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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

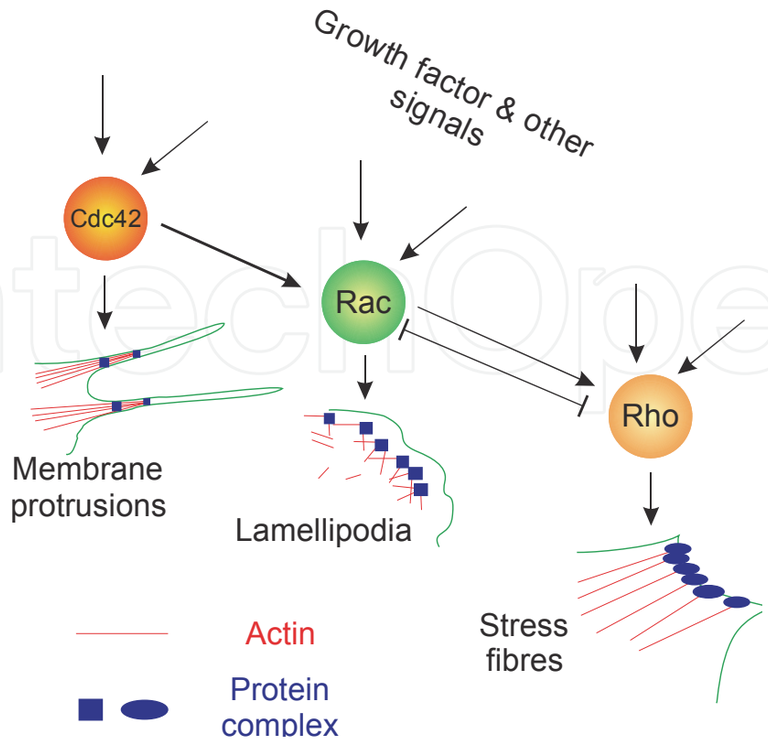

Fig. 1. Diagram of actin structures regulated by RhoA, Rac1 and Cdc42.

linear cascades, similarly to the classical Ras/Raf/MEK/ERK kinase cascade. However, recently a greater appreciation of the role of mechanical forces as fundamental influences in biology has emerged (Puceat et al. 2003). As central regulators of the actin-myosin cytoskeleton, an emerging concept is that many of the activities of Rho proteins may not be attributable to simple linear pathways, but instead are the product of modulating contraction and relaxation at the cellular and subcellular levels, with consequent effects on development and function at the tissue and organismal levels.

2. Embryonic Stem Cells

Pluripotent stem cells were first isolated from testicular teratocarcinoma (Pierce and Dixon 1959), a germ cell tumor type containing a population of pluripotent stem cells together with embryonic and extra-embryonic tissues that arise from these stem cells. Pluripotent stem cells of testicular teratocarcinomas are termed Embryonal Carcinoma (EC) cells and can give rise to collections of tumor cells having morphological characteristics of each of the three embryonic germ layers. In mice, EC cells have been demonstrated to be capable of contributing to every germ layer including the germ-line when injected into host blastocysts (Brinster 1974; Mintz and Illmensee 1975; Illmensee and Mintz 1976). Interestingly, under these conditions, EC cells are non-malignant, and chimeric mice containing tissues differentiated from EC cells are generally healthy. These observations formed the basis for the isolation of Embryonic Stem (ES) cells, which were derived from the pre-implantation embryo, arising when cells constituting the inner cell mass (ICM) of the pre-implantation blastocyst or the epiblast of the post-implantation blastocyst were placed in 2D-culture (Evans and Kaufman 1981; Martin 1981). Like EC cells, ES cells are pluripotent, being

capable of giving rise to all tissues of the adult organism originating from the three germ layers, upon injection into a host blastocyst (Bradley et al. 1984). The great similarities observed between EC cells and ES cells led to an appreciation of the importance of the tissue microenvironment in informing cell behavior and fate.

A major attraction of murine ESC (mESC) research stemmed from the realization that mutations introduced into the mESC genome would be readily transmitted through the germ-line, enabling the establishment of strains of mice harboring specific genetic mutations (Capecchi 1989), thereby facilitating the elegant functional characterization of virtually any gene of interest. The first gene to be targeted and inactivated in mES cells was the X-linked gene *Hprt*, which encodes hypoxanthine guanine phosphoribosyltransferase, an enzyme involved in purine metabolism (Thomas and Capecchi 1987). In turn, an *Hprt*-deficient ES cell line was engineered to re-introduce the *Hprt* coding sequence and used to produce knock-in gene-targeted mice for the first time, which faithfully recapitulated the wild-type *Hprt* expression pattern (Thompson et al. 1989). Following on from these pioneering studies, techniques for establishing gene-targeted mice have been considerably improved and refined. Gene targeting in mES cells to generate loss of function or gain of function mutations with an exquisite degree of subtlety and control is now an established tool in biological research.

2.1 Maintenance of pluripotency

ES cells express markers of their undifferentiated state such as the octamer binding protein 4 (Oct4) (Rosner et al. 1990; Scholer et al. 1990), the SRY-related HMG-box gene 2 (Sox2) (Yuan et al. 1995), signal transducer and activator of transcription 3 (Stat3) (Niwa et al. 1998), the homeobox protein Nanog (Chambers et al. 2003; Mitsui et al. 2003) and alkaline phosphatase (AP) (Hahnel et al. 1990) that denote their capacity for both self-renewal and pluripotency. Of these, Oct4 and Sox2 have key roles in the maintenance of ES cell self-renewing capacity such that their expression is essential for the maintenance of pluripotency and their ectopic expression in somatic cells contributes to the generation of induced pluripotent (iPS) cells (Takahashi and Yamanaka 2006; Yu et al. 2007; Nakagawa et al. 2008).

Oct4 is a POU-domain transcription factor also termed POU5F1 and is indispensable for plutipotency. Oct4 deficient embryos develop to the morula stage, but are unable to form an ICM (Nichols et al. 1998) and in vitro culture of Oct4 deficient embryos failed to yield ES cells (Nichols et al. 1998). These observations are further elaborated by more recent work showing that selective deletion of the Oct4 gene in primordial germ cells (PGC) results in their death by apoptosis (Kehler et al. 2004). Oct4 expression is very tightly regulated and its transient increase and decrease during early stages of embryonic development have been termed the totipotent cycle (Yeom et al. 1996). While evidence for the absolute requirement for Oct4 in the maintenance of ES cells is very strong, there is controversy on whether it is required for the maintenance of adult stem cells. Although there are numerous reports of Oct4 expression in adult stem cells including in hematopoietic and mesenchymal stem cells and stem cells of epithelial tissues such as the pancreas, kidney, breast, uterus, lung and skin, a recent study in which its expression was systematically abrogated in several of these tissues has revealed that Oct4 is required for neither the maintenance of adult stem cells nor for wound healing (Lengner et al. 2007).

Sox2 is a HMG-box containing transcription factor closely related to the Y-chromosome located sex determining gene SRY. Its main role in the maintenance of pluripotency is thought to be closely related to the regulation of Oct4 transcription. Indeed Sox2 and Oct4

can jointly bind regulatory chromosomal regions associated with both the *Oct4* and *Sox2* genes (Chew et al. 2005; Masui et al. 2007) as well as regulating Nanog expression (Kuroda et al. 2005; Rodda et al. 2005).

2.2 Culturing ES cells

Since the initial isolation of ICM-derived mESCs in the early 1980s (Evans and Kaufman 1981; Martin 1981), conditions for the culture of ESCs have been developed and progressively refined. mESCs are propagated on a feeder layer of murine embryonic fibroblasts (MEFs) or in media containing leukemia inhibitory factor (LIF), under which conditions they maintain a pluripotent state (Williams et al. 1988). Withdrawal of LIF or culture in the absence of fibroblasts results in spontaneous differentiation of mESCs into a variety of lineages (Evans and Kaufman 1981; Martin 1981; Williams et al. 1988). The dependence of mES cells on LIF is thought to be related to LIF mediated activation of STAT3 signaling (Smith et al. 1988) which together with Oct4/Sox2, has a possible role in the regulation of Nanog expression.

Human ESCs (hESCs), which have been isolated from the epiblasts of human blastocysts (Thomson et al. 1998; Reubinoff et al. 2000) are also propagated on a feeder layer of MEFs, but LIF has no role in maintaining their pluripotency (Thomson et al. 1998; Reubinoff et al. 2000). Instead, a balance between Tgfβ/activin/nodal signaling and suppression of BMP signaling together with the FGF signaling pathway are important for self-renewal and the maintenance of pluripotency in this system (James et al. 2005; Vallier et al. 2005; Xu et al. 2005). However, as yet no reliable defined medium has been developed to enable the culture of hES cells in the absence of feeder cells. Like mESCs, hESCs spontaneously differentiate if cultured in the absence of a feeder layer, but unlike mESCs they undergo blebbing and apoptosis when maintained in a dissociated state (Watanabe et al. 2007).

hESCs are not only a valuable tool for the study of human development, but also have applications in regenerative medicine, toxicology and the development of new drugs to target human disease (Murry and Keller 2008). mESCs and hESCs are thus examples of the two major types of pluripotent stem cells, derived as they are from the ICM and the epiblast respectively.

3. Rho family GTPases in embryonic stem cells

One of the most interesting recent developments in ES research is the revelation that signaling through RhoA plays a key role in the survival of human embryonic stem cells. This was first appreciated in 2007, following a cell-based screen of biologically active compounds that promoted survival and proliferation of dissociated hESCs that identified Y27632, a selective inhibitor of the Rho-effector protein ROCK (Watanabe et al. 2007). The ROCK1 and ROCK2 serine/threonine kinases are central and critical regulators of actomyosin contractility (Coleman et al. 2001). Typically, these kinases are activated by association with active GTP-bound Rho proteins. Active ROCK promotes actomyosin contractility through a dual mechanism of simultaneously phosphorylating and activating the contractile force-generating regulatory myosin light chain (MLC) and the LIM kinases (Sugihara et al. 1998), which modulate filamentous actin stability. In contrast to hESC, mES cells do not require ROCK inhibition for survival even when disaggregated to a single cell suspension. Since that initial study, subsequent screens have identified additional ROCK selective inhibitors that promote the survival of hESC (Andrews et al. 2010; Pakzad et al. 2010) and neural stem cells (Xu et al.

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

may reflect the critical importance of proper differentiation and spatial organization of the epiblast stage during embryonic development. If any individual cell in the epiblast layer were improperly positioned in the epithelial sheet, the potential consequences to the subsequent developmental stages and ultimately to the organism as a whole could be catastrophic.

3.2 Rac signaling in ES cells

The pro-apoptotic effect of Rho signaling in dissociated hESC is strongly counteracted by signaling through Rac. Indeed it has been shown that Rac1 is required for the survival of epiblast cells within the blastocyst during morphogenesis of the murine peri-implantation egg cylinder (He et al. 2010). During this process, the apoptosis mediated clearance of cells that are not in contact with the basement membrane (known as cavitation) is counteracted by signaling through Rac in those cells that remain apposed to the basement membrane (BM). In the absence of Rac1, cells in contact with the BM undergo apoptosis despite the survival signals that it normally provides (Kim et al. 2011). It is these BM-associated cells that give rise to the epiblast (He et al. 2010). Activation of Rac in the epiblast is mediated by the recruitment of the Crk adaptor protein and DOCK180 GEF (He et al. 2010). In turn, active Rac signals via PI3K and Akt to promote survival (He et al. 2010). Interestingly, a single dual-function protein, Abr, acts as Rho-GEF and Rac-GAP within dissociated hES cells in culture, simultaneously activating Rho and inactivating Rac upon cell dissociation, in a manner dependent on cell-cell interactions involving E-cadherin (Martin 1981; Ohgushi et al. 2010). The role of E-cadherin in hESC survival was also revealed in a chemical biology screen for small molecules that affected survival (Pakzad et al. 2010). One compound increased the survival of dissociated cells by reducing E-cadherin endocytosis, thus increasing the levels of cell-surface E-cadherin and consequently promoting cell-cell adhesions. In agreement with these observations, ectopic over-expression of E-cadherin was also sufficient to increase survival of dissociated hESCs (Rizzino 2010). However, when dissociated hESCs were grown on E-cadherin coated plates, they still underwent membrane blebbing and had significantly lower survival, indicating that homotypic E-cadherin interactions alone were not sufficient to promote survival (Ohgushi et al. 2010). These observations suggest the existence of a yet uncharacterized sensor that transmits a complementary signal derived from cell-cell adhesion that acts in concert with, or in parallel to, E-cadherin activation to repress actomyosin contractility and consequent cell death. Although mESCs are not sensitive to the same sort of dissociation-induced cell death, constitutive Rac1 deletion was found to induce membrane blebbing and eventual apoptosis of epiblast derived stem cells, possibly due to the lack of Rac1 activity to counter-balance the effect of RhoA activation (Kim et al. 2011). These Rac1 deleted cells also were defective in the formation of actin cytoskeleton structures such as lamellipodia and were significantly slower in migrating on collagen I coated dishes, revealing the critical role played by Rac1 in these biological activities. Similarly, Rac1 was found to be an important contributor to mESC migration on laminin (Li et al. 2010).

3.3 Cdc42 signaling in ES cells

Also implicated in murine peri-implantation development is the Cdc42 GTP-binding protein. Mouse embryoid bodies deficient for Cdc42 exhibited polarization defects characterized by aberrant adherens and tight cell-cell junction formation and failure of cavitation (Wu et al. 2007), in a process mediated by the atypical protein kinase C (aPKC) family of kinases. Despite the polarization defects, basement membrane formation, which requires polarized deposition and assembly of basement membrane components at the basal

side of a cell layer, was unaffected by deletion of Cdc42 (Wu et al. 2007). Interestingly mES cells lacking Cdc42 had lower levels of active Rac1 although total Rac1 protein levels were unaffected (Wu et al. 2007), suggesting that some of the observed defects could be the result of reduced Rac1 activity. However, unlike Rac1 deficient mES cells that would undergo apoptosis while in contact with the basement membrane (Kim et al. 2011), deletion of Cdc42 still allowed survival of cells in contact with the BM (Wu et al. 2007). Additional defects in PIP_2-induced actin polymerization and cytoskeletal organization were likely to also contribute to defective adhesion and migration of mESC deleted of Cdc42 (Chambers et al. 2003; Wu et al. 2007). The motility of mESC plated on plated on laminin also were dependent on Cdc42 as revealed by siRNA-mediated knockdown (Li et al. 2010). These morphological, polarization and motility defects almost certainly contributed to early embryonic lethality in Cdc42 deficient mice (Chambers et al. 2003). These tantalizing observations point to complementary functions for Rho, Rac and Cdc42 during the processes of cavitation and the appearance of the epiblast, and underscore the importance of these proteins in appropriately mediating the survival or apoptotic clearance of cells during early morphogenesis. It therefore appears that the activity of the Rho family GTPases crucially determines the fate of pluripotent stem cells within the early developing embryo.

4. Additional functions of Rho proteins in ES cells

An interesting aspect of ESC is that under the right conditions, such as hanging drop suspension leading to the formation of embryoid bodies (Kurosawa 2007) , differentiation results in the production of cardiomyocytes that spontaneously contact and relax (beating) as they would in an intact heart (Wobus et al. 1991). Human ESC can also be differentiated into cardiomyocytes, which has generated considerable excitement in the field because of their value in examining the role of specific proteins in cardiac disease phenotypes, and also due to the eventual possibility that they might have therapeutic utility (Brinster 1974). To examine the role of Rac1 in the differentiation of mESC into cardiomyocytes, ectopic expression of constitutively-active Rac 1 deficient in GTPase activity (Rac1V12) or dominant-negative Rac1 with reduced affinity for GTP (Rac1N17 was used to elucidate the consequences of Rac1 gain-of-function and loss-of-function, respectively (Puceat et al. 2003). Expression of active Rac1V12 blocked the characteristic beating of embryoid bodies, due to a differentiation defect as indicated by reduced expression of cardiomyocyte differentiation markers such as MEF2C and ventricular myosin light chain 2 (MLCv2). In contrast, expression of a constitutively active form of RhoA did not block cardiomyocyte differentiation. Previous research had revealed that Rac1 regulates the activity of the NADPH oxidase that generates reactive oxygen species (ROS) (Di-Poi et al. 2001) , and when H_2O_2 was added to embryoid bodies for up to 7 days the effect on blocking cardiomyocyte differentiation by active Rac1V12 was mimicked, while the ROS scavenger catalase reduced the differentiation block induced by active Rac1V12 (Puceat et al. 2003). Consistent with this conclusion, expression of a point-mutant form of Rac1 that does not activate the NADPH oxidase (Rac1V12D38) did not block cardiomyocyte differentiation. Expression of the dominant-negative Rac1N17 to examine loss-of-function did not affect differentiation but did impair beating by interfering with the organization of sarcomeric units required for contraction (Puceat et al. 2003). In contrast to what occurred when Rac1 was expressed early, when the MLCv2 promoter was used to express active Rac1 in differentiated cardiomyocytes, increased beating was observed due to a facilitation of differentiation and prolonged proliferation (Puceat et al. 2003). Expression of dominant-negative Rac1N17 from

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

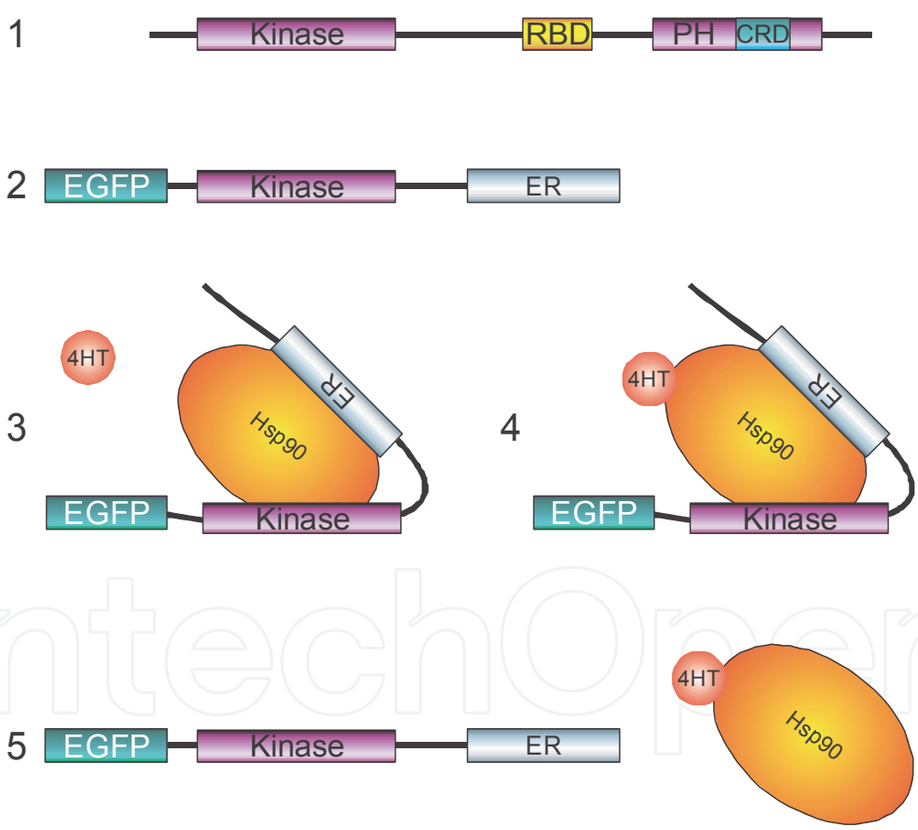

Fig. 2. Mechanism of conditional activation of ROCK. 1: Diagram of ROCK domains, RBD = Rho Binding Domain, PH = Pleckstrin Homology domain, CRD = Cysteine-Rich Domain. 2: Kinase domain of ROCK2 was fused to Enhanced Green Fluorescent Protein (EGFP) and the hormone-binding domain of Estrogen Receptor (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

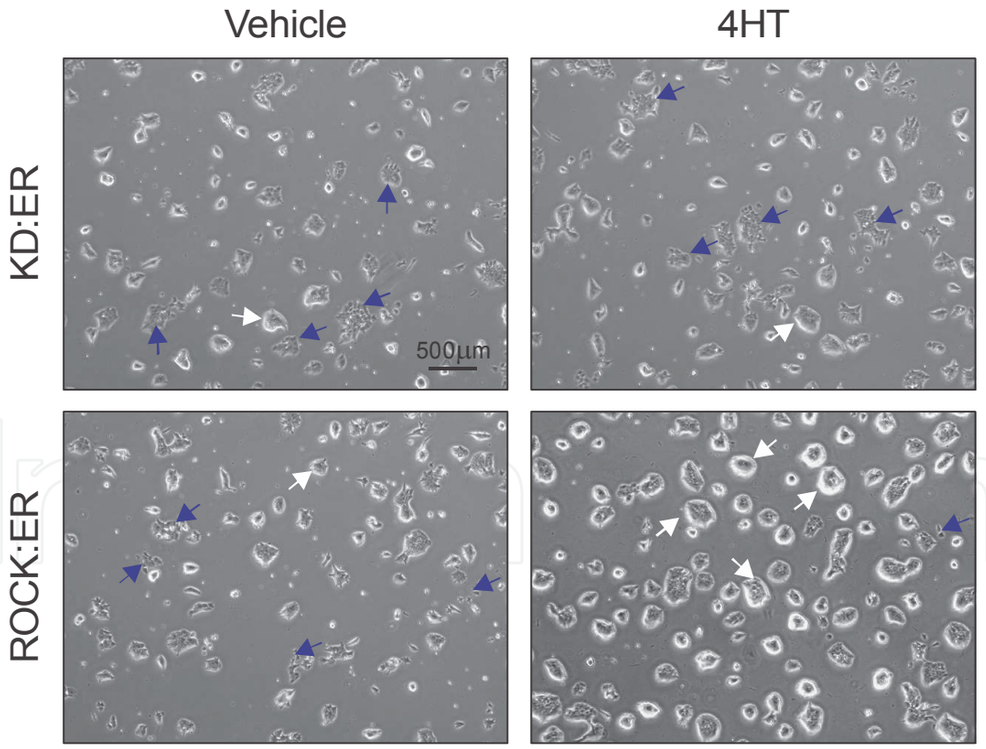

Fig. 3. Conditional ROCK activation in mES cells elicits a highly refractive colony morphology. Panels show brightfield images of pBabe-Puro-ROCK:ER and pBabe-Puro-KD:ER mES cells treated with Vehicle or 4HT. Flat colonies containing mainly differentiated cells (purple arrows) and raised colonies containing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

and pBabe-Puro-KD:ER mES cells (Figure 4A). To determine whether the increased ALP activity observed upon ROCK activation correlated with an increase in stemness, we then assessed the expression of two classical markers of pluripotency, Oct4 and Nanog. 4HT treated pBabe-Puro-ROCK:ER mES cells express significantly higher levels of Oct4 and Nanog than 4HT treated pBabe-Puro-KD:ER mES cells or vehicle treated pBabe-Puro-ROCK:ER and pBabe-Puro-KD:ER mES cells (Figure 4B). Consistent with this effect being mediated by the activity of ROCK, co-treatment of pBabe-Puro-ROCK:ER mES cells with 4HT and the selective ROCK inhibitor Y-27632 failed to induce Oct4 or Nanog expression (Figure 4B).

Taken together, these results strongly suggest that ROCK activation in mES cells promotes stemness and facilitates proliferation and survival. These observations are consistent with a previous report that inhibition of ROCK activity or silencing of ROCK expression in mESC causes a reduction in stem like properties including alkaline phosphatase activity and Oct3/4 expression, and increased expression of differentiation markers SOX-1, nestin and MAP2c when grown at high seeding densities (Chang et al. 2010). Interestingly, the effects of ROCK inhibition on morphology and colony formation were reversible if cells had been

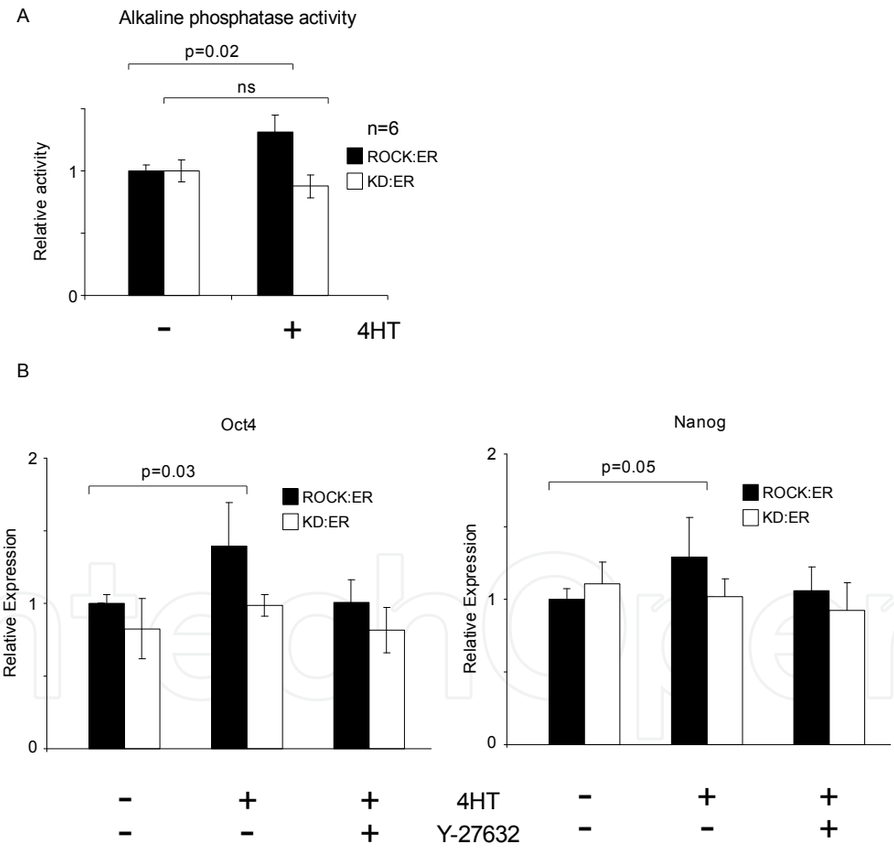

Fig. 4. Conditional ROCK activation in mES cells increases stemness. (A) Histogram shows alkaline phosphatase activity in pBabe-Puro-ROCK:ER and pBabe-Puro-KD:ER mES cells treated with Vehicle or 4HT. (B) Histograms show expression at the mRNA level of the stem cell markers Oct4 and Nanog in pBabe-Puro-ROCK:ER and pBabe-Puro-KD:ER mES cells treated with Vehicle or 4HT. All values are expressed as mean ± SD. P values were calculated using the Student's t-test.

grown at low initial densities where the reduction in stem like properties were not observed. However, at high cell densities where ROCK inhibition had repressed stem cell properties the effects were not reversible, suggesting that epigenetic reprogramming had occurred. It would be very interesting to determine whether the effects of ROCK activation on the maintenance of stemness would persist upon removal of tamoxifen and return of actomyosin contractility to basal levels.

6. Rho signalling in ES cell maintenance, proliferation, survival

There have been significant recent advances in our understanding of the requirement for specific Rho GTPases and downstream signaling pathways in ES cells from gene knockouts, RNAi and small molecule inhibitors. However, what has been missing is an understanding of where and when Rho proteins are activated and inactivated, for example during adhesion or differentiation. Activation-state sensitive fluorescent probes have been developed and used to characterize the temporal and spatial patterns of Rho activation during tumor cell migration and invasion (Vega et al. 2011). One exciting complementary area of research will be the determination of Rho protein activation with spatial and temporal resolution during ES cell growth and differentiation, ultimately through progressive developmental stages

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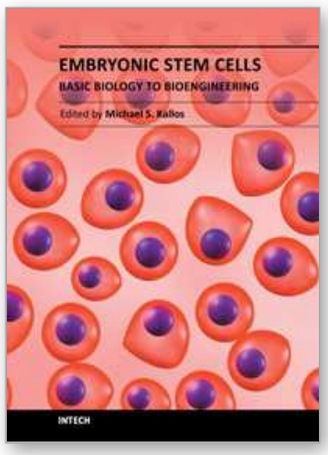

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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides 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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