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Comprehensive proteome quantification reveals NgBR as a new regulator for epithelial–mesenchymal transition of breast tumor cells

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ABSTRACT

Nogo-B receptor (NgBR) is a type I receptor and specifically binds to ligand Nogo-B. Our previous work has shown that NgBR is highly expressed in human breast invasive ductal carcinoma. Here, comprehensive proteome quantification was performed to examine the alteration of protein expression profile in MDA-MB-231 breast tumor cells after knocking down NgBR using lentivirus-mediated shRNA approach. Among a total of 1771 proteins feasibly quantified, 994 proteins were quantified in two biological replicates with RSD <50%. There are 122 proteins significantly down-regulated in NgBR knockdown MDA-MB-231 breast tumor cells, such as vimentin and S100A4, well-known markers for mesenchymal cells, and CD44, a stemness indicator. The decrease of vimentin, S100A4 and CD44 protein expression levels was further confirmed by Western blot analysis. MDA-MB-231 cells are

Abbreviations: NgBR, Nogo-B receptor; EMT, epithelial–mesenchymal transition; PI3K, phosphatidylinositol 3-kinase; GSK-3β, glycogen synthase kinase-3β; CNS, central nervous system; EC, endothelial cells; AmNogo-B, amino-terminal domain of Nogo-B; PS-DVB, polystyrene-divinylbenzene; FA, formic acid; $NaBH_3CN$, sodium cyanoborohydride; ACN, acetonitrile; NS, non-silencing; shNgBR, shRNAi/siRNA, shRNAi targeting NgBR; siNgBR, siRNA-targeting NgBR; NaCl, sodium chloride; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; Na_3VO_4, sodium orthovanadate; TEAB, triethyl ammonium bicarbonate; NaH_2PO_4, monosodium phosphate; Na_2HPO_4, disodium phosphate; SPE, solid-phase extraction; HPLC, high-performance liquid chromatography; MS, mass spectrometry; RP, reverse phase; SCX, strong cation exchange; CID, collision-induced dissociation; FDR, false detection rate; false positive, FP; true positive, TP; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PFA, paraformaldehyde; SEM, standard error of the mean; H/L, heavy/light; M/L, intermediate/light; M/H, intermediate/heavy; RSD, relative standard deviation; GO, gene ontology; PANTHER, protein analysis through evolutionary relationships; FSP1, fibroblastic marker; HMGA1, high mobility group A1; CFTR, cystic fibrosis transmembrane conductance regulator; MET, mesenchymal to epithelial transition; EpCAM, epithelial cell adhesion molecule.

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typical breast invasive ductal carcinoma cells showing mesenchymal phenotype. Cell morphology analysis demonstrates NgBR knockdown in MDA-MB-231 cells results in reversibility of epithelial–mesenchymal transition (EMT), which is one of the major mechanisms involved in breast cancer metastasis. Furthermore, we demonstrated that NgBR knockdown in MCF-7 cells significantly prevented the TGF-β-induced EMT process as determined by the morphology change, and staining of E-cadherin intercellular junction as well as the decreased expression of vimentin.

Biological significance

Our previous publication showed that NgBR is highly expressed in human breast invasive ductal carcinoma. However, the roles of NgBR and NgBR-mediated signaling pathway in breast tumor cells are still unclear. Here, we not only demonstrated that the quantitative proteomics analysis is a powerful tool to investigate the global biological function of NgBR, but also revealed that NgBR is involved in the transition of breast epithelial cells to mesenchymal stem cells, which is one of the major mechanisms involved in breast cancer metastasis. These findings provide new insights for understanding the roles of NgBR in regulating breast epithelial cell transform during the pathogenesis of breast cancer.

1. Introduction

Breast cancer is one of the most frequently diagnosed cancers and the leading cause of cancer death among females [1–3]. The current combinations of early detection with screening programs and the advent of more efficacious adjuvant systemic therapy successfully decrease breast cancer mortality [4]. Such effective pathway-specific targeted and patient-tailored therapeutics requests continued advances in our understanding of the molecular biology of breast cancer progression and discovery of new prognosis markers [4]. Breast cancer is the most common malignant disease among Western women, and its metastasis of distant sites is the main cause of death [5]. Epithelial–mesenchymal transition (EMT) is one of major mechanisms involved in breast cancer metastasis [5–8]. During EMT, the cells lose their epithelial characteristics and acquire more migratory mesenchymal properties. EMT is a well-recognized process in embryonic development to facilitate migration of neural crest cells out of the neuroectoderm [9,10]. EMT also happens in the formation of fibroblasts during wound healing and the transformation of epithelial cells into the invasive metastatic mesenchymal cells [9]. Here, we reveal the novel role of NgBR in promoting EMT of breast tumor cells by comprehensive proteome quantification approach.

EMT has been well documented in the progression of breast cancer [11,12]. EMT is a complicated molecular and cellular program by which epithelial cells lose their differentiated characteristics, such as cell–cell junctions and cell polarity due to decreased expression of E-cadherin, and gain the capabilities of motility and invasiveness by acquiring mesenchymal features demonstrated by the increased expression of vimentin, a typical marker for mesenchymal cells [6–8]. Activation of TGF-β signaling pathway has been demonstrated as an important regulator for the expression of epithelial genes and induction of mesenchymal genes [6–8,10]. In addition, cross-talk between TGF-β and PDGF, Wnt, and Notch signaling pathways also has been shown to contribute to EMT [6–8,10]. As a downstream signaling of TGF-β, PDGF, Wnt, and Notch signaling pathways, activation of the phosphatidylinositol 3-kinase (PI3K) and Akt promotes EMT [10,13]. Activated Akt phosphorylates glycogen synthase kinase-3β (GSK-3β) and results in inactivation of GSK-3β, which is a negative regulator for the activation of Snail that is essential signaling for EMT [13–17].

The Nogo isoforms-A, -B and -C are members of the reticulon family of proteins. Nogo-A and Nogo-C are highly expressed in the central nervous system (CNS), with Nogo-C uniquely found in skeletal muscle, while Nogo-B is found in most tissues [18,19]. Nogo-A (also called RTN4-A) binds its specific receptors, such as NgR and LiNGO1, and acts as a negative regulator of axon sprouting [20–23]. Nogo-B was previously identified as a protein that is highly expressed in caveolin-1 enriched microdomains of endothelial cells (EC) [24]. The amino terminus (residues1-200) of Nogo-B (AmNogo-B) serves as a chemoattractant for EC [24]. Mice deficient in Nogo-A/B show exaggerated neointimal proliferation, abnormal remodeling [24] and a deficit in ischemia induced arteriogenesis and angiogenesis [25]. NgBR was identified as a receptor specific for AmNogo-B by an expression cloning approach [26]. High affinity binding of AmNogo-B to NgBR is sufficient for AmNogo-B mediated chemotaxis and tube formation of endothelial cells [26]. We further demonstrated that NogoB-NgBR ligand–receptor pair is necessary for *in vivo* angiogenesis in zebrafish via the Akt pathway [27]. Genetic knockdown of either NogoB or NgBR by antisense morpholino abolished intersomitic vessel formation during developmental angiogenesis, and those defects can be rescued by constitutively activated Akt [27]. Our recent studies further demonstrated that NgBR is highly expressed in human breast invasive ductal carcinoma [28]. However, the exact roles of NgBR in the progression of cancer are still unclear. Here, we first utilized the on-column pseudo triplex stable isotope dimethyl labeling approach to quantify the different protein expression levels in both NgBR knockdown and control MDA-MB-231 breast cancer cells. Our results demonstrated that it is an effective approach to capture the unknown biological function of NgBR from the results of global protein alteration caused by NgBR deficiency.