Chapter 11

GENETIC ANALYSIS OF RHO PROTEIN FUNCTION IN MICE

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Abstract: The Rho small GTPases regulate a variety of cellular functions, including proliferation, differentiation, vesicle trafficking, gene expression, adhesion, and motility. Among their well established essential roles is that of organizing the actin cytoskeleton, and this aspect of Rho GTPase function has led to the identification of important roles for various Rho family GTPases in a variety of actin-dependent cellular processes, including cell shape change, adhesion, and migration. As critical regulators of these actin-mediated processes, the Rho GTPases also function as essential regulators of many developmental processes. The morphogenesis of tissues in all developing multi-cellular organisms requires precise changes in cell shape and cell movements that depend on the various Rho GTPases. This has been observed in studies using sevefigureral different developmental model systems, including flies, worms, frogs, and mice. Here, we review the literature reporting functions for Rho GTPases and their associated signaling components in the context of embryonic development, adult physiology, and pathogenesis. In particular, the focus of the studies described here is on the mouse system, where transgenic and "knockout" strategies have played an especially important role in elucidating the in vivo organization and function of the various Rho signaling pathways.

1. INTRODUCTION

The Rho family of small GTPases appears to mediate nearly all of the major cellular functions. These include proliferation, differentiation, vesicle trafficking, gene expression, adhesion, and motility (Etienne-Manneville and Hall, 2002). Among their well established essential roles is that of organizing the actin cytoskeleton, and this aspect of Rho GTPase function has led to the identification of important roles for various Rho family

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GTPases in a variety of actin-dependent cellular processes, including cell shape change, adhesion, and migration (Nobes and Hall, 1999). As critical regulators of these actin-mediated processes, it was not surprising to find that the Rho GTPases are essential regulators of many developmental processes as well. The morphogenesis of tissues in all developing multicellular organisms requires very precise changes in cell shape and cell movements that are undoubtedly dependent on the various Rho GTPases. This has indeed been confirmed in studies using several different developmental model systems, including flies, worms, frogs, and mice (Settleman, 1999). In other chapters in this volume, the signaling role of Rho GTPases is addressed largely in the context of cell culture systems, where much has been learned about the biochemical organization of Rho-mediated signaling pathways. In this chapter, we review the literature reporting functions for Rho GTPases and their associated signaling components in the context of embryonic development, adult physiology, and pathogenesis. In particular, the focus of the studies described here is on the mouse system, where transgenic and "knockout" strategies have played an especially important role in elucidating the in vivo organization and function of the various Rho signaling pathways.

2. REGULATORS AND TARGETS OF THE RHO GTPASES

As detailed in Chapter 3, the cycling of Rho GTPases, like that of other small GTPases, is largely controlled by three classes of regulatory proteins. These are the GAPs, the GEFs, and the GDIs. We will summarize studies of each of these various GTPase regulators in the context of mice. In addition to the regulators of the GTPases, the downstream targets of the GTPases, or effector proteins, play an essential role in mediating the actions of activated Rho proteins that ultimately elicit a biological response. The established signaling roles for these proteins, which typically interact with the GTP-bound, activated form of the GTPases, are also described in other chapters of this volume, and in this chapter, we will summarize findings from mouse studies that shed light on the role of these various Rho effectors in the context of the whole organism. Although a variety of other proteins appear to contribute less directly to the regulation and function of the Rho GTPases, in the interest of space, these will not be considered here.

2.1 Model organism systems for studying Rho GTPase function

Cell culture and biochemical studies have revealed much of what we now understand regarding the regulation and function of the various Rho-mediated signaling pathways. For example, the early micro-injection studies by Ridley and Hall provided important information regarding the role of the Rho family proteins as regulators of the actin cytoskeleton in cultured fibroblasts (Ridley and Hall, 1992a; Ridley and Hall, 1992b; Ridley et al., 1992). Additional biochemistry-oriented studies led to the identification of several direct binding partners for the activated Rho family GTPases, including p21-activated kinases (PAKs), Rho-kinases (ROCK), and the PKN family proteins (Burridge and Wennerberg, 2004). Thus, the power of these approaches has been clearly demonstrated. However, it can be argued that it is a considerably more difficult challenge to establish the regulatory mechanisms and functional requirements for the various Rho pathways in the context of whole organisms. These days, several experimental model systems, including Drosophila melanogaster, Xenopus laevis, C. elegans, and mice, are routinely used for such analysis. Each model provides a distinct set of advantages, and all share the property that their genomes encode several highly conserved Rho family GTPases as well as their regulators and targets. Thus, it should be possible to readily extrapolate findings from these relatively simple organisms to humans. Moreover, as complete annotated genome sequences are becoming available for each of these organisms, the power of a genetic approach to address the function of individual proteins in whole organisms has become even greater. We refer to previous review articles that have summarized the analysis of Rho GTPase function in several of these systems (Settleman, 2001; Settleman and Barrett, 2001), and here, we will focus exclusively on the use of the mouse system

2.1.1 Mouse models of Rho GTPase regulation and function

As a model system for studying the developmental function of particular genes, the mouse is particularly attractive because of its high degree of evolutionary relatedness to humans. The major aspects of embryogenesis, including fertilization, blastocyst development, embryo implantation, gastrulation, and organogenesis are remarkably similar in mice and humans. Moreover, all of the Rho family GTPases and their protein regulators and targets that have been identified in humans appear to have highly related orthologs in mice. Thus, while the use of classical genetic analysis is substantially more laborious in the mouse when compared to fruit flies and worms, the mouse provides a powerful model system in which the

developmental function of Rho-mediated signaling pathways can be examined.

Two methodologies account for the vast majority of the in vivo analysis of Rho signaling in the mouse. These are the introduction of transgenes under the control of tissue-restricted or inducible promoters, and the use of gene targeting in embryonic stem cells to disrupt particular genes of interest. As described below, the application of these methodologies to the analysis of Rho regulation and function in development has yielded a wealth of information regarding the organization of Rho pathways in vivo and their role in tissue morphogenesis during development. Moreover, mouse models are beginning to provide additional insights into the post-developmental role of Rho GTPase signaling in the physiology of the adult animal and, in some cases, in pathogenesis.

2.2 Expression studies of Rho GTPase pathway components in mice

When considering the roles for Rho GTPase signaling components in the context of a developing organism, it can be useful to determine the expression profile of a gene of interest in embryos. This is typically done using in situ hybridization of mRNA in intact tissues (whole-mounts) or tissue sections prepared on slides. It is reasonable to assume that detectable expression of a gene in a particular tissue correlates, to a first approximation, with a likely function for the gene product in that tissue. Of course, mRNA levels do not necessarily accurately predict protein expression levels. Moreover, levels of expression of a particular gene that do not exceed the threshold for detection may still be significant. Finally, a gene product does not necessarily play a significant role in a tissue where it is expressed, and redundant function provided by closely related family members is a considerable issue as well. Thus, expression analysis can loosely direct the focus of attention to a particular tissue type or a particular developmental stage, but this will rarely yield any definitive conclusions. For some pathway components, antibodies may be available that can be used for immunostaining of tissues directly. In mice, the analysis of embryonic expression of Rho pathway components has been rather limited, and overall, and has only provided some hints about developmental functions for Rho family GTPases and their numerous associated regulators and targets.

There are at least 20 GTPases that are classified as Rho family GTPases on the basis of primary structure relatedness (see Chapter 2). Of these, he expression of only a few has been examined during mouse evelopment. The expression of the RhoA GTPase in developing embryos has only been reported in the context of kidney development, where

immunohistochemistry revealed that RhoA was largely restricted to the mesonephric ducts and vesicles, and the periglomerular tubules, which exhibit intense staining (Bianchi et al., 2003). This finding suggest a potential role for RhoA in the epithelial-mesenchymal induction process required for kidney morphogenesis, and is consistent with additional studies that support a role for Rho GTPases in the epithelial-mesenchymal transition (Zondag et al., 2000). RhoB gene expression in mouse embryos has been examined in great detail, and it appears to be expressed widely throughout development (Henderson et al., 2000). Notably, as described below, RhoB knockout mice develop normally, highlighting the limited utility of expression analysis in predicting the developmental requirement for a gene of interest. Expression analysis of Rac1 revealed prominent widespread expression in very early embryos, a finding which, as described below, is consistent with its apparent role in early gastrulation (Moll et al., 1991). Cdc42 expression during development has not been characterized in detail, although the mRNA has been detected at very early stages of embryogenesis (Salas-Vidal and Lomeli, 2004), and as described later, this is consistent with a very early developmental requirement for Cdc42. The Rac-like RhoG gene has been examined in the developing brain. RhoG, which had been implicated in neurite outgrowth in cell culture studies (Katoh et al., 2000), is expressed throughout the ventricular zone in late neurogenesis, suggesting a role in newly generated neurons (Ishikawa et al., 2002). Postnatally, RhoG mRNA expression is enriched in white matter tracts of the corpus callosum, anterior commissure, and cerebellum, and this was determined to represent expression in oligodendrocytes (Ishikawa et al., 2002). Thus, RhoG may play a role in both glial and neuronal cells of the central nervous system.

The expression of relatively few of the numerous GTPase regulators has been examined in developing mice. One of these is FGD1, a GEF that specifically activates the Cdc42 protein, and which has been identified as the primary gene defect associated with faciogenital dysplasis, or Aarskog Syndrome (Gorski et al., 2000; Olson et al., 1996; Pasteris and Gorski, 1999). Notably, this congenital syndrome in humans is associated with defects in skeletal development, suggesting a likely function in developing bone. The mouse FGD1 ortholog was found to be first expressed during the onset of ossification, and is expressed in areas of active long bone formation (Gorski et al., 2000). Expression is also seen in osteoblasts at the onset of matrix mineralization, suggesting a role in differentiation of bone. Postnatally, FGD1 exhibits a broader expression pattern in skeletal tissues, suggesting that it regulates distinct Cdc42-mediated processes in bone development and in the mature skeleton. A more comprehensive analysis of the expression profiles of several GEFs for the Rho GTPases during mouse brain development has been reported, which indicates a complex expression

profile for this class of GTPase activators within the developing brain, and indicates the likelihood that particular GEFs may play unique functions in a subset of neural tissues (Yoshizawa et al., 2003).

Expression of another RhoGEF, LARG (leukemia-associated Rho GEF), has been examined in some detail in the context of developing mice (Kuner et al., 2002). LARG is first detected at embryonic day 14, and expression is seen in skin, intestinal epithelium, and smooth muscle layers of the bronchi, vasculature, and intestine. This specific pattern of LARG expression is maintained through development and persists in the adult animal, indicating a potential role for LARG both in the development and physiological function of these tissues. Interestingly, LARG was first identified as a translocation partner in a human myeloid leukemia, suggesting that the leukemic cells have usurped LARG to activate Rho inappropriately. LARG has previously been found to interact with the IGF-1 receptor, indicating a likely role in mediating the activation of Rho by IGF-1. In the expression study, LARG was found to co-localize with the IGF-1 receptor, indicating that LARG may specifically mediate the ability of IGF-1 to activate Rho in a subset of tissues that require IGF-1 for their proper development.

The RhoGAP, p190-A, is expressed predominantly in the developing central nervous system, and as detailed in a later section, mice lacking p190-A RhoGAP exhibit substantial defects in several aspects of neural development (Brouns et al., 2001).

Among the various Rho effector targets, relatively little detailed developmental expression analysis has been reported. The ROCKs exhibit prominent expression in developing cardiac tissues, which, as described below, is consistent with an apparent role for ROCKs in cardiac development (Nakagawa et al., 1996). Others have been detected in developing tissues at various embryonic stages using RT-PCR methodology, and we refer to an online database that provides substantial information regarding the developmental expression profile for several additional genes that encode Rho pathway components (web site adress: tbase.jax.org). Many more gene expression studies that have relied largely on northern blotting and western blotting of adult tissues have revealed tissue-restricted expression profiles for various regulators and targets of Rho GTPases. Collectively, these studies have not shed much light on likely developmental roles for individual pathway components, but rather, have reinforced the notion that while the GTPases themselves seem to be relatively broadly expressed, their regulators exhibit somewhat more restricted tissue expression patterns. This potentially provides a mechanism by which extracellular signals can be linked to the Rho GTPases via distinct sets of regulatory proteins depending on the tissue context. One notable exception to this is the Rac2 protein, which is largely restricted in its expression to

hematopoietic cells (Yu et al., 2001), and as described below, is specifically required in blood cells.

2.3 Rho GTPases in fertilization, preimplantaion, and early morphogenesis

Mammalian development is initiated with the fertilization of the egg by sperm, a process that has previously been shown to depend on actin polymerization and cytoskeletal rearrangement (Webster and McGaughey, 1990). Although a genetic analysis of this process in mice is technically challenging, the role of Rho GTPases in sperm-egg interaction has been examined using one of the Rho-inactivating toxins, Clostridium difficile toxin B (Kumakiri et al., 2003). Toxin B inhibits several of the Rho family GTPases, and treatment of eggs with toxin B results in a substantial inhibition of sperm fusion or sperm nucleus decondensation in the ooplasm. Rac1 and RhoB, but not Cdc42, are detected in ooplasm, suggesting that these GTPases may mediate the actin rearrangements required for fertilization.

Following fertilization, the mouse egg undergoes several cell divisions during its 4-5 day migration to the uterus. There, the multi-cellular blastocyst breaks free of its protective membrane, the zona pellucida, and implants itself within the uterine wall. The Rho GTPases have been implicated in the preimplantation mouse embryo. The ability to maintain preimplantation embryos in culture for several days facilitates the analysis of this early stage of development. Expression analysis revealed that Rac and Cdc42 genes are expressed in the preimplantation embryo, and immunostaining revealed that Rac1 protein translocates from plasma membrane to cytoplasm as this early embryo undergoes cleavage at the blastocyst stage (Natale and Watson, 2002). Possibly, these GTPases are required to mediate the turnover of adherens junctions, which are known to play an important role in the cell-cell adhesions that mediate blastocyst formation (Ohsugi et al., 1997).

Use of the Rho-inactivating Clostridium botulinum C3 toxin has also pointed to a role for Rho in preimplantation development (Clayton et al., 1999). Injection of the 4-cell blastomere with C3 disrupts cortical actin microfilament organization and prevents the requisite polarization of the 8-cell blastomere. This polarization process, referred to as compaction, requires both actin and microtubule reorganization, both of which are Rho-mediated. Thus, the ability of Rho to regulate the cytoskeleton, appears to be required for blastomere compaction. Notably, early cell divisions proceed normally in the presence of C3.

Mice containing a targeted disruption of the Cdc42 gene have been reported (Chen et al., 2000). Although Cdc42-deficient embryonic stem cells proliferate normally, homozygous mutant embryos fail some time before embryonic day E7.5, and exhibit a disorganized primary ectoderm and signs of degeneration by E5.5. In vitro analysis indicated that the Cdc42-deficient cells fail to support phosphatidylinositol 4,5-bisphosphate-induced actin assembly, suggesting a role for Cdc42 in regulating the actin cytoskeleton during preimplantation development. It is important to note that in such genetic studies of early embryo development, it is possible that some early phenotypes may be masked in homozygous mutant animals due to the contribution of maternal gene products. Thus, a disrupted gene may encode a protein that is required prior to the first signs of a defect.

Disruption of the Rac1 gene in mice also results in relatively early lethality (Sugihara et al., 1998). Mutant embryos collected between E6.5 and E8.5 exhibit clear defects in the process of gastulation. Gastrulation is the first major morphogenetic process in the implanted developing embryo, and ultimately gives rise to the three germ layers. This process is known to require precise cell shape changes, and regulated cell adhesion and cell migration. Thus, it was not surprising to find a role for Rho GTPases in gastrulation. In the absence of Rac1, gastrulating tissue appears misfolded, and numerous apoptotic cells are detected in early embryonic tissues. Rac1 gene expression is widely detected in E7.5 embryos, but the precise role of Rac1 in gastrulating tissues remains to be determined. Moreover, the identity of Rho GTPase regulators or effector targets that are required for gastrulation in early eambryos is unknown. Significantly, in the Drosophila system, a specific RhoGEF has been identified that regulates Rho-mediated cell shapes required for gastrulation (Barrett et al., 1997), and this GEF is regulated by heterotrimeric G-proteins (Hacker and Perrimon, 1998). In mammals, three related RhoGEFS, PDZ-RhoGEF, LARG, and p115 RhoGEF appear to function similarly in their ability to couple G-protein coupled receptor activation to Rho activation (Chikumi et al., 2004). Thus, it is possible that these RhoGEFs perform an evolutionarily conserved function in directing Rho-mediated cell shape changes in mammalian gastrulation.

2.4 Germ cell development

Expression studies have pointed to a likely role for Rho signaling in the development of male germ cells. For example, RhoB immunostaining indicated its expression in elongating spermatids, spermatocytes, and Sertoli cells (Lui et al., 2003). In addition, the RacGAP, MgcRacGAP, is known to be highly enriched in male germ cells, pointing to a likely specialized function for Rac in germ cell development (Naud et al., 2003). Several

mouse knockout studies have confirmed a role for Rho signaling in spermatogenesis. Specific disruption of the Rho-binding kinase, Citron, results in profound testicular impairment and a complete absence of mature spermatocytes. Cells exhibit a severe cytokinesis defect, and excessive apoptosis is detected. This defect appears to reflect the previously identified role for Citron in Rho-directed cytokinesis (Cunto et al., 2002). Notably, germ cell development in Citron-deficient females proceeds normally. Mice lacking Lim-kinase-2, one of two closely related Lim-kinases that have been implicated in linking several of the Rho family GTPases to the actin cytoseleton, are viable but exhibit a testes defect associated with partial degeneration of spermatogenic cells in the seminiferous tubules and increased apoptosis (Takahashi et al., 2002).

3. RHO GTPASES IN ORGANOGENESIS

3.1 The nervous system

A combination of cell culture and gene expression studies have strongly implicated the Rho family GTPases and their regulators and targets in many aspects of neural development and function. Again, this should not be surprising when considering that the processes of neuronal migration, neurite outgrowth and guidance, and synapse formation all require actin reorganization. Moreover, glial cells are also migratory and are likely to require Rho signaling for their proper development and function. Several studies have been conducted in which transgenic mice have been established that express gain- and loss-of-function forms of the various Rho GTPases specifically in neural cell types. In one study that utilized a promoter system that gives rise to specific gene expression in the glial cells of the peripheral nervous system, it was observed that constitutively active Rho prevents peripheral glial migration, associated with stalling, defective process extension, and defective axonal ensheathment (Sepp and Auld, 2003). The transgenic animals die embryonically, suggesting that RhoA is an important mediator of normal glial development. Expression of either dominant-negative or activated Rac1 also produced defects in glial migration and axon ensheathment (Sepp and Auld, 2003). In addition, these transgenes produced fasciculation defects in sensory axons, suggesting that RhoA and Rac1 play distinct roles in peripheral glia. Axon pathfinding was not obviously affected by any of these transgenes, indicating that axon guidance in the periphery is largely resistant to disruption of normal glial function. Significantly,

analogous studies of Cdc42 mutants revealed no apparent role for Cdc42 in glial development (Sepp and Auld, 2003).

In another transgene study, an activated form of Rac1 was expressed specifically in developing Purkinje cells of the developing cerebellum (Luo et al., 1996).The resulting mice were ataxic and their Purkinje cells exhibited a substantial reduction in axon terminals, and a reduction in size, but an increase in number, of dendritic spines. This finding suggests that Rac1 plays distinct roles in regulating neurite outgrowth in the context of axons and dendrites. In related studies using rat hippocampal slices, it was determined that expression of dominant-negative Rac1 causes elimination of dendritic spines, whereas an activated form of RhoA reduces the branching complexity through a ROCK-mediated process (Nakayama et al., 2000).Together, these results point to important and distinct roles for Rho and Rac GTPases in dendrite growth, number, and branching.

Several gene knockout studies have similarly supported a role for the Rho GTPases in various other aspects of neural development and function. Although no studies have reported neural phenotypes in mice containing specific disruptions of individual GTPases, this probably largely reflects the fact only a few such knockouts have been described and those animals tend to fail early in development, prior to neurogenesis. However, knockouts of some of the Rho regulators and targets have been described, and several of these exhibit neural defects. For example, Lim-kinase-1, which like Lim-kinase-2, mediates signals downstream of several Rho GTPases, is not essential for development, but is required for normal dendritic spine morphogenesis and synaptic function (Meng et al., 2003; Meng et al., 2002). Consequently, the adult animals exhibit learning defects and an altered fear response. A few additional knockout mouse studies of Rho pathway components have resulted in viable animals that exhibit defects in neural function. For example, disruption of WAVE1, a member of the WASP family of Cdc42 targets, results in mice with severe limb weakness, a resting tremor, and neuroanatomical malformations (Dahl et al., 2003). During late stages of development, WAVE-1 is largely restricted to the CNS, but does not seem to play a role in neurite growth. The precise role of WAVE1 in neurons is unknown. WAVE2-deficient mice fail at E12.5 and exhibit growth retardation and malformed ventricles (Yan et al., 2003).

Some of the Rho effector targets have been implicated in neural development and function in mouse studies. Mice lacking the Rho target, Citron kinase, are severely ataxic and eventually die of seizures (Di Cunto et al., 2003). Further analysis has revealed that Citron is required for cytokinesis of neuronal precursors, suggesting that the observed phenotype in mutant animals reflects a developmental defect in the CNS. Thus, defects in neural development do not necessarily lead to embryonic lethality, and it

can be difficult to distinguish between subtle defects in neural development and defects in neural physiology that may not be associated with any significant developmental defect. Considering that a variety of studies have suggested a role for Rho signaling in synapses, it is possible that Rho GTPases play an important role in synaptic transmission and synaptic remodeling in the mature brain. The Rac/Cdc42 target, Pak1, appears to play a role in dendrite formation in cortical neurons. Expression of a constitutively active form of Pak1 in developing mouse neurons caused an increase in dendrite number whereas a dominant-negative form of Pak1 decreased dendrite number (Hayashi et al., 2002). It has been difficult to use knockouts to confirm this function of Pak1 in knockout animals because there is evidence of substantial redundancy between some of the highly related Pak kinases. However, disruption of Pak4 results in embryonic lethality associated with differentiation and migration defects in spinal cord motor neurons and interneurons (Qu et al., 2003). These mice also exhibit a defect in the proper folding of the caudal region of the neural tube, suggesting that Pak4 plays a unique role among the Paks in both neuronal and neuroepithelial cells.

Some of the Rho regulators have also been implicated in neural development. The two RhoGAPs that constitute the p190 family of RhoGAPs (p190-A and p190-B) have each been disrupted in mice, and both mutants are associated with neonatal lethality that is associated with neural defects (Brouns et al., 2000; Brouns et al., 2001; Sordella et al., 2002). The p190-A knockout mice exhibit multiple neural defects that involve both neuropeothelial tissues as well as neurons themselves (Brouns et al., 2000; Brouns et al., 2001). Several defects in neuroepithelial fusion were observed, including a defect in closure of the anterior neural tube, defective fusion at the neural midline, resulting in a failure of midline commissure formation, and a defect in closure of the optic fissure, resulting in a small, malformed eye. Neuron-associated phenotypes included defective fasciculation (axon bundling) in the cranial nerves and in some of the major axon tracts in the forebrain, defective guidance of some axon tracts, and defective migration of cortical neurons, resulting in abnormal cortical layering. Despite the very similar overall structure of the p190-A and p190-B proteins, mice lacking p190-B exhibit a distinct neural phenotype (Sordella et al., 2002). Those mice exhibit a substantial reduction in some of the major midline crossing tracts, associated with a reduction in neuronal differentiation, a reduced striatum, and enlarges lateral ventricles (figure 1). The p190-A protein was identified as the major substrate of Src phosphorylation in the developing and adult nervous system, and in cellculture studies, both p190 proteins appear to mediate adhesion signals to the actin cytoskeleton (Brouns et al., 2001). Thus, these various defects in p190-deficient mice probably reflect

defects in the transduction of signals from various cell surface adhesion molecules to the actin cytoskeleton via the Rho GTPases.

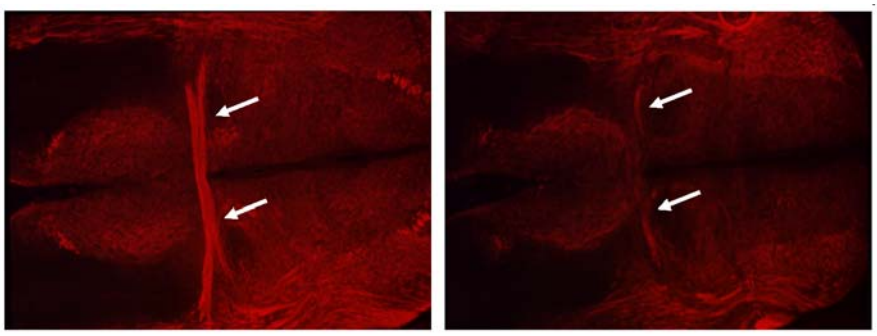

Figure. 1. In mouse embryos (E17.5) lacking the p190-B RhoGAP (-/-), the midline crossing axons of the anterior commissure (arrows) are severely reduced relative to wild-type embryos (+/+)

Several of the RhoGEFs have been disrupted in mice, and many of these are viable, suggesting the likelihood of functional redundancy among this relatively large class of GTPase regulators. In most cases, such animals have not been examined for even subtle defects in neural development. However, in mice lacking the Dbl RhoGEF, it was observed that distinct populations of cortical neurons exhibit a reduction in dendrite length, suggesting a highly specialized role for Dbl in some neurons (Hirsch et al., 2002). Such a finding highlights the fact that the developing brain consists of a relatively large number of distinct cell types, and that phenotypes that are limited to only one or a few of these cell types can easily go undetected in the absence of a detailed analysis.

In adult mice, Rho has been implicated in nerve regeneration following injury. It had been observed that microcrush lesions to the spinal cord of mice or rats results in a 10-fold activation of the RhoA GTPase (Dubreuil et al., 2003). That finding, together with numerous cell culture studies that indicated an important inhibitory role for Rho in neurite outgrowth, prompted studies to address the potential therapeutic utility of inactivating Rho following nerve injury. Several studies have made use of C3 toxin in such models, and it has been observed that C3 treatment of injured spinal nerves results in regeneration of axons and substantial functional recovery (Ellezam et al., 2002; Lehmann et al., 1999; Winton et al., 2002). A similar study using the ROCK inhibitor, Y-27632, indicated that inhibition of ROCK function could also promote recovery from nerve injury (Lehmann et al., 1999). Such findings suggest that activation of Rho and ROCKs

following nerve injury may prevent regeneration, and furthermore, that interfering with this pathway pharmacologically could be therapeutically beneficial

3.2 Eye and ear development

Inner ear development involves the morphogenesis of very complex and elaborate structures (the vestibule and cochlea) from a simple epithelium. Abnormal inner ear development in humans is associated with a variety of defects in balance and orientation as well as hearing. Rho GTPase signaling has been implicated both in developmental and physiological aspects of the mouse inner ear. Abr and Bcr are two closely related GAPs that regulate Rac and Cdc42 GTPases. Mice containing a targeted disruption of both of these genes (double knockouts) are viable, but exhibit behavioral defects including hyperactivity, circling behavior, and an inability to swim (Kaartinen et al., 2002). Such phenotypes are typically associated with vestibular defects, and these mice were found to display abnormal morphology of the saccule and ventricle of the inner ear, two structures that, together with the semicircular canals, constitute the vestibular system. The mice are not deaf, suggesting that these Rac/Cdc42 GAPs play a specific role in the morphogenesis of inner ear structures required for maintaining balance and orientation.

Within the cochlea, the hair cells are the essential structures for mechanosensation, and rely on a specialized cytoskeleton to transduce mechanical forces into signals for sensory neuron transmission. Interestingly, human mutations in the Rho-binding target, diaphanous, a regulator of actin polymerization, are associated with nonsyndromic deafness, confirming a likely role for Rho-directed actin assembly in cochlear hair cell function (Lynch et al., 1997). Gene knockouts of the mouse diaphanous genes have not yet been reported, and these should eventually be useful in determining whether diaphanous is required for developmental or physiological aspects of the cochlear hair cells.

Post-developmental hearing loss is often associated with damage to the cochlear hair cells by toxins or trauma, and the JNK signaling pathway has been implicated in the stress response in hair cells, raising the possibility that Rho GTPases (which can activate JNK) may be involved. Indeed, when explants of the organ of Corti, the cochlear structure that contains the hair cells, are treated with the bacterial C. difficile toxin B, which inactivates Rho, Rac, and Cdc42 proteins, a significant reduction in JNK-mediated hair cell death was observed upon exposure to toxic agents (Bodmer et al., 2002). This finding suggests that Rho GTPases may play a protective role in mature hair cells by mediating the activation of JNK in response to stress-inducing stimuli.

The potential role of Rho GTPases in the developing mouse eye has not received much attention thus far. However, a few reports have begun to implicate Rho signaling in eye morphogenesis. Cell culture studied have indicated that Rho signaling probably plays a role in lens epithelia both by regulating gene expression (alphaB-crystallin) and the actin cytoskeleton, and transgenic mice that express the Rho-inactivating C3 toxin in the developing lens exhibit lens cataracts and other ocular defects including defects in the focal attachments of the lens to the iris (Rao et al., 2002). Histological analysis also revealed that the lens defects included defective fiber cell differentiation and elongation, indicating a role for Rho in lens growth and maintenance of lens transparency.

The formation of the optic cup, which occurs between E11.5 and E13.5, gives rise to the retina, and requires dramatic morphogenesis of neuroepithelial tissue. The completion of the eye cup requires closure of an "optic fissure", in order to create a smooth seamless retina. In mice containing a targeted disruption of the p190-A RhoGAP gene, the optic fissure fails to close, leading to an abnormally formed eye structure associated with misfolded retinal tissue (Brouns et al., 2000). Thus, Rho is likely to play a role in the neuroepithelial fusion process required to close the optic fissure. This optic fissure defect appears to be related to other midline neuroepithelial fusion defects in p190-A RhoGAP knockout mice, including a failure to close the anterior neural tube and defective fusion of the forebrain hemispheres, suggesting that a similar Rho-mediated process regulates several neuroepithelial fusion events required for normal development.

3.3 Hematopoiesis and the immune system

Mouse models for studying the development of blood and lymphoid cells are particularly attractive for several reasons. A variety of cell surface markers that are routinely used to assess the various stages of hematopoietic differentiation are readily available. In addition, since many aspects of blood cell development are maintained throughout adult life, the development and physiology of blood cells can be easily examined in cases where gene disruption does not result in embryonic lethality. Finally, in cases where embryonic lethality is observed, fetal liver-derived stem cells can be used to reconstitute a complete hematopoietic system in irradiated host recipients. Surprisingly, of all of the developmental processes in mice, blood cell development has received, by far, the most attention in the context of Rho signaling. Hematopoiesis is generally not associated with the same kinds of tissue morphogenesis that play a major role in most other aspects of mammalian development. While blood cells do exhibit adhesive and

migratory behaviors, for the most part, they function as individual units and often exhibit a simple round morphology that does not appear to depend substantially on a dynamic actin cytoskeleton. In fact, as described blow, the vast majority of studies of Rho GTPases in hematopoiesis and immune function have revealed their role in the differentiation process and in signaling during the immune response. Thus, their role as cytoskeletal regulators may be less significant in the context of blood cells than it is in other tissues.

The rationale for the analysis of Rho GTPases in blood cell development and function can probably be traced, historically, to an early report that the Rac2 GTPase is largely restricted in its expression to myeloid cells (Didsbury et al., 1989), and subsequent findings that it is an essential component of the NADPH-mediated respiratory oxidative burst process that is used for microbial killing by neutrophils and macrophages (Bromberg et al., 1994). Indeed, several more recent reports that make use of mouse transgenics and knockouts have validated an important role for Rac2 in blood cells. Rac2-deficient animals are viable, and their neutrophils display defects in chemoattractant-stimulated superoxide production as well as in chemotaxis (Li et al., 2002; Roberts et al., 1999). The chemotaxis defect is associated with a reduction in F-actin formation, suggesting that Rac2 mediates distinct actin-dependent and -independent biological processes in neurtrophils. Interestingly, Rac1, which is 92% identical in sequence to Rac2, and is also expressed in neutrophils, cannot completely substitutre for Rac2. To address the role of Rac1 directly in neutrophils, a conditional Rac1 knockout mouse was generated in which Rac1 was specifically disrupted in the granulocyte/monocyte lineage (Glogauer et al., 2003). In those animals, neutrophils were observed to be profoundly defective in chemotactic migration and inflammatory recruitment, but interestingly, they exhibited normal levels of superoxide production. Thus, the closely related Rac1 and Rac2 GTPases both function in neutrophil chemotaxis, but Rac2 appears to function uniquely in regulating the respiratory burst.

The Rac GTPases are also detected in lymphocytes, and several mouse studies have addressed their role in lymphocyte development and function. An early transgenic study in which activated Rac2 was expressed specifically in thymocytes revealed an increase in apoptosis in thymus, suggesting a potential role in T cell selection (Lores et al., 1997).In another study, it was determined that Rac2 is selectively expressed in T helper cells of the TH1 class, which mediate cellular immunity (Li et al., 2000a). Using an inducible transgene system, it was demonstrated that expression of an activated Rac2 in mature T cells led to enhanced interferon-gamma production, while dominant-negative Rac2 inhibited interferon-gamma production, indicating a likely role for Rac2 in a key signaling pathway in

TH1 cells that involves NF-•B and MAP kinases (Lores et al., 1997). However, in a related study, no defects were observed in the ability of Rac2-deficient TH1 cells to respond to bacterial or viral infection in vivo (Croker et al., 2002b). In Rac2-deficient T cells, cytokine-stimulated proliferation is reportedly reduced, and a defect in MAP kinase activation has been noted, confirming a likely signaling role for Rac2 in some aspect of T cell activation. Rac1 also appears to play a role in developing thymocytes (Yu et al., 2001). Expression of an activated mutant form of Rac1 in pre-T cells can drive the differentiation process and promote proliferation of thymocyte precursors at the time of T cell antigen receptor beta selection (Gomez et al., 2000). An extension of this study revealed that Rac1 activation can switch thymocytes from a fate of positive to negative selection through an unknown mechanism (Gomez et al., 2001). The caveat with such studies that rely exclusively in the expression of a mutated form of the GTPase is that resulting defects may not correspond to the normal function of that GTPase.

Loss-of-function studies have pointed to roles for the Rac GTPases in B cell development and function as well. Rac2-deficient mice exhibit a significant reduction in peritoneal B lymphocytes, marginal zone B lymphocytes, and IgM-secreting plasma cells (Croker et al., 2002a). The isolated Rac2-deficient B cells also exhibited reduced chemotaxis in a chemokine gradient, indicating a likely role for Rac2 in both B lymphocyte differentiation and immune function. Using a conditional gene targeting approach, it was demonstrated that disruption of both Rac1 and Rac2 in the B cell lineage results in a nearly complete block in B cell development, indicating some degree of functional redundancy between Rac1 and Rac2 in this lineage (Walmsley et al., 2003). B lymphocyte precursors lacking both GTPases exhibited defects in the ability to transduce B cell receptor signals for proliferation and survival. Analysis of double-deficient hematopoietic stem/progenitor cells (HSC/Ps) also indicates a role for Rac1 and Rac2 in the movement of HSC/Ps from the marrow into the blood. In the absence of both GTPases, HSC/Ps exhibit a massive egress from the bone marrow, and are severely defective in the proliferative response to cytokines and adhesion to fibronectin (Gu et al., 2003).They also exhibit excessive apoptosis. Thus, these GTPases may perform redundant functions in the motility and engraftment of hematopoietic stem cells by mediating the response to both soluble and adhesive signals that direct motility, proliferation, and survival.

The Rho GTPase has also been implicated in lymphocyte development. In particular, several studies have revealed a role for Rho in the thymocyte lineage. By targeting the C3 toxin to the developing thymus in transgenic mice, it was determined that disruption of Rho function results in a severe reduction in thymic cellularity and is associated with defects in the maturation, proliferation, and survival of thymocytes, resulting in many

fewer mature peripheral T cells (Henning et al., 1997). A further study of these mice revealed that Rho plays distinct roles in both the survival and proliferation of thymic progenitors (Cleverley et al., 2000). Specifically, in pre-T cells, Rho is required for survival, but not for proliferation, whereas in late pre-T cells, Rho is required for proliferation but not survival. If C3 is expressed from a promoter that drives expression specifically in relatively late stages of T cell development, a block in T cell differentiation after the T cell receptor rearrangement stage was observed, suggesting a critical role for Rho at this important selection point in T cell development (Cleverley et al., 1999). Similarly, in transgenic mice in which an activated RhoA GTPase was expressed in thymocytes, an increase was observed in the positive selection of mature T cells bearing a rearranged T cell recept (Corre et al., 2001). Thus, Rho appears to play a significant role in the differentiation of both pre-T cells and mature T cells.

In addition to the studies of Rho and Rac GTPases in hematopoietic lineages, a single study has begun to address the role of Cdc42 in such cells. Specifically, in transgenic mice expressing an activated form of Cdc42 in late T cells, massive apoptosis was observed, resulting in a substantial decrease in the number of mature thymocytes and peripheral T cells (Na et al., 1999). Interestingly, one of the known Cdc42 targets, WASP, is specifically disrupted in patients with Wiscott-Aldrich Syndrome, in which patients exhibit immunodeficiency associated with defective T cells that appear to have a disorganized cytoskeleton (Schwartz et al., 1996). Thus, Cdc42 may play a role both in T cell development and in actin-mediated aspects of mature T cell function.

The nature of upstream regulation of the Rho family GTPases in hematopoiesis is not well established, but one regulator in particular, has received considerable attention. The vav1 protein, a member of the Rac/Cdc42 GEF family, is expressed almost exclusively in hematopoietic lineages, and was suspected of regulating GTPase function in such cells. In the first reported vav1 knockout, mice were observed to exhibit normal erythroid and myeloid development (Zhang et al., 1994). Subsequent studies, in which vav1-deficient embryonic stem cells were used to produce RAG2-deficient chimeras, resulted in thymic atrophy and B and T cells with reduced responsiveness to antigen receptors or stimulation with phorbol esters and calcium agonists (Gulbranson-Judge et al., 1999). B cells were found to respond normally to bacterial and other mitogens, indicating a selective role for vav1 in the development and function of lymphocytes. Additional analysis of vav1-deficient mice revealed that they exhibit a substantial defect in the positive selection of T cells, but do not seem to exhibit a defect in negative selection (Zhang et al., 1995). In light of the apparent role for the Rac GTPases in thymocyte selection, it seems likely

that activation of Rac by vav1 is required for some aspect of the positive selection of T lymphocytes. In vav1-deficient mice, conventional B cells appear to develop normally, however, it was reported that a subclass of mature B cells, the B1 B cells, are reduced in number, possibly due to an increased threshold for proliferative response to B cell receptor engagement (Zhang et al., 1995). Vav1 has also been implicated in the development of natural killer (NK) T cells. Vav1-deficient mice have a normal number of splenic NK cells, but the number of NK T cells is drastically reduced (Colucci et al., 2001). In addition, NK T cells from vav1-deficient mice do not produce IL-4 in response to in vivo activation, indicating a specific role for vav1 in NK T cell development and function (Chan et al., 2001).

Vav1 is closely related to two additional vav family members, vav2 and vav3, indicating the possibility of functional redundancy. Indeed, in two separate studies, it was determined that mice lacking both vav1 and vav2 exhibit a reduction in B lymphocyte number associated with an absence of a proliferative response to B cell antigen receptor engagement (Doody et al., 2001; Tedford et al., 2001). The defective response was associated with a failure to mobilize intracellular calcium, suggesting a specific and redundant function for vav1 and vav2 in B cell activation. A recent study has analyzed mice lacking all three vav proteins (Fujikawa et al., 2003). Those animals produce no functional B or T cells and fail to mount either T cell-dependent or T cell-independent humoral immune responses. Cells exhibit a defect in calcium mobilization in response to B cell receptor or T cell receptor engagement, but exhibit a normal MAP kinase activation, again consistent with a specialized and redundant role for the vav proteins in linking the activation of B and T cells to calcium-dependent responses.

Another RhoGEF that is highly enriched in hematopoietic cells is Lsc (Girkontaite et al., 2001). Lsc knockout mice have been produced, and mature lymphocytes were found to exhibit a reduction in motility in the absence of stimulation, whereas the marginal zone B cells exhibited enhanced migration in response to serum. Thus, Lsc appears to be another important activator of Rho GTPases within the immune system.

3.4 Fat, bone, skin, and muscle

A common mesenchymal stem cell gives rise to cells that form bone, fat, and muscle, and cell culture studies have implicated the Rho GTPases in each of these tissue types. The formation of bone, or osteogenesis, involves the differentiation of mesenchymal cells into osteoblasts, and is associated with a substantial change in cell shape. Although several cell culture studies have indicated a likely role Rho GTPase function in osteoblasts, relatively few reports have examined Rho signaling in animal models of bone

development. As described earlier, the FGD1 protein, a Cdc42 GEF, is expressed in developing bone, and loss of the human ortholog results in Aarskog Syndrome in humans, which is associated with skeletal defects (Pasteris and Gorski, 1999). The role of Src kinases in bone development and physiology is well established (Lowell and Soriano, 1996), and the ability of Src to modulate Rho-dependent actin reorganization is likely to play a role in bone development. However, this connection has yet to be established in the context of mouse models.

The role of Rho GTPases in skin development has not also not yet been addressed in mouse models, although Rho signaling has been shown to play a significant role in actin organization during keratinocyte differentiation (McMullan et al., 2003), which is associated with a substantial change in morphology and adhesion properties. In addition to development, two aspects of skin biology in adult mammals, wound healing, and oncogenesis are also likely to involve Rho signaling. Indeed, mice lacking the Rac activator, Tiam1 (a Rac-specific GEF), exhibit resistance to phorbol ester-promoted, Ras-induced skin cancers (Malliri et al., 2002; Sussman et al., 2000). This finding suggests that activation of the Rac GTPase by Ras is an important requirement for skin tumor formation. In a related study, it was observed that mice lacking RhoB, which develop normally, exhibit an increase in carcinogen-induced skin tumors, suggesting that RhoB is a negative regulator of oncogenesis in skin (Liu et al., 2001).

Adipogenesis, or the differentiation of fat cell precursors into mature adipose tissue, has been largely studied in the context of cell culture, where insulin/IGF-1 signaling appears to play a major role in this process. However, a recent report of the targeted disruption of the p190-B RhoGAP gene in mice revealed that this protein is required for adipogenic differentiation in vivo (Sordella et al., 2003). Further analysis, making use of embryo-derived cell cultures, indicated that Rho activity must be reduced in order for fat cell precursors to become mature adipocytes, thereby establishing a role for Rho signaling in adipogenesis. A specific pathway was identified in which excessive Rho activity leads to an increase in Rho-kinase-mediated phosphorylation of the insulin receptor substrate (IRS). This is an inhibitory phosphorylation that prevents the interaction of IRS with the insulin/IGF receptors, thereby down-modulating insulin/IGF signaling. It was also determined that insulin/IGF receptors can directly phosphorylate p190-B RhoGAP and thereby promote its activity by facilitating its translocation to membrane lipid rafts, where active Rho protein is enriched.

In that same analysis of p190-B RhoGAP, it was observed that while excessive Rho activity inhibits adipogenesis, it promotes myogenesis, or muscle cell differentiation. Myogenesis is also sensitive to IGF-1 signaling,

and the observation that Rho modulates the response to IGF-1 suggests that Rho probably plays a role in determining mesenchymal stem cell fate by determining how the cell will respond to IGF-1. Interestingly, IGF-1 signaling is also a major determinant of cell size and animal size, by promoting signals for cell growth, and mice lacking p190-B RhoGAP are approximately 30% reduced in size, and their tissues consist of cells that are reduced in size (Sordella et al., 2002). Thus, Rho appears to play an important role in several developmental processes that depend on IGF-1 signaling, and furthermore, this function of Rho appears to be independent of its ability to regulate the actin cytoskeleton. An important aspect of this analysis is that the developmental phenotypes observed in the knockout mice provided clues that led to the elucidation of a novel signaling role for the Rho GTPase. This provides a good example of the value of targeted gene knockouts in mice in establishing the molecular organization and function of GTPase-mediated signaling pathways in vivo.

Another Rho regulator, the RhoGEF, Trio, has also been implicated in muscle development (O'Brien et al., 2000). Trio knockout mice die in late embryogenesis and unusual spherical myofibers of skeletal muscle were detected at E18.5. The timing of the defect coincides with the so-called "second wave" of myogenesis, and was associated with a reduction in the number of the smaller secondary muscle fibers that are located adjacent to primary myofibers. Possibly, Trio regulates the alignment or fusion of secondary myoblasts. Interestingly Rac signaling has been directly implicated in the process of myoblast fusion in the Drosophila system (Erickson et al., 1997). However, the precise role of Rac signaling in that process is not clear.

3.5 Cardiac development and function

The formation of a mature heart, with its elaborate chamber and valve structure, and a purposeful asymmetry, is among the most remarkable of all tissue morphogenesis processes in mammals. Cell culture studies had implicated RhoA in the hypertrophic growth and cytoskeletal organization of cardiac muscle cells (Aikawa et al., 1999), and subsequent mouse studies have confirmed a likely role for Rho signaling in both cardiac development and physiology. Transgenic mice expressing wild-type RhoA or a constitutively activated RhoA mutant under the control of a cardiac-specific promoter exhibited atrial enlargement, and eventually, dilation of the left ventricular chamber and a consequent decrease in left ventricle contractility, ultimately leading to atrial fibrillation and death (Sah et al., 1999). Thus, excessive Rho activity can lead to ventricular failure. In a related study, an activated form of Rac1 was expressed specifically in the myocardium in

transgenic mice (Sussman et al., 2000). Those animals exhibited a range of postnatal cardiac phenotypes, whose severity was dependent on the level of transgene expression. In mice that died within two weeks of birth, a severe dilation phenotype was observed, which was associated with substantial enlargement of both ventricles and atria. Other mice exhibited a milder phenotype in which, at 3 weeks of age, no evidence of heart enlargement was seen, but did eventually appear by 2 months. These animals exhibited a progressive deterioration of ventricles, and their hearts were found to be hypercontractile in physiology studies. It was observed that the Rac target, PAK1, was translocated from a cytosolic to cytoskeletal localization in fractionated cardiomyocytes from transgenic animals. Moreover, immunostaining of paxillin indicated that focal adhesion formation may be affected in the Rac-expressing cardiomyocytes. Thus, Rac signaling appears to influence both cardiac dilation and hypertrophy, and Rac-regulated focal adhesions may be required for normal cardiac physiology. Notably, transgenic mice that overexpress the SRF transcription factor specifically in developing heart tissue exhibit cardiac hypertrophy (Zhang et al., 2001). Since SRF can be activated by Rho GTPases (Hill et al., 1995), this fnding suggests that the role of Rho GTPases in hypertrophy may also involve effects on SRF-dependent gene transcription.

Transgenic mouse embryos expressing RhoGDIα specifically in cardiomyocytes (starting at E8.5) fail at E10.5, and exhibit a disruption of cardiac morphogenesis associated with incomplete looping, lack of chamber demarcation, and ventricular hypoplasia (Wei et al., 2002). RhoGDIα can inhibit the activation of various Rho family GTPases, and so while this study suggests a likely role for Rho GTPases in early cardiac development, it is not clear from this analysis which of the Rho family members are involved.

Rho effector targets, including the ROCKs, have also been implicated in cardiac development. There are two ROCK genes in mammals, ROCK-I and ROCK-II, both of which are expressed in early embryonic heart. ROCK-II knockout mice have recently been reported, and the mutant animals die in late embryogenesis with apparent defects in blood coagulation and blood flow (Thumkeo et al., 2003). However, it is quite possible that there is significant functional redundancy between the two closely related ROCKs. Several other studies have made use of the pharmacological ROCK inhibitor, Y-27632, which effectively inhibits both forms of ROCK, and therefore, can potentially overcome such redundancy. To address a developmental requirement for ROCKs, early mouse embryos (E8.5-E9.5) were isolated and maintained in culture, and then treated with Y-27632 (Wei et al., 2001). The drug-treated embryos exhibited a block in the differentiation of both ventricles and atria. The treated embryos had smaller hearts and a dilated pericardium. A similar study by another group revealed

that Y-27632 treatment of early embryos blocked fusion of the bilateral heart primordial (Zhao and Rivkees, 2003). These finding suggest that the ROCKs play an essential role in several aspects of early cardiac development, and future studies using double ROCK-I/ROCK-II knockout mice will undoubtedly be performed to extend such findings.

The ability of ROCK inhibitors to prevent ROCK-mediated actomyosin contractility, and consequently affect vascular function has led to several studies in which such inhibitors appear to provide relief from vascular hypertension (Hu and Lee, 2003). Additional studies have now begun to address the consequences of ROCK inhibition in other aspects of cardiovascular disease. When adult mice fed on an atherogenic diet were injected daily with Y-27632, a substantial decrease in atherosclerotic lesion size in the aortic sinus and thoracic aorta was observed, suggesting that ROCKs may play a role in atherosclerotic plaque development, and furthermore, that ROCK inhibitors could provide effective treatments for atherosclerosis (Mallat et al., 2003). The potential therapeutic utility of ROCK inhibitors in cardiovascular disease was additionally revealed in a study in which it was shown that a high salt diet-induced left ventricular hypertrophy in rats could be prevented by chronic administration of Y-27632 (Satoh et al., 2003). This finding suggests that ROCKs may play a role in hypertension-induced cardiac hypertrophy, and indicates another potential therapeutic application of Rho pathway inhibitors in human disease.

The Rac/Cdc42 target, PAK4, is required for normal heart development. PAK4-null embryos fail prior to E10.5 with heart defects. Specifically, mutant embryos exhibited a thinning of the myocardial walls of the bulbus cordis and the ventricles. Most likely, this resulted in poor ventricular function, and embryonic lethality (Qu et al., 2003).

3.6 Vasculature and lungs

Angiogenesis is essential for vasculature formation during development, and plays an important role in wound healing and tumor growth in the mature organism. A variety of cell culture studies have revealed roles for Rho GTPases and their regulators and targets in several aspects of endothelial cell biology, including migration, cell-cell interactions, survival, and the response to adhesion molecules and growth factors, suggesting a likely role for the Rho GTPases in angiogenesis in vivo (Cascone et al., 2003). Thus far, however, the analysis of Rho function in this context in mouse models has been rather limited. Among the numerous Rho family GTPases, only RhoB has been directly implicated in angiogenesis in mouse studies (Adini et al., 2003). RhoB-deficient mice are viable, but exhibit a specific defect in retinal vascular development that is associated with abnormal sprout

morphology. This is consistent with the finding that RhoB plays a role in survival of sprouting endothelial cells, and suggests that RhoB might be a good therapeutic target for diseases associated with excessive sprouting angiogenesis, such as diabetic retinopathy and macular degeneration. In another study, the ROCK inhibitor, Y-27632, was used to specifically examine the role of Rho signaling in angiogenesis (Uchida et al., 2000). Mice were orally fed Y-27632, and angiogenesis in dorsal skin was quantified following local administration of VEGF delivered via transplanted VEGF-secreting cells. A significant inhibition of new vessel growth, without an effect on pre-existing vessels, was observed following Y-27632 treatment, suggesting that at least one Rho effector target plays a role in angiogenesis. A similar study using a different ROCK inhibitor, Wf-536, reported inhibition of tumor growth and angiogenesis in mice bearing xenotransplants of human prostate tumor cells (Somlyo et al., 2003). Thus, pharmacological inhibition of ROCK could potentially be an effective anti-cancer treatment.

As mentioned above, ROCK inhibitors have received a great deal of attention in the context of therapy for hypertensive disorders. Most of these studies have relied on rat models of hypertension, since, historically, this has been the model of choice for studying cardiovascular disease. However, in light of emerging conditional knockout technologies in mice, it is likely that future studies of specific gene function in cardiovascular disease will make more frequent use of mouse knockout models

3.7 The gastrointestinal system

Intestinal development begins relatively late in embryogenesis, with the conversion of a pseudo-stratified epithelium to a simple epithelial monolayer that undergoes differentiation and morphogenesis. This results in the formation of multiple discrete epithelial units known as crypts and villi that give rise to the mature intestine. The formation of these structures requires the differentiation of crypt stem cells, the migration of differentiating epithelial cells, and the morphogenesis of precisely folded epithelial tissue. The mature intestinal epithelium, once formed, undergoes periodic renewal throughout life, during which time, many aspects of the development process are recapitulated. The analysis of Rho GTPase signaling in intestinal morphogenesis has not yet been thoroughly addressed, although a few studies point to a likely role for Rho-mediated signaling. By expressing activated and dominant-negative forms of the Rac GTPase in regions of the developing small intestine, it was observed that activated Rac causes a cell-autonomous precocious differentiation of gut epithelia while dominant-negative Rac inhibits differentiation and migration of cells along crypt-villus

units (Stappenbeck and Gordon, 2000; Stappenbeck and Gordon, 2001). A follow-up mechanistic analysis of this defect revealed that expression of activated Rac in intestinal epithelia results in an accumulation of cytoplasmic phospho-JNK (Jun n-terminal kinase). Notably, the JNK substrate, c-jun, did not exhibit excessive phosphorylation, suggesting that the accumulation of cytoplasmic phospho-JNK may reflect a sequestering mechanism that suppresses JNK-mediated apoptosis in order for Rac signaling to promote cell proliferation without accompanying cell death.

IQGAP1, a Rac/Cdc42 effector targets that has been implicated in cadherin-based cell adhesion, also appears to play a role in the gastrointestinal system. Mice specifically lacking IQGAP1 appear to develop normally, however, homozygous mutant adults exhibit a late-onset gastric dysplasia (Li et al., 2000b). This suggests a function for Rac and/or Cdc42 signaling in maintaining the integrity of the gastric mucosa. IQGAP1 is highly related to IQGAP2, and it is possible that redundant functions between these proteins mask developmental phenotypes that would otherwise be associated with specific disruption of IQGAP1. However, a specific function for the Rho GTPases in maintaining the physiology of the epithelial lining of the mature gut is suggested by accumulating reports indicating that many of the pathogenic bacterial strains that infect the intestine produce toxins that specifically interefere with Rho GTPases and their normal regulation (Rudolph et al., 1999)

3.8 Mammary development and breast cancer

In the developing mammary gland, a variety of interactions between epithelial cells and surrounding matrix proteins play an important role in ductal morphogenesis, suggesting a likely role for Rho-mediated signaling. Similarly, changes in mammary epithelium that occur during pregnancy, lactation, and weaning may involve similar morphogenetic processes. The role of Rho GTPases has begun to be addressed in mouse models of mammary development and physiology. It had been observed that the Rac3 GTPase is frequently expressed at high levels in breast cancer cell cultures, and transgenic mice expressing an activated form of Rac3 were produced and found to exhibit defective postlactional involution and benign mammary lesions (Leung et al., 2003). These lesions consisted of epithelial islands that persisted during late stages of involution, at a time when elevated levels of apoptosis normally bring about the reduction in cell number. Thus, Rac3 may contribute to the involution process during weaning, and its apparent role in proliferation or survival of mammary epithelial cells, together with previously described expression studies, indicate a potential causative role in human breast cancer.

In a differential expression screen to identify genes whose expression is relatively high in the proliferating terminal end buds of developing mammary glands, the p190-B RhoGAP gene was detected (Chakravarty et al., 2003; Chakravarty et al., 2000). To examine a potential role for p190-B RhoGAP in mammary development, the mammary anlagen from p190-B knockout mice was rescued by transplantation into the cleared fat pad of recipient Rag1-deficient mice (Chakravarty et al., 2003).None of the p190-B-deficient epithelial transplants displayed any outgrowths in host recipients, indicating that p190-B RhoGAP is required for ductal morphogenesis. Interestingly, it has been established previously that IGF-1 signaling plays an important role in terminal end bud proliferation. Thus, the findings described above, that p190-B RhoGAP plays a critical role in regulating IGF-1 signaling during development, may be relevant to its role in mammary morphogenesis.

RhoC may also contribute to breast cancer. RhoC expression appears to be a reliable marker of human breast cancer invasiveness and metastasis potential (Kleer et al., 2002).In mice, a role for increased RhoC expression in the conversion of a primary tumor to a metastatic phenotype was reported (Clark et al., 2000), and several subsequent expression studies have confirmed a potential role for increased RhoC activity in tumor progression. The ability of Rho GTPase signaling to influence cell proliferation, adhesion and motility, and angiogenesis, indicates that these proteins could potentially play a role in tumorigenesis at multiple levels, and efforts are underway to explore the feasibility of using small molecule inhibitors of various Rho pathway components as therapeutic agents in the treatment of human cancers

4. CONCLUDING REMARKS

The mouse studies described above have revealed widespread and essential roles for several of the Rho family GTPases and their regulators and targets in embryonic development, as well as in the normal physiological function and pathology of adult animals. In some sense, this type of analysis is in its relative infancy, and in many cases, the phenotypes observed in transgenic and knockout mice have provided largely descriptive information regarding the requirement for the Rho GTPases in complex in vivo processes. However, the power of this technology is clear, and future studies, particularly those that make greater use of conditional gene targeting methods, will undoubtedly yield substantial new insights into the organization and function of Rho-mediated signaling pathways both in normal biology and in human disease

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