

Roles of RhoC in cancer

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Synonyms

Ras homologue gene family, member C; ARH9; ARHC

Summary

RhoC is a member of the Rho GTPase family that is very closely related to RhoA and RhoB. It is best known for its role in cancer cell migration, invasion and metastasis. Although RhoC was originally considered to act similarly to RhoA and share the same partners and functions, there is now good evidence that RhoC has unique functions in cancer cells and is probably regulated by specific partners. A RhoC biosensor has demonstrated that RhoC is active in invadopodia, which degrade extracellular matrix, in cancer cells. Unlike RhoA, RhoC is not essential for mouse development, and RhoC-null mice appear phenotypically normal, even though RhoC is widely expressed. However, in mouse models, RhoC has distinct functions to RhoA in cancer metastasis. RhoC is upregulated in many types of human cancer and it contributes to cancer progression and metastasis formation in mouse models. There is also evidence that it stimulates cancer proliferation and resistance to chemotherapy in cultured cancer cells. It is useful as a prognostic marker in many types of cancer and could be a target in cancer therapy.

Background

RhoC was originally identified, together with its homologues RhoA and RhoB, as a Ras-related small GTPase (Madaule and Axel 1985). RhoA, RhoB and RhoC together comprise the Rho subfamily of small GTPases characterized by their high homology within the Rho GTPase family. The human *RHOC* gene is on chromosome 1p13.1-p21. The *RHOC* gene is proposed to originate from a duplication of *RHOA* during evolution (Boueux et al. 2007).

RhoA and RhoC are 93% identical at the protein level; the divergence is mainly concentrated in the so called hypervariable region of the proteins at the C-terminus. Although multiple Rho subfamily GTPases exist in many eukaryotic organisms, specific RhoC orthologues do not exist outside vertebrates (Boueux et al. 2007). It was originally described to regulate reorganization of the actin cytoskeleton and cell shape, attachment, and motility in a similar way to RhoA. The focus of attention shifted when it was discovered that RhoC but not RhoA specifically contributes to melanoma metastasis (Clark et al. 2000). Since then many studies have investigated the different functions of RhoA, RhoB and RhoC in cell biology and cancer.

Tools for manipulating RhoC function

Classic tools for the study of Rho GTPases, including RhoC, took advantage of some conserved amino acids essential for GTP hydrolysis to make dominant negative (T19N substitution) or constitutively active (G14V or Q63L substitutions) forms of the protein (Wheeler and Ridley 2004). However, these mutations have the disadvantage that they probably do not allow the functional specificity of closely related Rho GTPases like RhoA and RhoC to be explored. Bacterial toxins, like *Clostridium Botulinum* exoenzyme C3 transferase or Toxin B, efficiently inhibit Rho activity by covalently modifying the protein on

key residues, but again they target all Rho subfamily proteins and, although they were central to establishing the role of these proteins in actin cytoskeletal organization, they can not be used to study RhoC-specific functions. RNA interference (RNAi) has been used to differentiate the functions for RhoA, RhoB and RhoC in cells. This has shown that RhoA and RhoC regulate cytoskeletal dynamics, cell morphology, migration and invasion in different ways (Bellovin et al. 2006; Wu et al. 2010; Bravo-Cordero et al. 2011; Vega et al. 2011). Spatio-temporal regulation of Rho GTPase activity is crucial to cell migration and invasion. The combined use of live-cell imaging and fluorescence biosensors is helping to decipher how the activity of RhoC and other Rho GTPases is tightly regulated on a subcellular level. For example, using a RhoC –specific biosensor in breast cancer cells, dynamic RhoC activation in invadopodia has been visualized (Bravo-Cordero et al. 2011). In addition, RhoC is active in protruding lamellipodia, but further away from the edge compared to RhoA (Zawistowski et al. 2013).

Regulation of RhoC activity

RhoC is a GTPase that act as a molecular switch, cycling between a GDP-bound inactive state and a GTP-bound active state, When active it interacts with effector proteins to regulate a variety of different processes (Wheeler and Ridley 2004). Its activation is positively regulated by guanine nucleotide exchange factors (GEFs), which stimulate the exchange of GDP for GTP on the protein, and negatively regulated by GTPase-activating proteins (GAPs), which stimulate its intrinsic GTPase activity. RhoC is also post-translationally modified at the C-terminus by prenylation with the addition of a geranylgeranyl group. This modification allows its anchorage to membranes and is likely to be essential for its biological function. Similar to several other Rho GTPases, RhoC associates with Rho GTPase

dissociation inhibitor proteins (RhoGDIs) which sequester the protein in the cytoplasm by interacting with the C-terminal geranylgeranyl group. These modes of regulation are shared with the other Rho proteins, RhoA and RhoB. The different functions of RhoC compared to RhoA and RhoB are thought to be achieved through RhoC-specific GEFs and/or GAPs and by its binding to specific effector proteins.

Most regulators of Rho subfamily proteins have only been tested on RhoA, and based on the sequence similarity with RhoC would be predicted to act on both proteins. The ones that have been specifically tested on RhoC are the GEFs Tim and Scambio, the GAPs p190RhoGAP, GRAF, p50RhoGAP and Myr5 and the 3 RhoGDI proteins (Bos et al. 2007). Few regulators have been compared for their activity on RhoA versus RhoC. One of the few examples of a specific regulator comes from studies of the RhoGEF ARHGEF3 (also known as XPLN), which acts on RhoA and RhoB but not RhoC, but no RhoC-selective GEFs or GAPs have been described so far (Arthur et al. 2002; Bos et al. 2007). A report described enhanced RhoC activity compared to RhoA in pancreatic carcinoma cells which correlated with an increase of RhoC membrane localization (Dietrich et al. 2009); this might reflect differential regulation of Rho isoform interaction with RhoGDIs.

Some Rho proteins are subject to post-translational modifications that regulate their stability or activity. RhoC can be phosphorylated by Akt and this phosphorylation is required for inflammatory breast cancer cell invasion (Lehman et al. 2012). RhoA is also ubiquitinated and thereby targeted for proteasomal degradation, but RhoC has not so far been reported to be ubiquitinated (Chen et al. 2009).

Regulation of RhoC expression

RhoC expression is widely expressed but its levels are variable between different tissues (GTEx consortium website, 53 human tissue sites; accessed 26/06/2016). Increased RhoC expression has been reported in a variety of more aggressive metastatic cancers, and is considered a marker for cancer progression. A recent example is acute myeloid leukaemia, in which RhoC was one of seven genes for which lower expression correlated with better outcome, and hence could be used to guide treatment (Marcucci et al. 2014).

RhoC expression and activity can be regulated in cancer cells is by the action of a variety of different microRNAs. In breast cancer, for example, the overexpression of the microRNA miRNA-10b induces the upregulation of RhoC by inhibiting the translation of the messenger RNA encoding HOXD10, and this in turn promotes invasion and metastasis (Ma et al. 2007). In squamous cell carcinoma, the downregulation of miRNA-138 induces metastasis by reducing direct RhoC mRNA degradation (Jiang et al. 2010). miRNA-93 also downregulates RhoC, and suppresses tumour growth and RhoC expression in an ovarian tumour xenograft model (Chen et al. 2015).

RhoC effectors

Because RhoA, B and C possess a high level of identity at the protein level, they share many common downstream effectors (Wheeler and Ridley 2004). The affinity of these interactions may vary due to their amino acid sequence differences. These interactions all involve the Rho switch I and II regions (see Figure 1) and Rho-binding domains (RBDs) of their effectors, which include Rho-associated kinase (ROCK-1 and-2), Protein kinase N (PKN1-3, also known as PRKs), Citron kinase, mDia1-3, Rhotekin and RhoGDI-1. RhoC and RhoA also interact with Phospholipase C ϵ (PLC- ϵ) via its catalytic core. Whether RhoC acts through

any of these targets specifically to regulate cancer progression is not clear. RhoC but not RhoA has been reported to bind to the Formin-like family members FMNL2 and FMNL3, which might contribute to RhoC function in cancer cell migration (Kitzing et al. 2010; Vega et al. 2011). RhoC also binds to IQGAP1, and this interaction is involved in gastric cancer cell migration (Wu et al. 2011).

RhoC has no known physiological function

The function of RhoC in humans is not known, and there are no known human phenotypes associated with RhoC variants (EBI Genetic Variation website; accessed 26/06/2016). Mice lacking RhoC have no detectable phenotype (Hakem et al. 2005). Notably, RhoC expression is not detectable in mouse macrophages (Königs et al. 2014), and RhoC does not have any effect on T- or B-cells in mice, nor does it affect mouse neutrophil migration (Hakem et al., 2005). This implies that targeting RhoC will not have a significant impact on the immune system although further immune cell types need to be analysed. RhoC-null mice also have no defect in osteoclast function or bone development (Charles et al. 2012).

RhoC functions in tumorigenesis

Formation of metastases

RhoC expression and activity is often increased in cancer and correlates with progression, metastasis formation and therefore a poor prognosis for patients (Vega and Ridley 2008). Increased RhoC expression in cancer was first identified in a screen for genes upregulated in melanoma metastases (Clark et al. 2000). RhoC expression was subsequently found to be upregulated in a variety of cancers including prostate cancer, breast cancer, gastric cancer, ovarian cancer, bladder cancer, hepatocellular cancer, pancreatic ductal adenocarcinoma,

non-small cell lung carcinoma (NLCLC), oesophageal squamous cell carcinoma, head and neck squamous cell carcinoma and skin squamous cell carcinoma (Karlsson et al. 2009). RhoC has been shown to play a causal role in metastasis in animal models. Initial studies found that over-expression of dominant and negative forms of RhoC correlated with the formation or the inhibition of experimental lung metastases, respectively (Clark et al. 2000). Subsequently it was shown using RhoC-null mice that RhoC was dispensable for breast cancer initiation and growth but confirmed that RhoC is critical for formation of metastases (Hakem et al. 2005). The inhibition of RhoC has since been described to reduce cancer cell invasion and metastasis in many in vitro and in vivo cancer models. Recent additions to the list include cholangiocellular carcinoma, in which RhoC is upregulated and is important for invasion in vitro in part through inducing matrix metalloprotease expression (Yang et al. 2016). RhoC is now proposed to be a marker for poor prognosis in many different cancers. Mutations of RhoC in cancer are very rare: the COSMIC database reports only 29 missense mutations and 5 deletions leading to frameshifts (accessed 26/06/2016). These mutations are scattered across the RhoC protein, indicating they are likely to be mostly passenger mutations. By contrast, RhoA is frequently mutated at a specific residue in angioimmunoblastic T cell lymphoma and at other residues in a variety of other lymphomas, as well as in stomach cancer (Chiba et al. 2015; Lin et al. 2015). Rare mutations have also been reported in the RhoA and RhoC downstream effector ROCK-1 (Lochhead et al. 2010).

Migration and invasion

RhoC has a unique role in cell migration, distinct from RhoA, which could underlie its specific contribution to cancer cell invasion and metastasis. For example, RhoC expression is increased during colon carcinoma cell epithelial-mesenchymal transition (EMT) and regulates EMT-induced migration (Bellovin et al. 2006), as well as TGF β -induced cervical

cancer EMT (He et al. 2015). RhoC mediates EGF-induced E-cadherin downregulation (a marker of EMT) in head and neck cancer (Tumur et al. 2015). Since EMT often occurs during epithelial cancer invasion, these results imply a causal role for RhoC in promoting cancer progression.

RhoC promotes polarized cell migration and invasion by controlling cell spreading and Rac1 activation around the cell periphery hence restricting lamellipodial broadening (Vega et al. 2011). RhoC regulates breast cancer cell adhesion to the extracellular matrix and motility and invasion by modulating the expression and co-localization of $\alpha 2$ and $\beta 1$ integrins on collagen I (Wu et al. 2011). RhoC is also implicated in the degradation of extracellular matrix as it is involved in the formation of matrix-degrading invadopodia in cancer cells: an active ring of RhoC restricts Cofilin activity and focuses invadopodial protrusion and matrix degradation (Bravo-Cordero et al. 2011). In addition, RhoC coordinates prostate cancer cell invasion in vitro by activating the protein kinases Pyk2, FAK, MAPK and AKT, which results in activation of the matrix-degrading metalloproteinases 2 and 9 (MMP2 and MMP9) (Iizumi et al. 2008). RhoC is also involved in the transcriptional program that controls the TGF β 1-induced switch from cohesive to single cell motility in breast cancer cells (Giampieri et al. 2009).

Angiogenesis and endothelial cells

RhoC can stimulate the production of pro-angiogenic factors by breast cancer cells (Merajver and Usmani 2005). RhoC is a downstream effector of vascular endothelial growth factor (VEGF) in endothelial cells and cancer cells. RhoC is thus essential for VEGF-mediated angiogenesis induced by hepatocellular carcinoma cells (Wang et al. 2008). RhoC is rapidly activated by VEGF and mediates VEGF-induced proliferation of human endothelial cells in vitro, although RhoC depletion inhibited migration (Hoeppner et al. 2015). These pro-tumoral

functions could potentiate the vascularisation of tumours that express RhoC and also may facilitate cancer cell intravasation and extravasation during tumour metastasis. Particularly relevant in this context is that RhoC in prostate cancer cells facilitates their attachment to endothelial cells in vitro and in vivo, and RhoC contributes to lung metastasis in an experimental metastasis model in vivo (Reymond et al. 2015).

Proliferation and apoptosis resistance

As well as inducing cancer cell invasion, RhoC often affects cancer cell proliferation. For example, RhoC depletion in human gastric carcinoma cells was reported to inhibit proliferation and increase apoptosis in vitro (Sun et al. 2007); and RhoC promoted human oesophageal squamous cell carcinoma and breast cancer cell proliferation in mice in vivo (Faried et al. 2006). Recent examples in which RhoC promotes proliferation include bladder cancer cells (Griner et al. 2015) and gastric cancer cells (Wu et al. 2012). On the other hand, RNAi-mediated suppression of RhoC in hepatocellular carcinoma cells showed that RhoC does not regulate cancer cell proliferation in mice and that depletion of RhoC in endothelial cells does not affect their apoptosis (Wang et al. 2008).

Chemotherapeutic resistance

Recent data indicate that RhoC can contribute to resistance to chemotherapies, at least in cultured cancer cell lines in vitro. For example, RhoC protein levels were higher in an etoposide-resistant lung cancer cell line than the non-resistant control, and RhoC contributed to etoposide resistance (Paul et al. 2016). RhoC expression was increased in human breast cancer samples following chemotherapy and was induced by etoposide in MCF7 breast cancer cells in vitro (Kawata et al. 2014). RhoC has been shown to be a direct target of the

transcription factor p53 in response to the chemotherapeutic agents doxyrubicin or cisplatin (Croft et al. 2011).

RhoC, transcription factors and cancer

RhoA is well known to regulate transcription through actin-dependent and actin-independent effects on a variety of transcription factors (Jaffe and Hall 2005). Recent evidence indicates that RhoC also plays a role in transcriptional regulation. RhoC is induced in melanoma cells by the transcriptional regulator ETS-1. RhoC then indirectly stabilizes the AP-1 family transcription factor c-Jun through the actin cytoskeleton (Spangler et al. 2011). C-Jun is an oncogene which is a critical mediator of tumour development.

Some Rho proteins are subject to post-translational modifications that regulate their stability or activity. RhoC can be phosphorylated by Akt and this phosphorylation is required for inflammatory breast cancer cell invasion (Lehman et al. 2012). RhoA is also ubiquitinated and thereby targeted for proteasomal degradation, but RhoC has not so far been reported to be ubiquitinated (Chen et al. 2009).

Conclusions

RhoC has the potential to be a target for therapeutic intervention in cancer and have few side effects, given that it does not appear to affect immune cell function or osteoclasts in the bone. In addition, because RhoC is very rarely mutated in human cancers, it is unlikely that clones resistant to RhoC inhibitors will be selected for during treatment because of RhoC mutation. As well as its well-defined role in metastasis, RhoC has now been implicated in chemotherapy resistance and hence RhoC inhibitors could potentially be combined with chemotherapy to reduce resistance, although this still needs to be tested using in vivo models.

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Figure 1

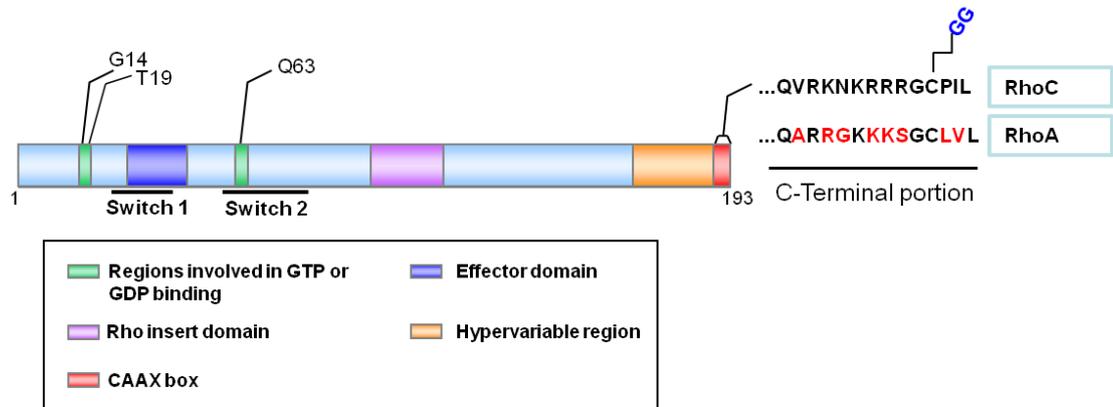


Figure 1. RhoC domain structure and features. Schematic of RhoC protein showing the different domains and residues important for its activity and used to create dominant negative and constitutively active mutations as described in the text. The C-terminal 14 amino acids including the CAAX box and comparison with the equivalent region of RhoA is shown. Red amino acids indicate non-conserved residues. GG depicts the prenylation site; following geranylgeranylation the last 3 amino acids are cleaved off.

Figure 2

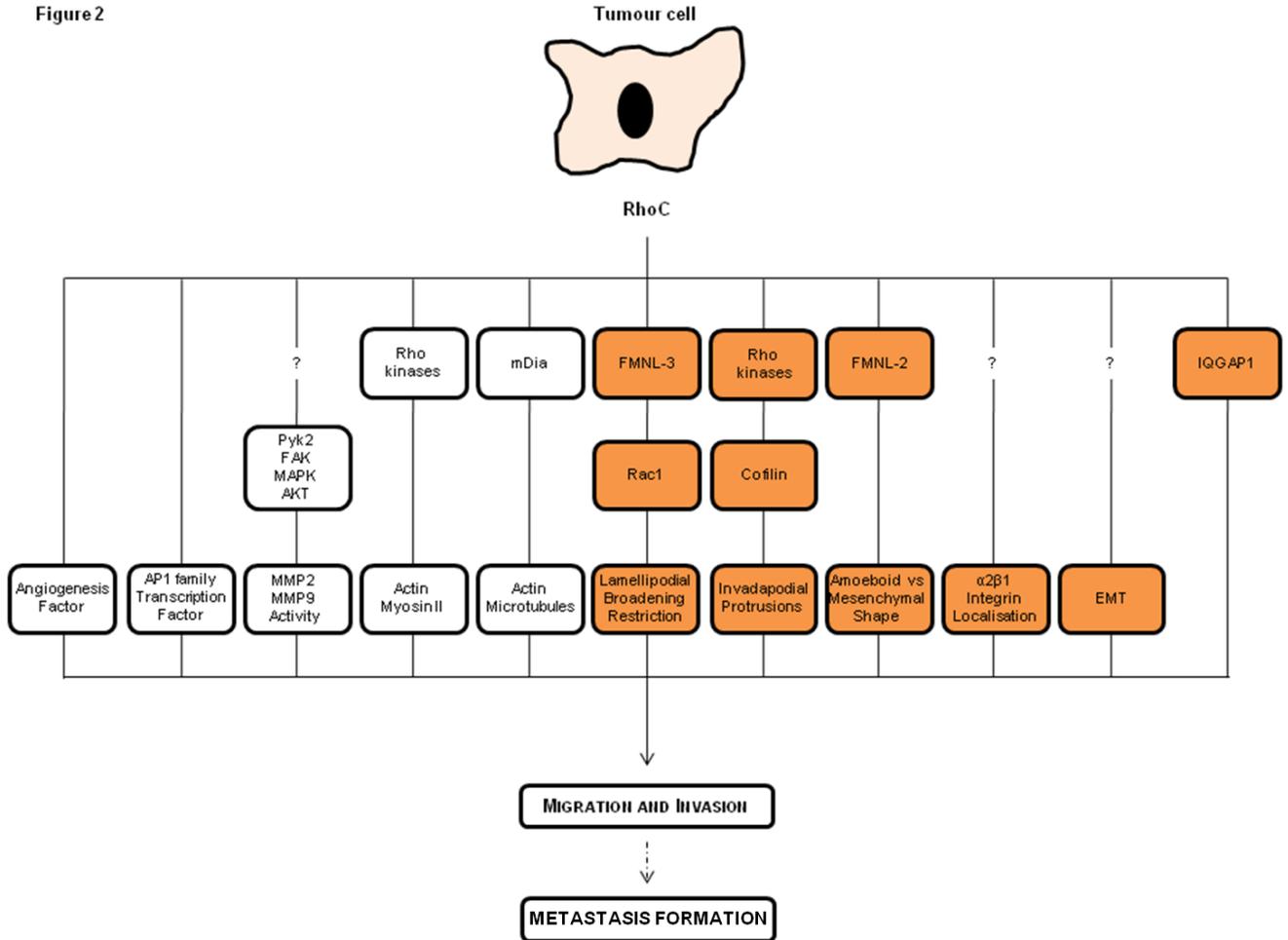


Figure 2. RhoC functions in cancer. Schematic of RhoC protein showing the different functions of RhoC in cancer and its downstream effectors. Non-coloured functions and interactors are shared with RhoA while orange-coloured functions and interactors are RhoC-specific. Note the arrow between migration/invasion and metastasis formation is a dotted line because not all of the RhoC effectors have been tested in vivo.