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COMMENTARY

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Pathological functions of the small GTPase Arf6 in cancer progression: Tumor angiogenesis and metastasis

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ABSTRACT

Although several lines of evidence have shown that the small GTPase ADP-ribosylation factor 6 (Arf6) plays pivotal roles in cancer progression of several types of cancers, little is known about the functions of Arf6 in tumor microenvironment. We demonstrated that Arf6 in vascular endothelial cells (VECs) plays a crucial role in tumor angiogenesis and growth using endothelial cell-specific *Arf6* conditional knockout mice into which B16 melanoma and Lewis lung carcinoma cells were implanted. It was also found that Arf6 in VECs positively regulates hepatocyte growth factor (HGF)-induced β1 integrin recycling, which is a critical event for tumor angiogenesis by promoting cell migration. Importantly, pharmacological inhibition of HGF-induced Arf6 activation significantly suppresses tumor angiogenesis and growth in mice, suggesting that Arf6 signaling would be a potential target for anti-angiogenic therapy. In this manuscript, we summarize the multiple roles of Arf6 in cancer progression, particularly in cancer cell invasion/metastasis and our recent findings on tumor angiogenesis, and discuss a possible approach to develop innovative anti-cancer drugs.

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The small GTPase ADP-ribosylation factor (Arf) was originally identified as a cofactor that promotes cholera toxin-catalyzed ADP-ribosylation of α-subunit of the heterotrimeric G protein Gs in the middle of 1980's.[1] Now, Arf is recognized as a signaling molecule regulating a wide variety of cellular events.[2] In mammals, Arf family consists of 6 related gene products, Arf1-6, which are classified into 3 classes based on their sequence homology.[3] Class I includes Arf1, Arf2 and Arf3, class II Arf4 and Arf5, and class III Arf6. Classes I and II of Arfs mainly localize at the Golgi and endoplasmic reticulum, and regulate vesicular trafficking between these intracellular organelles.[2] On the other hand, the sole member of class III, Arf6, is divergent from other classes of Arfs in its localization and functions; Arf6 localizes to the plasma membrane and several kinds of endosomes, and plays important roles in the membrane trafficking between these cellular compartments, *e.g.*, endocytosis, exocytosis and endosomal recycling.[2] In addition, Arf6 regulates actin cytoskeleton reorganization beneath the plasma membrane, thereby being involved in membrane dynamics-related cellular events such as membrane ruffle formation, neurite outgrowth, cell migration, and cancer cell invasion.[4-6] Thus, Arf6 is characteristic among Arf isoforms in its functions at the cell level.

Like other small GTPases, Arf6 functions as a molecular switch in cellular signaling by cycling between GDP-bound inactive and GTP-bound active forms, which is precisely controlled by Arf6-directed guanine nucleotide exchange factors (Arf6 GEFs) and GTPase-activating proteins (Arf6 GAPs). At the resting state of cells, Arf6 exists as the GDP-bound inactive form. In response to the stimulation of cells with agonists, such as hormones, neurotransmitters and growth factors, GDP on Arf6 is exchanged for GTP by the action of Arf6 GEFs, thereby for the inactive form of Arf6 being converted to the active. The activated Arf6 then interacts with its target molecules (effectors), modulates their activities or regulates their intracellular location to transduce signals downstream, and eventually cell functions are exerted. Thereafter, GTP on Arf6 is hydrolyzed to GDP by the action of Arf6 GTPase with support of Arf6 GAPs, which increase GTPase activity of Arf6, thereby for Arf6 reverting to the inactive state. Arf GEFs and Arf GAPs are

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classified into 3 families, cytohesin, EFA6 and BRAG families, and 4 families, GIT, SMAP, ARAP and ACAP families, respectively,[7-9] and 8 GEF and 8 GAP members have been identified to control Arf6 activity state.[7-9]

Significance of Arf6 in cellular functions has been thoroughly investigated as described above. Exploration for physiological and pathological functions of Arf6 at whole animal level recently began; Arf6 is expressed ubiquitously in mice[10] and plays a key role(s) in embryonic development as was demonstrated that *Arf6* knockout (*Arf6* KO) mice is embryonic lethal.[11] It is noteworthy that Arf6 plays important roles in pathological settings, especially in cancer development, as well as in physiological settings. It has been reported that the expression level of Arf6 is higher in several types of human cancers, such as prostate, non-small cell lung and squamous cell lung cancers.[12,13] In lung adenocarcinoma, triple positive expression of Arf6, phosphorylated epidermal growth factor receptor (EGFR) and the Arf6 GEF BRAG2/GEP100 is intimately associated with the increased risk of patient death.[14] Furthermore, a direct correlation between Arf6 protein expression levels and breast cancer invasiveness has been elucidated in a series of breast cancer cell lines with different invasive abilities.[15] Consistent with this observation, siRNA-mediated knockdown of *Arf6* in breast cancer, melanoma and glioma cells significantly suppresses their invasiveness.[15-17] In addition, it has been reported that melanoma cells ectopically expressed with the constitutively active Arf6 mutant (Arf6 Q67L) enhance their local invasive activity in mice.[18] Thus, Arf6 is recognized as a potential regulator for the cancer invasion/metastasis.

Process of cancer metastasis includes 5 steps; local tissue invasion of cancer cells, their intravasation into blood and lymphatic vessels, their survival in the circulation, extravasation and colonization in distal organs away from the primary tumor.[19] Investigation on the involvement of Arf6 in cancer metastasis has been focused on the step of local tissue invasion using cancer cell lines as a model system. To initiate local tissue invasion, transformed epithelial cells first have to detach from the primary tumor lesion site by disassembling their cell-cell junction, in which Arf6 has been shown to be involved. In Madin-Darby canine kidney (MDCK) cells, Arf6 induces the internalization of the major adherence molecule E-cadherin, thereby triggering disassembly of the cell-cell junction.[20] This Arf6-induced E-cadherin internalization appears to be mediated by the GTPase dynamin, which is a key player in the clathrin-dependent endocytosis, and by the nucleoside diphosphate kinase NM23-H1, which provides GTP to dynamin; the active form of Arf6 interacts with and recruits NM23-H1 to adherence junctions to induce dynamin-enhanced E-cadherin internalization.[21] Supporting the notion that Arf6 is involved in local tissue invasion of cancer cells by promoting E-cadherin internalization, MCF7 breast cancer cells with low invasive ability induce E-cadherin internalization and acquire a higher invasive phenotype when the cells are coexpressed with Arf6 GEF BRAG2/GEP100 and Arf6 and stimulated with epidermal growth factor (EGF).[22] Interestingly, the overexpressed Arf6 GAP SMAP1, which is involved in Arf6-mediated transferrin receptor endocytosis,[23] partially localizes along the adherence junction and regulates E-cadherin endocytosis,[24] suggesting that adequate cycling of Arf6 between active and inactive states is critical for adherence junction disassembly. This point would be clarified by further investigation in future.

After disassembly of cell-cell junction, cancer cells have to degrade extracellular matrix (ECM) barriers to invade into surrounding stroma.[19] Arf6 has been shown to be involved in ECM degradation by regulating the formation of invadopodia and the release of plasma membrane-derived microvesicles.[25] Invadopodia are actin-rich protrusive structures with ECM degradative capacity to promote cancer cell invasion.[26] Multiple molecules in cancer cells, such as actin binding proteins which reorganize actin cytoskeleton, adhesion molecules, and membrane-associated proteases, are involved in invadopodia formation.[26] Arf6 plays pivotal roles in the invadopodia formation by recruiting these molecules to the plasma membrane where invadopodia will be formed. In highly invasive MDA-MB-231 breast cancer cells, the actin binding protein cortactin and the ECM-cell adhesion molecule paxillin, which are integral molecules for invadopodia formation,[27] associate with the Arf6 effector AMAP1, and Arf6 activated by EGF stimulation recruits AMAP1 together with cortactin and paxillin to the plasma membrane, which in turn evokes the invadopodia formation.[22,28] In addition, Arf6 regulates intracellular trafficking of the membrane-associated protease MT1-MMP, which is indispensable for ECM degradation by invadopodia.[29] MT1-MMP has been shown to be transported from the sorting endosome to invadopodia via late endosome, although it cannot be totally ruled out that this molecule is transported via recycling endosome.[30,31] At the sorting endosome in MDA-MB-231 cells, MT1-MMP makes a complex with its inhibitory protein TIMP2, and this complex is transported to late endosome when Arf6 is inactivated by the Arf6 GAP.[32] Thereafter, MT1-MMP dissociates from TIMP2 at the late endosome and trafficked to invadopodia when Arf6 is activated.[33] Thus, MT1-MMP trafficking from the sorting endosome to invadopodia via the late endosome appears to require adequate cycling of Arf6 between

inactive and active states spatiotemporally controlled by Arf6 GEFs and GAPs.

In addition to the invadopodia formation, plasma membrane-derived microvesicles, which are shed from cancer cells by outward budding and fission of the plasma membrane and contain selective cargo proteolytic proteins, such as MT1-MMP, MMP2, and MMP9, are also important for ECM degradation.[34] Microvesicles degrade more distal ECM to create the invasion path, while invadopodia facilitate pericellular proteolysis of ECM.[34] Microvesicle formation is observed in various types of cancer cell lines including a melanoma cell line LOX cells, a colon carcinoma cell line SW480 cells, a prostate adenocarcinoma cell line PC3, and a breast cancer cell line MDA-MB-231 cells.[35] It has been demonstrated that an Arf6 signaling pathway regulates fission of the plasma membrane to release microvesicles in LOX cells: Arf6 activates the downstream effector phospholipase D, which in turn activate mitogen-activated protein kinase (MAPK) to promote MLCK phosphorylation and subsequently MLC phosphorylation, leading to actomyosin-based plasma membrane contraction to release microvesicles.[35] Given that the Arf6 GEFs, GEP100 and EFA6, activate MAPK pathway in MDA-MB-231 cells and the glioblastoma cell line U373 cells, respectively,[36,37] these GEFs might regulate shedding of microvesicles. Further studies on the mechanisms of cancer cell invasion focusing on Arf6 regulators would identify a novel target(s) for development of innovative anti-cancer drugs.

In addition to autonomous signaling in cancer cells, tumor microenvironment is also a critical cue to promote tumor growth and metastasis.[38] Tumor angiogenesis is one of the hallmarks underlying cancer progression.[39] Neovascularization in tumors is essential for tumors to grow beyond a relatively small size by supplying oxygen and nutrients, and inhibition of tumor angiogenesis leads to tumor stasis.[40] Although significance of Arf6 in cancer cell invasion has been well documented as described above, little is known about the functions of Arf6 in tumor microenvironment, particularly in tumor angiogenesis. To address this issue, we had generated the endothelial cell-specific *Arf6* conditional knockout (EC-*Arf6* cKO) mice by mating $Arf6^{flox/flox}$ mice with Tie2-Cre mice,[41] and analyzed the involvement of Arf6 in tumor angiogenesis and growth. Interestingly, neovascularization in and growth of B16 melanoma and Lewis lung carcinoma (LLC) tumors produced in EC-*Arf6* cKO mice were both significantly suppressed compared with those in control $Arf6^{flox/flox}$ mice.[42] In contrast, phenotype of developmental angiogenesis in EC-*Arf6* cKO mice which were overtly healthy through adulthood was very mild. These observations suggest that Arf6 in VECs is a key player in tumor angiogenesis, but not in developmental angiogenesis.

Tumor and stroma cells composing tumor microenvironment secrete angiogenic factors, such as vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF), to induce tumor angiogenesis.[43,44] It is of interest to clarify in which angiogenic factor-induced signaling pathways Arf6 is involved during tumor angiogenesis. We found that deletion of *Arf6* from VECs inhibits HGF-induced angiogenesis, but not VEGF- and bFGF-induced angiogenesis, as demonstrated by both *in vitro* tube formation and aorta ring assays.[42] These results, taken together with the observation that EC-*Arf6* cKO mice exhibit obvious defect in tumor neoangiogenesis without severe impairment in developmental angiogenesis,[42] indicate that Arf6 is indispensable for HGF-induced tumor angiogenesis but not VEGF-regulated developmental angiogenesis. This notion is strongly supported by our finding that Arf6 is activated upon HGF stimulation of VECs[42] and by the reports that HGF signaling in VECs is important for the postnatal angiogenesis after ischemia via HGF/c-met signaling rather than developmental angiogenesis[45] which absolutely requires VEGF.[46,47] However, Arf6 seems to be also involved in the VEGF-dependent signaling linking to endothelial cell behaviors; Arf6 is transiently activated by VEGF in VECs[42] and VEGF-induced cell migration is significantly suppressed by Arf6 knockdown or overexpression of the inactive Arf6 mutant Arf6 T27N.[48,49] The difference in manipulations blocking the Arf6 signaling may be responsible for the distinct results; long term ablation of *Arf6* by knockout may trigger compensatory mechanisms for Arf6 function(s) in the VEGF signaling pathway. Nonetheless, our data described above strongly suggest that Arf6 in VECs is indispensable for HGF-induced tumor angiogenesis.

The adherent molecule β1 integrin is important for tumor angiogenesis; β1 integrin in the plasma membrane adheres to extracellular matrix to promote VEC migration, which is essential for tumor angiogenesis.[50] Consistent with the previous report demonstrating the involvement of Arf6 in β1 integrin recycling,[51] ablation of *Arf6* from VECs almost completely blocked HGF-induced β1 integrin recycling to the plasma membrane, resulting in defective cell migration toward HGF.[42] From this result, taken together with the observation that Arf6 activity is persistently upregulated upon HGF treatment of VECs,[42] it is reasonable to expect the involvement of an Arf6 GEF in the Arf6 activation and subsequent β1 integrin recycling. Surprisingly, it was found that plural Arf6 GEFs including Grp1, GEP100, EFA6B and EFA6D are involved in the HGF-induced β1 integrin recycling,

leading us to speculate that different populations of Arf6 regulate distinct steps in the β1 integrin recycling process, probably due to the spatiotemporal activation of Arf6 by distinct Arf6 GEFs during recycling process. This speculation is supported by the fact that recycling of endosomal proteins to the plasma membrane involves multiple steps, *e.g.* budding of vesicles from endosomes, transport of the vesicles to the plasma membrane and tethering/fusion of the vesicles to/with the plasma membrane. Our speculation is also consistent with the report that Arf6 is implicated in several steps for recycling of glucose transporter 4 (Glut4) in differentiated 3T3-L1 adipocytes.[52] Furthermore, intracellular localization of Grp1 is divergent from that of other Arf6 GEFs; Grp1 localizes at the β1 integrin-positive intracellular compartment and other 3 GEFs at the plasma membrane in VECs. Thus, Grp1 might function at an early step of β1 integrin recycling, *e.g.*, a budding of vesicles at the intracellular compartment, and other GEFs at a later step(s) such as tethering/fusion of the vesicles to/with the plasma membrane.

In conclusion, Arf6 in VECs plays an important role in the HGF-induced tumor angiogenesis by controlling cell migration through promoting β1 integrin recycling to the plasma membrane (Fig. 1).[42] VEGF, of angiogenic growth factors secreted from tumor and tumor stroma cells, has been recognized to be most important for tumor angiogenesis. Therefore, during the past decade, anti-angiogenic drugs targeting VEGF receptor (VEGFR) signaling such as bevacizumab, a humanized VEGF-neutralizing monoclonal antibody, and sunitinib, a tyrosine kinase inhibitor targeting VEGFR and platelet-derived growth factor receptor (PDGFR), had been developed.[40] However, the clinical benefits obtained in anti-VEGFR signaling therapy have been relatively modest.[53] This is probably due to the tumors adaptation and re-initiation of tumor growth via a process referred to as angiogenic redundancy that compensatory angiogenic growth factors are produced to overcome VEGFR signaling inhibition.[53,54] Importantly, It has been demonstrated in a preclinical model that HGF can act as an alternative angiogenic factor when VEGF signaling is inhibited.[55]

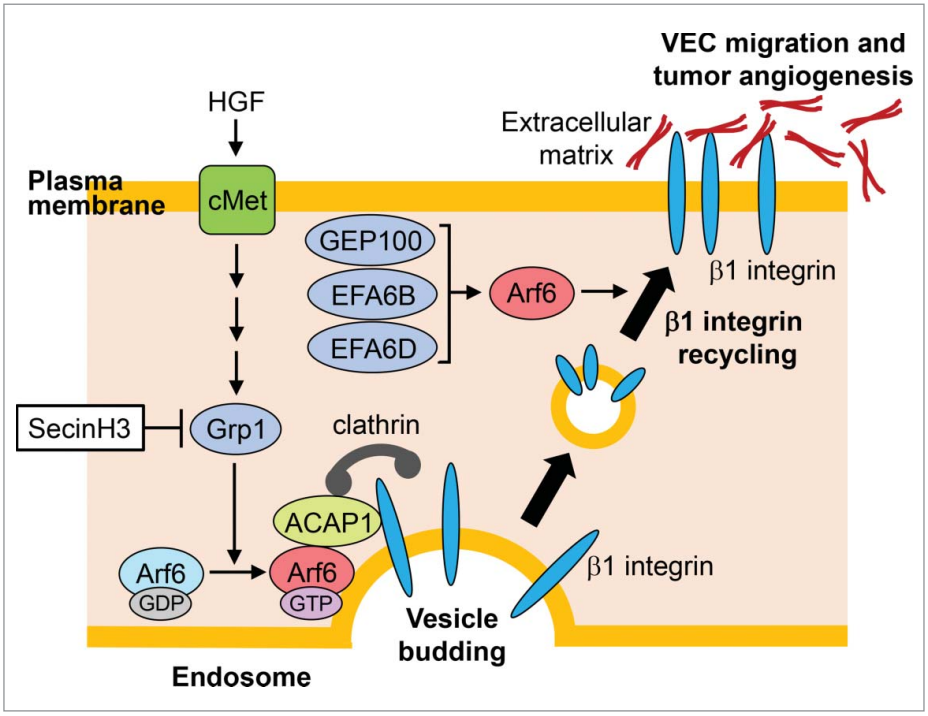

Figure 1. A model for the regulatory mechanism of β1 integrin recycling by Arf6 in vascular endothelial cells, which is critical for tumor angiogenesis. In response to HGF stimulation of vascular endothelial cells (VECs), Arf6 is activated by the Arf6 GEF Grp1 at the endosome, then interacts with the Arf6 GAP ACAP1 which binds to β1 integrin and recruits clathrin to the endosome to induce vesicle budding. Thus, in this case, ACAP1 functions as a downstream effector of Arf6 to form the β1 integrin-containing recycling endosome. Thereafter, the recycling endosome is tethered to and fused with the plasma membrane, when the distinct pool of Arf6 is activated by other Arf6 GEFs, GEP100, EFA6B and EFA6D, on the plasma membrane. β1 integrin recycled to the plasma membrane then adheres to extracellular matrix to promote VEC migration, which is essential for tumor angiogenesis. It is noteworthy that pharmacological inhibition of Grp1-induced Arf6 activation by SecinH3 interferes with β1 integrin recycling *in vitro*, thereby suppressing tumor angiogenesis in mice, suggesting that the Arf6-mediated signaling in endothelial cells could be a potential target for the development of innovative anti-angiogenic drugs.

Therefore, Arf6 signaling in VECs could be a potential target suppressing tumor angiogenesis and growth, particularly in combination with other anti-angiogenic therapeutics that target VEGFR and/or PDGFR.

In addition to significance of Arf6 in tumor angiogenesis, Arf6 in cancer cells plays important roles in cell invasion and metastasis as described above. Recent preclinical studies have suggested that anti-angiogenic therapy may promote cancer cell invasion and metastasis by inducing hypoxic signals of cancer cells.[56,57] The critical roles of Arf6 in both tumor vascularization and cancer cell invasiveness/metastasis might provide a novel cancer therapeutic opportunity to overcome the evasive resistance of cancer cells during the anti-angiogenic treatment. Consistent with this notion, SecinH3, a synthetic inhibitor for BRAG2/GEP100 and cytohesin family of Arf6 GEFs,[16,58] inhibits the HGF-induced Arf6 activation, β1 integrin recycling and tumor angiogenesis and growth,[42] and also suppresses the lung metastasis of melanoma tumor in mice.[16] Moreover, the phosphatidylinositol-3,4,5-triphosphate (PIP_3) antagonist, PIT-1, which inhibits the interaction of PIP_3 with Arf6 GEFs, ARNO and Grp1, suppress Arf6-mediated cell migration *in vitro*, and both tumor angiogenesis and metastasis in mice.[59] Thus, inhibitors of the Arf6 signaling pathway(s) would provide an innovative cancer therapeutic opportunity.

Since Arf6 is ubiquitously expressed in the whole body and regulates wide varieties of cellular functions,[2,10] targeting Arf6 itself may cause unwilling effects in the body. Targeting Arf6-related signaling molecules such as Arf6 GEFs or Arf6 effectors, which specifically regulate cancer invasion and tumor angiogenesis, but not physiological events, might be a very useful strategy for the development of innovative anti-cancer drugs with high specificity. In addition, Arf6 GAPs also could be considered as target molecules because of their involvement in the regulation of Arf6 activity and a wide variety of cellular functions. Most importantly, the tumor angiogenesis and growth of B16 melanoma and LLC tumors were significantly inhibited by SecinH3 treatment of mice without any obvious unwilling side effects, supporting our notion for the development of anti-cancer drugs described above.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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