Cell migration and invasion in human disease: the Tks adaptor proteins

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Abstract
Cell invasion plays a central role in a wide variety of biological phenomena and is the cause of tumour growth and metastasis. Understanding the biochemical mechanisms that control cell invasion is one of the major goals of our laboratory. Podosomes and invadopodia are specialized cellular structures present in cells with physiological or pathological invasive behaviours. These transient structures are localized at the ventral cell surface, contain an array of different proteins and facilitate cell-substrate adhesion, as well as the local proteolytic activity necessary for extracellular matrix remodelling and subsequent cellular invasion. We have shown previously that the adaptor proteins and Src substrates Tks4 and Tks5 are required for podosome and invadopodia formation, for cancer cell invasion in vitro, and for tumour growth in vivo. We have also defined a role for the Tks-mediated generation of ROS (reactive oxygen species) in both podosome and invadopodia formation, and invasive behaviour. Tks4 and Tks5 are also required for proper embryonic development, probably because of their roles in cell migration. Finally, we recently implicated podosome formation as part of the synthetic phenotype of vascular smooth muscle cells. Inhibitors of podosome and invadopodia formation might have utility in the treatment of vascular diseases and cancer. We have therefore developed a high-content cell-based high-throughput screening assay that allows us to identify inhibitors and activators of podosome/invadopodia formation. We have used this assay to screen for small-molecule inhibitors and defined novel regulators of invadopodia formation. In the present paper, I review these recent findings.

Podosomes and invadopodia
Podosomes and invadopodia can be defined as dynamic actin-rich protrusions of the ventral membrane of certain cell types [1]. They are the sites of attachment to, and degradation of, the ECM (extracellular matrix). Their presence correlates with migratory and invasive ability of cells, and they represent an increasingly important area of research. The term podosome is used to define the structures found in normal cell types, such as osteoclasts, macrophages, endothelial cells and vascular smooth muscle cells. The term invadopodia is used to describe the structures found in invasive cancer cells. Despite these different names, there are far more similarities than differences between the two structures. And where differences have been noted, for example in turnover time and length of protrusion, it is not clear whether these are intrinsic or related to the different culture conditions used for normal and cancer cells. Certainly, the key components of podosomes are shared with invadopodia. For a more detailed description of podosome and invadopodia components, see our recent review [1].

The Tks adaptor proteins
Our interest in podosomes and invadopodia began when we realized that a novel Src substrate and adaptor protein we had discovered, known as Tks5 (Figure 1), localized to invadopodia [2,3]. We went on to show that Tks5 is required for both invadopodia formation and invasive behaviour in a number of human cancer cell lines, as well as in the Src-transformed mouse fibroblasts (Src-3T3 cells) we use to study all aspects of Src transformation [4]. Tks5 thus joined a growing number of proteins shown to be necessary for invadopodia formation. Whereas most invadopodia components are broadly expressed in all cell types, we noticed that Tks5 is expressed in invasive cancer cells, but not in non-invasive cells. This suggested that Tks5 might play a central role in the initiation of invadopodia formation. To investigate this, we introduced Tks5 (along with Src to phosphorylate it) into a non-invasive breast cancer cell line and detected the robust formation of invadopodia [4]. In this assay, invadopodia formation was dependent on the PX (Phox homology) domain of Tks5, which suggests that lipid binding to the PX domain of Tks5 initiates invadopodia formation. In keeping with this, Oikawa et al. [5] subsequently showed that invadopodia are initiated at membrane sites rich in PtdIns(3,4)P2, a lipid known to bind to the PX domain of Tks5 [2]. Other studies have shown that recruitment of Tks5 and cortactin are the first events in invadopodia formation [6].

More recently, we have characterized the Tks5-related protein Tks4, which is also a Src substrate and adaptor protein, with a PX domain followed by four SH3 (Src homology 3) domains [7] (Figure 1). An examination of Tks4-null fibroblasts revealed that Tks4 is also required for Src-driven invadopodia formation. In the absence of Tks4, several invadopodia proteins, including Tks5, accumulate together at the membrane, but actin polymerization and ECM

Key words: atherosclerosis, cancer, embryonic development, reactive oxygen species (ROS).

Abbreviations used: Cdk, cyclin-dependent kinase; ECM, extracellular matrix; F-actin, filamentous actin; FTHS, Frank–ter Haar syndrome; MT1-MMP, membrane-type 1 matrix metalloproteinase; PX, Phox homology; ROS, reactive oxygen species; SH3, Src homology 3.
degradation do not take place. Over time in culture, Tks5 levels rise in Tks4-null cells, and actin polymerization is now visualized at these pre-invadopodia sites. But high levels of Tks5 cannot rescue ECM degradation. This is likely to be because Tks4 has a non-redundant role in the localization of MT1-MMP (membrane-type 1 matrix metalloproteinase) to invadopodia.

The defining properties of invadopodia and podosomes are the polymerization of F-actin (filamentous actin) and the degradation of the ECM. We are beginning to have a better understanding of how the Tks adaptor proteins participate in these processes. For example, Tks5 can associate with the actin regulatory proteins N-WASP (neuronal Wiskott–Aldrich syndrome protein) [5] and Nck [8]. Both Tks4 and Tks5 associate with ADAM (a disintegrin and metalloproteinase) family metalloproteases [2,7], and I have already mentioned the key role of Tks4 in recruiting MT1-MMP to invadopodia. Recently, we determined that both Tks proteins can act as organizers for the production of ROS (reactive oxygen species) by the NADPH oxidase family of enzymes [9,10]. ROS can be detected in invadopodia and are necessary for both podosome and invadopodia formation. Whereas the mechanisms by which ROS function in podosome and invadopodia assembly are not yet fully established, their known roles in promoting signal transduction, in transiently inhibiting the activity of some phosphatases and in increasing matrix metalloprotease synthesis are likely to prove important. Our current understanding of how the Tks adaptors might integrate diverse aspects of podosome and invadopodia formation and function are shown in Figure 2. Clearly it will be important to complete our understanding of the roles of Tks4 and Tks5 with more biochemical studies, to define the role of each SH3 domain, phosphorylation site and proline-rich motif.

**Tks proteins and embryonic development**

Cell migration and invasion is a property of specialized cell types in the adult organism and can also occur during pathological processes such as atherosclerosis. But during embryonic development, many cell types are migratory, and cell migration is required to pattern the developing embryo. We have been interested to determine whether the Tks adaptors are also involved in cell migration during embryonic development. To address this question for Tks5, we used zebrafish, since embryonic development is complete within 5 days, and the embryos of zebrafish are transparent, making the analysis of cell movements relatively facile. We used morpholino technology to reduce Tks5 expression in zebrafish embryos and observed a number of severe defects [11]. These include craniofacial and pigmentation abnormalities, heart malformations, lack of movement and oedema. Many of the defects observed are in cell types that arise from the differentiation of neural crest stem cells. We evaluated further the role of Tks5 using zebrafish expressing red fluorescent protein-tagged neural crest cells. We found that loss of Tks5 has little effect on neural crest cell number, but does impair their ventral migration,
coincident with a loss of protrusive structures in the migrating cells. In vitro studies using a mouse neural crest stem cell line showed the formation of Src and Tks5-dependent podosome-like structures in response to TGFβ (transforming growth factor β). Tks5 is also required for neural crest cell migration in vitro. Our data suggest that neural crest cell migration requires the formation of podosomes, implying that similar mechanisms are involved in cell migration during embryogenesis and cancer metastasis.

To evaluate embryonic roles for Tks4, we have analysed mice bearing a gene-trap cassette in the gene encoding Tks4, which fail to express any Tks4 protein [12]. Tks4-null mice are born at Mendelian ratios, but approximately 20% die in the first 3 weeks of life, from undetermined causes. At birth, Tks4-null mice are, on average, the same size as their wild-type and heterozygous littermates, but, by weaning, the surviving nulls are 40% smaller than normal littermates. They have severe craniofacial developmental defects, as well as other skeletal defects, heart abnormalities, glaucoma and a striking size reduction of WAT (white adipose tissue) depots. As we were characterizing the Tks4-null mice, van Bokhoven and colleagues used homozygosity mapping and copy number analysis to determine that mutations of the gene encoding Tks4 are associated with some cases of FTHS (Frank–ter Haar syndrome) (OMIM #249420), a rare, fatal, autosomal recessive disorder characterized by skeletal, cardiovascular and eye abnormalities. Analysis of seven FTHS families revealed five different homozygous mutations in the gene encoding Tks4 [12]. No Tks4 mutations were detected in six other FTHS families. However, we found that dermal fibroblasts from one of these individuals nevertheless express reduced levels of the Tks4 protein, suggesting a common mechanism underlying disease causation. These findings underscore the importance of Tks4 in embryonic development. Although it is not yet clear whether failure to produce podosomes underlies each of the developmental abnormalities observed in FTHS, the fact that several other podosome-associated proteins are mutated in disorders involving craniofacial and other skeletal abnormalities suggests that this is an idea worth pursuing in the future [13].

### Tks proteins and cancer progression

Most experiments on invadopodia are conducted in vitro. In the future, it will be important to visualize these structures in vivo and to characterize their roles in tumour progression in detail. To date, the roles for just a handful of invadopodia proteins have been probed in animal models. Expression of a form of AMAP1 [AMY-1 (associate of Myc-1)-associating protein-1] that cannot mediate association between cortactin and paxillin has little effect on breast cancer growth in mice, but a more pronounced effect on metastasis of these cells [14]. In contrast, knockdown of either MT1-MMP or cortactin affects the growth of implanted tumours [15,16]. The same is also true for Tks5: Src-3T3 cells with reduced Tks5 expression grow poorly when implanted subcutaneously, probably as a result of increased apoptosis and reduced tumour vascularization [17]. These results are not in keeping with a role for invadopodia solely in crossing basement membrane barriers in the process of metastasis, and suggest instead a broader role for invadopodia in tumour invasion and growth. Perhaps the pericellular proteolytic activity controlled by invadopodia is required not only for ECM degradation, but also for the local production and release of growth factors and other cytokines necessary to establish a productive tumour microenvironment.

### Isolating invadopodia regulators

The growing evidence for a role for invadopodia in tumour progression, as well as the role of vascular smooth muscle cell podosomes in atherosclerosis [18], suggests that targeting these structures might represent a valuable therapeutic approach. We set out to develop an assay to screen for invadopodia regulators. We opted for a high-content screening assay, using Src-3T3 cells stained with DAPI (4',6-diamidino-2-phenylindole) to visualize nuclei and rhodamine-labelled phalloidin to stain F-actin. We screened the LOPAC (Library Of Pharmacologically Active Compounds) collection; 1280 small molecules annotated to inhibit a variety of targets. We chose seven compounds for further analysis: two activators and five inhibitors [19]. The activators were cantharidin (a serine/threonine phosphatase inhibitor) and paclitaxel, a chemotherapeutic agent used in the treatment of a variety of solid tumours. We verified that paclitaxel promoted invadopodia formation and invasive behaviour in human cancer cell lines, including those resistant to the cytotoxic effects of the drug. These findings raise concerns about the use of paclitaxel in the neo-adjuvant setting (i.e. before the removal of the primary tumour) as well as once resistance has emerged. The most interesting inhibitors to emerge from our screen were annotated as Cdk (cyclin-dependent kinase) inhibitors. We ruled out a role for cell-cycle progression in the formation of invadopodia, which suggested that Cdk1 and 2 were unlikely to be the targets of the inhibitors. We therefore focused on Cdk5, a related kinase known to be involved in neuronal cell migration [20]. We validated Cdk5 as a positive regulator of invadopodia formation, and determined that Cdk5 and its associated protein, p35, are widely overexpressed in cancer cells. We determined that the mechanism by which Cdk5 promotes invadopodia formation is by phosphorylating and targeting for destruction the actin-binding protein caldesmon, which was described previously as a negative regulator of podosomes and invadopodia. Thus Cdk5 represents a validated invadopodia target.

### Future directions

Even though much more is now known about podosomes and invadopodia than just 5 years ago, there remain many important unanswered questions which will be the focus of our research in the coming years. What do invadopodia look like in vivo? What stimuli promote their formation? How do they regulate pericellular proteolysis? How common is it for
cancers to use invadopodia for invasion and metastasis? Will podosome and invadopodia inhibitors be of therapeutic use?

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