行政院國家科學委員會專題研究計畫 成果報告

基因轉殖鼠核心設施 II
研究成果報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-3112-B-002-017-
執 行 期 間 ：95 年 05 月 01 日至 96 年 04 月 30 日
執 行 單 位 ：國立臺灣大學醫學院醫學檢驗暨生物技術學系

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處 理 方 式 ：本計畫可公開查詢

中 華 民 國 96 年 09 月 26 日

行政院國家科學委員會補助專題研究計畫

■年度成果報告
□期中進度報告

基因體醫學國家型計畫

A4-基因轉殖鼠核心設施
A4-Transgenic mouse model core facility

計畫類別：□ 個別型計畫　□ 整合型計畫　■ 核心計畫
計畫編號：NSC 95－3112－B－002－017
執行期間：95 年 5 月 1 日　至　96 年 4 月 30 日

計畫主持人： 林淑華教授
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楊景雯、吳秋萍、林佳佳

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執行單位：台灣大學醫學檢驗暨生物技術學系

中　華　民　國　96　年　5　月

中文摘要

本核心計畫執行至今已 24 個月，執行進度摘錄如下：

一、服務(使用者分布與成果量化表列於 page 5).

(1) 本核心提供的服務及成果如下：

服務項目	委託案件數			完成案件數	待完成
	第一年	第二年	**合計**		
A4-1 Targeting Construct Production	7	23	**30**	24	6
A4-2 ES Cell Gene Targeting	2	3	**5**	5	0
A4-3 Chimera Production	16	27	**43**	25	18
A4-4 Cre-loxP Based Gene Targeting of ES Cell	10	21	**31**	24	7
A4-5 Mouse Embryo Cryopreservation and Recovery	0	1	**1**	1	0
A4-6 Sperm Cryopreservation	0	0	**0**	0	0
A4-7 Screening of Targeted ES Cells	21	57	**78**	55	23
A4-8 Pathogen Free Rederivation	0	1	**1**	0	1
A4-9 Mouse Embryonic Fibroblast (E13.5)	3	0	**3**	3	0
A4-10 Cre Recombinase Transfection	2	25	**27**	13	14

(2) 建立更好服務品質：

(a)建立 chromosome count: 為確保剔除的胚胎幹細胞株具有 40 條染色體，無第八染色體重復及 Y 染色體缺失等最常造成失敗的原因，本核心已完成技術的建立與人員的訓練。對每件胚胎幹細胞標的服務(A4-2) 作染色體數目鑑定，管控其品質。

(b) 建立 Mycoplasma testing: 為確保胚胎幹細胞(ES cell)及滋養層細胞品質，核心實驗室已執行定期 mycoplasma 檢測.

二、研究發展 (詳見 page 7).

(1) 建立 C57BL/6 小鼠品系胚胎幹細胞: 為縮短得到純品系基因剔除鼠的時間，本核心先前成功培育出 5 株 C57BL/6 品系胚胎幹細胞，並已利用近一年時間確定其中一株具高遺傳能力(germ-line transmission)。使用者委員會也已審訂使用此細胞株進行基因剔除實驗的相關規範。

(2) 改善核心服務內容技術研發:本核心引進"Recombinerring"的技術來製備標的載體，可大幅減低製備過程的難度及時間，減低使用者使用基因剔除鼠的進入障礙。此外，本核心除了提供囊胚注射法產製嵌合鼠，也完成利用胚胎幹細胞與桑甚胚聚集技術法(ES cells -morula aggregation)產製嵌合鼠的測試。將來可提供須要此技術的研究人員。

(3) RNA 干擾技術(RNAi): 本核心也建立胚胎顯微注射 RNA 干擾技術(RNAi)，可協助使用者於胚胎時期以 RNA 干擾技術(RNAi)探討基因的功能。初步成果詳見合作研究(下文)。

三、合作研究方面 **(詳見 page 8).**

(1) 依核定的計畫書本核心將運用 knock-in 技術與 ENU 計畫的研究人員(本校 Dr.李建國)合作完成基因剔除的人類疾病小鼠，此合作研究持續進行。

(2)與本校許輝吉教授合作(NRPGM 計畫主持人，計畫名稱"基因剔除動物模式研究人類 L2DTL/ramp 基因")，協助其建立 L2DTL/ramp 基因剔除小鼠，接著以胚胎顯微注射 RNA 干擾基因探討 L2DTL/ramp 在胚胎發育所扮演的角色，確認其基因剔除表現型(knockout phenotype)為"cell autonomous"，相關的研究成果已發表 (*J Biol Chem* 282(2)：1109-18) 。

四、訓練與推廣活動(詳見 page 8).

(1) 核心設施使用說明會：本核心於 2005 年 10 月 19 日於台南成功大學、高雄醫學大學、高雄長庚醫院，2006 年 2 月 22 日於中山醫學大學、中國醫藥大學、中興大學，2006 年 7 月 3 日於國防醫學院，2006 年 3 月 28 日於台大藥理所，2006 年 4 月 14 日於國家衛生研究院，共舉辦九場說明會，參加人數分別為 58、51、50、67、50、67、 60、43 及 75 人。說明會內容包含基因剔除小鼠產製原理、核心設施使用願景及申請服務說明等。

(2) 實驗課程教學：本核心研究人員及助理協助主持人於 2006 年八月舉辦為期一週，由教育部補助的"Biotechnology Education Improvement Program"計畫的實驗教學課程，協助推廣 RNAi 及胚胎顯微相關技術及原理，共有 21 為來自國內不同單位的人員接受訓練。

(3) 攝製實驗內容教學錄影帶：本核心已完成幾項實驗的教學錄影帶，將來可幫助訓練與推廣。

(4) 架設網站：本核心已完成且開放專屬網站(http://140.112.133.74)，提供大眾瀏覽，並將與 user 相關的重要訊息(包括服務進度、排序等)，利用密碼方式開放給使用本核心的使用者。

五、使用者委員會

本核心分別於 2005 年 7 月 24 日，11 月 9 日，2006 年 3 月 1 日，7 月 19 日及 11 月 28 日共舉辦過五次使用者委員會。第一次會議確認核心設施服務收費標準及委員會會議次數及日期。第二次會議完成"核心設施服務使用規範"的修訂。第三次會議完成新增第十項服務使用規範及收費標準的修訂。第四次會議完成會議召集人選舉及標準作業程序的審核。第五次會議決議核心與其他 PI 的合作需經使用者委員會同意才可進行。第二年度相關會議記錄請參閱附件。

英文摘要

The following summaries the progress of the core facility executed for 18 months.

1. Service (see page 5 for description of users and detail).

(1). The table shows the services offered by this core and the number of cases submitted to and completed by this core.

Service item	Cases Submitted			Finish	On going
	Fist year	Second year	Total		
A4-1 Targeting Construct Production	7	23	**30**	24	6
A4-2 ES Cell Gene Targeting	2	3	**5**	5	0
A4-3 Chimera Production	16	27	**43**	25	18
A4-4 Cre-loxP Based Gene Targeting of ES Cell	10	21	**31**	24	7
A4-5 Mouse Embryo Cryopreservation and Recovery	0	1	**1**	1	0
A4-6 Sperm Cryopreservation	0	0	**0**	0	0
A4-7 Screening of Targeted ES Cells	21	57	**78**	55	23
A4-8 Pathogen Free Rederivation	0	1	**1**	0	1
A4-9 Mouse Embryonic Fibroblast (E13.5)	3	0	**3**	3	0
A4-10 Cre Recombinase Transfection	2	25	**27**	13	14

(2). Chromosome count. In order to assure the quality of the targeted ES cell clones, we have trained our technician capable of counting the chromosome numbers of the ES cells. Before used for blastocyst injection, every targeted ES cell line will be subjected to this counting to assure no triplication of any somatic chromosomes or lost of the X or Y chromosomes.

(3)Mycoplasma Test. In order to assure the quality of the targeted ES cell clones, we have trained our technician to check if our ES clones are contaminated by mycoplasma.

2. R&D activities (see page 7 for detail).

(1) Derivation of ES cell lines of C57BL/6 origin. We have generated five C57BL/6 ES cell lines and identify at least one with germ-line transmission competency. The users committee has helped the core to set the guideline of using this ES cell line for targeting. We will be able to prove whether this approach will speed up knockout mice production.

(2) Development of other techniques to improve core services. By introducing “recombinerring” technology to produce targeting vector, we can construct the targeting vector faster and easily. This technology can reduce user’s obstacle to use knockout mouse for their study. We have set up ES cell-morula aggregation technique as a different choice of producing chimeric mice. Users can request using this technique for producing their KO’s.

(3) RNAi microinjection in mouse embryo: We also set up microinjection procedure for RNAi in mouse embryo. This technology can help users to investigate their interesting gene in embryo

stage by using RNA interference.

3. Collaboration Research activities. (see page 8 for detail).

Our core has been cooperating with Professor Hey-Chi Hsu of National Taiwan University, also the project leader of NRPGM. The research project is entitled "Gene knock-out animal model for the study of human L2DTL/ramp gene". Our team has helped Professor Hsu build up L2DTL/ramp knockout animal model, and has used the embryo-microinjection technology, combined with application of RNA interference technology, to assist Professor Hsu in clarifying the function of the L2DTL/ramp gene during the growth of mouse embryo. Relevant research results have been published(*J Biol Chem* 282(2)：1109-18). We have proposed in our initial grant application to collaborate with Dr. Lee's working on ENU mice to use knock-in for production of their disease mice. The collaboration is continued by training technicians for making KO constructs and breeding mice.

4. Training and Dissemination activities (see page 8 for detail).

(1). Disseminations: We have held nine facility illustration meetings at the National Cheng Kung University in Tainan, Kaohsiung Medical University, and Chang Gung Memorial Hospital in Kaohsiung on October 19, 2005; Chung Shan Medical University, China Medical University, National Chung Hsing University on February 22, 2006; National Defense Medical Center on July 3, 2006; National Taiwan University Shool of Medicine Department of Pharmacology on March 28, 2007; National Health Research Institutes on April 14, 2006. The numbers of participants were 58, 51, 50, 67, 50, 67, 60, 43 and 75 people, respectively. We have made general introductions to knockout mice technology, its future and impact on research and also introduction to the execution of the core and how to use the core, etc. The director of the R&D department of the Kaohsiung Medical University and the Chairman of the Department of Pediatrics of the Chang Gung Memorial Hospital were excited about the core services.

(2) Training: We have held a one week workshop on August 2006. (PI's grant "Biotechnology Education Improvement Program" from the Advisory Office of MOE). Twenty one people from nationwide were trained to use RNAi microinjection technology.

(3) Videos for KO experiments. We are in the process of taping all the experimental procedures of making KO mice. This video will be very useful both in training new technicians and postdoct's and helping our users to know what to expect from the core.

(4) Internet facility. The core has completed and opens its internet network for users to visit and get informed.

5. Users Committee activities (see Appendix for second year records).

The users committees have met four times on July 24, Nov. 9 2005 and Mar. 1, Jul. 19 2006 since this project granted. The outcome of the four meetings is (1) The committee have agreed and confirmed the service charges this core has claimed for each service item; (2) The committee has suggested to invite Dr. SeTu from the Graduate Institute of Medical Sciences, National Defense Medical Center to be on the committee; and (3) meeting frequency and dates of each meeting of the committee; and (4) Settle the issues of guideline of the core and application forms for the users; and (5) elect the convener; and (6) to examine the SOP for core service.

目錄

報告內容

1. 前言

Introduction of genes into mammalian germ line is one of the major technology advances in biology. These transgenic animals have been influential in providing new insights into mechanisms of development and developmental gene regulation. Among these, transgenic mouse is one of the best model organisms for studying gene functions and for dissecting the pathological mechanisms contributing to various human diseases[2-3]. This is not only because it is mammalian origin but also a lot of powerful techniques have become available, which allow various manipulations of mouse genome and breeding of the manipulated creatures. Of these, the most advantageous is the establishment of many mouse embryonic stem (ES) cell lines which allows the transmission of any genetic alternations introduced in culture to the gene pool[22-25] and in vitro screening for rare genetic events prior to germ-line transmission. There are two major advances in this filed: one involves the search for genes and mutations during development based on random insertion of reporter constructs into the genome of the ES cells; another is the direct introduction of specific mutation into the germ-line, following homologous recombination in ES cells and knockout of the gene in the ES cells, to produce knockout mice. Both advances contribute to make the mouse model standing out for genetic analysis[22-25].

Gene targeting in ES cells, i.e. introduction of exogenous DNA into ES cells in culture, can be achieved by homologous recombination using different methods. Figure 2A depicts the targeting constructs that have been used to introduce loss-of-function mutations (gene knockout) into genes in ES cells. Sometimes, other gene may compensate function of the lost gene and no phenotype is observed in gene-knockout mice. Many studies have been documented to support this phenomenon, so called "gene redundancy"[22-25]. Another obstacle confront in knockout studies is embryonic lethality. Knockout of certain genes, unexpected to play a role in embryonic development based on previous experience and knowledge, is doomed to result in the death of embryos and thus abrogates further functional study of the gene in the mouse.

The situation is now amendable using conditional and tissue-specific gene knockout technology, which, instead of knocking out the gene expression ubiquitously, allows one to disrupt gene functions only at certain time point and/or only in specific tissues and organs as designed. Two systems for tissue-specific gene knockouts have been established for mouse gene targeting experiments, the Cre-loxP and the Flp-FRT systems. These systems are originated from bacteriophage P1[26] and the budding yeast *Saccharomyces cerevisiae*[27], respectively. These techniques have proven to be very useful in conditionally activating and inactivating specific genes in spatially and temporally restricted patterns[14-17]. Basically, these systems consist of two basic elements: the recombinase enzyme (Cre, or Flp) and a consensus DNA sequence (loxP or FRT) recognized by the recombinase. The recombinase catalyzes recombination between two of the consensus recognition sites, and consequently results in the modification of the associated DNA in between. The 34-bp consensus recognition sequence consists of two 13-bp inverted repeats and an 8-bp non-palindromic core, which defines the orientation of the overall sequence of the recognition site (loxP or FRT) (Fig. 2B). Mediated by the excision function of the Cre recombinase, the flanked region can be deleted or reverted depending on the orientation of loxP (or FRT). When the ES cell clone obtains the loxP-floxed allele, it serves dual purposes depending on at what circumstances the Cre is introduced. When the Cre recombinase is introduced into the ES cell clone carrying the conditional allele, a gene segment (or the entire gene) flanked by the two recognition target sites (*loxP* or *FRT*), it is essentially the same as generating conventional (whole body) KO mouse line (Fig. 2B). When the Cre is introduced into the mouse line carrying the conditional allele

by breeding, it is for conditional tissue-specific KO; gene excision is dependent on breeding of two transgenic lines expressing the recombinase (Cre or Flp) in selected cell lineages or tissues at a specific stage of embryonic or postnatal development. After intercrossing of the two mouse lines, cells expressing the recombinase in the double transgenic offspring delete/modify the conditional alleles by recombination of the two consensus sequences (Fig. 2C).

It is usually required to study gene function in a pure genetic background to avoid epigenetic influences (and modifiers). Because most of the available ES cells are derived from the strain 129 of mice, one would breed the chimeras with the wild-type 129 mice to obtain KO mice. Unfortunately, the 129 mice are basically bad breeders and their genetic background in terms of H-2 locus etc., are not well characterized, which prohibit researchers from using this mice as breeding partners. Backcrossing of 129-derived chimeras to the other inbred strains, such as C57BL/6 and Balb/Cc mice, to generate congenic strains is mostly adopted by researchers to circumvent such problems. However, it usually takes more than one year to obtain a KO mouse line of nearly pure genetic (C57BL/6 or Balb/C) background. One way to shorten the long duration required for generating KO mice of pure genetic background is to use the ES cells of C57BL/6 origin. With the C57BL/6 derived ES cells, one round of backcrossing of chimeras to C57BL/6 will produce heterozygous KO mice of pure C57BL/6 background, which takes only 1-2 months. Although C57BL/6 ES cells are available for some time, it is not commonly used. One of the major reasons is the limited source of blastocytes, which have to be of the same genetic background as C57BL/6 but different coat colors. Now this problem is solved by the availability of the white C57BL/6 strains from both the Jackson Laboratory and the National Cancer Institute at Bethesda, USA[18]. We have imported such strain into the Laboratory Animal Center, NTU. In addition, the P.I. has generated five C57BL/6 ES cell lines during her 3-months sabbatical at UNC-CH (at Dr. Oliver Smithies and Dr. Maeda's laboratory). Once their competence in germ-line transmission is proven in the lab, these cells can be useful in accelerating the whole process.

In 1998, at the same year of the completion of the *Caenorhabditis elegans* genome project, Andrew Fire and Craig Mello described a new phenomenon, RNA interference (RNAi), which was based on the silencing of specific genes by double-stranded RNA (dsRNA). Since then, RNAi has become a powerful reverse genetic approach to study gene functions. For example, in *C. elegans*, if you inject worms with dsRNA or soak them in a solution of dsRNA, the target gene is silenced. In mammal, gene targeting by homologous recombination is commonly used to determine gene function, but it is costly, laborious, and time-consuming. Long dsRNAs can kill human cells through induction of interferon response. Short RNA segments (of 21-22 base pairs) do not activate this killing process, but still possess enough sequence complexity to specify the silencing of a particular gene[30]. Many strategies have been developed for siRNA based gene silencing. The use of chemically synthesized siRNA, allowing the inexpensive and rapid analysis of gene function in mammals, has speed up the somatic cell genetics. Retrovirus delivery and hydrodynamic infusion of siRNA into primary tissues also allow the analysis of gene function in a physiological context without the production of knockout mice. Lentiviral delivery of hairpin RNA to ES cells or blastocysts for the production of knockdown mice has allowed the rapid analysis of gene function through stable and heritable gene silencing. This siRNA-based gene silencing not only offers the potential for gene-function analysis, but also holds promise for the development of therapeutic gene silencing[31-32]. Therefore, we would like to establish this technique for analysis of murine gene function. In addition, we would like to help development of therapeutic treatment for virus infections or autoimmune diseases in mouse model.

Generation of knockout (KO) mice by disruption of gene functions in the murine embryonic stem cells is a very important tool to study gene functions. Nevertheless, it is not so popularly

adopted by researchers in Taiwan. There are some imaginary obstacles. Although there are many commercially available transgenic facilities inside and outside of Taiwan, they are not readily applicable to the P.I.s in Taiwan either due to communication difficulties (those outside of Taiwan) or academically not knowledgeable (those inside this island). Comprehensive consultation is a major determinant and requirement for inexperienced researchers to obtain the KO mice in a reasonable time. Conceivably, there is a need for transgenic mouse core facility with academic knowledge to promote this technology before it goes to biotechnology companies. Thus, one of the long term objectives of the transgenic mouse model core we are proposing here is to serve as a catalyst for development of life sciences and biotechnology research in the biomedical research community in Taiwan. The principle investigator (P.I., Dr. Shu-Wha Lin, 林淑華) would like to convince the reviewers and the community that we are capable of carrying out this mission because of the following factors. First, we have many years of experiences in transgenic and knockout mice technologies. Secondly, the institute (College of Medicine, National Taiwan University) has just built a new mouse facility that can host more than 3,000 cages especially for Tg and KO mice. Third, Dr. Lin' group has experiences in running core facilities. Lastly and most importantly, Dr. Lin has invited Dr. Chun-Nan Lee (Dr. 李君男), Chair of the Department of Medical Technology, as Co-P.I. of the project, to help coordinating among the Core, the Animal Center and the Department. A talented PostDoc. (I-Shing Yu, projected to receive his Ph.D. by year 2005) who has over 10 years of experiences in KO technology and has published more than 3 KO mouse lines has been the manager of the core .

2. 研究目的

(1) Providing services for generating knockout (KO) mice.
(2) Providing services for accessory mouse reproduction technologies.
(3) Establishing powerful techniques for efficient generation of KO mice.
  (a) To circumvent problems of positional effect and shearing of large DNA fragments.
  (b) To circumvent the problem of embryonic lethality.
  (c) To shorten the duration of generating KO mice with pure genetic background.
  (d) To fine-tune the gene expression level by knockin technology.
  (e) To conditionally activate gene expression by excision of loxP-floxed translational stop codon.
(4) Collaborative research on supporting ENU mice research by generating mice with knockin point mutant alleles and by RNAi technique.
(5) Organizing activities for training and Dissemination.

3. 研究方法

(a)Targeting transgene at the HPRT locus. Transgenic mice can be generated using homologous recombination in embryonic stem (ES) cells to produce a single-copy transgene insertion at a defined site in the mouse genome[40]. As shown in Fig. 1, site-specific integration of the transgene can be achieved at the hypoxanthine phosphoribosyl transferase (*Hprt*) locus on the X chromosome based on HAT selection. The vector can be obtained from Dr. Maeda (one of our advisory committee). In the targeting vector, the 5' homology region consists of 4 kb of DNA derived from the 5'upstream region flanking the *Hprt* locus and the 3' homology region contains proximal portions of the *Hprt* gene including sequences which could restore *Hprt* gene function in the BK4 ES cell line (E14Tg2a). Both the targeting vector and the BK4 ES cell line can be

obtained from Dr. Maeda. This ES cell line has an inactivating deletion of the proximal portion of the *Hprt* gene. Because the ES cell line is male (XY) and the *Hprt* locus is on X chromosome, it is hemizygous for the abnormal *Hprt* locus, ie functionally *Hprt* deficient and 6-thioguanine resistant. One can insert the transgene at the site flanked by the 5' and 3' homologous arms. A successful homologous recombination event will restore *Hprt* gene function in the *Hprt*-deficient BK4s ES cells[40]. The surviving ES cells can then be used to generate Tg mice by blastocyst injection.

(b) Cre-loxP system for generating both conventional and conditional tissue specific KO mice. The use of the Cre-loxP system to knockout genes in a conditional and tissue-specific manner has been established in the P.I.'s laboratory. We will be able to assist the community with the Cre-loxP techniques. Moreover, we will advise our clients to create a Cre-loxP construct for both conventional and conditional gene knockouts (see "Background and Fig. 2"). This will eliminate the waiting for the revealing of lethality. As shown in Fig. 2B and that mentioned previously, we will use vector of dual purposes, i.e., one ES cell clone for both conventional and conditional tissue-specific gene KO, by monitoring the timing of the Cre. Since the minimal 34-bp target site is very unlikely to occur randomly in the mouse genome, the excision is rather specific. In addition, another advantage of using this system is that the sequence is also small enough to be considered as a "neutral" sequence when integrated into chromosomal DNA. Finally, the major advantage is the recombinase functions autonomously with high fidelity and no cofactors or additional sequence elements are required for efficient recombination[47]. Moreover, recombination can occur over large distances (Mb) and in a wide range of cell types, both in vitro and in vivo, including undifferentiated as well as postmitotic cells[27,48,49]. Taken together, these properties make the Cre-*loxP* system very useful for site specific DNA modifications, and for generating conditional KO in mice. Information about tissue specific Cre mice can be obtained through internet searches (http://www.mshri.on.ca/nagi/).

(c) Derivation of ES cell lines of C57BL/6 origin or others. C57BL/6 ES cells are commercially available. We have also obtained Bruce 4 ES cells from Dr. Collin Stewart at the National Cancer Institute, USA. These will be used for core services as well. Also, the P.I. has generated five C57BL/6 ES cell lines during her 3-months sabbatical at UNC-CH (at Dr. Oliver Smithies and Dr. Maeda's laboratory). We are in the process of testing their competency of germ-line transmission. These ES cell lines will be very useful once their germ-line transmission is proven. When using ES cells of C57BL/6 for gene targeting experiments, it is critical to have isogenic blastocysts as donor host to increase the germ-line transmission rate[18]. We have imported such white C57BL/6 strains (from both the Jackson Laboratory and the National Cancer Institute at Bethesda, USA) into the Laboratory Animal Center in campus. We will attempt to derive new ES cell lines, such as Balb/C and the protocol for ES cell derivation will the protocol described by Hogan et al[36].

(d) Generation of mice expression point mutation alleles. Two strategies for making allele-specific point mutations in ES cells have been described in Fig. 4 & 5. Other techniques (knockin) for generating knockout mice expressing gene products with point mutations will also be developed in this project. This approach is similar to Fig. 3 except that a fragment with point mutation can be inserted.

(e) Conditional gene activation by excision of loxP-floxed translational stop codon integrated to suppress gene expression. There are various modifications for conditional gene silencing and

activation. The core will dedicate itself to develop a system for conditional gene activation by using the Cre-loxP system in Fig. 6. A cassette containing a loxP-stop codon-loxP DNA fragment can be inserted in front of the translation initiation codon of a gene to prevent synthesis of the gene. Expression of the downstream gene depends on the presence of the Cre recombinase, whose expression can be designed in particular tissues at desired time intervals. The stop codon can be poly A sequences from bGH gene or the small T antigen of the SV 40 virus (fragments easily obtained from commercially available cloning vectors such as pCR3-Uni from the Invitrogen Inc. etc.). Moreover, there are two possibilities for making conditional gene activation, one is to express an exogenous gene by pronucleus injection of the coding region of the gene and the other is to knockin the coding region to the endogenous gene locus. In either cases, the loxP-stop-loxP is inserted upstream of the translation initiation codon of the gene.

(f) Development of other techniques to improve core services. There are at least two other ways to generate KO mice, one is tetraploid-ES cell fusion[50] and the other is nuclear transfer (NT)[51]. After targeting the ES cells, the selected hemizygous KO ES cells can be injected into blastocysts with 4N cells to generate KO mice. Rossant et al. has used this technique and observed that, after development of the fusion embryos, the embryo proper was all derived from the ES cells and the placenta from the 4N cells of the blastocyst[50]. The production of knockout mice using the tetraploid-ES cells fusion technique becomes very promising. We will explore the possibility of using this technique for our KO service[50]. The advantage of this approach is that one can shorten the duration of breeding chimeras and also avoid the problem of block at the germ-line transmission. Although we don't have the experiences in tetraploid fusion, Dr. WTK. Cheng, one of our user's committee and one of the P.I.'s long-term collaborators at our University, is familiar with the tetrapoid embryos-ES cell fusion of livestock and will advice us in performing this experiment. In terms of nuclear transfer (NT), the P.I. has experiences on ICSI, which employs a very similar technique to NT. Therefore, we will try to develop NT in our lab. Other techniques, such as cryopreservation of embryos and sperm, will be established in the Core. Although we have experiences in preserving mouse embryos of several knockout mouse lines, preservation of mouse sperms is not an easy task. We will follow the protocol by Dr. Iwakura (University of Tokyo, Shinagawa campus) who has been one of the P.I.'s long-term consultants and has provided us protocols for in vitro fertilization (IVF) of mouse oocytes. All these important techniques need a lot of practicing and modifications. We think that we will be able to solve the encounter problems by asking help and advices from our long-term collaborators and colleagues.

4. 結果與討論

Progress in Service Activities

(1). This core has received many requests for knockout mice production. We are proud to say that comparing to the core facility at the Pasteur Institute we have made better achievement (information from the 6th Transgenic Technology meeting held in Spain in September 2005). The quantity and distribution of the users of all services are listed below:

Service item	Geographical distribution	# Users	# Cases
Targeting Construct Production	Nation Taiwan University	12	18
	National Cheng Kung University	5	8

	Academia Sinica	2	3
	Tri-Service General Hospital	1	1
	Subtotal	**20**	**30**
ES Cell Gene Targeting	Nation Taiwan University	1	2
	National Yang Ming University	2	2
	Non-academic's	1	1
	Subtotal	**4**	**5**
Chimera Production	Nation Taiwan University	12	22
	National Cheng Kung University	5	7
	National Yang Ming University	3	8
	Academia Sinica	2	3
	Non-academic's	1	3
	Subtotal	**23**	**43**
Cre-loxP Based Gene Targeting of ES Cell	Nation Taiwan University	12	16
	National Cheng Kung University	5	7
	National Yang Ming University	2	3
	Academia Sinica	2	3
	Non-academic's	1	2
	Subtotal	**22**	**31**
Mouse Embryo Cryopreservation and Recovery	Non-academic's	1	1
	Subtotal	1	1
Screening of Targeted ES Cells	Nation Taiwan University	12	44
	National Cheng Kung University	3	12
	Academia Sinica	2	7
	Non-academic's	1	15
	Subtotal	**18**	**78**
Pathogen Free Rederivation	Academia Sinica	1	1
	Subtotal	**1**	**1**
Mouse Embryonic Fibroblast (E13.5)	Academia Sinica	1	3
	Subtotal	**1**	**3**
Cre Recombinase Transfection	Nation Taiwan University	12	15
	National Cheng Kung University	5	7
	Academia Sinica	2	3
	Non-academic's	1	2
	Subtotal	**20**	**27**

(2). Chromosome count. In order to assure the quality of the targeted ES cell clones, we have trained our technician capable of counting the chromosome numbers of the ES cells. Before used for blastocyst injection, every targeted ES cell line will be subjected to this counting to assure no triplication of any somatic chromosomes or lost of the X or Y chromosomes.(a)

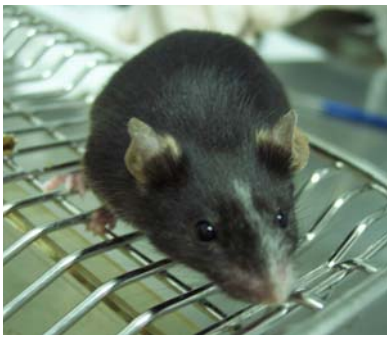
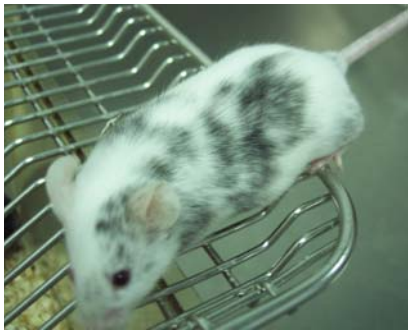
(a)chromosome count  (b)mycoplasma testing 

(3). **Mycoplasma Test.** In order to assure the quality of the targeted ES cell clones, we have trained our technician to check if our ES clones are contaminated by mycoplasma. We use PCR method for testing (above PCR data as an example). (b)

Progress in R&D Activities

(1) Derivation of ES cell lines of C57BL/6 origin.

We have generated five C57BL/6 ES cell lines and identify at least one (the chimeric mice on the left) with germ-line transmission competency. The ES cell lines will be very useful for gene targeting experiments, because they will greatly shorten the time to obtain KO mice of pure C57BL/6 genetic background. To use the cell line, it is critical to have isogenic blastocysts as donor host to increase the germ-line transmission rate. The core has obtained a white C57BL/6 strain (from both the Jackson Laboratory and the National Cancer Institute at Bethesda, USA) from the Laboratory Animal Center on campus.

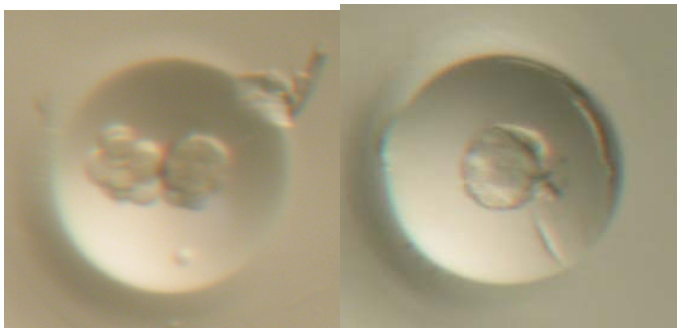



Above figures show 2 chimeric mice, derived from the C57BL/6 ES cell clone no.7, with high (left) and low (right) coat colors. The mouse on the left gave germ line transmission as predicted.

(2) Development of other techniques to improve core services.

1. We have set up ES cell-morula aggregation technique as a different choice of producing chimeric mice. Users can request using this technique for producing their KO's.

2. By introducing "recombinerring" technology to produce targeting vector, we can construct the targeting vector faster and easily. This technology can reduce user's obstacle to use knockout mouse for their study.



Above figures show two morula aggregate with ES cell (left), and after aggregation (right).

(3) **RNAi microinjection in mouse embryo:** We also set up microinjection procedure for RNAi in mouse embryo. This technology can help users to investigate their interesting gene in embryo stage by using RNA interference. Relevant research results have been submitted for publication.

Progress in Collaboration Research Activities

1. We have proposed in our initial grant application to collaborate with Dr. Lee's working on ENU mice to use knock-in for production of their disease mice. We have established an easy, sensitive, and reliable two-step screening method for ENU-mutagenized mice that displayed abnormal responses to IFNα and IFNγ. Through screening some 2800 ENU-mutagenized mice from 134 pedigrees, we have identified a pedigree 117 that displayed altered responses to IFNα. We have finished inheritance test and genetic mapping for the pedigree 117. The inheritance test suggested that the hyporesponsiveness to IFNα was a recessive trait. We have outcrossed pedigree 117 (C57BL/6) to wild-type C3H mice for genetic mapping. Base on Chi square ($\chi 2$) value and the lowest score of non-recombinant from unaffected group, the mutation is most likely linked to Chromosome 10. Interestingly, we have also found that the protein of STAT2 was completely undetectable in the mutant mice despite that the mRNA was still present at a lower level and was inducible by the treatment of IFNα suggesting that the defect might be in the translational or post-translational level. Since mouse STAT2 gene is physically located in Chromosome 10 (127.7 Mb) closed to where the genetic marker was used for the mapping, we speculate that the impaired IFNα response in pedigree 117 is most likely due the loss of function of STAT2. We are currently sequencing genomic DNA of STAT2 to identify the mutation site(s). Characterization of the pedigree 117 mutant mice and generation of a knock-in mouse harboring the mutation found in the pedigree 117 in the transgenic core facility will be our future directions for confirming that the phenotypes seen in the ENU-mutagenized mouse indeed are due to the sole mutation in the STAT2 gene.

2. Our core has been cooperating with Professor Hey-Chi Hsu of National Taiwan University, also the project leader of NRPGM. The research project is entitled "Gene knock-out animal model for the study of human L2DTL/ramp gene". Our team has helped Professor Hsu build up L2DTL/ramp knockout animal model, and has used the embryo-microinjection technology, combined with application of RNA interference technology, to assist Professor Hsu in clarifying the function of the L2DTL/ramp gene during the growth of mouse embryo. Relevant research results have been submitted for publication.

Progress in Training and Dissemination Activities

(1). **Disseminations**: We have held seven facility illustration meetings. Participants and posters are listed below. We have made general introductions to knockout mice technology, its future and impact on research and also introduction to the execution of the core and how to use the core, etc. The director of the R&D department of the Kaohsiung Medical University and the Chairman of the Department of Pediatrics of the Chang Gung Memorial Hospital were excited about the core

services.

Propagating poster

Dates (時間)	**Venue (地點)**	**Number (參與人數)**
2005/10/19 10:30-11:30	National Cheng Kung University	58
2005/10/19 14:00-15:00	Kaohsiung medical University	51
2005/10/19 16:00-17:00	Kaohsiung Cheng-Gung Memorial Hospital.	50
2006/02/22 10:30-11:30	Chung Shan Medical University	67
2006/02/22 14:00-15:00	China Medical University	50
2006/02/22 16:00-17:00	National Chung Hsing University	67
2006/07/03 10:00-11:30	National Defense Medical Center	60
2007/03/28 11:00-12:30	National Taiwan University Shool of Medicine Department of Pharmacology	43
2007/04/14 10:00-11:30	National Health Research Institutes	75

(2) **Training:** We have held a one week workshop on August 2006. (PI's grant "Biotechnology Education Improvement Program" from the Advisory Office of MOE). Twenty one people from nationwide were trained to use RNAi microinjection technology.

(3) **Videos for KO experiments:** We are in the process of taping all the experimental procedures of making KO mice. This video will be very useful both in training new technicians and

postdoct's and helping our users to know what to expect from the core.

(4) **Internet facility:** The core has completed and opens its internet network (http://140.112.133.74) for users to visit and get informed.

5. 圖/表

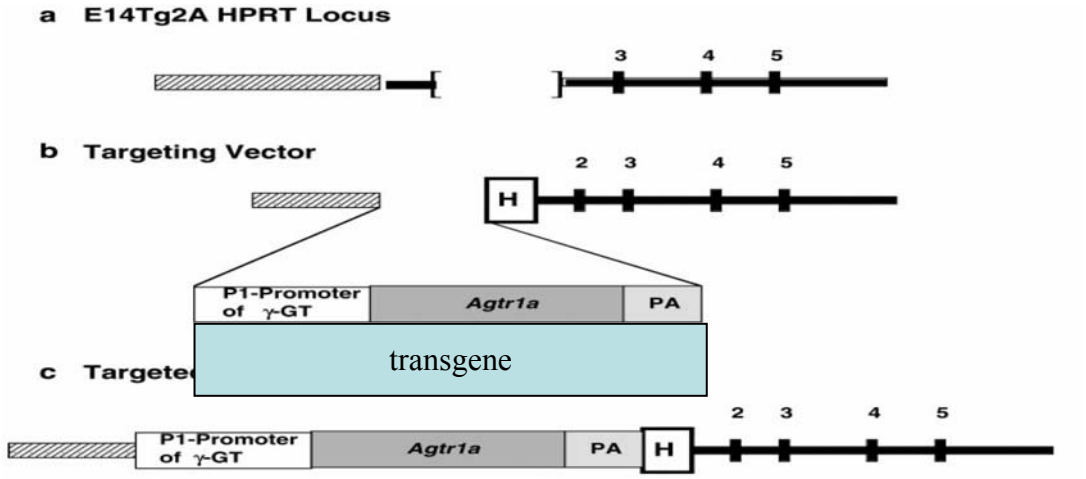
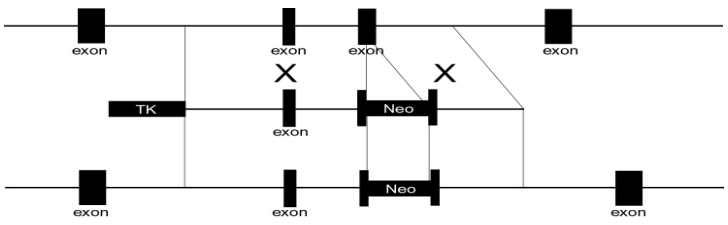
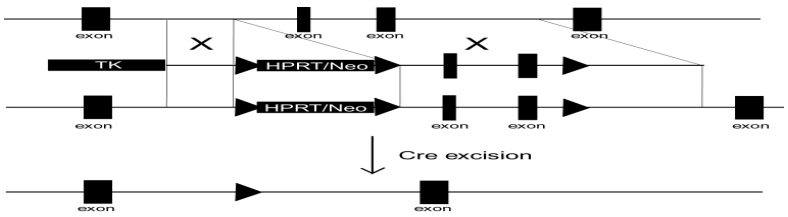


Fig. 1. Schematic diagram of the strategy used for creating single copy transgenic mice with site specific integration at the *Hprt* locus (adopted from ref. 40). *a*: the *Hprt* locus from BK4s embryonic stem (ES) cells has a deletion proximal to exon 3. *b*: the targeting vector containing the transgene along with the complementary HPRT sequences (the human HPRT promoter and exon 1) (H). *c*: The targeted locus with the transgene integrated in a position that is 5' to the now functional *Hprt* locus.

A. Conventional KO.



B. Dual-purpose targeting construct (for both conventional and conditional KO).



C. Breeding scheme for conditional tissue-specific KO.

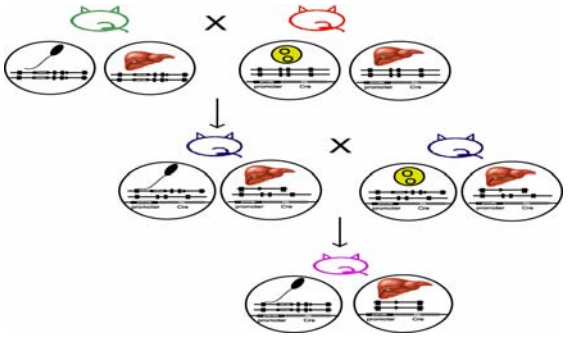


Fig.2 Targeting vector based on positive-negative selection for conventional (A) and conditional tissue-specific knockout (B and C) in mouse ES cells. Panel A. Top is wild-type genome in ES cells, middle is the targeting construct with positive selection marker for neomycin resistance and negative selection for absence of thymidine kinase (TK) gene, and bottom is the genomic structure of the ES clones with knockout gene due to homologous recombination between wild-type gene and targeting vector after transfection into the ES cells by electroporation. In this figure, the integrated neo gene disrupts the reading frame of the 3rd exon and beyond. **Panel B.** Targeting vector for generating both conventional and conditional tissue-specific KO. Also based on positive-negative selection for presence of HPRT gene and absence of TK. Filled triangle: loxP sequence. The loxP-floxed HPRT cassette and a single loxP sequence is integrated into introns without disrupting the exons. The effect of the loxP cassettes is supposed to be minimal, and therefore, mice generated from this ES clone are very close to wilt-type. When Cre recombinase is introduced into the ES cells, the loxP-floxed allele can be deleted. And the ES cells can be injected into blastocyst to generate conventional whole-body KO mice. Alternatively, the ES cells can be injected without treatment of Cre to generate mice carry a loxP-floxed allele. The mice will be bred as shown in panel C. **Panel C.** Breeding scheme for generating conditional tissue-specific KO mice. The genotypes of the breeding pair are depicted at the bottom of each mouse. Top left is mouse with the loxP-floxed allele. Top right is mouse expressing Cre recombinase at specific tissue. Breeding of these two strains will generate mice with deletion of one allele of the gene at tissues where Cre is actively expressed (middle mouse lines, conditional and tissue specific). Breeding of the heterozygous line will generate homozygous deletions in the offspring.

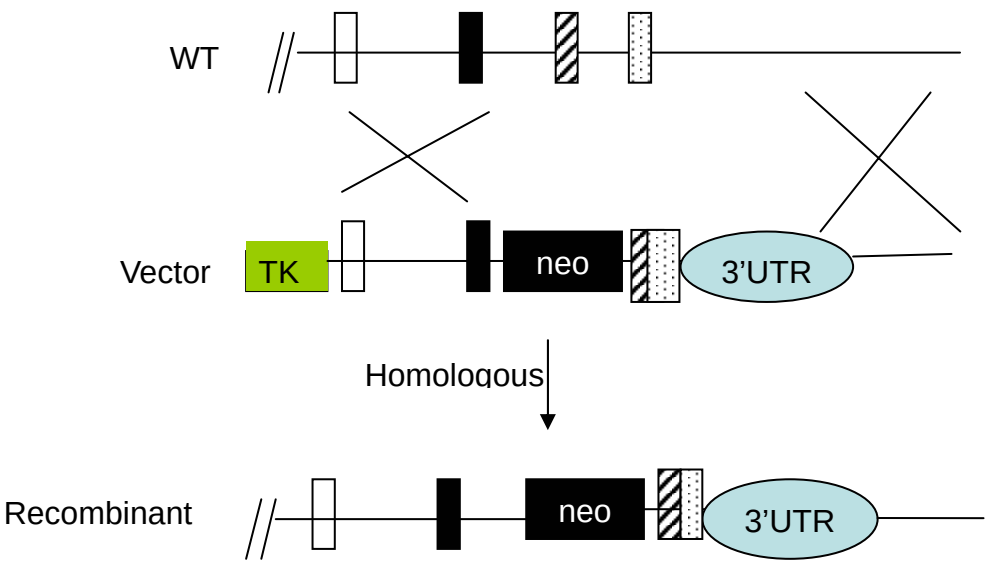


Fig. 3. Schematic diagram of one of the strategies used for creating mice with strong or weak 3'untranslated region (3'UTR). Part of the genomic structure of a gene with its last 4 exons (boxed) is shown (WT). Neo and TK are positive and negative selection markers. Boxes are exons. Dashed and dotted boxed are the last 2 exons. Targeting vector contain the last 4 exons of the modified gene in a minigene configuration. After homologous recombination, the 3'UTR will replace the endogenous one and influence the expression of the gene depending on the strength of the 3' UTR.

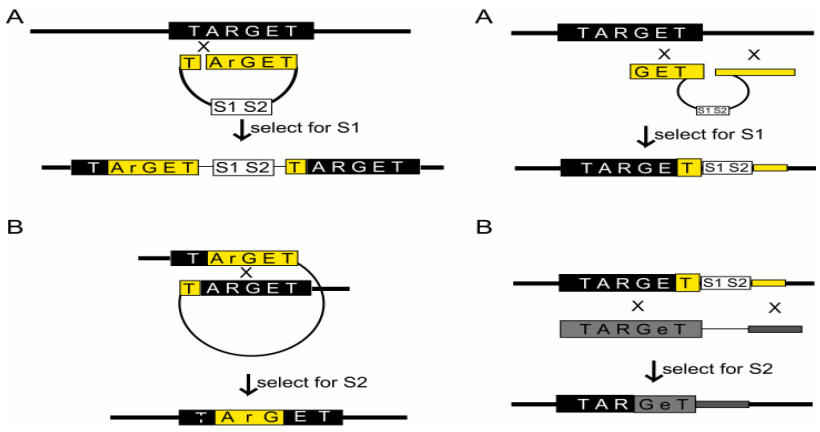


Fig. 4 Strategies for generating allele-specific point mutations (adopted from ref. 33) by knockin of the mutated allele. This is also called the plug-socket method. The 2-step procedure on the right is more commonly used. In this procedure, a selection marker is introduced first by replacement type of vector instead of insertion type (left). The allele with point mutations is then introduced at the 2nd step (B) to excise out the selection marker.

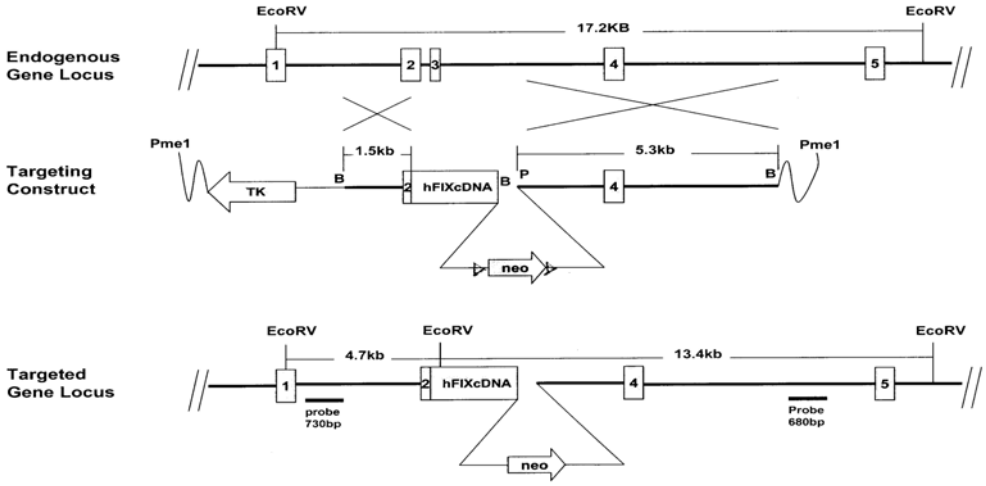


Fig. 5 Strategies for generating allele-specific point mutations (adopted from ref. 34) by knockin of the mutated allele. (Top): Endogenous gene locus. Exons 1-5 of the endogenous mouse factor IX gene are shown. (Middle): Targeting construct. The construct was linearized with Pme I and used for homologous recombination is shown. Recombination between the 5' (1.5 kb) and 3' (5.3 kb) regions of homology is depicted. The Pme I site is within the plasmid vector, 29 nucleotides from the BamHI site. The human factor IX cDNA is depicted in black. (Bottom): Targeted gene locus: The mouse factor IX locus after homologous recombination is shown. The regions from which the 730 bp and 680 bp probes were generated are shown. After EcoR V cleavage of mouse genomic DNA, the 730 bp probe gives a 4.7 kb fragment and the 680 bp probe a 13.4 kb fragment when homologous recombination has occurred. Mouse genomic DNA is shown as a thick line and the inserted neo sequences as a thinner line. B, BamHI; P, PacI.

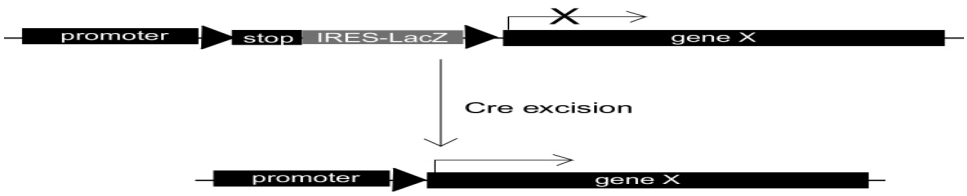


Fig. 6. Conditional gene activation. A construct for generating transgenic mice is depicted. The stop DNA can either be a stop signal (poly A addition signal) plus stop codons or stop codons only. The loxP-floxed DNA containing stop signals and a reporter gene integrated (IRES-LacZ, IRES: internal ribosome entry site) in front of the ATG codon of the target gene. Expression of the target gene is activated upon Cre excision of the stop signal. Filled triangle:loxP.

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計畫成果自評

(a) What was the percentage effort of the Core distributing to the "Service", "R&D" and "Collaboration Research" activities approved and/or recommended by the Review Committee for the current funding period? How did Core fulfill the plans original proposed and/or recommended in the competing application for each activity?

We have proposed in our original grant application that the core would spend 70% of our efforts on routine service, 20% on technological research and development, and 10% on collaborative research. We believe that we have fulfilled this proposition.

(b) What were the significant unexpected outcomes (positive or negative) of your Core scientific activities?

The core has received unexpected amount of requests for targeting construct production and Southern Screening. It will take a lot of time and effort to complete the task. We therefore ask for more manpower to carry out this service.

(c) What were the challenges or obstacles your Core encountered in reaching its goal?

Due to the distinct nature of the service items of this project, well-experienced technical personnel are required to carry out the proper procedures. The training of a technical staff usually takes a long time. However, the domestic "research assistant" system is poorly designed that it does not provide sufficient welfare, thus caused high circulation of assistants. It is a common scene that assistants decide to leave for career planning reasons, notwithstanding the fact that they have received long-term training in the lab and have become capable of working independently. This often causes great loss to the research team. We would like to propose that the personnel system for NRPGM core facility be re-examined in order to attract assistants' longer commitment to the research project, which may thus maintain the steady development of core technologies; in the meantime, providing quality services to domestic researchers.

(d) What were the best products of your Core (service items, technology platform, training courses, Web sites, scientific findings, other)?

Our service items (such as blastocyst injection) are the best products of our Core. We are the first Core facility to provide targeting construct production service in Taiwan (even in the word, except one biotech company owned by Dr. Francis Stward). By introducing "recombinerring" technology

to produce targeting vector, we can construct the targeting vector faster and easily. This technology can reduce user's obstacle to use knockout mouse for their study.

(e) What were the major accomplishments occurring in your Core and their (potential) significance to NRPGM and the biomedical research in Taiwan?

We have established a nation-wide mouse model core to serve the academic community in Taiwan. The ultimate goal of this project is to facilitate the use of mice as models for studying gene functions and phenotypes of human diseases. We wish to help the community to do better science and not "me too" projects.

附錄

List of scientific accomplishments

1. Su KY, Chien WL, Fu WM, Yu IS, Huang HP, Huang PH, Lin SR, Shih JY, Lin YL, Hsueh YP, Yang PC, and Lin SW. (2007) Mice deficient in collapsin response mediator protein-1 exhibit impaired long-term potentiation and impaired spatial learning and memory. J Neural Science **27(10)**:2513-24.
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