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Abstract

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γ-Actin regulates cell migration and modulates the ROCK signaling pathway

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ABSTRACT Cell migration plays a crucial role in numerous cellular functions, and alterations in the regulation of cell migration are required for invasive transformation of a tumor cell. While the mechanistic process of actin-based migration has been well documented, little is known as to the specific function of the nonmuscle actin isoforms in mammalian cells. Here, we present a comprehensive examination of γ-actin’s role in cell migration using an RNAi approach. The partial suppression of γ-actin expression in SH-EP neuroblastoma cells resulted in a significant decrease in wound healing and transwell migration. Similarly, the knockdown of γ-actin significantly reduced speed of motility and severely affected the cell’s ability to explore, which was, in part, due to a loss of cell polarity. Moreover, there was a significant increase in the size and number of paxillin-containing focal adhesions, coupled with a significant decrease in phosphorylated paxillin in γ-actin-knockdown cells. In addition, there was a significant increase in the phosphorylation of cofilin and myosin regulatory light chain, suggesting an overactivated Rho-associated kinase (ROCK) signaling pathway in γ-actin-knockdown cells. The alterations in the phosphorylation of paxillin and myosin regulatory light chain were unique to γ-actin and not β-actin knockdown. Inhibition of the ROCK pathway with the inhibitor Y-27632 restored the ability of γ-actin-knockdown cells to migrate. This study demonstrates γ-actin as a potential upstream regulator of ROCK mediated cell migration.—Shum, M. S. Y., Pasquier, E., Po’uha, S. T., O’Neill, G. M., Chaponnier, C., Gunning, P. W., Kavallaris, M. γ-Actin regulates cell migration and modulates the ROCK signaling pathway. FASEB J. 25, 4423–4433 (2011). www.fasebj.org

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Actin is the most abundant and highly conserved protein in eukaryotic cells. Polymerized actin participates in a wide range of cellular activities, including cell motility, cell shape maintenance, polarity, and chemotaxis, as well as endocytosis and phagocytosis (1). In the cytoplasm of mammalian cells, there are two isoforms of nonmuscle actin, termed β- and γ-actin. They are highly conserved across species and differ by only 4 amino acids at the N terminus. Questions have often been raised about why it has been necessary for a cell to maintain two near-identical proteins throughout the process of evolution. Several observations have been made to suggest that β- and γ-actin are functionally distinct via differences in cellular localization of their mRNAs (2, 3), differential effect on cell morphology (4) and protein localization (5–8), and expression levels in different tissues (9, 10). The published data indicate an intricate system of regulation in nonmuscle actin isoforms, governing spatial segregation and tissue-specific expression. This system demonstrates the cell’s ability to recognize, sort, and modulate the expression of β- and γ-actin. Recently, alterations in nonmuscle γ-actin have been reported in several human illnesses. For instance, mutations in γ-actin have been linked to a specific type of dominant progressive deafness in humans (11, 12). Data from our laboratory have indicated that alterations of γ-actin, whether through mutations and/or altered expression, play a significant role in mediating antimicrotubule drug resistance in childhood acute lymphoblastic leukemia (13, 14). Although functional evidence exists for γ-actin in mediating human disease, the mechanisms by which these diseases occur are still unclear. To better understand γ-actin’s role in disease, an in-depth understanding of the functional and mechanistic role of γ-actin is required. Here, we present a series of experiments using RNAi to elucidate γ-actin’s role in cell adhesion, polarity, and migration, and the mechanism by which it regulates these cellular events.

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MATERIALS AND METHODS

Cell culture

Human neuroblastoma SH-EP cell line was maintained as monolayers in DMEM supplemented with 10% FCS. Human lung fibroblast MRC5 cell line was maintained as monolayers in MEM supplemented with 10% FCS, 1% nonessential amino acid, 1% sodium pyruvate, 2% sodium bicarbonate and 1% L-glutamine. Both cell lines were grown at 37°C in a humidified atmosphere with 5% CO₂ and were routinely screened and free of mycoplasma.

γ-Actin gene silencing by small interfering RNA (siRNA)

The knockdown of γ-actin by siRNA was carried out as described previously (14), with minor modifications. Briefly, SH-EP and MRC5 cells were plated onto 6-well plates at 1.2 × 10⁵ cells/well and transfected with γ-actin siRNA (5′-AA-GAGATGCGCCGGCTGTGCTA-3′; Qiagen, Valencia, CA, USA) or All Star negative control siRNA (Qiagen), using Lipofectamine 2000 reagent (Invitrogen), with Lipofectamine Plus (Invitrogen) before electroporation. Immunoblotting was performed using anti-p-cofilin (phospho-Ser3), anti-cofilin, anti-GAPDH (Abcam, Cambridge, UK), anti-paxillin (BD Transduction Laboratories, Lexington, KY, USA), anti-phospho-paxillin (Invitrogen), anti-phospho-myosin light chain 2 (Cell Signaling Technology, Beverly, MA, USA), polyclonal anti-γ-actin, anti-total actin (Sigma-Aldrich, St. Louis, MO, USA) (9) and monoclonal anti-β- and anti-γ-actin (7). The membranes were incubated with HRP-conjugated IgG secondary antibodies, and proteins were detected with ECL plus (GE Healthcare Life Sciences, Uppsala, Sweden). The blots were scanned using the Typhoon 9400 (GE Healthcare), which provides linear dynamic range for quantitative measurement and densitometric analysis performed as described previously (14).

Immunofluorescence

Immunofluorescence was carried out as described previously (14). Cells were grown on glass coverslips or 2- and 4-well chamber slides prior to fixing with formaldehyde (3.7% final concentration), permeabilized with 0.1% Triton X-100, and stained with anti-α-tubulin (Sigma-Aldrich), anti-pericentrin (Abcam), or anti-paxillin to visualize the microtubule network, microtubule organizing center (MTOC), and focal adhesions, respectively. Secondary antibody staining with Alexa Fluor 488-conjugated anti-mouse antibody and Alexa Fluor 568-conjugated anti-rabbit antibody (Molecular Probes; Invitrogen) was applied. Immunofluorescence staining protocol for β- and γ-actin antibody was previously described (7). Briefly, cultured cells were rinsed in cultured medium with HEPES (20 mM), prewarmed at 37°C, and then fixed with 1% paraformaldehyde (PFA) in DMEM (pseudowarmed at 37°C) for 15 min at room temperature. Cells were quickly rinsed with PBSs and then permeabilized with −20°C methanol for 5 min. Permeabilized cells were incubated with anti-β- and γ-actin monoclonal antibody for 1 h at room temperature. Secondary antibody staining with Alexa Fluor 488-conjugated IgG1 anti-mouse and Alexa Fluor 568-conjugated IgG2b anti-mouse antibody (Invitrogen) was applied to visualize β- and γ-actin, respectively. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was used to visualize nuclear material. Images were captured using an Axiovert 200 M fluorescent microscope (Zeiss, Oberkochen, Germany) and a ×63 1.4-NA Plan lens, coupled to an AxioCamMR3 camera and driven by the Axio Vision software (Zeiss). Pixel intensity values were calculated using the ImageJ pixel intensity analysis algorithm (15). Confocal microscopy images were acquired using an Olympus Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan) equipped with an oil-immersion Plan apochromat 1.4-NA ×100 objective lens. A Z series of images of 0.4 µm/section was collected using sequential scanning. Images were processed using the Olympus Fluoview software (Olympus).

Wound-healing, cell polarity, and transwell migration assays

SH-EP neuroblastoma cells were seeded at 48 h posttreatment with control or γ-actin siRNA in Ibbidi culture inserts (Ibbidi, Lübeck, Germany) mounted on 6-well plates precoated with fibronectin (10 µg/ml). At 24 h after seeding, the culture inserts were removed, leaving a definite cell-free gap of ~400 µm. Wells containing cells were washed, new culture medium was added, and cultures were placed in a 37°C humidified incubator for 2 h to allow the cells to initiate migration. The wound was then imaged at every hour up to 10 h after wounding using the ×5 objective of the same microscope device used for immunofluorescence experiments.

The cell polarity assay was carried out essentially in the same manner as a wound-healing assay. At 2 h after initial wounding, cells were fixed and stained for the MTOC as described above. Correct polarization is defined as the reorientation of the MTOC within 120° to the front of nucleus, in the direction of the wound.

Transwell migration was carried out according to the manufacturer’s guidelines (BD Transduction Laboratories). Briefly, SH-EP cells were serum starved for 2 h at 48 h siRNA transfection and seeded onto cell culture inserts in serum-free medium. Cells were driven to migrate toward the underside of the porous membrane (8.0 µm) in medium containing 10% FCS. Cells were placed in a 37°C humidified incubator overnight to allow migration through the pores. At 72 h after initial siRNA transfection, cells were fixed with 100% methanol for 20 min, and then were air dried for 1 h. Cells fixed onto the transwell membrane were stained with May-Grünwald and Giemsa stain (Sigma-Aldrich). The transwell membrane was dry mounted on glass slides, and migrated cells were visualized using the Olympus BH-2 microscope. Migration index was calculated as the number of migrated cells divided by the number of migrated cells, plus the number of nonmigrated cells.

Adhesion assay

siRNA-transfected cells were harvested and seeded at a cell density of 5 × 10⁵ cell/well in 96-well plates coated with increasing log concentration of fibronectin (0.1–10.0 µM). Cells were allowed to adhere for 45 min at 37°C in a humidified incubator with 5% CO₂. Nonadherent cells were washed away, and remaining cells were fixed with 50% crystal violet/methanol. Fixed cells were washed in PBS and lyzed.
with 10% SDS. Colorimetric reading at 570 nm was carried out on a Bio-Rad microplate reader.

**Live cell imaging**

γ-Actin- and control siRNA-transfected cells were grown on a FluoroDish (World Precision Instruments, Sarasota, FL, USA) in phenol red-free DMEM culture medium (Invitrogen) supplemented with 10% FCS. Immediately before imaging, 100 mM of ascorbic acid (Invitrogen) was added to the cells to prevent photobleaching.

**Random motility assay**

Random motility assay was carried out as described by Pourroy et al. (16). Briefly, SH-EP cells transfected with either control or γ-actin siRNA were seeded sparsely on a FluoroDish coated with fibronectin (10 μg/ml; Invitrogen) and imaged every 10 min for 4 h (16, 17). Cells were manually tracked, and their trajectories were recorded using AxioVision software (Zeiss). The random motility coefficient was calculated as previously demonstrated by Pasquier et al. (17). Persistence ratio was expressed as a sum of vectoral distance over total distance traveled (18).

**Rho-associated kinase (ROCK) signaling inhibition**

ROCK signaling was interrupted through the use of a specific ROCK inhibitor, Y-27632 (Sigma-Aldrich). Y-27632 (10 μM) was added to siRNA-transfected cells at 48 h post-transfection, and remained in culture medium for a further 24 h and during wound-healing experiments. Suppression of ROCK signaling was visualized via Western blotting using antibody against phosphorylated myosin regulatory light chain (MLC).

**Statistical analysis**

Results are expressed as means ± s.e of ≥3 independent experiments. For statistical comparisons, results were analyzed by 2-sided Student’s unpaired t test. Values of P < 0.05 were considered statistically significant.

**RESULTS**

**γ-Actin siRNA knockdown affects wound healing and transwell migration**

As a first step in investigating the functional role of γ-actin, siRNA was used to knock down γ-actin expression in human neuroblastoma SH-EP cells. Partial knockdown (34±7%, P=0.0092) was achieved in SH-EP cells compared to control cells at 72 h post-transfection (Fig. 1A). β-Actin expression was also examined to investigate whether there was isoform expression compensation. No significant change in the relative protein expression of β-actin was found in the γ-actin-knockdown cells compared to control siRNA-transfected cells (Fig. 1B). A small reduction in total actin was observed γ-actin-knockdown cells; however, this change was not statistically significant when compared to the control siRNA-transfected cells (Supplemental Fig. S1A).

One of the key functional features of actin is its role in cell motility. To determine whether γ-actin had a distinct role in this process, we examined the effects of γ-actin knockdown on cell motility using the wound-healing assay (Fig. 1C). A significant reduction in the percentage of wound closure was observed (26.8±5.2 vs. 62.5±8.1% in γ-actin-knockdown and control siRNA-treated cells, respectively, P=0.0022) after 10 h of migration (Fig. 1D). To further validate defects in migration, the transwell assay was employed to examine the effects of γ-actin knockdown on directed migration. γ-Actin-knockdown cells displayed a significant reduction in their ability to migrate through the transwell, as represented by the significant difference in the migration index (35.9±2.1 vs. 49.9±2.1% in γ-actin and control siRNA-treated cells, respectively, P<0.0001; Fig. 1E). Similar results were observed in human lung fibroblast MRC5 cells, where the knockdown of γ-actin (Supplemental Fig. S1B) significantly affected transwell migration (Fig. 1F).

**Knockdown of γ-actin affects random motility**

Suppression of γ-actin expression can reduce cell proliferation (14). To confirm that the effect of γ-actin knockdown of on cell migration is not due to reduced cell proliferation, a random cell motility assay was employed. Individual moving cells, excluding those undergoing mitosis, were tracked over a 4-h period, and their motility behavior was analyzed using the persistent random-walk model (19). The individual random-walk trajectory of 5 representative cells is shown in Fig. 2A. In cells treated with γ-actin siRNA, the migration pattern appears to be less random, and the cells do not explore their environment with the same efficiency as their control counterparts (Fig. 2A). This observation was confirmed by mathematical analysis of the cells’ random motility, where we observed a significant increase in the persistence ratio (0.41±0.05 and 0.24±0.02 in γ-actin and control siRNA-treated cells, respectively, P=0.018) coupled with a significant reduction in cell speed (0.49±0.02 and 0.62±0.03 μm/min in γ-actin and control siRNA-treated cells, respectively, P = 0.0001) in γ-actin-knockdown cells compared to the control cells. This led to a significant reduction in the random motility coefficient (1.4±0.1 and 2.0±0.1 μm²/min in γ-actin and control siRNA-treated cells, respectively, P=0.0025), which reflects the capacity of the cells to explore their microenvironment (Fig. 2B).

**Loss of polarity in γ-actin siRNA-treated SH-EP cells**

Intrinsic polarization of the cytoskeletal components is required for directed movement. A hallmark of cell polarity is the reorientation of the MTOC toward the front of the nucleus, in the direction of movement (20). To test the hypothesis that the depletion of γ-actin affects cell migration through alterations in cell polarity, the reorientation of the MTOC during directed migration was examined. Using pericentrin as a marker for the MTOC, and α-tubulin for the microtubule network, we observed that in the control siRNA-
treated cells at 2 h postwounding, the MTOC relocates to the front of the cells, toward the migration edge, in the majority of the cells. However, this process appears to be disrupted in γ-actin-knockdown cells (Fig. 3A, B). Interestingly, there was no obvious visible change to the microtubule network in γ-actin-siRNA-treated cells as compared to the control siRNA-treated cells. Quantitating the percentage of cells with correct MTOC orientation, as defined by reorientation to the front of the nucleus within 120° of the direction of the wound edge, we found a significant reduction in the percentage of cells with correct MTOC orientation in γ-actin-siRNA-treated cells (43.1 ± 1.2 and 64.6 ± 1.3% in γ-actin and control siRNA-treated cells, respectively, P<0.0001; Fig. 3C). This result demonstrates that partial knockdown of γ-actin significantly affects cell polarization at the initiation stage of cell migration.

**γ-Actin is involved in focal adhesion, distribution, and morphology, and reduces the level of phosphorylated paxillin at steady state**

To determine whether partial knockdown of γ-actin affects cell attachment, γ-actin and control siRNA-treated cells were examined for their ability to attach to extracellular matrix. Attachment to fibronectin was significantly decreased at low concentrations of fibronectin in the γ-actin-knockdown cells compared to control (Supplemental Fig. S2). As cellular attachment is, in part, governed by focal adhesions, SH-EP cells were immunostained for paxillin and counterstained with phalloidin to examine the distribution of focal adhesions and actin stress fibers, respectively. In control siRNA-treated cells, the distribution of paxillin is predominantly localized to the leading edge and the trailing edge. However, in the γ-actin-knockdown cells, such ordered localization of paxillin appears to be disrupted, following the localization of altered actin stress fibers, resulting in a random distribution of paxillin along the cell periphery (Supplemental Fig. S3).

SH-EP cells immunostained for paxillin demonstrated altered distribution of focal adhesions along with visible focal adhesion morphology changes (Fig. 4A). The knockdown of γ-actin led to an increase in the average size of focal adhesions (2.0 ± 0.1 and 1.1 ± 0.06 μm² in γ-actin and control siRNA-treated cells, respectively, P<0.0001; Fig. 4B), as well as a
significant increase in the average number of focal adhesions per cell (63.9 ± 4.8 and 40 ± 3.1 in γ-actin and control siRNA-treated cells, respectively, \( P < 0.0002 \); Fig. 4C).

To address whether the changes in focal adhesion distribution and morphology were due to changes in paxillin protein expression, a Western blot on siRNA-treated cells was performed. No significant difference in paxillin protein levels was observed between γ-actin and control siRNA-treated cells (Fig. 4D). In contrast, there was a small and statistically significant reduction in the levels of phosphorylated paxillin at tyrosine residue 118 in the γ-actin siRNA-treated cells (\( P = 0.0085 \)), indicating that the activity of paxillin is affected by γ-actin protein levels.

**γ-Actin knockdown alters actin isoform localization, drives stress fiber formation, and affects the phosphorylation of key actin regulatory proteins**

Spatial distribution of actin isoforms is important for their function. Using specific monoclonal antibodies against β- and γ-actin and immunofluorescence staining of SH-EP cells, we examined the localization of the nonmuscle actin isoforms. Confocal microscopy imaging of β- and γ-actin in SH-EP cells revealed that the two distinct isoforms occupy specific regions of the cell. We observed that in SH-EP cells, β-actin is predominantly localized to stress fibers (Fig. 5A), whereas γ-actin is observed as a fine meshwork of actin filaments throughout the cell body. Comparing the localization of the two nonmuscle actin isoforms, it appears that γ-actin is the isoform predominantly found in the cell periphery, near lamella and lamellipodial-like structures. These data complement a recent study in HSCF and HaCaT cells, where γ-actin was found to be localized as a meshwork in cortical and lamellipodial structures (7). However, when γ-actin is knocked down in SH-EP cells, an increase in stress fiber bundles is observed, resulting in a stress fiber phenotype that lacks lamella and lamellipodial-like structures. Such a change in stress fibers is similar to previous published results obtained using phalloidin staining (14). This is further supported by pixel-value line graphs in control and γ-actin siRNA-treated cells (Supplemental Fig. S4). The data show that in γ-actin-knockdown cells, the distribution between β- and γ-actin differs from control siRNA-transfected cells and reflects the prominent stress fibers described above (Supplemental Fig. S4).

The increase bundling of stress fibers in γ-actin-knockdown cells is suggestive of a change in the expression and/or phosphorylation of actin regulatory proteins. Hence, we examined the phosphorylation of MLC and cofilin. MLC is responsible for the regulation of nonmuscle myosin II, an actin motor protein that plays an essential role in actin filament organization and actin-based motility (21). Similarly cofilin regulates the actin cytoskeleton via its severing of filamentous

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**Figure 2. γ-Actin knockdown affects random motility.** A) Random migration traces for control and γ-actin siRNA-treated cells (5 representative cell shown). B) Histogram shows average (control, \( n = 100 \); γ-actin, \( n = 80 \)) persistence ratio, random motility coefficient, and cell speed. Error bars = se; \( n = 3 \). **\( P < 0.01 \), ***\( P < 0.001 \); Student’s \( t \) test.
actin, and hence, it increases the pool of free actin monomers to enable further filament formation at other cellular sites (22). Using Western blot analysis, we found that both phospho-MLC (Fig. 5B) and phospho-cofilin (Fig. 5C) were up-regulated in SH-EP cells when treated with \(\gamma\)-actin siRNA, demonstrating that expression levels of \(\gamma\)-actin can, in turn, change the activities of actin regulatory proteins. The increases in both phosphorylated paxillin and MLC is unique to \(\gamma\)-actin knockdown, and was not observed when SH-EP cells are treated with \(\beta\)-actin siRNA (Supplemental Fig. S5), suggesting that the mechanism involved in the alteration of stress fibers is unique to \(\gamma\)-actin and not through \(\beta\)-actin regulation.

**Inhibition of ROCK signaling rescues \(\gamma\)-actin-knockdown motility phenotype**

As the phosphorylation of cofilin and MLC share an upstream regulatory pathway through the small GTPase RhoA and its downstream effector ROCK (23), we examined whether \(\gamma\)-actin participates in activating ROCK signaling. SHEP cells transfected with control and \(\gamma\)-actin siRNA were thus treated with a selective ROCK inhibitor, Y-27632 (24), at 10 \(\mu\)M for 16 h prior to protein harvest. This concentration of Y-27632 did not affect cell proliferation or viability (data not shown). Western blot analysis revealed that the addition of Y-27632 was sufficient to negate the increase in phosphorylated MLC in \(\gamma\)-actin-knockdown cells (Fig. 6A). The suppression of ROCK signaling significantly enhanced cell motility in the wound healing assay, and it was able to, in part, compensate for the inhibition of motility caused by the knockdown of \(\gamma\)-actin (Fig. 6B). We observed a significant increase in the percentage of wound closure in cells treated with both \(\gamma\)-actin siRNA and Y-27632, as compared to siRNA alone. Collectively, these data show that \(\gamma\)-actin affects cell migration, in part, through ROCK signaling.

**DISCUSSION**

The functional diversity of the cytoplasmic actins has not been fully defined. Herein, we demonstrate that partial suppression of cytoplasmic \(\gamma\)-actin interferes with the cell’s ability to control cell motility via its effect on ROCK-mediated signaling. \(\gamma\)-Actin has a direct role in mediating key features of cellular movement and adherence.
The data presented here demonstrate an intricate level of regulation by γ-actin. Alterations in γ-actin protein levels interfere with the cell’s ability to control cell motility. Interestingly, these phenotypic changes occur at a knockdown level ranging between ~27 and 42%. Despite a relatively modest reduction in expression, numerous cellular components crucial in mediating migration are severely affected. A clear example is in the initiation of motility, in both directed migration, as seen from the results in wound healing and random migration. In the case of wound healing, cells at the wound edge are bound by their physical confinement to migrate in a uniform direction but are still unable to successfully migrate when γ-actin expression was knocked down. In random migration, where cells are allowed to migrate without the influence of external chemotactic stimuli, we find that knocking down γ-actin severely affects their overall cell speed and random motility coefficient due to the lack of initiation of movement. We believe that this lack of initiation is most likely due to several factors, including effects on cell polarity and cell adhesion dynamics.

Cell polarity is a process in which the asymmetric organization of cellular components and structures enables the establishment of directionality. During polarized migration, the MTOC reorients to a position between the nucleus and the leading edge (25, 26). This reorientation of the centrosome is controlled by a common set of signaling factors, including Cdc42 and the Par complex proteins (27, 28). In this position, the MTOC radiates microtubules toward the leading edge, carrying adhesion molecules to the site of new adhesions. In this study, we demonstrate that the knockdown of γ-actin significantly prevents the reorientation of the MTOC between the nucleus and the leading edge. The lack of a polarized cytoskeleton can, in part, explain the lack of migration initiation in γ-actin-knockdown cells. This is the first demonstration that γ-actin can regulate microtubule organization. While the mechanism in which γ-actin regulates MTOC reori-
entation is unclear, we hypothesize that crosstalk between the effectors of the Rho GTPases may play a deciding factor in the modulation of polarity (29). We observed a small but statistically significant increase in the phosphorylation levels of cofilin and MLC, both of which are regulated by RhoA signaling through ROCK. Since cells are homogeneous, a small change in the levels of phosphoproteins may reflect a larger effect on specific locations in the cells. The fact that there is a small, but statistically significant, increase in phosphocofilin must mean that there are significant differences in some locations in the cell. It is unlikely that the difference within the cell will be uniformly distributed.

Here, we propose that activated RhoA signaling inhibits Cdc42 and Rac1 signaling that governs cell polarity proteins, such as the Par complex. It has been previously shown that ROCK can phosphorylate Par3 in its aPKC-binding region, which results in disruption of the Par3-Tiam1-aPKC-Par6 complex, leading to altered cell migration (30). It has previously been demonstrated in astrocytes that during polarization, Cdc42 localizes and activates Par6 and aPKC at the leading edge, where aPKC phosphorylates GSK3β, thereby suppressing its activity and inhibits RhoA signaling (31). Our results have demonstrated that in SH-EP neuroblastoma cells, γ-actin is the predominant non-muscle actin isoform found localized toward the cell periphery in lamellar and lamellipodial-like structures. It is possible that such spatial occupation of γ-actin in the cell periphery may play an important role in the activity of the Par complex and that the displacement of γ-actin from the cell periphery caused by the treatment of γ-actin siRNA is likely to affect such interactions.

Figure 5. γ-Actin knockdown drives stress fiber formation. A) Representative images of control or γ-actin siRNA-transfected SH-EP cells, stained for β-actin (green), γ-actin (red), and DAPI (blue); highlighted box shows magnified view. Scale bars = 5 μm or as indicated. B, C) Western blot analysis of phospho-MLC expression (B) and phospho-cofilin expression (C) in SH-EP neuroblastoma cells 72 h posttreatment with control and γ-actin siRNA. Histograms show relative protein levels of expression, determined by densitometry from triplicate repeats and normalized to GAPDH. Error bars = se; n = 3, *P < 0.05, **P < 0.001; Student’s t test.
Interestingly, we demonstrate that the loss of cofilin activity through phosphorylation resulted in the loss of cell polarization (32). Interestingly, we demonstrate that γ-actin knockdown significantly increases the phosphorylation of cofilin leading to a nonpolarized cell state. Enhanced cofilin phosphorylation causes a reduction in the dynamics of the actin cytoskeleton (32). The reduction in dynamicity, as seen by the increase in ventral stress fibers and increased phosphorylation of MLC results in increase actomyosin tension (33). The inactivation of cofilin via phosphorylation prevents the turnover of actin filaments back into monomers, which are required for the formation of new filaments. Such increase in cellular tension also affects focal adhesion turnover at the trailing edge of migration, a necessary process in polarized migration (34).

It is well known that focal adhesions play an important role in providing a physical connection between actin stress fibers and extracellular matrix necessary for cellular movement (35). While focal adhesions are important components of cell migration, the inability to turn over established adhesions prevents the formation of new adhesion in membrane protrusions (36). Dynamic focal adhesions are required for fibroblastic cell migration, which relies on the cells’ ability to form new adhesion sites while recycling old adhesions to enable cell migration. In γ-actin-knockdown cells, paxillin morphology underwent significant alterations, such as increased size and number of paxillin-containing adhesions compared to the control siRNA-treated cells. The fact that the expression level of paxillin was not affected by γ-actin knockdown suggests that, without the turnover of old adhesion sites, there may not be sufficient paxillin to form new adhesions, thus leading to a decrease in cell motility. The effect is compounded by the significant reduction in phosphorylated paxillin in the γ-actin-knockdown cells, indicating that the paxillin activity is significantly perturbed.

The recruitment of focal adhesion kinase responsible for the phosphorylation of paxillin is required for adhesion disassembly (37). γ-Actin knockdown leads to dephosphorylation of paxillin, which, in turn, reduces the cell’s ability to turn over existing adhesion sites. However, it is not clear whether the alteration to turnover of paxillin adhesion is a direct consequence of γ-actin knockdown or whether it is secondary event through the loss of specific protein-protein interactions. Indeed, it is possible that the interaction between actin and actin binding proteins, such as tropomyosin, may affect paxillin phosphorylation and the turnover of focal adhesion complexes (18). Similarly, it is not known at this stage whether the changes in paxillin localization resulted from the altered stress fiber bundling caused by γ-actin knockdown, or whether the knockdown of γ-actin directly affected paxillin localization, thus contributing to increased actin stress fiber bundling.

What has been demonstrated is that the knockdown of γ-actin leads to increase in ROCK activity, as demonstrated by increased levels of phospho-MLC and phospho-cofilin. The specific inhibition of ROCK activity through the use of Y-27632 increased cell motility in γ-actin-knockdown cells, restoring it to control cell levels. These data indicate that γ-actin potentially acts either upstream of, or directly through, ROCK signaling to influence cell migration (Fig. 7); however, the γ-actin-knockdown effect on paxillin adhesion alterations may occur via a separate signaling pathway.

Given different cell types have specific functions in the body, it is highly likely that differences in actin isoform localization within the literature may reflect differences in cell type (38, 39). Earlier studies examining the effect of differential gene expression of β- and γ-actin demonstrated that in myoblast cells, overexpression of the β-actin gene promoted an increase in stress fiber bundling and cell surface area, whereas the
overexpression of γ-actin at the gene level produced a decrease in cell surface area and cell membrane changes, as well as changes in the localization and expression of adhesion proteins, vinculin and talin (4, 40). These alterations to cell morphology under the influence of actin isoform modulation in myoblast cells demonstrate a remarkable similarity to the results in this study, where a reduction in γ-actin protein levels via RNA interference alters cell morphology through changes in paxillin and actin stress fiber bundling, leading to disruptions in cell adhesion and motility. More importantly, these examinations conducted in SH-EP cells demonstrate that the alterations in the phosphorylation of paxillin and MLC were only found in γ-actin knockdown and not β-actin knockdown, further segregating the distinct functional regulation of the two isoforms of nonmuscle actin.

Recent data by Dugina et al. (7) focused on the differential regulation of cell motility by the modulation of nonmuscle β- and γ-actin. The results shown in our study further expand on the previous study by identifying the unique role of γ-actin in modulating cell motility through multiple levels of cytoskeletal regulation, not just in terms of localization of the actin isoforms, but also on cell polarity and adhesion dynamics. From the changes in cell migration through alterations in cell polarity, actin dynamics, as well as Rho GTPase signaling, we have discovered that γ-actin exerts a distinctive role in mediating cellular functions that is unique and not shared with β-actin.

Our results strengthen the notion that after the initial duplication of the actin gene, each has served a specific role, and the loss of function through mutations or altered expression leads to the development of human diseases (12, 14). Zhang et al. (41) recently reported a mechanism in which the cell is able to regulate differential degradation of nonmuscle β- and γ-actin due to the preferential arginylation of β-actin leading to a more stable protein structure. This result provides a potential explanation for the preferential localization of β-actin to stable actin stress fibers, as compared to the localization of γ-actin to the cell periphery where high levels of dynamic actin turnover are required. Since arginylated γ-actin is more likely to be degraded via ubiquitination, it may imply a unique method of localization regulation (41).

γ-Actin is associated with disease states, such as inherited deafness (12) and cancer (14). Our study has demonstrated the importance of γ-actin in mediating key cellular events, such as cell migration through modulation of cell polarity and focal adhesion dynamics. γ-Actin may mediate these effects, either directly or indirectly, via the Rho GTPase signaling pathway upstream of ROCK activity. Increased understanding of the role of γ-actin in key cellular events will lead to advances in tackling diseases associated with this actin isoform.

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REFERENCES


Figure 7. Proposed model of cell migration regulation by γ-actin. For details, see text. Briefly, formation of actin stress fibers is regulated by Rho GTPase signaling, in particular the activation of MLC and the inactivation of coflin via ROCK signaling. We propose that γ-actin functions to negatively regulate ROCK, as demonstrated when by the knockdown of γ-actin in SH-EP cells. Knockdown of γ-actin increases ROCK signaling, leading to an increase in the phosphorylation of MLC and coflin, which results in an overabundant actin stress fiber phenotype, leading to the repression of cell motility. When a specific ROCK inhibitor, Y-27632, is employed in γ-actin-knockdown cells, phospho-MLC is reduced, and normal cell motility is restored.

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