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RESEARCH ARTICLE



## Krüppel-like factor 4 mediates cellular migration and invasion by altering RhoA activity

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### ABSTRACT

Krüppel like factor 4 (KLF4) is a transcription factor that regulates genes related to differentiation and proliferation. KLF4 also plays a role in metastasis via epithelial to mesenchymal transition. Here, we investigate the function of Klf4 in migration and invasion using mouse embryonic fibroblasts and the RKO human colon cancer cell line. Compared to wild-type, cells lacking *Klf4* exhibited increased migration-associated phenotypes. In addition, overexpression of *Klf4* in *Klf4*<sup>-/-</sup> MEFs attenuated the presence of stress fibers to wild-type levels. An invasion assay suggested that lack of *Klf4* resulted in increased invasive capacity. Finally, analysis of RhoA showed elevated RhoA activity in both RKO and MEF cells. Taken together, our results strongly support the novel role of KLF4 in a post-translational regulatory mechanism where KLF4 indirectly modulates the actin cytoskeleton morphology via activity of RhoA in order to inhibit cellular migration and invasion.

### ARTICLE HISTORY

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### KEYWORDS

Krüppel-like factor 4; actin cytoskeleton; migration; invasion; RhoA; G protein

### Introduction

KLF4 is a zinc-finger transcription factor belonging to the Krüppel-like family that is involved in the regulation of a diverse group of biological processes such as apoptosis, differentiation, and proliferation (Garrett-Sinha et al. 1996; Shields et al. 1996; Hagos et al. 2009). While the function of KLF4 as a tumor suppressor has been demonstrated in multiple forms of cancers, its role as an oncogene has also been proposed due to its upregulation in squamous cell carcinoma and breast cancer, which suggests that its regulatory role in tumorigenesis is context-dependent (Ohnishi et al. 2003; Pandya et al. 2004; Zhao et al. 2004; Huang et al. 2005; Wei et al. 2005; Rowland and Peeper 2006; Zammarchi et al. 2010). We have previously shown that *Klf4* knockout in MEFs is associated with genomic instability and impaired autophagy during oxidative stress, resulting in increased DNA damage and apoptosis (Hagos et al. 2009; Liu et al. 2014; Liu et al. 2015). The genomic instability associated with lack of *Klf4* was additionally found to be reversible by transfecting KLF4-expression plasmids to *Klf4*<sup>-/-</sup> MEFs (El-Karim et al. 2013). Furthermore, KLF4 prevents epithelial to mesenchymal transition and metastasis of breast cancer both by

inhibiting Snail and activating E-cadherin (Yori et al. 2011). However, the specifics of the many potential tumor suppressor pathways in which KLF4 is involved are yet to be precisely defined.

Evidence suggests that KLF4 deficiency may be associated with cell migration and adhesion, which are important characteristics of tumor cells (Li et al. 2012; Lv et al. 2016). The organization of the actin cytoskeleton is closely related to the overall shape and morphology of the cell. In MEFs, more migratory cells have elongated phenotypes, which have been linked to morphological changes and alterations in cell motility (Goulimari et al. 2005; Thievensen et al. 2015). This raised a question as to whether KLF4 might play a role in the regulation of actin reorganization, a critical step in regulation of cell motility (Pollard and Borisy 2003; Yamazaki et al. 2005).

With respect to the regulation of cytoskeletal dynamics, proteins in the Rho GTPase family were considered to be attractive candidates, as Rho A, B, and C expression have been extensively shown to modify actin polymerization (Ridley and Hall 1992; Raftopoulou and Hall 2004). Specifically, RhoA activity is known to induce transformation and motility-associated actin

structures, including stress fibers, which can promote metastasis (Ridley 2013; Lei et al. 2014). The activity of Rho proteins, as in other small GTPases, is controlled via binding to either GTP, to induce an activated conformation, or GDP, for the inactivated state (Boguski and McCormick 1993). This process is regulated by Guanine nucleotide exchange factor (GEF), which acts as a catalyst for stimulating the release of GDP in exchange for GTP, and GTPase-activating protein (GAP), which increases the rate of GTPase activity with respect to Rho proteins (Van Aelst and D'Souza-Schorey 1997).

In the present study, we sought to investigate the mechanism by which Klf4 inhibits migration and invasion. Here, we show that MEFs and RKO cells lacking *Klf4* display increased frequency of morphology and actin reorganization favorable to migration. The morphological variation was supported by increased rates of migration and invasion for cells without *Klf4* expression. We further propose that this phenomenon involves a mechanism downstream of Klf4 that affects GAP or GEF proteins with respect to RhoA activity rather than directly affecting RhoA expression at the transcriptional or translational level.

## Materials and methods

### Cell culture, reagents, and drug treatment

Spontaneously immortalized primary MEFs, either wild-type (*Klf4*<sup>+/+</sup>) or null (*Klf4*<sup>-/-</sup>) for Klf4, were derived from terminated 13.5-day old embryos of crossbred mice heterozygous for Klf4 (*Klf4*<sup>+/-</sup>) on a C57BL/6 background to generate the homozygous knockout and littermate wildtype (Katz et al. 2002). Cells were passed about every three days following seeding with the 3T3 protocol (Todaro and Green 1963). All experiments were performed post-senescence and cells were discarded past passage 25 to maintain genomic integrity. The RKO (RKO-EcR-KLF4) cell line was derived from a human colon cancer cell line and stably transfected with the *pAdLoxEGF-KLF4* plasmid, as previously described (Chen et al. 2001). The plasmid contains the ecdysone-inducible promoter (EcRE), which is not naturally expressed by the human cell line, and a full-length *KLF4* gene that is naturally expressed at undetectable levels (Dang et al. 2001). The EcRE gene is conjugated to an enhanced GFP gene and accompanied by an internal ribosomal entry site. Therefore, KLF4 is conditionally expressed in RKO cells via the addition of Ponasterone-A (PA) (Sigma-Aldrich, St. Louis, MO, #P3490) and EtOH served as the solvent control (Dang et al. 2003). Both cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1%

penicillin/streptomycin. Cells were incubated at 37 °C in a 5% CO<sub>2</sub> environment. Cells were grown to appropriate confluency (60–70%) and then treated with 5 μM PA in EtOH for 3 days. For ROCK inhibition, the cells were treated with 10 μM Y-27632 (Sigma-Aldrich, St. Louis, MO, #Y0503). For serum starvation, the cells were washed with PBS and then incubated in 1% FBS.

### Western blotting

Cells were grown to appropriate confluency after which protein samples were extracted in lysis buffer, ground up with acid-treated glass beads, and then denatured by heating to 98 °C for 5 minutes. The lysates were loaded onto Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup> Precast Gels (Bio-Rad Laboratories, Hercules, CA, #4561096) in Tri/Glycine/SDS buffer (Bio-Rad Laboratories, Hercules, CA, #1610732) for electrophoresis. The gel was then transferred to a nitrocellulose membrane in Tris/glycine transfer buffer (Bio-Rad Laboratories, Hercules, CA, #1610734) with 10% methanol. The membrane was immunoblotted with primary antibodies, which in this study consisted of: GAPDH (housekeeping control, #97166), RhoA, (Cell Signaling, Danvers, MA, #2117), and RhoC (AbCam, Branford, CT, #ab64659). The HRP-conjugated secondary antibodies used were anti-mouse and anti-rabbit (Cell Signaling, Danvers, MA, #7076, #7074, respectively). The protein expression was then visualized by an Immun-Star<sup>™</sup> HRP Chemiluminescence Kit (Bio-Rad Laboratories, Hercules, CA, #1705070) and ChemiDoc<sup>™</sup> XRS + System (Bio-Rad Laboratories, Hercules, CA, #1708265) and imaged through Image Lab on a Gel Doc system.

### Phalloidin staining and morphology identification

Cells were fixed with 3.7% formaldehyde and then stained with DAPI and 100 nM Acti-stain 555 Phalloidin (Cytoskeleton, Inc., #PHDH1-A) for 30 minutes. Stained cells were visualized on an Olympus U-RFL-T fluorescent microscope and imaged using Infinity Analyze. For phenotypic analysis, actin stress fibers were qualified by the presence of directional non-uniform, filamentous structures limited to the interior of the cell. Filopodia were identified as thin actin structures that protruded beyond the cell body. Lamellipodia were identified as semicircular actin structures on the leading periphery of the cell body (Lehtimäki et al. 2017). Cells were plated at low confluency and allowed to incubate under standard cell culture conditions. After treatment conditions, cells were imaged at 10 random locations within the plate using brightfield microscopy on an Olympus U-RFL-T. In each image, the total cell number was

counted ( $n > 300$  cells per condition), and each individual cell was manually determined positive or negative for stress fiber, filopodia, and lamellipodia to create percentages. In conditions with plasmid transfection, only GFP positive cells were counted ( $n > 150$ ). For bright-field morphology analysis, migratory phenotypes were identified based on cell elongation for MEFs and a non-polar shape for RKO cells as supported by primary literature (Goulimari et al. 2005; Takahashi et al. 2014).

### Wound-healing assay

Cells were grown to high confluency (about 90%) and then switched to 1% FBS conditions before being scraped with the tip of a 200  $\mu$ L pipette adjacent to a reference mark. Brightfield microscopy was used to image initial size of scrape at time 0. Migratory rates were determined by comparing the area at initial and final time using ImageJ calculations. Serum starvation was utilized in order to compensate for the effects of proliferation (Liu et al. 2015).

### Invasion assay

QCM ECMatrix Cell Invasion Assay kit (EMD Millipore, Darmstadt, Germany; #ECM550) was used to examine the invasive characteristics of the cells. Cells were counted and plated in equal numbers ( $1 \times 10^6$  cells/ml) onto an invasion chamber which consisted of tissue culture plates with cell culture inserts that contain polycarbonate membranes with 8  $\mu$ M pores layered with thin ECMatrix<sup>TM</sup>. During 72 hours of incubation in the nutrient gradient, invasive cells were allowed to migrate from the 0.1% FBS top layer through the thin ECM layer into the lower 10% FBS supplemented DMEM and cling to the bottom of the membrane. The invasive cells were stained with Crystal Violet and then imaged via bright-field microscopy. The number of stained cells in each well was counted and the average number of cells for each data point was calculated.

### Transient transfection

MEF cells were transfected with 5  $\mu$ g/ $\mu$ L of DNA, either encoding GFP or Klf4 conjugated to GFP on a EGFP plasmid backbone, using the Lipofectamine 3000 Transfection Reagent Protocol (Thermo Fisher, Waltham, MA, #L3000015) at 70% confluency as previously described (Liu et al. 2015). The efficiency of the transfection was observed under an Olympus IX51 microscope 24 hours post-transfection.

### RhoA activation assay

Cells grown under normal conditions were treated in serum starvation condition (1% FBS) for 6 hours, after which the samples were collected and snap frozen in liquid nitrogen to be preserved. After the protein quantity was made equivalent across samples by measuring the protein concentration using a Bradford reagent via the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA), Rhotekin-RBD beads and other reagents from the RhoA pulldown activation assay Biochem Kit (Cytoskeleton, Inc., Denver, CO, #BK036-S) were added to the protein lysates and then centrifuged as described in the product protocol provided by the company. A Western Blot was then performed on the proteins that remained bound to the beads using a special RhoA primary antibody provided by the company.

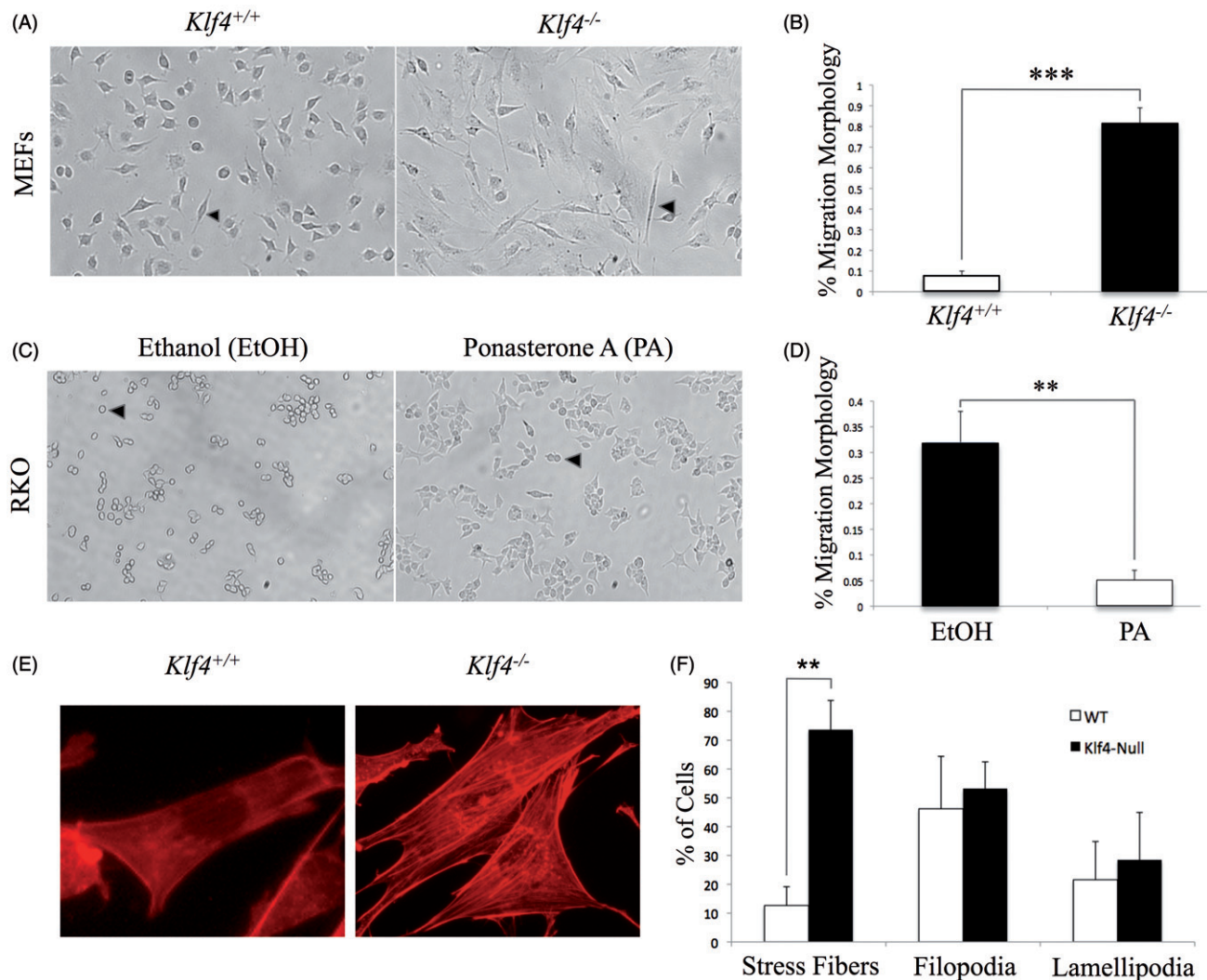
### Statistical analysis

GraphPad QuickCalcs software was used to perform an unpaired  $t$  test to evaluate the difference between trials where indicated.  $p$  Values below .05 ( $p < .05$ ) were considered statistically significant. Graphpad prism was used to generate some figures. In all figures, error bars indicate standard deviation.

## Results and discussion

### Klf4-deficient cells exhibit an altered morphology associated with migration when compared to cells expressing Klf4

A visual comparison of  $Klf4^{+/+}$  and  $Klf4^{-/-}$  MEFs under brightfield revealed consistent differences in morphology.  $Klf4^{+/+}$  cells showed a less protruding morphology typical of fibroblasts while  $Klf4^{-/-}$  cells were more elongated (Figure 1(A)). Quantification of individual cell morphologies between  $Klf4^{+/+}$  and  $Klf4^{-/-}$  MEFs showed that the difference was statistically significant at a  $p$  value of  $<.0001$  (Figure 1(B)). Furthermore, our data are consistent with previous research that found an association of elongated morphology with greater cell motility in MEFs (Goulimari et al. 2005). In order to determine if the elongated, migratory phenotype was also present in the established RKO colon cancer cell line, containing an inducible *KLF4* construct, the *KLF4*-deleted RKO cells were treated with PA or with EtOH as a control for three days prior to visualization. The PA-treated cells expressing *KLF4* presented a greater number of protrusions compared to the EtOH-treated cells, which exhibited a more typical compact epithelial shape (Figure 1(C)). Quantification of the differences revealed that they were statistically significant



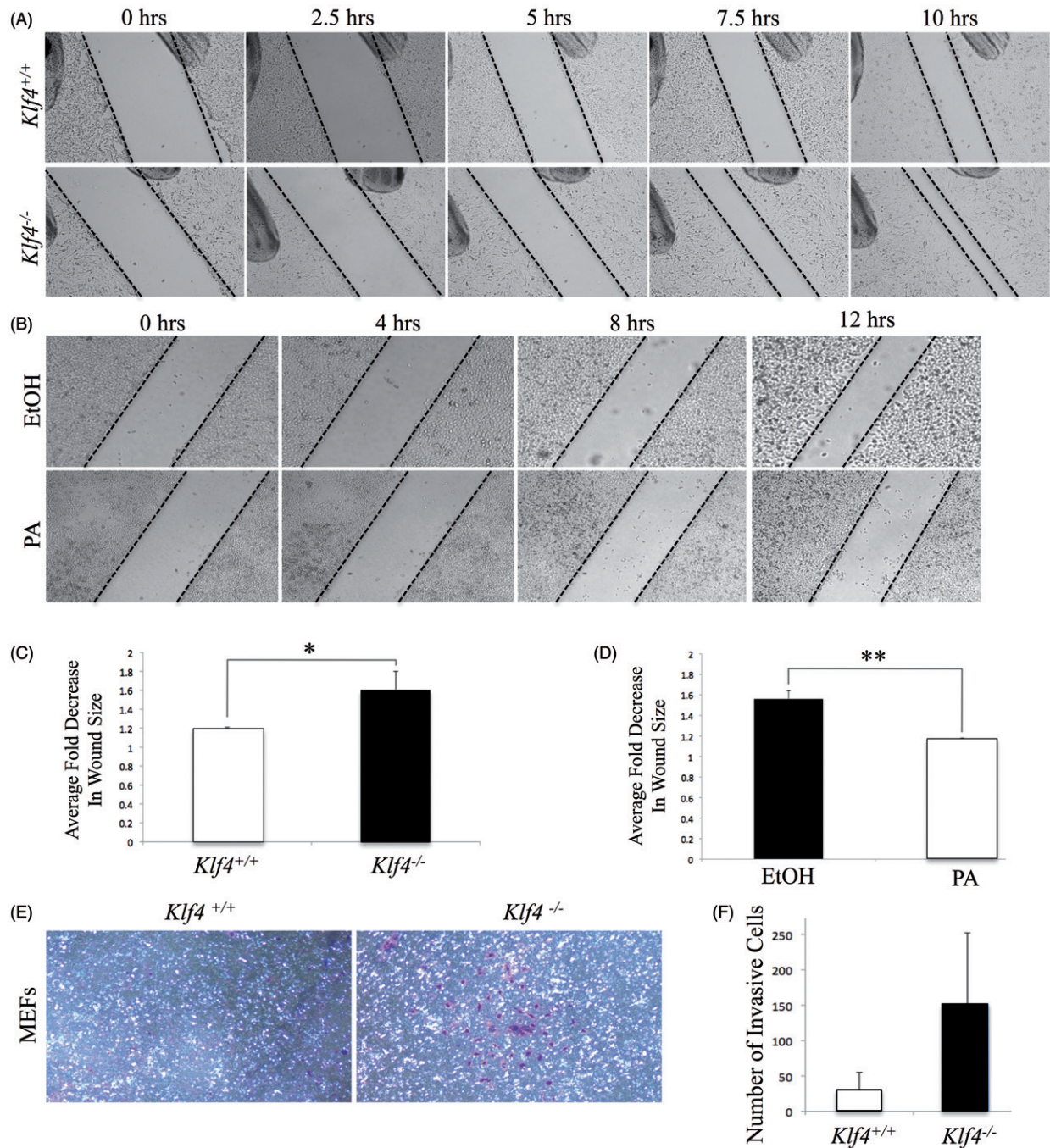
**Figure 1.** *Klf4* deficient cells exhibit an altered morphology associated with migration when compared to cells expressing *Klf4*. (A) Representative wild type and *Klf4*-null MEFs morphology. Early passage cells were imaged using brightfield at 10× magnification. (B) Quantification of migratory morphology in A with  $n = 9$  and  $p \leq .0001$ . (C) Repeat of procedure in A, but using ethanol and PA-treated RKO cells. (D) Quantification of migratory morphology in C with  $n = 3$  and  $p = .0036$ . (E) Representative wild type and *Klf4*-null MEFs stained with phalloidin 555 nm at 40× magnification. (F) Quantification of phalloidin-stained actin structures for wild type and *Klf4*-null MEFs,  $n = 3$  and  $p = .0071$  (stress fibers),  $p = .7481$  (filopodia), and  $p = .7598$  (lamellipodia). Arrows indicate a migratory phenotype. \*\*Indicates  $p \leq .01$ , \*\*\* indicates  $p \leq .001$ . Error bars indicate standard deviation of replicates.

with a  $p$  value of .0036 (Figure 1(D)). Previous work by Takahashi et al. (2014) has demonstrated that the non-polar morphology in RKO cells was correlated with increased migration. Given the role of actin polymerization in cell morphology and migration, the MEFs were stained with phalloidin to visualize the actin cytoskeleton (Figure 1(E)). We found no significant difference in the percentage of cells expressing lamellipodia and filopodia between wild-type and *Klf4*-null MEFs, with  $p$  values equal to .7598 and .7481, respectively (Figure 1(F)). However, there was a significant difference in the presence of stress fibers with a  $p$  value equal to .0071. These findings led us to hypothesize that the particular increase in stress fibers in *Klf4*-null cells may

result in more motility-enabled cell populations (Goulimari et al. 2005) (Figure 1).

#### *Klf4* null cells exhibit increased rate of migration

In order to confirm the role of *Klf4* in migration, scratch assays were performed with both MEFs and RKO cells. Wild-type and *Klf4*-null MEFs were plated to confluence, cultured in reduced serum condition to synchronize cell cycles and limit cellular proliferation, and then scratched. The change in wound area caused by the cells migrating into the empty space, relative to a reference mark, was documented via brightfield microscopy at specific time points after generation of the



**Figure 2.** *Klf4* deficient cells present with increased migration as well as invasive trend compared to cells expressing *Klf4*. (A) Wild type and *Klf4*-null MEF monolayer scraped with a pipette in 1% serum starvation and imaged at 4× magnification. (B) Ethanol and PA treated RKO cell monolayer in 1% serum starvation imaged at 4× magnification. (C) Quantification of averaged fold decrease in area of A between  $t=0$  and  $t=7.5$ ,  $n=5$  with a  $p$  value of .041. (D) Quantification of averaged fold decrease in area of B between  $t=0$  and  $t=12$ ,  $n=3$  with a  $p$  value of .0028. \* indicates  $p \leq .05$ , \*\* indicates  $p \leq .01$ . (E) Matrigel invasion assay after 72-hour incubation period, after which invasive cells were stained and documented via brightfield microscopy at 10× magnification. (F) Quantification of number of cells present in each respective well;  $n=2$  for MEFs and  $p=.196$ . Error bars indicate standard deviation of replicates.

wound (Figure 2(A)). *Klf4*<sup>-/-</sup> MEFs demonstrated increased wound closure compared to wild-type over the time period with a  $p$  value equal to .041 (Figure 2(C)). After treatment with PA and EtOH under reduced serum conditions, the RKO cells were scratched,

following the identical procedure as the MEFs (Figure 2(B)). Comparing 0 hours to 12 hours post scratch indicated that the RKO cells treated with EtOH were more migratory compared to those treated with PA. The quantification of the difference in migration was

statistically significant with a  $p$  value of .0028 (Figure 2(D)). Our findings are consistent with previously published data involving colon cancer cell model that demonstrated overexpression of *Klf4* inhibits migration (Dang et al. 2003). Here, we demonstrate in a non-transformed, MEF cell line that *Klf4* expression inhibits migration (Figure 2).

#### ***Klf4* deficient cells show a greater invasion trend compared to cells expressing *Klf4***

To determine the invasive capacity of cells null for *Klf4*, an invasion assay utilizing matrigel was performed. MEFs were seeded in the matrigel invasion assay wells and incubated for 72 hours. Documentation of Crystal Violet-stained membranes through brightfield microscopy revealed that *Klf4* null cells have higher invasive potential (Figure 2(E)). Quantification of all the invasive cells in the wells showed an average of 152 *Klf4*<sup>-/-</sup> MEFs were capable of passing through the synthetic membrane into 10% FBS supplemented media. In contrast, only an average of 32 *Klf4*<sup>+/+</sup> displayed an invasive phenotype (Figure 2(F)), showing almost a fivefold increase with loss of *Klf4*. The trend, of MEFs lacking *Klf4* showing increased invasive capability, is consistent with previous findings demonstrated in RKO cells (Dang et al. 2003). However, our invasion assays carried high variability, which may have been due to the absence of stronger chemoattractant across the basal polycarbonate membrane. *In vivo*, metastasis is driven by chemoattraction, to which cells with invasive phenotypes can respond, an aspect our assay is lacking (Zhou et al. 2014; Orellana et al. 2015). The observed data in combination with Dang et al. (2003) suggests the ability of cells lacking *Klf4* to more easily cross the matrigel membrane due to the function of KLF4 in inhibiting invasion.

#### ***KLF4* overexpression reduces stress fiber formation in *Klf4*<sup>-/-</sup> MEFs**

To confirm the percentage of cells expressing migration-associated stress fibers, mock transfected MEFs wild type or null for *Klf4* were co-stained with phalloidin and DAPI (Figure 3(A)). Visualization by fluorescent microscopy of mock transfected *Klf4*<sup>-/-</sup> MEFs revealed a statistically significant increased level of stress fibers, 68.2%, compared to mock transfected *Klf4*<sup>+/+</sup> MEFs, 7.1%. The calculated  $p$  value was equal to .00731 (Figure 3(B)). To confirm the function of *Klf4* in stress fiber formation, *Klf4*-deficient MEFs were either transfected with plasmids *GFP* or *GFP* conjugated to *Klf4* before being fixed and stained with phalloidin and DAPI (Figure 3(A)). Scoring cells based on the presence

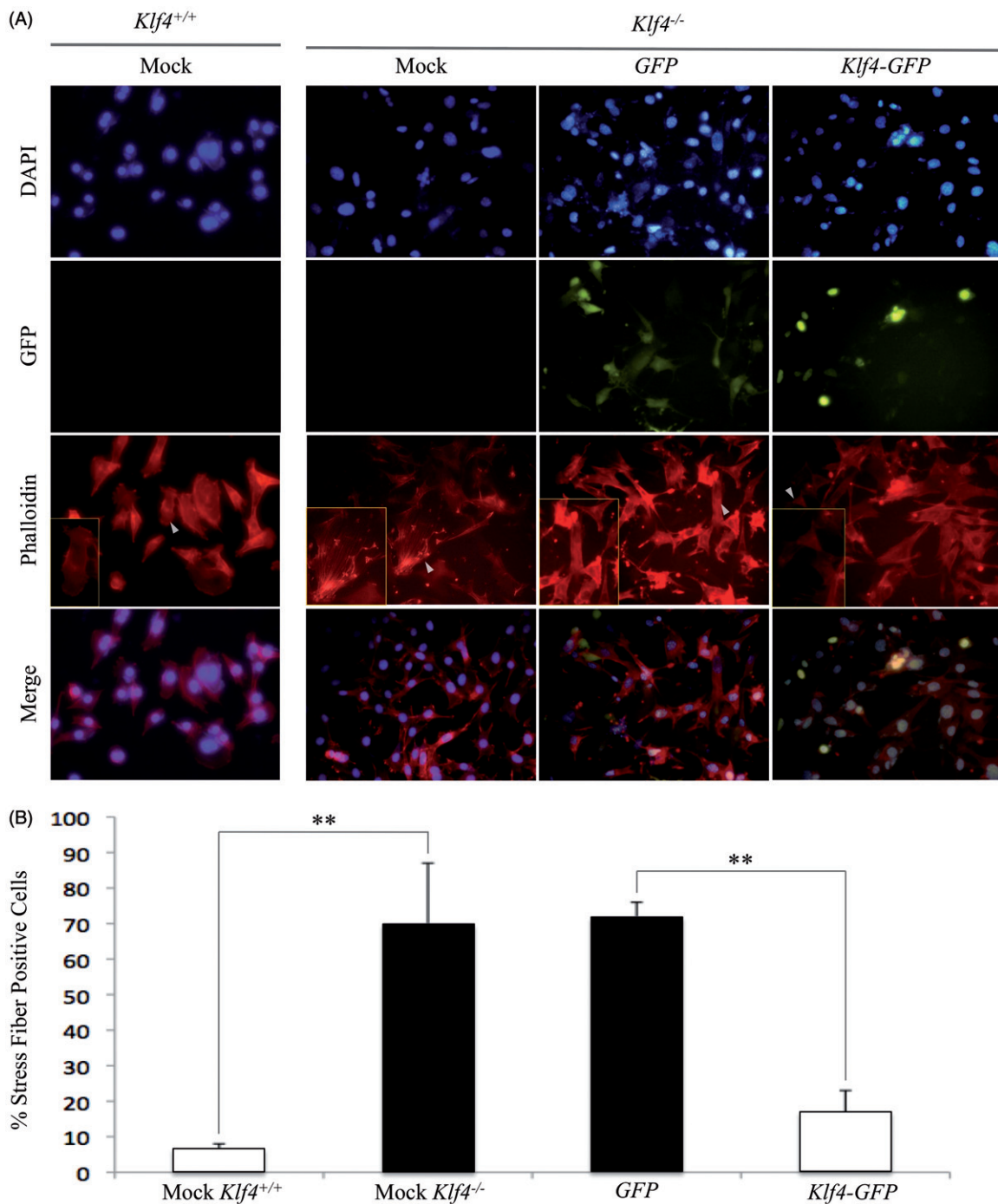
or absence of stress fibers indicated that 71.5% of *GFP*-transfected MEFs expressed the migratory phenotype compared to only 17% of *Klf4*-*GFP* transfected MEFs (Figure 3(B)). The difference in stress fibers between *GFP* and *Klf4*-*GFP* transfected cells was statistically significant with  $p$  equal to .0097. The similar rate of stress fibers for *Klf4* null MEFs and *GFP*-transfected MEFs compared to *Klf4* wild-type cells and *Klf4*<sup>-/-</sup> cells transfected with *Klf4*-*GFP* suggests that *Klf4* is sufficient to regulate stress fibers (Figure 3).

#### ***Lack of *Klf4* does not alter *RhoA* or *RhoC* gene expression***

Taken together, our data and previous research demonstrates that *Klf4* inhibits motility-associated phenotypes. We therefore wanted further insight into the mechanism by which *Klf4* mediates the dynamics of the actin cytoskeleton. Since Rho GTPases are known to coordinate the dynamics of the actin cytoskeleton, our efforts were focused on identifying a potential relationship between KLF4 and Rho GTPases (Ridley 2013; Lei et al. 2014). We began by testing the expression of *RhoA/B/C* family proteins with an antibody that targets a shared epitope, finding increased *RhoA/B/C* expression in *Klf4*<sup>-/-</sup> compared to *Klf4*<sup>+/+</sup> (data not shown). Further testing with *RhoA* and *RhoC* was selected following a decrease in stress fibers after treatment with ROCK inhibitor (Figure 4(A)), which is a key effector in signal transduction of the *RhoA* and *RhoC* pathways in stress fiber formation and motility (O'Connor and Chen 2013). *RhoB* was not considered for testing due to its role in cell survival and not motility (Wheeler and Ridley 2004; Ridley 2013). Probing with *RhoA* (Figure 4(B)) and *RhoC* (Figure 4(C)) specific antibodies demonstrated no discernible difference in relative protein expression. Our findings are consistent with prior microarray data as no differences were observed between *Klf4*<sup>+/+</sup> and *Klf4*<sup>-/-</sup> MEFs for *RhoC* at the mRNA level (Hagos et al. 2011). However, this microarray data identifies upregulated expression of *RhoA* mRNA, a difference that does not translate to protein expression as shown in our western analysis. The lack of a translational regulation by KLF4 is also supported with previously published ChIP-seq data for KLF4 demonstrating no significant binding score between KLF4 and regulatory regions controlling *RhoA* or *RhoC* expression (Chen et al. 2008) (Figure 4).

#### ***Klf4*-deficient cells exhibit increased *RhoA* activity as demonstrated by upregulated GTP-*RhoA***

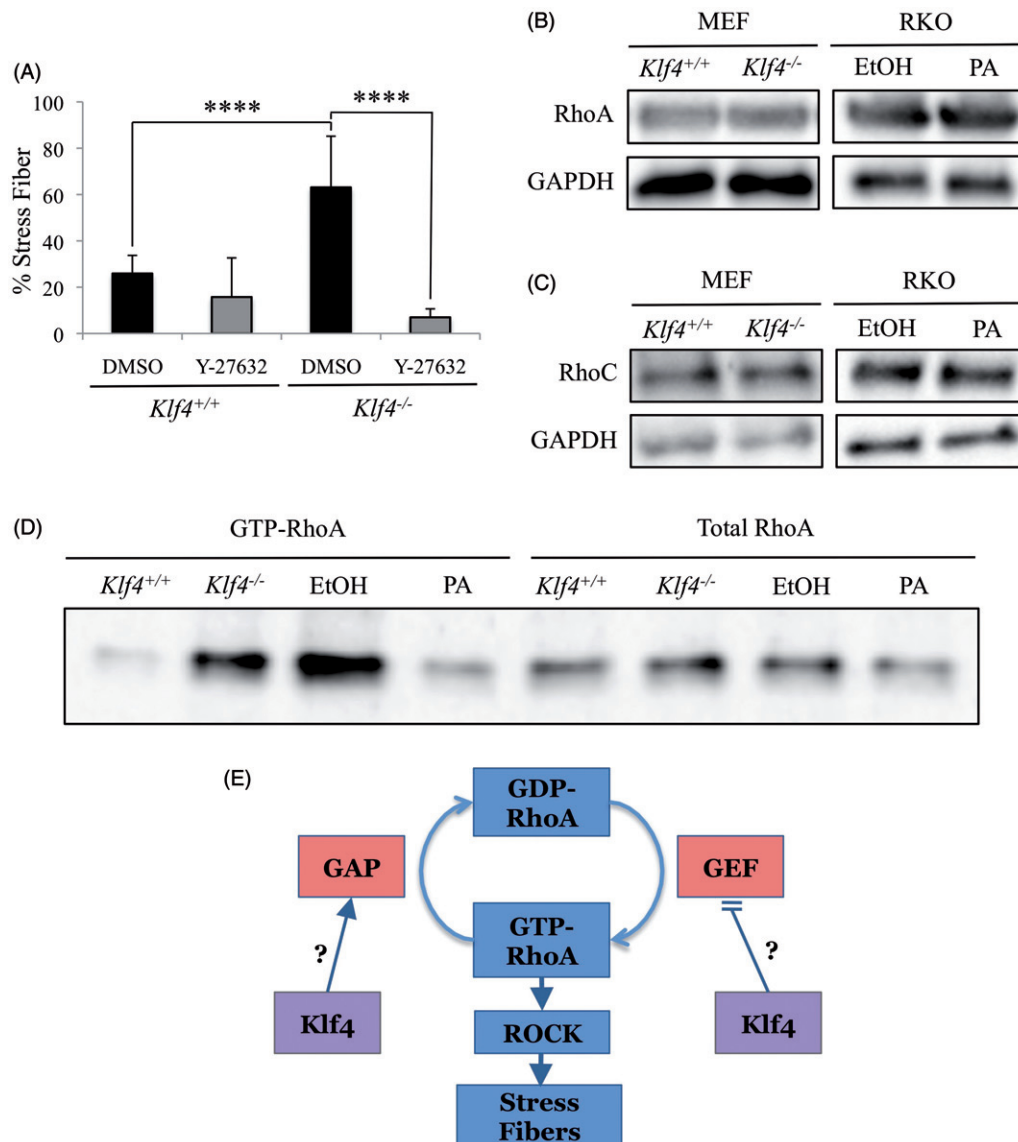
*RhoA* is a GTPase that has been shown to induce migration via stress fiber formation when activated



**Figure 3.** Reintroduction of *Klf4* reduces stress fiber formation in *Klf4*-null MEFs. A) Representative images of DAPI and Phalloidin 555 stained images at 20 $\times$  magnification. Arrows indicate boxed in cells showing representative cells of the population. (B) Quantification of stress fibers in A with  $n=3$  and  $p=.0097$  for *Klf4*-GFP and GFP-transfected MEFs and  $n=3$  and  $p=.00731$  for mock wildtype and mock *Klf4*-null transfected MEFs. \*\* indicates  $p \leq .01$ . Error bars indicate standard deviation of replicates.

(Chen et al. 2014). RhoA activity is regulated by its binding to GTP or GDP, which is in turn regulated by GAP and GEF (Boguski and McCormick 1993; Van Aelst and D'Souza-Schorey 1997). The relationship raises the possibility that RhoA regulation may happen posttranslationally rather than through the gene expression. In order to examine if the presence or absence of *Klf4*

correlates with GTP-bound RhoA, we assayed the binding of RhoA to the Rho-binding domain of Rhotekin, a Rho effector protein with a high affinity for the GTP-bound form of RhoA (Ren et al. 1999). MEFs and RKO cells were starved at 1% FBS to maintain a controlled state prior to RhoA pulldown and subsequent western blotting. We found that both *Klf4*<sup>-/-</sup> MEFs and RKO



**Figure 4.** *Klf4* deficient cells have elevated GTP-RhoA. (A) Quantification of phalloidin acti-stain 555 and DAPI co-stained MEFs, either wildtype or *Klf4*-null, treated with Y-27632 for 24 hours at 10 $\mu$ M;  $n = 3$ . \*\*\*\* indicates  $p < .0001$ . (B) Representative images of western blots. RhoA primary (21 kDa) antibody, GAPDH (37 kDa) antibody. (C) Representative western blots hybridized with RhoC (19 kDa) antibody, GAPDH (37 kDa) antibody. (D) Representative RhoA activity assay and western blotting of lysate from the RhoA activity assay prior to GTP-RhoA isolation with  $n = 3$ . (E) Proposed mechanism of Klf4 in stress fiber formation. Error bars indicate standard deviation of replicates.

cells lacking *KLF4* expression exhibited elevated levels of GTP-RhoA compared to *Klf4*<sup>+/+</sup> MEFs and *KLF4*-induced RKO cells (Figure 4(D)). Quantification of these results reveals a fourfold elevation in RhoA activity in both RKO and MEF cells lacking *Klf4* expression (data not shown). Our findings are consistent with previous work where GTP-RhoA is increased in MEFs that are more migratory (Goulimari et al. 2005). The elevated levels in the absence of *Klf4* expression indicate a possible mechanism in which *Klf4* regulates either GEF or GAP to alter RhoA activity. To increase GTP-bound RhoA, either GEF may be upregulated, or GAP downregulated. Our data suggests that in wild-type cells, *KLF4*

either inhibits GEF or activates GAP to decrease the level of GTP-RhoA, such that a decrease in the rate of stress fiber formation, associated with migration, would be observed (Figure 4(E)). Compatible with our findings, the ChIP-seq data present numerous GEFs and GAPs to whose regulatory regions *KLF4* significantly binds (Chen et al. 2008).

Future work is required to determine the specific GEF or GAP that is transcriptionally regulated by *Klf4*. Luciferase assays could be used to determine direct *KLF4* binding to GEF or GAP regulatory gene sequences. Expanding our understanding of the molecular mechanism may prove valuable in better describing the

metastatic process and identifying specific druggable targets further downstream. Designing a drug to prevent migration and invasion of *Klf4* negative cancers, by targeting selected components of the pathway, could enhance the effectiveness of current cancer treatments.

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## Disclosure statement

All authors declare that they have no competing interests at this point of time.

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