附件一

行政院國家科學委員會補助專題研究計畫

□期中進度報告
■期末報告

（果蠅 Tricornered/Dmob2 基因與細胞形態發生的研究）

計畫類別：■個別型計畫　□整合型計畫
計畫編號：NSC 98－2311－B－029－002－MY3
執行期間： 100 年 8 月 1 日至 101 年 10 月 31 日

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計畫主持人：范聖興
共同主持人：
計畫參與人員：陳淑芬、萬雅玄

本計畫除繳交成果報告外，另含下列出國報告，共 _1_ 份：
□移地研究心得報告
■出席國際學術會議心得報告
□國際合作研究計畫國外研究報告

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中　華　民　國　101 年　10 月 30　日

Introduction

Mps-one-binder (MOB) family proteins belong to a member of a conserved gene family found in yeast, *Drosophila*, *Trypanosoma brucei*, vertebrates, and humans (Luca and Winey, 1998; Salimova et al., 2000; Colman-Lerner et al., 2001; Devroe et al., 2004; Bichsel et al., 2004; Hammarton et al., 2005; He et al., 2005; Lai et al., 2005). All Mob proteins contain Mob/phocein domain, which its function is currently unclear (Hergovich, 2011). Mob1 protein is originally identified as a regulator of mitotic exit and cytokinesis in yeast (Luca et al., 1998). *Drosophila* Mob1/Mats protein acts as a tumor suppressor to regulate cell proliferation and apoptosis (Lai et al., 2005). Similarly, the function of Mob1 in yeast is responsible for mitotic checkpoint regulation (Luca and Winey, 1998). In *Drosophila*, the Mob2 protein physically binds to Tricornered, an orthologue of NDR kinase in vertebrate (Emoto et al., 2004). Study also shows that Dmob2 involves in photoreceptor morphogenesis by regulating the subcellular localization of Crumbs, a cell polarity gene, and phosphorylated Moesin (Liu et al., 2009). Yeast MOB2 promotes polarized cell growth and induces asymmetric cell fate (Colman-Lerner, 2001). In addition, mouse MOB2 is found to widely express in many tissues including brain, intestine, and kidney. Using ectopic expression, MOB2 was found to promote neurite formation in neuronal cells. Knocking down the MOB2 expression suppressed the neurite formation. We further provide evidence that MOB2 regulates the dynamic of actin cytoskeleton in developing neuronal cells (Lin et al., 2011). MOB2 in yeast is critical for promoting polarized cell growth and induce asymmetric cell fate in yeast (Racki et al., 2000; Colman-Lerner et al., 2001; Hou et al., 2003; Weiss et al., 2002). Study also shows that *Drosophila* Mob4 is required for spindle focusing during mitosis (Trammell et al., 2008). In human, there are six members in MOB protein family including MOB1A, hMOB1B, hMOB2, hMOB3A, hMOB3B, and hMOB3C). Currently, the functions of hMOB1A/B are well known to their putative tumor-suppressive functions through the regulation of NDR/LATS kinases (Kohler et al., 2010). The functions of other human MOB protein are less well defined. Although the growing number of researches have begun to address the molecular mechanism of MOB1 protein in diverse biological system, the signals involve in regulating MOB2 proteins remain unclear. Thus, it will be interesting to study the functions and its molecular regulation of MOB2 protein.

In this study, we begin with the study of how mob2 regulate rhabdomere morphogenesis in *Drosophila*. We found that *Drosophila* mob2 play a critical role in regulating actin cytoskeleton and thus participating in rhabdomere formation. With the results form *Drosophila*, we then extend our study by investigating the role of Mob2 protein in mammalian cells. We found that mouse Mob2 protein plays a similar role in organizing actin filaments and regulating neurite formation during development. We used neuro 2A cells (N2A), a mouse neuroblastoma cell line, to study how Mob2 participates in neuritogenesis. Immunocytochemical study indicated that Mob2 localization in cytoplasm and on the tip of neurite protrusion. Using RNA interference to knockdown Mob2 expression and found that neurite formation was suppressed in low-serum induced N2A cells whereas overexpression of Mob2 induced neurite formation in normal serum growth N2A cells. In addition, we also investigate Mob2 protein in regulating migrating cells. We found that knockdown or overexpression of Mob2 in human HT1080 cells have dramatic effects

in their migrating behavior. Taken together, these studies have found the role of Mob2 protein in regulating actin based cell activity.

Results

1. *Drosophila* mob2 in rhabdomere morphogenesis

To analyze the function of Dmob2 in photoreceptor morphogenesis, We used generating transgenic flies to express *Dmob2* double strand RNA (dsRNA). After downregulation of the Dmob2 expression, we asked how it affected eye development. A total of three distinct transgenic flies that targeted different *Dmob2* RNA fragments (*pWIZ-Dmob2-IR1*, *pWIZ-Dmob2-IR2*, *pWIZ-Dmob2-IR3*) were generated (Fig. 1a). To assay the ability of RNAi flies to knockdown the Domb2 expression, we crossed each RNAi transgenic flies with *GMR-Gal4*, an eye specific activator, to drive the transcription of *Dmob2* dsRNA. We then isolated pupal eyes and assayed for their expression of Dmob2 using Western blot. The results showed that Dmob2 expression in *GMR>Dmob2-IRs* was reduced by roughly 90% as compared to the wild type (Fig. 1b). Immunocytochemical evidence was consistent to the immunoblot result which showed that *GMR>Dmob2-IRs* flies could downregulate Dmob2 expression in photoreceptor cells (Fig 1c-j).

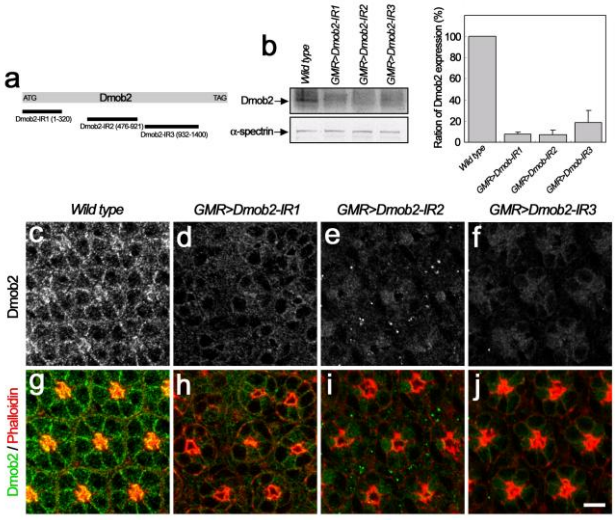

Fig. 1 Downregulation of Dmob2 expression in GMR>Dmob2-IR flies
(a) Schematic drawing shows that three double-stranded RNAs are used to disrupt the Dmob2 expression. (b) Western blot analysis shows the expression of Dmob2 in the wild type and GMR>Dmob2-IRs flies. Quantitative analysis shows that Dmob2 expression decreases roughly 90% as compared to the wild type. (c) Confocal micrographs show that the expression of Dmob2 in 50% pd of wild type and GMR>Dmob2-IRs photoreceptor cells. Immunocytochemical evidence is consistent to the Western blot showing that GMR>Dmob2-IRs flies could downregulate Dmob2 expression in photoreceptor cells. Scale bar= 10 μm.

With these transgenic flies, we then studied whether the downregulation and overexpression of *Dmob2* expression affected eye development using scanning electron microscope (SEM) and transmission electron microscope (TEM). In SEM, the control adults (*GMR-Gal4/+*) eye consists of hexagonal array of approximately 750 dome-shaped ommatidia; small mechanosensory bristles project from alternate vertices over most of eye (Fig. 2a). When we crossed the *GMR-Gal4* to *pWIZ-Dmob2-IRs* (*GMR>Dmob2-IRs)* flies, we found the regular array of ommatidia disappeared; a cavernous structure often appeared in the center of ommatidia and the bristles projected randomly on the eyes. Since all *Dmob2-IRs* showed similar eye phenotypes, we only showed *GMR>Dmob2-IR1* phenotype here (Fig. 2b)

and for further analysis. In addition, we also examined the eye phenotype by overexpression of Dmob2 in developing eyes using *GMR-Gal4* activator. In *GMR>Dmob2* flies, the external eye phenotype was subtle, only slightly abnormal array of ommatidia was found (Fig. 2c). In TEM, the photoreceptor cells in the control eyes (*GMR-Gal4/+*) were arrayed as a typical trapezoid (Fig. 2d). The rhabdomeres appeared as oval and faced toward the interrhabdomere space (IRS, asterisk). In addition, we also found that rhabdomeres were confined to the center of the apical surface of photoreceptor cells. In *GMR>Dmob2-IR1* adults, the array of photoreceptor cells was not trapezoid (Fig 2e). The shape and size of rhabdomeres became irregular with some being missing (Fig. 2e, arrowheads) and some being widely expanded (Fig. 3e, arrows). In *GMR>Dmob2* adults, the array of photoreceptor cells was irregular (Fig. 2f). We often found that some photoreceptor with multiple domains of rhabdomere (Fig. 2f, arrow) or some being missing rhabdomere in their apical surface (Fig. 5f arrowhead).

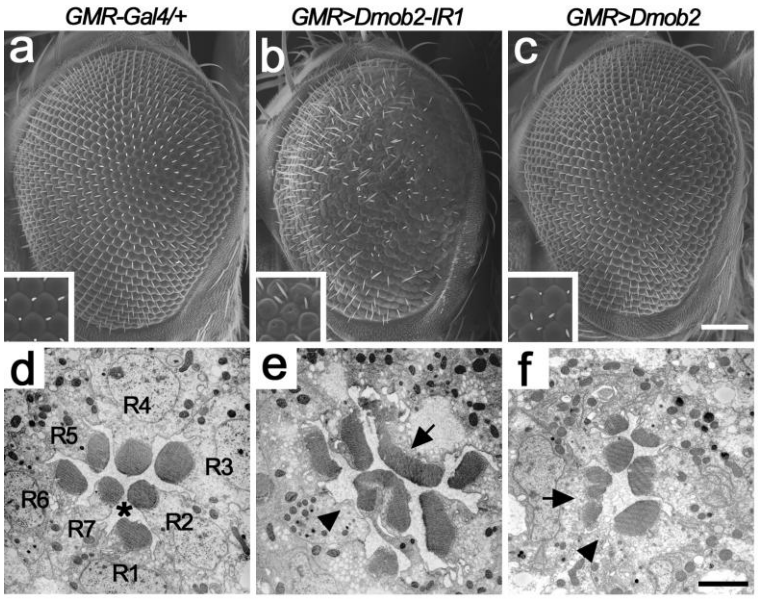

Fig. 2 Dmob2 is required for normal eye development

Scanning electron micrographs show control (*GMR-Gal4/+*), *GMR>Dmob2-IR1*, and *GMR>Dmob2* adult eyes. (a) In *GMR-Gal4/+* fly, regular dome-shaped ommatidia make up the entire eye field. The mechanosensory bristles project from alternate facet vertices over most of eye. The inset shows the higher magnification of compound eye. (b) In *GMR>Dmob2-IR1* fly, the regular array of ommatidia disappeared; a cavernous structure often appears in the center of ommatidia (inset).The mechanosensory bristles project randomly on the eyes. (c) In *GMR>Dmob2* fly, the external eye is quite normal as in the control eye. Transmission electron micrographs shows internal eye structure of *GMR-Gal4/+*, *GMR>Dmob2-IR1*, and *GMR>Dmob2* adults. (d) In *GMR-Gal4/+* fly, the rhabdomere appears oval and is composed of a stack of microvilli. The rhabdomere localizes specifically in the center of apical membrane, which is marked by the two adherens junctions (arrowheads). All rhabdomeres face each other toward the IRS (asterisk). (e) In *GMR>Dmob2-IR1* fly, the shape of rhabdomere is irregular; some are widely expanded (arrow) and some are missing (arrowhead). (f) In *GMR>Dmob2* fly, the shape of rhabdomere become irregular; some appear as multiple domain (arrow) and others are almost diminished (arrowhead). Scale bar= 100 μm (a, b), 20 μm (c, d).

2. Mouse Mob2 in neurite morphogenesis

To study the function of Mob2 in neurite formation, RNA interference was used to downregulate Mob2 expression. N2A cells were transfected with two distinct shRNAs that targeted the *mob2* sequence and control scramble shRNAs, individually. After puromycin selection and low-serum induction, the level

of Mob2 expression in N2A cells decreased to 72% and 73%, respectively, when compared to control shRNA (three experiments, Student's *t*-test, ***p<0.001) (Fig. 3A). To study whether downregulation of Mob2 affects neuritogenesis, N2A cells were individually transfected with *mob2* and control shRNAs. The transfected cells were stained with anti-Mob2, rhodamine-phalloidin, and sytox to assay for their ability of neurite formation. When N2A cells were treated with control shRNA, the cells expressed Mob2 and extended neurites normally as the untransfected cells (Fig. 3B). When cells were treated with mob2-shRNAs, the expression of the Mob2 protein was significantly reduced in the N2A cells (Fig. 3B). Those cells lacking Mob2 expression usually had short or no neurites (Fig. 3B).

To determine the specificity of mob2-shRNAs, N2A cells were cotransfected with mob2-shRNAs and a mutant version of mob2 gene, which it expresses a protein same as wild type Mob2 protein but do make the mRNA resistant to shRNA (Fig. 3C). If the phenotype observed in N2A cells was mob2 specific, we were then able to observe that expression of the mutant version of mob2 gene should rescue the mob2-shRNAs induced phenotype. Indeed, the results indicated that the simultaneous expression of mob2-shRNA and mutant version of mob2 were able to induce neuritogenesis (Fig. 3D, arrows). Quantitative analysis showed that 75.5 ± 8.5% of cells bore neurites in the control shRNA while the percentage of cells bearing neurites in mob2-shRNA treated cells was significantly reduced to 19.4 ± 3.5% and 16.1 ± 2.9% (Fig. 3E). When N2A cells were cotransfected with mob2-shRNAs and mutant version of mob2 gene, the percentage of cells bearing neurites increased to 67.9 ± 0.4% and 68.2 ± 2.5%, respectively (Fig. 3E). Statistical analysis indicated that the number of cells bearing neurites was significantly reduced in Mob2-knockdown cells whereas expression of mutant version of mob2 gene in mob2-shRNA background increased the percentage of cells bearing neurites (three experiments, n>300 neurons per experiment, Student's *t*-test, ***p<0.001) (Fig.3E). Together, these results demonstrate that the Mob2 protein plays a significant role in neurite formation in N2A cells.

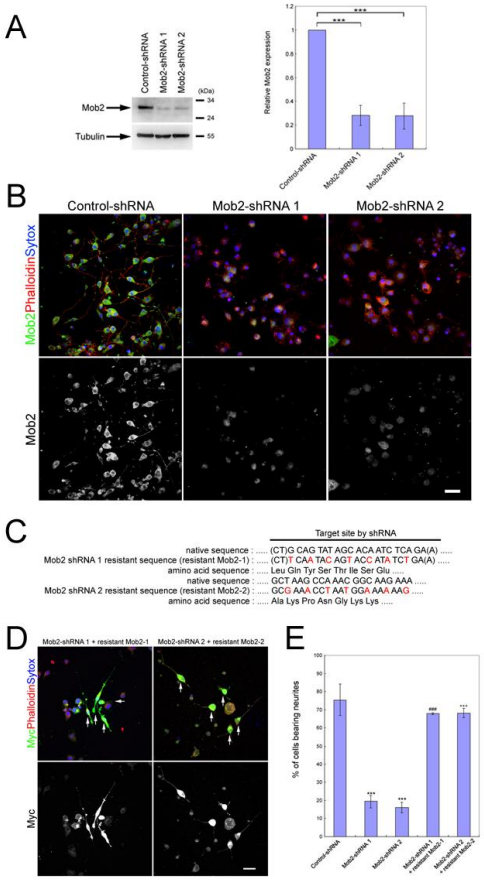

Fig. 3 Downregulation of Mob2 expression in shRNA treated N2A cells

N2A cells were transfected with control scrambled shRNA and mob2-shRNAs. After 36 hours of transfection, the cells were harvested and processed for immunoblot and immunocytochemistry. (A) Western blot analysis indicated that Mob2 expression was reduced significantly in mob2-shRNA treated N2A cells. Statistical analysis indicated that the expression of Mob2 in control and mob2shRNAs-treated cells were significantly different (three experiments, *Student's* t-test *** indicates p<0.001). (B) The shRNA transfected N2A cells were stained for the anti-Mob2 antibody (green), for F-actin with rhodomine-phalloidin(red), and for nucleus with Sytox (blue). When N2A cells were treated with control shRNA, Mob2 expressed normally and

large number of cell grew neurites. When N2A cells were treated with mob2-shRNAs, Mob2 expression decreased significantly. At the same time, the percentage of neurite formation (as indicated by phalloidin staining) reduced significantly. (C) Two mutant versions of mob2 gene which encoded wild type Mob2 protein but was not targeted by mob2-shRNAs were designed to cotransfect with mob2-shRNAs. (D) When mob2-shRNAs were cotransfected with mutant version of *mob2* gene (indicated by anti-cMyc, green) in N2A cells, most of cotransfected cells extended neurites (arrows). (E) Quantitative analysis indicated that the percentage of cells bearing neurites in mob2-shRNAs and control shRNA treated cells were significantly different (*Student's t-test*, *** indicates $p<0.001$). When mutant version of mob2 gene was transfected simultaneously with mob2-shRNAs, the percentage of cells bearing neurites increased (Student's *t*-test, *** indicates $p<0.001$). Arrows indicate mob2-shRNA and mutant version of mob2 gene cotransfected cells. Scale bars: 50 μm.

3. MOB2 protein participates in migration of human cells

To study the function of hMOB2, we used anti-Mob2 antibody to stain HT1080. Immunocytochemistry revealed hMOB2 protein was mainly localized at the cytoplasm and at the leading edge of HT1080 cells. The localization of hMOB2 detected at the leading edge colocalized with actin cytoskeleton at the lamellipodia (Fig. 4). These results suggest a potential function for the hMOB2 protein in cell spreading and migration.

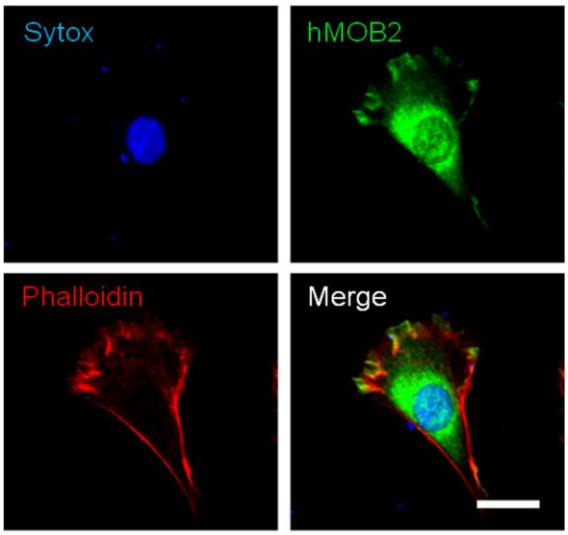

Fig. 4 Subcellular localization of hMOB2 in HT1080 cells. Confocal images showed HT1080 cells stained with anti-MOB2 (green), rhodamine-phalloidin (red), and nucleus (blue). hMOB2 expression was mainly concentrated at cytoplasm. However, we found hMOB2 also co-localized with rhodamine-phalloidin at the plasma membrane of leading edges. Scale bar: 20μm.

To study the function of hMOB2 in cell spreading, RNA interference method was used to downregulate hMOB2 expression. HT1080 cells were infected with two distinct shRNAs virus which targeted the *hmob2* sequence and control scramble shRNAs. After puromycin selection, we established two hmob2 downregulated cell lines. Western blot analysis revealed hMOB2 expression in transfected cells deceased to 70% and 69%, respectively, when compared to control shRNA (three experiments, *Student's t*-test, *** $P<0.001$) (Fig. 5).

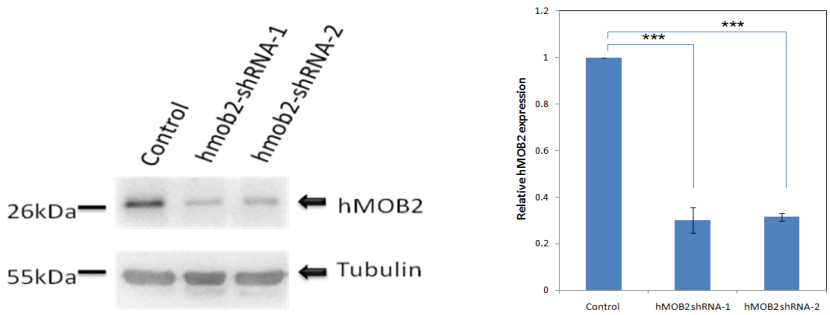

Fig. 5. Downregulation of hMOB2 expression in HT1080 cells. HT1080 cells were infected with control scrambled shRNA and hmob2-shRNAs virus. Western blot analysis indicated that hMOB2 expression was reduced significantly in hmob2-shRNA treated HT1080 cells. Statistical analysis indicated that the expression of hMOB2 in control and hmob2shRNAs-treated cells were significantly different (three experiments, *Student's t*-test *** indicates $P<0.001$). Tubulin acts as loading control.

To study whether downregulation of hMOB2 affects cell spreading, we analyzed the ability of cell spreading according to Brugmans et al., 1982. We defined the cells to three categories; cell area < 400 μm^2 (Class 1, round cells weakly adhered onto the substrate), 400 μm^2 < cell area < 800 μm^2 (Class 2, cells in the course of spreading) and cell area > 800 μm^2 (Class 3, flat and well-spread cells with extended pseudopodia) (Fig.6A). Quantitative analysis was used to analyze the cells categories after two hours seeding on the petri dish. In control cells, 60% of cells were in class 2 and around 10 % of cells has entered class 3 (Fig. 6). However, the cells were mainly stayed at the class 1, some entered class 2, and only few were in class3 in hMOB2 downregulated cells (Fig. 6). These results suggest that downregulation of hMOB2 expression suppressed cell spreading in HT1080 cells. To further investigate whether downregulation of hMob2 affect cell migration, we performed wound healing assay to determine the cell mobility in control and hmob2-shRNA treated cells. As clearly indicated in the Figure 7, the wound healing occurred about six hours after wound in control cells. In hmob2-shRNA treated cells, there was still a big gap between the wound. This result suggested that hMob2 plays a critical role in cell migration.

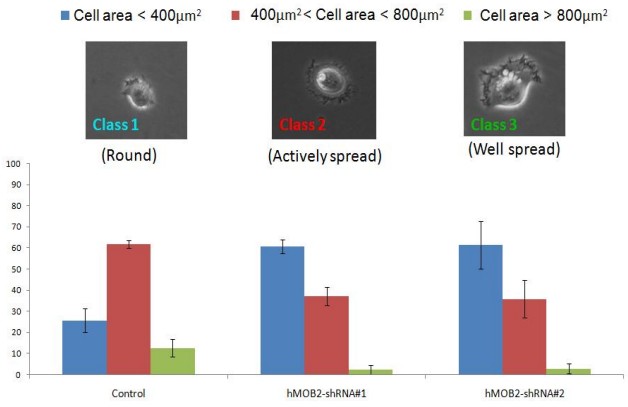

Fig. 6 Downregulation of hMOB2 expression affected cell spreading. Confocal images showed three categories (Class 1, Class 2, and Class 3) of cells according to their surface area. Quantitative analysis showed the percentages cells in the three classes in control and *hmob2*-shRNA cells.

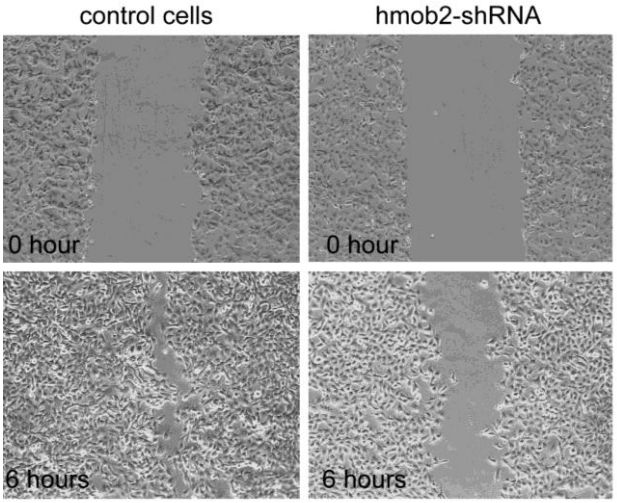

Fig. 7 Downregulation of hMOB2 expression reduced the rate of cell migration Phase-contrast images showed the migration of HT1080 cells after wound formation. In control cells, cells closure at around 6 hours. In *hmob2*-shRNA treated cells, there was a gap remained.

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計畫自評：

This grant proposal has completed the identification of the role of *Drosophila* mob2 in rhabdomere morphogenesis. We found that mob2 plays an important role in photoreceptor morphogenesis. Our results show that *Drosophila mob2* (*Dmob2*) is responsible for regulating the subcellular localization of phosphorylated-Moesin and Crumbs in photoreceptor cells. During development, the subcellular localization of Dmob2 is in the entire apical membrane of photoreceptor cells in early-pupal stage and then specifically confines to the subrhabdomere domain of photoreceptor cells in the late-pupal stage. Downregulation of Dmob2 expression in developing photoreceptor cells disrupts P-Moesin and Crumbs localization at photoreceptor cells and thus results in the formation of aberrant rhabdomeres. In addition, we find that Dmob2 binding protein, Tricornered, a serine-threonine kinase, may participate in Moesin phosphorylation. Expression of Tricornered kinase dead domain in photoreceptor cells results in the rhabdomere phenotype, which is similar to Dmob2-downregulated cells. In summary, our findings suggest that Dmob2 may coordinate with Tricornered to phosphorylate Moesin and participates in photoreceptor morphogenesis. The part of results has been published in Liu et al., 2009, Cell Tissue Research 338:377-389.

We also found that Mob2 protein plays a significant role in promoting neuritogenesis in neuronal cells. Our results show that Mob2 is highly expressed during neuritogenesis in the Neuro-2A (N2A) cells. Downregulation of Mob2 expression using RNA interference impairs the neurite formation both in N2A cells. Overexpression of Mob2 in N2A cells promotes the neurite formation. Furthermore, our results show that deletion of N-terminal 28 to 143 amino acids of Mob2 protein fails to promote neuritogenesis suggesting its importance in neurite formation. In summary, these results extend our pervious knowledge about molecular regulation of neuritogenesis and provide additional information on the role of Mob2 in mediating neurite formation. The part of results is published in the Lin et al., 2011, FEBS Lett 585:523-530.

In addition to the publication of research results, this grant has supported two Ph. students and one master students to complete their research thesis.

1. Liu LY, Lin CH, Fan SS. (2009). Function of Drosophila mob2 in photoreceptor morphogenesis. Cell Tissue Res. 338, 377-389.
2. Lin CH, Hsieh M, Fan SS (2011). The promotion of neurite formation in Neuro2A cells by mouse Mob2 protein. FEBS Lett. 585:523-30.

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