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以基因剔除技術研究 cullin 4B 的功能

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計畫主持人：林淑華
共同主持人：
計畫參與人員：

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中文摘要

Cullin 4B(Cul 4B) 屬於 cullin 基因族，它是在 1996 年由 Dr. Kipreos 等人比對基因庫中與 cullin 1 相似的基因時發現的，Cul 4B 在許多組織中大量表現（包括整個胚胎），其功能仍不明。大部分 Cullin 基因的功能是參與 ubiquitin-proteasome 降解蛋白質路徑，因此為 ubiquitin 連接酶複合體的組成分子之一， Cul 1 和 Cul 3 參與 cyclin E 的降解過程，在調控 cell cycle 及其他細胞活動上扮演重要角色，而 Cul 4B 是否參與 cell cycle 相關蛋白質的降解仍有待釐清。本計劃的目標是利用基因剔除技術研究 Cul 4B 的功能，藉以了解其缺失後所造成的影響。本實驗室先前已與美國德州大學伍焜玉教授合作，著手進行老鼠的 Cul 4B 基因剔除實驗。首先是得到小鼠同源 Cul 4B 基因，進行標的質體的構築，初步成果發現當將標的質體轉感入小鼠胚胎幹細胞（embryonic stem cell; ES cells）中，抗生素篩選後，共得到 400 多個存活的 ES 細胞株，然而並未得到 Cul 4B 破壞的細胞株。由反轉錄聚合酶鏈反應(RT-PCR)證實 Cul 4B 在 ES 細胞已表現，而且當 Cul 4B 被定位於 X 染色體上後，解答了上述的疑惑(本實驗室進行上述實驗時 Cul 4B 的染色體定位尚未發表)。由於本實驗室所使用的 ES 細胞為 XY 性染色體，而 Cul 4B 位於 X 染色體上，被剔除後 ES 細胞即無正常的 Cul 4B 功能支持，可能因此導至 ES 細胞死亡，以至於先前無法得到 Cul 4B 剔除的 ES 細胞株。所以本計劃採用組織特異性(tissue specific)基因剔除鼠技術，進行 Cul 4B 的基因剔除，以得到 Cul 4B 基因剔除鼠。組織特異性剔除將採用 Cre-LoxP 系統，而目標是肝臟的 Cul 4B 基因剔除，第一年的成果是構築好標的 DNA，經兩次轉染實驗篩選得到 264 個 ES 細胞株，經南方墨點法確認，有 6 個在 cul4B 基因序列中具有 loxP 序列的細胞株，希望能將此 ES 細胞株以顯微注射的方式，打入囊胚以得到嵌合鼠。

關鍵詞：cullin 4B，ubiquitin 連接酶，組織特異性基因剔除鼠，Cre-LoxP

英文摘要

The cullin proteins are subunit of ubiquitin ligase and involved in mediating protein degradation through ubiquitin-proteasome pathway. There are at least six different cullins found in mammalian cells. Each cullin protein seems to form different ubiquitin ligase complex and regulates the level of key proteins in many cellular processes. For example, Cullin1 and Cullin3 are introduced in the degradation of cyclin E, and therefore, play significant roles in cell cycle regulation and other essential biological processes. Cullin 4B (Cul 4B) is a member of the cullin gene family and the function of Cul 4B is not yet characterized. It is highly expressed in various tissues including whole embryo. Whether Cul 4B is involved in degradation of cell cycle proteins remains to be determined. In collaboration with Dr. Kenneth Wu at the University of Texas, we have been investigating the function of Cul 4B by gene targeting in mouse embryonic stem cells. Our preliminary results led us to propose that Cul 4B may be important for the ES cell survival. We have knocked-out Cul 4B by traditional positive-negative selection strategy and find that the homologous recombination efficiency was extremely low compared to our regular found targeting efficiency of more than 10%, indicating that Cul 4B might be essential for the viability of the ES cells. This is proven to be true by the fact that the Cul 4B is expressed in the ES cells and that Cul 4B was mapped to the X chromosome, figured out later from the human genome sequencing database. Since our ES cells are male (XY) origin and Cul 4B is expressed in these cells, one round of targeting would generate Cul 4B null ES cells, which conceivably may lead to lethality of the ES cells supposing Cul 4B is important for survival. Based on this finding, it is essential to knockout Cul4B conditionally and tissue specifically to avoid lethality. We have used the Cre-LoxP system to target mouse liver and preliminary results are summarized below. We have constructed several targeting vectors with Cul 4B gene flanked by lox P sites, and transfected 10^7 ES cells with the constructs. Of 264 clones isolated with antibiotics selection and southern blot analysis, 6 candidate clones were obtained. Injection of blostocysts with these ES clones and generation of the chimeric mice are underway. By mating the loxP-floxed Cul 4B heterozygous mice with mouse strains that express Cre in liver or germ cells, we will generate mouse strain with deletion of Cul 4B in these two organs. The future goal of this project is to study Cul 4B function by analyzing the phenotypes of the Cul 4B knockout mice.

Keywords：Cullin 4B, ubiquitin ligase, tissue specific knockout mice, Cre-LoxP

前言

Targeted protein degradation by the ubiquitin-proteasome pathway plays a vital role in monitoring the abundance of many short-lived regulatory proteins. Through the action of a multienzyme system consisting of the E1 ubiquitin activating enzyme, the E2 ubiquitin conjugating enzyme, and the E3 ubiquitin ligase, multiple ubiquitin moieties are delivered to the target protein to form a polyubiquitin chain and then the target protein was recognized and degraded by the 26S proteasome. The SCF subclass of E3s is a group of multimeric protein complexes that are assembled around a core module composed of Skp1, a cullin family member, the Rbx1 (Roc1/Hrt1) protein and a F-box protein. Each cullin protein seems to form different E3 complex and plays a role in regulating the level of key protein in many cellular processes. Cul 4B is a novel member of this family and its function is unkown. A preliminary study in my lab, in collaboration with Dr. Kenneth Wu, revealed that whole-body knockout of the *Cul 4B* gene might lead to embryonic lethality. A conditional tissue-specific knockout is, conceivably, an appropriate strategy to study the function of Cul 4B. This project will focus on the roles of Cul 4B in cell growth and embryo development.

研究目的

The specific aims of this proposal are as follows:
(1) To create conditional and tissue-specific Cul 4B knockout mice using Cre-LoxP system.
(2) To study the function of Cul 4B and its role in cell growth during embryogenesis and adulthood.

文獻探討

Cullin 4B

Cullin 4B (Cul 4B) is a member of the cullin gene family as defined by sequence homology (1). The full-length cDNA of human Cul 4B have been isolated, and the sequence analysis indicated that the Cul 4B is expressed as a 716-amino acid protein (2). Human cullin 4A(Cul 4A) and Cul 4B are orthologs of nematode Cul 4. The partial C-terminal amino acid sequences of Cul 4A and Cul 4B share 88% identity (1). Cul 4B is expressed in a wide variety of tissues including whole embryo. Recently, the gene coding for human Cul 4B was identified to locate on X chromosome Xq23 by sequence analysis in GenBank and shown that it spans over 36 Kb and contains 20 exons (3).

There are at least 6 identified mammalian cullins (1), and they share extensive sequence homology in a region of approximately 200 amino acid residues, designated the cullin homology domain (CH) (4, 5). Cullins interact with the RING-H2 domain protein Rbx1/Roc1/Hrt1 through their CH domains to form core ubiquitin ligase (E3) modules as SCF complex. Among members of the cullin family, Cul 1, Cul 2 and Cul 3 have been demonstrated to mediate the selective degradation of regulators of cell cycle and signaling pathways (see section "cullins"). Recently, the associated proteins and the proteolytic substrates of Cul 4A and Cul 5 were demonstrated. However, little is known about the Cul 4B.

Cullins

Cul1 was originally identified in nematodes in a screen for genes inhibiting hyperplasia (1) and is homologous to Cdc53 (also called CulA) in yeast. A family of four additional *Caenorhabditis elegans* and six human genes were identified in databases by searching with the Cul1 sequence. Cul1 is the best characterized member of the family and is the only one that interacts with Skp1 (6). The name cullin comes from the verb 'to cul' in the sense of 'to sort'. Cul1 has three domains that mediate its association with other components of the SCF (reviewed in ref 7). The least conserved domain among cullin members is the N-terminal region which in Cul1 mediates its binding to Skp1. The second and most highly conserved domain is present in the extreme C-terminus of all cullins and mediates the attachment of a small ubiquitin-like protein, Nedd8 (Rub1 in yeast). Covalent modification of cullins by the Nedd8 pathway appears to enhance the ubiquitin-ligating activity of some SCF ligases (see next section) (reviewed in ref 8). A third domain downstream to this Skp1-binding region is called the cullin homology (CH) region or Cdc34/RING-H2 subunit-recruiting domain. This domain is conserved among all cullins and binds to Rbx1/Roc1 (Hrt1 in yeast) (ref 9 and reviewed in ref 10, 11). Other cullins also bind to Rbx1 and form different ligases with different specificity. Cul-2 associates with a complex of elongin C (a protein similar to Skp1), and elongin B (an ubiquitin-related protein), and either the VHL (Von Hippel Lindau) tumor suppressor protein or a Socs (suppressor of cytokine signaling)-box-containing protein. Together, this complex is referred to as the VBC-like ligase, in which VHL and probably Socs proteins carry out the role of the substrate-targeting subunit performed by F-box proteins in SCF complexes (reviewed in ref 10, 11). It is likely that other cullins form similar complexes and that the SCF pathway is a prototype system for a larger class of multi-component cullin-based ubiquitin ligases. Interestingly, APC/C contains a cullin subunit (Apc2) and RING finger protein (Apc11) that targets a specific set of substrates.

Targeted deletion of the *Cul1* gene in *C. elegans* and in mice has provided useful genetic tools for the analysis of the function of this gene. In *C. elegans*, the loss of Cul1 results in increased proliferation and hyperplasia in all larval tissues examined (1). This suggests that Cul1 is an essential negative regulator of cell cycle. Additionally, Cul1 is required for developmentally regulated cell cycle exit (G1 to G0 transition), however the loss of function of Cul1 in quiescent cells does not have an effect on cell cycle re-entry (G0 to G1 transition). Mice carrying a deletion in *Cul1* die around embryonic day 6.5 and cyclin E protein levels, but not mRNA are increased in these cells (as determined by immunohistochemistry and in situ hybridization, respectively) (12, 13). The mechanism of developmental arrest in $Cul1^{-/-}$ embryos is unknown. The accumulation of cyclin E is not likely to be the cause, since cells have a tolerance for high levels of this cyclin (14, 15). It is likely that the emerging role of Cul1 in SCFs is as a core component that supports the targeting of multiple substrates, which probably accumulate in these $Cul1^{-/-}$ cells. Very recently, it has been reported that *Cul1* is a direct transcriptional target of Myc and that enforced expression of Cul1 or antisense p27

oligonucleotides is capable of overcoming the slow growth phenotype of Myc-deficient mouse embryonic fibroblasts (16). This indicates that p27 is a major obstacle to cell cycle progression in the absence of Cul1.

In addition to Cul1, Cul2 and Cul3 also have been demonstrated to mediate the selective degradation of regulators of cell cycle and signaling pathways. Cul2 associates with elongin C, elongin B, VHL protein and Rbx1 proteins to form VCBCR complex. The VCBCR complex binds HIF-1alpha, transcription factors critically involved in cellular responses to hypoxia, and targets it for ubiquitin-mediated proteolysis (17). Okuda et al. also showed that a member of the atypical protein kinase C group, PKClambda, is ubiquitinated by the VCBCR complex (18). Cul3 may be a core component of an E3 ubiquitin ligase complex that targets free cyclin E for destruction. Genetic evidence from *cul3*$^{-/-}$ mice (19) is also consistent with the specific involvement of Cul3 in the ubiquitination of cyclin E. Mice homozygous for the *cul3* deletion die at or before day 7.5, with disorganized (or in some cases absent) extraembryonic tissues, abnormal development of the trophectoderm, and a failure to undergo proper gastrulation. Importantly, cyclin E levels in *cul3*$^{-/-}$ embryos are elevated in the ectoplacental cone and the extraembryonic ectoderm (but not embryonic ectoderm) relative to wild-type mice, and this increase correlated with a dramatic increase in the number of S-phase cells as determined by BrdU incorporation (19). Thus, Cul3 is likely to be directly required for destruction of cyclin E in specific tissues in the developing mouse embryo. However, it seems unlikely that the developmental defects observed are solely due to cyclin E accumulation, as Cul3 complexes will likely regulate the levels of other proteins in addition to cyclin E.

Recently, the association proteins and proteolysis substrates of Cul4A and Cul5 were identified. Cul4A can physically associate with the damaged DNA binding protein (DDB), which is composed of two subunits, p125 and p48. DDB binds specifically to UV-damaged DNA and is believed to play a role in DNA repair. Chen et al. reported that Cul4A stimulates degradation of p48 through the ubiquitin-proteasome pathway, resulting in an overall decrease in UV-damaged DNA binding activity (20). Kamura et al. demonstrated that the Cul5/Rbx1 module associates with the Elongin BC complex and a novel elongin BC-box/Leucine-rich repeat-containing protein MUF1 to form a functional ubiquitin ligase (21).

The versatility of protein ubiquitination as a cellular regulatory mechanism is now well established and appears to be comparable to that of other protein modifications such as phosphorylation. Indeed, protein phosphorylation and ubiquitination go hand in hand in the regulation of many cellular pathways. However, the enzymology of the ubiquitination reaction is more complex than that of protein phosphorylation, and the mechanism of polyubiquitin chain formation is unclear yet. It is hoped that the recent characterization of the APC and SCF complexes, and their reconstitution, will allow further progress on this topic to be made. We can expect in the near future is the identification of an ever-increasing number of substrates of the ubiquitin system and of their specific E2/E3 complexes. It remains to be seen whether all the new E3s will belong to one of the known classes, or whether new types of E3s will be

identified. The ubiquitin system is involved in many cellular processes, some of which are linked to human pathologies, such as malignancies, hypertension, the inflammatory response and skeletal muscle breakdown (review in ref 22). Therefore, modulation of the activity of the ubiquitin system may be expected to serve in the future as a target for therapeutic intervention. Angelman's syndrome, a human genetic disease with features of severe motor and intellectual retardation, is due to mutations in the ubiquitin ligase E6-AP (23, 24). Liddle's syndrome, in which patients develop a severe form of hypertension, is due to a mutation in the kidney epithelial Na^+ channel (ENaC) that does not allow interaction of the channel with its ubiquitin ligase, Nedd4. Stabilization of the channel leads to excess reabsorption of Na^+ and H_2O (25). It is likely that, in addition to these two syndromes, other human genetic diseases will be linked to genes encoding ubiquitin system components. One intriguing possibility is the existence of slightly altered-activity variants of ubiquitin system genes. In some cases, small differences in steady-state levels of regulatory proteins can have significant effects on the organism; for example, p27Kip1 was shown to be haploinsufficient for tumor suppression, i.e., even heterozygous mice displayed an increased incidence of cancer (26). Thus, it is conceivable that even minor alterations in the normal degradation of p27Kip1 or of other regulatory proteins by the ubiquitin system can contribute to a disease phenotype. This type of mutations in ubiquitin system genes leading to specific pathologies may be uncovered with the new mapping methods for quantitative trait loci.

In the light of other cullin members involved in the regulation of key proteins of cellular processes, it is possible that Cul 4B also play a role in regulating cell function. Our preliminary results indicated that the disruption of Cul 4B may lead to lethality of the ES cells, so we change to create conditional tissue-specific knockout mice to study the function of Cul 4B. This study will provide an insight into the physiological roles of Cul 4B in cellular processes including cell growth and embryo development. The results of this study will expand the knowledge on the regulation of cell function through ubiquitin-mediated degradation pathway.

研究方法

Designs

Mouse with deletion of the *Cul 4B* gene in specific tissues will be generated as an animal model for dissecting the functional roles of Cul 4B. The Cre/LoxP strategy derived from the bactriophage P1 will be used to perform conditional and tissue specific knockout. We will create mouse lines with the 1.9 kp LoxP sequences integrated in introns 9 and 11 of *Cul 4B* gene. When the cre recombinase is introduced into these mice by CMV driven-Cre gene expression or by breeding the LoxP carrying mice with the transgenic mice expressing Cre enzyme, we will be able to delete exons 9-11 of *Cul 4B* in the mouse. The morphology and physical examination of the generated Cul 4B knockout mice will be performed to evaluate consequences of deletion of Cul 4B.

Methods

(1) Construction of LoxP containing targeting vector:

Two targeting vectors will be designed based on selection by neomycin resistance gene and HPRT gene, respectively. As shown in fig. 1, a 7-kb *Bam*HI fragment containing exons 6-13 and a 3.7-kb *Bam*HI-*Eco*RI fragment containing exons 14-17 of mouse *Cul 4B* gene will be subcloned into the plasmid pPNT-HPRT ($HPRT^+$) or pKO-loxP (neo^r) containing 2 LoxP sequences. The selection gene is inserted into the intron 13 and flanked by 2 loxP sites. The third loxP site is inserted into the intron 10 in the 7 kb fragment. The cre recombinase will direct homologous recombinantion of any pairs of the LoxP sites, and excision of some LoxP combinations will result in deletion of exons 11-13.

(2) Culturing ES cells and gene targeting at the Cul 4B locus.

Wild type ES cell lines (from Dr. Babinet, Pasteur Institute, France) and E14 ($HPRT^-$) ES cell lines (from Dr. Hung Li, Academia Sinica) were cultured and propagated as described (66) in DMEM media supplemented with 15% heat inactivated fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol. To maintain the ES cells in their undifferentiated state, mitomycine C treated primary fibroblasts are used as feeder cells, and the leukemia inhibitory factor (LIF) was added to the media at a final concentration of 1000 U/ml. Transfection of the ES cells with the targeting construct was performed by electroporation at 220 V and 960 μF (Gene Pulser, BioRads) using a 1 ml cuvvete filled with 20 μg/ml of DNA and 107 ES cells. The targeting plasmid will be linerized at one end of homology prior to its intruduction into the cells. The desired ES clones will be selected with G418 (or HAT) and Gancyclovir and survival colonies were isolated and established as clones for further analysis (27).

(3) Generation of conditional Cul 4B knockout mice.

The ES cells harboring the trargeting construct of Fig. 2, will be identified by Southern blotting, and injected into blastocysts to generate chimeric mice. Breeding of the chimera will be carried out as described (28, 29) to obtain germline transmission. Crossing the mouse line with Cre transgenic mice will generate mice with tissue-specific KO of the *Cul 4B* gene. We intend to use CMV (cytomegalovirus)- or albumin-driven Cre transgenic mice provided by Dr. Frank Koentgen (Director and CEO, Ozgene, the Australian Gene Targeting Center) and Dr. Iwakura at the Univeristy of ToKyo. Mice with no Cul 4B in liver and in kidney will be investigated.

(4) Using electrofusion to generate knockout mice.

Electrofusion is used to generate tetraploid embryos, which can then be used to make aggregation chimeras with ES cells. Tetraploid embryos normally develop very poorly, and in the chimeras, they give rise only to trophoblast cells in the blastocyst and planceta and to a few extraembryonic endoderm cells. The majority of the cells in the embryo, and all of the cells in the newborn mouse, are therefore derived from the donor ES cells. It means we don't need to generate chimeric mice by injection ES cells into blastocyst, then mating chimera mice to

generate chimeric mice by injection ES cells into blastocyst, then mating chimera mice to generate KO mice. We can obtain KO mice by aggregation using electrofused embryo and ES cells. To do so, we need the eletrofusion machine (e.g. Multiporator, Eppendorf, Germany) as list in the budget for equipment. We will follow the methods described in ref 30. Briefly, collect two-cell embryos and fuse these embryos by electrofusion, then make aggregation with ES cells. The formed blastocysts are implanted into foster mice.

(5) Determination of Cul 4B expression in tissues from knockout mice.

The expression of Cul 4B in the tissues will be determined by RNA and protein levels by methods established in our laboratory as described previously (29). *In-situ* hybridization and immunohistochemistry (30) will be performed to assess the Cul 4B expression pattern in tissues first, then quantitated by RT-PCR and Western blot analysis respectively.

(6) Morphology and functional study of Cul 4B knockout mice.

The morphology of organs of Cul 4B knockout mice will be analyzed by histochemical methods. We will evaluate functional assays depending on the results of phenotype manifestations of Cul 4B knockout mice.

(7) Production of antibodies.

Antibodies to Cul 4B are very important tools for this project. We anticipate generating polyclonal and/or monoclonal antibodies by immunize animals with synthetic peptides. Another strategy is to express the human Cul 4B cDNA in the T7 phage system (31) to obtain the full-length protein for immunization. We will express the Cul 4B protein using the pET15 vector in the *E. coli* BL21(DE3)/pLysS strain. The fusion protein will be purified and used to raise polyclonal as well as monoclonal antibodies. We are experienced in making antibodies (32-34).

結果與討論

In an attempt to study the function of Cul 4B, a novel gene with unknown function, by creating *Cul 4B* null mice, we have spent nearly a year to clone the mouse homolog of human Cul 4B, generating a positive-negative selection based-targeting vector, and used the vector to disrupt *Cul 4B* in mouse embryonic stem (ES) cells. Of nearly 400 ES clones survived in HAT and Gancyclovir selections, none showed disruption of the *Cul 4B* gene by Southern blotting analysis. It was not known until recently that human *Cul 4B* was mapped to the X chromosome. Since the genes 1 Mb nearby the human *Cul 4B* gene were also mapped to the X chromosomes in mouse, we imagine that mouse *Cul 4B* is likely to locate in the X chromosome. The ES cells we used to disrupt the *Cul 4B* is XY genotype and single targeting event will generate *Cul 4B* null ES cells. This can explain our preliminary result that we were not able to isolate *Cul 4B* null ES cells. Cul 4B is expressed in the ES cells (by the analysis of RT-PCR, we can detect its message). This provides a clue that Cul 4B is functioning in the very early stage of embryonic development. Whole body disruption right from the ES cell stage might be impossible to achieve. Conditional and tissue-specific knockout is an appropriate approach in this regard.

(1) Cloning and analysis of mouse *Cul 4B* genomic DNA.

Isogenic *Cul 4B* genomic DNA was obtained by screening a 129/sv-mouse genomic library (a gift of Dr. Begue, Pasteur Institute, France) with a human Cul 4B cDNA. A total of 14 positive clones were obtained. One of the clone, Clone Q with an insert size of approximately 12.25 kb was isolated and further characterized (data not shown). By analogy to human Cul 4B, physical mapping showed that it contains 15 (exons 3-17) of the 20 exons of the mouse *Cul 4B* gene (fig. 2).

(2) **Construction of targeting vector for whole-body KO of the *Cul 4B* gene.**

Disruption strategy was to interrupt and delete from the 430^{th}-500^{th} codon of Cul 4B by replacing it with the hprt gene from pPNT-HPRT. The genomic DNA used for constructing pPNT-HPRT-Cul 4B are two fragments, that are the 5' 4.3 kb of *Bam*HI-*Apa*I (flanking by *Xab*I and *Kpn*I sites) fragment containing the sequences of exons 6-11, and the 3' 3.7 kb of *Bam*HI-*Eco*RI fragment (flanking by *Xho*I/SalI site) containing exons 14-17. The 2.8 kb fragment containing the exons 12-13 and its flanking sequences was replaced by a 3.2 kb HPRT gene. The construct was approximately 16.3 kb long. The final construct was shown in Fig. 3.

(3) Targeting of ES cells by pPNT-HPRT-Cul 4BKO.

The transfection experiments were quite successful and nearly 400 clones were isolated and subjected to Southern blot analysis with appropriate combinations of restriction enzyme digestion and probe hybridization. No candidate clones were obtained (Fig 4). Compared to the case of thromboxane A2 synthase, its targeting efficiency was 10 %, the homologous recombination between ES cell and targeting vector might have lead to loss function of Cul 4B which is required for ES cell growth. We also found that Cul 4B is expressed in the ES cells by the analysis of RT-PCR (Fig 5.) . We assume that the knockout ES cells can't survive without Cul 4B.

(4) Construction of targeting vector for tissue specific KO of the *Cul 4B* gene

Two targeting vectors will be designed based on selection by neomycin resistance gene and HPRT gene, respectively. As shown in fig. 1

(5) Targeting of ES cells by tissue specific pPNT-HPRT-Cul 4B-3 loxP.

The transfection experiments were quite successful and nearly 264 clones were isolated and subjected to Southern blot analysis with appropriate combinations of restriction enzyme digestion and probe hybridization. 6 candidate clones were obtained (Fig 6).

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計畫成果自評

本研究自得到小鼠同源 Cul 4B 基因後即開始進行研究，首先進行標的質體的構築，發現當將標的質體轉感入小鼠胚胎幹細胞（embryonic stem cell; ES cells）中，抗生素篩選後，共得到 400 多個存活的 ES 細胞株，然而並未得到 Cul 4B 破壞的細胞株。由反轉錄聚合酶鏈反應(RT-PCR)證實 Cul 4B 在 ES 細胞已表現，而當 Cul 4B 被定位於 X 染色體上後，解答了上述的疑惑(本實驗室進行上述實驗時 Cul 4B 的染色體定位尚未發表)。由於本實驗室所使用的 ES 細胞為 XY 性染色體，而 Cul 4B 位於 X 染色體上，被剔除後 ES 細胞即無正常的 Cul 4B 功能支持，可能因此導至 ES 細胞死亡，以至於先前無法得到 Cul 4B 剔除的 ES 細胞株。所以改用組織特異性(tissue specific)基因剔除鼠技術，進行 Cul 4B 的基因剔除，以得到 Cul 4B 基因剔除鼠。

目前已很快地完成所有標的 DNA 之建構，至並經兩次轉染實驗，篩選得到 264 個 ES 細胞株經南方墨點法確認，有 6 個在 cul4B 基因序列中具有 loxP 序列的細胞株，可將此 ES 細胞株以顯微注射的方式打入囊胚，由於本實驗室對基因剔除已有良好的經驗，相信很快可以拿到嵌合鼠進行分析。

Figures:

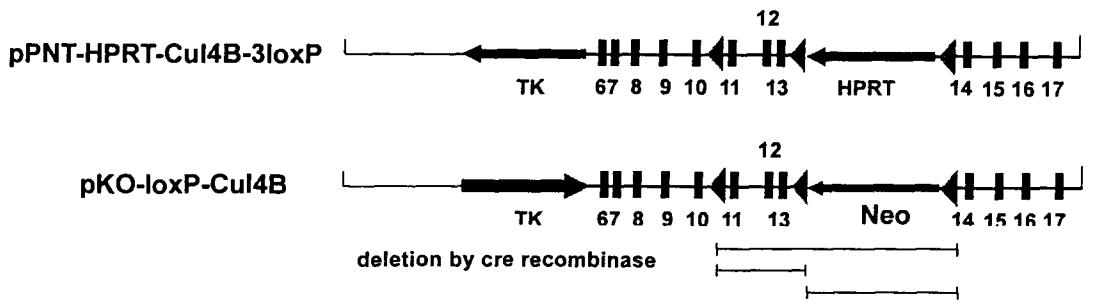
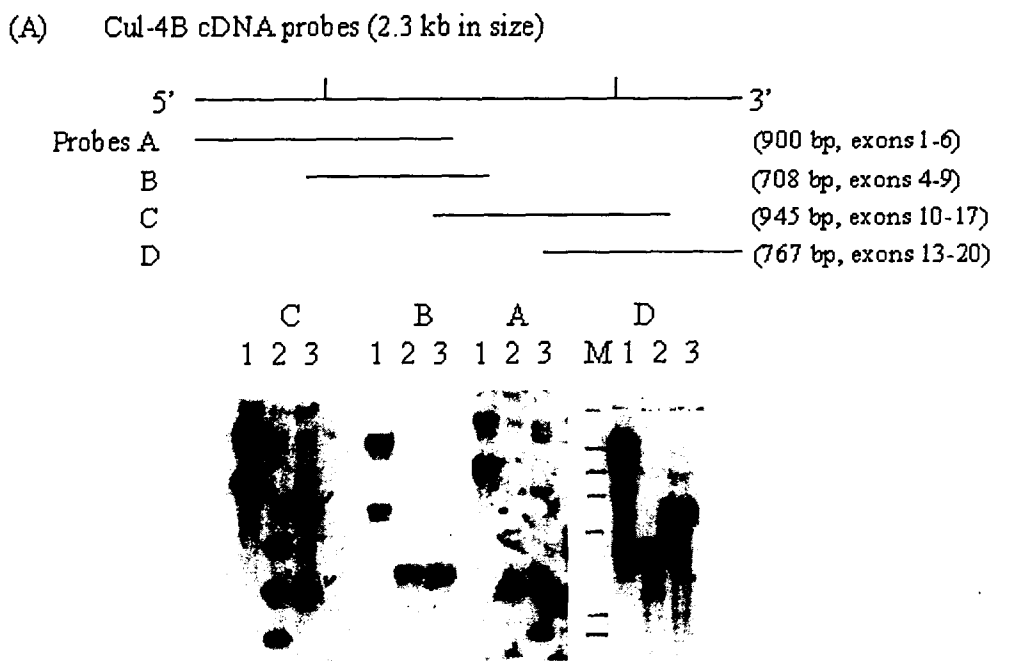


Fig. 1. Targeting Vectors for tissue specific Cul 4B KO. Two vectors based on different selection atrategies, HPRT and Neor, will be constructed. TK is thymidine kinase gene used for negative selection. Filled boxes are exons of Cul 4B gene. Treatment with Cre enzyme will delete LoxP-flanked DNA regions. In this case exons 11-13 will be deleted. ◀ : loxP



1:phage Q digested with *Bam*HI. 2: digested with *Bam*HI and *Eco*RI. 3: digested with *Eco*RI. M: phage lambda DNA digested with *Hin*dIII. Note that as references of sizes of the signals, the signals in probe C, lane 3 are 5.5 kb and 2.7 kb, in probe B is 2.7 kb and in probe A are 5.5,2.3 and 1.65 kb, respectively.

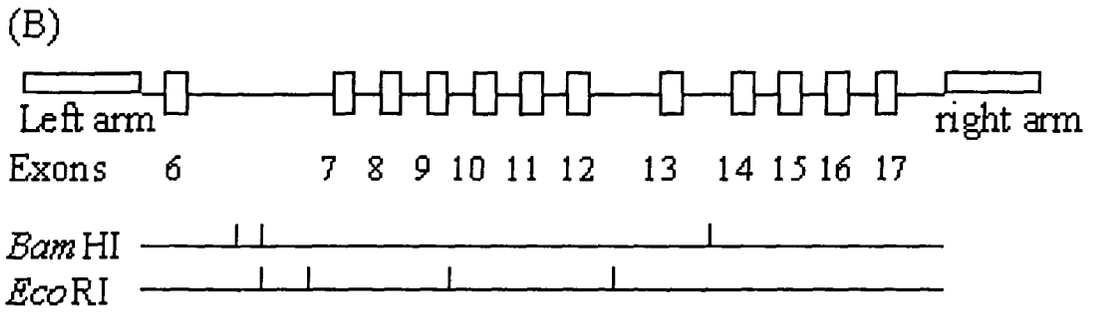


Fig. 2. Restriction enzyme mapping of the DNA structure of mouse cullin 4B genome. A human cullin 4B cDNA was used to screen a mouse genomic DNA library constructed by inserting the *Bam*HI treated total genomic DNA from the 129sv strain mice into lambda phages. More than 10 phage clones carrying different and overlapping fragments were identified and isolated. Phage clone Q containing a 12.25 kb insert covering exons 6-17 was identified by sequencing with exon specific primers and by Southern blotting of the *Eco*RI and *Bam*HI single- or double-digested DNA probed with 3 cDNA subfragments (probes A-C) of cul-4B (panle A). The 12.25 kb insert was subjected to detailed restriction enzyme mapping using more than 15 different enzymes. Only results of the mapping of *Eco*RI and *Bam*HI were shown below. The 12.25 kb insert can be divided in to 5 *Eco*RI fragments or 4 *Bam*HI fragments. The *Eco*RI fragments were, from 5' to 3', 1.65 (fused to the left arm of the lambda vector), 0.65, 2.3, 2.7, and 5.5 kb (fused to the right arm of the vector) in size. The *Bam*HI fragments were, from 5' to 3', 1.3, 0.2-0.3, 7.0 and 3.7 kb. The sizes of the introns are not to scale (panel B).

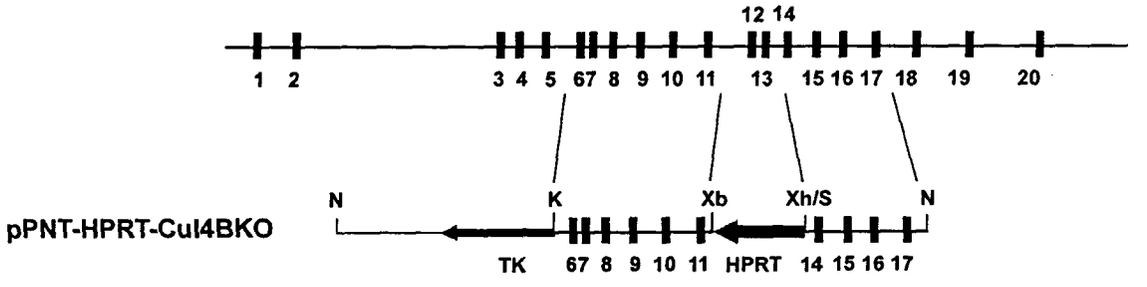


Fig.3. Genomic structure of mouse *Cul 4B* gene and the targeting construct for whole - body knockout of the *Cul 4B* gene in mice. TK is thymidine kinase gene, HPRT: hypoxanthine phosphoribosyl transferas. Filed boxes are exons. Restriction sites are N: NotI, K: KpnI, Xb:XbaI, Xh: XhoI, and S:SalI. Top: genomic structure of the mouse *Cul 4B* gene. Bottom: targeting construct based on HPRT and TK genes.

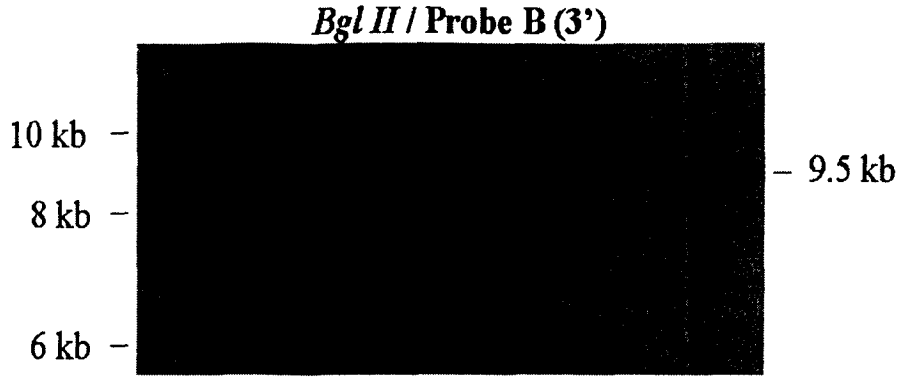


Fig 4. Southern blot analysis of ES clones

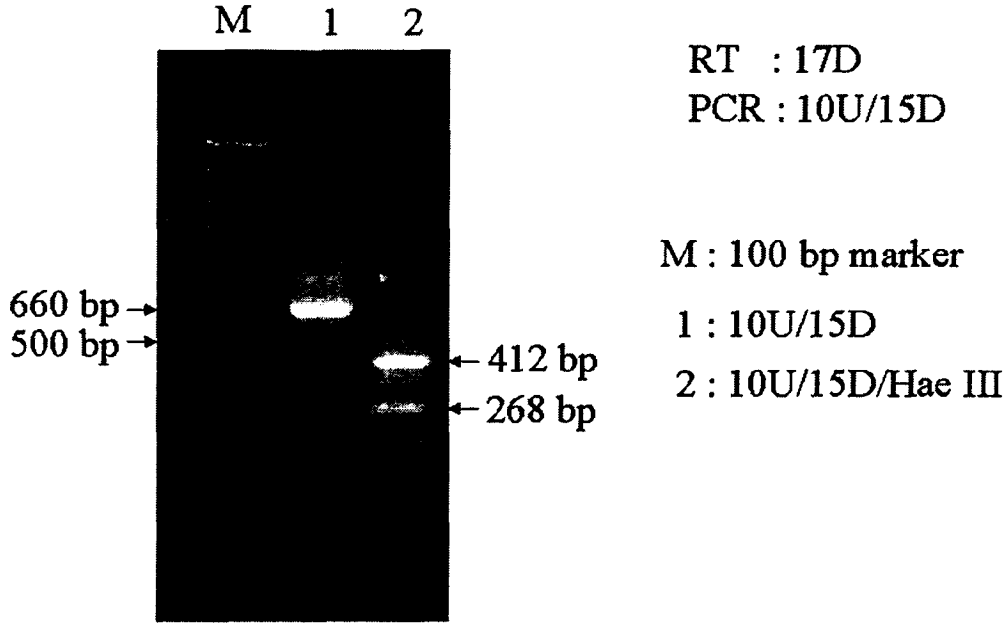


Fig 5. ES cell *Cullin 4B* RT-PCR Result

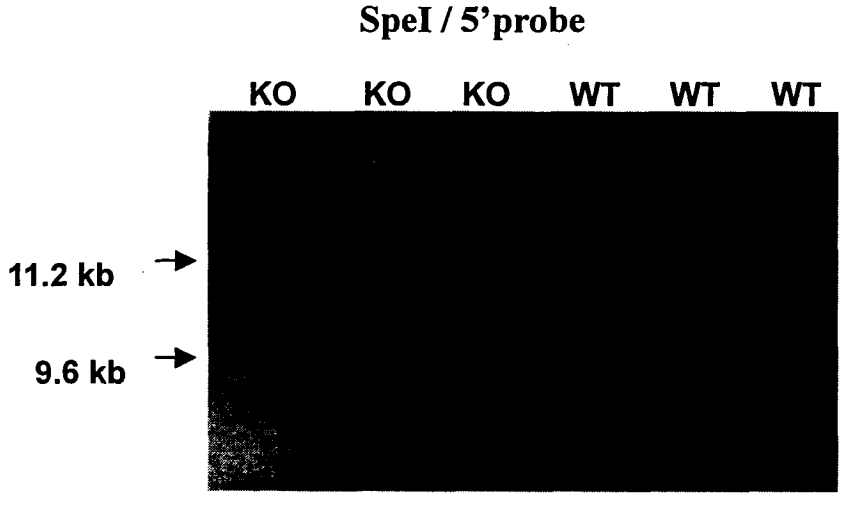


Fig 6. Southern blot analysis of ES clones