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Mass Spectrometry-Based Proteomic Profiling of Pseudopodia of Metastatic Cancer Cells

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Abstract : Pseudopodia are dynamic actin cytoskeleton-based membrane protrusions of cells that enable directional cell migration. Pseudopodia of cancer cells play key roles in cancer metastasis. Recent studies using pseudopodial subcellular fractionation methodologies combined with mass spectrometry-based proteomic profiling have provided insight into the pseudopodiome that control the protrusions of invasive metastatic cancer cells. This review highlights how to characterize the protein composition of pseudopodia and develop strategies to identify biomarkers or drug candidates that target reduction or prevention of metastatic cancer.

Keywords : Pseudopodia, Cancer metastasis, Proteomics, Mass spectrometry

Introduction

Cell migration plays an important role in numerous physiological and pathological processes, including embryogenesis, inflammatory response, and metastasis.[1,2] For their mobility, cells have to sprout and extend adhesive cytoplasmic protrusions called pseudopodia. Pseudopodia are actin-mediated membrane structures of cells that facilitate directional migration by response to chemoattractant signals. Such signal responses drive actin polymerization and extension of leading pseudopodia in the direction of the stimulus. Many molecular components involved in cell migration are being identified in various biological fields, including cancer cell biology, stem-cell biology, immune-biology, and neuro-biology. In cancer cells, metastasis is an important process that leads to severe organ failure and ultimately death. Invasion of surrounding tissues by movement of cancer cells is the first step in metastasis. This invasion of surrounding tissues occurs through the adjacent basement membrane.[3] The invasive cancer cells generate invadopodia to attach and degrade extracellular matrix (ECM) and then transform into larger pseudopodia.[4-6] This remodeling from invadopodia to pseudopodia initiates invasive membrane protrusions, which play a key role in driving cancer cell seeding in the body.

Mass spectrometry-based proteomic approaches have provided significant biological- functional information on the composition and organization of proteins in cells and tissues. Pseudopodial proteomics has revealed comprehensive identification of pseudopodiome and key molecules involved in cancer invasion and metastasis.[2,7-9] This review discusses how profiling pseudopodia of cancer cells by mass spectrometry-based proteomic approach helps to reveal important signatures of cancer metastasis and identify possible therapeutic drug targets.

Isolation of pseudopodia from cancer cell body for proteomic analysis

For large-scale pseudopodial proteomics, dissection of pseudopodial fractions from cells is one of the most important steps. This is because the quality (or purity) of the pseudopodia sample determines the significance of the proteomic results. Klemke group established an efficient method for pseudopodia isolation from cell bodies which successfully can be used for proteomic profiling of pseudopodiome in metastatic cancer cells (Figure 1 A).[2,10] To extend pseudopodia, cancer cells (1.5×10^6) are starved overnight and then plated on the surface of the upper microporous (3 mm) membrane trans-well chamber (24 mm). Chemoattractant factors such as lysophosphatidic acid (LPA), serum, or extracellular matrix proteins are placed in the lower chamber to create a diffusion gradient. The cells sense the diffusion gradient from the lower chamber and extend pseudopodia to the lower chamber through the membrane pores (Figure 1B and C) while the

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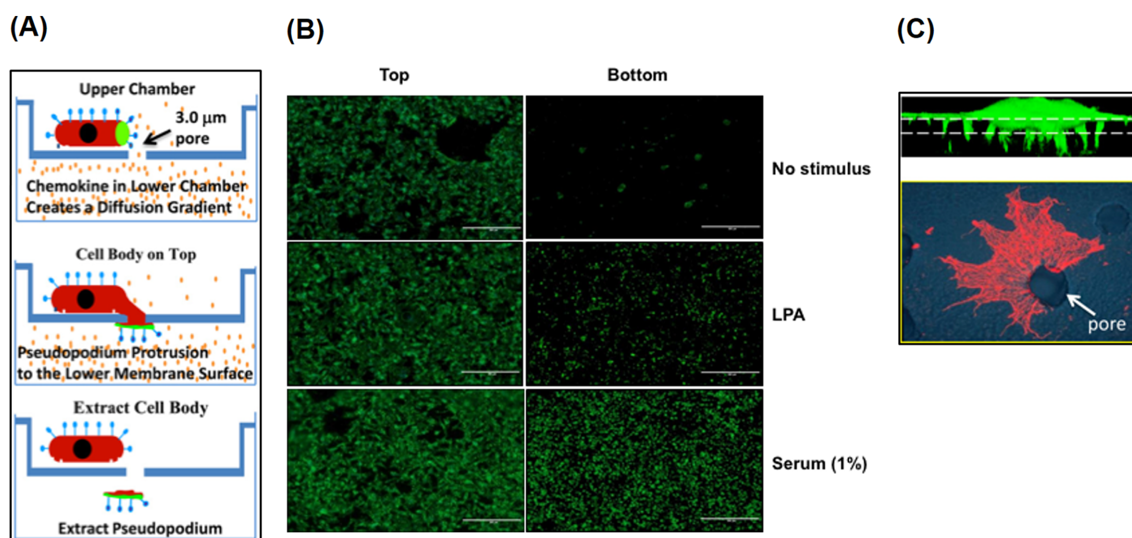

Figure 1. Pseudopodia purification using microporous membranes. (a) Schematic illustration of pseudopodia purification by microporous (3mm) membrane trans-well chamber. Cells are allowed to attach in the upper chamber to attach and chemoattractant factors are placed in the lower chamber to create a diffusion gradient. The cells sense the gradient and extend pseudopodia through the pore to the lower chamber in the directional of the gradient. The pseudopodia are isolated from cell bodies for proteomic analysis by mass spectrometry.[4] (b) Extension of pseudopodia by metastatic cancer cells in response of lysophosphatidic acid (LPA) and serum.[7] (c) Confocal image of a pseudopodia through the microporous filter.[4]

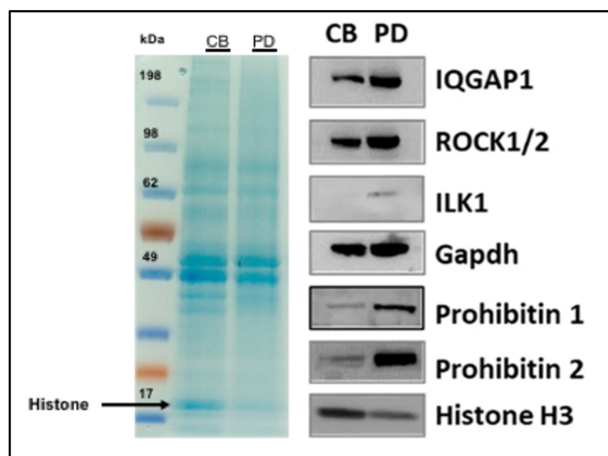

Figure 2. Different enrichment between pseudopodia (PD) fraction and cell body (CB) fraction. (left) SDS-PAGE and Coomassie blue staining of PD and CB. Note that the histone is highly enriched in the CB fraction not PD fraction. (right) Western blot analysis of proteins from PD fraction and CB fraction.[7]

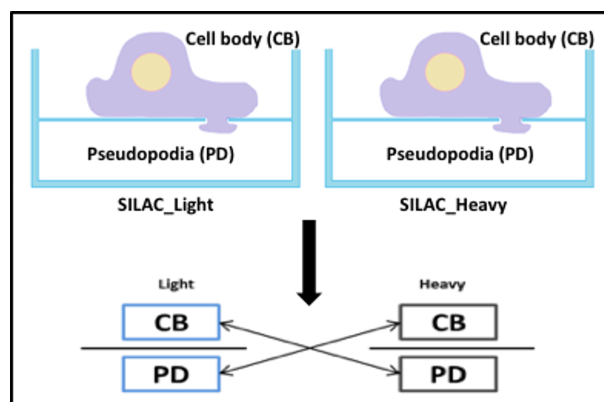

Figure 3. Schematic illustration of quantitative proteomics profiling for pseudopodia of metastatic cancer cells using SILAC. The isolated pseudopodia fraction and cell body fraction are mixed 1:1 in a label swapping scheme and then profiled using quantitative mass spectrometry-based proteomics.[7]

cell bodies remain on the upper membrane surface. For physical isolation of the pseudopodia from cell bodies, ice-cold methanol is used to fix the cell structures firstly, and then the cell bodies on the upper chamber are removed by a cotton swab or glass coverslip in prior to collecting the pure pseudopodia fraction to reduce contamination with proteins. Then, the pseudopodia are scraped by a glass coverslip. For solubilization of hydrophobic proteins, including cytoskeletons and membrane components, 1% sodium dodecyl sulfate (SDS) is employed. The enriched pseudopodiome are fractionated by SDS gel electrophoresis (Figure 2) and then each fraction is subjected to in-gel digestion for further proteomics analysis. To characterize the protein composition of pseudopodia, stable isotope labeling methods such as stable isotope labeling using amino acids in cell culture (SILAC) (Figure 3),[7] tandem mass tags (TMT), and isobaric tags for relative and

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absolute quantitation (iTRAQ) can be applied to compare pseudopodiome with the proteome of the cell body via quantitative proteomic analysis.

Proteomic analysis of pseudopodiome for cancer metastasis study

Many pseudopodial studies have reported a number of pseudopodial proteins as key molecules or drug target candidates leading to new insight in the field of cancer metastasis (Table 1).[2,7,9-12] Particularly, quantitative proteomic analysis of pseudopodia determined the differences of accurate protein composition between pseudopodia and cell bodies.[7,13] In SILAC, stable isotope labeled amino acids ($^{14}N_2$ $^{12}C_6$-lysine and $^{14}N_4$ $^{12}C_6$-arginine or $^{15}N_2$ $^{13}C_6$-lysine and $^{15}N_4$ $^{13}C_6$-arginine) are incorporated into cellular proteomes by replacing the natural amino acids through metabolic processes. Trypsin digested peptides are quantified from MS scans by comparing light and heavy peptides at the same elution time from the liquid chromatography (LC) separation. TMT and iTRAQ are two commonly used quantitative proteomic methods utilizing amine-reactive isobaric stable isotope (^{13}C and ^{15}N). The reagents of TMT and iTRAQ consist of three parts; reporter groups, balance groups, and reactive groups. The isobaric mass labels are covalently attached at the N-termini and lysine side chains of tryptic peptides derived from individual biological samples. After chemically labeling each digest mixture with specific isobaric tags, the samples are pooled and run in a mass spectrometer. The differentially labeled peptides with identical sequence have the same molecular weight and same retention time; as such, the pooled labeled peptides appear in the same peak in the mass spectrum and are chromatographically indistinguishable. However, when the reporter ion group of labeled peptides gets dissociated by collision energy, it produces ion signals in the MS/MS scans, that can be used to distinctly quantify individual peptides of a multiplex set.

Two receptor tyrosine-kinases (RTKs), epithermal growth factor receptor (EGFR) and ephrin type-A receptor 2 (EPHA2) are well known to be key regulators of cancer migration and metastasis.[14,15] Some of the proteins involved in cytoskeleton remodeling also play a major role in cancer cell migration. Expectedly, a number of cytoskeleton associated proteins, actin-related proteins 2/3 (ARP2/3), cortactin, paxillin, transforming protein RhoA (RhoA), rho-associated protein kinase 1/2 (ROCK1/2), neural Wiskott-Aldrich syndrome protein (N-WASP), a-actinin, ras-related C3 botulinum toxin substrate 1/2 (RAC1/2), and pseudopodium-enriched atypical kinase 1 (PEAK1) were identified as key regulators of cancer cell migration or metastasis.[8,16-21] Proteins associated with

Table 1. Major components of pseudopodia in metastatic cancer cells.

Gene_symbol	Protein_name	Biological function	References
PAX	Paxillin	Cell adhesion, actin cytoskeleton regulation	16, 18
RAC1 and 2	Ras-related C3 botulinum toxin substrate 1 and 2	Focal adhesion, actin cytoskeleton regulation	16, 18
SRC	Proto-oncogen tyrosine-protein kinase Src	Cell adhesion, tyrosine kinase	32, 33
FAK	Focal adhesion kinase	Tyrosine kinase	16, 18
CTTN	Src substrate cortactin	Focal adhesion, actin cytoskeleton regulation	16, 18, 32, 33
CDC42	Cell division control protein 42 homolog	Cytoskeleton regulation	16, 18
PHB 1 and 2	Prohibitin 1 and 2	Cell migration, myelination	7, 13
PEAK1	Pseudopodium-enriched atypical kinase 1	Focal adhesion, cytoskeleton regulation	8
EGFR	Epidermal growth factor receptor	Receptor tyrosine kinase	15, 34
RHOA	Transforming protein RhoA	Cell migration, cell adhesion	16, 18
ACT	Actin	Cell structure	16, 18, 32
ACTN1	Alpha-actinin 1	Focal adhesion, cytoskeleton regulation	32
WASL	Neural wiskott-aldrich syndrome protein	Cytoskeleton regulation	16, 18
ARRB	Beta-arrestin	Scaffold protein, signal transduction	34
ITGB	Beta-integrin	Cell adhesion	16
CAV1	Caveolin-1	Cell membrane signaling	16, 18
FSCN	Fascin	Cytoskeleton regulation	5, 16
IQGAP1	Ras GTPase-activating-like protein IQGAP1	Cytoskeleton regulation	35
ARP2/3	Actin-related protein 2/3 complex subunit 2	Cytoskeleton regulation, cell migration	36
EPHA2	Ephrin-type-A receptor2	Receptor tyrosine kinase	14
ILK	Integrin-linked protein kinase	Focal adhesion, cell migration	31, 37

adhesion, including E-cadherin and b-catenin are key components of cancer cell migration.[22] Particularly, one of the membrane proteins, called integrin aids in elongating leading edges in pseudopodia and invadopodia. Some of the epithelial-to-mesenchymal transition (EMT) related proteins were also identified in pseudopodia fractions. EMT is the transformation of epithelial cells to mesenchymal cells by loss of cell polarity and cell-cell adhesion. These cells obtain migratory and invasive properties.[23,24] Interestingly, another major group of identified proteins in the pseudopodia fractions are regulators of vesicle-mediated transport, including small guanosine triphosphatase (GTPase), adenosine diphosphate-ribosylation factor 6 (ARF6), ras-related protein 1 (RAB1), ras-related proteins 34 (RAB34), and coat protein complex 1 (COP1).[25-28] These key proteins are important candidates for identification of potential drug targets and development of specific anti-metastatic treatments.

Biomarker and clinical drug development

Biomarkers or therapeutic drug targets of cancer metastasis can be identified through pseudopodial proteomics. One study revealed a novel and previously uncharacterized non-receptor tyrosine kinase, named PEAK1 by comprehensive profiling of pseudopodia fractions using proteomic approaches.[8] PEAK1 is highly expressed in human metastatic pancreatic ductal adenocarcinoma (PDAC), which correlates with poor patient survival. Overexpressed PEAK1 in human PDAC cells enhances pseudopodia formation. Therefore, this study showed that PEAK1 regulates cancer cell migration and metastasis and that this protein can be an attractive target for treating primary and secondary tumor growths as well as a potential biomarker for diagnosis of cancer metastasis.[8]

Prohibitin (PHBs) proteins were also identified as components of pseudopodia. These proteins localize at the plasma membrane and significantly affect migratory ability in cancer cells.[7] In another proteomics study of pseudopodia in neuronal cells, it has been reported that PHBs are highly localized in pseudopodia fractions and are necessary for myelination by axo-glial interactions in Schwan cells.[13] A chemical inhibitor of PHBs, Roc-A (Rocaglamide, (1R,2R,3S,3aR,8bS)-2,3,3a,8b-Tetrahydro-1,8b-dihydroxy-6,8-dimethoxy-3a-(4-methoxyphenyl)-N, N-dimethyl-3-phenyl-1H-cyclopenta[b]benzofuran-2-carboxamide) was found to efficiently decrease cancer cell migration. As a result, Roc-A was suggested as a drug candidate targeting cancer metastasis reduction or prevention.[7] Integrin-linked protein kinase (ILK) is a key modulator of actin cytoskeletal dynamics and highly enriched in pseudopodia.[7,29] ILK family members are upregulated in several types of cancers. Several studies show that knockdown of ILK and a chemical inhibitor of ILK, Cpd22 (compound 22, N-Methly-3-(1-(4-(piperazin-1-yl)-5-(4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)-1H-pyrazol-3-yl)propanamide) decrease cancer cell proliferation and migration.[7,30,31] Therefore, inhibition of ILK may provide an effective clinical treatment for several types of cancers, including high-metastatic cancer.

Conclusions

In summary, cancer metastasis involves a complex cascade of biological events and metastatic cells are refractory to current standard chemotherapies. However, metastatic cells need to produce membrane protrusions for metastasis, so these membrane protrusions are very exploitable to identify and inhibit specific proteins associated with metastasis. A strategy combining purification of the membrane protrusions such as pseudopodia, coupled with mass spectrometry-based proteomic approaches, shows comprehensive biological signatures of metastatic cells and reveals important candidates to decrease metastatic ability in cancer cells.

Conflicts of Interest

The authors declare no conflict of interest.

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