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Atypical RhoV and RhoU GTPases control development of the neural crest

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Abbreviations: NC, neural crest; BMP, bone morphogenic protein; FGF, fibroblast growth factor; EMT, epithelial-mesenchymal transition; EGFR, epidermal growth factor receptor.

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This review addresses the developmental roles of 2 GTPases of the Rho family, RhoV/Chp and RhoU/Wrch. These two GTPases form a distinct subfamily related to Rac and Cdc42 proteins and were detected in a screen for Rho members that are particularly expressed in the neural crest, an embryonic tissue peculiar to vertebrates. The neural crest represents a physiological model of normal epithelial to mesenchymal transition (EMT), in which epithelial cells at the border of neural and non-neural ectoderm differentiate, lose their intercellular connections and migrate throughout the embryo. We showed that RhoV, transiently induced by the canonical Wnt pathway, is required for the full differentiation of neural crest cells, while RhoU, induced later by the non-canonical Wnt pathway, is necessary for the migration process. These two GTPases, which are highly conserved across vertebrates, are thus tightly functionally linked to Wnt signaling, whose implication in embryonic development and cancer progression is well established. In the light of the recent literature, we discuss how RhoV and RhoU may achieve their physiological functions.

Introduction

Development of the Neural Crest (NC) is probably the most dramatic morphogenetic event of vertebrate embryogenesis. Originating at the boundary between neural and non-neural ectoderm, NC cells differentiate in response to complex inductive cues emanating from the surrounding tissues.[1] At this early stage, NC cells express a set of transcription factors, such as Snai1/Snail, Snai2/Slug or Twist, which are known for their pro-invasive activities in stem cells and cancer cells.[2] After commitment (specification stage), NC cells migrate throughout the embryo and differentiate to form a broad range of terminal derivatives, including pigment cells, craniofacial skeleton, cartilage, neurons or glia of the peripheral nervous system.[3] Among the morphogens required for proper NC development, BMP, FGF, Notch and canonical Wnt pathways have prominent roles in NC induction, while non-canonical Wnt is required for NC migration.[4,5] Prior to migration, NC cells undergo a delamination phase, characterized by the loss of epithelial adherens junctions and the acquisition of invasive properties. This developmental process, known as epithelial to mesenchymal transition (EMT), has been proposed to mimic very early events of malignant progression, in which adherent adenoma cells switch to an invasive carcinoma phenotype.[6]

Because of their impact on adhesion and migration dynamics of many cell types,[7] GTPases of the Rho family were suspected to be involved in NC cell dynamics, and several studies pointed to a role of the major Rho family members Rho and Rac1 in NC formation in the Xenopus embryo.[8-10] Xenopus represents a model of choice for experimental embryology, mostly because of its rapid embryonic development and the large size of its eggs, which makes them amenable to microinjection and microdissection. Another major advantage of this model is the possibility of manipulating just one side of the embryo, while the other side serves as an internal control of development. Xenopus is also ideal because it

contains orthologues for 18 of the 20 Rho family members found in placentals.[11] We performed a comprehensive in situ hybridization screen to identify Rho members that are preferentially expressed in NC. Apart from RhoB and Rnd1, we identified RhoV/Chp and RhoU/Wrch, as being expressed sequentially at distinct NC developmental stages.[12,13]

RhoV and RhoU form an ancient Rho subfamily related to Rac1 and Cdc42 GTPases.[11] RhoV and RhoU are atypical in this family as they display a high intrinsic guanine nucleotide exchange activity and are thus thought to be constitutively active whenever they are expressed.[14,15] In keeping with their spontaneous activation, they are expressed at very low levels (in particular RhoV) in various tissues and organs.[11] Furthermore, they are palmitoylated and not prenylated like most Rho members, suggesting that they act at distinct subcellular locations,[16] and they contain additional N-terminal and C-terminal extensions, critical for their activities.[15,16] Despite the knowledge of their biochemical properties, little was known about the physiological function of these 2 GTPases, and the work we performed on Xenopus embryos unveiled their roles in NC development.

RhoV

RhoV is induced in the prospective NC territory as a canonical Wnt response gene, expressed as early as Snai1.[13] RhoV induction in response to Wnt is independent of Snai1, since expression of a dominant negative Snai1 mutant in Wnt1-treated embryos did not impair RhoV expression, whereas it blocked the subsequent induction of the Snai2 or Sox9 genes (unpublished data). RhoV expression is transient and is no longer detected at the migration stage. RhoV knockdown by antisense morpholino injection perturbs NC differentiation: while having no effect on the early Snai1 expression, it impaired induction of the Snai2, Twist or Sox9 genes. Consequently, NC-derived cranial structures are strongly inhibited in morphant embryos. Conversely, RhoV overexpression expands the NC territory and increases the expression of Snai1, Snai2 and Twist, indicating that RhoV feeds positively the canonical Wnt pathway. RhoV was shown to activate PAK1,[17] a member of a family of versatile kinases involved in cell migration and invasion.[18] PAK1 itself can phosphorylate and activate Snai1.[19] Since Snai1 activity is critical for NC induction,[20] RhoV might thus participate in the propagation and amplification of the canonical Wnt pathway. RhoV activity is also probably relies on its activity on cell adhesion, as recently shown in the zebrafish embryo, wherein RhoV is required for proper localization of E-cadherin and β-catenin at adherens junctions.[21] Along the same line, we observed that the neural plate was expanded upon RhoV inhibition and restricted upon moderate RhoV overexpression. This supports a role of RhoV in cell motility since folding of the neural plate is sensitive to the medial migration of NC cells.[22]

RhoU

As a non-canonical Wnt response gene,[23,24] RhoU was expected to be involved in NC cell migration[5] and its expression was indeed detected only from the migration stage in NC cells. RhoU depletion impaired NC migration and the subsequent formation of craniofacial cartilages.[12] NC cells from RhoU-depleted explants adopted a rounded phenotype and showed reduced adhesion to the substrate. Intriguingly, these effects are in contradiction with the increased density of integrin-dependent adhesive structures observed in RhoU-silenced mammalian cells.[25,26] Moderate RhoU overexpression also inhibited NC cell migration but with a distinct mechanism; RhoU-expressing explants readily adhered to the substrate and migratory NC cells scattered at an even higher rate than control cells. However, instead of being polarized, the scattering was isotropic and the persistence of NC cells migration was reduced, indicative of a defect in sensing polarity cues. Overall, these experiments suggest that RhoU controls NC migration through the regulation of polarized cell adhesion.

RhoV and RhoU Signaling in NC Development

Although the signaling pathways used by the 2 GTPases in NC cells remain to be fully determined, several candidates have emerged from the recent literature (**Fig. 1**). RhoU was shown to associate with EGFR in a Grb2-dependent manner and mediate changes in cell adhesion and migration.[27] Grb2[28] and EGFR[29,30] were themselves described as critical for NC adhesion, migration and late differentiation. Another potent RhoU regulator is Src, which can phosphorylate RhoU at its C-terminus thereby modifying its subcellular location.[31] Src and its substrate Tks5 are also required for NC migration in zebrafish development.[32] Several effectors have been identified for RhoV and RhoU,[33] in particular PAKs. PAK1 and PAK2 are expressed in migrating NC cells and indeed their activation or inhibition mimicked the phenotypes observed upon RhoU expression and depletion, respectively.[12] The proline-rich tyrosine kinase Pyk2 may also mediate RhoU activity in NC cell migration; indeed Pyk2 interacts with RhoU and the 2 partners cooperate with Src in cytoskeletal dynamics.[34] Furthermore, Pyk2 activation triggers EGFR signaling and epithelial cell motility during wound healing.[35] Last, RhoU might control polarized migration through interaction with Par6,[33,36] a RhoU and Cdc42 partner required for Cdc42-dependent cell polarity.[37]

Specific Roles of RhoV and RhoU in NC Development

The specific roles of RhoV and RhoU in NC development remain to be determined. RhoU can rescue RhoV depletion,[13] while the reverse is not true.[12] Thus RhoU in NC might exert the same functions as RhoV does, plus other functions probably linked to its specific domain; RhoU contains an SH3-binding proline-rich region in its NH2 terminus, that is responsible for its binding to Grb2.[15] Another difference between the 2 proteins is the tyrosine that is phosphorylated by Src, which is present at position 254 in RhoU[31] but absent in RhoV.

Given the functional differences between the 2 GTPases, one can thus propose that RhoV, induced early by the canonical Wnt pathway, initiates the cellular effects necessary for NC formation. These effects are then prolonged by RhoU which is induced later by the non-canonical Wnt pathway and which in addition triggers migration by interacting with partners through its SH3-binding domain. Two clues nevertheless suggest that RhoV may also have specific properties not held by RhoU: i) in the Xenopus embryo, RhoV mRNA is no longer detected in migrating cells,[13] indicating the presence of an active shutdown mechanism; ii) the RhoV protein displays an extremely high turnover in mammalian cells (unpublished data), suggesting that its activity is tightly controlled. This strongly suggests that RhoV must not be expressed during migration, which therefore suggests that RhoU cannot substitute for all activities of RhoV.

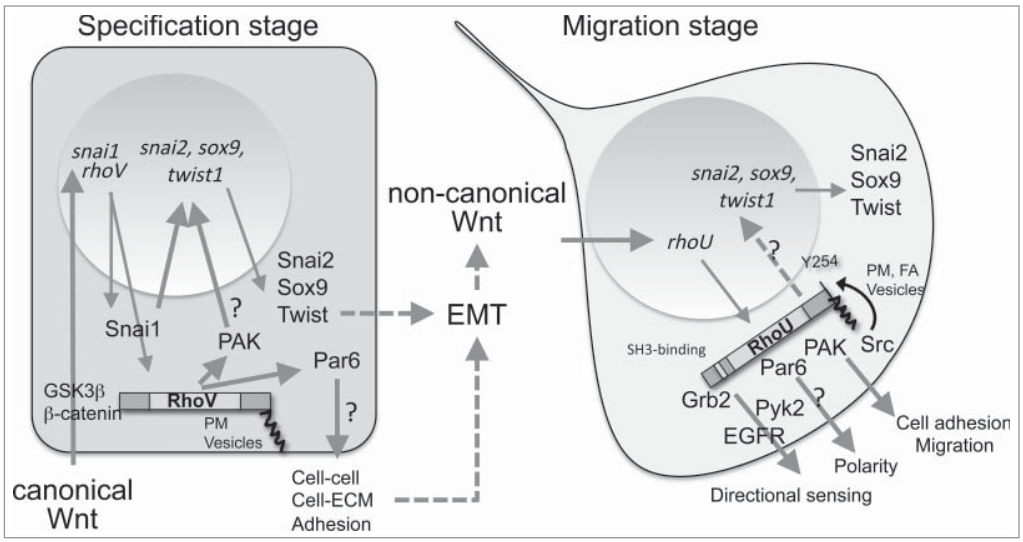

Figure 1. Demonstrated and putative roles of RhoV and RhoU in the developing neural crest. At specification stage, RhoV is induced early by the Wnt canonical pathway (*via* GSK3β/β−catenin). RhoV exhibits N-terminal and C-terminal extensions (dark gray boxes) required for its activity and are located through palmitoylation at the plasma membrane (PM) and vesicles. RhoV and Snai1 cooperate for the induction of *snai2, sox9* and *twist*, required for EMT. This may be mediated through enhanced PAK activity, shown to phosphorylate and enhance Snai1 transcriptional activity. RhoV might also disrupt NC cell adhesion through Par6 activation. During EMT, RhoV expression is shutdown while the non-canonical Wnt pathway induces RhoU. RhoU also exhibits N-terminal and C-terminal extensions, which contain an SH3-binding domain (white box) and a tyrosine phosphorylated by Src (Y254). RhoU is located at PM, vesicles and focal adhesions (FA). RhoU is involved in NC cell adhesion and migration in a PAK-dependent pathway. RhoU is also required for NC polarity - potentially through its interaction with Par6 - and may participate in directional migration by regulating focal adhesion turnover through interaction with Grb2 or Pyk2 and through phosphorylation by Src. Question marks indicate pathways or interactions not yet validated in NC.

The sequential expression of the 2 GTPases may therefore be envisioned as follows (**Fig. 1**): As a canonical Wnt response gene, RhoV cooperates with Snai1 in the induction of NC-specific markers and is probably responsible for disrupting epithelial junctions and modifying cell polarity, potentially through its binding to Par6, as proposed for RhoU in MDCK cells.[36] Disruption of cell-cell contacts might then activate the non-canonical Wnt pathway[37] and therefore RhoU expression, which in turn could promote polarized cell migration through its SH3-binding domain.

Concluding Remarks

In conclusion, functional analysis of RhoV and RhoU in the Xenopus embryo has revealed their specific roles during development of the neural crest. Although the 'big 3' GTPases (RhoA, Rac1 and Cdc42) have already been implicated in Wnt signaling, mostly in non-canonical pathways,[38,39] recent literature showed that the conditional invalidation of Rac1 or Cdc42 in mouse NC only induced mitotic and survival defects in post-migratory NC cells. This excludes a role for Rac1 and Cdc42 at early stages of NC development, i.e. in the specification, EMT and migration stages.[40,41] This further emphasizes the unique roles of RhoV and RhoU in the high dynamics of this embryonic tissue. Moreover, due to their sensitivity to canonical and non-canonical Wnt pathways, these 2 GTPases might well take a significant contribution in Wnt-related pathologies, in particular tumorigenesis.[42]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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