The Limch1, a novel microfilament associated protein, associated with the sarcomeric type stress fiber

Abstract
Limch1 as a novel protein engaged in cell migration was identified in a systematic screening of misexpressing proteins in migratory border cells during Drosophila oogenesis. This novel protein comprises an N-terminal calponin homologous (CH) domain, and a C-terminal LIM domain flanking its central undefined coiled-coil stretches. Depletion of Limch1 protein with specific siRNA in Hela cells enhances the cell migration and results in morphology change. Immunofluorescence microscopy investigation in subcellular localization indicated that Limch1 mainly distributes on stress fibers. Either CH domain or LIM domain of — Limch1 is able to coordinate with the first coiled-coil stretch in Limch1 localization by domain characterization with overexpression of GFP tagged Limch1 deletion mutants in U2OS cells. In contrast, the GFP fused central coiled-coil stretch alone is randomly distribution in the cytoplasm. Indeed, the Limch1 is associated with the sarcomeric type stress fibers (SSFs) in the non-muscle cells. The association of Limch1 with SSFs was dissected by costaining with α-actinin as a Z-disc marker. While the inhibiting ROCK activity leads to Limch1 disassociation from the SSFs, it suggested Limch1 might be a downstream effector of ROCK signaling. However, the ROCK is a crucial regulator involved in SSFs assembly and mediated actomyosin contraction. The results of ROCK
inhibitor treatment from SSFs may imply that Limch1 likely plays an important role in the contractile SSFs assembly and function. Furthermore, SSFs contraction is an essential force generator in migratory cell. Take together, the misexpression of Limch1 delayed the border cell migration during oogenesis in Drosophila, it hinted that association of Limch1 with SSFs may manage contractile SSFs assembly, and operate SSFs dependent force generation for cell migration.
**Introduction**

Cell migration plays a critical role for many physiological and pathological processes, such as development of organ and neuron, wound healing and immune function [1-3]. In pathological studies, tumors formation and cancer cells move within tissues need their own motility to promote metastasis and angiogenesis. However, cell migration is very important problem in cancer therapy[4]. Cell migration involves multiple processes by various signaling molecules. These signals lead cell to perform migration cycle which is polarization, protrusion and translocation. And this behavior is regulated by small Rho family GTPases, which are Rho, Rac and Cdc42[5].

From our collaboration lab screens 83 novel human genes from differential expression level between liver carcinoma, hepatoma cells and normal cells, using cDNA microarray, serial analysis of gene expression (SAGE) and differential display analysis (DD). We further used *Drosophila* migration system to identify two know genes and three novel genes that interrupt border cells migration. Border cells migration in *Drosophila* oogenesis provides us a tractable genetic model to study cancer invasion [6]. LIMCH1 (KIAA1102) is a novel protein among these three genes, which affect border cells migration. Further, we over-express Limch1 to MDCK cells and detect that Limch1 can increase MDCK cell mobility. Further work, we investigated ability of Limch1 in promoting cell migration.

Limch1 protein is predicted containing one calponin homology domain at its N-terminus and one LIM domain at its C-terminus. It is
module of 1083 residues and predicted molecular weight as 121 kD.

Calponin homology domains contain about 100 residues and are identified in the variety of protein ranging from cytoskeleton and signaling protein, either as actin binding motif or functional regulation[7]. Calponin is first described containing single CH domain, an actin binding protein. Calponin has been identified to direct target for ROCK to regulate actomyosin in smooth and non-muscle cells[7]. Furthermore, single CH domain of calponin is neither sufficient nor necessary for F-actin binding[8]. Other proteins such as Vav and IQGAP are also including single CH domain at its N-terminus. These proteins involve in regulation of Rho family GTPases [7]. Generally, proteins containing two tandem CH domains are called actin binding domain which must bind to actin, such as actinin [9].

LIM domain is a tandem zinc finger structure that serves as a modular protein-binding interface mediating protein-protein interaction in the cytoplasm and the nucleus. LIM domains can interact with other domain, such as homeodomains, catalytic domains, cytoskeleton binding domains and other domains. But no single binding motif has been identified as a common to target for LIM domains [10, 11]. Several proteins associated with cytoskeleton which contained LIM domain have been identified to play a role in signal transduction and organization of the actin filaments [11]. LIM domain has no catalytic function, but it mediate many process by interacting with target proteins. Function of containing LIM domain protein can be as molecular adaptors, competitors, autoinhibitors or localizers [10].
Materials and methods

Antibodies

The following antibodies were used: rabbit anti-Limch1 polyclone; mouse anti-vinculin monoclon e (Santa cruz); mouse anti-actin monoclon e (Chemicon); mouse anti-actinin-1 BM-75.2 monoclon e (Sigma); goat anti-actinin-4 N-17 polyclone; mouse anti-phospho-myosin light chain 2 (Ser-19) monoclon e.

Cell Culture and immunofluorescence staining

Mahlavu, J7, and Hela cells were cultured in DMEM with 10% fetal bovine serum (complete medium), and transfection was performed by using jetPEI™ (Invitrogen), according to the manufacturer’s instructions. Mahlavu and J7 obtained from Dr. Kwang-Huei Lin Lab. Stable clones selection used complete medium containing G418 (1.5mg/ml, Sigma). In immunofluorescence staining, cells were fixed at indicated periods with 4% formaldehyde in PBS containing 0.5% Triton X-100 for 20 min at room temperature, followed blocking with 2% BSA in PBS. The cells were observed with a confocal laser microscope (BIO-RAD RADIANCE 2100).

Cell motility assay

Cell migration was evaluated by transwell (Millicell) composed of a polycarbonate membrane with 8μ m pores. Cells were suspended in 0.25% trypsin, neutralized with trypsin inhibitor (Gibco), and harvested
in serum free medium. The cells were seeded on the upper chamber at 1x10^5 cells and were incubated for 24 hr, chemotactic medium contained 10% fetal bovine serum on the lower chamber. Cells that migrated to the lower side of membrane were fixed and stained with Giemsa solution (Sigma) or DAPI. Migrating cell were evaluated by counting cells with inverted or fluorescent microscope.

**Western blotting**

Cells were lysed at 5x10^7 cells/ml with RIPA buffer (50 mM Tris, pH 8.5, 150 mM NaCl, 0.1% SDS, 0.2% Na-Deoxcholic acid sodium salt, 2 mM EDTA, 1% Triton X-100) containing protease inhibitor for 20 min on ice. Cell lysate was centrifugation at 12,000 rpm for 10 minutes to remove insoluble material. Proteins were separated by SDS-PAGE. After transfer to PVDF membrane and incubation with primary antibodies, the bound antibody was detected with HRP-conjugated anti-mouse IgG and ECL western blotting detection reagents.

**Time-lapse microscopy.**

Phase contrast: cells were plated on the coverslip for 24 h before imaging. Cells were incubated at 37 °C with 10% CO2 in DMEM containing 10% FCS during imaging. Fluorescent imaging: MDCK cells stably expressing Limch1–GFP and imaged using a Zeiss Fluorescent microscope.
Result and Discussion

**ΔN-Limch1 causes border cell migration delay in Drosophila.**

During *Drosophila* oogenesis, a cohesive cluster of border cells migrates through the nurse cells to reach the oocyte. It provides us a tractable genetic model to study cancer invasion [12]. To study functions of genes critical for cell migration, we screened thirty-five transgenic fly, which generated with human cDNA by collaborative labs, and selected the genes from different expression levels between normal and cancer tissues. Then, ectopic expressing these genes in border cells resulted in interrupting border cell migration, in which two known and two novel human genes were identified as PUMH2, A10-KIAA0704, A52-KIAA0981 and A54-KIAA1102/LIMCH1 (table.1). Among these genes, Limch1 markedly caused a group of border cells migration defect (Fig.1). Further, we sequenced the DNA construct of Limch1 transgenic fly and found that the cDNA of Limch1 is lacking of its N-terminus (ΔN-Limch1) while BLAST with genome database. Misexpressing ΔN-Limch1 in border cells in two different transgenic fly lines resulted in 18% and 23% migration defect, and protein expression levels were determined by Western blots (Fig.2 and table.2).

Border cell migration needs precise adhesion regulation to gain traction at the front and detaching at the rear. Maintenance of DE-cadherin (encoded *shotgun*) between border cells is crucial for border cell migration, because loss of DE-cadherin in border cells disrupts migration process [13]. We attempted to determine whether
DE-cadherin was implicated in migration defect caused by expression of ∆N-Limch1. High levels of DE-cadherin is enriched at the interface of border-border cells and border-polar cells in WT. In contrast, ectopic expressing ∆N-Limch1 in border cells leaded to reduction of DE-cadherin levels between border cells, but no effect on the interface of border-polar cells (Fig.3). These results indicated that ∆N-Limch1 may affect border cell migration in disturbing DE-cadherin location or expression between border cells.

**Limch1 expression profile**

Misexpression of Limch1 in border cells impaired the cell migration, leading us to explore the role of Limch1 in this process. The *Limch1* is not encoded homologous genes in *Drosophila*, thus, we used the mammalian cells for further studies. We cloned a open reading frame of the predicted human Limch1 protein (amino acid 1-1083, accession number NM_014988), which contains single CH (calponin homology) domain at the N-terminus (amino acid 23-124) and a LIM domain at the C-terminus (amino acid 1012-1070)(Fig.4A). The CH domain is considered as actin binding domain and the LIM domain is a protein-protein interaction domain which was occurred in the certain nuclear proteins [14, 15]. To confirm whether the isolated cDNA encoding full length Limch1 correspond to endogenous Limch1 in the mammalian cell lines, the HA-tagged Limch1 full length protein was expressed in Hela cells and detected as an approximate 150 kD major band with the anti-HA monoclonal and anti-Limch1 polyclonal antibody by Western blots (Fig.4B). The specificity of anti-Limch1
antibody was further confirmed by small interference RNA knockdown (Fig 9.). The protein mass of exogenous and endogenous Limch1 in the SDS-PAGE is higher than predicted Limch1 molecular weight of about 121 kD in the databases. We guess that Limch1 may be modified by certain post-translational modification, such as glycosylation could cause drastic changes at molecular weight. We assessed Limch1 expression in different cell lines by Western blots. The Limch1 was highly expressed in Mahlavu and Hela cell, and slightly expressed in Huh7 and MDA-MB-231 (selected stably expressing EGF receptor), but not detected in U2OS cell line (Fig.5). We also detected the other cancer cell lines and found Limch1 was not expressed in most cell lines (Fig.6).

**Limch1 associates with sarcomeric type stress fibers.**

To study the subcellular location of Limch1 in the cells, we examined the Limch1 expressing cell lines— Hela, Huh7 and Mahlavu cells with anti-Limch1 polyclone antibody by immunofluorescence staining. These results revealed a dotted line pattern consistent in all cell types. Furthermore, this specificity of staining signal was confirmed by depleting Limch1 protein with siRNA (Fig. 15). The double labeling with phalloidin and anti-Limch1 antibody in Hela cells indicated that Limch1 was localized on actin stress fibers and displayed periodic distribution pattern along these filaments (Fig 16 A). To analyze this distribution in more detail, the Limch1 does not exist at the end of actin filaments and is not overlapped with the focal adhesion marker vinculin (Fig 16 B). We also found that Limch1 was
not located at the dorsal stress fibers, peripheral actin filaments and cleavage furrow during cell division (Fig 17A-C). In addition, to treat the cells with the actin depolymerization agent cytochalasin D. While the actin depolymerized, the periodic punctuate pattern of Limch1 was disrupted and overlapped with the aggregated actin structures (Fig 18). These data indicated that Limch1 was specific localized at the contractile SSFs.
Reference


Figure 1. Overexpressing human genes in border cells during oogenesis results in migration defect. These stage 10A egg chambers were stained with phalloidin to reveal the cell outline. Arrow indicated that the border cells were expressed human genes and GFP by slbo-GAL4.

(A) In WT egg chamber, border cells translocates from anterior side (left) toward posterior side (right) through the nurse cells and arrived the oocyte. (B) PUMH2 showed 17% group delay. (C) A54-KIAA1102/Limch1 showed 17-19% group delay. (D) A52-KIAA0981 showed 5-32% group delay. (E) A10-KIAA0704 showed a little cell detaching from border cells group.
Figure 2. Expression level of ΔN-Limch1 protein in the transgenic fly lines. Misexpressing ΔN-Limch1 protein in *Drosophila* ovary by GR1-GAL was detected with anti-Flag antibody by Western blot. Arrow indicated the ΔN-Limch1 protein. Open arrow head indicated the non-specific bands.
Figure 3. Overexpressing ΔN-Limch1 genes in border cells alters the distribution of DE-cadherin during oogenesis. (A-B) WT egg chamber showed the DE-cadherin enriched at the interface between border cells (arrow) and polar cells (arrow head). (C-D) Expressing ΔN-Limch1 protein in the border cells resulted in that DE-cadherin protein was disappeared from the interface between border cells and polar cells. The cell outline was stained with phalloidin, and arrow head indicated the border cells in A,C. The egg chambers were stained with anti-DE-cadherin antibody in B, D.
Figure 4. Recognition of Limch1 protein in Hela cells. (A) The protein structure of Limch1 was obtained from SMART database. It is module of 1083 amino acid and containing Calponin homology domain at N-terminus (CH) and LIM domain at C-terminus (LIM). The coiled-coil domains at the central region (green). Purple zone indicates complex region. (B) Exogenous and endogenous Limch1 proteins exhibited ~150 kD major band (arrow) with anti-HA monoclonal and anti-Limch1 polyclonal antibodies by Western blots.
Figure 5. Expression pattern of Limch1 protein in different cancer cell lines. Limch1 protein was highly expressed in Mahlavu and Hela cells, and slightly expressed in Huh7 and MDA-MB-231 cells, and not detected in U2OS cells. This result was performed with anti-Limch1 antibody by Western blot.
Figure 6. Expression pattern of Limch1 protein in different cancer cell lines. Endogenous Limch1 protein was expressed in J7, Mahlavu, MDA-MB-231, KB and Detroit cancer cell lines. The cell lysates of different cancer cell lines were detected by Western blot. Arrow indicated endogenous Limch1 protein. J7, Mahlavu, Huh7 are liver cancer cells. SCM1, AZ521 are gastric cancer cells, MDA-MB435S, MDA-MB-231, MCF7 are breast cancer cells. SAS, OECM1 are oral cancer cells. KB, BM1, NPC are nasopharyngeal cancer cells. Detroit is a pharynx cancer cell.
Figure 15. Limch1 protein exhibits dotted-line pattern in the cancer cell lines. Immunofluorescence data was stained with anti-Limch1 antibody (A-C) Endogenous Limch1 of Hela cells, Mahlavu cells and Huh7 cells revealed dotted-line pattern and nucleus signal. (D-E) Transfection of Hela and Mahlavu cells with Limch1 siRNA-1 causes the dotted-line pattern disappear. It suggests that nucleus signal is non-specific.
Figure 16. Limch1 protein associates with SSFs. Immunofluorescence results were stained with anti-Limch1 antibody in Hela cells (A-D, F-G, green). (A) The Limch1 showed the periodic pattern along central F-actin bundle by labeling with phalloidin. (B) The Limch1 did not extend to the vinculin marked focal adhesion.
Figure 17. Limch1 protein does not localize at peripheral actin filament, dorsal stress fibers and cleavage furrow. (A) Lamelipodium of spread Hela cell was marked with anti-actinin-4 antibody. (B) J7 cells were stained with anti-actinin-1 antibody. Arrow indicated the dorsal stress fibers. (C) Arrow head indicated the cleavage furrow during cell division by labeling with phalloidin in J7 cells. Scale 10 μm in A, 20 μm in B-C.
Figure 18. Activity of ROCK and Myosin II is required for Limch1 recruitment to stress fibers. Hela cells were fixed after treatment with indicated drug and stained with phalloidin (Red) and anti-Limch1 antibody (Green). (A) DMSO treatment as the control. (B) Inducing actin depolymerization by cytochalasin D. Arrow indicates actin asters which colocalized with Limch1. Scale, 20 μm.