Construction of Mouse Annexin II Gene Knockout Recombinant and Generation of Mouse Annexin $II^{-/-}$ Cell Line

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Abstract Annexins are Ca^{2+} and phospholipids binding proteins forming an evolutionary conserved multigene family with members of the family being expressed throughout animal and plant kingdoms. Some members of the family participate in the regulation of membrane organization, membrane traffic, and the regulation of ion (Ca^{2+}) currents across membranes or Ca^{2+} concentration within cells. Recently, several cellular or animal annexin knockout models as well as dominant-negative mutants of the family have been established. As an important member of annexin family, annexin II has been proved to be involved with and play some important roles in the development of some human diseases. The paper reports that a gene knockout recombinant (pPNT/annexinII/LacZ) was constructed for blocking the mouse annexin II expression, and two cell clones (D4 and E2) were screened out as the annexin $II^{-/-}$ L5178Y cell clones that will be confirmed further more using PCR. The annexin II knockout recombinant and the annexin $II^{-/-}$ cell line can be used to generate annexin II null mice and further researches on annexin II in future.

Key words annexins; gene knockout; cellular model

Annexins are Ca^{2+} dependent phospholipids-binding proteins forming an evolutionary conserved multigene family expressed throughout animal and plant kingdoms excepting yeasts and prokaryotes[1-3]. Some members of the family participate in the regulation of membrane organization, membrane traffic and the regulation of ion (Ca^{2+}) currents across membranes or Ca^{2+} concentration within cells[2]. Annexin II is an important member of annexin family, and plays important roles in the membrane traffic and organization, regulation of ion channel activity, and binding to a number of different molecules as a surface-bound receptor. There are evidences indicate that annexin II is involved in the development of some human diseases, for examples, heart and cardiovascular diseases, disorders of hemoagglutination, and cancer, etc[2]. Some experimental results show that annexin II can bind to endothelial cell surface, mediate or regulate the interactions between the endothelial cell surface receptors and different molecules[4, 5].

Because of the importance of the family for some physiological or biochemical mechanisms and the development of some diseases, a number of investigators focus their researches on annexin family, and thus, the cellular or animal annexin knockout models as well as dominant-negative mutants have recently been established for a number of members of the family [6-11]. So far, there is no gene knockout animal or cellular model was reported for annexin II. To develop an annexin II knockout cellular model, generate annexin II null mice for further research on annexin II in future, we constructed annexin II gene knockout recombinant and successfully developed the annexin II knockout cell line, annexin $II^{-/-}$ L5178Y cell line.

1 Materials and Methods

1.1 Construction of the annexin II knockout DNA recombinant, pPNT/annexinII/LacZ

The plasmid vector, pPNT (Fig. 1)[12] and mouse embryonic stem cell genomic DNA, 129sv were kindly

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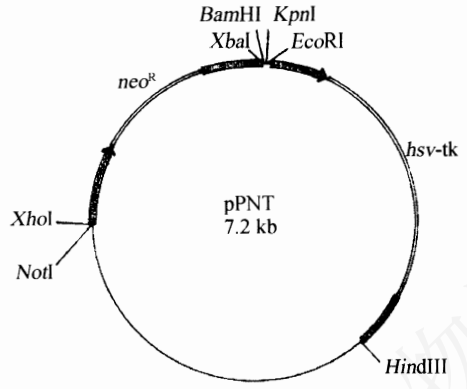

Fig.1 Map of pPNT[12]

The shaded arrows represent the PGK-1 promoter, the hatched boxes represent the PGK-1 poly-A addition sequences, the open boxes are the *neo*^{R} and *hsv*-tk genes as labeled.

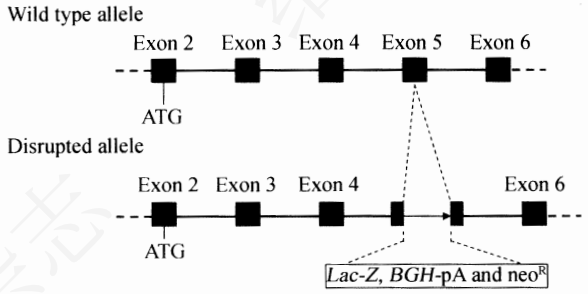

Fig. 2 Disruption of the mouse annexin II gene locus

The inserts from mouse annexin II gene locus were amplified from 129sv. The inserts of *Lac*-Z and *BGH*-pA were amplified from plasmid pcDNA4/TO/Lac-Z (Invitrogen).

provided by Dr. Ye-Shih Ho (Institute of Environmental Health Sciences, Wayne State University, USA). Two genomic fragments (3.2 kb, 3.4 kb) of mouse annexin II were amplified from the 129sv by the kit of Platinum PCR Supermix High Fidelity (Invitrogen). The 3.2 kb fragment (intron 4–5 and exon 5) was inserted into the *Not*I and *Xho*I site at upstream of the *neo*^{R} gene of the vector, pPNT. The 3.4 kb fragment (exon 5, intron 5-6 and exon 6) was inserted into the *Bam*H I and *Eco*R I site at downstream of the *neo*^{R} gene. The exon 5 is disrupted by inserting the sequences of *Lac-Z, BGH*-pA, and *neo*^{R} into it following the 3.2 kb sequence (Fig. 2). By the steps described as above, the annexin II knock-out DNA recomninant, pPNT/annexinII/LacZ was constructed.

1.2 Transfection and cell selective culture

L5178Y tk$^{+/-}$ cell line, a mouse thymus lymphoma cell line, was purchased from American Tissue Collection Center (ATCC). The growth medium was composed of the follows: 500 ml RPMI 1640 medium (Invitrogen), 5 ml penicillin and streptomycin (10 000 IU/ml and 10 000 μg/ml, Mediatech Cellgro), 5 ml sodium pyruvate solution (100 mmol/L, 11 mg/ml, Mediatech Cellgro), 2.5 ml pluronic F-68 (100 mg/ml, Invitrogen), and 50 ml horse serum (Invitrogen). pPNT/annexinII/LacZ was linearized by *Not* I and transfected into L5178Y tk$^{+/-}$cells by using BTX electroporation system (BTX) with an improved procedure: Take the cells in logarithmic growth phase, wash the cells twice with PBS and transfection medium (same to growth medium but contains no serum), then resuspend the cells with 2–5 ml serum free medium and prepare 2×10^7 cells/ml cell suspension. Take 250 μl (5×10^6 cells) into a 1.5 ml tube and add 25 μg (or less) linear DNA, mix by gently vortexing the tube. Put on ice for 10 min. Transfer the cells into the pre-cold electroporation cuvette (0.4 cm), and keep on ice. Reset the parameters of the BTX electroporator as the follows: 250 V, 950 μF, 125 Ω. Put the cuvette back on ice for 10 min after transfection, then put into flask and culture in incubator at 37 ℃ and 5 % CO_2. After 48 h, add G418 into the culture (1 000 μg/ml).

1.3 Selection of the stably transfected cell clones

Keep the transfected cells cultured with G418 for 6 weeks. Clone the cells in 96-wells plate using limit dilution method: to remove the dead cells, centrifuge the cells at 700 r/min for 1 min, re-suspend the cells with growth medium and dilute the cell concentration to 3 cells/ml, add 0.3 ml into each well, incubate the plate in the incubator for 1 h, check each well under scope and mark the wells that contain one cell only. Transfer them to 6-wells plate when the marked wells grow up to 500 cells/well or more, and continue to culture for expanding cells. To select the stably transfected clones, X-gal staining was used to each clone. To remove the dead cells centrifuge the cells at 700 r/min for 1 min. Re-suspend the cells to 10^4 cells/ml in PBS. Take 250 μl and centrifuge the cells down to the Poly-L-Lysine (Sigma, MO) coated slide (800 –1 000 r/min, 5 min). Fix the cells with 0.05% glutaraldehyde at room temperature for 5–15 min.

Wash the cell dot with PBS 3 times thoroughly. Dry up the cell dot in air. Put staining solution (250 μl 0.1 mol/L K. Ferricyanide, 250 μl 0.1mol/L K. Ferrocyanide, 50 μl 0.1 mol/L $MgCl_2$, 100 μl 50 mg/ml X-gal, add PBS up to 5 ml) to cover the cells dot on the slide and incubate the slide at room temperature in a wet box for overnight. Wash the cell dot using water and dry out in air. Positive cells should be stained blue.

1.4 Synchronization of cells

Because the highest annexin II detection level is at the end of G_1 phase and beginning of S phase, we used 5-Fluoro-2'-deoxyuridine (FUdR) (Sigma) to form synchronization of the cells of each clone that had been confirmed as stably transfected clones by X-gal staining. FUdR can stop the cells proliferation at the beginning of S phase. The procedure is as the follows: Seed 10^5 cells into each well in 6-wells plate with medium including no thymine deoxy-ribonucleoside (TdR), add the follows after 40–48 h: 1.0 μg/ml FUdR, 0.5 μg/ml uridine (Sigma), continue to culture 16–20 h. Harvest all cells of each well into a 1.5 ml tube.

1.5 Western blotting

Re-suspend the harvested cells with 100 μl SDS-PAGE sample buffer (3 ml 1 mol/L pH 6.8 Tris-HCl, 1 g SDS, 5 ml glycerol, 50 μl 100 mmol/L DTT, 10 mg bromophenol blue, water up to 50 ml). Boil the samples for 5 min, load and run a 10 % SDS-PAGE gel, transfer the gel to nitrocellulose membrane at 4 ℃ for overnight. Wash the membrane with TBST (0.2– 0.25 mol/L pH 7.6 –8.0 Tris, 1.25 –1.37 mol/L NaCl, 0.025 %–0.1 % Tween 20) once for 10 min. Incubate the membrane with blocking buffer (4 %–5 % low fat dry milk in TBST) at room temperature for 1 h. With 1 ∶ 1 500 ratio in blocking buffer, incubate the membrane with goat anti-annexin II (Santa Cruz Biotechnology, Inc.) at 4 ℃ for overnight with gently shaking. Wash with TBST three times for 15 min each time. Incubate with the blocking buffer containing horseradish peroxidase (HRP) labeled anti-goat (Santa Cruz Biotechnology, Inc.) at 4 ℃ for overnight. Wash the membrane with TBST three times. Put the membrane on a glass plate. Mix ECL Western blotting detection reagents 1 and 2 with equal volume (Amersham Pharmacia Biotech), add the mixed reagents on to the membrane and make sure it is cover every where. Incubate at room temperature for 1 min. Drain away the solution and take image with Luminescent Image Analyzer (Fuji Photo Film Co., Ltd.).

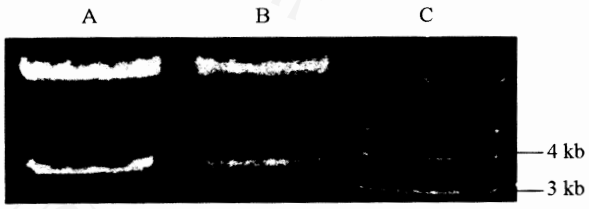

Fig. 3 Digestion analysis of the 17.3 kb pPNT/annexinII/LacZ
(A) Inserted part (3.3 kb) of the *Lac*-Z and *BGH*-pA cut out by *Not*I and *Sal*I. (B) Inserted part (3.5 kb) of the exon 5, intron5–6 and exon 6 cut out by *Bam*HI and *Eco*RI. (C) 1 kb DNA ladder.

2 Results

2.1 Construction of pPNT/AnnexinII/LacZ

Fragment (3 240 bp) from intron 4–5 and exon 5, fragment (3 171 bp) of *Lac*-Z gene, and fragment (249 bp) of *BGH* poly-A were just inserted into the upstream of the *neo*^{R} gene of the vector, pPNT. Fragment (3 430 bp) from exon 5, intron 5–6 and exon 6 was just inserted into the downstream of the *neo*^{R} gene. These recombination steps resulted in the construction of the pPNT/annexinII/LacZ (17.3 kb) successfully (Fig. 2). To make sure of the recombinant constructed correctly, the inserted parts from mouse annexin II genomic sequence were cut out and analyzed by an agarose gel as Fig. 3.

2.2 Generation of the stably transfected cell clones

The pPNT/annexinII/LacZ linearized by *Not*I was transfected into L5178Y tk$^{+/-}$ cell line. After culturing with G418 selective growth medium for 6 weeks, the transfected cells were cloned by using limit dilution method. The stably transfected clones were selected by using X-gal staining method. Selected stably transfected clones are as the follows: A3, A10, B1, B11, C3, C8, C11, D4, D8, D12, E2, E3, E10, G4, G5, G11, G12, and H9.

2.3 Identification of the annexin II$^{-/-}$ L5178Y clones

The stably transfected clones were synchronized and stopped proliferating at the beginning of S phase by

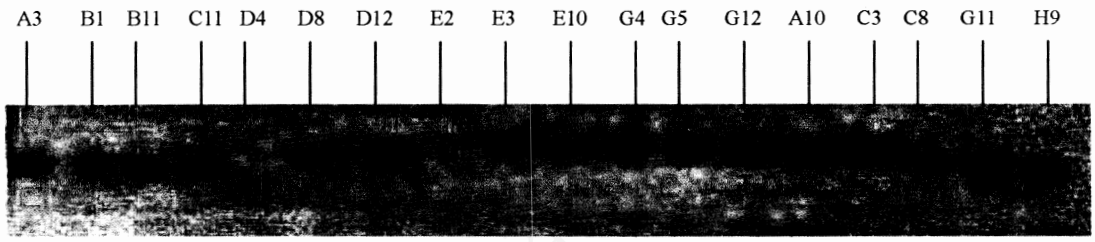

Fig. 4 Detection of annexin II expression for each stably transfected clones by Western blotting
The molecule weight of annexin II is around 37 kDa. Clones D4 and E2 have no annexin II detected.

incubating with the growth medium containing FUdR. To identify out the annexin $II^{-/-}$ clones, the western blotting was used to detect the annexin II expression for each clone. The detection result is shown as Fig. 4. The clones, D4 and E2, have no annexin II detected at 37 kDa (The molecule weight of mouse annexin II is around 37 kDa), so the clones D4 and E2 were established as annexin $II^{-/-}$ clones.

3 Discussion

Annexin II is involved in a number of human diseases and intermediate the regulation of membrane organization, membrane traffic, ion (Ca^{2+}) currents across membranes or Ca^{2+} channel activity, and binding to a number of different molecules as a surface-bound receptor [2, 5, 13, 14]. Annexin II is composed of heavy (p36) and light (p11) chains. It can be found *in vivo* as p36, p36p11, and $p36_2p11_2$. The heavy and light chains are commonly lost in prostate cancer and in some of its precursor, prostate intraepithelial neoplasia [15]. Annexin II may facilitate tumor invasion metastasis because some data shows that human procathepsin B interacts with the annexin II tetramer on the surface of tumor cells [16]. Tyrosine phosphorylation of annexin II could be part of the internalization and sorting mechanism of the insulin receptor and plays role in insulin signal transduction [17], also, the insulin receptor and its signaling pathways may participate in molecular mechanisms mediating annexin II secretion[18]. Annexin II regulates volume-sensitive Cl^- channels, and annexin II-p11 complex formation is either directly or indirectly involved in the activation of $I_{Cl,vol}$ in the endothelial cells[19]. In summary, annexin II has multiple functions or roles involved in the development of some human diseases and its other functions are currently investigated.

The gene knockout animal or cellular models of annexins I, VI, and VII have been developed[6-11], but annexin $II^{-/-}$ animal or cellular models have not been reported yet. In our study, we constructed a gene knockout recombinant, pPNT/annexinII/LacZ, for the generating of the annexin $II^{-/-}$ animal or cellular models. As a primary development, we tried to generate the annexin $II^{-/-}$ mouse cellular model. We chose exon 5 to be disrupted because it is located near to the center of the annexin II genomic sequence that is easy to be recombined when the wild type allele combines to disrupted allele. *Lac*-Z, *BGH*-pA and neo^R were inserted into the center of the exon 5 sequence resulting in the disruption of the annexin II. The insertion of *Lac*-Z and *BGH*-pA is just for the convenience to select the stably transfected clones. The neo^R is from the knockout vector for cell selective culture. In the pPNT/annexinII/LacZ, a 3.2 kb mouse annexin II genomic sequence (partly from intron 4–5 and exon 5) was inserted into the upstream of the neo^R gene and a 3.4 kb mouse annexin II genomic sequence (partly from exon 5, intron 5–6 and exon 6) was inserted into the downstream of the neo^R gene. The agarose gel analysis shows that the insertion is correct (Fig. 3). After G418 culturing and X-gal staining selection, eighteen clones were established as the pPNT/annexinII/LacZ stably transfected clones. To detect the annexin II expression at a highest expression level, FUdR was used to make the synchronization of the proliferation of the 18 stably transfected clones at the beginning of S phase. Western blotting result shows that clones D4 and E2 have no annexin II detected and indicates that annexin II has been completely blocked in the both clones (Fig. 4). As the result, clones D4 and E2 have been established

as annexin II$^{-/-}$ L5178Y cell clones that will be confirmed further more using the PCR with the primers based on the sequences of the inserts and annexin II genome.

In summary, the annexin II knockout recombinant, pPNT/annexinII/LacZ was constructed using pPNT as vector, the pPNT/annexinII/LacZ was transfected into L5178Y tk$^{+/-}$ cell line, eighteen L5178Y cell clones were selected as pPNT/annexinII/LacZ stably transfected clones, and clones D4 and E2 were established as the annexin II$^{-/-}$ cellular clones. The pPNT/annexinII/LacZ and the annexin II$^{-/-}$ cell line can be used to generate annexin II null mice and make further researches on annexin II in future.

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小鼠膜联蛋白 II 基因剔除重组子的构建及小鼠无膜联蛋白 II 细胞系的建立

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摘要 为了便于对膜联蛋白 II (annexin II)的进一步研究以及今后进一步发展膜联蛋白 II$^{-/-}$ 动物模型，构建了膜联蛋白 II 基因封闭重组子(pPNT/annexinII/LacZ)，筛选了细胞(L5178Y)克隆，并获得了膜联蛋白 II$^{-/-}$ 稳定细胞克隆(D4，E2)。所获膜联蛋白 II$^{-/-}$ L5178Y 克隆有待于以 PCR 做进一步鉴定。

关键词 膜联蛋白；基因剔除；细胞系

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