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Rho-GTPases in Embryonic Stem Cells

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1. Introduction

The Rho family of small GTP-binding proteins is comprised of 22 members, including the most well characterized members RhoA, Rac1 and Cdc42 (Jaffe and Hall 2005). The Rho family proteins share a high degree of homology with the Ras proto-oncogene, and indeed were first identified as a result of this similarity (*R*as *ho*mologue). Activity of these proteins is dependent upon their nucleotide binding state; inactive when associated with GDP but active following exchange of GDP for GTP, which induces conformational changes that promote association/activation of downstream effector proteins. The GDP/GTP cycle is regulated by GAPs that accelerate GTP hydrolysis by providing a critical catalytic amino acid leading to a return to the inactive state (Bernards and Settleman 2005), and GEFs that promote guanine nucleotide exchange and consequent Rho activation (Rossman et al. 2005). The number of GAPs and GEFs far exceeds the number of Rho proteins, and the roles of individual GAPs and GEFs in specific cell types and biological processes is currently an intensively studied field.

Although united by homology and function as regulators of the actin cytoskeleton, each of RhoA, Rac1 and Cdc42 has a distinct role in the organization of actin structures (Figure 1). RhoA is principally involved with the production of actin-myosin bundles and the generation of actomyosin contractile force. Rac1 contributes to the formation of actin meshworks that result in the emergence of large protrusive structures that lead to spreading or, if occurring in a polarized manner, will contribute to motility. Cdc42 promotes the formation of actin-rich filopodia. Together, coordinated programs of RhoA, Rac1 and Cdc42 activation/inactivation play prominent roles in processes such as endocytosis/exocytosis, adhesion and motility, which may subsequently impact upon proliferation and death/survival. Recent advances in the development of activation-state sensitive fluorescent probes have allowed temporal and spatial analysis of Rho protein activation, which has added significantly to our appreciation of Rho regulation and function (Hodgson et al. 2010). Much of the early research on Rho protein function relied upon over-expression of dominant-negative mutants that reduced affinity for GTP and constitutively-active mutants that reduced GTP hydrolysis; however, more refined analysis has become possible with the rise of RNAi and knockout methodologies (Heasman and Ridley 2008).

The study of Rho family proteins has historically focused on their roles as molecular switches acting downstream of cell surface receptors to regulate the actin cytoskeleton (Jaffe and Hall 2005). Significant effort has gone into classifying signaling from Rho proteins into

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2010), thereby independently validating the role of ROCK as a key regulator of ESC survival. The addition of Y27632 to the culture media is now standard practice and has greatly improved the reliability of hES cell survival (Olson 2008; Krawetz et al. 2009). The addition of Y-27632 can be directly to the cell culture medium or into the extracellular matrix upon which the hESCs are plated (Danovi et al. 2010). ROCK inhibitors have also been shown to improve recovery of cryopreserved ESC (Scott and Olson 2007; Wickman et al. 2010) and increase the efficiency of adenovirus-mediated gene transfer (Patwari and Lee 2008).

3.1 Rho signaling in ES cells

Recently, it has become clear that the actomyosin machinery downstream of Rho activation is essential for the blebbing and apoptosis that follow dissociation of hESCs (Martin 1981; Chen et al. 2010; Ohgushi et al. 2010), as inhibition of the myosin heavy chain ATPase with Blebbistatin, the use of actin disruption drugs or selective knock-down of ROCK1, ROCK2 or the myosin heavy and light chains all prolong survival of dissociated hESCs. Rho activation, coupled with Rac inhibition, was determined to be the driver of dissociation-induced hESC apoptosis via ROCK-mediated myosin light chain phosphorylation (Ohgushi et al. 2010). Activation of ROCK1 by caspase-mediated cleavage (Buecker et al. 2010) does not appear to contribute to apoptosis induced in this manner (Ohgushi et al. 2010). Overexpression of an active form of Ezrin, which strengthens the physical coupling between the plasma membrane and cortical actin cytoskeleton, was sufficient to block blebbing but not the dissociation-induced cell death, indicating that apoptosis was not caused by blebbing itself but the result of actomyosin contraction (Ohgushi et al. 2010). Although the dissociation-induced cell death was linked back to mitochondrial depolarization and cytochrome c release, further study will be required to determine how actomyosin contractility is coupled to the mitochondrial pathway of apoptosis (Ohgushi et al. 2010). It is also becoming clear that the particular sub-embryonic origin of the embryonic stem cell line determines whether Rho signaling is detrimental to survival on dissociation. While epiblast-derived hESCs are acutely sensitive to Rho signaling following dissociation, ICM-derived mESC have the capacity to survive dissociation without the need for inhibition of the actomyosin machinery (Ohgushi et al. 2010), a characteristic they share with human induced pluripotent stem cells (hiPSC), which display mESC-like morphological features (Evans and Kaufman 1981). On the other hand, epiblast-derived murine epiblast stem cells (mEpiSC) or mESCs differentiated into epiblast-like cells acquire a dependence on ROCK-inhibition in order to survive dissociation (Ohgushi et al. 2010). One theoretical possibility to account for these observations is that external pulling forces from adjacent cells in an epithelial sheet counteract the internal actomyosin contractile forces within individual cells such that the internal and external mechanical forces become balanced in all directions along the epithelial plane, thereby limiting their pro-apoptotic effects. Since mESCs are derived from the ICM prior to differentiation into epithelial-type cells and grow in disorganized three-dimensional cell collectives similar to the *bona fide* inner cell mass, they may not be dependent on external tension derived from cell-cell adhesions, such as those that occur in an epithelial sheet, for survival. In contrast, hESCs grow as tightly adherent two-dimensional sheets similar to the epiblast where pulling forces from adjacent cells would be sensed. In agreement with this model, when human induced pluriopotent stem cells (hiPSCs) were reprogrammed from fibroblasts through the expression of five reprogramming factors plus LIF, they acquired the ability to grow at low density or in suspension in parallel with changed in vitro growth characteristics to mESC-like disorganized three-dimensional structures (Tashiro et al. 2010). This exquisite sensitivity of epiblast and epiblast-like stem cells

the MLCv2 promoter had a similar effect as early expression on the organization of sarcomeric units. These results revealed that the role of Rac1 in cardiac differentiation is likely dependent on the developmental stage. Given the availability of mESC in which Rac1 can be conditionally deleted (Yuan et al. 1995), more refined analysis of the role of Rac1 in cardiac differentiation and disease should be possible.

5. Activating ROCK in mouse ICM-derived ES cells

Mechanical forces are increasingly appreciated as major influences in embryonic development. External mechanical forces can be produced by physical alterations to the microenvironment. These external forces are sensed by cells, leading to responses that allow the cell to adapt to the changed environmental circumstances. One way that cells respond to mechanical force is via integrin-mediated activation of Rho and ROCK resulting in increased cellular stiffness via increased actomyosin contracility, which is also known as reinforcement (Guilluy et al. 2011). There is considerable evidence that suppression of

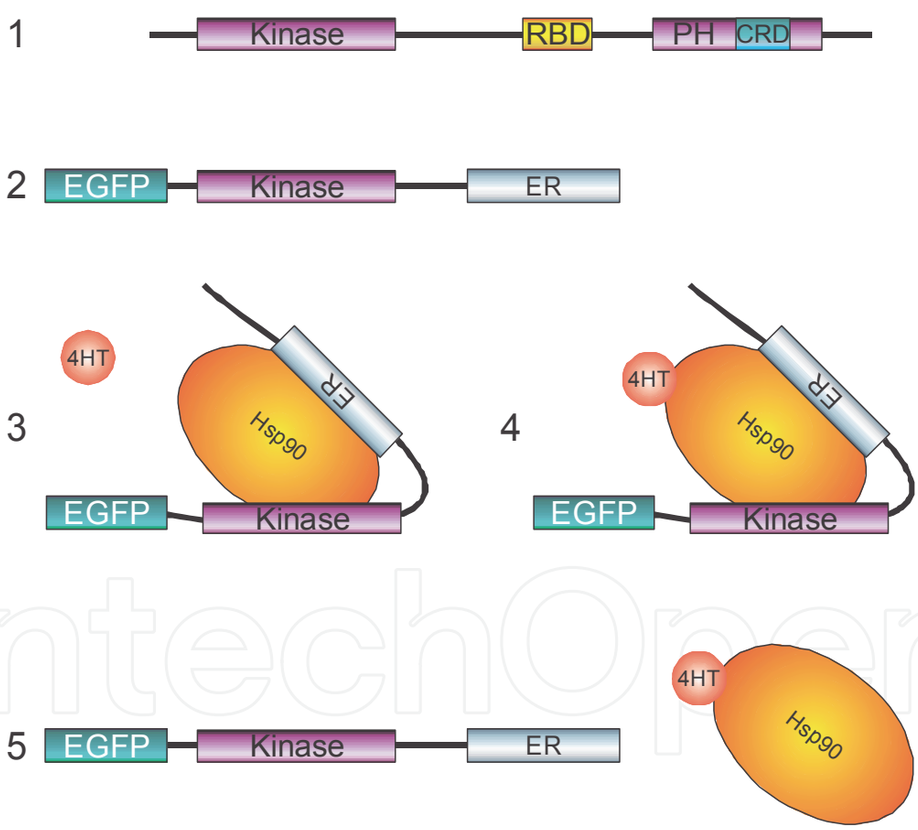

Fig. 2. Mechanism of conditional activation of ROCK. 1: Diagram of ROCK domains, RBD = Rho Binding Domain, PH = Pleckstrin Homology domain, CRD = Cysteine-Rich Domain. 2: Kinase domain of ROCK2 was fused to Enhanced Green Fluorescent Protein (EGFP) and the hormone-binding domain of Estrogen Receptor (ER) to create conditionally regulated ROCK:ER. 3: In the absence of ligand, Heat Shock Protein 90 (Hsp90) binds to the ER domain and represses catalytic activity. 4: Upon binding of estrogen analogues such as 4-hydroxytamoxifen (4HT), 5: Hsp90 is displaced thereby allowing for ROCK catalytic activity.

actomyosin contractility by inhibition of ROCK promotes the survival and continued proliferation of epiblast-derived hES cells. It is suggested, however, that this signaling axis is less important in ICM-derived mES cells. We therefore decided to take advantage of a system to conditionally activate ROCK within mES cells to determine whether ROCK activation and consequent actomyosin contractility had a role in their proliferation, survival and/or maintenance of pluripotency. Accordingly, we transduced G4 mES cells (George et al. 2007) with a pBabe-Puro retroviral vector (Morgenstern and Land 1990) encoding a conditionally-active version of ROCK fused to the hormone-binding domain of the estrogen receptor (Figure 2) (Croft and Olson 2006) to establish the pBabe-Puro-ROCK:ER mES cell line in which ROCK activity could be elicited by treatment with the estrogen analog 4-hydroxytamoxifen (4HT). As a negative control, cells were transduced with pBabe-Puro encoding a kinase-dead counterpart (KD:ER) to produce control pBabe-Puro-KD:ER mES cells that express of catalytically inactive control ROCK protein.

When maintained in 4HT, pBabe-Puro-ROCK:ER mES cells exhibited robust growth and a large number of colonies exhibiting a refractive colony morphology under transmitted light and fewer colonies exhibiting a differentiated morphology, consistent with a high degree of pluripotency (Figure 3). Consistent with this observation, 4HT treated pBabe-Puro-ROCK:ER mES cells express significantly higher levels of the pluripotency marker alkaline phosphatase (ALP) than 4HT treated pBabe-Puro-KD:ER mES cells or vehicle treated pBabe-Puro-ROCK:ER

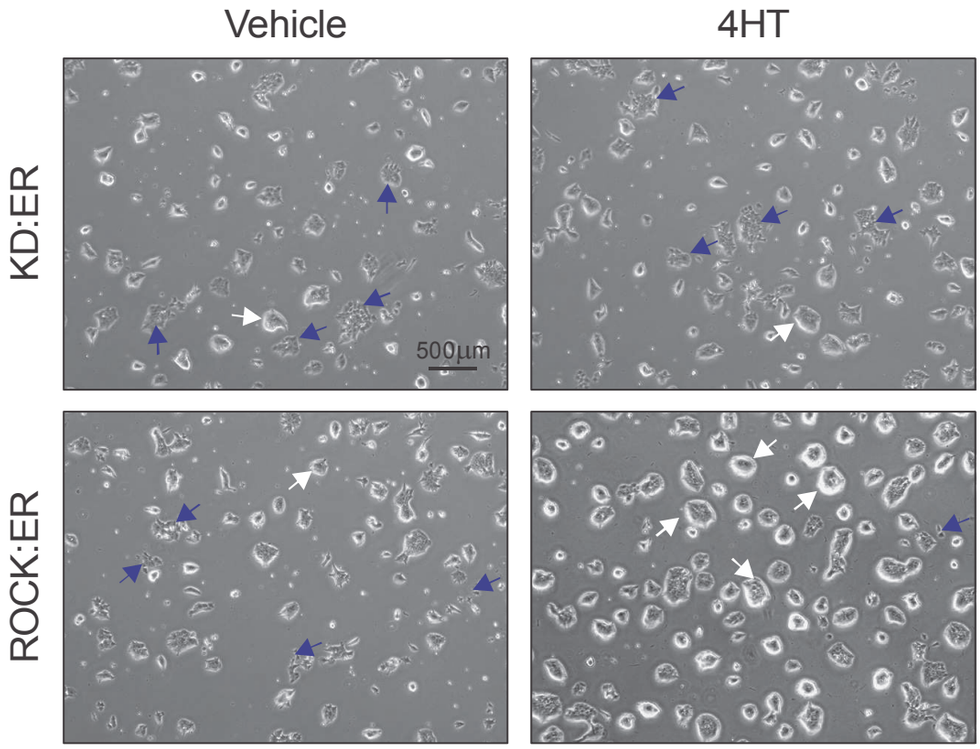

Fig. 3. Conditional ROCK activation in mES cells elicits a highly refractive colony morphology. Panels show brightfield images of pBabe-Puro-ROCK:ER and pBabe-Puro-KD:ER mES cells treated with Vehicle or 4HT. Flat colonies containing mainly differentiated cells (purple arrows) and raised colonies containing mainly undifferentiated cells (white arrows) are indicated. Scale bar denotes 500µm.

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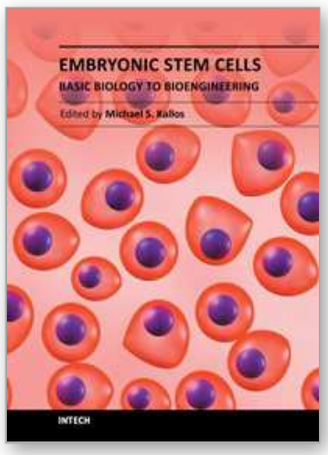

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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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