Preparation of ORP4L/ApoE double-gene knockout mice

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Abstract

ORP4L, a member of the Oxysterol-binding protein (OSBP) related-proteins (ORPs) family, has been shown crucial in cell survival. But its role in macrophage function and effect in atherosclerosis are not clear. The mouse lack of ApoE spontaneously develops atherosclerosis lesions on a high-fat diet, and the distribution of the atherosclerosis plaques is similar to human. Thus ApoE knockout mouse is an ideal animal model for the research of atherosclerosis. In this study, ORP4L heterozygous mice were mated with ApoE knockout mice and ORP4L/ApoE heterozygous mice were generated, then the ORP4L/ApoE heterozygous mice mated with each other and ORP4L/ApoE double gene knockout (DKO) mice were obtained. The model with ORP4L/ApoE double-gene knockout will be used for further observation of ORP4L role in atherosclerosis.

Keywords

Oxysterol, ORP4L, ApoE, knockout.

1. Introduction

Oxysterol-binding protein (OSBP) related-proteins (ORPs) is a proteins family that all members containing an oxysterol binding domain, which has been shown to accommodate a variety of oxysterols, cholesterols, ergo sterol, but also phosphatidylinositol-4-phosphate (PI4P). OSBP is the prototype member of this family; it binds 25-hydroxycholesterol (25OHC) with a Kd of 10nM which is higher than the affinity for other oxysterols. The other researches about ORPs display different affinity for various oxysterols, and like OSBP, some of them have been demonstrated to bind cholesterol. The capacity of ORPs to bind both oxysterols and cholesterols imply that ORPs can mediate the function of oxysterols, and they also play an important role in lipid metabolism and transport [1].

In addition, many of the mammalian ORPs have an ER targeting determinant. This can be either a peptide motif (two phenylalanines in an acidic tract, FFAT) that interacts with VAMP-associated proteins (VAPs), integral membrane proteins of the ER [2], or an ER-targeting carboxy-terminal trans-membrane segment [3, 4]. Most of them also contain an amino-terminal region having a pleckstrin homology (PH) domain which interacts with phosphatidylinositol phosphates (PIPs) [5-8]. ORPs containing the PH domain are called “Long ORPs”, while those lacking a PH domain are called “short ORPs”. In several cases the binding of PIPs by ORPs is crucial for their targeting to specific subcellular membranes, while the role of sterol binding has remained somewhat mysterious.

ORPs’s function have not been illustrated completely yet. However, several ORPs exist at membrane contact sites and regulate the activity of enzymatic effectors or assembly of protein complexes, with impacts on signaling, vesicle transport, and lipid metabolism. More and more protein interaction partners of ORPs have been identified, providing hints of their involvement in multiple aspects of cell regulation.

ORP4L is a member of the ORP family, it is a full-length version that includes an N-terminal PH domain [9]. The mRNA and protein of ORP4L expression has a strict specificity of tissue distribution, only in brain, heart, testis, retina and other tissues [10]. The research of mouse showed that deficiency of ORP4 causes male infertility due to severe OAT in mice. In ORP4-deficient testis, postmeiotic spermatids underwent extensive apoptosis, leading to a severely reduced number of spermatozoa.

These results suggest that ORP4 is essential for the postmeiotic differentiation of germ cells [11]. The study of leukemia found that ORP4L only expressed in peripheral blood leukocytes of leukemia, but no expression in normal human peripheral blood leukocytes [12-15]. Many studies about a variety of cancer cells reported that ORP4L highly expressed in liver cancer, chronic myeloid leukemia and acute T lymphoblastic leukemia and other cancers [12, 16]. Thus, the expression of ORP4L is considered a potential sign of tumor metastasis and poor prognosis. Recent study about ORP4 indicated that Silencing ORP4L or all ORP4 isoforms triggers growth arrest and apoptosis, which is suppressed by H-ras and involves the C-terminal lipid binding domain [17]. This result imply that ORP4 is required for survival and proliferation of immortalized and transformed cells. But, the role of ORP4L in macrophage survival has not been accessed yet and ORP4 role in vivo in atherosclerosis has not been reported also.

As an important component of plasma lipoproteins, ApoE can regulate plasma cholesterol levels, and it is also a molecular target in the development of hyperlipidemia and atherosclerosis (AS) [18].Mice usually have a strong ability to resist atherosclerosis, even C57BL/6J mice, the most sensitive strain, just formed slight fatty streaks when fed a high fat diet. Therefore, while mice experiments have many advantages, its research on atherosclerosis is still limited. Until 1992, University of North Carolina and Rockefeller University apply embryonic stem cell gene knockout technology successfully bred ApoE knockout mice, and the mice spontaneously develop atherosclerotic lesions on a normal chow diet or high-fat diet with pathology similar to advanced human atherosclerotic lesions [19, 20]. This mouse thus is served as a model to study atherosclerosis widely.

In this study, we generated ORP4L/ApoE double-gene knockout (DKO) mice and provided the tool to study potential role of ORP4L in atherosclerosis.

2. Materials and methods

2.1. Materials.

ORP4L knockout (ORP4LFlox/-) mice were produced by our lab;

ApoE knockout (ApoE-/-) mice were obtained from the Jackson Laboratory;

Taq EX kit was purchased from Takara Bio;

Trans 2K plus II DNA marker was purchased from TransGen Biotech;

Protein markers were purchase from Thermo Fisher Scientific;

Rabbit antibodies against human ORP4L were purchased from Sigma-Aldrich;

Anti-actin monoclonal antibody was purchased from Proteintech Group;

All primers were produced by Invitrogen;

2.2 Methods.

Generation of ORP4L/ApoE DKO mice. ORP4L heterozygous (ORP4LFlox/-) mice were mated with ApoE knockout (ApoE-/-) mice and ORP4L/ApoE heterozygous(ORP4LWT/-ApoEWT/-) mice(referred to as the F1 generation) were born, then the ORP4L/ApoE heterozygous mice mated with each other and ORP4L/ApoE DKO(ORP4L-/-ApoE-/-) mice(referred to as the F2 generation) were obtained.

Genotyping. Polymerase chain reaction (PCR) with tail snip DNA was used to identify ORP4L/ApoE DKO mice and ApoE KO mice. The information of primers is in Table 1.

Western blot analysis. Murine tissues were homogenized in quadruple volumes (w/v) of SET buffer (containing 0.25 mol/L sucrose, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 7.4) with protease inhibitors(Roche). After centrifugation at 1000 g at 4 °C, the supernatants were used as the total protein extracts. Each total protein extract was separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% (w/v) skim milk in TBS-T buffer (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.05% (w/v) Tween-20) and incubated with the monoclonal

anti-human ORP4L antibody. After incubation with horseradish peroxidase-conjugated anti-rat IgG antibody, ORP4L was detected by enhanced chemiluminescence.

Table 1 The information of primers

Primer Name	Sequence
5armORP4L(m)sen1932	AGTGTCCTCAAGGGCTCA
3armORP4L(m)anti40	GTGCGTCCAACCAAGTCA
oIMR0180	GCCTAGCCGAGGGAGAGCCG
oIMR0181	TGTGACTTGGGAGCTCTGCAGC
oIMR0182	GCCGCCCCGACTGCATCT

3. Results

3.1 Generation of ORP4L/ApoE heterozygous mice

Because deficiency of ORP4 causes male infertility, we used ORP4L heterozygous ($ORP4L^{Flox/-}$) mice crossed with ApoE KO ($ApoE^{-/-}$) mice. There were two genotypes ($ORP4L^{Flox/WT}ApoE^{WT/-}$ and $ORP4L^{WT/-}ApoE^{WT/-}$) in offspring mice, referred to as the F_1 generation. ApoE gene only had one type ($ApoE^{WT/-}$), so we just identified the type of ORP4L gene (Fig. 1) using primer 5armORP4L(m)sen1932 and 3armORP4L(m)anti40. Flox amplified fragment of 803 bp, WT amplified fragment of 579 bp, and ORP4L KO amplified fragment of 283 bp.

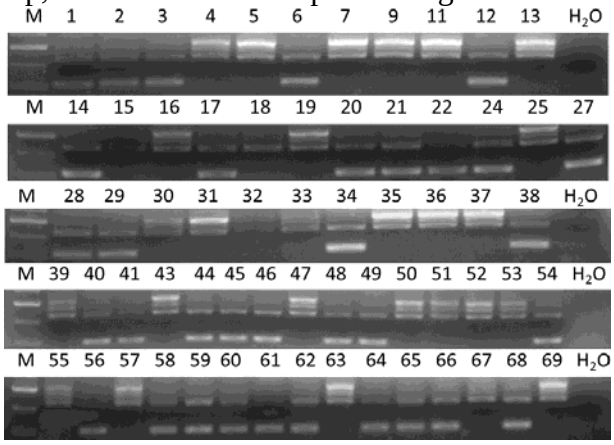

Fig. 1 PCR results of ORP4L gene in F1 generation mice. M: DNA marker; 1-69: the group with DNA from F1 generation mice; H2O: the negative control.

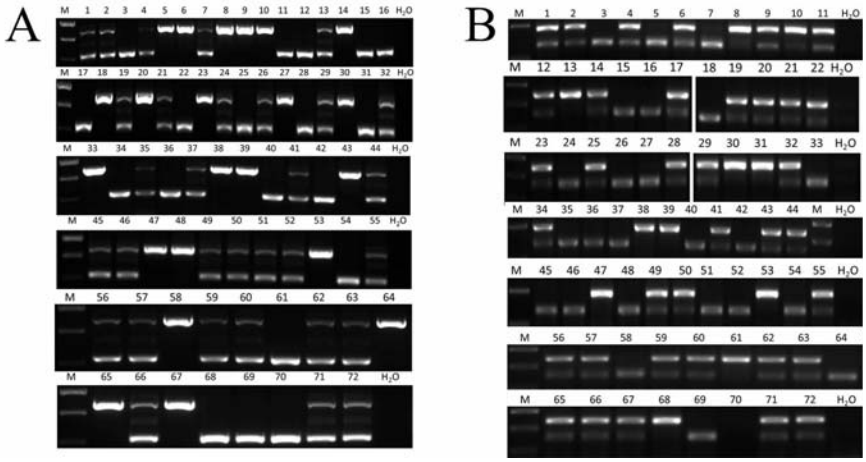

Fig. 2 PCR results of ORP4L (A) and ApoE (B) gene in F2 generation mice. M: DNA marker; 1-72: the group with DNA from F2 generation mice; H2O: the negative control.

3.2 Generation of ORP4L/ApoE DKO mice

We selected ORP4L$^{WT/-}$ApoE$^{WT/-}$ mice in F_1 generation, these mice were then crossed with each other to produce 9 kinds of genotypes mice (referred to as the F_2 generation) which include ORP4L/ApoE DKO (ORP4L$^{-/-}$ApoE$^{-/-}$) mice. Identification method of ORP4L is same as before. According to identification strategies from The Jackson Laboratory, we identified the type of ApoE gene using primer oIMR0180, oIMR0181 and oIMR0182. ApoE KO amplified fragment of 245 bp and WT amplified fragment of 155 bp. The identification of ORP4L and ApoE gene by PCR were showed in Fig. 2.

3.3 Western blot analysis determined the deficiency of ORP4L in DKO mice

To confirm ORP4L had been knockout, we detected the protein expression of ORP4L in brain, heart, liver, lung and testis from ApoE KO and ORP4L/ApoE DKO mice. The data showed DKO mice had no ORP4L expression (Fig. 3).

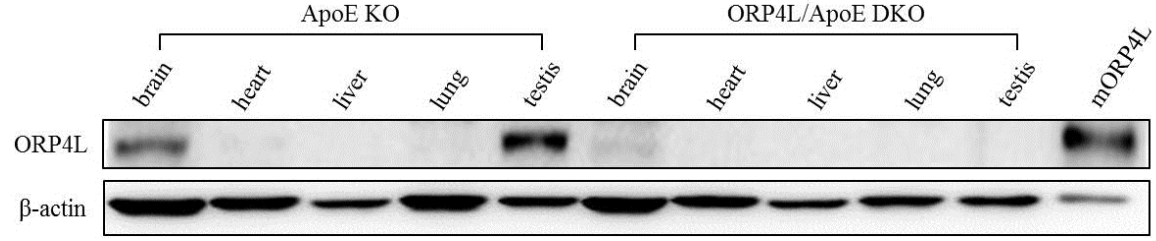

Fig. 3 The expression of ORP4L in different tissues of ApoE KO and ORP4L/ApoE DKO mice. mORP4L: the group with lysis of cells transfected with murine ORP4L gene.

4. Discussion

In this study, We found ORP4L KO male mice do not have fertility corresponding to the research about ORP4 in spermatids of mouse, and the reproductive capacity of ORP4L heterozygous mice are reduced. We use 15 pairs of F_1 generation mice to cross with each other, and 100 offspring mice are expected to give birth. However, there are only 72 offspring mice and 3 ORP4L/ApoE double-gene knockout mice. So we speculate that lack of ORP4L may also affect fertility of male mice and lead to reduction of reproduction rate. In view of ApoE-null mice are the suitable animal model to study atherosclerosis, we can use the ORP4L/ApoE double-gene knockout mice, fed with high fat diet to establish atherosclerosis model for accessing ORP4L role in atherosclerosis in vivo.

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