intracellular levels of divalent cations may be increased. Divalent cations such as for example calcium may be comprised in the activation medium. Preferably, the cations may enter the reconstructed embryo,

15

particularly upon subjecting the reconstructed embryo to an electric pulse. In a preferred embodiment the electric pulse may cause entering of calcium into the reconstructed embryo.

The application of an electrical pulse using direct current may be an activation step.

20

However, in a preferred embodiment the electrical pulse applied for activation may also serve as an additional fusion step.

Prior to applying an electrical pulse using direct current the at least one cytoplasm and the at least one reconstructed embryo may be aligned by the application of alternating current. The alternating current may be in the range of the range of 0.06 to 0.5 KV/cm, such as 0.1 to 0.4 KV/cm, for example 0.15 to 0.3 KV/cm. In a preferred embodiment alignment of the at least one cytoplasm and the donor cell may be performed using alternating current at 0.2 KV/cm.

25

Activation may be induced by the application of direct current across the fusion plane of the at least one cytoplasm and the donor cell. Direct current in the range of 0.2 to 5 KV/cm, such as 0.4 to 5 KV/cm, for example 0.5 to 5 KV/cm.. Another preferred embodiment of the present invention is the application of direct current in the range of 0.5 to 2 KV/cm. In a further preferred embodiment the direct current may be 0.7 KV/cm.

30

The direct current may preferably be applied for in the range of 10 to 200 micro seconds, such as 25 to 150 micro seconds, for example 50 to 100 micro seconds. A particular embodiment may be 80 micro seconds.

5 In an especially preferred embodiment fusion with direct current may be using a direct current of 0.7 KV/cm for 80 micro seconds.

An especially preferred embodiment of activation according to the present invention may be use of an electrical pulse in combination with subjecting the reconstructed embryo to agents that inhibit protein synthesis, spindle body formation, and divalent cations.

10 Activation may be performed by any combination of the methods described above.

In vitro culture of embryos

One aspect of the invention relates to a method of in vitro culturing embryos, whereby the blastocyst rate increased to 25.3%. Thus, a method of culturing a reconstructed
15 embryo is within the scope of the present invention, comprising the steps of a) establishing at least one oocyte having at least a part of zona pellucida, b) separating the oocyte into at least two parts obtaining an oocyte having a nucleus and at least one cytoplasm, c) establishing a donor cell or cell nucleus having desired genetic properties, d) fusing at least one cytoplasm with the donor cell or membrane surrounded cell
20 nucleus, e) obtaining the reconstructed embryo, f) activating the reconstructed embryo to form an embryo, and g) culturing said embryo.

Another aspect of the invention relates to a method of cell nuclear transfer in which a step of culturing the embryo is included.

25

In a preferred embodiment in relation to the methods described herein embryos are cultured in a sequential set of media. Preferably the blastocysts are grown in traditional medium such as for example NCSU37 or equivalent medium as known to a person skilled in the art, wherein glucose is removed and substituted by other agents. One
30 agent may be pyruvate. Another agent may be lactate. The agents may also be combined and replace glucose in the traditional medium.

The embryos may be cultured in the substituted media as described above from Day 0 to Day 3, such as from Day 0 to Day 2.

5 The pyruvate concentration may range from 0.05 to 1 mM, such as 0.1 to 1 mM, for example 0.125 to 1 mM, such as 0.15 to 1 mM. However the concentration of sodium pyruvate may also range from 0.05 mM to 0.9 mM, such as 0.05 to 0.8 mM, for example 0.05 to 0.7 mM, such as 0.05 to 0.6 mM, for example 0.05 to 0.5 mM, such as 0.05 to 0.4 mM, for example 0.05 to 0.3 mM, such as 0.05 to 0.2 mM. Preferably the concentration ranges between 0.05 to 0.17 mM. A preferred concentration of sodium
10 pyruvate is 0.17 mM.

The lactate concentration may range from 0.5 to 10 mM, such as 0.75 to 10 mM, for example 1 to 10 mM, such as 1.5 to 10 mM, such as 1.75 to 10 mM, for example 2 to 10 mM, such as 2.5 to 10 mM. However the concentration of sodium lactate may also
15 range from 0.5 mM to 9 mM, such as 0.5 to 8 mM, for example 0.5 to 7 mM, such as 0.5 to 6 mM, for example 0.5 to 5 mM, such as 0.5 to 4 mM, for example 0.5 to 0.3 mM. Preferably the concentration ranges between 1 to 5 mM, such as 2 to 4 mM, for example 2 to 3 mM. A preferred concentration of sodium lactate is 2.73 mM.

20 After the initial glucose-free incubation medium glucose is again replacing the pyruvate and lactate. The embryos may be cultured in the glucose containing medium from Day 4 to Day 3, preferably from Day 3 to Day 7. The glucose concentration may range from 1 to 10 mM, such as 2 to 10 mM, for example 3 to 10 mM, such as 4 to 10 mM, for example 5 to 10 mM. However, the glucose concentration may also range from 1 to 9
25 mM, such as 2 to 8 mM, for example 3 to 7 mM, such as 4-6 mM. A preferred concentration of glucose according to the present invention is 5.5 mM of glucose.

Organ or tissue donation

In one embodiment, the animals of the invention may be used as a source for organ or
30 tissue donation for humans or other animals, either animals of the same species or animal of other species. Transfer between species is usually termed xenotransplantation. Entire organs that may be transplanted include the heart, kidney, liver, pancreas or lung. Alternatively, parts of organs, such as specific organ tissues may be transplanted or transferred to humans or other animals. In a yet further
35 embodiment, an individual cell or a population of individual cells from an animal of the

invention may be transferred to a human being or another animal for therapeutic purposes.

Cryopreservation

5 The term 'cryopreserving' as used herein can refer to vitrification of an oocyte, cytoplasm, a cell, embryo, or pig of the invention. The temperatures employed for cryopreservation is preferably lower than -80 degree C, and more preferably at temperatures lower than -196 degree C. Oocytes, cells and embryos of the invention can be cryopreserved for an indefinite amount of time. It is known that biological
10 materials can be cryopreserved for more than fifty years.
It is within the scope of the present invention that embryos may be cryopreserved prior to transfer to a host pig when employing methods for producing a genetically engineered or transgenic non-human mammal. Such cryopreservation prior to transfer may be at the blastocyst stage the of embryo development. Vitrification is a form of
15 cryopreservation where living cells are rapidly cooled so that the fluid of the cell does not form into ice. Thus, vitrification relates to the process of cooling where cells or whole tissues are preserved by cooling to low sub-zero temperatures, such as (typically) -80 C or -196 C
In particular the invention relates to the vitrification of an oocyte, however, the invention
20 also relates to the vitrification of embryos, preferably embryos at the blastocyst stage. In one embodiment, the embryo is cultured to blastocyst stage prior to vitrification. Especially pig embryos are covered by the present invention. Also vitrified cytoplasm are covered by the present invention, as are cells.

25 Yet another aspect of the invention relates to the cryopreservation of a pig embryo derived by a method for cell nuclear transfer as described herein comprising a step of vitrifying a pig embryo. A further aspect of the invention relates to pig embryos obtained, or obtainable by the methods provided herein.

30 Mitochondria

Cells of the tissue of the genetically modified non-human mammals and/or non-human embryos obtainable by the present invention may harbour mitochondria of different maternal sources. In a preferred embodiment the non-human mammals and/or non-human embryos may harbour mitochondria from only one maternal source, However,
35 in another preferred embodiment the non-human mammals and/or non-human

embryos may harbour mitochondria from at least two maternal sources, such as three maternal sources, for example four maternal sources, such as five maternal sources, for example six maternal sources, such as seven maternal sources, for example eight maternal sources, such as nine maternal sources, for example ten maternal sources.

- 5 The probability of having a specific number of maternal sources can be calculated based on the observed types of mitochondria.

Evaluation of treatment and methods for diagnosis

- 10 The present invention offers a method for screening the efficacy of a pharmaceutical composition, wherein the method comprises the steps of i) providing the pig model of the present invention, ii) expressing in said pig model the genetic determinant and exerting said phenotype for said disease, iii) administering to the pig model a pharmaceutical composition the efficacy of which is to be evaluated, and iv) evaluating the effect, if any, of the pharmaceutical composition on the phenotype exerted by the
- 15 genetic determinant when expressed in the pig model. In one preferred embodiment the preclinical testing of drugs targeting plaque stability and superimposed thrombosis is within the scope of the present invention.

- 20 Furthermore, within the scope of the present invention is a method for evaluating the response of a therapeutical treatment of atherosclerosis, wherein the method comprises the steps of i) providing the pig model of the present invention, ii) treating said pig model with a pharmaceutical composition exerting an effect on said phenotype, and iii) evaluating the effect observed. Based on the evaluation one could further advise on the treatment based on the observed effects.

- 25 In addition, the present invention relates to a method for treatment of a human being suffering from atherosclerosis, wherein the method comprises the initial steps of i) providing the pig model of the present invention, ii) expressing in said pig model said genetic determinant and exerting said phenotype for said disease, iii) administering to
- 30 said pig model a pharmaceutical composition the efficacy of which is to be evaluated, and v) evaluating the effect observed, and v) treating said human being suffering from atherosclerosis based on the effects observed in the pig model. In a preferred embodiment the treatment comprises treating a human being suffering from familial hypercholesterolemia.

35

It is therefore appreciated that the pig model according to the present invention may also receive medicaments for diseases other than atherosclerosis in order to test the combined effect of a drug for atherosclerosis and other drugs administered to the pig.

5 Furthermore, the pig model of the present invention also allows for the development of bioimaging technology for diagnosis of atherosclerosis. Thus, the present invention offers a method for evaluating a bioimaging technology for the diagnosis of atherosclerosis, wherein the method comprises the steps of i) providing the pig model of the present invention, ii) expressing in said pig model the genetic determinant and
10 exerting said phenotype for said disease, iii) bioimaging the pig model and iv) evaluating the result, if any, of the bioimaging technology of the phenotype exerted by the genetic determinant when expressed in the pig model.

The genetically modified pig of the present invention may also be used in order to
15 improve catheter-based therapies.

Examples

Lipoprotein metabolism in pigs

Lipoprotein metabolism in pigs is reasonably well described. The distribution and
20 composition of plasma lipoproteins in swine are similar to those in humans, and LDL is the major cholesterol transporting lipoprotein in both species.¹² Like humans, all VLDL particles secreted by the liver contain the long non-edited form of apoB100 rather than the truncated apoB48 form, which is dominant in mice¹³.

Apolipoprotein B-containing lipoproteins are cleared by hepatic uptake via 1) the LDL
25 receptor that binds to apolipoprotein E in IDL particles and to apolipoprotein B100 in LDL particles¹⁴, and 2) the LDL receptor-related protein (LRP) that binds to apolipoprotein E in apoB48-containing chylomicron remnants¹⁵. The relative importance of these pathways varies between species. In mice deficiency of apolipoprotein E has a more severe atherosclerotic phenotype than LDL receptor
30 deficiency, whereas the opposite is true in humans. The impact of apoE and LDL receptor deficiency on lipid metabolism in pigs is not known.

EXAMPLE 1

Production of ApoE knockout pigs

ApoE knockout pig by homologous recombination

The pig apolipoprotein E gene (GenBank accession no. U70240) has been sequenced.

^{11,16} A targeting vector construct containing the gene and downstream non-coding sequence is created with a promoter-less neomycin resistance gene cassette inserted into one of the exons to disrupt gene function. The targeting vector is linearized and transfected into Yucatan fetal fibroblasts, isolated from new born Yucatan ear biopsies. Plasmids encoding zinc finger nucleases constructed to recognize and cleave a site in the targeted genomic sequence may be used to increase efficiency of homologous recombination. Fibroblasts are cultured in the presence of G418. Resistant clones are screened for homologous recombination by PCR. Yucatan minipigs with knockout of one or both apoE alleles are created from recombinant cells by "hand-made" cloning.

1. Cloning of targeting constructs

A 1.6 kb fragment containing the 5'-end of the ApoE gene, a 3.2 kb fragment containing the entire ApoE gene, and a 6.1 kb fragment containing the 3'-end of the ApoE gene were all amplified by PCR using genomic DNA extracted from fibroblasts isolated from newborn Yucatan minipigs as template (see below). Upon subcloning and sequencing, parts of the resulting PCR products were used for further cloning into the targeting vector pKO Scrambler NTKV-1903 (Stratagene) comprising a neomycin resistance gene.

1.6 kb ApoE fragment (5'-end of ApoE) - primers used for amplification is underlined:

5'-

gcctgggaatgagtgccagctcctccagttccacgtggcctcaccacacacctcaactctgagctctgggagtcgtgtaac
 agggctgctgggggatgggggggtgcagtcagcgctcaccaatctgtcacagaagtaactggaactgttcttgttctatc
 cccgatgatgggttaaatgcaaccattttcccgctcttagtgaccgagaaacaatgttcagagaggctaggtcattg
 ctcaaggtcacacagctgacaacccgcagagcctggattcaggcctggaggcttgggtccagagttcacagtccgaac
 caggcgacgggacaggaacactcccaggcctgtggaaggcgcggtatgcaggccgcgagctcctggaatgcgcaa
 ggcttatgtggggcagagagctgcatcctcattgcacaaatcaggaaagcggtcagagaagcactcagatgtgccc
 aaggctacggccctcgagaggagtgagggttaaaactctgtggtgcaacggaaacgaatccaactgggaacatga
 ggctgtgggttgatccccggcctcgctcaatgggttaaggatccagcacggcgctgccgtgagctgtggtgtaggtcgc
 agacgaggcttgatcccacttggtgtggctgtggctgtggctgtggctgtaggccgcagctgtaactgtaattcgacccc
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 agcctaaactttcagcaaaaagaacactctcaaagcctaaactttgagcagatgccttacaccgccccacgccttcat
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ccagcccacccccaccagtggtatggagaaaggctcgggagtactgggtgagagacacctctttccatgggggctgg
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 gagggcagcccacctcgtgacccgacgggggtggcccagctggccccagttctggaggagtggcggggcgggg
 ggagccctataattggccgaatctgggctccctgaatcctactcagccccggaggaggaaggaggaaggaggagga
 5 ggaagcaaccggtgaggagcagacctgggggcacagagatgggctcggggctcgggtgtgggaggggtgggctgtag
 ggggaggaggaaatgacctggccccccggggccaccaccgaggcaggagtggggatgaggctagagcccaggg
 actggacctagaaggaggggtgggcagcaggaggaggttatccgccttggtggaaggggaggtcaggggaagcagc
 gggacctgtaggaagaaccagacgagccagagccgacgaattgtactggc-3'

10 **3.2 kb ApoE fragment (entire ApoE gene) - primers used for amplification is underlined:**

5'-

gcccagctggccccagttctggaggagtggcggggcggggggagccctataattggccgaatctgggctccctgaat
 cctactcagccccggaggaggaaggaggaaggaggaggaggaagcaaccggtgaggagcagacctgggggcac
 agagatgggctcggggctcgggtgtgggggggtgggctgtagggggaggaggaaatgacctggccccccggggcca
 15 ccaccgaggcaggagtggggatgaggctagagcccagggactggacctagaaggaggggtgggcagcaggagga
 ggttatccgccttggtggaaggggaggtcaggaagcagcgggacctgtaggaagaaccagacgagccagagcc
 gacgaattgtactggcaggtatggcgcatctactcaagtttgagcacactaagagctccatcgaggagaccaggggt
 ggcggcgaccaggggtgacctcgaccgggctggcggcagggtagctagagcgttggtggaaggacatgtaaagag
 gattaaattaggaatgagtggaaaacaggggttagatgtgaagttggagcttggaaatgtgaaggtaccaggaagaacg
 20 tgagcttgagcccagaaagcaaggctggggctcacatgggactccagggtggaggggggtggggggcgacgtgggt
 ggaatttgaaccctgggagagaggggaaggcttttggccgcagccgacctggggatggggagataggagaagacaat
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 25 tgggggtggggctgtcaggttccctgcccctccccatccccggctgtaccgggtgcccctcctcatccctgggtctcttc
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 aagcccacccaccagggctgggggagcacctgctctgtaccaggtaccgtgtgctgggagggggcagaggacctgat
 ggctgtgaactggctcgggtgcaggatgccggacagaggacgagccggggccgcccggagggtgcacgtgtgggtgg

[illegible]

20 **6.1 kb ApoE fragment (3'-end of ApoE) - primers used for amplification is underlined:**

5'-
attcatcaagcttcacagcagcttctgggtgtccccgtgtgatttctcagctccagcctcagttccctttccttcctgcactga
ccaccagttctctgtctctgccctctgccctgtgtgtctctattgtctcttctcccccttttcttttttggccgagcccatggcatgc
ggaagttccccggccagggattgaacccatgccacagccgccacaacgaaggatccttaactactaggccaccaggg
25 aactccatcctttctaactctgtctttgctttcccttttttagcgtttagggctgcaccctcagcatgtggaagtccccagggtag
gggtcaaattggcgctacagctgccagcctacaccacagcccagcaacgcaggatccaagccacatctttgacctac
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30 attgccatgagctccagtataggtaacagaaatgtcttgcacacaccgctgtggctgtgacgtaggctggcagtttagct
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5 cgaccaggaatcgaaccctagccatagaagtcacaatgccagatccttagctactagcccaccaggagggttccatctcc
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15 aaataaataaatgcatacgtactaaatacacatacacatacacataataaaaaataaaaaaattaaaagctggatctc
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25 ccagggacctggaggcttcagaggcaggaacccagcgtcggggaacaggggttctggtggacctcagtgaggggc
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gaaggaagattgggggctgtgaaattaaggctccagagagcgtccaggaggcctgggagctcccagatgcagag
agagggggagaatggaagggttagtcggacagtgatattggagatggcatggtgggcagggtgtgtggaggcagactat
35 gagacccagactcctgaagagctttagctgaagagacctactaagaaggggaggaggaggttgcaccttggtca

gtggtaacgaatccgacgaggaacatgaggtgctgggttcgatccccggcccctgctcagtgggtaaggatccggc
 gttgccatgagctgtggttaggtgcagacgtggctcggatcccgtgtggctgtggctctggcgtaggccggcggctaca
 gctccgattggaccctagcctgggaacctccatagccatgggagcggcccaagaaatggcaaaaagacaaaaa
 aaaaagactcctccaagaacttgggtgctatgcactattaaggccatgaggggtaataacctcagagggcccagagat
 5 gtaaagtcacacagccagcatgctggacaactggatcggggccccccagcctcaggcaatcactccactaccctcctcc
 tgggctgggctgcccagataaggaacattatcttgggctgattcaccaccaggcacacagaaggcatttattacacttctt
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 aacaccggatccttaacccactgagttagggccagggatcgaacgaacctacgtcctcatggatgctactcaggttcattt
 10 ccgctgagccacgatgggaactcctgttgattacactttcaaggataatgaagggggatgtgagagaggtcaaagggtg
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 atgtcaggcataaaccagcacatgactaggcctggcacgggcactcattatttggtgaaacgagtatgtacatgca
 15 aagaaaataaataaacatgacattttcataaaacctctgaggtagattgtttccactgagccacgatgggagctccattt
 aaaaatttttttaggagttcccgctcatggcgagtggttaaccaatccgactaggaacctatgaggtgctgggttcgatccct
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 20 gtaggttcagatgaggctcagatccacattgtctgtggctctggcatgggctggcggctacagctccaattcgaccctca
 gcctgggaacctccatagccgtgggagcggctctagaaatggcaaaaagacccaaaagaaaaagaaaaagaaa
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 cttcttttttagggccgcaccggcggttatggacatatggaggtcccaggctaggggtcgaatcggagctgcacctgggt
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 25 acgtatcatgccattcctccgggattggctgtctgttttacagctaagaaaggtggggctcctggtctagtgtgagag
 gaaagcacgtcacagcctcttgagccccacctggctcgtctagtaccctctctacattttaacacatgacccccaagac
 tcacattcaaggatctcctttaccatccctggagctcaccccaagagctcccaatactgaatgtttgcacccctgcccccttt
 ctgggttaggctcagccccagcctaggtgacccag-3'

30 2. Transfection and screening of targeted porcine cells

Porcine fibroblasts from newborn or fetal Yucatan minipigs are cultured from ear
 biopsies. Cells are grown to 50% confluence in a 75 cm² flask (TPP, Switzerland),
 trypsinized and resuspended in 40 ml medium (DMEM, Lonza, Switzerland). One
 fourth (10 ml) were subsequently seeded in a 10 cm² petri dish. The cells are

transfected with 6 ug of vector DNA in FuGENE 6 transfection reagent according to the protocol of the manufacturer (Roche Applied Science). The cells are grown under geneticin (Gibco Invitrogen) selection (1 mg/ml) for 8 days, neomycin resistant cell colonies were isolated, and each colony was transferred to a 1.9 cm² well (24-well plate, TPP, Switzerland) and grown to 80% confluence. Each colony is transferred to 9.4 cm² well (6-well plate TPP, Switzerland), grown to 80% confluence, and 1/3 of the cells is used for RNA isolation whereas 2/3 of the cells is transferred to a 25 cm² flask, grown to 80% confluence and stored at -135°C in DMEM containing 10% DMSO until further use in handmade cloning.

Screening for locus-specific targeting events is performed by PCR and Southern Blotting using genomic DNA extracted from the neomycin-resistant clones as template. For PCR, a forward primer situated 5'-upstream for the ApoE homology region and a reverse primer located within the neomycin resistance gene, or a forward primer situated within the neomycin resistance gene and a reverse primer located 3'-downstream for the ApoE homology arm, is used.

Southern blot is performed to verify the locus-specific targeting events. Genomic DNA was digested and the blots probed with a DNA-probe situated outside the region of ApoE homology.

The resulting transgenic ApoE knockout porcine fibroblasts are subsequently used for somatic cell nuclear transfer (SCNT) by handmade cloning.

EXAMPLE 2

ApoE knockout pig created by AAV-mediated homologous recombination

1. Cloning of targeting constructs

Part of ApoE intron 2 (left homology arm) and intron 3 (right homology arm) was amplified by PCR and used together with a gel extracted fragment of the vector pNeDaKO-Neo (containing a neomycin resistance gene) in a 3-fusion PCR as described by Kohli et al. (Nucleic Acids Research 2004, vol. 32, no. 1).

The resulting PCR product comprising the ApoE gene sequences flanking exon 3 and a neomycin resistance gene was cloned into the adeno-associated virus (AAV) vector pAAV-MCS (Stratagene). Packaging of this targeting construct (see below) and

subsequent production of viral lysate for infection of porcine cells were performed as described by Kohli et al. (2004).

5 AAV-MCS/ApoE KO targeting construct - sequence (4055 bp)

(shaded region = Neo-sequence from pNeDaKO-Neo, unshaded regions = ApoE gene sequences, primer sequences are underlined)

10 atacatacgcggccgcggatctgctcgggaaatatctgcttggattaggctgatgcagataaggggggtgcaaggcttg
gaaggctgtgactggacagggtgggctctgggtgggaggagcgagccccgcgctgttgagtacaatttctccctct
gcaggttggccaatcgcaagccagaagatgaggggtctgtgggttgggtgtaaccctcctcgaggtatgggggtg
gggctgtcaggttccctgccccccccatccccggctgtaccgggtccccctctcatccctgggtctcttctgctggtct
ctctccccctgaggagaggcctagatgtgaggcctctctggcactccttgccttgaacagctcgttttactctctgagcctca
15 gttcccccatctttaaagtgggagttatgttgagagattccagctgtggctcagcagggttaagaacccgactagatccatga
ggaagaggggtcaatccctggctcgctcagcgggttaaggatccggcgttgccatgagctgcggcataagtcgcagat
gcagctcgaatcgggtgtgtgtgtggctgtgggtcagggctggcagctatcgcttccatcggaacccctgcctgggaacttcc
acgtatgccactggtgcagccctaaaagacaaacaaacaaaaacgaaagaaagagaaaagaaaggaaaggggg
cttctgtttctaagcgtgtgtgctggcagggcgtgagcattagatacgtgtcagctgtgactagcgtgcacggagcacac
20 aatccatgctgtccagtaattagacaggctgggtgtccttccacccccctccctgcccaccagtgtcttagagaagcccac
ccaccaggggtgggggagcacctgtctgtaccagggtaccgtgtgctgctaaagggaaacaaaagctggagctccac
cgcgataacttctatagcatacattatacgaagtatcgcgccctaccgggtaggggagggcgcttttcccaaggcagtc
tgagcatgcgcttagcagccccgtgggcacttggcgctacacaagtggcctctggcctgcacacattccacatcca
ccggtaggcgccaaccggctccgttcttgggtggcccccttcgcccaccttctactcctccctagttaggaagttcccccc
25 cggccgcagctcgcgctgtgcaggacgtgacaaatggaagtagcacgtctcactagtctcgtgcagatggacagcac
cgctgagcaatggaagcgggtaggccttggggcagcggccaatagcagcttggctccttcgcttctgggtcagagg
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gccaatatgggatcggcattgaacaagatggattgcacgcagggtctccggccgcttgggtggagaggctattcggcta
30 tgactgggcacaacagacaatcggctgtctgatgccgccgtgtccggctgtcagcgcaggggcgcccgggtcttttgtc
aagaccgacctgtccggtgccctgaatgaactgcaggacgaggcagcgcggtatcggtgggtggccacgacggggcgt
tccttgccagctgtgctcgacgtgtcactgaagcgggaagggaactggctgtattgggcgaagtgcgggggcaggat
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ctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactcggtggaagccgggtctgtcaatca
35 ggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgcgcatgcccgc
ggcgaggatctcgtcgtgacctatggcgatgcctgcttgcgaatatcatggtggaatggccgcttttctggattcatcg

actgtggccggctgggtgtggcggatcgctatcaggacatagcgttggctacccgtgatattgctgaagagcttggcggc
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 gttcttctgaggggatcaattctctagagctcgctgatcagcctcgactgtgccttctagttgccagccatctgtgttggccct
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 tggcatcgccagctcaggagccctccttctccccccccccgccccgcccctcttgcgcccaggagctgatagagga
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 cgccaccctgagtaccaggggccggccagccgtgcgcgagcgcgcgaagcctggggccagaagctgcgcggac
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 aagtggaggagcagggcagccagttgcgcctgcaggccgagggcggccgcgtatgtat

25

2. Transduction and screening of targeted porcine cells

Transduction of porcine fibroblasts from newborn or fetal Yucatan minipigs was
 30 performed in 75 cm² flasks as described by Kohli et al. (2004). Briefly, cells were
 infected with the virus for 2-3 hours and subsequently allowed to grow for 48 hours.
 The cells were harvested by trypsinization 48 hours post transduction and seeded into
 96-well plates with media containing geneticin to select for targeted cells and allowed
 to grow for 2-3 weeks.

35

Initial screening for locus-specific targeting events was performed by PCR using genomic DNA extracted from the neomycin-resistant clones as template. For this PCR, a forward primer situated outside the left ApoE homology arm and a reverse primer located within the neomycin resistance gene, or a forward primer situated within the neomycin resistance gene and a reverse primer located outside the right ApoE homology arm, was used.

Southern blot was performed to verify the locus-specific targeting events. Genomic DNA was digested and the blots probed with a DNA-probe upstream for the left ApoE homology arm or downstream for the right ApoE homology arm.

The resulting transgenic ApoE knockout porcine fibroblasts were subsequently used for somatic cell nuclear transfer (SCNT) by handmade cloning.

EXAMPLE 3

Proprotein convertase subtilisin/kexin type 9 (PCSK9)

In most cases of atherosclerosis the genetic component is complex, but in some cases the inheritance of the disease is monogenic. These cases are mostly caused by mutations in genes coding for proteins involved in lipoprotein trafficking, and the most severe in humans are caused by mutations affecting LDL receptor-mediated lipoprotein uptake (recessive and autosomal dominant familial hypercholesterolemia). Recently, a gain-of-function mutation in the PCSK9 gene was described as the cause of autosomal dominant familial hypercholesterolemia (17) PCSK9 binds to the LDL receptor leading to its degradation (18). Therefore, gain-of-function mutations in humans and overexpression of PCSK9 transgenes in mice leads to functional LDL receptor deficiency (19).

1. Cloning of constructs

Donor cells transgenic for human proprotein convertase subtilisin/kexin type 9 (PCSK9) were produced using the DNA transposon-based vector pSBT-HCR-hAAT-PCSK9-bpA. Briefly, human PCSK9 was amplified by PCR from a PCSK9 cDNA clone (OriGene Technologies, Rockville, USA) using Pfx polymerase (Invitrogen) and the following primers.

Fw1 5'- AAAGGCGCGCCACCATGGGCACCGTCAGCTCCAGG -3'

Rv1 5'-

AAAGGCCGGCCTCACTCACTTGTCATCGTCGTCCTTGTAAGTCCTGGAGCTCCTGG
GAGGCCTG -3'

5 The forward primer includes an *Ascl* restriction site, consensus Kozak sequence and the beginning of the coding sequence of the human PCSK9 gene. The reverse primer includes a C-terminal FLAG tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys), a stop codon and an *FseI* restriction site.

10 The human hepatocyte control region (HCR) of the apolipoprotein E gene followed by the human α_1 -antitrypsin (hAAT) promoter was amplified by PCR from the plasmid pBS-apoEHCR-hAATp-hFIXmg-bpA (Miao CH et al. Mol Ther. 2000;1(6):522-532) (Pfx polymerase, 25 cycles, Invitrogen) using primers Fw2 and Rv2.

Fw2: 5' - AAATTAATTAAGTAGT CTGCA GGCTC AGAGG - 3'

15 Rv2: 5'- GGCCGGCC GTTTAAAC GGCGCGCC GCAGATTGTGAAAGTGGTTCG - 3'

The bovine growth hormone polyadenylation signal (bpA) was amplified by PCR from pBS-apoEHCR-hAATp-hFIXmg-bpA using primers Fw3 and Rv3 (25 cycles, Pfx polymerase).

20

Fw3 5'- GGCGCGCC GTTTAAAC GGCCGGCC TCAGC CTCGA CTGTG CCTTC -3'

Rv3 5'- GCGC TTAATTAA AGCCCCAGCTGGTTCCATAG -3'

25 The Rv2 and Fw3 primers contain a complementary region with *Ascl* and *FseI* restriction enzyme sites, and the HCR-hAAT and bpA segments were joined by fusion PCR using the primers Fw2 and Rv3. The fused PCR product was cloned into the unique *PacI* site of the Sleeping Beauty transposon-based vector pSBT-PGK-puro.(Yant and Kay. Mol Cell Biol. 2003;23(23):8505-18). The human PCSK9 cDNA was thereafter cloned into the unique *Ascl* and *FseI* restriction sites.

30

Vector construct for liver-specific overexpression of a native human PCSK9 transgene

35 A Sleeping Beauty transposon-based vector was created containing the human hepatocyte control region (HCR) of the apolipoprotein E gene,²³ human α_1 -antitrypsin (hAAT) promoter, human PCSK9 cDNA with a consensus Kozak sequence and an C-terminal FLAG tag,¹⁸ and a bovine growth hormone polyadenylation signal (bpA)

(pSBT-PCSK9). The construct includes a puromycin resistance gene under the control of the PGK promoter.

2. Transfection

5 Porcine fibroblasts from newborn or fetal Yucatan minipigs were cultured in DMEM (Lonza, Switzerland) containing 15% fetal calf serum (Lonza). Cells were seeded in a 10 cm Petri dish at 1%, 0.25% or 0.1% confluency on day 0. On day 1, pSBT-HCRapoE-hAAT-PCSK9-BpA was co-transfected with a plasmid expressing the hyperactive Sleeping Beauty transposase mutant HSB3 (pCMV-HSB3, Yant SR et al. 10 Mol Cell Biol. 2004;24(20):9239-47) or the inactive transposase mutant mSB (pCMV-mSB, Yant et al. Nat Genet. 2000;25(1):35-41). From day 3, the cells were selected in 0.6 microgram/ml puromycin (Sigma) for 14 days. Number of resistant clones were counted (Figure 1) and HSB3-tagged colonies were then cultured in 15%FCS in DMEM until hand-made cloning.

15

To confirm the integrity of the pSBT-HCRapoE-hAAT-PCSK9-BpA construct it was transiently transfected into the hepatocarcinoma cell line, HepG2. After 72 hours, the cells were stained for the PCSK9-FLAG protein using a biotinylated anti-FLAG antibody 20 (1:1000, Sigma) and an Alexa 594-conjugated streptavidin (1:400) (Figure 2).

3. Transgene expression and copy number

Blood samples are obtained from newborn cloned pigs, and the presence and level of the transgenic PCSK9-FLAG protein in plasma is measured by ELISA using an anti- 25 FLAG antibody (Sigma). Genomic DNAs of blood samples of the transgenic piglets and the surrogate mother are extracted according to the Chemagen DNA-extractor protocol. Southern blotting using a puromycin resistance gene (pac) probe is used to determine the number of integration sites.

30 **EXAMPLE 4**

LDL receptor knockout pig by homologous recombination

Part of the pig LDL receptor coding sequence has been sequenced (GenBank accession no. AF065990). A targeting vector containing part of the gene sequence is created with a promoter-less neomycin resistance resistance gene cassette inserted 35 into one of the exons to disrupt gene function. The targeting vector is linearized and

transfected into Yucatan fetal fibroblasts. Fibroblasts are cultured in the presence of G418. Resistant clones are screened for homologous recombination by PCR. Yucatan minipigs with knockout of one or both apoE alleles are created from recombinant cells by “hand-made” cloning.

5

EXAMPLE 5

LDL receptor knockdown pig by RNA interference

Part of the pig LDL receptor coding sequence has been sequenced (GenBank accession no. AF065990). Efficient targets for RNA interference will be determined empirically by transient expression of vectors containing 1) a short hairpin RNA sequence expressed under a U6 or H1 promoter targeting a 19 nucleotide sequence in LDL receptor mRNA.

For stable short hairpin RNA expression, a transposon-based or retroviral vector is constructed with 1) a short hairpin RNA sequence expressed under an U6 or H1 promoter targeting a 19 nucleotide sequence in LDL receptor mRNA, 2) a puromycin resistance gene cassette, and 3) two flanking insulator sequences. The transposon-based targeting vector is cotransfected with transposase-expressing plasmid into Yucatan fetal fibroblast cultures. The retroviral vector is transduced into Yucatan fetal fibroblast cultures. Fibroblasts are cultured in the presence of puromycin. Resistant clones are screened for efficient down-regulation of LDL receptor mRNA and protein. LDL receptor knockdown Yucatan minipigs are created from transgenic cells by “hand-made” cloning.

Identification of effective shRNAs

In this example, a human-sized model of atherosclerosis is created by development of genetically engineered Yucatan minipigs in which RNA effector molecules directed against the endogenous LDL-receptor induce reduce lipoprotein clearance and hypercholesterolemia.

Ten shRNA-expressing plasmids targeting different 19-nucleotide sequences in Yucatan minipig LDL receptor mRNA and one control pSUPER.retro.puro expressing an irrelevant shRNA was created in pSUPER.retro.puro (Oligoengine) using manufacturer's recommendations.

Table 2.

Targets for shRNA directed LDL receptor knockdown

	First base in pig cDNA AF065990 sequence	Sequence
T1	763	tgtaaagcgcgagtgca
T2	889	tcccatatctgcaatgacc
T3	1150	accctggaccgtagtgagt
T4	1308	tgacaccattattggcgaa
T5	1309	gacaccattattggcgaa
T6	1439	agactcttccaagagaa
T7	1553	tgaacggagtggacgtcta
T8	1814	tcacaggctcggacataca
T9	858	CCAACGAGTGTCTGGACAA
T10	1109	CCTACCTCTTCTTCACCAA

To measure the capacity of these shRNAs to target and facilitate degradation of LDL-receptor RNA we set up a Renilla luciferase screening-assay based upon targeting of a Renilla luciferase-LDL-receptor fusion mRNAs in HEK293 cells. Part of the coding sequence of the porcine LDL receptor was amplified by PCR from DNA obtained from Yucatan minipig fibroblasts using the following primers.

Fw 5' AAAACTAGTGCCAAGACGGGAAATGCATC 3'

Rv 5' AAAACGGGTGCTGTTGATGCTCTTAAGCC 3'

The forward primer contained a SpeI site and the reverse an AgeI site and the LDL receptor segment was cloned into unique SpeI and AgeI sites in the 5'UTR region of the Renilla luciferase gene in a modified version of the pSiCheck-2 vector (Promega) to make the pSiCheck2-LDLR vector.

HEK293 cells were seeded at 19000 cells per well in 24-well plates (TPP) in DMEM containing 10% fetal calf serum (Lonza) on day 0. On day 1, cells were co-transfected with 0.04 µg pSiCheck2-LDLR and 0.36 µg pSUPER.retro.puro.T1-T10 or pSUPER.retro.puro.shScrambled using FuGene 6 transfection reagent (Roche Applied Science). On day 3, luciferase activity was measured using the Dual-Luciferase® Reporter Assay System from Promega. The assay was performed in triplicates. The experiment was repeated twice. Results are given in Figure 4.

To facilitate stable expression of effective shRNAs in Yucatan minipig fibroblasts, shRNA sequences are cloned into one or both linkers of the plasmid pSBT/cHS4.H1p.PGK-puro.U6p.cHS4, in which shRNAs are expressed under an U6 or H1 polymerase III promoter, see Figure 3.

Transfection

Porcine fibroblasts from newborn or fetal Yucatan minipigs are cultured in DMEM (Lonza, Switzerland) containing 15% fetal calf serum (Lonza). Cells are seeded in a 10 cm Petri dish at 1%, 0.25% or 0.1% confluency on day 0. On day 1, pSBT-shLDLR plasmid is co-transfected with a plasmid expressing the hyperactive Sleeping Beauty transposase mutant HSB3 (pCMV-HSB3, Yant SR et al. Mol Cell Biol. 2004;24(20):9239-47). From day 3, the cells are selected in 0.6 microgram/ml puromycin (Sigma) for 14 days. Number of resistant clones are counted (Figure 4) and HSB3-tagged colonies are then cultured in 15%FCS in DMEM until hand-made cloning.

Transgene expression and copy number

Fibroblasts are cultured from newborn cloned pigs in DMEM supplemented with 10% fetal calf serum. For analysis of the level of LDL receptor knockdown, fibroblasts are cultured in DMEM supplemented with 10% lipoprotein-deficient serum for 48 hours and the level of LDL receptor mRNA is determined by quantitative PCR. Genomic DNA is isolated from fibroblasts by standard procedures. Southern blotting using a puromycin resistance gene (pac) probe is used to determine the number of integration sites.

25 **EXAMPLE 6**

Handmade cloning (HMC) and establishment of pregnancies for examples 1, 2, 3, 4 and 5.

For the cloning and delivery of transgenic fibroblasts are used in HMC. Recipient sows receive a total of in the range of 60-70 of a mixture of day 5 and/or 6 blastocysts.

30 Except where otherwise indicated all chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Oocyte collection and in vitro maturation (IVM)

Cumulus-oocyte complexes (COCs) are aspirated from 2 to 6 mm follicles from slaughterhouse-derived sow ovaries and matured in groups of 50 in 400 µl IVM medium consisting of bicarbonate-buffered TCM-199 (GIBCO BRL) supplemented with 10% (v/v) cattle serum (CS), 10% (v/v) pig follicular fluid, 10 IU/ml eCG, 5 IU/ml hCG (Suigonan Vet; Skovlunde, Denmark) at 38.5 °C in 5% CO₂ in humidified air in the Submarine Incubation System (SIS; Vajta et al., 1997) for 41–44 h.

HMC is performed by a procedure based on partial digestion of the zona pellucida, as described earlier (Du et al., 2005 and 2007). Matured COCs are freed from cumulum cells in 1 mg/ml hyaluronidase in Hepes-buffered TCM-199. From this point (except where otherwise indicated) all manipulations are performed on a heated stage adjusted to 39 °C, and all drops used for handling oocytes are of 20 µl covered with mineral oil. Zonae pellucidae of are partially digested with 3.3 mg/ml pronase solution dissolved in T33 (T for Hepes-buffered TCM 199 medium; the number means percentage (v:v) of CS supplement, here 33%) for 20 s, then oocytes are washed quickly in T2 and T20 drops. Oocytes with distended and softened zonae pellucidae are lined up in T20 drops supplemented with 2.5 µg/ml cytochalasin B. With a finely drawn glass pipette, oocytes are rotated to locate the polar body on the surface. By oriented bisection with an Ultra Sharp Splitting Blade (AB Technology, Pullman, WA, USA) less than half of the cytoplasm close to the polar body is removed manually from the remaining putative cytoplasm.

Transgenic donor fibroblasts grown to a confluent monolayer in DMEM supplemented with 10% FCS were trypsinized and kept in T20 (Kragh et al., 2004). Fusion is performed in two steps. For the first step, 50% of the available cytoplasts are transferred into 1 mg/ml of phytohemagglutinin (PHA; ICN Pharmaceuticals, Australia) dissolved in T0 for 3 s, then each one was quickly dropped over a single transgenic fibroblast. After attachment, cytoplast-fibroblast cell pairs are equilibrated in fusion medium (0.3 M mannitol and 0.01% PVA) for 10 s and transferred to the fusion chamber (BTX microslide 0.5 mm fusion chamber, model 450; BTX, SanDiego, CA, USA). Using an alternating current (AC) of 0.6kV/cm and 700 kHz, pairs are aligned to the wire of a fusion chamber with the somatic cells farthest from the wire, then fused with a direct current of 2.0 kV/cm for 9 µs. After the electrical pulse, cell pairs are incubated in T10 drops to observe whether fusion has occurred.

Approximately 1 h after the first fusion, each pair is fused with another cytoplasm and activated simultaneously in activation medium (0.3 M mannitol, 0.1 mM MgSO_4 , 0.1 mM CaCl_2 and 0.01% PVA). By using an AC of 0.6 kV/cm and 700 kHz, one fused pair and one cytoplasm was aligned to one wire of the fusion chamber, with fused pairs contacting the wire, followed by a single DC pulse of 0.85 kV/cm for 80 μs . When fusion has been observed in T10 drops, reconstructed embryos are transferred into porcine zygote medium 3 (PZM-3; Yoshioka et al., 2002) supplemented with 5 $\mu\text{g}/\text{ml}$ cytochalasin B and 10 $\mu\text{g}/\text{ml}$ cycloheximide. After a 4 h incubation at 38.5 °C in 5% CO_2 , 5% O_2 and 90% N_2 with maximum humidity, embryos are washed three times in PZM-3 medium before culture

Embryo culture and transfer

Embryos are cultured at 38.5 °C in 5% CO_2 , 5% O_2 and 90% N_2 with maximum humidity in PZM-3 medium in the well of well system (WOWs; Vajta et al., 2000). Day 5 and 6 blastocysts with clearly visible inner cell mass are surgically transferred to Danish landrace sows on day 4 or 5 after weaning. Pregnancies are diagnosed by ultrasonography on day 21 and confirmed every second week. Piglets are delivered by Caesarean section on day 114, 24 h after treatment with prostaglandin F2.

EXAMPLE 7

Examination of phenotype of transgenic atherosclerotic pigs

On conventional diets (~3% fat, w/w), Yucatan minipigs have low plasma cholesterol levels (~2 mmol/l) and do not develop atherosclerosis. Transgenic minipig models of atherosclerosis has an expected total cholesterol level of >5 mM total cholesterol on normal pig diet, which can be further accentuated by feeding a diet containing 20% saturated fat and even more by feeding a diet containing 20% saturated fat and 1% cholesterol.

To characterize the level of *hypercholesterolemia* that can be obtained in transgenic Yucatan minipigs, transgenic and normal Yucatan minipigs are maintained on normal pig diet, pig diet supplemented with 20% saturated fat or pig diet supplemented with 20% saturated fat and 1% cholesterol. Blood samples are taken at time of weaning and every 3 months thereafter.

Plasma total cholesterol and triglycerides are measured on a Vitros 950 analyzer (Ortho-Clinical Diagnostics). HDL cholesterol (HDL-C) is measured enzymatically on a

Kone 30 analyzer (Thermo) using kits from ABX (Triolab, Copenhagen, Denmark). The distribution of cholesterol within lipoprotein fractions is analyzed by fast protein liquid chromatography.

5 To characterize the level of *atherosclerosis* induced by the hypercholesterolemia, pigs are examined by intravascular ultrasound (IVUS) at 6 months, 9 months and 12 months of age. After the last examination, the minipigs are killed and the heart and all major arteries are removed and opened longitudinally to identify atherosclerotic lesions. Atherosclerotic lesions are fixed in 4 % formaldehyde for 24 hours and embedded in paraffin. Specimens are serially sectioned, stained by elastin-trichrome and analyzed
10 microscopically. Atherosclerotic lesions are characterized according to the classification described by Virmani et al. Transgenic pigs are expected to exhibit pathological intimal thickening and fibrous cap atheromas.

EXAMPLE 8

15 The use of a porcine model of atherosclerosis in testing preventive and therapeutic strategies

Molecular imaging of atherosclerosis:

Diagnostic imaging of atherosclerosis, which is becoming theoretically possible with the advent of new high-resolution imaging technology, is a promising new tool for risk
20 stratification of asymptomatic persons. However, to develop tracers/contrast agents and imaging sequences that are able to visualize atherosclerotic plaques and atherosclerotic disease activity, we need a human-sized animal model of the disease that can be examined in patient CT, MR, and PET-scanners.

Example: Substance X is administered intravenously and accumulation in
25 atherosclerotic plaques is analyzed by MR scanning, CT scanning, SPECT scanning, PET scanning or intravascular ultrasound. After the scanning, the minipigs are killed. Arterial specimens containing atherosclerotic lesions are fixed, paraffin-embedded, sectioned, stained and microscopically analyzed. Results obtained by imaging are compared to the histological findings.

30 Effect of stenting on atherosclerotic plaques: Plaques in the coronary arteries of a one-year old transgenic pig is mapped by intravascular ultrasound (IVUS) and a drug X-eluting stent and control stent is placed at the locations of two fibrous cap atheromas.

After 3 months, pigs are killed and the pathology of the stented atherosclerotic lesions is compared by histological techniques.

Drug study:

- 5 Atorvastatin (3 mg/kg per day) or placebo is given to transgenic minipigs (6 months of age) for 3 months. Blood samples are obtained at the initiation of the study and every month hereafter. IVUS examinations of the coronary arteries are carried out at the initiation of the study and after 3 months. After the last IVUS examination, the minipigs are killed and atherosclerotic lesions are processed for histological analysis. The effect
10 of atorvastatin on atherosclerosis is determined by comparing the serial IVUS data and the histology of atherosclerosis at study end between atorvastatin and placebo-treated minipig groups.

EXAMPLE 9

- 15 Alternatively, the shRNA and promoter cassette will be inserted into fetal fibroblasts using the "*Master pig system*"

Based on the well-described mechanisms of SB transposition (4-8) and Flp recombination (9, 10), the present invention discloses a new target vector for site-
20 specific integration into the genome. This vector carries within the context of a SB transposon vector a bicistronic gene cassette containing (i) the FRT recombination site embedded in the coding sequence of eGFP and (ii) an IRES-driven puromycin resistance gene. We demonstrate efficient selective plasmid insertion into SB-tagged genomic loci. In an attempt to further improve the performance of these vectors, we
25 have analyzed the effect of insulator elements, believed to protect inserted foreign genes against transcriptional silencing, within the context of SB vectors. Our investigations indicate that insulators flanking the FRT gene expression cassette may serve to maintain and stabilize gene expression of Flp-inserted transgenes.

- 30 Two nonviral integration technologies are employed in the present invention, the SB transposon system and the Flp recombinase, in a combined effort to achieve active locus detection, mediated by SB, and site-directed insertion at an attractive site, mediated by Flp. A bi-phased technology is disclosed in which an integrating SB vector, carrying a reporter gene and a selective marker gene, may first serve as a
35 reporter for continuous gene expression and hence as a target for gene insertion (Fig.

- 5). By using an actively integrated vector as opposed to plasmid DNA that is randomly recombined into the genome we certify (i) that only a single copy, and not concatemers, of the vector are inserted and, moreover, (ii) that the reporter cassette is not flanked by sequences derived from the bacterial plasmid backbone which may have a detrimental effect on the locus activity over time. In a second modification step this vector may serve as a target for insertion of one or more gene expression cassettes in a well-characterized locus.

Vector construction

- The SB transposon-based vector used in this study was derived from the pSBT/SV40-GFIP.loxP vector. This vector contains, within the context of a SB transposon, a bicistronic FRTeGFP-IRES-puro (GFIP) cassette flanked upstream by an ATG start codon and downstream by a poly A sequence. Moreover, the vector contains a recognition site for the Cre recombinase (loxP) located between the upper inverted repeat of the vector and the SV40 promoter driving expression of the FRTeGFP-IRES-puro cassette.

Construction of pSBT/SV40-GFIP.loxP vector

- The pSBT/RSV-GFIP vector contains the terminal inverted of the SB DNA transposon flanking a FRT-GFP.IRES.puro bicistronic gene cassette driven by a promoter derived from Rous sarcoma virus (RSV). The eGFP sequence was amplified from peGFP.N1 (Clontech) using a forward primer containing the 48-bp FRT sequence. To analyze FRT-GFP functionality, the FRT-eGFP fusion was inserted into an expression vector containing the SV40 promoter. The PCR-fragment containing FRT-tagged eGFP fusion gene was digested with MluI and XmaI and inserted into MluI/XmaI-digested pSBT/RSV-hAAT (pT/hAAT in ref. (8), obtained from Mark Kay, Stanford University, USA), generating a transposon vector with RSV-driven eGFP expression (pSBT/RSV-eGFP). An IRES-puro cassette was PCR-amplified from pecoenv-IRES-puro (provided by Finn Skou Pedersen, University of Aarhus, Denmark), digested with XmaI, and inserted into XmaI-digested pSBT/RSV-eGFP, generating pSBT/RSV-GFIP (see Fig 6). Alternative versions of this vector containing the SV40 promoter (pSBT/SV40-GFIP) and the promoter derived from the human ubiquitin gene (pSBT/Ubi-GFIP), were generated. In addition, by inserting a Cre recombination target site (loxP) into the MluI site located between the left inverted repeat of the transposon and the SV40 promoter of pSBT/SV40-GFIP, the vector pSBT/SV40-GFIP.loxP was created. The donor plasmid pcDNA5/FRT, containing a FRT-hygro fusion gene without a start codon, was

obtained from Invitrogen. The Flp-encoding plasmid, pCMV-Flp was obtained from A. Francis Stewart, University of California San Francisco, USA). This plasmid encodes the enhanced Flp variant designated Flpx9 (11). A SB-vector containing two copies of the 1.2-kb chicken DNase hypersensitive site 4 (cHS4)-derived insulator element (12, 13) was generated by inserting PCR-amplified cHS4 sequences and an intervening linker into NotI/Spel-digested pSBT/PGK-puro (obtained from Mark Kay, Stanford University, USA). The PGK-puro cassette was cloned back into construct by using restriction sites located in the linker, generating pSBT/cHS4.PGK-puro.cHS4

For further use in pigs an alternative Cre recognition site (loxP-257) was inserted into a unique AscI site that was created by mutagenesis at a position located between the poly A sequence and the lower inverted repeat of the vector. This vector was designated pSBT/loxP.SV40-GFIP.loxP257. The presence of two Cre recombination sites allows Cre recombinase-mediated cassette exchange after Flp-based plasmid insertion, thereby facilitating, if needed, removal of plasmid sequences and selection genes.

SB transposition in primary pig fibroblasts

The SB transposon vectors, either SBT/PGK-puro or the target transposon SBT/loxP.RSV-GFIP.loxP257, were inserted into the genome of pig fibroblast by co-transfecting (using Eugene-6 from Roche) 1.5 µg pSBT/loxP.RSV-GFIP.loxP257 (or pSBT/PGK-puro) with 1.5 µg pCMV-SB (or 1.5 µg pCMV-mSB as a negative control). pCMV-SB (rights held by Perry Hackett, University of Minnesota, Minnesota, USA) encodes the Sleeping Beauty transposase reconstructed from fossil DNA transposable elements of salmoid fish. pCMV-SB, pCMV-mSB, and the hyperactive variant pCMV-HSB3 were obtained from Mark Kay, Stanford University, USA. SB-tagged cell clones appeared as a result of selecting transfected cells with puromycin (0.5 µg/ml). Colonies were fixed and stained in methylene blue in methanol and subsequently counted.

Solid SB transposition in primary pig fibroblasts

SB transposes efficiently in most mammal cells but with higher efficacy in human cells than in murine cells. Transposition of SB vectors has never been analyzed in porcine cells, and we therefore initially tested activity in primary pig fibroblasts. We utilized a standard transposon encoding a puromycin resistance gene (SBT/PGK-puro) and found decent levels of transposition, resulting in about 75 drug-resistant colonies in

cultures of fibroblasts co-transfected with pSBT/PGK-puro and pCMV-SB (Fig. 7). Less than 3 colonies appeared after transfection with pSBT/PGK-puro and pCMV-mSB, the latter which encodes an inactive version of the transposase. Interestingly, a mean of almost 140 colonies was obtained using the hyperactive transposase variant HSB3, indicating that HSB3 also in porcine cells mediates higher levels of transposition compared to the original SB transposase.

Efficient insertion of a FRT-tagged SB vector in pig fibroblasts

To generate SB-tagged cell clones containing a Flp recombination target site for site-specific gene insertion, we co-transfected the pSBT/loxP.SV40-loxP257 plasmid with pCMV-mSB, pCMV-SB, and pCMV-HSB3, respectively. HSB3 again showed the highest activity, resulting in about 30 drug-resistant colonies after transfection of 3×10^4 fibroblasts (Fig. 8).

Puromycin-resistant colonies were isolated and expanded. Clone analysis by fluorescence microscopy demonstrated efficient FRT-eGFP expression (Fig. 9), demonstrating vector functionality and easy FRT-eGFP detection in pig fibroblasts. These fluorescent cell clones carrying the Flp FRT recombination sequence are currently being used for creation of cloned transgenic animals by hand-made cloning.

Verification of SBT/loxP.SV40-eGFP-loxP257 as target for Flp recombination

Due to limitations of long-term growth of primary pig fibroblasts in tissue culture we were not able to demonstrate Flp-based gene insertion into FRT-tagged SB vectors in pig fibroblasts. We therefore chose to test functionality of the FRT-containing vector by a standard set of recombination experiments carried out in HEK-293 cells. We generated clones of HEK-293 cells containing the transposed SBT/loxP.SV40-eGFP-loxP257 vector. By co-transfection of such clones with (i) a pcDNA5/FRT-derived substrate plasmid containing a FRT-hygro fusion gene and a red fluorescent protein (RFP) expression cassette and (ii) a plasmid encoding the Flp recombinase (pCMV-Flp_{x9}), we subsequently identified hygromycin B resistant colonies. By fluorescence microscopy we observed that site-specifically engineered clones, as expected, turned-off eGFP expression and turned-on RFP expression (data not shown). This 'green-to-red' phenotypic change indicates that the integrated SB-derived target vector serves as acceptor site for Flp-based recombination.

In conclusion, the Sleeping Beauty DNA transposon-based vector of the present invention serves in its integrated form as a target for recombinase-based gene insertion. The SB vector is efficiently transferred by cut-and-paste transposition into the genome of primary porcine fibroblasts and therefore is not flanked by plasmid-derived bacterial sequences. Use of these genetically engineered primary cells in for example microinjection and hand-made cloning allows subsequent detailed analyses of SB vector-derived eGFP expression in cloned pigs and identification of animals with attractive expression profiles (e.g. ubiquitous, tissue-specific). Primary fibroblasts from such 'master pigs' is further modified by Flp-based recombination, allowing site-directed gene insertion in a SB vector-tagged locus which is not silenced in the tissue of interest. Cloned pigs harboring a site-specifically inserted disease gene of interest or a shRNA expression cassette for downregulation of endogenous genes can be generated by a second round of animal cloning.

EXAMPLE 10

Production of disease model by handmade cloning

Except where otherwise indicated all chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Oocyte Collection and in vitro maturation (IVM)

Cumulus-oocyte complexes (COCs) were aspirated from 2-6 mm follicles from slaughterhouse-derived sow or gilt ovaries. COCs were matured in groups of 50 in 400 μ l bicarbonate-buffered TCM-199 (GIBCO BRL) supplemented with 10% (v/v) cattle serum (CS), 10% (v/v) pig follicular fluid, 10 IU/ml eCG, 5 IU/ml hCG (Suigonan Vet; Skovlunde, Denmark) at 38.5°C in the "Submarine Incubation System" (SIS; Vajta, et al. 1997) in 5% CO₂ in humidified air for 41-44 hours.

In vitro fertilization (IVF)

IVF experiments were performed with *in vitro* matured oocytes in 3 identical replicates. After maturation, COCs were washed twice with mTBM containing 2mM caffeine (mTBM_{fert}) and transferred in groups of 50 to 400 μ l mTBM_{fert}. Freshly ejaculated semen was treated as described previously (Booth, et al., in press). After 2 h capacitation at 38.5°C and in 5% CO₂ in humidified air, sperm was added to the oocytes with the adjusted final concentration of 1×10^5 sperm/ml. Fertilization was performed at 38.5°C and in 5% CO₂ in humidified air in the SIS for 3 h. After the

insemination, the presumptive zygotes were vortexed in mTBM_{fert} to remove cumulus cells before washing in IVC medium and placing in culture dishes (see *Embryo culture and evaluation*).

5 Handmade cloning (HMC)

The applied HMC method was based on our previous work in cattle and pig (Kragh, et al., 2004; Peura and Vajta, 2003; Vajta, et al., 2003), but with significant modifications. Briefly, at 41 h after the start of maturation, the cumulus investment of the COCs was removed by repeated pipetting in 1mg/ml hyaluronidase in Hepes-buffered TCM199.

10 From this point (except where otherwise indicated), all manipulations were performed on a heated stage adjusted to 39°C, and all drops used for handling oocytes were of 20 µl volume covered with mineral oil. Oocytes were briefly incubated in 3.3mg/ml pronase dissolved in T33 (T for Hepes-buffered TCM 199 medium; the number means percentage (v/v) of CS supplement, here 33%) for 5 s. Before the oocytes started to
15 become misshaped in pronase solution, they were picked out and washed quickly in T2 and T20 drops. Oocytes with partially digested but still visible zona were lined up in drops of T2 supplemented with 3 mg/ml polyvinyl alcohol (TPVA) and 2.5 µg/ml cytochalasin B. Trisection instead of bisection was performed manually under stereomicroscopic control with Ultra Sharp Splitting Blades (AB Technology, Pullman,
20 WA, USA; Fig. 10a). Fragments of trisected oocytes were collected and stained with 5 µg/ml Hoechst 33342 fluorochrome in TPVA drops for 5 min, then placed into 1 µl drops of the TPVA medium on the bottom of a 60 mm Falcon Petri dish covered with oil (3-4 fragments per drop). Using an inverted microscope and UV light, positions of fragments without chromatin staining (cytoplasts) were registered and later collected
25 under a stereomicroscope in T10 drops until the start of the fusion.

Fetal fibroblast cells were prepared as described previously (Kragh, et al., *in press*).

Fusion was performed in two steps where the second one included the initiation of activation, as well. For the first step, one third of the selected cytoplasts (preferably the
30 smaller parts) were used. With a finely drawn and fire-polished glass pipette, 10 cytoplasts were transferred as a group to 1 mg/ml of phytohaemagglutinin (PHA; ICN Pharmaceuticals, Australia) for 3 s, then quickly dropped onto one of the few fibroblast cells individually that were sedimented in a T2 drop. After attachment, 10 cytoplast-fibroblast cell pairs were equilibrated in fusion medium (0.3 M mannitol and 0.01%
35 PVA) for 10 s. Using an alternative current (AC) of 0.6KV/cm and 700 KHz, cell pairs

were aligned to the wire of a fusion chamber (BTX microslide 0.5 mm fusion chamber, model 450; BTX, SanDiego, CA, USA) with the donor cells farthest from the wire (Fig. 10b), then fused with a direct current (DC) of 2.0 KV/cm for 9 μ s. After the electrical pulse, cell pairs were removed carefully from the wire, transferred to T10 drops and incubated to observe whether fusion had occurred.

Approximately 1 hour after the first fusion, fused pairs together with the remaining two thirds of cytoplasts were equilibrated in activation medium drops separately (0.3 M mannitol, 0.1 mM MgSO_4 , 0.1 mM CaCl_2 and 0.01% polyvinylalcohol (PVA)). Under a 0.6KV/cm AC, cytoplast - fused pair - cytoplast triplets were aligned sequentially to the wire in groups of 10, with fused pairs located in the middle (Fig. 10c). A single DC pulse of 0.7 KV/cm for 80 μ s was used for the second fusion and initiation of activation. The triplets were then removed from the wire and transferred carefully to T10 drops to check the fusion (Fig. 10d). Reconstructed embryos were incubated in culture medium (see *Embryo culture and evaluation*) supplemented with 5 μ g/ml cytochalasin B and 10 μ g/ml cycloheximide for 4 h at 38.5°C in 5% CO_2 , 5% O_2 and 90% N_2 with maximum humidity, then washed thoroughly for 3 times in IVC medium before culture.

Parthenogenetic activation (PA)

Parthenogenetically activated oocytes were produced either separately or in parallel with HMC. Oocytes were denuded in the same way as above except that a longer incubation in pronase was used to get the zona pellucida completely removed. Zona free (ZF) oocytes were then equilibrated for 10 s in activation medium (0.3 M mannitol, 0.1 mM MgSO_4 , 0.1 mM CaCl_2 and 0.01% PVA) and transferred to the fusion chamber (BTX microslide 0.5 mm fusion chamber, model 450; BTX, SanDiego, CA, USA). A single DC pulse of 0.85 KV/cm for 80 μ s was generated with a BLS CF-150/B cell fusion machine (BLS, Budapest, Hungary) and applied to ZF oocytes. For zona intact (ZI) oocytes, a single DC pulse of 1.25 KV/cm for 80 μ s was used (according to our unpublished preliminary experiments, these parameters resulted in the highest activation and subsequent in vitro development for ZI and ZF oocytes, respectively). The procedure after the electrical pulse was the same as for HMC reconstructed embryos.

Embryo culture and evaluation

All porcine embryos produced by the above treatments were cultured in a modified NCSU37 medium (Kikuchi, et al., 2002) containing 4 mg/ml BSA at 38.5°C in 5% O₂, 5% CO₂ and 90% N₂ with maximum humidity. The culture medium was supplied with 0.17 mm sodium pyruvate and 2.73 mm sodium lactate from Day 0 (the day for fertilization and activation) to Day 2, then sodium lactate and sodium pyruvate was replaced with 5.5mm glucose from Day 2 to Day 7. All ZF embryos were cultured in the WOW system (Vajta, et al., 2000) in the same culture medium and gas mixture as used above, with careful medium change on Day 2 without removing the embryos from the WOWs. The blastocyst rate was registered on Day 7. To determine total cell numbers, blastocysts were fixed and mounted to a glass microscopic slide in glycerol containing 20 µg/ µl Hoechst 33342 fluorochrome. After staining for 24 h, embryos were observed under a Diaphot 200 inverted microscope with epifluorescent attachment and UV-2A filter (Nikon, Tokyo, Japan).

10. 1

Differences in developmental competence between sow (2.5 years, 170Kg in weight) derived oocytes and gilt (5.5~6 months, 75Kg in weight) derived oocytes were investigated through ZF and ZI PA after 44 h in vitro maturation. Four combined groups were investigated in 3 identical replicates: (1) ZF oocytes from sows (2) ZI oocytes from sows (3) ZF oocytes from gilts (4) ZI oocytes from gilts. For ZF activation, a single DC pulse of 0.85 KV/cm for 80 µs was applied, while a single 1.25 KV/cm pulse was used to activate ZI oocytes. Following 7 days culture as described above, the percentage of blastocysts per activated embryo was determined.

The *in vitro* developmental competence of parthenogenetically activated oocytes derived from either sows or gilts was investigated. As shown in Table 3, the blastocyst rates of parthenogenetically activated oocytes from sows were significantly higher than those from gilts, either after ZF or ZI PA.

Table 3

Blastocyst development of Day 7 parthenogenetically activated sow and gilt oocytes

<i>Zona Free</i>		<i>Zona Intact</i>	
<i>No. of activated oocytes</i>	<i>No. of blastocysts (%)</i> *	<i>No. of activated oocytes</i>	<i>No. of blastocysts (%)</i> *

sow	103	43(42±4) ^a	110	61(55±6) ^c
gilt	85	17(20±2) ^b	137	36(26±5) ^d

^{a,b} Different superscripts mean significant differences ($p < 0.05$).

^{c,d} Different superscripts mean significant differences ($p < 0.05$).

* Percentage (Mean $\pm$ S.E.M) of embryos developed to blastocysts.

5 The difference in oocytes developmental competence between sows and gilts has been examined in in vitro production (IVP) and somatic cell nuclear transfer (SCNT) embryos separately, resulting in a similar conclusion as in the earlier publication of other research groups (Sherrer, et al., 2004; Hyun, et al., 2003), i.e. that embryos from sow-derived oocytes are superior to those from gilt-derived oocytes in supporting

10 blastocyst development. Although gilts used in our study were at the borderline of maturity, the difference between Day 7 blastocyst rates after PA was significant, proving the superior developmental competence of sow oocytes.

10.2

15 The feasibility of modified porcine HMC was investigated in 6 identical replicates, with IVF and in parallel ZF PA as controls. The more competent sow oocytes (according to Example 1) were used in Example 2. Seven days after reconstruction and/or activation, the number of blastocysts per reconstructed embryo and total cell numbers of randomly selected blastocysts were determined.

20 More than 90% of oocyte fragments derived from morphologically intact oocytes could be recovered for HMC after the trisection. In average, 37 embryos could be reconstructed out of 100 matured oocytes. The developmental competence of all sources of porcine embryos is shown in Table 4. On Day 7, the development of reconstructed embryos to the blastocyst stage was 17±4% with mean cell number of

25 46±5, while the blastocyst rates for IVF, and ZF PA were 30±6% and 47±4% (n=243, 170, 97) respectively.

Table 4

In vitro development of embryos produced by HMC, IVF and ZF PA

<i>Embryo origins</i>	<i>No. of embryos/oocytes in culture</i>	<i>No. of blastocysts</i>	<i>blastocyst rates (Mean $\pm$ S.E.M).</i>	<i>Mean cell number of blastocysts</i>
HMC	243	41	17±4 ^a	46±5 ^d

IVF	170	52	30±6 ^b	74±6 ^e
ZF PA	97	46	47±4 ^c	53±7 ^d

^{a,b,c} Different superscripts mean significant differences ($p < 0.05$).

^{d,e} Different superscripts mean significant differences ($p < 0.05$).

Although the theoretical maximum efficiency was still not approached, the integration of zona partial digestion and oocyte trisection almost doubled the number of reconstructed embryos compared to our earlier system (Kragh, et al., 2004 Reprod. Fertil. Dev 16, 315-318). This increase in reconstruction efficiency may have special benefits in porcine cloning since oocyte recovery after aspiration is more demanding and time-consuming than in cattle. An even more important point is the high embryo number required for establishment of pregnancies following porcine nuclear transfer. IVC in pigs is also regarded as a demanding and inefficient procedure (Reed, et al., 1992 Theriogeneology 37, 95-109). A disadvantage of ZF systems is that the embryos have to reach at least the compacted morula or early blastocyst stage *in vitro* to avoid disintegration in the oviduct without the protective layer of the zona pellucida. On the other hand, once in the blastocyst stage, zona free embryos can be transferred successfully as proved by calves born after either embryonic or somatic cell nuclear transfer (Peura et al., 1998; Tecirlioglu et al., 2004; Oback et al., 2003; Vajta, et al., 2004) and also by the piglets born after zona-free IVP of oocytes (Wu, et al., 2004). NCSU37 medium has been the most widely and successfully used medium for the culture of pig embryos. However, despite the improved embryo development compared with other media, the viability of IVP porcine embryos is still compromised after IVC. Some reports suggested that glucose is not metabolized readily by early porcine embryos before the eight-cell stage but used in higher amounts in embryos between the compacted morula and blastocysts stages (Flood, et al., 1988). The replacement of glucose with pyruvate and lactate in NCSU37 for the first 2 days culture resulted in a blastocyst rate of 25.3% for IVP porcine embryos in Kikuchi's study (Kukuchi, et al., 2002), which was further corroborated by our present studies with an IVP blastocysts rate of 30% in average. Moreover, the first evaluation of this sequential culture system on porcine HMC and ZF PA embryos has resulted in blastocyst rates of 17% and 47% respectively. Sometimes, the blastocyst rate of ZI PA could even reach levels as high as 90% (Du, unpublished)

Statistical analysis

ANOVA analysis was performed using SPSS 11.0. A probability of $P < 0.05$ was considered to be statistically significant.

5 10.3

Vitrification of hand-made cloned porcine blastocysts produced from delipated in vitro matured oocytes.

10 Recently a noninvasive procedure was published for delipation of porcine embryos with centrifugation but without subsequent micromanipulation (Esaki *et al.* 2004 Biol Reprod. 71, 432-6).

Cryopreservation of embryos/blastocysts was carried out by vitrification using Cryotop (Kitazato Supply Co, Fujinomiya Japan) as described previously (Kuwayama *et al.* 2005a; 2005b). At the time of vitrification, embryos/blastocysts were transferred into
15 equilibration solution (ES) consisting of 7.5% (V/V) ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO) in TCM199 supplemented with 20% synthetic serum substitute (SSS) at 39°C for 5 to 15 min. After an initial shrinkage, embryos regained their original volume. 4~6 embryos/blastocysts were transferred into 20 ul drop of
20 vitrification solution (VS) consisting of 15% (V/V) EG and 15% (DMSO) and 0.5M sucrose dissolved in TCM199 supplemented with 20% SSS. After incubation for 20 s, embryos were loaded on Cryotop and plunged into liquid nitrogen. The process from exposure in VS to plunging was completed with 1 min.

Embryos/blastocysts were thawed by immersing Cryotop directly into thawing solution
25 (TS) consisting of 1.0M sucrose in TCM199 plus 20% SSS for 1min, then transferred to dilution solution (DS) consisting of 0.5 M sucrose in TCM199 plus 20% SSS for 3 min. To remove cryoprotectant, embryos/blastocysts were kept twice in a washing solution (WS; TCM199 plus 20% SSS), 5 min for each time. Survival of vitrified blastocysts was determined according to reexpansion rates after 24 h recovery in culture medium
30 supplemented with 10% calf serum (CS).

The non-invasive delipation method was applied to in vitro matured porcine oocytes and further development of delipated oocytes after parthenogenetic activation was investigated in 4 identical replicates. Oocytes were randomly separated into delipation
35 and control groups.

For delipation, oocytes were digested with 1mg/ml pronase in the presence of 50% cattle serum (CS) for 3 min, and washed in Hepes-buffered TCM-199 medium supplemented with 20% CS which results in partial zona pellucida digestion (fig. 11a). Subsequently 40-50 oocytes were centrifuged ($12000 \times g$, 20 min) at room temperature in Hepes-buffered TCM-199 medium supplemented with 2% CS, 3 mg/ml PVA and 7.5 $\mu\text{g/ml}$ cytochalasin B (CB) (fig. 11b). Zonae pellucida of both centrifuged and intact oocytes were removed completely with further digestion in 2mg/ml pronase solution. For activation, a single direct current of 85Kv/cm for 80us was applied to both groups, followed by 4 h treatment with 5 $\mu\text{g/ml}$ CB and 10 $\mu\text{g/ml}$ cycloheximide (CHX). All embryos were then cultured in the modified NCSU37 medium. Day 7 blastocysts were vitrified and warmed by using the Cryotop technique (Kuwayama et al., RBM Online, in press) at 38.5°C. Survival of vitrified blastocysts was determined according to reexpansion rates after 24 h recovery in culture medium supplemented with 10% CS. Cell numbers of reexpanded blastocysts from both groups were determined after Hoechst staining. Results were compared by ANOVA analysis. Partial zona digestion and centrifugation resulted in successful delipation in 173/192 (90%) of oocytes. The development to blastocysts was not different between delipated and intact oocytes ($28 \pm 7\%$ vs. $28 \pm 5\%$ respectively; $P > 0.05$). However, survival rates of blastocysts derived from delipated oocytes were significantly higher than those developed from intact oocytes ($85 \pm 6\%$ vs. $32 \pm 7\%$ respectively; $P < 0.01$). There is no difference in average cell number of reexpanded blastocysts derived from either delipated or intact oocytes (36 ± 7 vs. 38 ± 9 , respectively; $P > 0.05$). The results demonstrate that the simple delipation technique does not hamper the in vitro development competence of activated porcine oocytes, and improves the cryosurvival of the derived blastocysts without significant loss in cell number.

After delipation, zona pellucida of oocytes from both groups was removed completely. The same parameters as described above for electrical activation were applied to both groups. Seven days after activation, blastocyst rates and blastocyst cell numbers were determined.

The feasibility of applying a non-invasive delipation technique to in vitro matured porcine oocytes was investigated. 90% (173/192) oocytes can be delipated successfully. As shown in table 5, the development to blastocysts was not different between delipated and intact oocytes ($28 \pm 7\%$ vs. $28 \pm 5\%$ respectively; $P > 0.05$).

However, survival rates of blastocysts derived from delipated oocytes were significantly higher than those developed from intact oocytes ($85\pm6\%$ vs. $32\pm7\%$ respectively; $P<0.01$). There is no difference in average cell number of reexpanded blastocysts derived from either delipated or intact oocytes (36 ± 7 vs. 38 ± 9 , respectively; $P>0.05$).

5

Table 5. Developmental competence and cryosurvival of vitrified-thawed embryos from delipated and intact activated oocytes.

Oocyte treatment	Activated oocyte	Blastocyst rate (%)	Reexpanded blastocyst after warming (%)	Mean cell number of reexpanded blastocysts
Delipated	173	28 ± 7	85 ± 6	36 ± 7
Intact	156	28 ± 5	32 ± 7	39 ± 9

10

Handmade Cloning of delipated oocytes

Delipated oocytes were used for HMC in 5 replicates. Four identical replicates of non-delipated oocytes for HMC were used as a control system. Seven days after reconstruction, blastocysts produced from both groups were vitrified with Cryotop.

15

Survival rates and cell numbers of re-expanded blastocysts were determined as described for the blastocysts produced by PA.

20

Except where otherwise indicated, all manipulations were performed on a heated stage adjusted to 39°C , and all drops used for handling oocytes were of 20 μl volume covered with mineral oil. For somatic cell nuclear transfer, the handmade cloning (HMC) described in our previous work (Du, et al., 2005) was applied with a single modification: for enucleation of both delipated and control oocytes, bisection instead of trisection was applied.

25

Briefly, after the removal of cumulus investment, control oocytes were incubated in 3.3mg/ml pronase dissolved in T33 for 10 s. Before the oocytes started to become misshaped in pronase solution, they were picked out and washed quickly in T2 and T20 drops. Delipated oocytes after centrifugation were digested in the 3.3mg/ml pronase solution for an additional 5 s.

30

Both control and delipated oocytes with partially digested, distended and softened zonae pellucidae were lined up in T2 drops supplemented with 2.5 $\mu\text{g/ml}$ cytochalasin B. Bisection was performed manually under stereomicroscopic control (Fig 11c) with

Ultra Sharp Splitting Blades (AB Technology, Pullman, WA, USA). Halves were collected and stained with 5 µg/ml Hoechst 33342 fluorochrome in T2 drops for 5 min, and then placed into 1 µl drops of T2 medium on the bottom of a 60 mm Falcon Petri dish covered with oil (3-4 halves per drop). Using an inverted microscope and UV light, positions of halves without chromatin staining (cytoplasts) were registered. Cytoplasts were later collected under a stereomicroscope and stored in T10 drops.

Porcine foetal fibroblast cells were prepared with trypsin digestion from monolayers as described previously (Kragh, et al., 2005). Fusion was performed in two steps where the second one included the initiation of activation, as well. For the first step, 50% of the available cytoplasts were transferred into 1 mg/ml of phytohaemagglutinin (PHA; ICN Pharmaceuticals, Australia) dissolved in T0 for 3 s, then quickly dropped over single fibroblast cells. After attachment, cytoplast-fibroblast cell pairs were equilibrated in fusion medium (0.3 M mannitol and 0.01% PVA) for 10 s and transferred to the fusion chamber. Using an alternating current (AC) of 0.6KV/cm and 700 KHz, pairs were aligned to the wire of a fusion chamber with the somatic cells farthest from the wire (Fig 11d), then fused with a direct current of 2.0 KV/cm for 9 µs. After the electrical pulse, cell pairs were removed carefully from the wire, transferred to T10 drops and incubated to observe whether fusion had occurred.

Approximately 1 hour after the first fusion, each pair was fused with another cytoplast in activation medium. AC current and a single DC pulse of 0.7 KV/cm for 80 µs were applied as described above. Fusion was detected in T10 drops, then reconstructed embryos were transferred into IVC0-2 medium (see *Embryo culture and evaluation*) supplemented with 5 µg/ml cytochalasin B and 10 µg/ml cycloheximide. After a 4 h incubation at 38.5°C in 5% CO₂, 5% O₂ and 90% N₂ with maximum humidity, embryos were washed 3 times in IVC0-2 medium before culture.

Table 6. Developmental competence and cryosurvival of vitrified-thawed embryos of SCNT porcine embryos derived from delipated and intact oocytes.

HMC group	No. of reconstructed embryos	Blastocyst rate (%)*	Reexpanded blastocyst after warming (%)*	Mean cell number of reexpanded blastocysts*
Delipated	240	21±6 ^a	79±6 ^b	41±7 ^d
Intact	150	23±6 ^a	32±8 ^c	39±5 ^d

Different superscripts mean significant differences ($p < 0.05$).

*: mean $\pm$ S.E.M.

5 In vitro developmental competence was observed in HMC with delipated oocytes when Day 7 blastocyst rates were compared with control HMC group ($21\pm6\%$ vs. $23\pm6\%$ respectively; $P>0.05$; Table 6). Cryosurvival rate after vitrification of cloned blastocysts derived from delipated oocytes was significantly higher than those developed from intact oocytes ($79\pm6\%$ vs. 32 ± 8 , respectively; $P<0.01$).

10 10. 4

Chemically assisted handmade enucleation (CAHE) and comparison to existing methods

15 After 41-42 h maturation *in vitro*, COCs were further cultured for 45 min in the same solution supplemented by $0.4\text{ }\mu\text{g/ml}$ demecolcine. Cumulus cells were then removed by pipetting in 1 mg/ml hyaluronidase dissolved in Hepes-buffered TCM-199. From this point (except where otherwise indicated), all manipulations were performed on a heated stage adjusted to 39°C . All drops used for handling oocytes were of $20\text{ }\mu\text{l}$ in volume, and were covered with mineral oil.

20 Basic steps of the HMC procedure have been described elsewhere herein. Briefly, oocytes without cumulus cells were incubated in 3.3 mg/ml pronase dissolved in T33 (T for Hepes-buffered TCM 199 medium; the number means percentage [v/v] of CS supplement, here 33%) for 20 s. When partial lyses of zonae pellucidae and slight deformation of oocytes occurred, they were picked up and washed quickly in T2 and
25 T20 drops. Nine oocytes were lined up in one T2 drop supplemented with $2.5\text{ }\mu\text{g/ml}$ cytochalasin B (CB). By using a finely drawn and fire-polished glass pipette, oocytes were rotated to find a light extrusion cone and/or strongly attached polar body on the surface, and oriented bisection was performed manually under stereomicroscopic control with a microblade (AB Technology, Pullman, WA, USA). Less than half of the
30 cytoplasm (close to the extrusion or PB) was separated from the remaining part (Fig. 12). After bisection of all 9 oocytes in the drop, larger parts and smaller parts (with the extrusion or attached PB) were collected and placed into separate drops of T2, respectively.

Oriented handmade enucleation without demecolcine treatment (OHE)

All steps were similar to the previously described procedure, but demecolcine preincubation was not applied.

5 *Random handmade bisection for enucleation (RHE)*

Demecolcine preincubation was omitted from the pretreatment of this group, as well. After removal of cumulus cells, zonae pellucidae were partially digested by pronase as described above. Random handmade equal bisection was applied in drops of T2 supplemented with 2.5 µg /ml CB. All demi-oocytes were selected and stained with 10
10 µg /ml Hoechst 33342 in T2 drops for 10 min, then placed into 1 µl drops of T2 medium covered with mineral oil (three demi-oocytes into each drop). Using an inverted microscope and UV light, the positions of chromatin free demi-oocytes, i.e. cytoplasts were registered. These cytoplasts were later collected under a stereomicroscope and stored in T2 drops before further manipulations.

15

Fusion and initiation of activation

Porcine fetal fibroblast cells were prepared as described previously (Kragh, et al., 2005, Du, et al., 2005). Fusion was performed in two steps, where the second one included the initiation of activation as well. For the first step, with a finely drawn and
20 fire-polished glass pipette, approximately 100 somatic cells were placed into a T2 drop, and 20-30 cytoplasts were placed into a T10 drop. After a short equilibration, groups of 3 cytoplasts were transferred to 1 mg/ml of phytohaemagglutinin (PHA) for 2-3 sec, then each was quickly dropped over a single somatic cell. Following attachment, cytoplast-somatic cell pairs were picked up again and transferred to a fusion medium
25 (0.3 M mannitol supplemented with 0.01% [w/v] PVA). By using an alternative current (AC) of 0.6 KV/cm and 700 KHz, equilibrated pairs were aligned to one wire of a fusion chamber (BTX microslide 0.5 mm fusion chamber, model 450; BTX, San Diego, CA) with the somatic cells farthest from the wire, then fused with a single direct current (DC) impulse of 2.0 KV/cm for 9 µsec. Pairs were then removed carefully from the wire to a
30 T10 drop, and incubated further to observe whether fusion had occurred.

Approximately 1 h after the fusion, fused pairs and the remaining cytoplasts were separately equilibrated in activation medium (0.3 M mannitol, 0.1 mM MgSO₄, 0.1 mM CaCl₂, supplemented with 0.01% [w/v] PVA). By using a 0.6 KV/cm AC, one pair and
35 one cytoplast was aligned to one wire of the fusion chamber, with fused pairs

contacting the wire. A single DC pulse of 0.86 kV/cm for 80 μ sec was used for the second fusion and initiation of activation. Fusion was checked in after incubation in T10 drops.

5 *Traditional Cloning (TC)*

Micromanipulation was conducted with a Diaphot 200 inverted microscope (Nikon, Tokyo, Japan), as described before (Chen et al., 1999; Zhang et al., 2005). Briefly, after 42-44 h *in vitro* maturation, the cumulus cells were removed as described above. All manipulations were performed on a heated stage adjusted to 39°C. A single 50 μ L micromanipulation solution drop was made in the central area on a lid of 60 mm culture dish and covered with mineral oil. Groups of 20-30 oocytes and fetal fibroblast cells were placed in the same drop. After incubation for 15-30 min, the oocyte was secured with a holding pipette (inner diameter = 25-35 μ m and outer diameter = 80-100 μ m). After being placed at the position of 5-6 o'clock, the first polar body and the adjacent cytoplasm (approx. 10% of the total volume of the oocyte) presumptively containing metaphase plate were aspirated and removed with a beveled injection pipette (inner diameter = 20 μ m). A fetal fibroblast cell was then injected into the space through the same slit. After nuclear transfer (NT), reconstructed couplets were transferred into drops of media covered with mineral oil for recovery for 1 - 1.5 h until fusion and activation was conducted. The recovery medium was NCSU-23 supplemented with 4 mg/mL BSA and 7.5 μ g/mL CB. Reconstructed couplets were incubated in fusion medium for 4 min. Couplets were aligned manually using a finely pulled and polished glass capillary to make the contact plane parallel to electrodes. A single, 30 μ sec, direct current pulse of 2.0 kV/cm was then applied. After culture in drops of IVC0-2 (specified in "Embryo culture and evaluation") supplemented with 7.5 μ g/mL CB for 30-60 min, fusion results were examined under a stereomicroscope. Fused couplets were subjected to a second pulse in activation solution. After 30 min incubation in T10 they were transferred to IVC0-2 to evaluate *in vitro* development.

30 *Further steps of activation*

After the activation impulse, all reconstructed embryos were incubated in IVC0-2 supplemented with 5 μ g/ml CB and 10 μ g/ml cycloheximide at 38.5°C in 5% CO₂, 5% O₂, and 90% N₂, with maximum humidity.

Embryo culture and evaluation

4 h later, all reconstructed and activated embryos were washed and cultured in Nunc four-well dishes in 400 μ l IVC0-2 covered by mineral oil at 38.5°C in 5% CO₂, 5% O₂, and 90% N₂, with maximum humidity. IVC0-2 was a modified NCSU37 medium (Kikuchi, et al., 1999), containing 4 mg/ml BSA, 0.17 mM sodium pyruvate, and 2.73 mM sodium lactate from Day 0 (the day for activation) to Day 2. Sodium pyruvate and sodium lactate were replaced with 5.5 mM glucose from Day 2 to Day 7 (IVC2-7). All zona free embryos were cultured in the Well of the Well (WOW) system (Vajta et al., 2000) in the same culture medium and gas mixture as used above, with careful medium change on Day 2 without removing the embryos from the WOWs. TC embryos were cultured in groups of 15 to 30 in wells of four-well dishes by using the same medium amount and composition. Cleavage and blastocyst rates were registered on Day 2 and Day 7, respectively. To determine total cell numbers, blastocysts were fixed and mounted to a glass microscope slide in a small amount (<2 μ l) of glycerol containing 10 μ g/ml Hoechst 33342. After staining for several hours at room temperature, embryos were observed under a Diaphot 200 inverted microscope with epifluorescent attachment and UV-2A filter (Nikon, Tokyo, Japan).

Comparison of efficiency of CAHE vs. OHE

The efficiency and reliability of CAHE was tested in 12 identical replicates by using a total of 620 oocytes. After 41-42 h maturation, oocytes were subjected to demecolcine incubation. Oriented bisection was performed in oocytes where an extrusion cone and/or a strongly attached PB was detected after partial pronase digestion. Percentages of bisected vs. total oocytes and surviving vs. bisected oocytes were registered. Subsequently both putative cytoplasts and karyoplasts were collected separately and stained with Hoechst 33342 (10 μ g/ml in T2 for 10 min). The presence or absence of chromatin was detected under an inverted fluorescent microscope (Fig. 13).

The efficiency and reliability of OHE was investigated in 9 identical replicates using a total of 414 oocytes. After 42-43 h in vitro maturation, oriented bisection was performed in matured oocytes where an extrusion cone and/or a PB was detected after partial pronase digestion. Results were evaluated as described in the previous paragraph.

The results are shown in Table 7.

Table 7: The efficiency of chemically assisted handmade enucleation (CAHE) and oriented handmade enucleation (OHE)

Groups	No. of treated oocytes	Bisected/total oocytes (%) [*]	Cytoplasm/bisection (%) [*]	Cytoplasm/total oocyte (%) [*]
CAHE	620	96±1 ^a	94±2 ^b	90±3 ^c
OHE	414	92±2 ^a	88±3 ^b	81±4 ^d

*: mean ± A.D. (absolute deviations)

5 Different superscripts mean difference (P<0.05)

No differences between groups regarding extrusion cones and/or attached polar bodies allowing oriented bisection or in the lysis rates were detected, and the successful enucleation per bisected oocyte ratio was also similar. However the overall efficiency of the procedure measured by the cytoplasm per total oocyte number was higher in the CAHE than in the OHE group.

Comparison of in vitro development of embryos produced with CAHE, RHE and TC

15 In 8 replicates, a total of 468 in vitro matured oocytes were randomly distributed and subjected to three of the enucleation procedures described above. Fusion rates between cytoplasm and donor fibroblasts were registered. Reconstructed embryos were activated and cultured as described earlier. Cleavage and blastocyst rates were determined on Day 2 and Day 7, respectively. Stereomicroscopic characteristics of the developed blastocysts were compared between groups.

Table 8: Developmental competence of embryos derived from chemically assisted handmade enucleation (CAHE), random handmade enucleation (RHE) and traditional, micromanipulator based cloning (TC).

25

Groups	No. of reconstructed embryos	Fusion rate (%) [*]	Cleavage rate (%) [*]	Blastocyst rate (%) [*]	Cell no. of blastocysts (Day 7)
CAHE	150	87±7 ^a	97±6 ^b	28±9 ^d	57±6 ^e
RHE	86	81±4 ^a	87±8 ^b	21±9 ^d	49±7 ^e
TC	178	81±10 ^a	69±9 ^c	21±6 ^d	53±6 ^e

*: mean $\pm$ A.D. (absolute deviations)

Different superscripts mean difference ($P < 0.05$).

Fusion rates after enucleation were similar between CAHE, RHE and TC, respectively.

5 The second fusion and activation resulted in negligible ($<1\%$) losses in the first two groups. Although TC resulted in lower cleavage per reconstructed embryo rates than the other two groups, this difference was not present in the blastocyst per reconstructed embryo rates.

10 Stereomicroscopic characteristics (size; estimated proportion and outlines of the inner cell mass) did not differ between groups. Cell numbers (57 ± 6 vs. 49 ± 7 vs. 53 ± 6) of the produced blastocysts from CAHE, RHE and TC are shown in Table 8, Fig. 14 and Fig. 15.

15 Statistical analysis

AVEDEV was performed by Microsoft XP Excel software and ANOVA was performed by SAS system. A probability of $P < 0.05$ was considered to be statistically significant.

10.5

20 Production of piglets

Handmade cloning (HMC)

Forty one hrs after the start of *in vitro* maturation, the cumulus investment of the COCs was removed by repeated pipetting in 1mg/ml hyaluronidase in Hepes-buffered

25 TCM199. From this point (except where otherwise indicated) all manipulations were performed on a heated stage adjusted to 39°C , and all drops used for handling oocytes were of 20 μl volume covered with mineral oil. Oocytes were briefly incubated in 3.3mg/ml pronase dissolved in T33 (T for Hepes-buffered TCM 199 medium; the number means percentage (v/v) of calf serum (CS) supplement, here 33%) for 20 sec
30 and then quickly washed in T2 and T20 drops. Oocytes with partially digested but still visible zona were lined up in drops of T2 supplemented with 2.5 $\mu\text{g/ml}$ cytochalasin B (CB). With a finely drawn and fire-polished glass pipette, oocytes were rotated to find the polar body (PB) on the surface, and oriented bisection was performed manually under stereomicroscopic control with a microblade (AB Technology, Pullman, WA,
35 USA). Thus, less than half of the oocyte cytoplasm (close to the extrusion or PB) was

removed from the remaining putative cytoplasm. Cytoplasm was washed twice in T2 drops and collected in a T10 drop.

Fetal fibroblast cells were prepared as described previously (Kragh, P.M. *et al. Theriogenology* 64, 1536-1545 (2005)).

- 5 Fusion was performed in two steps where the second one included the initiation of activation, as well. For the first step, halves of putative cytoplasm were used. With a finely drawn and fire-polished glass pipette, 10 cytoplasm were transferred as a group to 1 mg/ml of phytohaemagglutinin (PHA; ICN Pharmaceuticals, Australia) for 3 sec, then quickly dropped individually onto one of the few fibroblast cells that were
- 10 sedimented in a T2 drop. After attachment, 10 cytoplasm-fibroblast cell pairs were equilibrated in fusion medium (0.3 M mannitol and 0.01% PVA) for 10 sec. Using an alternative current (AC) of 0.6KV/cm and 700 KHz, cell pairs were aligned to the wire of a fusion chamber (BTX microslide 0.5 mm fusion chamber, model 450; BTX, SanDiego, CA, USA) with the somatic cells farthest from the wire, then fused with a
- 15 direct current (DC) of 2.0 KV/cm for 9 μ sec. After the electrical pulse, cell pairs were removed carefully from the wire, transferred to T10 drops and incubated to observe whether fusion had occurred.

- Approximately 1 hr after the first fusion, fused pairs together with the remaining cytoplasm were equilibrated in activation medium drops separately (0.3 M mannitol, 0.1
- 20 mM MgSO_4 , 0.1 mM CaCl_2 and 0.01% PVA). Under a 0.6KV/cm AC, cytoplasm - fused pair were aligned sequentially to the wire in groups of 10, with fused pairs far from the wire. A single DC pulse of 0.7 KV/cm for 80 μ sec was used for the second fusion and initiation of activation. The pairs were then removed from the wire and transferred carefully to T10 drops to check the fusion. Reconstructed embryos were incubated in
- 25 PZM-3 medium supplemented with 5 μ g/ml CB and 10 μ g/ml cycloheximide for 4 hr at 38.5°C in 5% CO_2 , 5% O_2 and 90% N_2 with maximum humidity, then washed thoroughly before culture.

Traditional Cloning (TC)

- 30 Micromanipulation was conducted with a Diaphot 200 inverted microscope (Nikon, Tokyo, Japan). Cumulus cells were removed as described above after 42 to 44 hr maturation. All manipulations were performed on a heated stage adjusted to 39°C. A single 50 μ L drop of micromanipulation solution (NCSU-23 supplemented with 4 mg/mL BSA and 7.5 μ g/mL CB) was made in the central area on a lid of 60 mm culture dish
- 35 and covered with mineral oil. Groups of 20 to 30 oocytes and fetal fibroblast cells were

placed in the same drop. After incubation for 15 to 30 min, one oocyte was secured with a holding pipette (inner diameter = 25-35 μm and outer diameter = 80-100 μm). After being placed at the position of 5-6 o'clock, the first polar body and the adjacent cytoplasm (approx. 10% of the total volume of the oocyte) presumptively containing metaphase plate were aspirated and removed with a beveled injection pipette (inner diameter = 20 μm). A fetal fibroblast cell was then injected into the space through the same slot. After nuclear transfer (NT), reconstructed couplets were transferred into drops of media covered with mineral oil for recovery for 1 to 1.5 hrs until fusion and activation was conducted. Reconstructed couplets were incubated in fusion medium for 4 min. Couplets were aligned manually using a finely pulled and polished glass capillary to make the contact plane parallel to electrodes. A single, 30 μsec , direct current pulse of 2.0 kV/cm was then applied. After culture in drops of PZM-3 medium supplemented with 7.5 $\mu\text{g/mL}$ CB for 30-60 min, fusion results were examined under a stereomicroscope. Fused couplets were subjected to a second pulse in activation solution. After 30 min incubation in T10 they were transferred to PZM-3 medium to evaluate *in vitro* development.

Embryo Culture and Transfer

Reconstructed embryos were cultured in PZM-3 medium (Dobrinisky, J.T. et al. *Biol Reprod* 55, 1069-1074 (1996) supplemented with 4 mg/ml BSA. Zona-free embryos produced from HMC were cultured in the modified WOWs system (Feltrin, C. Et al. *Reprod Fertil Dev* 18, 126 (2006). Two different cell lines (LW1-2 for HMC, LW2 for TC) were used as nuclear donor cells for HMC and TC to allow the identification of the offspring from the two procedures. LW1-2 and LW2 originate from fetuses from a cross (with Duroc) and pure Danish landrace, respectively.

The average blastocyst per reconstructed embryo rate after *in vitro* culture for 7 days was $50.1 \pm 2.8\%$ (mean $\pm$ S.E.M), which is significantly higher ($p < 0.01$) for HMC than that of TC performed in parallel in our laboratory (Table 9) and also the highest one that has ever been reported in pig cloning.

Table 9

In vitro development of embryos produced from handmade cloning and traditional cloning

Group	Somatic cell donor	No. of reconstructed embryos	Cleavage rate (%)	Blastocyst rate (%)
HMC	LW1-2	643	83.7±4.90 ^a	50.06±2.80 ^a
TC	LW2	831	74.86±13.16 ^b	28.98±2.84 ^b

^{a, b}, Values of different superscripts within columns are significantly different ($p < 0.05$).

*: mean±S.E.M.

- 5 Mixed blastocysts produced from both HMC and TC were surgically transferred to 11 naturally synchronized sows on Day 4 or 5 of estrous cycle. Six (55%) recipients were diagnosed pregnant by ultrasonography, 2 aborted and by the time of writing 2 have delivered 3 and 10 piglets, respectively. A litter size of 10 cloned piglets is, according to our knowledge, the largest litter size so far achieved in pig cloning. All of them are healthy and behave normally except one showed rigid flexure of distal joint of one foreleg. %).

Preliminary results suggest that when embryos of similar stages were transferred, recipients on Day 4 of the estrous cycle supported pregnancy establishment better than those of Day 5 (Table 10).

Table 10. *In vivo* development of cloned porcine embryos

Recipient number	Embryos transferred		Embryo stage (Day)	Recipient cycle (Day)	Pregnancy status	No. of piglets born		Gestation length (Day)
	HMC embryo	TC embryo				piglets from HMC	No. piglets from TC	
1327	22	10	D5,6,7	4	Y	2	1	116
1539	36	10	D7	4	Y	8	2	115
1309	30	28	D5,6	4	Y			
1553	45	44	D5,6	4	Y			
1668	48	18	D5,6	5	Y, aborted			
1428	78	22	D5,6	5	Y, aborted			
1725	44	4	D5,6,7	5	N	-	-	-
1643	22	11	D5,6,7	4	N	-	-	-
1520	30	26	D5,6	4	N	-	-	-
1363	37	7	D6,7	5	N	-	-	-
1560	99	42	D5,6,7	5	N	-	-	-

Microsatellite Analysis

Parental analysis using 10 different porcine microsatellite markers confirmed the identical genotype of cloned piglets and donor cells used for nuclear transfer.

Identification was done by microsatellite analysis of genomic DNA from each of the newborn piglets, the surrogate sow, and the donor skin fibroblasts LW1-2 and LW2 originating from two fetuses that represent Danish landrace and Duroc, respectively.

Ten polymorphic microsatellite loci (SW886, SW58, SW2116, SW1989, SW152, SW378, KS139, SO167, SW1987, SW957) located on different porcine chromosomes were amplified by 3-color multiplex PCR and the products analyzed on the Genetic Analyzer 3130 X1 (Applied Biosystems) using the program Gene Mapper 3.7.

For the second recipient, the offspring per embryo rate (22%) was the highest one ever reported so far in pig cloning (Walker, S.C. *et al. Cloning Stem Cells* 7, 105-112 (2005); Hoshino, Y. *et al. Cloning Stem Cells* 7, 17-26 (2005)). Comparable live birth/transferred embryo efficiencies were obtained in HMC (17%) and TC (15%).

Statistical Analysis

Differences between the experimental groups were evaluated using independent-samples t-test by SPSS 11.5. $P < 0.05$ was considered significant.

Sequences**SEQ ID NO: 1: Sus scrofa ApoE gene sequence****SEQ ID NO: 2: Sus scrofa ApoE protein**

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DEFINITION Sus scrofa apolipoprotein-E (Apo-E) gene, complete cds.

ACCESSION U70240

40 VERSION U70240.1 GI:2388608

KEYWORDS .

SOURCE Sus scrofa (pig)

ORGANISM Sus scrofa

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
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Sus.

REFERENCE 1 (bases 1 to 4267)
5 AUTHORS Ramsoondar, J.J., Rucker, E.B., Vasquez, J.C., Gallagher, D.S.,
 Grimm, D.R., Lunney, J.K., Schook, L.B. and Piedrahita, J.A.
 TITLE Isolation and genetic characterization of the porcine
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 JOURNAL Anim. Genet. 29 (1), 43-47 (1998)
10 PUBMED 9682450
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ApoE

Sus scrofa apolipoprotein-E (Apo-E) gene, complete cds.

ACCESSION U70240

VERSION U70240.1 GI:2388608

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KEYWORDS .

SOURCE Sus scrofa (pig)

ORGANISM Sus scrofa

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
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Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla;
 Suina; Suidae;

Sus.

REFERENCE 1 (bases 1 to 4267)

AUTHORS Ramsoondar, J.J., Rucker, E.B., Vasquez, J.C.,

20

Gallagher, D.S.,

Grimm, D.R., Lunney, J.K., Schook, L.B. and Piedrahita, J.A.

TITLE Isolation and genetic characterization of the porcine
 apolipoprotein E gene

JOURNAL Anim. Genet. 29 (1), 43-47 (1998)

25

PUBMED 9682450

REFERENCE 2 (bases 1 to 4267)

AUTHORS Ramsoondar, J.J. and Piedrahita, J.A.

TITLE Direct Submission

JOURNAL Submitted (10-SEP-1996) VAPH, Texas A&M University,
 College

30

Station, TX 77843, USA

FEATURES Location/Qualifiers

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Homo sapiens

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    REFERENCE  1  (bases 1 to 5491)
        AUTHORS Nickerson,D.A., Taylor,S.L., Fullerton,S.M., Weiss,K.M.,
45  Clark,A.G., Stengard,J.H., Salomaa,V., Boerwinkle,E. and
        Sing,C.F.
        TITLE    Sequence diversity and large-scale typing of SNPs in the
        human
                apolipoprotein E gene
50  JOURNAL    Genome Res. 10 (10), 1532-1545 (2000)
        PUBMED    11042151
    REFERENCE  2  (bases 1 to 5491)
        AUTHORS  Nickerson,D.A.
        TITLE    Direct Submission
55  JOURNAL    Submitted (27-APR-2000) Department of Molecular
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                University of Washington, Box 357730, Seattle, WA 98195,
        USA

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LOCUS NM_000041 1223 bp mRNA linear PRI
 18-FEB-2007
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 SOURCE Homo sapiens (human)
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 Haplorrhini;
 Catarrhini; Hominidae; Homo.
 35 REFERENCE 1 (bases 1 to 1223)
 AUTHORS Mullick,A.E., Powers,A.F., Kota,R.S., Tetali,S.D.,
 Eiserich,J.P.
 and Rutledge,J.C.
 40 TITLE Apolipoprotein E3- and nitric oxide-dependent modulation
 of endothelial cell inflammatory responses
 JOURNAL Arterioscler. Thromb. Vasc. Biol. 27 (2), 339-345 (2007)
 PUBMED 17138935
 REMARK GeneRIF: data suggest a role of vascular wall apoE3 to
 45 balance the intracellular redox state in injured endothelial cells via
 NO-dependent pathways
 REFERENCE 2 (bases 1 to 1223)
 50 AUTHORS Gupta,V., Narayanaswami,V., Budamagunta,M.S., Yamamoto,T.,
 Voss,J.C. and Ryan,R.O.
 TITLE Lipid-induced extension of apolipoprotein E helix 4
 correlates with

- low density lipoprotein receptor binding ability
JOURNAL J. Biol. Chem. 281 (51), 39294-39299 (2006)
PUBMED 17079229
REMARK GeneRIF: analysis of the LDLR recognition properties of
5 apoE
REFERENCE 3 (bases 1 to 1223)
AUTHORS Pei,W.D., Zhang,Y.H., Sun,Y.H., Gu,Y.C., Wang,Y.F.,
Zhang,C.Y.,
10 Zhang,J., Liu,L.S., Hui,R.T., Liu,Y.Q. and Yang,Y.J.
TITLE Apolipoprotein E polymorphism influences lipid phenotypes
in
JOURNAL Circ. J. 70 (12), 1606-1610 (2006)
PUBMED 17127808
15 REMARK GeneRIF: ApoE polymorphism appears to be associated with
variance
of the lipoprotein phenotype in Chinese families with
familial
20 combined hyperlipidemia.
REFERENCE 4 (bases 1 to 1223)
AUTHORS Olarte,L., Schupf,N., Lee,J.H., Tang,M.X., Santana,V.,
Williamson,J., Maramreddy,P., Tycko,B. and Mayeux,R.
TITLE Apolipoprotein E epsilon4 and age at onset of sporadic and
25 familial
Alzheimer disease in Caribbean Hispanics
JOURNAL Arch. Neurol. 63 (11), 1586-1590 (2006)
PUBMED 17101827
REMARK GeneRIF: Individuals with a family history of AD and the
30 APOE
epsilon4 allele, additional genetic or environmental
factors may
accelerate the onset of dementia.
REFERENCE 5 (bases 1 to 1223)
AUTHORS Christidis,D.S., Liberopoulos,E.N., Kakafika,A.I.,
35 Miltiadous,G.A.,
Cariolou,M., Ganotakis,E.S., Mikhailidis,D.P. and
Elisaf,M.S.
TITLE The effect of apolipoprotein E polymorphism on the
40 response to
lipid-lowering treatment with atorvastatin or fenofibrate
JOURNAL J. Cardiovasc. Pharmacol. Ther. 11 (3), 211-221 (2006)
PUBMED 17056835
REMARK GeneRIF: There was a clear association between
45 apolipoprotein E
polymorphism on lipid-lowering response to treatment with
atorvastatin and fenofibrate in patients with different
types of
dyslipidemia.
REFERENCE 6 (bases 1 to 1223)
50 AUTHORS Lohse,P., Brewer,H.B. III, Meng,M.S., Skarlatos,S.I.,
LaRosa,J.C.
and Brewer,H.B. Jr.
TITLE Familial apolipoprotein E deficiency and type III
55 hyperlipoproteinemia due to a premature stop codon in the
apolipoprotein E gene
JOURNAL J. Lipid Res. 33 (11), 1583-1590 (1992)
PUBMED 1361196
REFERENCE 7 (bases 1 to 1223)

- AUTHORS Moriyama,K., Sasaki,J., Matsunaga,A., Arakawa,F.,
Takada,Y.,
Araki,K., Kaneko,S. and Arakawa,K.
- 5 TITLE Apolipoprotein E1 Lys-146----Glu with type III
hyperlipoproteinemia
JOURNAL Biochim. Biophys. Acta 1128 (1), 58-64 (1992)
PUBMED [1356443](#)
REFERENCE 8 (bases 1 to 1223)
- 10 AUTHORS Feussner,G., Funke,H., Weng,W., Assmann,G., Lackner,K.J.
and
Ziegler,R.
TITLE Severe type III hyperlipoproteinemia associated with
unusual
apolipoprotein E1 phenotype and epsilon 1/'null' genotype
- 15 JOURNAL Eur. J. Clin. Invest. 22 (9), 599-608 (1992)
PUBMED [1360898](#)
REFERENCE 9 (bases 1 to 1223)
AUTHORS Schuler,G., Hambrecht,R., Schlierf,G., Niebauer,J.,
Hauer,K.,
Neumann,J., Hoberg,E., Drinkmann,A., Bacher,F., Grunze,M.
- 20 et al.
TITLE Regular physical exercise and low-fat diet. Effects on
progression
of coronary artery disease
- 25 JOURNAL Circulation 86 (1), 1-11 (1992)
PUBMED [1617762](#)
REFERENCE 10 (bases 1 to 1223)
AUTHORS Utermann,G., Pruin,N. and Steinmetz,A.
- 30 TITLE Polymorphism of apolipoprotein E. III. Effect of a single
polymorphic gene locus on plasma lipid levels in man
JOURNAL Clin. Genet. 15 (1), 63-72 (1979)
PUBMED [759055](#)
COMMENT REVIEWED [REFSEQ](#): This record has been curated by NCBI
staff. The
reference sequence was derived from [BU848796.1](#) and
35 [BC003557.1](#).
On Jun 16, 2004 this sequence version replaced [gi:4557324](#).
- 40 lipoprotein
Summary: Chylomicron remnants and very low density
(VLDL) remnants are rapidly removed from the circulation
by
receptor-mediated endocytosis in the liver. Apolipoprotein
E, a
45 main apoprotein of the chylomicron, binds to a specific
receptor on
liver cells and peripheral cells. ApoE is essential for
the normal
catabolism of triglyceride-rich lipoprotein constituents.
- 50 The APOE
gene is mapped to chromosome 19 in a cluster with APOC1
and APOC2.
Defects in apolipoprotein E result in familial
55 (HLP III),
dysbetalipoproteinemia, or type III hyperlipoproteinemia
in which increased plasma cholesterol and triglycerides
are the
consequence of impaired clearance of chylomicron and VLDL
remnants.

the Publication Note: This RefSeq record includes a subset of
 the publications that are available for this gene. Please see
 5 the Entrez Gene record to access additional publications.
 COMPLETENESS: complete on the 3' end.

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Sus scrofa low density lipoprotein receptor (LDLR) mRNA, LDLR-N
allele, partial cds.

ACCESSION AF065990
VERSION AF065990.1 GI:3153894
KEYWORDS .
SOURCE Sus scrofa (pig)
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Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Euteleostomi;
Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla;
Suina; Suidae;
10 Sus.
REFERENCE 1 (bases 1 to 2403)
AUTHORS Hasler-Rapacz,J., Ellegren,H., Fridolfsson,A.K.,
Kirkpatrick,B.,
15 Kirk,S., Andersson,L. and Rapacz,J.
TITLE Identification of a mutation in the low density
lipoprotein
receptor gene associated with recessive familial
hypercholesterolemia in swine
20 JOURNAL Am. J. Med. Genet. 76 (5), 379-386 (1998)
PUBMED 9556295
REFERENCE 2 (bases 1 to 2403)
AUTHORS Hasler-Rapacz,J., Ellegren,H., Fridolfsson,A.K.,
Kirkpatrick,B.,
25 Kirk,S., Andersson,L. and Rapacz,J.
TITLE Direct Submission
JOURNAL Submitted (16-MAY-1998) Genetics & Animal Science,
University of
Wisconsin, 1675 Observatory Drive, Madison, WI 53706, USA
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 40681 agaggttgca gtgagccgag atcgcgccac tgcattccag cccgggaggc aagagcaaaa
 5 40741 ctccatctca aaaaaaaaaa aaaaaggagt tcagaggccc ggcatggtgg ttcacacatg
 40801 tgatcccaga acttggggag gttgaggcag gagaatcacc tgagctcaga gttcaagacc
 40861 agcctgggca gcacagcaag acccatctc tgcaaaaaat aaaaatttag ccagtggtg
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 40981 ggtaggagat tgagactgca gtgacttggt attgcgtcac tgcgctccag cctgggtgac
 10 41041 agagcaagcc cttgtctctt aaaaaaaaaa aaaaattcaa agaagggttt ccagagggcc
 41101 aggaggagg aagggagagg aggtgtttta ttttttgct tttattttt attttgagac
 41161 agagtctctc tctgtcacc aggttgagg gtgagtgtgt gatcttggct cactgcaact
 41221 tctgcctcct ggggtcaagc aattcttatg cctcagcctc agcctcctga gtagctggga
 41281 ttacaacact atgcccgggt aatttttgta tttttagtag agacgaggtt tcgccatgtt
 15 41341 gccagactg gtctcgaact cctgacctca agtgatccac cgccttggtc ctccccactg
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 41521 cctgttgggt ttgaggccgt tgtagctgga gcaaacagag agaggggtaa aaaggcagga
 41581 ggctaccagg caggttggtc agagcctgt gggccactgg ggaggacttt ggcttttggc
 20 41641 ctgagagcgg tgggaagtga ctgaatccgg tactcaccgt ctccctctgg cggctcctgg
 41701 gggaaacatgc ttggggatca ggctggggga ggctgccagg ccagagggt gagaagtagg
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 41821 ggcagagaca gatggtcagt ctggaggatg acgtggcgtg aacatctgcc tggagtcccg
 41881 tccctgccc gaacccttcc tgagacctcg ccggccttgt tttattcaa gacagagaag
 25 41941 accaaagcat tgccctccag agctttgttt tatatattta ttcactctgg aggcagaaca
 42001 ggcttcggac agtgcccatg caatggcttg ggttgggatt ttggtttctt cctttcctcg
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 42181 gatgtcaggc ccagagaagc aagtggcttt caacacacaa cagcagatgg caccaacggg
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 42361 cccgaatca tgaccaccc agtgtctttc gaggtgggtt tgtaccttc ttaagccagg
 42421 aaagggattc atggcgctcg aatgatctg gctgaatccg tgggtggacc gagaccaaac
 42481 tcattcacca aatgatgcca cttcccagag gcagagcctg agtactggt caccctaat
 35 42541 atttattaag tgccctgagc acccggttac cttggccgtg aggacacgtg gcctgcacc
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 42721 gtgtttattat ttgtcactgt ttctgtcgt gtgtgttggg atgggatccc aggccaggga
 42781 aagcccggtg caatgaatgc cggggacaga gaggggcagg ttgaccggga cttcaaagcc
 40 42841 gtgatcgtga atatcgagaa ctgccattgt cgtctttatg tccgccacc tagtgcttcc
 42901 acttctatgc aatgcctcc aagccattca cttcccaat cttgtcgttg atgggtatgt
 42961 gtttaaaaca tgcacggtga ggccgggcgc agtggctcac gcctgtaatc ccagcacttt
 43021 gggaggccga ggcgggtgga tcatgaggtc aggagatcga gaccatcctg gctaacacgt
 43081 gaaaccccgct ctctactaaa aatacaaaaa attagccggg cgtggtggcg ggcacctgta

43141 gtcccagcta ctccgggaggc tgaggcagga gaatggtgtg aaccgggaa gcggagcttg
 43201 cagtgcgccc agattgcgcc actgcagtc gcagctctggc ctgggcgaca gagcgagact
 43261 ccgtctcaaa aaaaaaaaaa aaaaaaaaaa catgcatggt gcatcagcag cccatggcct
 43321 ctggccaggc atggcgaggc tgagggtgga ggatggtttg agctcaggca tttgagctg
 5 43381 tcgtgagcta tgattatgcc actgctttcc agcctgggca acatagtaag acccatctc
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 43501 ggctgagctg gatcacttga gttcaggagt tggagaccag gcctgagcaa caaagcgaga
 43561 tcccatctct acaaaaacca aaaagttaa aatcagctgg gtacggtggc acgtgcctgt
 43621 gatcccagct acttgggagg ctgaggcagg aggatcgcc ctgagccagga ggtggaggtt
 10 43681 gcagtgcgcc atgatcgagc cactgcactc cagcctgggc aacagatgaa gaccctatit
 43741 cagaaataca actataaaaa aataaataaa tcctccagtc tggatcgttt gacgggactt
 43801 cagggttctt ctgaaatcgc cgtgttactg ttgactgat gtccggagag acagtgcag
 43861 cctccgtcag actcccgcgt gaagatgtca caagggttg gcaattgtcc ccagggacaa
 43921 aacactgtgt cccccagc gcagggaacc gtgataagcc tttctggtt cggagcacgt
 15 43981 aaatgcgtcc ctgtacagat agtggggatt tttgttatg tttgacttt gtatatgtg
 44041 tgaaactgtt atcacttata tatatatata tacacacata tatataaat ctatttatit
 44101 ttgcaaacc tggttgctgt attgttcag tgactattct cggggccctg ttagggggg
 44161 tattgcctct gaaatgcctc ttctttatgt acaaagatta tttgcacgaa ctggactgtg
 44221 tgcaacgctt tttgggagaa tgatgtccc gttgtatgta tgagtggct ctgggagatg
 20 44281 ggtgtcactt tttaaaccac tgtatagaag gttttttag cctgaatgtc ttactgtgat
 44341 caattaaatt tcttaaatg

Human LDL receptor cDNA sekvens (NM_000527)

25 Coding region underlined.

1 gccccgagtgc caatcgccggg aagccagggt ttccagctag gacacagcag gtcgtgatcc
 61 gggtcgggac actgcctggc agaggtgcgc agcatggggc cctgggctg gaaattgcgc
 121 tggaccgtcg ccttgcctcc cgccgcggcg gggactgcag tgggcgacag atgtgaaaga
 30 181 aacgagttcc agtgccaaga cgggaaatgc atctcctaca agtgggtctg cgatggcagc
 241 gctgagtgcc aggatggctc tgatgagtc caggagacgt gcttgcctgt cacctgcaaa
 301 tccggggact tcagctgtgg gggccgtgtc aaccgctgca ttcctcagtt ctggagggtc
 361 gatggccaag tggactgcga caacggctca gacgagcaag gctgtcccc caagacgtgc
 421 tcccaggacg agtttcgctg ccacgatggg aagtgcatt ctcggcagtt cgtctgtgac
 35 481 tcagaccggg actgcttggg cggtcagac gaggcctcct gccggtgct cacctgtggt
 541 cccgcagct tccagtgcaa cagctccacc tgcattcccc agctgtgggc ctgcgacaac
 601 gaccccgact gcgaagatgg ctccgatgag tggccgcagc gctgtagggg tctttacgtg
 661 ttccaagggg acagtagccc ctgctcgccc ttcgagttcc actgcctaag tggcgagtgc
 721 atccactcca gctggcgtg tgatggtgg cccgactgca aggacaaatc tgacgaggaa
 40 781 aactgcgtg tggccacctg tcgcctgac gaattccagt gctctgatgg aaactgcac
 841 catggcagcc ggcagtgtga ccgggaatat gactgcaagg acatgagcga tgaagtggc
 901 tgcgtaaatg tgacactctg cgagggaccc aacaagtta agtgtcacag cggcgaaatgc
 961 atcacctgg acaaagtctg caacatggct agagactgcc gggactggtc agatgaacc
 1021 atcaaagagt gcgggaccaa cgaatgcttg gacaacaacg gcggtgttc ccacgtctgc

	1081	aatgacctta agatcggtta cgagtgcctg tgccccgacg gcttcagct ggtggcccag
	1141	cgaagatgcg aagatatcga tgagtgtcag gatcccagaca cctgcagcca gctctgctg
	1201	aacctggagg gtggctacaa gtgccagtgt gaggaaggct tccagctgga cccccacacg
	1261	aaggcctgca aggctgtggg ctccatcgcc tacctcttct tcaccaaccg gcacgaggtc
5	1321	aggaagatga cgctggaccg gagcgagtac accagcctca tccccaacct gaggaacgtg
	1381	gtcgctctgg acacggaggt ggccagcaat agaatctact ggtctgacct gtcccagaga
	1441	atgatctgca gcaccagct tgacagagcc caccgctct cttcctatga caccgtcatc
	1501	agcagggaca tccaggcccc cgacgggctg gctgtggact ggatccacag caacatctac
	1561	tggaccgact ctgtcctggg cactgtctct gttgcggata ccaagggcgt gaagaggaaa
10	1621	acgttattca gggagaacgg ctccaagcca agggccatcg tggtgatcc tgttcatggc
	1681	ttcatgtact ggactgactg gggaaactccc gccaaagatca agaaaggggg cctgaatggt
	1741	gtggacatct actcgctggt gactgaaaac attcagtggc ccaatggcat caccctagat
	1801	ctcctcagtg gccgcctcta ctgggttgac tccaaacttc actccatctc aagcatcgat
	1861	gtcaatgggg gcaaccggaa gaccatcttg gaggatgaaa agaggctggc ccacccttc
15	1921	tccttgccg tctttgagga caaagtattt tggacagata tcatcaacga agccattttc
	1981	agtgccaaac gcctcacagg ttccgatgtc aacttgttgg ctgaaaacct actgtcccca
	2041	gaggatatgg tcctcttcca caacctcacc cagccaagag gagtgaactg gtgtgagagg
	2101	accacctga gcaatggcgg ctgccagtat ctgtgcctcc ctgccccgca gatcaacccc
	2161	cactgcccc agtttacctg cgctgcccg gacggcatgc tgctggccag ggacatgagg
20	2221	agctgcctca cagaggctga ggctgcagtg gccaccagg agacatccac cgtcaggcta
	2281	aaggtcagct ccacagccgt aaggacacag cacacaacca cccggcctgt tcccagacc
	2341	tcccggtgc ctggggccac ccctgggctc accacggtgg agatagtac aatgtctcac
	2401	caagctctgg gcgacgttg tggcagagga aatgagaaga agcccagtag cgtgagggt
	2461	ctgtccattg tcctcccat cgtgtcctc gtcttcttt gcctgggggt cttccttcta
25	2521	tggagaact ggcggttaa gaacatcaac agcatcaact ttgacaaccc cgtctatcag
	2581	aagaccacag aggatgaggt ccacatttgc cacaaccagg acggctacag ctaccctcg
	2641	agacagatgg tcagtctgga ggatgacgtg gcgtgaacat ctgctggag tcccgccct
	2701	gccagaacc cttcctgaga cctcgccggc cttgttttat tcaaagacag agaagaccaa
	2761	agcattgcct gccagagctt tgttttatat atttattcat ctgggaggca gaacaggtt
30	2821	cggacagtgc ccatgcaatg gcttgggttg ggattttggt ttcttcttt cctgtgaagg
	2881	ataagagaaa caggcccggt gggaccagga tgacacctc atttctctc aggaagttt
	2941	gagtttctct ccaccgtgac acaatcctca aacatggaag atgaaagggc aggggatgtc
	3001	aggcccagag aagcaagtgg ctttcaacac acaacagcag atggcaccaa cgggaccccc
	3061	tggccctgcc tcatccacca atctetaagc caaacccta aactcaggag tcaacgtgtt
35	3121	tacctttct atgcaagcct tgctagacag ccaggttagc ctttgcctg tcacccccga
	3181	atcatgacct acccagtgtc ttctgaggtg ggtttgtacc ttcttaagc caggaaagg
	3241	attcatggcg tcgaaaatga tctggctgaa tccgtggtgg caccgagacc aaactcattc
	3301	accaaatgat gccacttccc agaggcagag cctgagtcac cggtcacct taatatattat
	3361	taagtgcctg agacaccgg ttaccttggc cgtgaggaca cgtggcctgc acccaggtgt
40	3421	ggctgtcagg acaccagcct ggtgccatc ctcccagccc ctaccactt ccattcccgt
	3481	ggtctccttg cactttctca gttcagagtt gtacactgtg tacatttggc atttgtgtta
	3541	ttattttgca ctgtttctg tcgtgtgtgt tgggatggga tcccaggcca gggaaagccc
	3601	gtgtcaatga atgccgggga cagagagggg caggttgacc gggacttcaa agccgtgatc
	3661	gtgaatatcg agaactgcca ttgtcgtctt tatgtccgcc cactagtgc ttccacttct

3721 atgcaaatgc ctccaagcca ttcacttccc caatcttgtc gttgatgggt atgtgtttaa
 3781 aacatgcacg gtgaggccgg ggcagtgagg ctcacgcctg taatcccagc actttgggag
 3841 gccgaggcgg gtggatcatg aggtcaggag atcgagacca tcttggttaa caaggtgaaa
 3901 ccccgctctc actaaaaata caaaaaatta gccgggagcg gtggtgggca cctgtagtcc
 5 3961 cagctactcg ggaggctgag gcaggagaat ggtgtgaacc cgggaagcgg agcttgagct
 4021 gagccgagat tgcgccactg cagtccgcag tctggcctgg gcgacagagc gagactccgt
 4081 ctcaaaaaaa acaaaacaaa aaaaaacat gcattggtgca tcagcagccc atggcctctg
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 10 4261 aaaaatgaat ttggccagac acaggtgcct cagcctgta atcccagcac tttgggaggg
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 20 4861 aaactgttat cacttatata tatatatata cacatatata taaaatctat ttatttttgc
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 4981 gcctctgaaa tgcctcttct ttatgtacaa agattatttg cacgaactgg actgtgtgca
 5041 acgctttttg ggagaatgat gtccccgttg tatgtatgag tggcttctgg gagatgggtg
 5101 tcacttttta aaccactgta tagaagggtt ttgtagcctg aatgtcttac tgtgatcaat
 25 5161 taaatttctt aaatg

Human LDL receptor proteinsekvens

NP_000518

30 Protein sequence of precursor protein (1-860).

1 mgpwgwkrlw tvalllaaag tavgdrcern efqcqdgkci sykvwcdgsa ecqdgdsdesq
 61 etclsvtcks gdfscggrvn rcipqfwrcd gqvdcnngsd eqgcpkctcs qdefrchdggk
 121 cisrqfvcds drdclgdsde ascpvltcgp asfqcnstc ipqlwacdnd pdcedgsdew
 35 181 pqrccrglyvf qgdsspscaf efhclsgeci hsswrcdggp dckdksdeen cavatcrpde
 241 fqcsdgnclh gsrqcdreyd ckdmdevgc vnvltcegnp kfkchsgeci tldkvcnmar
 301 dcrdwsdepi kecgtnecl d nnggcshvcn dlkigyecld pdgqqlvaqr rcedidecqd
 361 pdtcsqlcvn leggykcqce egfqlphtk ackavgsiay lfftnrhevr kmtldrseyt
 421 slipnlrnvv aldtevasnr iywsdlsqrm icstqldrah gvssydtvis rdiqapdgla
 40 481 vdwihsniyw tdsvlgtvsv adtkgvkrkt lfrenskpr aivvdpvhgf mywtldwgtpa
 541 kikkgglnv diyslvteni qwpngitld lsgrylwvds klhsissidv nggnrktil
 601 dekrlahpfs lavfedkvfw tdiineaifs anrltgsdvn llaenllspe dmvlfhnlqt
 661 prgvnwcert tlnsggcqyl clpapqinph spkftcacpd gmlardmrs clteaaava
 721 tqetstvrk vsstavrtqh ttrpvpdts rlpqatpglt tveivtmshq algdvagrgn
 45 781 ekkpssvral sivilpivllv flclgvflw knwrlknins infdnpyyqk ttedevhich

841 nqdgysypsr qmvsleddva

ShRNA sequences

5 SiRNAs with target sites i porcine LDL receptor mRNA sequence.

	Startbase in porcine cDNA AF065990 sequence	Sequence
Target 1	763	TGTCAAAGCGGCGAGTGCA
Target 2	889	TCCCATATCTGCAATGACC
Target 3	1150	ACCCTGGACCGTAGTGAGT
Target 4	1308	TGACACCATTATTGGCGAA
Target 5	1309	GACACCATTATTGGCGAAG
Target 6	1439	AGACTCTCTTCCAAGAGAA
Target 7	1553	TGAACGGAGTGGACGTCTA
Target 8	1814	TCACAGGCTCGGACATACA

pSUPER.retro.puro sequence

10 Used restriction sites

BglII: 1447

XhoI: 1420

Numbers refer to the original pSuper.retro.puro sequence

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pSUPER retro.puro SEQUENCE:

1 TGAAAGACCC CACCTGTAGG TTTGGCAAAGC TAGCTTAAAGT AACGCCATTT TGCAAGGCAT
 61 GGAAAATACA TAACTGAGAA TAGAGAAAGTT CAGATCAAGG TTAGGAACAG AGAGACAGCA
 20 121 GAATATGGGC CAAACAGGAT ATCTGTGGTA AGCAGTTCCT GCCCCGGCTC AGGGCCAAGA
 181 ACAGATGGTC CCCAGATGCG GTCCCGCCCT CAGCAGTTTC TAGAGAACCA TCAGATGTTT
 241 CCAGGGTGCC CCAAGGACCT GAAATGACCC TGTGCCTTAT TTGAACTAAC CAATCAGTTC
 301 GCTTCTCGCT TCTGTTTCGCG CGCTTCTGCT CCCCAGAGCTC AATAAAAAGAG CCCACAACCC
 361 CTCACCTGGC GCGCCAGTCC TCCGATAGAC TGCGTCGCCC GGGTACCCGT ATTCCCAATA
 25 421 AAGCCTCTTG CTGTTTGCAT CCGAATCGTG GACTCGCTGA TCCTTGGGAG GGTCTCCTCA
 481 GATTGATTGA CTGCCCACCT CGGGGGTCTT TCATTTGGAG GTTCCACCGA GATTTGGAGA
 541 CCCCTGCCCA GGGACCACCG ACCCCCCCGC CGGGAGGTAA GCTGGCCAGC GGTCTTTTCG
 601 TGTCTGTCTC TGTCTTTGTG CGTGTTTGTG CCGGCATCTA ATGTTTGC GCCTGCTGT
 661 ACTAGTTAGC TAACTAGCTC TGTATCTGGC GGACCCGTGG TGGAACTGAC GAGTTCTGAA
 30 721 CACCCGCGCG CAACCCTGGG AGACGTCCCA GGGACTTTGG GGGCCGTTTT TGTGGCCCGA
 781 CCTGAGGAAG GGAGTCGATG TGGAATCCGA CCCCCTCAGG ATATGTGGTT CTGGTAGGAG
 841 ACGAGAACCT AAAACAGTTC CCGCCTCCGT CTGAATTTTT GCTTTCGGTT TGGAACCGAA
 901 GCCGCGCGTC TTGTCTGCTG CAGCGCTGCA GCATCGTTCT GTGTTGTCTC TGTCTGACTG
 961 TGTTTCTGTA TTTGTCTGAA AATTAGGGCC AGACTGTIAC CACTCCCTTA AGTTTGACCT
 35 1021 TAGGTCACTG GAAAGATGTC GAGCGGATCG CTCACAACCA GTCGGTAGAT GTCAAGAAGA
 1081 GACGTTGGGT TACCTTCTGC TCTGCAGAAT GGCCAACCTT TAACGTCGGA TGGCCGCGAG
 1141 ACGGCACCTT TAACCGAGAC CTCATCACCC AGGTAAAGAT CAAGGTCTTT TCACCTGGCC
 1201 CGCATGGACA CCCAGACCAG GTCCCTTACA TCGTGACCTG GGAAGCCTTG GCTTTTGACC
 1261 CCCCTCCCTG GGTCAAGCCC TTTGTACACC CTAAGCCTCC GCCTCCTCTT CCTCCATCCG
 40 1321 CCCCGTCTCT CCCCTTGAA CCTCCTCGTT CGACCCCGCC TCGATCCTCC CTTTATCCAG
 1381 CCCTCACTCC TTCTCTAGGC GCCGGAATTA GATCGATCTC

a) original pSUPER.retro.puro sequence

TCGAGGTCGA CGGTATCGAT AAGCTTA

45

b) After cleavage (BglII og XhoI) and insertion of T1 DNA oligo

TCGAGAAAAA TGTCAAAGCG GCGAGTGCAT TCAAGAGATG CACTCGCCGC
TTTGACAGGG

c) Similar for for T2-T8

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GAT CTGTGGTCTC ATACAGAACT TATAAGATT CCAAATCCAA AGACATTTC A
1501 CGTTTATGGT GATTTCCAG AACACATAGC GACATGCAAA TATTGCAGGG CGCCACTCCC
1561 CTGTCCCTCA CAGCCATCTT CCTGCCAGGG CGCACGCGCG CTGGGTGTTC CCGCCTAGTG
1621 AACTGGGCC CGCGATTCTT TGGAGCGGGT TGATGACGTC AGCGTTCGAA TTCTACCGGG
1681 TAGGGGAGGC GCTTTTCCCA AGGCAGTCTG GAGCATGCGC TTTAGCAGCC CCGCTGGGCA
1741 CTTGGCGCTA CACAAGTGGC CTCTGGCCTC GCACACATTC CACATCCACC GGTAGGCGCC
1801 AACCGGCTCC GTTCTTTGGT GGCCCTTCG CGCCACCTTC TACTCTCCC CTAGTCAGGA
1861 AGTTCCCCC CGCCCCGAG CTCGCGTCGT GCAGGACGTG ACAAATGGAA GTAGCACGTC
1921 TCACTAGTCT CGTGCAGATG GACAGACCG CTGAGCAATG GAAGCGGGTA GGCTTTGGG
1981 GCAGCGGCCA ATAGCAGCTT TGCTCCTTCG CTTTCTGGGC TCAGAGGCTG GGAAGGGGTG
2041 GGTCCGGGGG CGGGCTCAGG GGCGGGCTCA GGGGCGGGGC GGGCGCCCGA AGGTCTCCG
2101 GAGGCCCGGC ATTCTGCACG CTTCAAAAGC GCACGTCTGC CGCGCTGTTC TCCTCTTCCT
2161 CATCTCCGGG CTTTTCGACC TGCAGCCCAA GCTAGCTTAC CATGACCGAG TACAAGCCCA
2221 CGGTGCGCCT CGCCACCCGC GACGACGTCC CCAGGGCCGT ACGCACCTTC GCCGCCCGT
2281 TCGCCGACTA CCCC GCCACG CGCCACACCG TCGATCCGGA CCGCCACATC GAGCGGGTCA
2341 CCGAGCTGCA AGAACTCTTC CTCACGCGCG TCGGGCTCGA CATCGGCAAG GTGTGGGTG
2401 CGGACGACGG CGCCGCGGTG GCGGTCTGGA CCACGCCGGA GAGCGTCGAA GCGGGGGCGG
2461 TGTTCCGCCA GATCGGCCCC CGCATGGCCG AGTTGAGCGG TTCCCGGCTG GCCGCGCAGC
2521 AACAGATGGA AGGCCTCCTG GCGCCGACC GGCCCAAGGA GCGCGCTGG TTCCTGGCCA
2581 CCGTCGGCGT CTCGCCCCGAC CACCAGGGCA AGGGTCTGGG CAGCGCCGTC GTGCTCCCCG
2641 GAGTGGAGGC GGCCGAGCGC GCCGGGGTGC CCGCTTCTT GGAGACCTCC GCGCCCCGCA
2701 ACCTCCCTT CTACGAGCGG CTCGGCTTCA CCGTCACCGC CGACGTCGAG GTGCCCCAAG
2761 GACCGCGCAC CTGGTGCATG ACCCGCAAGC CCGGTGCCTG ACGCCCGCCC CACGACCCGC
2821 AGCGCCCGAC CGAAAGGAGC GCACGACCCC ATGCATCGAT AAAATAAAAG ATTTTATTTA
2881 GTCTCCAGAA AAAGGGGGA ATGAAAGACC CCACCTGTAG GTTTGGCAAG CTAGAGAACC
2941 ATCAGATGTT TCCAGGGTGC CCCAAGGACC TGAAATGACC CTGTGCCTTA TTTGAATAA
3001 CCAATCAGTT CGCTTCTCGC TTCTGTTCGC GCGCTTCTGC TCCCGAGCT CAATAAAAGA
3061 GCCACAACC CCTCACTCGG CGCGCCAGTC CTCCGATAGA CTGCGTCGCC CGGGTACCCG
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 Start codon
 FRT site
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 Puro

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 gtgagtactcaaccaagtcattctgagaatagtgatgcggcgaccgagttgctcttggccggcgtcaatacgggataata
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 45 gcgtatcacgaggcccttcgtc

pSBT/SV40-GFIP, sequence

50 SB inverted repeats
 SV40 promoter
 Start codon

FRT site
eGFP
Puro

5 tcgcgcggttcggtgatgacgggtgaaaacctctgacacatgcagctcccggagacgggtcacagcttgctgtgaagcggat
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 tc

pSBT/SV40-GFIP.loxP, sequence

SB inverted repeats
 35 SV40 promoter
 Start codon
 FRT site
 eGFP
 Puro
 40 tcgcgcgtttcgggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaagcggat
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PCSK9 human coding sequence preceded by a Kozak sequence and constructed with a C-terminal FLAG tag.

[illegible]

[illegible]

cctgtcgtgccagctgcattaatgaatcgGCCAACGCGCGGGGAGAGGCGGTTTgcgtattgggcgctctccgcttctc
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 actcttcttttcaatattatgaagcatttatcagggttattgtctcatgagcggatacatattgaatgtatttagaaaaataaa
 25 caaataggggttcgcgcacatttccccgaaaagtgccacctgacgtctaagaaaccattattatcatgacattaacctat
 aaaaataggcgatcacgagggcccttctgc
 Left inverted repeat

hAAT – human alpha-antitrypsin promoter
 30 PCSK9 – Coding sequence for proprotein convertase subtilisin/kexin type 9 (PCSK9)
 with C-terminal FLAG-tag
 BGH pA – Bovine growth hormone polyA region
 PGK – phosphoglycerate kinase promoter
 Puro – Coding sequence for puromycin-N-acetyl transferase (PAC)
 35 Right inverted repeat

pSBT/cHS4.H1p.PGK-puro.U6p.cHS4, sequence
 SB inverted repeat
 40 cHS4 insulator
 H1 promoter with linker (reverse orientation)
 PGK promoter
 Puro ORF
 U6 promoter with linker
 45

tcgcgcgtttcgggtgatgacggtgaaaacctctgacacatgcagctcccgagacgggtcacagcttgtctgtaagcggat
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Claims

1. A genetically modified pig as a model for studying atherosclerosis, wherein the pig model expresses at least one phenotype associated with said disease
5 and/or
a modified pig comprising at least one mutation in the
i. endogenous ApoE gene or part thereof and/or LDL gene
or part thereof, and/or
ii. endogenous ApoE gene or part thereof and/or
10 iii. endogenous LDL receptor gene or part thereof,
transcriptional and/or translational product or part thereof
and/or
a modified pig comprising at least one human and/or porcine PCKS9 gene or part
thereof,
15 transcriptional and/or translational product or part thereof.
2. The modified pig according to claim 1, wherein the pig is a mini-pig.
3. The modified pig according to claim 2, wherein the mini-pig is selected from the
20 group consisting of Goettingen, Yucatan, Bama Xiang Zhu, Wuzhishan and Xi
Shuang Banna, including any combination thereof.
4. The modified pig according to claim 3, wherein the pig is a Yucatan minipig.
- 25 5. The modified pig according to claim 1, wherein the pig is not a mini-pig.
6. The modified pig according to claim 1, wherein the pig belongs to the species of
S. domesticus.
- 30 7. The modified pig according to claim 5, wherein the pig is selected from the group
consisting of Landrace, Yorkshire, Hampshire, Duroc, Chinese Meishan,
Berkshire and Piêtrain, including any combination thereof.
8. The modified pig according to claim 1, wherein the pig is an inbred pig.
35

9. The modified pig according to any of the preceeding claims, wherein said pig comprises at least one mutation in an endogenous ApoE and/or LDL gene or part thereof, transcriptional and/or translational product or part thereof.
- 5 10. The modified pig according to any of the preceeding claims, wherein said pig comprises at least one mutation in an endogenous ApoE gene or part thereof, transcriptional and/or translational product or part thereof.
- 10 11. The modified pig according to any of the preceeding claims, wherein said pig comprises at least one mutation in an endogenous LDL gene or part thereof, transcriptional and/or translational product or part thereof.
- 15 12. The modified pig according to any of claims 1 to 11, wherein said mutation is introduced into the endogenous porcine ApoE or LDL gene by homologous recombination.
- 20 13. The modified pig according to any of claims 1 to 11, wherein said mutation is introduced into the endogenous porcine ApoE or LDL gene, transcriptional and/or translational product or part thereof by siRNA.
- 25 14. The modified pig according to any of the preceding claims, wherein said pig comprises at least one human and/or porcine PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.
- 30 15. A genetically modified porcine blastocyst derived from the genetically modified pig model as defined in claim 1
and/or
a modified porcine blastocyst comprising at least one mutation in the
- i. endogenous ApoE gene or part thereof and/or LDL gene or part thereof, and/or
 - ii. endogenous ApoE gene or part thereof and/or
 - iii. endogenous LDL receptor gene or part thereof,
- transcriptional and/or translational product or part thereof.
and/or

a modified blastocyst comprising at least one human and/or porcine PCKS9 gene or part thereof,
transcriptional and/or translational product or part thereof.

- 5 16. A genetically modified porcine embryo derived from the genetically modified pig model as defined in claim 1
and/or

a modified porcine embryo comprising at least one mutation in the

- 10 i. endogenous ApoE gene or part thereof and/or LDL gene
or part thereof, and/or
 ii. endogenous ApoE gene or part thereof and/or
 iii. endogenous LDL receptor gene or part thereof,

transcriptional and/or translational product or part thereof.

and/or

- 15 a modified embryo comprising at least one human and/or porcine PCKS9 gene or part thereof, transcriptional and/or translational product or part thereof.

17. A genetically modified porcine fetus derived from the genetically modified pig model as defined in claim 1

20 and/or

a modified porcine fetus comprising at least one mutation in the

- i. endogenous ApoE gene or part thereof and/or LDL gene
or part thereof, and/or
 ii. endogenous ApoE gene or part thereof and/or
25 iii. endogenous LDL receptor gene or part thereof,

transcriptional and/or translational product or part thereof.

and/or

a modified fetus comprising at least one human and/or porcine PCKS9 gene or part thereof, transcriptional and/or translational product or part thereof.

30

18. A genetically modified porcine donor cell and/or cell nucleus derived from the genetically modified pig model as defined in claim 1

and/or

a modified porcine donor cell and/or cell nucleus comprising at least one mutation
35 in the

- i. endogenous ApoE gene or part thereof and/or LDL gene or part thereof, and/or
 - ii. endogenous ApoE gene or part thereof and/or
 - iii. endogenous LDL receptor gene or part thereof,
 - transcriptional and/or translational product or part thereof.
 - and/or
 - a modified donor cell comprising at least one human and/or porcine PCSK9 gene or part thereof, transcriptional and/or translational product or part thereof.
- 10 19. The modified pig according to any of the preceding claims, wherein said at least one phenotype is hypercholesterolemia.
20. The modified pig according to claim 19, wherein said hypercholesterolemia is characterized by an at least 10% increase in total cholesterol level in the plasma
- 15 as compared to a standard level of the pig.
21. The modified pig according to any of the preceding claims, wherein said at least one phenotype is atherosclerosis.
- 20 22. The modified pig according to claim 21, wherein said atherosclerosis is determined by intravascular ultrasound, PET scanning, CT scanning and/or MR scanning.
23. The genetically modified pig model, porcine blastocyst, embryo, fetus and/or donor cell according to any of claims 1 to 22 obtainable by nuclear transfer comprising the steps of
- 25
- i) establishing at least one oocyte having at least a part of a modified zona pellucida,
 - ii) separating the oocyte into at least two parts obtaining an oocyte

30 having a nucleus and at least one cytoplasm,

 - iii) establishing a donor cell or cell nucleus with desired genetic properties,
 - iv) fusing at least one cytoplasm with the donor cell or membrane surrounded cell nucleus,

35 v) obtaining a reconstructed embryo,

- vi) activating the reconstructed embryo to form an embryo;
culturing said embryo; and
vii) transferring said cultured embryo to a host mammal such that the
embryo develops into a genetically modified fetus,
5 wherein said genetically modified embryo obtainable by nuclear transfer
comprises steps i) to v) and/or vi),
wherein said genetically modified blastocyst obtainable by nuclear transfer
comprises steps i) to vi) and/or vii),
wherein said genetically modified fetus obtainable by nuclear transfer comprises
10 steps i) to vii).
24. A method for producing a transgenic pig, porcine blastocyst, embryo, fetus and/or
donor cell as a model for atherosclerosis comprising:
i) establishing at least one oocyte
15 ii) separating the oocyte into at least three parts obtaining at least one
cytoplasm,
iii) establishing a donor cell or cell nucleus having desired genetic
properties,
iv) fusing at least one cytoplasm with the donor cell or membrane
20 surrounded cell nucleus,
v) obtaining a reconstructed embryo,
vi) activating the reconstructed embryo to form an embryo;
culturing said embryo; and
vii) transferring said cultured embryo to a host mammal such that the
25 embryo develops into a genetically modified fetus
wherein said genetically modified embryo obtainable by nuclear transfer
comprises steps i) to v) and/or vi),
wherein said genetically modified blastocyst obtainable by nuclear transfer
comprises steps i) to vi) and/or vii),
30 wherein said genetically modified fetus obtainable by nuclear transfer comprises
steps i) to vii).
25. The method according to claim 23 or 24, said method comprising one or more of
the features as defined in any of the preceding claims, wherein the method for
35 activation of the reconstructed embryo is selected from the group of methods

consisting of electric pulse, chemically induced shock, increasing intracellular levels of divalent cations and reducing phosphorylation.

5 26. The method according to claim 23 or 24, said method comprising one or more of the features as defined in any of the preceding claims, wherein steps d) and f) are performed sequentially or simultaneously.

10 27. The method according to claim 23 or 24, said method comprising one or more of the features as defined in any of the preceding claims, wherein the embryo is cultured in vitro.

28. The method according to claim 27, wherein the embryo is cultured in sequential culture.

15 29. The method according to claim 23 or 24, said method comprising one or more of the features as defined in any of the preceding claims, wherein the embryo is cryopreserved prior to transfer to a host mammal.

20 30. The method according to claim 29, wherein the embryo is at a blastocyst stage.

31. The method of claim 23 or 24, wherein the pig is not a mini-pig.

25 32. The method of claim 23 or 24, wherein the pig belongs to the species of *S. domesticus*.

33. The method of claim 23 or 24, wherein the pig is selected from the group consisting of Landrace, Yorkshire, Hampshire, Duroc, Chinese Meishan, Berkshire and Piétrain, including any combination thereof.

30 34. The method of claim 23 or 24, wherein the pig is an inbred pig.

35. The method of claim 23 or 24, wherein the pig is a mini-pig.

36. The method of claim 35, wherein the mini-pig is selected from the group consisting of Goettingen, Yucatan, Bama Xiang Zhu, Wuzhishan and Xi Shuang Banna, including any combination thereof.
- 5 37. The method of claim 36, wherein said minipig is a Yucatan minipig.
38. A method for evaluating the effect of a therapeutical treatment of atherosclerosis, said method comprising the steps of
- 10 i) providing the pig model according to any of claims 1 to 22,
 ii) treating said pig model with a pharmaceutical composition exerting an effect on said phenotype, and
 iii) evaluating the effect observed.
39. The method of claim 38 comprising the further step of advising on medical
- 15 treatment based on the afore-mentioned observed effects.
40. A method for screening the efficacy of a pharmaceutical composition, said method comprising the steps of
- 20 i) providing the pig model according to any of claims 1 to 22,
 ii) expressing in said pig model said genetic determinant and exerting said phenotype for said disease,
 iii) administering to said pig model a pharmaceutical composition the efficacy of which is to be evaluated, and
 iv) evaluating the effect, if any, of the pharmaceutical composition on the
- 25 phenotype exerted by the genetic determinant when expressed in the pig model.
41. A method for treatment of a human being suffering from atherosclerosis, said method comprising the initial steps of
- 30 i) providing the pig model according to any of claims 1 to 22,
 ii) expressing in said pig model said genetic determinant and exerting said phenotype for said disease,
 iii) administering to said pig model a pharmaceutical composition the efficacy of which is to be evaluated, and
- 35 iv) evaluating the effect observed, and

v) treating said human being suffering from atherosclerosis based on the effects observed in the pig model.

5 42. The method according to any of claims 38 to 41, wherein said genetic determinant is as defined in any of claims 9 to 14.

 43. The method according to any of claims 38 to 41, wherein said phenotype is as defined in any of claims 19 to 22.

10

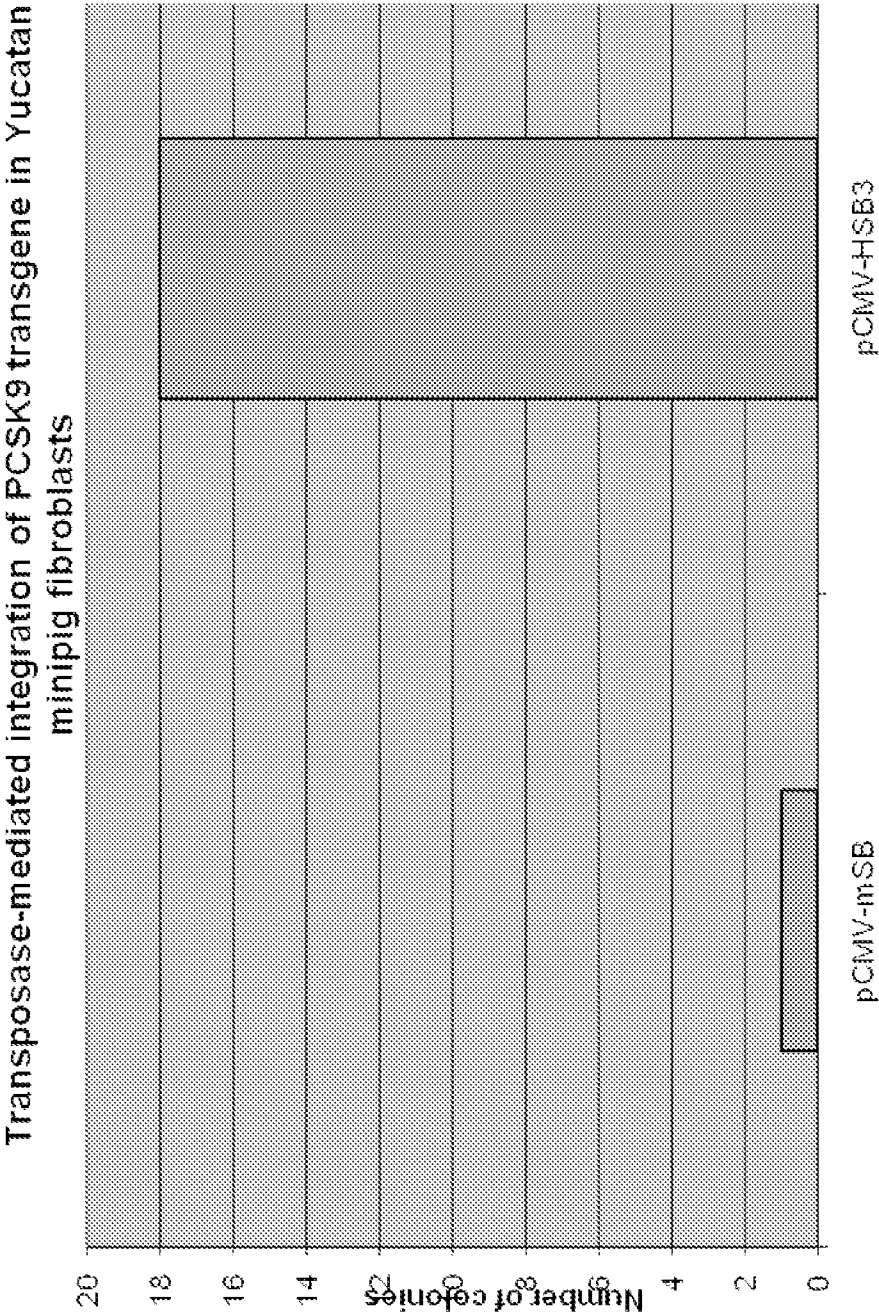


Fig. 1

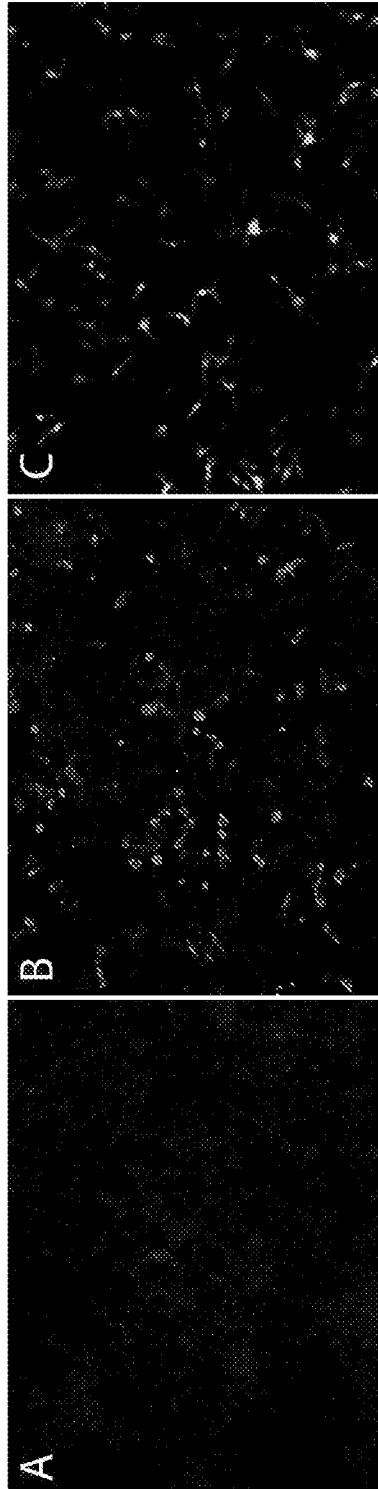


Fig. 2

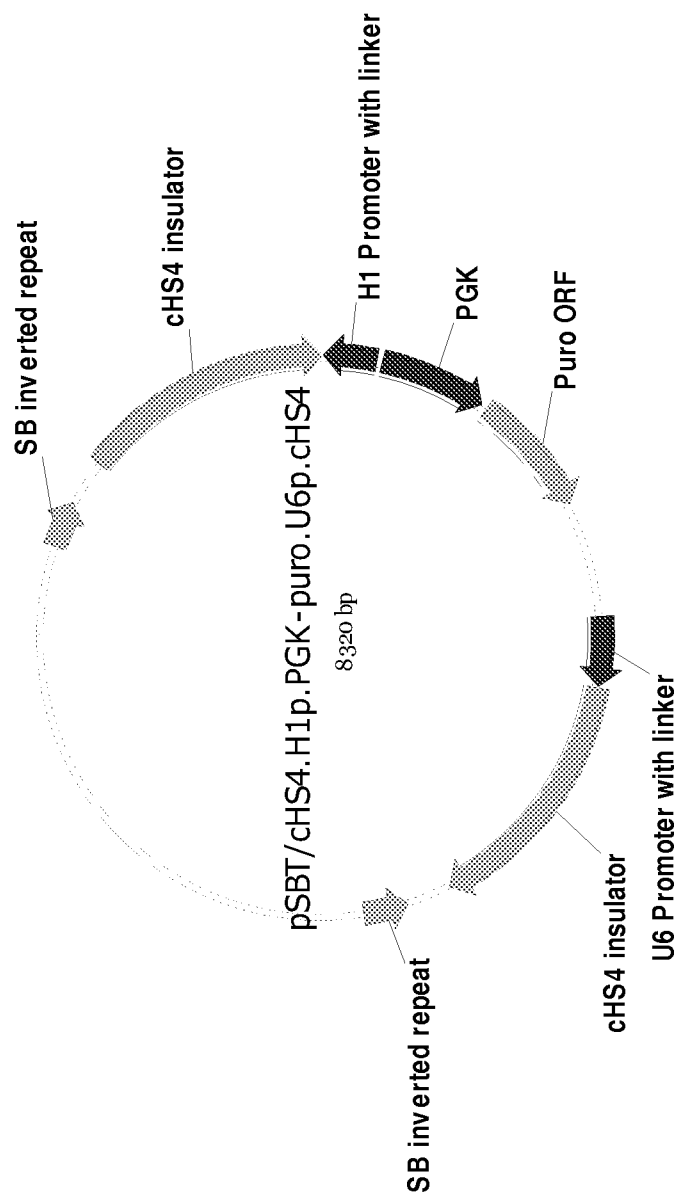


Fig. 3

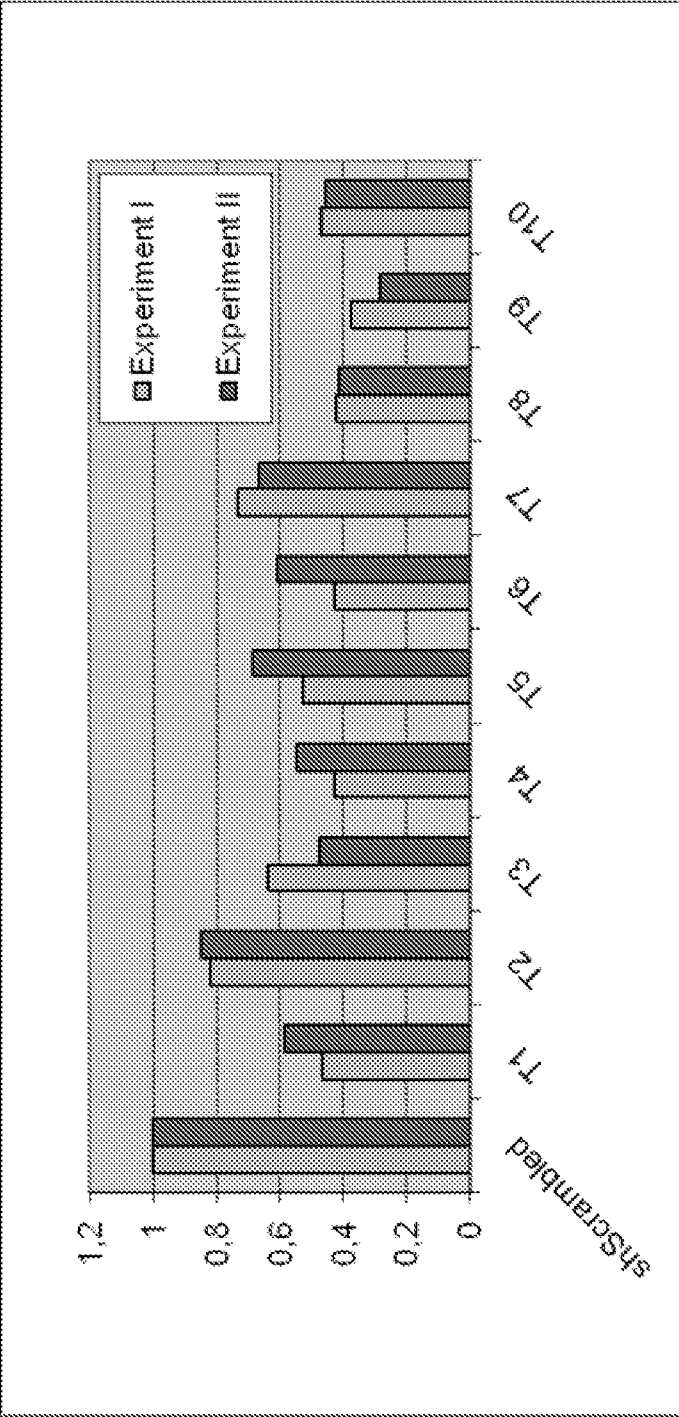


Fig. 4

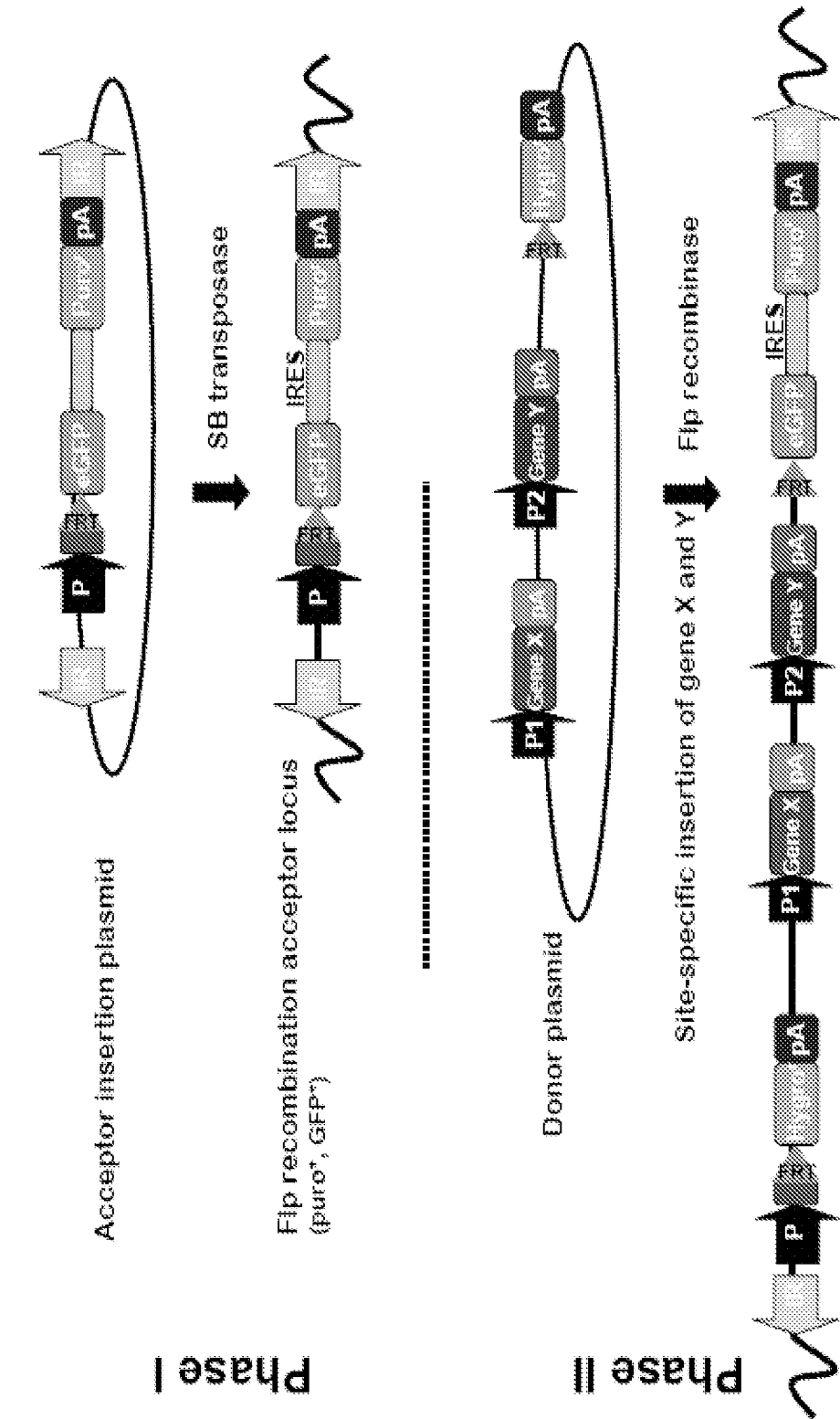


Fig. 5

Map of pSBT/RSV-GFIP

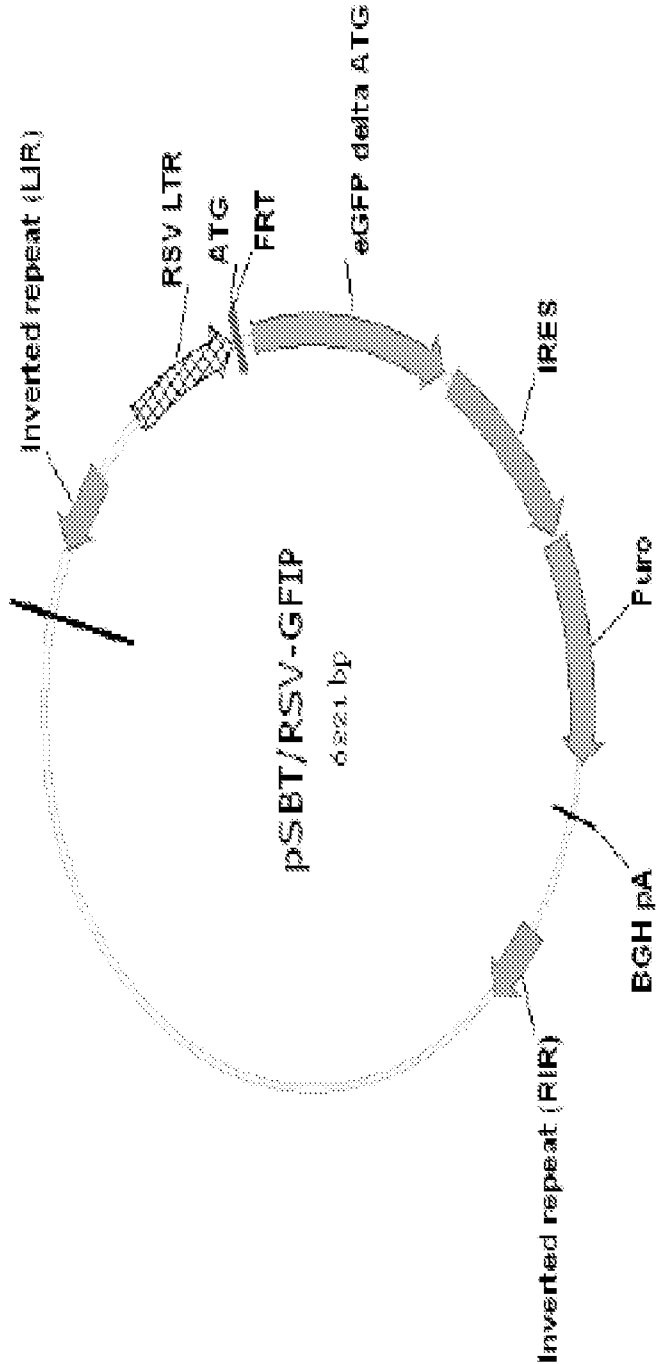


Fig. 6

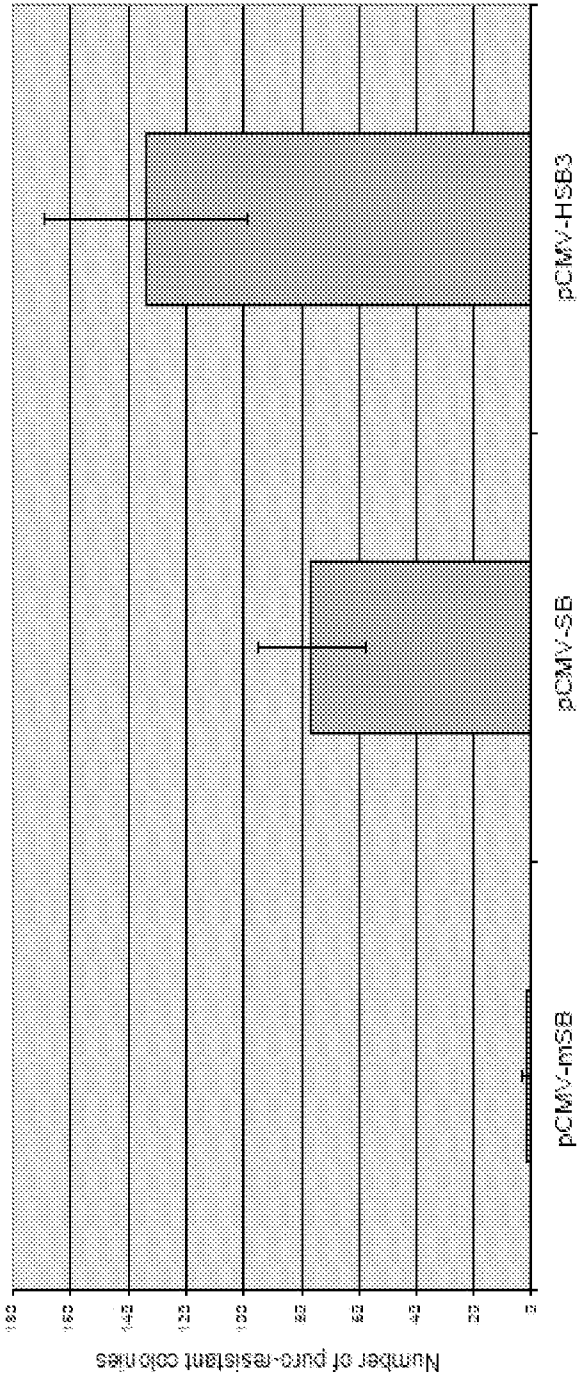
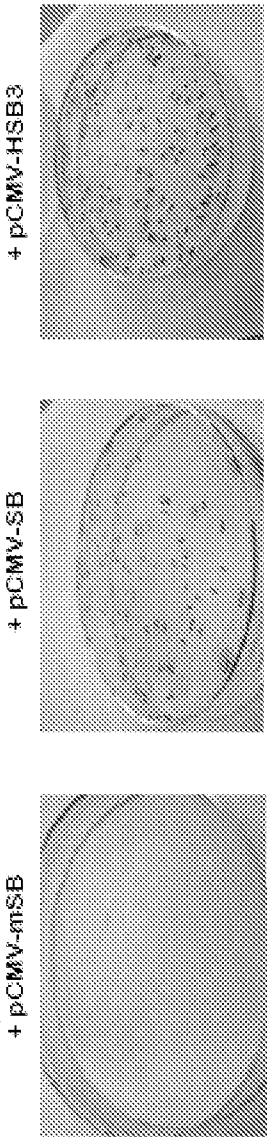


Fig. 7

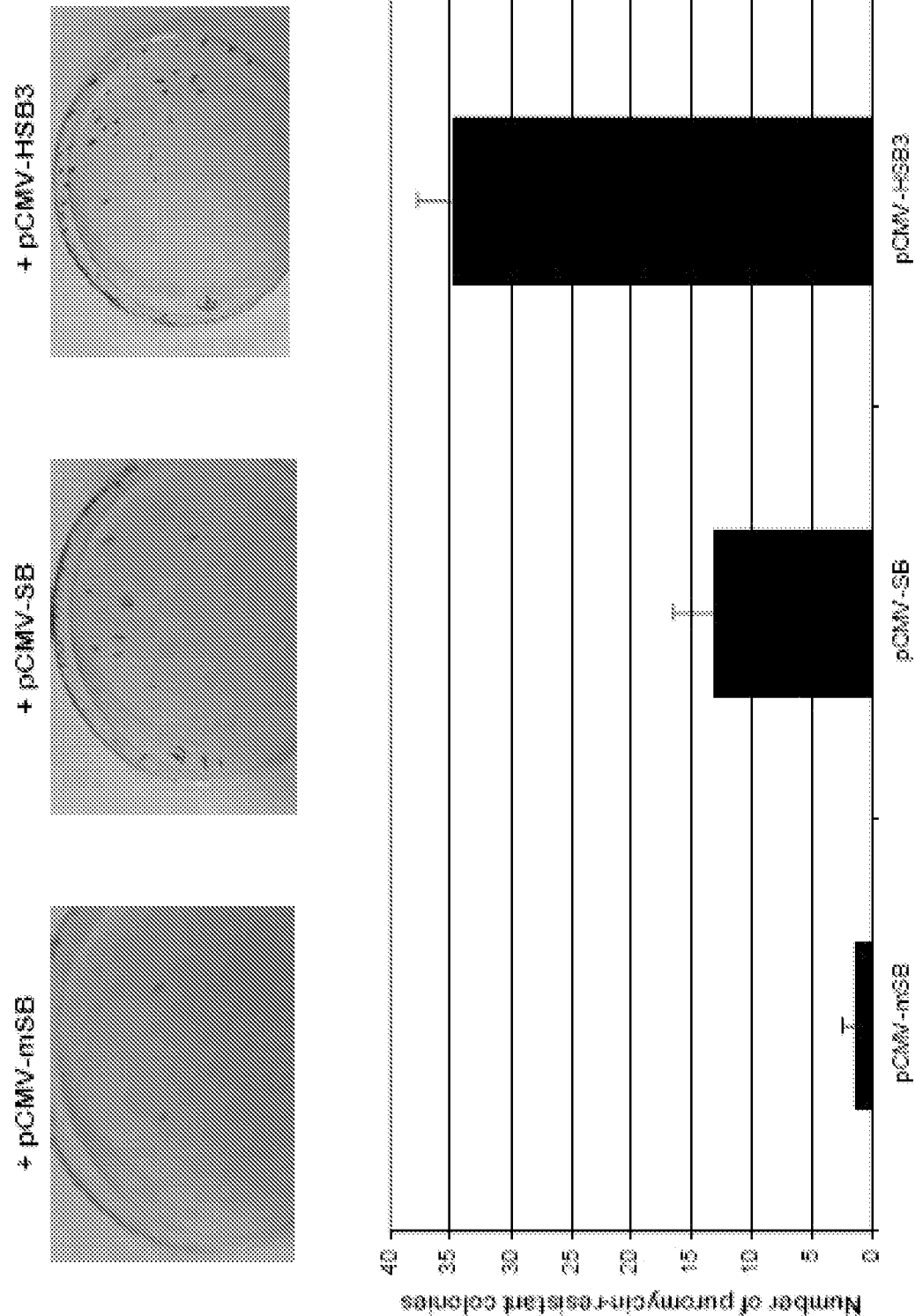


Fig. 8

9 / 15

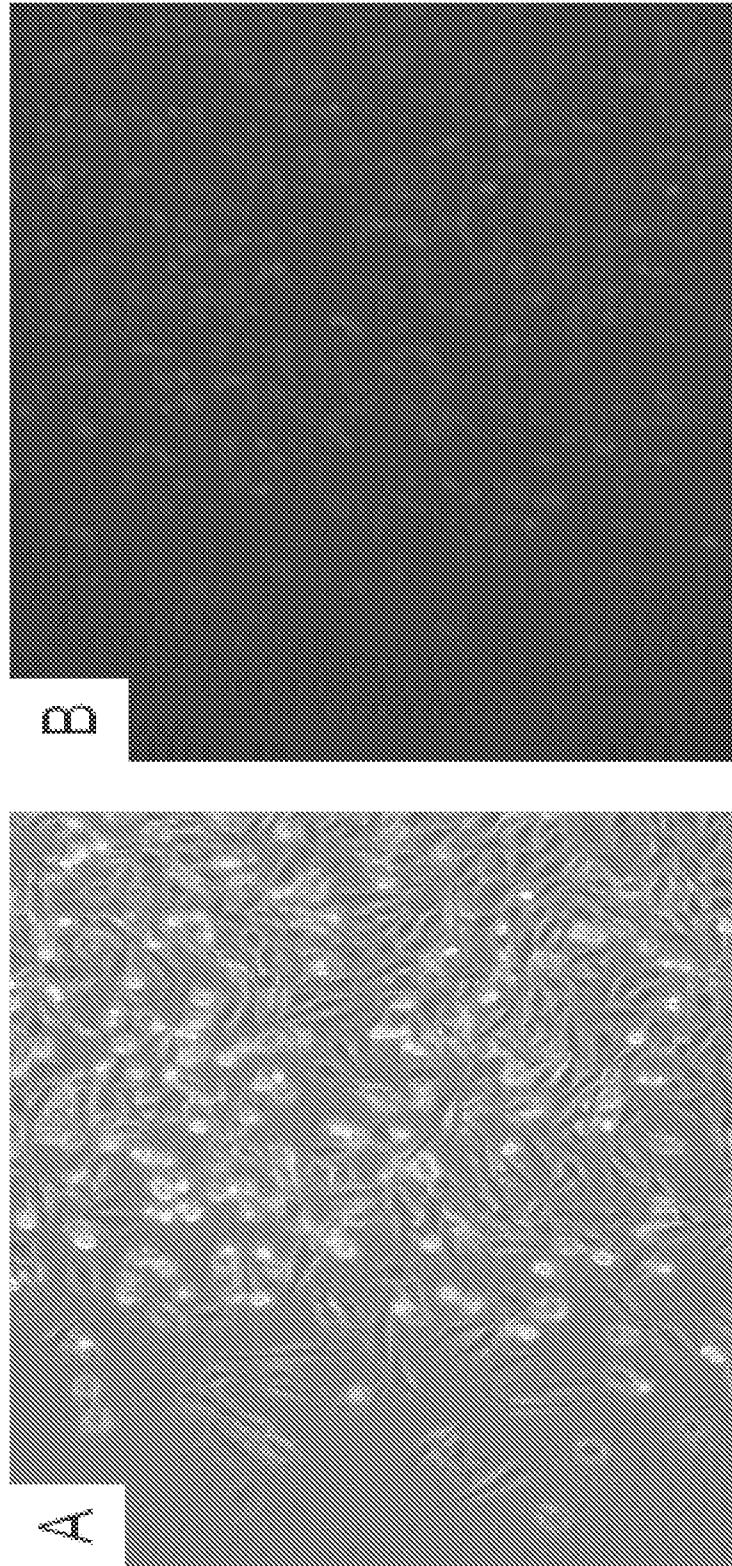


Fig. 9

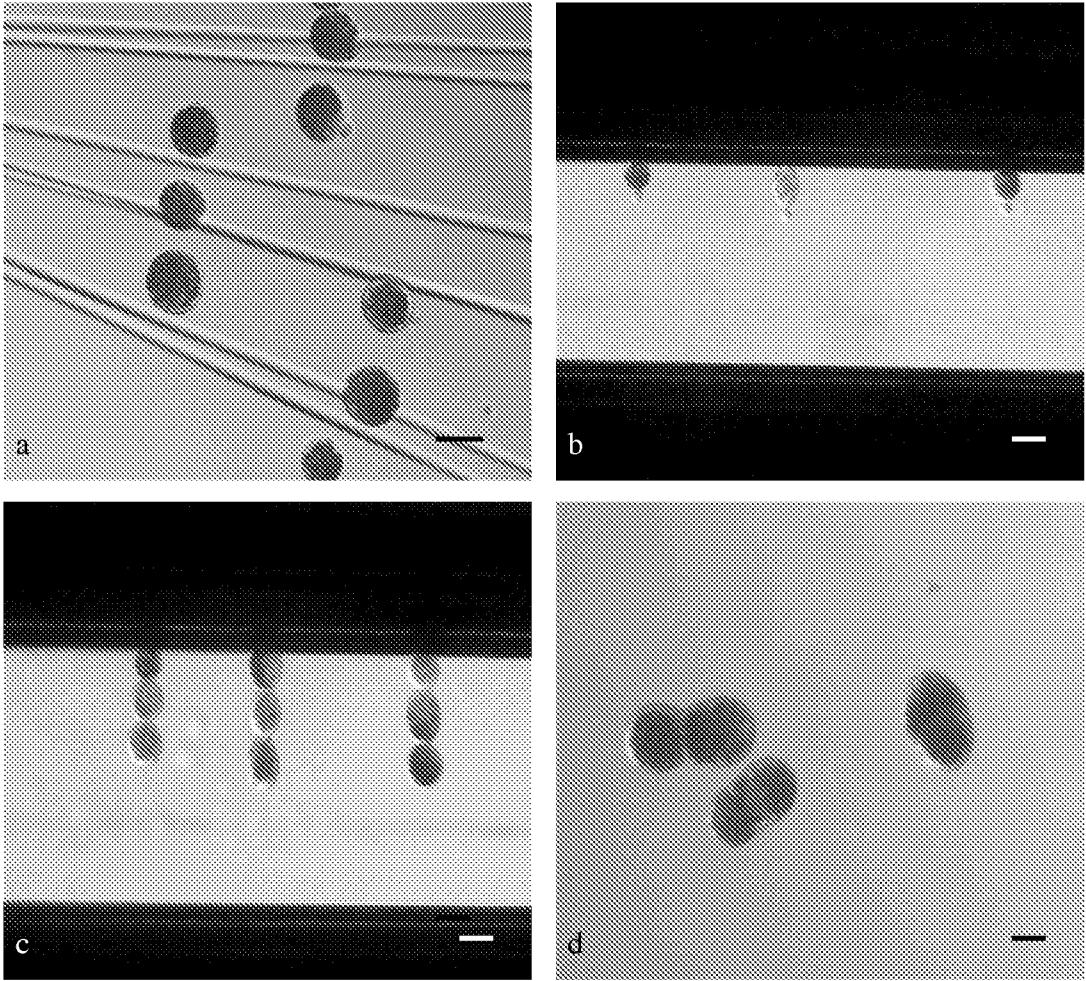


Fig. 10

11 / 15

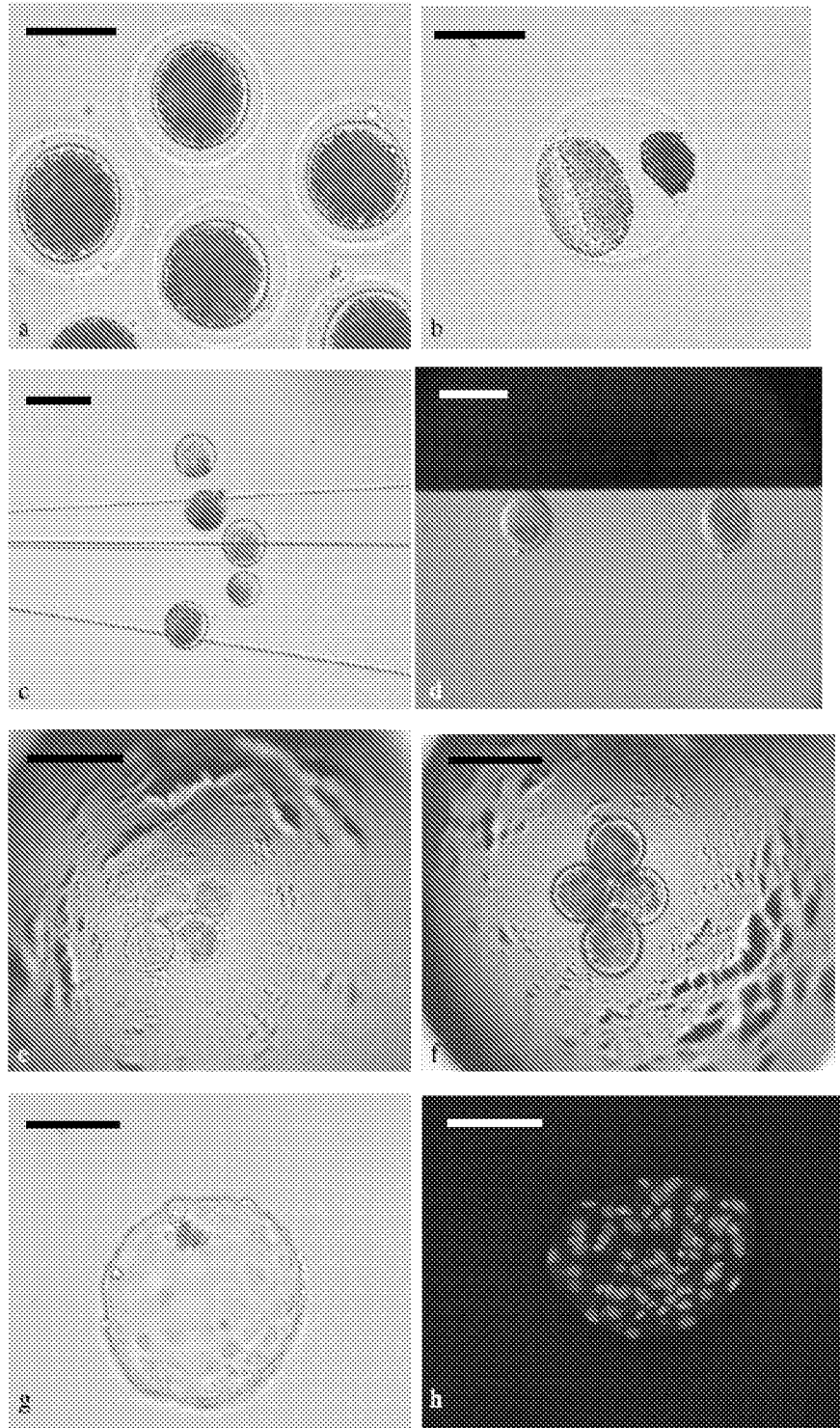


Fig. 11

12 / 15

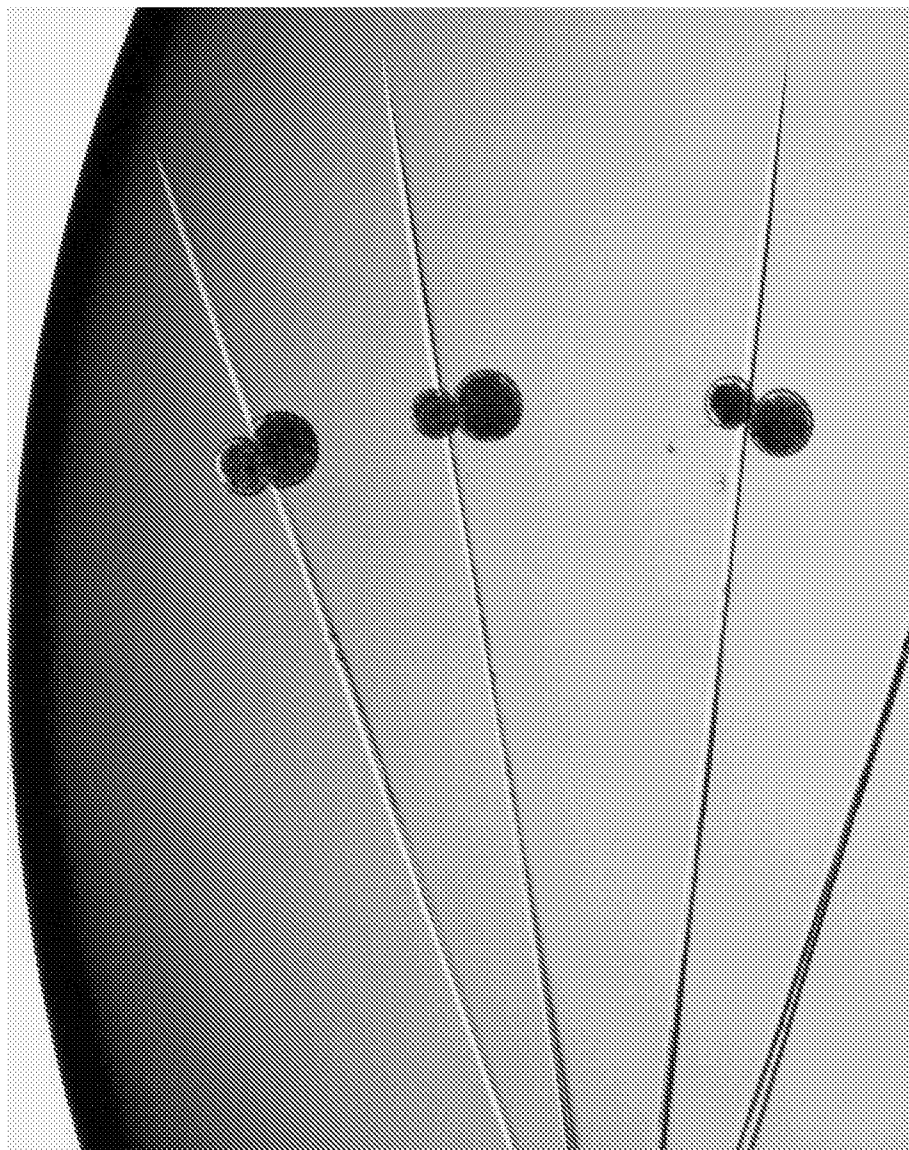
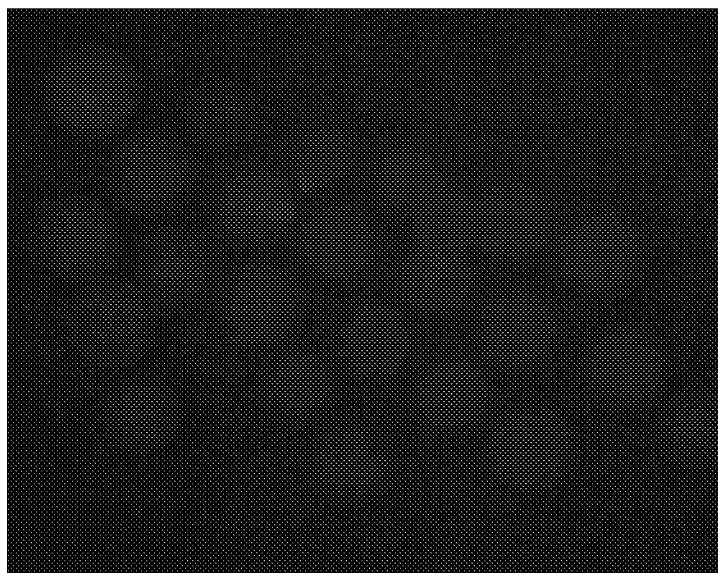


Fig. 12

13 / 15

A



B

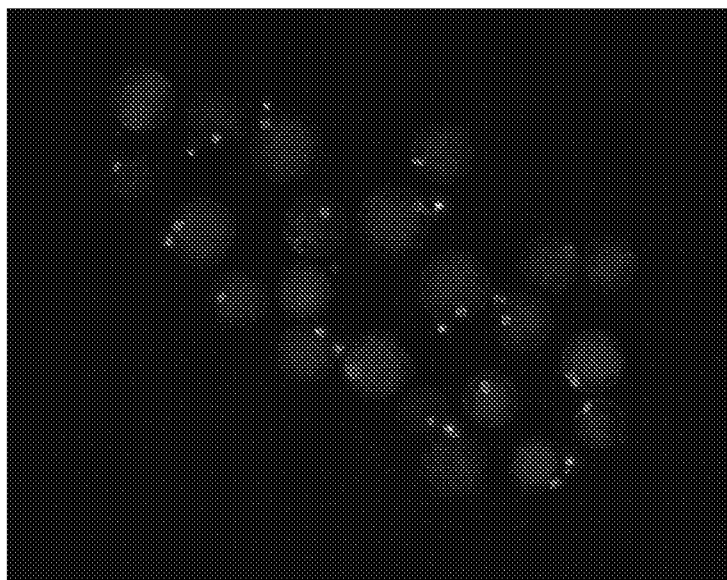


Fig. 13

14 / 15

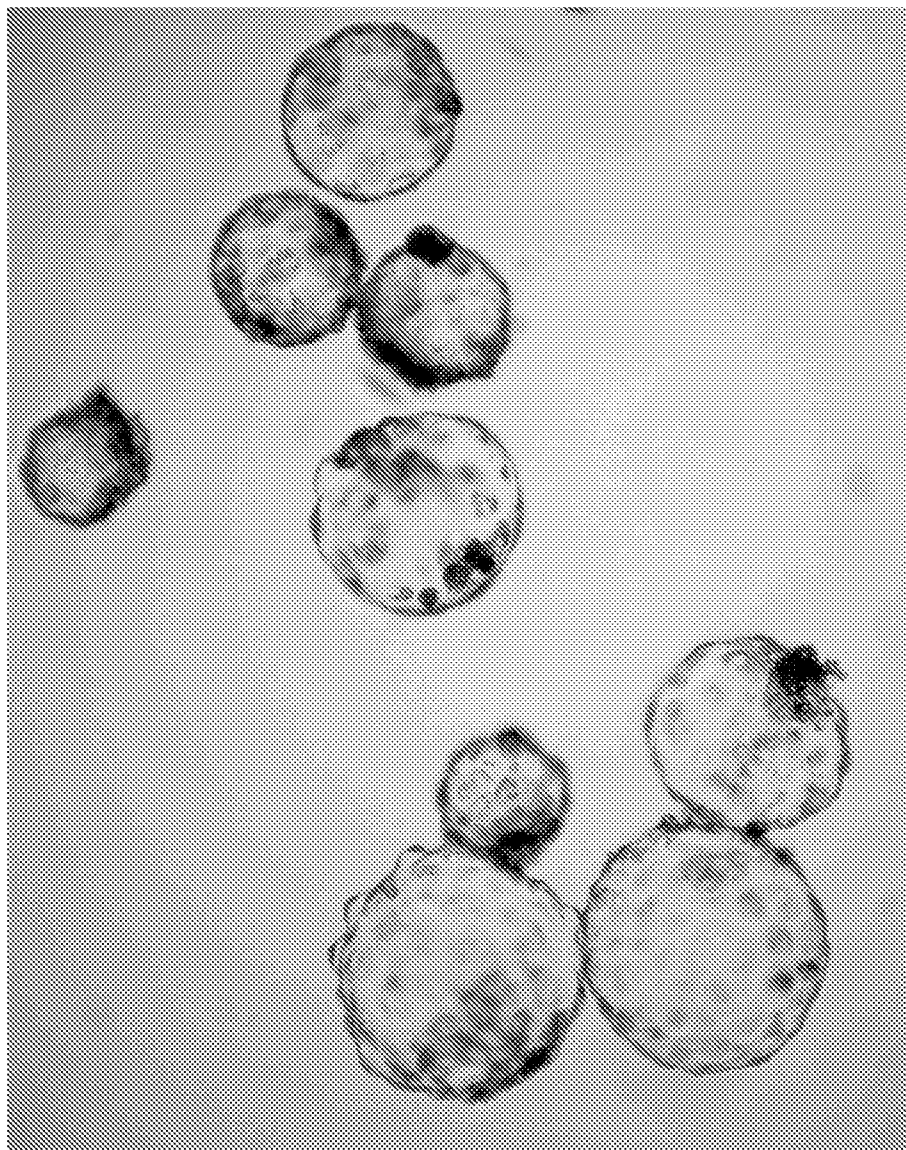


Fig. 14

15 / 15

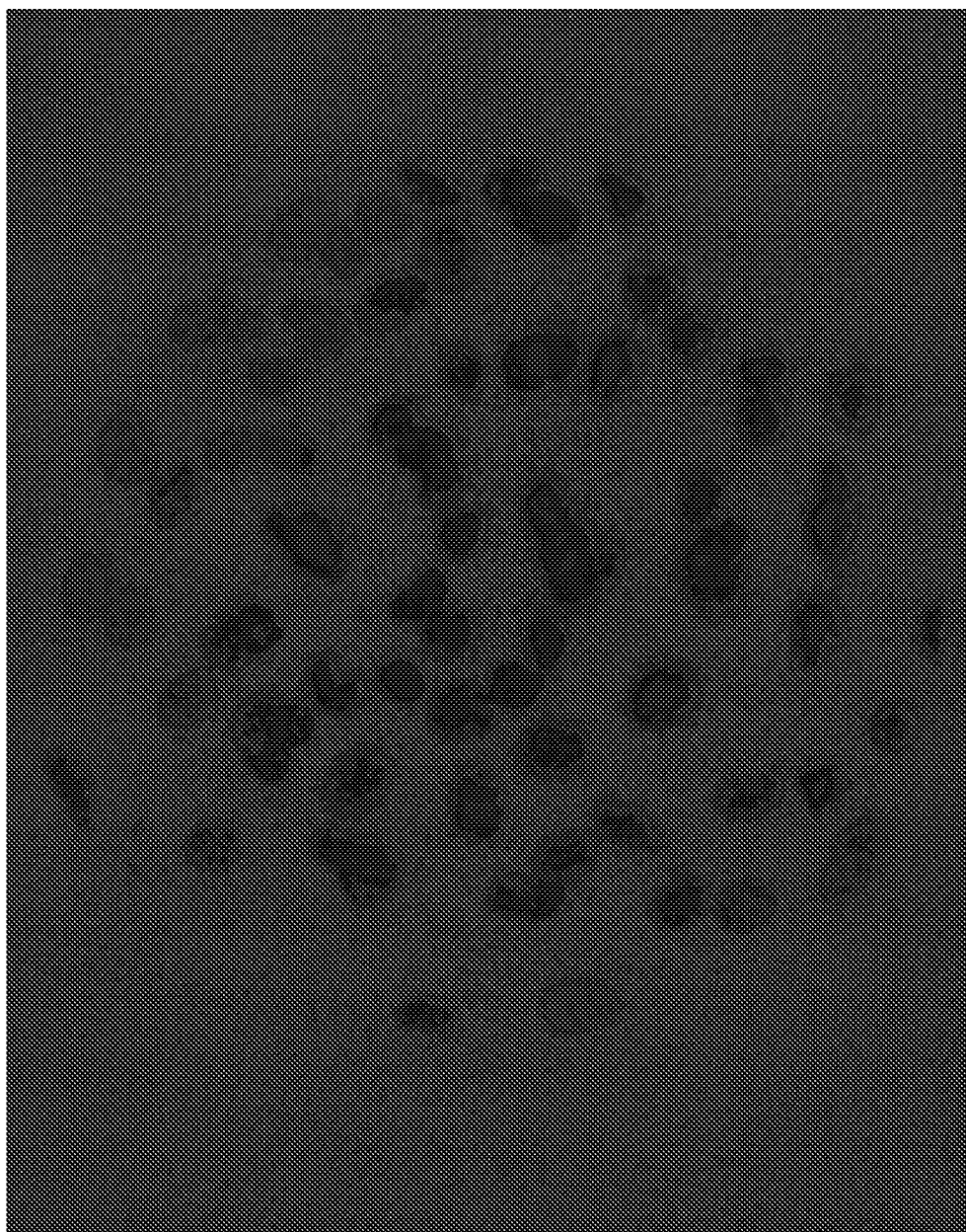


Fig. 15