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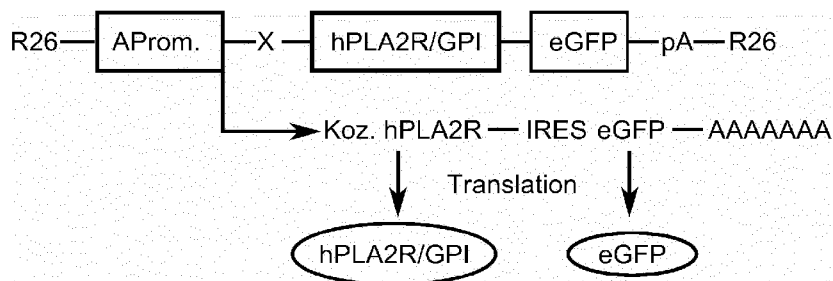
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(54) **Title:** GENETICALLY ENGINEERED MURINE ANIMAL AS A MODEL FOR PRIMARY MEMBRANEOUS NEPHROPATHY



**Fig. 2**

(57) **Abstract:** The invention relates to a genetically engineered murine animal, in particular a gene knockin mouse, as animal model for human disease. The technical problem to be solved is to provide a suitable means for assisting research for the causes and the development of and possible therapies and/or therapeutic agents for primary membranous nephropathy. For this purpose, the invention provides, in a first aspect, a genetically engineered murine animal, having expressibly integrated into the genome of all its germ cells and somatic cells a nucleic acid sequence coding for a fusion protein comprising the extracellular domains of the human phospholipase A2 receptor (hPLA2R), an ER targeting signal peptide and a membrane anchoring domain.



**{Description}****{Title of Invention}**

{0001} Genetically engineered murine animal as a model for Primary Membraneous Nephropathy.

**{Technical Field}**

{0002} The invention relates to a genetically engineered murine animal, in particular a gene knockin mouse, as animal model for human disease.

**{Background}**

{0003} In human kidneys, the phospholipase A2 receptor (PLA2R) is expressed on podocytes and probably the major auto-antigen in primary membranous nephropathy (MN, also called membranous glomerulonephritis, MGN) [1]. Binding of circulating autoantibodies to the receptor leads to the formation of immune complexes and initiates podocyte injury and proteinuria. Even though sufficient indirect evidence suggests that this pathomechanism is involved in primary MN, the ultimate experimental proof has yet to be provided. There is still a need for a means enabling further insight into the causes and the development of this disease, and for testing possible therapies or therapeutic agents.

**{Summary of Invention}**

{0004} The technical problem to be solved is to provide a suitable means for assisting research for the causes and the development of and possible therapies and/or therapeutic agents for primary membranous nephropathy.

{0005} In order to solve this problem the invention provides, in a first aspect, a genetically engineered murine animal, having expressibly integrated into the genome of all its germ cells and somatic cells a nucleic acid sequence coding for a fusion protein comprising the extracellular domains of the human phospholipase A2 receptor hPLA2R, an ER targeting signal peptide and a membrane anchoring domain.

{0006} In a second aspect the invention provides a genetically engineered murine animal, obtainable by transfecting an embryonic stem cell of a murine animal with a vector comprising a nucleic acid encoding a fusion protein comprising the extracellular domains of hPLA2R, an

ER targeting signal peptide and a membrane anchoring domain, integrating the transfected embryonic stem cell into a blastocyst of a non-transfected murine animal, crossing the chimeric animal resulting from the blastocyst with a non-transfected murine animal and selecting a transfected murine animal from the offspring.

{0007} In a third aspect the invention provides a genetically engineered murine animal, expressing exclusively in its podocytes a fusion protein comprising the extracellular domains of the human phospholipase A2 receptor hPLA2R, an ER targeting signal peptide and a membrane anchoring domain.

{0008} In a fourth aspect the invention provides a process for manufacturing a genetically engineered murine animal, the process comprising the steps of transfecting an embryonic stem cell of a murine animal, preferably a mouse or rat, further preferred a mouse, with a vector comprising a nucleic acid encoding a fusion protein comprising the extracellular domains of the human phospholipase A2 receptor hPLA2R, an ER targeting signal peptide and a membrane anchoring domain, integrating the embryonic stem cell into a blastocyst of a non-transfected murine animal, crossing the chimeric animal resulting from the blastocyst with a non-transfected murine animal and selecting a transfected murine animal from the offspring.

{0009} The invention provides murine animal models for e.g. research and drug testing regarding MN, i.e. genetically modified murine animals, e.g. mice, that express or can be made to express the human PLA2R. Since rodents normally do not express PLA2R on glomerular podocytes, it was previously impossible to investigate concepts for treating primary membranous nephropathy in wild-type rats or mice. Existing mouse models of MN are often unsatisfactory [7]. The animal models of the current invention are useful not only for answering the principal question of whether PLA2R can be a target in the initiation of MN, but also as a very useful tool for analyzing the formation and resolution of immune complexes and the humoral and cellular mediation of this disease. These models will allow researchers to characterize in more detail the pathogenesis of membranous nephropathy and will help to develop more specific therapies.

### **{Brief Description of Drawings}**

**{Fig. 1}**

{0010} Construction of the target vector used to produce hPLA2R/GPI mice. R26 = Rosa26 locus, AProm = chicken actin promoter, eGFP = enhanced green fluorescent protein, pA = poly(A) tail

**{Fig. 2}**

{0011} Podocyte-specific activation of gene expression after breeding with Podo-Cre mice. R26 = Rosa26 locus, AProm = chicken actin promoter, eGFP = enhanced green fluorescent protein, pA, AAAAAAA = poly(A) tail, Koz. = Kozak sequence, IRES = internal ribosome entry site

**{Fig. 3}**

{0012} Glomerular human PLA2R expression in Podo-Cre hPLA2R/GPI mice. Arrows: PLA2R staining in cell-membranes and cytoplasm of podocytes.

**Description of Embodiments**

{0013} The current invention provides a novel and realistic animal model for Primary Membranous Nephropathy (MN) in humans.

{0014} In a first aspect the invention relates to a genetically engineered murine animal, having expressibly integrated into the genome of all its germ cells and somatic cells a nucleic acid sequence coding for a fusion protein comprising the extracellular domains of the human phospholipase A2 receptor (hPLA2R), an ER targeting signal peptide and a membrane anchoring domain.

{0015} The nucleic acid sequence encoding the fusion protein is stably and expressibly integrated into the genome of the murine animal of the invention, “stably” meaning that the nucleic acid remains a part of the genome of the host animal and is inheritable in a Mendelian fashion, “expressibly” meaning that the nucleic acid is conditionally expressed, i.e. is functionally linked to a promoter driving transcription of the nucleic acid in a living cell, but is only expressed under specific circumstances, e.g. when expression is switched on or enabled by a specific measure, e.g. the activation of a recombinase in the cell. An example is a nucleic acid the expression of which is prevented by a stop cassette downstream of the promoter, e.g. a floxed neomycin stop cassette, the removal of which by e.g. Cre recombinase enables expression (see. e.g. [8]).

{0016} Thus, the genetically engineered murine animal of the present invention is prepared to express the human phospholipase A2 receptor (hPLA2R), because it stably contains a nucleic acid coding for it in its genome in expressible form, but it essentially does not express the human phospholipase A2 receptor (hPLA2R) in any of its cells unless the expression is deliberately initiated or enabled.

{0017} The phospholipase A2 receptor (PLA2R) is a 180 kDa type I transmembrane glycoprotein, binding several types of phospholipase A2 (sequence see NCBI NM\_007366.4). In humans, it is mainly expressed in podocytes of kidney glomerula. The sequence of the extracellular domains comprising the nt 273–4184 of the above sequence is represented in SEQ ID NO: 1.

{0018} The term “ER targeting signal peptide” or “leader sequence” relates to an amino-terminal (N-terminal) signal sequence mediating the targeting of a nascent secretory and membrane protein to the endoplasmic reticulum (ER). An example for such an ER targeting signal peptide is a CD8 leader sequence. A nucleotide sequence encoding such a CD8 leader sequence is presented in SEQ ID NO: 13.

{0019} The term “membrane anchoring domain” as used herein means a carboxy-terminal (C-terminal) amino acid sequence with which a protein is anchored in a cell membrane. The term here comprises the term “GPI-addition signal peptide” (GPIsp), relating to a C-terminal amino acid sequence signaling the addition of a GPI anchor to the partially folded nascent protein. Usually, the GPIsp is removed and directly replaced by a GPI precursor, which is further processed, i.a. by carbohydrate side-chain modifications, to the final GPI anchor. The term “GPI anchor” relates to a glycosylphosphatidylinositol structure having an ethanolamine phosphat residue, with which the structure is covalently bound to the C-terminal of a protein, and being anchored in a cell membrane via a lipid component, e.g. an inositolphospholipid [see e.g. [9]]. Most GPI anchors have the following core structure:  $\text{Man}\alpha 1-4\text{GlcN}\alpha 1-6\text{myo-inositol-1-PO}_4\text{-lipid}$ , e.g.  $\text{ethanolamine-PO}_4-6\text{Man}\alpha 1-2\text{Man}\alpha 1-6\text{Man}\alpha 1-4\text{GlcN}\alpha 1-6\text{myo-inositol-1-PO}_4\text{-lipid}$ . This core structure may be modified by various substitutions. An example of a coding sequence for a “GPI-addition signal peptide” is given in SEQ ID NO: 14.

{0020} The term “murine animal” refers to animals of the subfamily Murinae in the family Muridae. In particular, the term refers to animals of the genus *Mus* and *Rattus*. Examples for murine animals are animals of the species *Mus musculus* (house mouse) and *Rattus norvegicus* (brown rat, Norway rat). In case the current invention refers to crossing steps between murine animals, it is understood that murine animals are meant being able to produce fertile offspring. In particular, crossing a murine animal with another murine animal therefore means crossing murine animals of the same species. The terms “mouse” and “rat” or “laboratory mouse” and “laboratory rat” used herein are meant to refer to the house mouse (*Mus musculus*) and brown rat (*Rattus norvegicus*), respectively.

{0021} In a preferred embodiment of the invention the membrane anchoring domain is or comprises a GPI-addition signal peptide domain, and/or the ER targeting signal peptide is or comprises a human CD8 leader sequence. Preferably, the GPI-addition signal peptide domain is encoded by a nucleic acid having the sequence of SEQ ID NO: 14.

{0022} In a further preferred embodiment of the invention the nucleic acid further encodes a reporter protein. A reporter gene is a gene not natively present or expressed in a cell under study and which is introduced and expressed together with the gene of interest, thus functioning as a marker for the successful uptake and/or expression of the gene of interest. For this purpose, a reporter gene is usually arranged in immediate vicinity of the the gene of interest. It may be placed under the control of the same promoter as the gene of interest. An example of a preferred reporter gene is eGFP (enhanced Green Fluorescent Protein), causing cells expressing it to glow green under blue light. In the nucleic acid the eGFP coding sequence is preferably located downstream of the fusion protein coding sequence. The reporter gene sequence may be preceded by an internal ribosome entry site (IRES), i.e. a nucleotide sequence being able to bind the eukaryotic ribosome, thus allowing translation initiation to occur in the middle of a messenger RNA (mRNA).

{0023} In a further preferred embodiment, the genetically engineered murine animal of the invention is a murine animal being transfected with a vector comprising or having a sequence according to SEQ ID NO: 2. The vector represented by SEQ ID NO: 2 is a modified Rosa26-

CAG-Stop-eGFP vector (No.: 15912, Addgene, Cambridge, Ma, USA; [5]), containing a strong chicken actin promoter (AProm), a floxed neomycin-stop cassette (Stop), a nucleic acid coding for a hPLA2R/GPI fusion protein and an IRES triggered fluorescence reporter protein (GFP), which specifically integrates into the mouse Rosa26 (R26) locus through its R26 sites allowing targeted integration by homologous recombination.

{0024} In a second aspect the invention relates to a genetically engineered murine animal, preferably a mouse or rat, obtainable by transfecting an embryonic stem cell of a murine animal with a vector comprising a nucleic acid encoding a fusion protein comprising the extracellular domains of hPLA2R, an ER targeting signal peptide and a membrane anchoring domain, integrating the transfected embryonic stem cell into a blastocyst of a non-transfected murine animal, crossing the chimeric animal resulting from the blastocyst with a non-transfected murine animal and selecting a transfected murine animal from the offspring. The ER targeting signal peptide is preferably a CD8 leader sequence, e.g. being encoded by nucleic acid having a sequence of SEQ ID NO: 13, the membrane anchoring domain is preferably a GPI-addition signal peptide, preferably being encoded by nucleic acid having a sequence of SEQ ID NO: 14. Preferably, the nucleic acid further encodes a reporter protein, for example eGFP. The reporter protein coding sequence is preferably located downstream of the fusion protein coding sequence.

{0025} It is known to the skilled person how to transfect embryonic stem cells of murine animals and how to produce murine animals therefrom, harboring a transgene in all of its germ and somatic cells. A vector may, for example, be introduced into a pluripotent embryonic stem cell by electroporation. The introduced vector than is inserted into the genome by homologous recombination, thus, e.g. “knocking in” a functional gene into a desired locus. Transfected cells are placed in a selective medium and surviving clones are isolated and grown in culture. Cells tested positive for homologous recombination, e.g. via a reporter gene product, are injected into blastocysts from a donor murine animal, e.g. a laboratory mouse, e.g. a C57BL/6 mouse, and implanted into pseudopregnant foster mothers. Chimeric offspring are backcrossed, e.g. with C57BL/6 females, to generate heterozygous animals. Heterozygous animals are then intercrossed to generate homozygous gene-targeted animals.

{0026} Suitable vectors are known in the art, and include, for example, Rosa26 vectors, i.e. vectors targeting the genomic mouse locus Rosa26 in order to knockin e.g. cDNA constructs for ubiquitous or conditional gene expression in transgenic mice. A exemplary Rosa26 vector is a CAG-STOP-eGFP-ROSA26 targeting vector (e.g. plasmid 15912, Addgene, Inc., Cambridge MA 02139, USA; [5]), CAG standing for the CAG promoter (chicken beta-actin promoter with CMV enhancer).

{0027} Especially preferred, the genetically embryonic stem cell is transfected with a vector comprising or having a sequence according to SEQ ID NO: 2.

{0028} In a further aspect the current invention relates to a genetically engineered murine animal, preferably a mouse or rat, further preferred a mouse, expressing exclusively in its podocytes a fusion protein comprising the extracellular domains of the human phospholipase A2 receptor (hPLA2R), an ER targeting signal peptide and a membrane anchoring domain. In this embodiment the human PLA2 receptor is essentially exclusively expressed in the podocytes of kidney glomerula of the murine animal of the invention, which thus represent an animal model coming very close to the situation in humans. Preferably, the nucleic acid further comprises a coding sequence for a reporter protein, e.g. eGFP, preferably downstream of the fusion protein coding sequence.

{0029} Exclusive expression of the fusion protein in podocytes can, for example, be achieved by a recombinase, being expressed in a podocyte-specific manner, e.g. under the control of a podocyte-specific promoter, the recombinase removing a stop cassette present in a nucleic acid and preventing the expression of the fusion protein comprising the extracellular domains of the human phospholipase A2 receptor (hPLA2R), the ER targeting signal peptide and the membrane anchoring domain.

{0030} The genetically engineered murine animal of this aspect of the invention may be obtained by crossing a murine animal according to the first aspect of the invention, or a murine animal according to the second aspect of the invention, with a genetically engineered murine animal expressing a recombinase exclusively in its podocytes. An example for a suitable recombinase is the Cre recombinase. The Cre recombinase is a 38kDa tyrosine recombinase

enzyme derived from the bacteriophage P1 catalyzing the site specific recombination between two DNA recognition sites (loxP sites). Preferably, the genetically engineered murine animal is a mouse. Transgenic mice that express Cre recombinase exclusively in podocytes are known (see [6]).

{0031} In still another aspect the invention relates to a process for manufacturing a genetically engineered murine animal comprising the steps of transfecting an embryonic stem cell of a murine animal, preferably a mouse or rat, further preferred a mouse, with a vector comprising a nucleic acid encoding a fusion protein comprising the extracellular domains of the human phospholipase A2 receptor (hPLA2R), an ER targeting signal peptide and a membrane anchoring domain, integrating the embryonic stem cell into a blastocyst of a non-transfected murine animal, crossing the chimeric animal resulting from the blastocyst with a non-transfected murine animal and selecting a transfected murine animal from the offspring.

{0032} In a preferred embodiment the process further comprises the step of crossing the transfected murine animal produced with a genetically engineered murine animal expressing a recombinase, preferably a Cre recombinase, exclusively in its podocytes.

### **{Examples}**

{1. General description of the generation of the hPLA2R-Knockin mice of the invention.}

{0033} In a first step, a nucleic acid encoding a fusion protein was generated, consisting of the complete extracellular part of the human PLA2R (NCBI accession number: NM\_007366; bp 273–4184; [2]; SEQ ID NO: 1), a GPI-anchor domain for efficient integration into cell membranes [3] and a CD8-leader sequence used in other artificial systems [4]. To generate this fusion protein the pCMVSPORT6-MfeDANOD-KV-FS vector (DANOD, [4]) containing the CD8-leader as well as the GPI-anchor domain was used as the primary vector. The complete extracellular human PLA2R domains were specifically amplified by PCR from a commercially available full-length cDNA construct (No. SC308948, OriGene, Rockville, MD, USA). Subsequently, this PCR fragment was cloned in frame between the CD8-leader and the GPI anchor (1.1).

{0034} In a second step the nucleic acid coding for the fusion protein produced in step one was subcloned into a knockin target vector, called Rosa26-CAG-Stop-eGFP (No.: 15912, Addgene, Cambridge, Ma, USA; [5]) to obtain a hPLA2R/GPI carrying construct (figure 1, 1.2.). This modified target vector specifically integrates into the mouse Rosa26 (R26) locus through its R26 sites that allow targeted integration by homologous recombination. Furthermore, this vector contained a strong chicken actin promoter (AProm), a floxed neomycin-stop cassette (Stop), the nucleic acid coding for the hPLA2R/GPI fusion protein and an IRES triggered fluorescence reporter protein (GFP). R1-ES-cells (1.7.) were transfected with the hPLA2R/GPI construct (1.8.) to obtain pluripotent ES cells having stably integrated foreign DNA into their R26 locus. Next, these ES cells were injected in blastocysts while the modified blastocysts were implanted in false pregnant females to create chimeric littermates (1.14.). Further breeding of the chimera with C57BL/6 females resulted in littermates with a stable hPLA2R/GPI positive genotype. In the following, these animals are called “hPLA2R/GPI” mice.

{0035} For podocyte-specific protein expression the hPLA2R/GPI mice were finally bred with Podo-Cre mice [6]. These mice express the Cre recombinase under the control of the podocin promoter. Due to this highly specific promoter the Cre recombinase expression is strictly confined to podocytes. Consequently, the floxed stop-cassette is removed exclusively in podocytes and the strong actin promoter activates transcription of hPLA2R/GPI as well as eGFP, leading to translation of hPLA2R/GPI and eGFP (figure 2). These mice specifically express hPLA2R in their podocytes (figure 3), and are therefore called “Podo-Cre hPLA2R/GPI” mice. These mice are especially useful for establishing human PLA2R-dependent MN models.

{2. Detailed description of the generation of the hPLA2R-Knockin mice of the invention.}

{2.1 Generation of the GPI-PLA2R fusion construct}

{0036} A sequence-verified construct of the full-length PLA2R cDNA (NM\_007366, TrueClone No.: SC308948, OriGene,) was used as the source to amplify the extracellular part [2] of the human PLA2R by PCR. The PCR-fragment was cloned into the DANOD-vector [4].

{0037} For cloning, cut 5 µg of the DANOD vector with Mfe-I (No.: R0144; NEB, Ipswich, MA, USA) and Bgl-II (No.: R0589, NEB) in NEB-buffer 2 at 37° C over night (10 U of each enzyme in 50 µl). Separate the vector-backbone from the former insert in a 1 % agarose/TAE gel at 90 V until the fragments are clearly distinguishable. Cut out the vector by a scalpel and purify the DNA through the QIAquick Cell Extraction Kit (No.: 28704, Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

{0038} For PLA2R-specific PCR amplification, use 5 ng of the original construct (No.: SC308948 Origene) add specific forward (FW) and reverse (Rev) DANOD primers containing Bgl-II or Mfe-I restriction-sites at their 5' ends respectively (3.1.1.), deoxynucleotide-mix (8 mM, No.: R0241, Fermentas/Fisher Scientific, Schwerte, Germany), Phusion-DNA-polymerase with proof-reading activity (No.: F-530, Fermentas) and specific 5x polymerase-buffer in a total volume of 50 µl. Use the following amplification-protocol in a thermal cycler (LifeTouch, Biozym, Hessisch Oldendorf, Germany): Once 3 minutes, 98° C; 35x 15 s, 98° C; 15 s, 66° C; 6 minutes, 72° C and a final extension step for 6 minutes at 72° C. Add 10 U of Mfe-I and Bgl-II respectively as well as 10x NEB-buffer 2. Directly cut the PCR fragment (60 µl) for 2 h at 37° C in a water bath (U3, Julabo, Seelbach, Germany). Purify the fragment using the PCR Purification Kit (No.: 28106, Qiagen). Check the integrity of both DNA-fragments in a 1 % agarose/TAE gel and estimate the approximately DNA concentration by comparing the intensity of each fragment with the known concentrations of the DNA-ladder bands (1 kb Plus DNA Ladder No.: 10488095 Life Technologies, Darmstadt, Germany).

{0039} Ligate 100 ng vector-DNA with different molar ratios of the PCR-fragment (3:1, 1:1, 1:3) and 1 U T4-DNA Ligase (No.: M0202, NEB) in specific 10x ligation-buffer containing 10 mM ATP in 10 µl at 12° C over night by using a thermal cycler (LifeTouch, Biozym). Add 90 µl 1xTE and perform transformation with 5 µl ligation-reaction/30 µl chemical competent XL10 E. coli (No.: 200315, Agilent Technologies, Santa Clara, Ca, USA) for 30 minutes on ice. Then heat shock E. coli 45 s at 42° C, put on ice 2-3 minutes, add 220 µl LB-Medium (No.: 3002-031, MP-Biomedical, Solon, OH, USA) and incubate E. coli for 1 h at 37° C in a shaking-incubator (GFL). Strike out the bacteria suspension on LB-Agar plates supplemented with 100 µg/ml ampicillin (No.: 8495222, Böhringer Mannheim, Mannheim, Germany) and incubate

these plates over night at 37° C (B 6030, Kendro/Heraeus, Langenselbold, Germany) for selection of vector-DNA containing, ampicillin resistant colonies.

{0040} Pick some colonies with a toothpick, transfer to 50 µl 1 x TE and mix thoroughly. Identify positive, entire vector-construct carrying E. coli by screening PCR. Mix together (20 µl end volume) 5 µl E. coli suspension, 1 µl of each FW- and Rev-DANOD Screening primers (10 mM, 4.1.2.), 0.4 µl deoxynucleotid mix (8 mM, Fermentas), 0.2 µl Dream-Taq polymerase (No.: EP0712, Fermentas) and 2 µl 10x Dream-Taq buffer. Use the amplification protocol in a thermo cycler (LifeTouch, Biozym) as follows: Once 5 minutes, 95° C; 40x 30 s, 95° C; 30 s 64° C; 6 minutes 72° C and once 6 minutes 72° C for final extension. Check the fragments in a 1 % agarose gel at 90 V for 45 minutes. Transfer positive clones with a 10 µl pipet-tip in LB-Medium (50 ml) and incubate for about 1 day at 37° C in a shaking incubator (GFL). Isolate vector-DNA using the PureLink Plasmid Midi Kit (No.: K210005, Life Technologies) according to the manufacturer's recommendations. Further check integrity by restriction enzyme digestion (Mfe-I/Bgl-II) and complete sequencing of the cloned construct including CD8-leader and GPI-anchor.

#### {2.2. Generation of the R26 PLA2R-specific Target Vector}

{0041} A clone without any mutations and correct in frame cloning of the CD8 leader and GPI-anchor was used as starting material (2.1.). There is one Asc-I restriction site to clone the foreign-DNA of interest between the floxed stop-cassette and the GFP-reporter. Therefore, the complete fusion cDNA construct was re-amplified from the DANOD vector by PCR.

{0042} Cut 5 µg of the R26-Stop-eGFP Vector [5] with 25 U Asc-I (No.: R0558, NEB) in 10x NEB-buffer 4 in 50 µl at 37° C over night. Add 10 U calf intestine alkaline phosphatase (No.: M0290, NEB) for 1 h at 37° C to dephosphorylate the vector ends. Add 50 µl 10 mM Tris/Cl pH 8.0 and perform phenol (Tris/Cl pH 8.0 saturated, No.: A980-1, C. Roth, Karlsruhe, Germany) extraction twice. Add 10 µl 3 M Na-Acetate pH 5.2 (No.: 1.06267, Merck, Darmstadt, Germany) and 300 µl 100 % ethanol (No.: 9065.1, C. Roth) to precipitate DNA 1-2 h at -80° C. Centrifuge 20 minutes at 20,000xg (4° C, 5417R, Eppendorf, Hamburg, Germany) and wash the pellet three times in 70 % ethanol, dry 3-5 minutes in a Speed Vac (Concentrator

5301, Eppendorf) and dissolve in 12 µl 10 mM Tris/Cl pH 8.0. Estimate DNA concentration on a Nano Drop (ND-2000, Peqlab, Erlangen, Germany).

{0043} For PCR amplification, use 5 ng of the DANOD-construct, add specific FW and Rev R26-primers containing Asc-I restriction-sites at their 5' ends (3.1.3.) All other components are identical as used in chapter 2.1. "PLA2R-specific amplification". Add 10 U Asc-I in 10x NEB-buffer 4 for 2 h at 37° C.

{0044} Ligation, Transformation, PCR-screening as well as the final verifications are performed as mentioned in chapter 2.1., with the exception that R26-Screening FW and Rev primers were used (3.1.4., SEQ ID NO: 9, 10).

{0045} Finally, linearize 70 µg verified PLA2R-specific target vector with 100 U AsiSI (No.: R0630, NEB) at 37° C over night in 300 µl 10 mM Tris/Cl pH 8.0. Purify DNA through one phenol (C. Roth) and one chloroform (No.: 4423.1, C. Roth) extraction and precipitate DNA by adding 30 µl 3 M Na-Acetate pH 5.2 (Merck) and 1 ml 100 % ethanol (C. Roth). Further steps see 2.1. Invert the tube several times during the extractions because the 20 kb fragment is very sensitive. The linearized DNA is now ready to be transfected in ES-cells (2.8.). The sequence of the target vector is presented in SEQ ID NO: 2.

{2.3. Generation of Mouse Embryo Fibroblasts (MEF)}

{0046} Use 13.5-14.5 dpc (day post coitum) old embryos of FVB mice. For this purpose three pregnant females (8-10 embryos/female) are required.

{0047} Dissect the uterus, wash out the embryos with 1 x PBS and remove the embryo's envelope. Remove all organs and intensively wash the torso with 1 x PBS. Transfer the torso in 2-3 ml of suspension-medium to a new Petri dish, mince it by a scalpel and press the shredded tissue through 20 G and 26 G cannulas to complete homogenization. Put the homogenate into a 15 ml tube, add a small amount of DNase powder (No.: D-4527, Sigma) and 5 ml of 0.05 % Trypsin/EDTA. Incubate for 10 minutes at 37° C, mix occasionally. Let the suspension settle down for one minute and collect the supernatant in 20 ml MEF-Medium. Digest the remaining

suspension again for one minute in 5 ml trypsin mixture, put the supernatant to the first and repeat this step 2-3 times. Count the cells (exclude the small erythrocytes) and cultivate  $5 \times 10^6$  cells/15 cm Petri dish (No.: 639160, Greiner, Frickenhausen, Germany) in 25 MEF-Medium supplemented with 15 % FCS. Change the medium every day. Three to four days later the MEF should be confluent. Split 1:3-1:5 or freeze  $2.5 \times 10^6$  MEF in 2 x Freeze (see 3.10).

#### {2.4. Gelatin treatment}

{0048} Incubate dishes with 0.1 % gelatin for 30 minutes at room temperature. Be sure that the bottom of the different dishes is totally covered by the liquid (the volume depends on the diameter of the dish), discard the solution and let them air dry. The dishes can be stored at 4°C.

#### {2.5. MEF cell culture}

{0049} Quickly thaw  $2.5 \times 10^6$  MEF at 37° C in a water bath, dilute in 25 ml MEF-Medium and cultivate them for 3 days until confluence in a 15 cm Petri dish (Greiner). To split the MEF 1:3 to 1:4 discard medium and wash with 25 ml 1 x PBS, add 3 ml of 0.05 % Trypsin/EDTA for 3 - 5 minutes at 37° C. Pipette up and down until cells are scattered. Stop digestion by adding 12 -17 ml MEF-Medium (depends on dilution ratio), thoroughly mix the suspension and put 5 ml of this suspension to a new plate equipped with 15 ml MEF-Medium. Let them grow another 3 days.

#### {2.6. Inactivation of MEF cells}

{0050} Replace the medium (15 ml) of a confluent 15 cm MEF-plate, add 150 µl Mitomycin C (10 µg/ml) and incubate 2-3 h at 37° C. Discard the medium and wash twice with 20 ml 1 x PBS. Add 3 ml of 0.05% Trypsin/EDTA for 3-5 minutes, pipette up and down, stop digestion with 7 ml MEF-Medium. Collect the suspension and count cells. Centrifuge and suspend the MEF  $1-5 \times 10^6$ /ml freeze medium on ice. Freeze the inactivated MEF in 1 x Freeze or dilute to  $5 \times 10^4$  MEF/ml in MEF-Medium and cultivate them in gelatinated Petri-dishes for ES cell cultures.

### {2.7. ES cell culture}

{0051} R1-ES cells were obtained from Prof. Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital Toronto. They were obtained at passage 11 and expanded to passage 14 and 15, respectively. For details of ES-strain see:

[http://www.informatics.jax.org/mgihome/nomen/strain\\_129.shtml](http://www.informatics.jax.org/mgihome/nomen/strain_129.shtml). Alternatively, R1/E cells ATCC SCRC-1036 (LGC Standards GmbH, Mercatorstr. 51, 46485 Wesel, Germany; <http://www.lgcstandards-atcc.org>) could be used.

{0052} Thaw ES-cells quickly in a water bath at 37° C, dissolve them in 5 ml ES medium and centrifuge cell-suspension 5minutes at 300xg at room temperature. Suspend the ES-cell pellet in ES medium (the volume depends on the number of frozen cells ) and plate on gelatinated cell culture plates supplemented with a confluent layer of inactivated MEF cells (feeder cells) Change the ES medium every day and split confluent ES-cells in a ratio of 1:4 to 1:6 every second day. To perform splitting, wash cells with 1 x PBS/EDTA and subsequently trypsinize with 1/5 volume of 0.25 % Trypsin/EDTA for 3-5 minutes at 37° C. Pipette cells up and down to get a homogeneous single cell suspension. Stop trypsin activity by adding 2 x volumes of ES medium and centrifuge for 3 minutes at 300xg at room temperature. Suspend the pellet in the appropriate volume of ES medium depending on the cell culture plate and dilution factor of choice. Plates must be gelatinated and plated with an appropriate number of inactivated MEF the day before use.

### {2.8 Electroporation and Selection of ES-Cells}

{0053} Use  $1 \times 10^7$  ES-cells/electroporation and change ES medium 2 h before trypsinization. After trypsination (2.7) suspend the pellet in 10 ml 1 x PBS and centrifuge for 3 minutes at 300xg (room temperature). Suspend the pellet in 0.8 ml 1 x PBS, add linearized DNA (25-80 µg, 2.2.) and transfer into a 0.4 cm gap-electroporation-cuvette (No.: 65-0032, Life Technologies/Gibco). Use the Electroporator (Gene Pulser II, Biorad, Hercules, CA, USA) with the settings as follows: Double-pulse 250 V, 500 µF. Store electroporated cells 10 minutes at room temperature and transfer them into 10 ml ES medium before spreading on 5-6 10 cm dishes (No.: 664160, Greiner, gelatinated + inactivated MEF). For positive selection change ES

medium supplemented with 150-300 µg/ml Geneticin (G418, Life Technologies/Gibco) every day for one week. During this week G418 resistant ES cell clones (colonies) should grow that stably incorporate the foreign DNA in their genome. Pick the colonies with 10-20 µl pipet-tips by using a binocular (SZH10 equipped with DF PLANAPO IX, Olympus, Hamburg, Germany) transfer each colony into one well of a round bottom 96-well plate (650180, Greiner) supplemented with 40 µl 0.25 % trypsin solution and incubate 3-5 minutes at 37° C. During this incubation replace the MEF-Medium with 160 µl of ES medium in a gelatinated, feeder cell layered flat bottom 96-well plate (550180, Greiner). Afterwards transfer the total amount of trypsinized and singulated cells to the new plate and mix suspension by pipetting up and down carefully. 2-3 days later the cells should be confluent. For splitting, wash the well with 200 µl 1 x PBS/EDTA and trypsinize with 40 µl 0.25 % trypsin for 3-5 minutes at 37 °C, pipette up and down until cells are single. Stop enzyme activity by adding 160 µl of ES medium. Distribute cell-suspension on two gelatinated, feeder cell layered flat bottom 96-well plates as well as one gelatinated 96-well plate without any feeder cells. Again, 2-3 days later the cells should be confluent. Freeze both plates that include feeder cells and prepare the other plate without feeders for DNA-isolation.

#### {2.9. Freeze of ES cells}

{0054} Change ES medium 2 h before freezing. Wash each well with 200 µl PBS/EDTA, trypsinize the cells with 40 µl 0.25 % trypsin for 3-5 minutes at 37 °C, pipet up and down, stop enzyme-activity by adding 60 µl ES medium. Put the plate on ice, add 100 µl 2 x Freeze and gently mix. Overlay suspension with 50 µl mineral-oil, wrap the plate with adhesive tape and freeze in a Styropor-box at -80 °C. Two days later remove the box and store the plates at -80 °C.

#### {2.10. DNA-Preparation}

{0055} Discard ES medium of confluent grown ES cell layers, wash with 200 µl 1 x PBS and discard. Add 100 µl DNA lysis buffer and incubate over night at 55 °C in a moist chamber. Add 10 µl 8 M LiCl and 100 µl 2-propanol. To precipitate DNA, gently shake the plate over night in a moist chamber (Important: Tightly seal the plate with parafilm). Centrifuge the plate for 30

minutes at 200xg at 4 °C. Discard supernatant and wash twice with 70 % ethanol (centrifuge between the washes, 20 minutes, 2000xg, 4 °C).

#### {2.11. Southern Blot, Random-Prime-Labeling and Hybridization}

{0056} Run a 1 % agarose/TAE gel with EcoRI digested DNA-samples (see 2.10.) using DNA loading buffer. Use fresh 1 x TAE Buffer and a clean chamber. To facilitate transfer of DNA-fragments longer than 3 kb incubate the gel for 5 minutes in 0.25 N HCl at room temperature, discard HCl and wash extensively with deionized water. Add 0.4 N NaOH and incubate for 15 minutes. Prepare filter papers (5 pieces) and the XL-1 membrane for blotting and moisten them with 0.4 M NaOH. Put the gel onto a glass-plate, then place the Hybond N+ membrane (No.: RPN119B, GE Healthcare/Amersham, Freiburg, Germany) and the filter papers without any air-bubbles on the gel. Put a stack of dry paper-towels then a second glass plate onto the filter papers and wrap the whole with saran wrap and place a weight at the top. Incubate over night at room temperature. Float membrane with 2 x SSC not more than 10 minutes and bake it for 2 h at 80 °C.

{0057} Label the DNA probe (Rosa probe-A 693 Bp, EcoR-I/Pac-I fragment) [5, supporting online materials] with alpha 32P dATP using the Megaprime DNA Labeling System (No.: RPN1604, GE Healthcare/Amersham) according to the manufacturer's recommendations.

{0058} For hybridization put membrane tightly in a hybridization bottle, add pre-warmed hybridization buffer and roll it 1-2 h at 68 °C. Discard buffer, cook the random prime labeled probe ( $2.5 \times 10^5 - 5 \times 10^5$  cpm/ml) for 5 minutes, add fresh hybridization buffer (about 5 ml, depends on the bottle) including the labeled DNA probe and let it roll over night.

{0059} Discard hybridization buffer and wash the membrane in 2 x SSC 0.5 % SDS. Pull out the membrane and wash it in 2 x SSC 0.5 % SDS for 15 minutes at 68 °C. Discard wash buffer and repeat once. Further wash membrane in 0.2-0.1 x SSC several times for 10-15 minutes until no radioactivity can be detected in the wash buffer. Put the membrane between saran wrap and expose.

### {2.12. Generation of blastocysts}

{0060} Sacrifice donor mice (3.5 dpc) and place on back. Spray abdomen with skin disinfectant solution (Braunoderm<sup>®</sup>, B. Braun Melsungen AG, Melsungen, Germany). Pick up the skin with a blunt forceps, cut through the skin, pull at both ends of the cut to expose the internal fascia and then cut through the internal fascia. Expose uterine horns, ovaries, oviduct and cervix and pick up the fat pad on the ovary on one side of the mouse. Remove the fatty mesotarium along the uterus on both sides and then cut it out. Place the uterus in a dish containing 1 x PBS. Place the uterus on a lid of a 10 cm dish (Greiner) under the binocular (Olympus) and remove any tissue and fat which may be still attached to the uterus. Then cut off the oviducts, insert an eye scissor in the opening of the uterus to widen it. Place the uterus for an additional wash in a new dish with 1 x PBS. Fill a 1 ml syringe with KSOM/HEPES attach a 30 G needle, place the uterus cervix facing towards you on the edge of a depression dish under the binocular (Olympus), insert the needle through the cervix. Rinse each horn with 0.5 ml of medium. Collect the blastocysts with a mouth controlled glass capillary and place them in a drop of KSOM covered with mineral oil at 37 °C, 5 % CO<sub>2</sub>.

### {2.13. Injection of ES cells in blastocysts}

{0061} Start with a confluent well (6-well plate, No.: 657160, Greiner) of the desired ES cell clone, change ES medium 2 h prior trypsination. Wash cells with 3 ml 1 x PBS, add 0.5 ml 0.25 % trypsin and incubate for 2-3 minutes at 37 °C, pipette up and down gently. Stop enzyme reaction in 5 ml ES medium. Don't mix the solution. At this stage 2-10 cell aggregates are recommended. Centrifuge 3 minutes at 300xg, carefully suspend the pellet in 0.8 ml HEPES buffered ES medium and store on ice at least 30 minutes.

{0062} Prepare injection set up (Olympus IX70; Eppendorf: PatchMan NP2, TransferMan NK2, Cell tram air, Cell tram vario), i.e. turn on cooling water, fill holding pipette with 1 x PBS, make sure the oil in the injection capillary is smoothly moving. To start, fill injection slide with 0.5 ml cold KSOM/HEPES flick tube with the cells and pipette 3-6 blastocysts on the upper part of the injection slide. Add 50 µl of the ES-cell suspension to the lower part of the injection slide, put the slide on the microscope stage and bring both capillaries in the medium

(not to the bottom). Start to collect ES-cells with clearly visible, small, round nuclei without any vacuoles and inject 15 ES cells per blastocyst. Use the holding pipette to fetch and fix a blastocyst. Turn the fixed blastocyst until you can inject the ES-cells between the border of trophoblast without hurting the inner cell mass. Place the ES cells on the top of the inner cell mass. Soak of additional volume carefully and place the injected blastocyst separate from the others. As soon as the blastocysts on the slide are injected transfer them to the incubator (Innova CO-14, Eppendorf), in a pre-equilibrated (37 °C) drop of KSOM.

#### {2.14. Implantation of blastocysts}

{0063} Female mice mated with vasectomized males at 2.5 dpc are used. Weight of the mouse is determined. Injection of Ketamin/Rompun for anesthesia: 120/16 mg/kg KG. Check pedal reflex, inject Rimadyl (5 mg/kg s.c.) for analgesia. Shave back carefully, wipe with tissue soaked with Braunoderm. Put mice on a clean Kimwipe and place it under the binocular (Olympus) on a heating mate. Make a small incision on the back parallel to the last rib. Wipe incision with a fresh tissue soaked with Braunoderm and slide the skin laterally until the ovary can be seen under the fascia. Make a small incision over the ovary and grasp the ovaries fat pad and use a blunt forceps to pull fat pad, ovary and the upper part of the uterine horn. Clamp the fat pad with a serrafine and place it and the ovary and uterus on a small sterile filter paper. The corpus luteum must be clearly visible otherwise the mouse is not pseudo pregnant. Use a mouth pipette with glass capillary attached to transfer the blastocysts to a fresh drop of medium and then soak up to 8 of them in the capillary like pearls on a string. Gently grasp the uterine horn with a fine forceps, take a 27 or 30 G needle and make a hole in the uterus. Insert the capillary containing the blastocysts and slowly expel them into the uterus. Withdraw the capillary, release the serrafine and put the fat pad, ovary and uterus back. Close the inner skin with sutures and repeat on the other side. Clip the outer skin with wound clips, disinfect using Braunoderm, wrap mouse in a sterile tissue and place it in a fresh type 2 long cage on a heating mat until it is awake and moving. Health control is done daily up to five days before delivery, cages and water is changed once a week. 5 days before delivery mice are placed in a fresh cage, with lots of nesting material and their old nest. Five days after delivery pups are counted and sexed and after five additional days chimerism can be determined. They are mated to wildtype females as soon as they are mature. Brown offspring is then genotyped.

### {2.15. PLA2R-GPI genotyping}

{0064} Isolate genomic DNA from small pieces of tail-tissue in 150 µl of Direct-PCR-Tail (No.: 31-102T, Peqlab) supplemented with 0.25 mg/ml Proteinase K (4.12.) at 55° C over night. Stop reaction at 85° C for 45 minutes.

{0065} Add 1 µl of gDNA-solution, 1 µl of PLA2R/GPI-genot. FW and Rev. primers respectively (10 mM each; 3.1.5.), 0.4 µl dNTP mix (8 mM, Fermentas) and 0.2 µl Dream-Taq (Fermentas) in the appropriate buffer in a total volume of 20 µl. Use the amplification protocol in a thermo cycler (LifeTouch, Biozym Scientific GmbH, 31840 Hessisch Oldendorf, Germany) as follows: Once 5 minutes, 95° C; 40 x 30 s, 95° C; 30 s 64 °C; 1 minutes 72 °C and once 3 minutes 72 °C for final extension. Check the fragments in a 1.5 % agarose gel at 90 V for 45 minutes.

### {3. Material}

#### {3.1. PCR-Primers}

##### {3.1.1. DANOD-Primers:}

{0066} FW: 5' TTTTTTAGATCTGAGGGTGTGGCGGCGGCGCTT 3' (SEQ ID NO: 3)

{0067} Rev: 5' TTTTTTCAATTGTTCTTACTGTTACCATCAAATTGAGC 3' (SEQ ID NO: 4)

##### {3.1.2. DANOD-Screening-Primers:}

{0068} FW: 5' CTTGCTCCTGCCGCTAGCCTT 3' (SEQ ID NO: 5)

{0069} Rev: 5' CAAGGAACAGGGATACACAGCTT 3' (SEQ ID NO: 6)

##### {3.1.3. R26-Primers:}

{0070} FW: 5' AAAAAGGCGCGCCTTAGCGATGGCCTTACCAGTGACCGCCT 3' (SEQ ID NO: 7)

{0071} Rev: 5' AAAAAAGGCGCGCCCTACGGCTCAGCAAGACAAAGCA 3' (SEQ ID NO: 8)

{3.1.4. R26-Screening-Primers:}

{0072} FW: 5' AATAGAACTCTTGCTTGCTTTGCTA 3' (SEQ ID NO: 9)

{0073} Rev: 5' TCTTCAAGAAGCTTCCAGAGGAA 3' (SEQ ID NO: 10)

{3.1.5. PLA2R/GPI genot.-Primers:}

{0074} FW: 5' TTAACAATCAAGGATGAGGCT 3' (SEQ ID NO: 11)

{0075} Rev: 5' TCTTCAAGAAGCTTCCAGAGGAA 3' (SEQ ID NO: 12)

{3.2 50x TAE buffer:}

Tris base 242 g, dissolve in 750 ml deionized water

+ 57.1 ml glacial acetic acid

+ 100 ml 0.5 M EDTA pH 8.0

adjust volume to 1 L

working solution is 1x

{3.3 1x TE:}

10 mM Tris/Cl pH 8.0

1 mM EDTA pH 8.0

{3.4 10x PBS:}

10.9 g Na<sub>2</sub>HPO<sub>4</sub> anhydrous

+ 3.2 g NaH<sub>2</sub>PO<sub>4</sub>

+ 90 g NaCl

dissolve just under 1 L deionized water, then adjust pH to 7.4

working solution is 1x

{For PBS/EDTA:}

500 ml 1 x PBS

+ 0.1g EDTA

## {3.5. Trypsin:}

Trypsin/EDTA MEF cells (0.05 %, No.: 25300054 Life Technologies/Gibco)

Trypsin/EDTA ES cells (0.25 %, No.: 25200056 Life Technologies/Gibco)

## {3.6. MEF Medium:}

DMEM No.: 6196, 5026 Life Technologies/Gibco		500 ml:
FCS (Gold) No.: A11-649, PAA	9 %	50 ml
MEM (100x) No.: 111400 Life Technologies/Gibco	1x	5.5 ml
Pen/Strep (100x) No.: 15140122 Life Technologies/Gibco	1x	5.5 ml

## {3.7. ES medium:}

DMEM, No.: 42430025 Life Technologies/Gibco		500 ml:
FCS (Gold), A11-649 PAA	15%	92.0 ml
Glutamin (100x), No.: 25030024 Life Technologies/Gibco	1x	6.2 ml
MEM (100x), No.: 11140035 Life Technologies/Gibco	1x	6.2 ml
Pen/Strep (100x), No.: 15140122 “	1x	6.2 ml
Nucleosidmix (100x), see 4.8.	1x	6.2 ml
Sodium pyruvate, No.: 1136003 “	1x	6.2 ml
2-Mercaptoethanol (50 mM), No.: 31350010 “	2x	1.24 ml
LIF (107 U/ml), No.: ESG 1107 Chemicon Int.	1000 U/ml	62 µl
For selection add:		
G418 (50 mg/ml) No.: 10131019 Life Technologies/Gibco		4-6 µl

## {3.8. Nucleoside Mix 100x:}

Adenosine	No.: A-4036	Sigma	80 mg
Guanosine	No.: G-6264	Sigma	85 mg
Cytidine	No.: C-4654	Sigma	73 mg
Uridine	No.: U-3003	Sigma	73 mg
Thymidine	No.: T-1895	Sigma	24 mg

## {3.9. Electroporation buffer:}

HEPES	20 mM
NaCl	137 mM
KCl	5 mM
Na <sub>2</sub> HPO <sub>4</sub>	0.7 mM
Dextrose	6 mM
Adjust pH to 7.05 using 0.5 N NaOH	

## {3.10. Freeze media:}

1 x Freeze:

FCS	40 %
Appropriate Medium	50 %
DMSO No.: D-2650, Sigma	10 %

## {2 x Freeze:}

FCS	80 %
DMSO	20 %

## {3.11. DNA lysis buffer:}

Tris/Cl pH 7.5	10 mM
EDTA	10 mM
NaCl	10 mM
SDS	0.5 %
Proteinase-K	20 mg/ml

## {3.12. KSOM:}

			100 ml:
NaCl	No.: S-5886	Sigma	555 mg
KCl	No.: P-4505	Sigma	18.5 mg
KH <sub>2</sub> PO <sub>4</sub>	No.: P-5655	Sigma	4.75 mg
MgSO <sub>4</sub>	No.: M-7774	Sigma	4.95 mg
CaCl <sub>2</sub>	No.: C-7902	Sigma	25 mg
NaHCO <sub>3</sub>	No.: S-5761	Sigma	210 mg

Glucose	No.: G-6152	Sigma	3.6 mg
Na-Pyruvate	No.: P-4562	Sigma	2.2 mg
DL-Lactic-Acid	No.: L-1375	Sigma	174 µl
EDTA, 10 mM	No.: E-6635	Sigma	100 µl
Streptomycin	No.: S-9137	Sigma	5 mg
Penicillin	No.: P-7794	Sigma	6.3 mg
Phenol Red 0.5 %	No.: P-0290	Sigma	100 µl
L-Glutamine	No.: G-8540	Sigma	14.6 mg
MEM es. AA	No.: 11130-051	Gibco	1 ml
MEM non es. AA	No.: M-7145	Sigma	0.5 ml
BSA	No.: A-4378	Sigma	0.1 g

{0076} KSOM/HEPES:

add to KSOM:

NaHCO <sub>3</sub>	15 mM
HEPES pH 7.4	20.85 mM

{3.13. 20 x SSC:}

3 M NaCl  
0.2 M sodium citrate

{3.14. 20 x SSPE:}

3 M NaCl  
0.2 M NaH<sub>2</sub>PO<sub>4</sub>  
0.02 M EDTA

{3.15. Hybridization buffer:}

7 % (w/v) SDS	No.: L-6026	Sigma
10 % (w/v) Polyethylenglykol 6000	No.: 81255	Sigma
1.5 x SSPE (20x)		
200 µg/ml denatured herring sperm DNA	No.: D1816	Promega

## {3.12. Other Material}

Mineral oil	No.: M-8410	Sigma
0.1% Gelatin 100 ml,	No.: G1393	Sigma
Mitomycin C	No.: M-0503	Sigma
Proteinase-K	No.: 0311584401	Roche
DNase	No.: D-4527	Sigma

## {4. Glomerular expression of hPLA2R in knockin mice of the invention}

{0077} hPLA2R/GPI mice of the invention were bred with Podo-Cre mice [6], in order to generate “Podo-Cre hPLA2R/GPI” mice according to the invention. To confirm expression of hPLA2R in podocytes of these mice, immunohistological investigations were performed on kidney tissue sections. It turned out that both the eGFP gene and the hPLA2R gene, which had been introduced into the mice via the target vector into the Rosa26 locus, were expressed in the glomerulus. Moreover, the location of the stained cells corresponds to the typical distribution of podocytes in the glomerular tuft (figure 3, arrows).

{0078} For further testing, “Podo-Cre hPLA2R/GPI” mice and “hPLA2R/GPI” mice of the invention were actively immunised with recombinant human PLA2R, and the generation of specific immune complexes was investigated. Immune complexes were only found in “Podo-Cre hPLA2R/GPI” mice along the glomerular basement membrane, but not in hPLA2R/GPI mice that do not express hPLA2R due to the lacking Cre recombinase (not shown). This also proves the excellent functionality of the invention.

**{Sequence overview}**

{0079} Overview of sequences referred to in the application:

SEQ ID NO:	Type	Annotation
01	mRNA	PLA2R1, extracellular domains
02	DNA	hPLA2R/GPI target vector
03	DNA	DANOD fw primer
04	DNA	DANOD rev primer
05	DNA	DANOD screening fw primer

06	DNA	DANOD screening-rev-Primer
07	DNA	R26 fw-primer
08	DNA	R26 rev primer
09	DNA	R26 screening fw primer
10	DNA	R26 screening rev primer
11	DNA	PLA2R/GPI genot. fw primer
12	DNA	PLA2R/GPI genot. rev primer
13	DNA	CD8 leader sequence
14	DNA	GPI anchor sequence

**{Citation List}**

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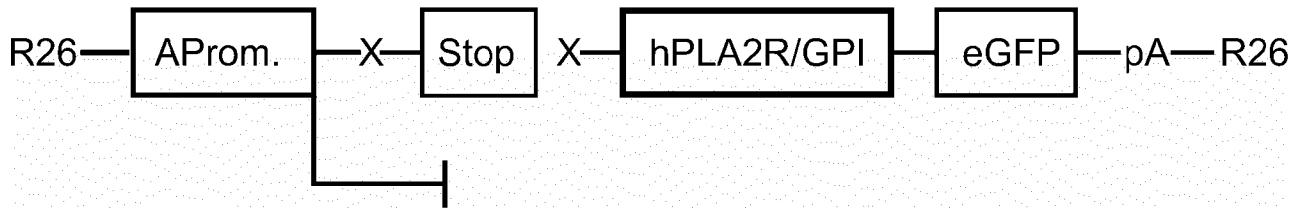
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## CLAIMS

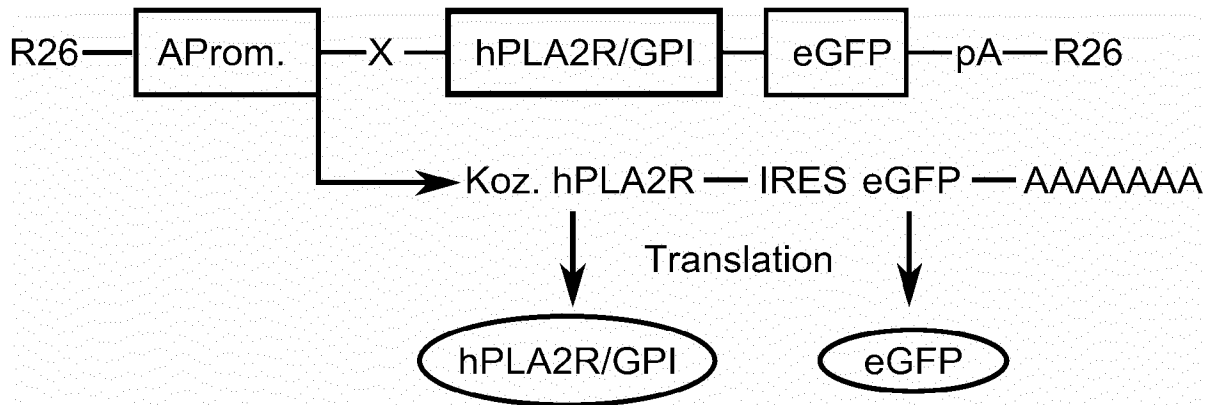
1. A genetically engineered murine animal, having expressibly integrated into the genome of all its germ cells and somatic cells a nucleic acid sequence coding for a fusion protein comprising the extracellular domains of the human phospholipase A2 receptor hPLA2R, an ER targeting signal peptide and a membrane anchoring domain.
2. The genetically engineered murine animal of claim 1, wherein the membrane anchoring domain is or comprises a GPI-addition signal peptide domain, and/or wherein the ER targeting signal peptide is or comprises a human CD8 leader sequence.
3. The genetically engineered murine animal of one of claims 1 or 2, wherein the nucleic acid further encodes a reporter protein, preferably eGFP.
4. The genetically engineered murine animal of one of claims 1 to 3, being transfected with a vector comprising or having a sequence according to SEQ ID NO: 2.
5. A genetically engineered murine animal, obtainable by transfecting an embryonic stem cell of a murine animal with a vector comprising a nucleic acid encoding a fusion protein comprising the extracellular domains of hPLA2R, an ER targeting signal peptide and a membrane anchoring domain, integrating the transfected embryonic stem cell into a blastocyst of a non-transfected murine animal, crossing the chimeric animal resulting from the blastocyst with a non-transfected murine animal and selecting a transfected murine animal from the offspring.
6. The genetically engineered murine animal of claim 5, wherein the nucleic acid further encodes a reporter protein, preferably eGFP.
7. The genetically engineered murine animal of one of claims 5 or 6, wherein the embryonic stem cell is transfected with a vector comprising or having a sequence according to SEQ ID NO: 2.

8. A genetically engineered murine animal, expressing exclusively in its podocytes a fusion protein comprising the extracellular domains of the human phospholipase A2 receptor hPLA2R, an ER targeting signal peptide and a membrane anchoring domain.
9. The genetically engineered murine animal of claim 8, further expressing a recombinase under a podocyte-specific promoter.
10. The genetically engineered murine animal of claims 8 or 9, obtained by crossing a murine animal according to one of claims 1 to 4, or a murine animal according to one of claims 5 to 7, with a genetically engineered murine animal expressing a recombinase exclusively in its podocytes.
11. The genetically engineered murine animal of claim 10, wherein the recombinase is a Cre recombinase.
12. The genetically engineered murine animal of one of claims 1 to 11, wherein the murine animal is a mouse or rat, preferably a mouse.
13. Process for manufacturing a genetically engineered murine animal, comprising the steps of transfecting an embryonic stem cell of a murine animal with a vector comprising a nucleic acid encoding a fusion protein comprising the extracellular domains of the human phospholipase A2 receptor hPLA2R, an ER targeting signal peptide and a membrane anchoring domain, integrating the embryonic stem cell into a blastocyst of a non-transfected murine animal, crossing the chimeric animal resulting from the blastocyst with a non-transfected murine animal and selecting a transfected murine animal from the offspring.
14. Process for manufacturing a genetically engineered murine animal according to claim 13, further crossing the transfected murine animal produced with a genetically engineered murine animal expressing a recombinase, preferably a Cre recombinase, exclusively in its podocytes.

15. Process for manufacturing a genetically engineered murine animal according to one of claims 13 or 14, wherein the murine animal is a mouse or rat, preferably a mouse.



**Fig. 1**



**Fig. 2**



**Fig. 3**

INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2015/061325

A. CLASSIFICATION OF SUBJECT MATTER  
INV. A01K67/027 C07K14/705 C12N15/85  
ADD.  
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED  
Minimum documentation searched (classification system followed by classification symbols)  
A01K C07K C12N  
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZAHNER G, ET AL.: "The Generation of Inducible Podocyte Specific Human PhospholipaseA2-Receptor-1 Transgenic Mice", J AM SOC NEPHROL, vol. 23, FR-P0874, 30 October 2012 (2012-10-30), pages 568A-568A, XP002732007, the whole document ----- -/--	1-15

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents :

<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>
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Date of the actual completion of the international search  15 July 2015	Date of mailing of the international search report  23/07/2015
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  Brero, Alessandro
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## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2015/061325

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BORZA DORIN-BOGDAN ET AL: "Mouse models of membranous nephropathy: the road less travelled by.", AMERICAN JOURNAL OF CLINICAL AND EXPERIMENTAL IMMUNOLOGY 2013, vol. 2, no. 2, 2013, pages 135-145, XP002732006, ISSN: 2164-7712 cited in the application page 142</p> <p style="text-align: center;">-----</p>	1-15
A	<p>BECK L H ET AL: "M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy", NEW ENGLAND JOURNAL OF MEDICINE, MASSACHUSETTS MEDICAL SOCIETY, US, vol. 361, no. 1, 2 July 2009 (2009-07-02), pages 11-21, XP002553808, ISSN: 0028-4793, DOI: 10.1056/NEJMOA0810457 cited in the application the whole document</p> <p style="text-align: center;">-----</p>	1-15