行政院國家科學委員會補助專題研究計畫成果報告

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kif3 與 *lefty* 基因組與胚胎左右軸不對稱性發生之探討

The role of *kif3* and *lefty* genes in the development of left-right asymmetry during mammalian embryogenesis

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計畫主持人： 謝豐舟
共同主持人： 施景中

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一、中文摘要

在脊索動物的胚胎最主要有 3 個身體的軸要次第發育:背腹、前後、及左右軸。其中左右軸的不對稱最晚發育也最少被研究。哺乳類的內臟有一非隨意的不對稱安置型態,如心、胃、肝、脾等各發育於身體中軸的兩側。哺乳類左右軸的分化機轉仍不完全清楚，它的機轉既深奧同時也充滿挑戰，哺乳類不對稱的發展首先見于原心臟管的環狀化,但事實上已可由體節的早期生成時看見如 *nodal*,*shh* 和 *pitx2* 的不對稱分佈而測得(最主要均表現在胚體左側)。然而,這些左右分化的訊號如何去傳遞及誘導器官發育則仍然大部份未知，本計劃的目的即在設計新的方法去探討 *kif3* 及 *lefty* 基因組在形成左右軸的特殊角色。

關鍵詞：左右軸不對稱性，*kif3a, kif3b, lefty*, 人類，老鼠，模版 DNA**，表現型態，螢光立體顯微鏡。**

Abstract

In vertebrates, there are three major body axes formation during embryogenesis: dorsal-ventral, antero-posterial and left-right (L-R) axes. Of the three, the origin of left-right asymmetry is the last developed and also has been the least studied. In mammals, there are a specific non-random asymmetry arrangement with respect to the midline of the body (*situs solitus*), including heart, stomach, liver, spleen, and so forth. Little is known about how regulates the L-R axis formation, and indeed the theoretical problems are deep, challenging and still confusing. The L-R asymmetry first becomes anatomically apparent in the orientation of the heart tube looping, but is already detectable at the somitogenesis stage by asymmetric expression of several genes, such as *nodal, shh* and *pitx2*, with expression being observed in the left side in most of the cases. However, how these L-R signals are transmitted and as the asymmetric morphogenic inducers are still unknown. The purpose of this study is to determine the specific roles of kif3 and lefty genes in the formation of L-R axis.

First of all, human and mouse sequences of *kif3a, kif3b* and *lefty* of cDNA and genomic clones, are retrieved from computer search (NCBI/Gene Bank). Using human and mouse genomic DNA as templates, human and mouse *kif3a, kif3b*, and *lefty* cDNA sequences will be obtained by PCR strategies. Then we isolate the cDNA fragments cloned by PCR under long-wave UV guide. If the sizes of fragments are identical to our expected result, extract DNA from the gel fragments and ligate with vecotr. The next step is E. coli transformation. The DNA products obtained by *E. coli* transformation are further examined and confirmed by specific enzymatic restriction reactions. Finally the purified DNA are sequenced and prepared in a large amount.

The next step is integration of GFP (green fluorescence protein) into these DNA sequence. We also use β-geo as the reporter gene to study the promoter function of these genes. Then we isolate and culture the post-implanted embryos in an *ex utero* environment and inject the DNA construct into the cultured embryos. For the purpose of tracing the dynamic changes of these specific genes expression, we examine these embryos under fluorescence stereomicroscopy to see the fluorescence illumination of GFP-tagged DNA. Because the ex *utero* cultured embryo cannot survive more than 3 days, we examine the embryos dating between 3-12 days with whole mount *in situ* hybridization. The purpose of this step is to see when the gene expression "turn-on" and "turn-off" and correlate how they intervene the events of L/R formation. The final step is to localize these genes in the human chromosomal map by fluorescence *in situ* hybridization (FISH).

Our new designed method is helpful to examine the beginning and pathway of L/R determination and also elucidate the interrelationship of *kif3* and *lefy* genes in cascade of L/R formation events. More human homologies of other L/R determining genes will be obtained in this way. We may also apply these data in the discovery and screening of the human lateralization defects in the future.

Keywords: Left-right asymmetry, *kif3a*, *kif3b*, *lefty*, mouse, human, cDNA, expression patterns, fluorescence stereomicroscopy.

二、緣由與目的

In all vertebrates, there are three major body axes formation during embryogenesis: dorsal-ventral, antero-posterial and left-right axes. Of the three, the origin of left-right asymmetry has been the least studied. In mammals, there are a specific non-random asymmetry arrangement with respect to the midline of the body (*situs solitus*), including positioning of the cardiac apex, stomach, liver and spleen. The apex of heart invariably points to the left side, the right and left lung display differences in lobulation. The liver is on the right side and spleen on the left, and the large intestine curls from right to left.

Little is known about how regulates the L-R axis formation. Experimental analysis of vertebrate laterality may date back to the 19th century when reversals in asymmetry organ placement (*situs inversus*) were reported following unilateral warming of chick embryo on the left side (Dareste, 1877). Another three sets of experiments, generation of twinned embryos by ligature, inversion of the middle part of the medullar plate, and unilateral ablation, resulted in defined and predictable laterality defects. From these experiment data, Wilhelmi concluded that "the left side of the germ has something that the right side does not have" (Wilhelmi, 1921).

Recent advances in molecular technique have elucidated a couple of problems in developmental biology. Similarly, many advances have been made in understanding specification of L-R axis formation in recent years. Nonetheless, the initiating events in vertebrates are still unknown.

三、研究方法

I. ISOLATION OF MOUSE/HUMAN *kif3a, kif3b, AND lefty* cDNA CLONES

Mouse *kif3a* and ***kif3b*** cDNA and genomic clones, **human *kif3a*** and ***kif3b*** cDNA and genomic clones, and **human and mouse *lefty*** cDNA and genomic clones, will be retrieved from computer search. Using human and mouse genomic DNA as templates, human and

mouse *kif3a, kif3b*, and *lefty* cDNA sequences will be obtained by PCR strategies.

1. Medline/Gene Bank searches
2. Through Medline and NCBI Gene Bank (http://www.ncbi.nlm.nih.gov) searches, the sequences of *kif3a, kif3b,* mouse *lefty* and human *lefty* cDNA sequences and proteins can be retrieved as follows.
3. Design primers Polymerase chain reaction (PCR)
4. DNA Gel extraction
5. DNA ligation and *E. coli* Transformation.
6. Miniprep and large scale preparation
7. Nucleotides sequencing

II. GREEN FLUORESCENCE PROTEIN (GFP)-TAGGED KIF3A/KIF3B IN THE MOUSE DEVELOPMENT

1. Construction of GFP-tagged KIF3A/KIF3B and KIF3A/KIF3B reporter constructs

Mouse KIF3a and KIF3b full-length cDNAs will be cloned into appropriate GFP expression vectors (*p*EGFP, Clontech). Besides, *kif3a* and *kif3b* promoters (from mouse genomic clones) will be assayed by fusing with a reporter β-geo gene cassette. Cloning sequences will be reconfirmed in frame by DNA sequencing.

2. Microinjection of KIF3a and KIF3b constructs into mouse zygotes

Zygotes (one cell stage embryos) will be collected from the superovulated females. The standard protocol suggested by Robertson will be followed.

3. Isolation and culture of whole postimplantation embryos

Different stages of developing mouse embryos will be collected to examine the expression patterns of KIF3a and KIF3b constructs in a serial time course. Mouse embryos will be cultured in M2 medium. Standard protocols for isolation and culture of whole postimplantation embryos was suggested by Sturm and Tam in *Methods in Enzymology, Vol 225, Guide to Techniques in Mouse Development, pp164-189.*

4. Analysis of the dynamic expression patterns of *kif3a* and *kif3b* in mouse embryos

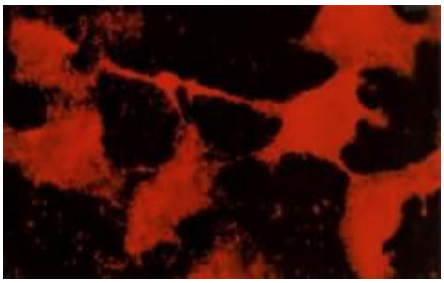
III. WHOLE MOUNT *IN SITU* HYBRIDIZATION

IV. HISTOLOGICAL ANALYSIS

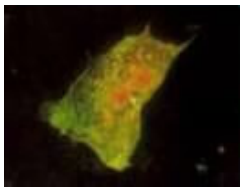
V. FLUORESCENT *IN SITU* HYBRIDIZATION (FISH) FOR THE LOCATION OF KIF3A/KIF3B/LEFTY IN THE MOUSE/HUMAN CHROMOSOMES

四、結果與討論

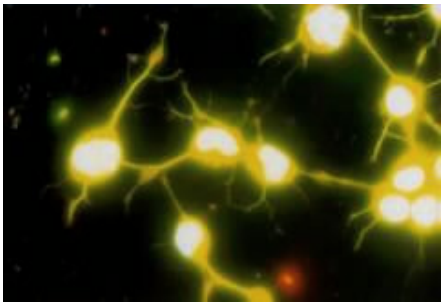
1. We have completed the DNA sequencing of mouse clones of *kif3a, kif3b, from* mouse brain via RT-PCR and partial clone of *lefty* from designed PCR primers.
2. Confirmation the DNA sequence of mouse clones of *kif3a, kif3b, lefty* genes with previously published data.
3. Partial clone of human kif3a, kif3b and lefty were also isolated from human term placenta. However, complete sequences of them were not available at present. Thus localization of *kif3a, kif3b, lefty* and related left-right determining genes in human chromosomal map is not finished yet.
4. Study the gene expression patterns and investigate how they intervene left-right formation:



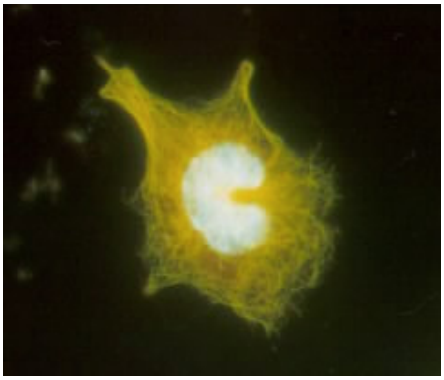
(Cos7 [fibroblast] cell line – non-transfected)



(Cos7 transfected with kif3b, anti-tubulin [staining microtubule] was decorated with FITC. kif3b was tagged with GFP and thus identified by polyclonal anti-GFP [red]. In the green area, the microtubule was not colocalized with kif3b. In the orange area, kif3b was colocalied with the distribution of microtubule.)



(Neuro2A [neuronal cell line; with abundant endogenous kif3a, kif3b and possibly other kinesin superfamily]



(Neuro2A cell line with kif3b overexpression. The distribution of kif3b was colocalized with microtubule).

According to our observation, *kif3b* is predominantly worked with microtubule (colocalized with microtubule) and must bind with *kif3a* to form the dimmer then work together. Since Cos 7 (fibroblast) is lack of endogenous kif3a, the overexpression of kif3b is diffusely distributed in the cytoplasm (i.e. cannot work appropriately with microtubule). Hence we believed the beginning of left-right axis formation is possibly the early meiosis during blastocyst formation. The point of origin of L/R pathway is unknown. The normal laterality observed in mouse morula aggregation chimeras suggested that L/R in this species becomes fixed after the morula stage. Besides, microtubue arrays aligned in an anteroposterior direction appear to help define the coordinates against which the L/R axial process is oriented. Since the KIF3A and KIF3B are attributed to microtubule-associated proteins, we would like to observe the GFP-tagged KIF3A and KIF3B proteins from early embryonic cleavage stage in the subsequent experiment. Through examining the living embryos, the mysteries of when and how the microtubule-directed events on L/R determination may be elucidated.

五、計畫成果自評

Basically, it is a big project that is not possible to be finished within one year. We take almost four to five months to isolate and confirm the mouse clones of kif3a/3b/lefty, and human clones of kif3a/kif3b/lefty was obtained in partial sequence. Currently, we are still worked with the in vitro experiment to study the functions of kinesin superfamily in various cell

lines. The FISH, ISH studies are still nedd more time to carry on. As for the in vivo experiment, such for the knock out study, it at least needs 3 more years to go through.

In the subsequent experiment, we hope we can explain the following question:

a. Relation between *kif3* genes and *lefty* genes, and also the related left-right determination genes.

We know that at 10-20 L/R determining genes have now been identified in vertebrates. Nonetheless, how is the interrelationship between them is still unclear. After we controlling most common L/R genes, we may simultaneously observe the cascades of these gene expression patterns by the LEICA MZ FLIII through the aid of GFP. The relationships between them are then hopefully to solved.

b. Identified the homology of these L/R determining genes between human and other species.

We know that L/R asymmetry is a universal phenomenon in vertebrates. Thus we believed there are a couple of homologies of these L/R genes between vertebrates. Through this study, we can study the *kif3* and *lefty* genes homology between human and mouse. This is probably helpful to find more genes in their superfamily that have been identified to play a role in L/R determination in mouse or other vertebrates

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