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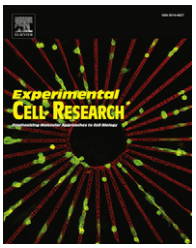

Research Article

Perturbation of invadolysin disrupts cell migration in zebrafish (*Danio rerio*)

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ABSTRACT

Invadolysin is an essential, conserved metalloprotease which links cell division with cell migration and is intriguingly associated with lipid droplets. In this work we examine the expression pattern, protein localisation and gross anatomical consequences of depleting invadolysin in the teleost *Danio rerio*. We observe that invadolysin plays a significant role in cell migration during development. When invadolysin is depleted by targeted morpholino injection, the appropriate deposition of neuromast clusters and distribution of melanophores are both disrupted. We also observe that blood vessels generated via angiogenesis are affected in invadolysin morphant fish while those formed by vasculogenesis appear normal, demonstrating an unanticipated role for invadolysin in vessel formation. Our results thus highlight a common feature shared by, and a requirement for invadolysin in, these distinct morphological events dependent on cell migration.

Introduction

The proteins that control cell migration are vital not only for normal health and development, but are also frequently implicated in the progression of disease. It is fundamentally necessary for cells to be able to move from one place to another when an organism is developing, e.g. this process is required for nervous and vascular system development, and for appropriate responses to immune challenges. However, when the mechanisms that control cell migration go awry, the consequences can be catastrophic, as when cancer cells become metastatic or endothelial cells form vasculature inappropriately.

Invadolysin was originally identified in a screen designed to discover mutations affecting mitosis and higher order chromosome structure in *Drosophila melanogaster*. Its drastic effects on cell cycle dynamics, chromosome structure, nuclear envelope protein accumulation in mitotically active tissues, and germ cell migration in developing embryos are thought to be the cause of late larval lethality [1]. The gene belongs to the leishmanolysin class of proteases [2] and encodes a conserved zinc metalloprotease that represents the metazoan counterpart of the M8 family of metzincins. The name *invadolysin* also reflects the localisation to structures resembling invadopodia.

Invadolysin is conserved from bacteria to plants and higher vertebrates and is phylogenetically distinct from the Matrix MetalloProtease (MMP) and A Disintegrin and Metalloprotease (ADAM) families [3]. We have identified one variant of invadolysin in fruit flies, four alternatively-spliced forms in human cells, and the zebrafish contain two splice variants of this gene. Analysis in a number of human cell lines has shown that invadolysin is associated with lipid droplets [4], and that *Drosophila* lacking the invadolysin protein also contain less triglycerides.

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In order to gain a better understanding of invadolysin function in a vertebrate, we decided to analyse this novel metalloprotease in *Danio rerio* (zebrafish). Zebrafish presents an excellent system in which to study gene function in a developing vertebrate, and has been used extensively in genetic screens and developmental studies [5,6]. Furthermore, this model system has been used to analyse many diverse regenerative processes including retinal development [7], those following spinal chord crush injuries [8], and more recently, heart regeneration [9].

The development of gene-specific morpholino oligonucleotides (MO) has facilitated the analysis of depletion, or knock-down, phenotypes in both zebrafish and *Xenopus* embryonic development [10–12]. In this study, we have capitalised on the use of MO-induced blockade of translation and GFP/dsRed reporter lines to analyse the in vivo consequences of depleting invadolysin. We observed a reduction in the number of mechanosensory cell clusters known as neuromasts, defects in melanophore migration, and inhibition of blood vessel formation through angiogenesis. As already demonstrated in *Drosophila*, these results lend additional support to a role for invadolysin in cell migration, and further extend this function to the likely involvement of particular vertebrate chemokine signalling pathways.

Materials and methods

Ethics statement

All experiments were approved by the local ethics committee and conducted in accordance with the Animals (Scientific Procedures) Act 1986 in a UK Home Office approved establishment. Under these guidelines no specific ethical approval is required for work on zebrafish younger than 120 hpf (hours post fertilisation).

Danio rerio Husbandry

Zebrafish (*Danio rerio*) of the WIK and AB strains were raised and maintained under constant temperature of 28 °C, a 14 h light/10 h dark cycle, and fed on *Artemia*. Embryos were collected using marble filled containers and staged according to [13] or by hpf. *Tg*(*flk*1:*GFPnls*;*GATA*1:*dsRed*) line was a kind gift from Dr. Tim Chico, University of Sheffield.

Morpholino sequences

The following anti-sense morpholino oligonucleotides (obtained from Gene Tools LLC, Philomath, USA) were generated to target the initiating ATG of zebrafish LMLN (XM_684004). ATG-MO: 5′ GAG GCT GCC GCC ATC CTG ACG CCA T 3′ [fluorescein]. Con-MO: 5′ GAc GCT cCC GCg ATC CTG AgG CgA T 3′ [fluorescein]. The Con-MO oligo contains 5 mis-matched base pairs (lower case) and served as a toxicity and phenotype control. The Ex6-MO morpholino is designed to target the exon/intron boundary at the 3′ end of Exon 6: 5′-TCC TTA AAT ATC TCC GTT ACC TGT C-3′ [lissamine rhodamine]. The oligos were manufactured to incorporate either a 3′ fluorescein or lissamine rhodamine moiety to aid visualisation of morpholino uptake in the developing embryo. All morpholinos were re-suspended in dH_2O to a concentration of 1 mM and stored at −80 °C.

Morpholino micro-injection

Prior to injection, the morpholino oligos were diluted to the appropriate concentration in dH_2O. 0.1% aqueous phenol red was added to assist visualisation of injected bolus. A bolus of approximately 5 nL was injected into freshly collected embryos at the 1–2 cell stage. A range of concentrations was used to establish the best survival to phenotype ratio. In this study, a concentration of 100 nM was found to be optimal, thus the injected dose was approximately 5 ng. Embryos were aligned in rows on a plastic plate and injected using a micro-manipulator and micro-injector. The injected embryos were washed into a petri-dish with salted system water (conductivity 400 microsiemens) containing 0.1% methylene blue, and allowed to develop in a 28 °C incubator.

4-Di-2-asp staining

4-(4-(Diethylamino)styryl)-*N*-methylpyridinium iodide (4-Di-2-Asp, Sigma) was used to visualise neuromast clusters. A 1:1000 dilution of 5 mg/ml stock in dH_2O was added to 35 mm petri-dishes containing fish of the appropriate stage. The fish were allowed to swim freely in the solution for 10 min. Anaesthetic (MS-222) was added after staining, the neuromast cells were then visualised using 460 nm light on a Leica ZX microscope and photographed using a Leica RFX camera.

Immunofluorescence and live imaging

All immunofluorescence staining was carried out as follows: anaesthetised fish of the appropriate stage were placed in fresh fixation buffer (PBS+4% formaldehyde+2% Triton X-100) overnight at 4 °C. The fish were washed 3 × 5 min with PBS+0.1% Triton X-100 (PBS-Tx) and then blocked in PBS-Tx+3% BSA for 1–2 h at room temperature. Fish were washed 2 × 5 min in PBS-Tx, and the primary antibodies (diluted in PBS-Tx+0.3% BSA) were added, then left to incubate overnight at 4 °C. The fish were then washed 3 × 5 min in PBS-Tx and the secondary antibodies (plus 0.5 μg/ml of DAPI if using) were added (diluted in PBS-Tx) and incubated at room temperature for 2 h. The fish were then washed 4 × 5 min in PBS-Tx, and finally mounted in PBS containing 50% glycerol.

The primary antibodies used in this work include: two custom made rabbit anti-zebrafish LMLN antibodies (A6970 and A6971) raised against peptide sequence YCDSVRSAPLQLTC (Genosphere Biotechnologies) which were used at 1:250 dilution, and mouse anti-acetylated tubulin clone 6–11 B-1 (T6796, Sigma) used at 1:500 dilution. Secondary antibodies were diluted at 1:500 and include: Alexa Fluor 594 donkey anti-rabbit IgG (A21206), Alexa Fluor 488 donkey anti-mouse IgG (A21202), and Alexa Fluor 488 Phalloidin (A12379)—all from Invitrogen. DAPI was used at 0.5 μg/ml. Control experiments were performed using pre-immune sera and secondary antibody alone. The staining patterns shown herein were not observed in control experiments.

Slides were viewed on an Olympus Provis microscope, equipped with epifluorescence optics. Images were captured using an Orca II CCD camera (Hamamatsu) and SmartCapture 2 software (Digital Scientific). The resulting files were processed using Adobe Photoshop.

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