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Deficiency in ubiquitin-like protein Ubl4A impairs migration of fibroblasts and macrophages

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ABSTRACT

Ubiquitin-like protein Ubl4A is a small, multi-functional protein with no ubiquitination activity. We have previously demonstrated that Ubl4A directly interacts with actin-related protein 2/3 complex (Arp2/3) and promotes Arp2/3-dependent actin branching, thereby accelerating plasma membrane translocation of protein kinase Akt upon insulin stimulation. Here, we show that Ubl4A is critical for plasma membrane protrusion and cell migration. Ubl4A, F-actin and Arp2/3 are co-localized at the cell leading edges during wound closure. Knockout of Ubl4A significantly reduces actin-mediated membrane protrusion and delays wound healing by primary mouse embryonic fibroblasts. Consistently, the ability of fibroblasts to migrate out of corneal tissue *ex vivo* is also impaired in *Ubl4A*-deficient mice. Furthermore, cell motility, but not phagocytosis, is significantly decreased in *Ubl4A*-deficient macrophages compared with wild-type controls. These results imply an important role for Ubl4A in cell migration-associated pathophysiological processes.

1. Introduction

Cell migration plays a crucial role in many biological events, including embryogenesis, chemotaxis, and wound healing [1,2]. Migration is a complicated and integrated process that relies on coordinated dynamics of the cytoskeleton [3,4]. Actin filaments reorganize in the leading edges of the cell membrane and form dynamic protrusions known as lamellipodia and filopodia, which are essential for cell sensory perception, cell-cell adhesion, endocytosis and phagocytosis [5,6]. One of the most critical prominent functions of lamellipodia and filopodia is to mediate directional cell migration [1,4,7].

The formation of membrane protrusions is driven by actin polymerization to form a meshwork beneath the cell plasma membrane [8–11]. The integral building block of the actin meshwork is the Y-shaped actin branch. The daughter actin filaments (new filaments) extend at an angle of 70° from the mother filaments [9,12,13]. The nucleation of the branch point is mainly composed of the actin-related protein 2/3 complex (Arp2/3), which consists of 7 subunits [14,15]. Inhibition of the Arp2/3 by small interfering RNA significantly decreases the filament branching density and reduces lamellipodia formation [16]. Arp2/3-dependent actin branching at the leading edge appears to control both the direction and the speed of cell migration [3].

Ubiquitin-like protein 4A (Ubl4A) is a small protein of 157 amino acids encoded by a housekeeping gene located on the X chromosome (Xq28) [17]. The Ubl4A NH_2-terminus is 43% identical to that of ubiquitin, and its COOH-terminus is unique [17]. However, despite being a ubiquitin family member, Ubl4A has no ubiquitination activity [18]. It is known that Ubl4A is involved in delivery of tail-anchored proteins to the endoplasmic reticulum [19–22]. Other known functions of Ubl4A include its involvement in tumor suppression and in cell death in response to DNA damage [23], indicating a versatile capability of this protein. Our recent work revealed that Ubl4A is critical for insulin-induced plasma membrane translocation and subsequent activation of Akt, a key regulator of many cellular signaling pathways [24]. Interestingly, the underlying mechanism by which Ubl4A performs this function is through interaction with Arp2/3 and promotion of actin branching. *Ubl4A*-deficient mice showed not only defective Akt signaling but also impaired neutrophil directional migration [24]. In this study, we found that Ubl4A is critical for migration of fibroblasts and macrophages, suggesting that Ubl4A may have a broader impact on the actin meshwork-related cellular activities.

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2. Materials and methods

2.1. Animals

All experimental procedures with mice were permitted by the Institutional Animal Care and Use Committee (IACUC). *Ubl4A*-deficient (KO) C57BL/6 mice and their wild-type (WT) littermates were generated and characterized as described previously [24]. All tissues were harvested according to the guidelines approved by the IACUC.

2.2. Wound-healing assay

Primary fibroblasts from *Ubl4A*-deficient mice and WT littermate embryos were isolated and characterized as described previously [24]. Cells were cultured in regular Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS with 5.0% CO_2 at 37 °C and passaged every 3 days. All cells used in the experiments were within 5 passages. For *in vitro* wound-healing assays, primary fibroblasts were cultured to 100% confluence and scratched across the monolayer with a pipette tip to create a wound gap. The detached cells were washed away. The wound-closure process was monitored and documented with a phase-contrast microscope at various time points, as indicated in the figure legends.

2.3. Corneal isolation and migration assay

Eyeballs were isolated from 3-month-old mice after sacrificed by humane euthanasia. Eyeballs were enucleated, and corneas were surgically excised around the conjunctiva at the limbus [25]. Corneas were rinsed with sterile phosphate-buffered saline (PBS) and placed onto 6-well plates coated with 0.1% gelatin, with the stromal side facing down. After 10 min of dry-attachment, the cornea was cultured in regular DMEM with 10% FBS. Each cornea was imaged under a microscope in 4 different regions at three different time points per region, as indicated in the figure legends. The same area was imaged through the time course for each group. ImageJ software (version 1.48) was used to analyze the area on each dish covered by migrated cells. The percentage of area covered by the cells was calculated by dividing the migrated cell-covered area by the total area of the imaged field.

2.4. Preparation of murine bone-marrow-derived macrophages

Femurs and tibias were collected from 3 to 4 month-old WT littermates and *Ubl4A*-deficient mice. Excess of tissues and muscles were removed. The isolated bones were sterilized with 70% ethanol for 1–2 min and rinsed with PBS. Both epiphyses of each bone were removed carefully. The bone marrow was flushed out using a syringe filled with 2 ml of bone-marrow-derived macrophage (BMDM) differentiation medium (50% of DMEM, 30% of L-929 supernatant, 20% of FBS, 100 U/ml penicillin, and 100 U/ml streptomycin) [26]. Bone marrow cells were collected by centrifugation, and red blood cells were lysed with ammonium-chloride-potassium (ACK) lysing buffer. Cells were seeded after being washed or were frozen for future experiments. For macrophage differentiation, cells were seeded in a 10-cm cell culture dish containing BMDM differentiation medium and maintained for 7–10 days. The medium was changed every 3 days until the bone marrow cells differentiated into macrophages.

2.5. Macrophage phagocytosis assay

The standard bead-based phagocytosis assay was performed as described previously [27]. Briefly, macrophages from both WT littermates and *Ubl4A*-deficient mice were cultured (~0.6×10^6 cells per group) in 6-well cell culture plates and serum starved in plain DMEM containing 0.5% FBS overnight. After starvation, latex beads (5 μm diameter) were added into each well (1×10^7 beads) and incubated for various lengths of time, as indicated in the figure legends. The engulfment process was stopped by washing the plates with ice-cold PBS and then fixing with 4% Paraformaldehyde (PFA) for 15 min at room temperature. The cells were visualized by staining with 0.05% crystal violet for 20 min at room temperature. The percentage of bead-positive cells and the number of beads per cell were counted in five random fields per sample under a microscope.

2.6. Macrophage migration assay

The frozen bone marrow cells were thawed and cultured in BMDM differentiation medium as described above. After 10 days, the differentiated macrophages were serum starved overnight. The cells were trypsinized and washed with plain DMEM before the experiment. Cells (~1×10^6 cells) were seeded in the upper chamber of a trans-well insert in a 6-well plate in plain DMEM. The lower chamber was filled with BMDM differentiation medium. The inserts were then cultured for different lengths of time, as indicated in the figure legends. After incubation, cells in both the upper and lower chambers were fixed with 4% PFA for 15 min at room temperature, and then washed with PBS. Cells were stained with 0.05% crystal violet for 20 min, and cells in the upper chamber were removed with a cotton swab. The migrated cells attached to the bottom membrane of the trans-well insert were imaged and quantitated from 5 random fields under a microscope.

2.7. Live imaging

Primary fibroblasts within 5 passages were cultured on a 35 mm poly-D-lysine-coated glass bottom micro-well dish and transfected with mCherry-Lifeact-7 (a gift from Michael Davidson, Addgene plasmid # 54491) for 16 h. After serum starvation for 3 h, cells were subjected to insulin stimulation (2 μg/ml) and immediately placed in a heated chamber with 5.0% CO_2 at 37 °C. Time-lapse movies were captured at 5-s intervals for 10 min with a 63x objective under Marianas fluorescence confocal microscope using a 488 nm solid-state laser with SlideBook 5.0 software. Trajectory analysis was performed using the ImageJ plugin ADAPT (Automated Detection and Analysis of ProTrusion). The speed and average motion of cells were quantitated by ImageJ software with a cell-ruffle quantitative analysis-macro developed by the facility of University of Chicago.

2.8. Immunofluorescence staining

Primary fibroblasts within 5 passages were grown on 22 × 22-mm glass coverslips coated with 0.1% Gelatin and placed in 6-well plates. After initiation of the wound-healing assay, the cells were incubated for 30 min and then fixed with 4% paraformaldehyde for 15 min, followed by permeabilization in PBS containing 0.3% Triton X-100 for 15 min. Primary antibodies against Ubl4A and ArpC2 were incubated overnight and detected using secondary antibodies conjugated with either Alexa Fluor 488 or Alexa Fluor 647. Actin was detected using Alexa Fluor 594 Phalloidin. Images were captured using a Leica SP5-II-AOBS Tandem Scanner confocal microscope equipped with Leica LAS-AF software. Multiple-line scans of fluorescence intensity were analyzed using ImageJ.

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