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Knockdown of kinesin KIF11 abrogates directed migration in response to epidermal growth factor-mediated chemotaxis

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ABSTRACT

Establishment of microtubule polarity is critical for directional cell migration involved in morphogenesis, differentiation, cell division, and metastasis. Current models, involving iterative microtubule capture and inactivation of microtubule depolymerizing mechanisms at the leading edge, cannot account for the biased migration exhibited by cells in culture in the absence of directional cues, suggesting central mechanisms governing microtubule polarity remain unknown. We engineered two human MDA-MB-231/IMP1 breast carcinoma cell lines, denoted kdKIF11-1 and kdKIF11-2, in which the kinesin KIF11 (also known as Eg5) was stably knocked down by two different shRNAs. Western blot analysis showed knockdown by each shRNA decreased KIF11 expression by 58% and 79% for kdKIF11-1 and kdKIF11-2, respectively, whereas Rac1 expression was unaffected. All cell lines retained a well-defined microtubule structure. Compared to cells infected with the control viral vector, both KIF11 knockdown cell lines displayed a 14–45% increase in cell motility in a scratch wound healing assay. In contrast, KIF11 knockdown decreased invasion by 70%, compared to the control, as measured by invasion through Matrigel-coated transwells. To determine whether the reduction in invasion was due to reduced chemotaxis, we substituted collagen for Matrigel in the transwell assay and similarly observed a 44–54% reduction in migration, using EGF as the chemoattractant. However, when including EGF in both the upper and lower chambers of the transwell to stimulate migration but eliminate chemotaxis, transwell migration decreased for the control cell line only, indicating that KIF11 knockdown did not impair migration, but severely impaired chemotaxis. We conclude KIF11 is a key downstream molecule that responds to directional cues in chemotaxis to govern the direction of migration.

1. Introduction

Directional cell migration is critical for morphogenesis, differentiation, cell division, and metastasis. Much is known about the involvement of the actin cytoskeleton in providing the motive force for migration, including the activation of actin filament nucleation at the leading edge by CDC42- and Rac1-dependent mechanisms, cleavage and nucleation by gelsolin and formins, and polymerization of actin by profilin to propel the leading edge of the cell forward [1]. Although the motive force and velocity of movement is provided by these mechanisms, these mechanisms alone do not account for the directional persistence of movement displayed by cells in the absence of chemotactic signals. The importance of polarity in chemotactic response and directional persistence is reflected in *in vivo* observations demonstrating similar cell velocity, but random migration of non-metastatic tumor cells vs. the chemotactically driven, blood vessel-directed migration of metastatic breast cancer cells [2].

Microtubules have long been known to determine cell polarity [3]. Treatment of fibroblasts with microtubule-disrupting drugs disrupts polarity without inhibiting membrane protrusion or membrane ruffling [4,5]. In addition, microtubules can generate force to push the leading edge forward, possibly through a kinesin-1 sliding mechanism following microtubule capture at the leading edge [6–8], and deliver both membranes [9,10] and signaling proteins, including APC, Rac, Cdc42, and IQGAP [6,11,12], for self-sustaining leading edge protrusion. However, all of these mechanisms presuppose microtubule polarity and are generally directed toward persistent activation of actin dynamics at the leading edge.

Mechanisms regulating the establishment of microtubule polarity are not well known. However, kinesins are likely candidates for regulating microtubule polarity. KIF1C, a kinesin-3 family member,

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and KIF9, a kinesin-9 family member, promote the interaction of microtubule plus ends with podosomes, and control podosome turnover and localization [13,14]. Both cell mitosis and cell migration are polar processes and commonly involve centriole positioning. During mitosis, duplicated centrioles migrate to form an oriented bipolar structure such that cell division occurs specifically either in the plane or perpendicular to the tissue axis, and disruption of mitotic polarity in stratifying epithelia can result in disorders such as polycystic kidney disease, over-proliferation and neoplasia [15–17]. Centrioles also translocate to the side of the nucleus facing the new leading edge in cells undergoing a change in migratory direction [18,19]. Due to the involvement of the kinesin KIF11, a kinesin-5 family member, in the orientation and initial separation of centrosomes to form the spindle poles at the beginning of mitosis [20], we hypothesized that KIF11 acts as a sensor to orient the centrosome and polarize the direction of migration in cells undergoing a chemotactic response. We show that knockdown of KIF11 in MDA-MB-231(GFP-IMP1) cells accelerates cell motility in a wound healing assay, but reduces epidermal growth factor (EGF)-mediated chemotaxis in collagen-coated transwell assays, and serum-induced invasion in Matrigel-coated transwell assays. These results suggest that KIF11 is required for the response to directional cues in migrating cells.

2. Material and methods

2.1. Cell culture and reagents

For our experiments, we chose the MDA-MB-231(GFP-IMP1) cell line to examine effects of KIF11 on cell motility. We previously showed these cells display an intermediate migratory phenotype [21], enabling us to detect increases or decreases in cell motility. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Hyclone, Guangzhou, China) and 100 U/ml penicillin and 100 U/ml streptomycin. Epidermal growth factor was purchased from Sino Biologicals (Beijing, China).

2.2. Knockdown of KIF11 by shRNA

Modified V3LHS391759 and V3HLS643569 pGIPz lentiviral vectors (Thermo Scientific Open Biosystems, Pittsburgh, PA), encoding KIF11 shRNA sequences AACAATCTACATGAACTACAA and CCGAAGTGTTGTTTGTCCA, were used to engineer the kdKIF11-1 and kdKIF11-2 cell lines, respectively. Initially, the TurboGFP was deleted from the plasmid vector, then KIF11 shRNA-encoding lentivirus was generated, according to the manufacturer's instructions, and used directly to infect cells. Three days after infection, cells were selected in growth medium supplemented with 8 μg/ml puromycin for approximately 2 weeks, until a stably growing population arose, after which cells were continually cultured in the absence of puromycin. Control cells were similarly selected following infection with a lentivirus encoding the puromycin selection gene alone. Although most experiments were performed within 8 passages after selection, we observed no significant difference in results at up to 20 passages in our transwell invasion and migration assays.

2.3. Wound healing assay for cell migration

Confluent cultures in 35 mm dishes were incubated in DMEM + 1% FBS for 8 h to achieve quiescence. Circles 3 mm in diameter were marked on the bottom of each dish to identify the areas for image capture and ensure that measurements were taken at the same locations. A wound was made by scratching the monolayer, with a sterile pipette tip through the circled areas, then cells were rinsed twice with phosphate-buffered saline and refed with DMEM + 10% serum. Phase-contrast micrographs of the circled areas were taken immediately after refeeding and after 12-h and 20-h incubations at 37 °C. The distance of migration was calculated from 6 images, using ImageJ analysis. Cell velocity was calculated by taking five random, evenly-spaced measurements along each wound length and averaged to determine wound width (μm).

2.4. Transwell assays for invasion and chemotaxis

Cell invasion assays were performed using commercially available Boyden chamber transwells (8 μm pore size, Millipore, Bellarica, MA) that we layered with 100 μl of either 50 μg/ml collagen (chemotaxis assays) or 100 μg/ml Matrigel (BD Biosciences, San Jose, CA). Confluent cultures were incubated in DMEM + 1% serum for 8 h to achieve quiescence. For invasion assays, 750 μl DMEM + 10% serum was added to the lower chamber. Then cells were resuspended in DMEM + 1% FBS to a concentration of 5×10^4 cells/ml, 500 μl cell suspension was added to the upper chamber, and cells were allowed to invade for 14 h at 37 °C under a humidified 5% CO_2 atmosphere. Transwell chemotaxis assays were similarly performed except that 750 μl DMEM + 1% serum with 10 ng/ml EGF (Sino Biologicals, Beijing, China) was added to the lower chamber and cells were allowed to migrate for 6 h. Cells remaining in the upper chamber were scraped with a flattened cotton swab, and lower cells were fixed with 4% formaldehyde and stained in crystal violet. Micrographs of 10 fields were randomly taken using an inverted microscope and a 20X objective. Images shown are representative of at least three independent experiments. Cell numbers were counted and averaged for each cell line in a particular experiment. Results are expressed as mean ± SEM. Significance was determined using Student's t test.

2.5. Immunocytochemistry for microtubules

Cells were inoculated onto poly-D-lysine-coated coverslips and allowed to attach for 24 h. Monolayers were fixed in −30 °C methanol, air-dried, then immunofluorescently stained by standard methods using primary murine anti-tubulin antibody (1:1000, Sigma) and secondary Cy3-conjugated goat anti-mouse IgG (1:800, EarthOx, San Francisco, CA), and counterstained with DAPI.

2.6. Western blot analysis for KIF11 and Rac1

Western blots were performed by standard methods as previously described [22], and probed with mouse anti-Eg5 (1:500, Novus Biologicals, Littleton, CO), mouse anti-Rac1 (1:500, Millipore), and mouse anti-β-actin (1:3000, Sigma, St. Louis, MO). Horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:1000, Wuhan Boster, Wuhan, China) was used as the secondary antibody. Results shown are representative western blots of at least two independent experiments. Relative protein expression was quantified using ImageJ software.

3. Results

3.1. Characterization of KIF11 knockdown cell lines

We initially knocked down KIF11 by infecting cells separately with lentivirus encoding two different KIF11 shRNAs and quantified KIF11 expression by western blot analysis. Quantification and normalization of band intensities to β-actin indicated a 58

and 79% reduction of KIF11 in the kdKIF11-1 and kdKIF11-2 cell lines, respectively (Fig. 1(A), upper panel and Fig. 1(B)). Cell death, resulting from KIF11 inhibition, has been shown to require Rac1 [23]. Resistance of our KIF11 knockdown cell lines, due to changes in Rac1 levels, would be an obvious mechanism by which KIF11 knockdown could affect cell motility. However, western blot analysis showed no significant difference in Rac1 levels following KIF11 knockdown compared to control (Fig. 1(A), lower panel).

Although there was no gross alteration in cell morphology following KIF11 knockdown, since KIF11 is a microtubule-binding protein, we performed immunocytochemistry for microtubules to identify possible effects on intracellular microtubule structure. Cells in both control and KIF11 knockdown cell lines possessed well-defined microtubule networks with no gross alterations in microtubule structure Fig. 1(C). These results are similar to those previously observed following chemical inhibition of KIF11 [24]. These results show that KIF11 knockdown did not result in changes in microtubule structure or in the level of Rac1.

3.2. KIF11 knockdown reduces invasion through Matrigel-coated transwells

Migration through a three-dimensional Matrigel matrix offers a stringent test to detect combined differences in attachment, cell velocity, and ability to degrade the matrix through secretion of matrix-metalloproteases. Therefore, we initially chose to examine whether knockdown of KIF11 alters invasion. Knockdown of KIF11 reduced invasion by 70% for both KIF11 knockdown cell lines (control = 428.8 ± 18.1 cells/field vs. 128.2 ± 46.9 cells/field for kdKIF11-1 ($^{**}p < 0.01$ vs. control) and 126.95 ± 19 cells/field for kdKIF11-2 ($^{**}p < 0.01$ vs. control)) (Fig. 2). Thus invasion was inhibited by KIF11 knockdown.

3.3. KIF11 knockdown increases migration in a wound healing assay

The decrease in invasion, observed in our Matrigel transwell assay could be due to decreased cell attachment or motility. We measured the rate of cell attachment, over a 2-h period, onto collagen-coated dishes and found no differences between control and KIF11 knockdown cell lines (not shown). Therefore, we determined the effects of KIF11 knockdown on cell motility in an *in vitro* wound healing assay (Fig. 3). Both kdKIF11 cell lines closed the wound by 20 h, whereas a visible gap remained in the control culture Fig. 3(A). Using the 12-h time point, calculation of velocities revealed control cells migrated at 9.1 μm/h vs. 13.2 μm/h for kdKIF11-1 and 10.3 μm/h for kdKIF11-2 Fig. 3(B).

3.4. KIF11 knockdown reduces epidermal growth factor-mediated chemotaxis

Decreased invasion through Matrigel-coated transwells could be due to either defects on chemotaxis or reduced secretion of matrix metalloproteases that degrade the basement membrane. It has been previously shown that the parental MDA-MB-231 cells possess EGF receptors and respond to EGF-mediated chemotaxis [25]. Due to the importance of EGF receptors in metastasis

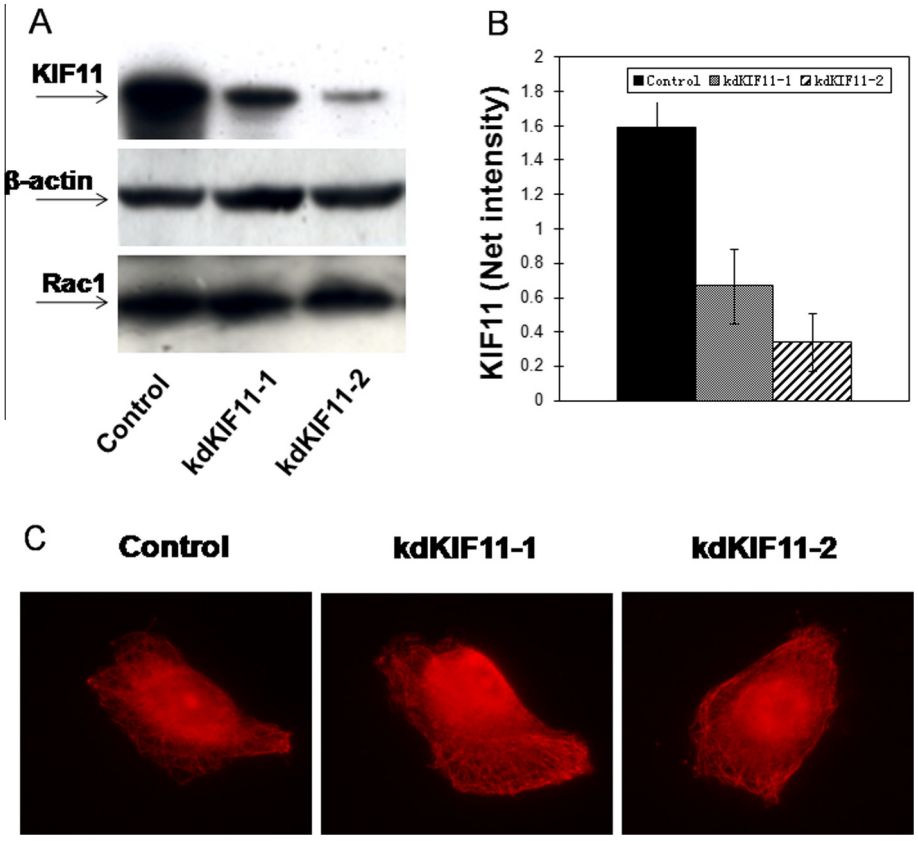

Fig. 1. KIF11 knockdown by western blot and immunocytochemistry for microtubules of human breast carcinoma cells. (A) Representative western blot for control, kdKIF11-1, and kdKIF11-2 MDA-MB-231(GFP-IMP1) human breast carcinoma cells. The KIF11 bands are intentionally over-exposed to display the KIF11 band in the kdKIF11-2 cell line. Note the dramatic decrease in the levels of KIF11, but unchanged expression of Rac1. (B) Quantification of KIF11 band densities after normalization to β-actin ($n = 4$). (C), Immunocytochemistry for microtubules (red) in control, kdKIF11-1 and kdKIF11-2 cell lines. Note the normal appearance of microtubule structure in all cell lines (400X).

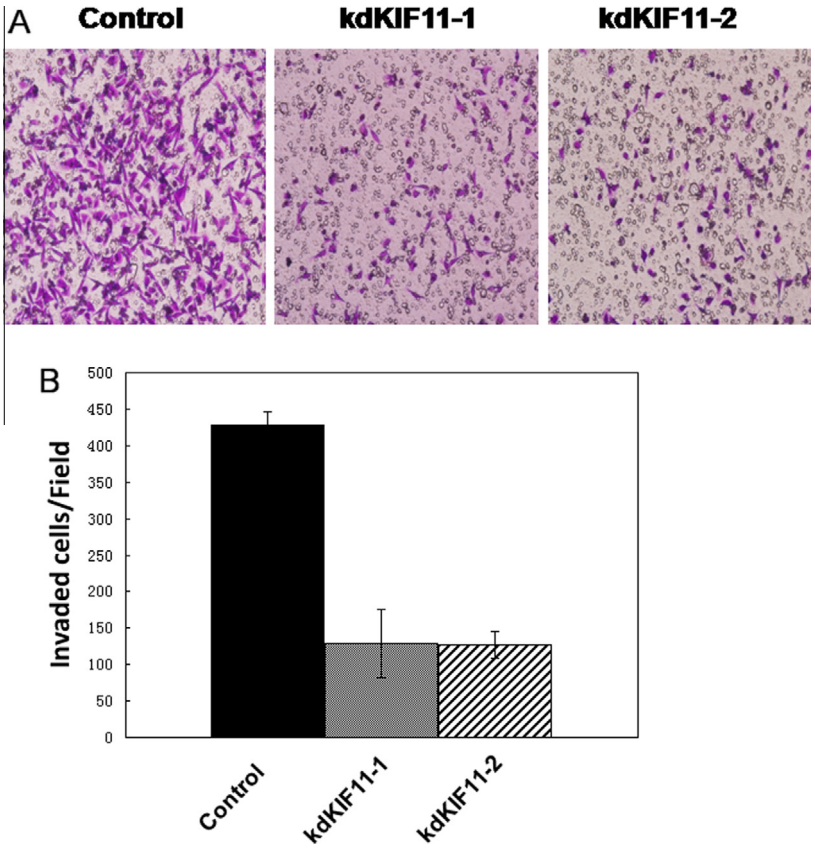

Fig. 2. KIF11 knockdown decreases invasion of human breast carcinoma cells. (A) and (B), invasion was measured by allowing cells to migrate through Matrigel-coated transwells over a 14-h duration. (A) Representative fields of crystal violet-stained cells (200X). (B) The average number of cells counted per 20X-objective field is shown. Note that KIF11 knockdown decreased invasion of human breast carcinoma cells by 65–70%. $^{*}p < 0.05$.

[26,27], and to determine whether KIF11 knockdown affects invasion or chemotaxis, we performed a 6-h transwell migration assay using collagen-coated transwells. Control cells averaged 337.9 ± 56.0 cells/field migrated vs. 187.6 ± 66.4 cells/field for kdKIF11-1 ($^{**}p < 0.01$ vs. control) and 154.7 ± 36.3 cells/field for kdKIF11-2 ($^{**}p < 0.01$ vs. control) (Fig. 4). Thus, both KIF11 knockdown cell lines displayed a 44–54% reduced migration through collagen-coated transwells, suggesting that chemotaxis was inhibited by KIF11 knockdown. To further differentiate the effects of KIF11 knockdown on chemotaxis vs. cell motility, we added EGF to both upper and lower chambers to stimulate migration in the absence of chemotaxis. Addition of EGF to both upper and lower chambers resulted in 186.0 ± 6.5 control cells/field migrating through the transwell vs. 191.8 ± 6.1 kdKIF11-1 cells/field ($p = 0.6$ vs. control) and 239.95 ± 31.2 kdKIF11-2 cells/field ($^{**}p < 0.01$ vs. control). Importantly, in contrast to control cells, transwell migration of the kdKIF11 cell lines was not decreased when EGF was added to the upper chamber, suggesting that chemotaxis was severely impaired in the KIF11 knockdown cell lines.

4. Discussion

We report a novel role for KIF11 in sensing chemotactic signals to direct cell migration. KIF11 has been shown to play a role in cell migration under conditions of myosin IIA inhibition, although under normal conditions the velocity of cell migration did not appear to be affected by KIF11 inhibition, based on time-lapse images captured every 10 min [24]. In contrast, our wound healing results and our transwell migration assay, with EGF in the upper chamber, suggest that knockdown of KIF11 can result in increased cell velocity, but with reduced persistence of migration in any one direction, leading to the possibility that in the prior study, KIF11-inhibited cells could have traveled longer distances from initial location to final destination. In preliminary experiments using live-cell imaging, we have been unable to accurately measure cell velocity due to the rounded morphology and tendency of our cells to become spherical following tail retraction during migration, thus obscuring the nucleus, whose center is normally used for measurement. Our results also suggest that the current model of microtubule capture at the cell cortex of the leading edge [6] alone is not sufficient for directed migration, although KIF11 could be required for polarized capture.

It is possible that the changes we observed could be due to general effects resulting from subtle disruption of microtubule function due to a lack of KIF11 binding. However, prior reports showing chemical inhibition of KIF11 [24], and we show here by KIF11 knockdown that KIF11 inhibition does not negatively affect cell motility, which is highly dependent on microtubule function. The more rapid migration of both KIF11 knockdown cell lines and the greater EGF-stimulated transwell migration, in the absence of an EGF gradient, displayed by the lowest KIF11-expressing kdKIF11-2 line, suggests a second possibility that KIF11 regulates sensitivity to chemotactic agents. Therefore, we conclude that KIF11 specifically plays an important role in sensing chemotactic signals to govern the direction and persistence of cell migration.

The function of KIF11 in chemotaxis is unclear. Our original reasoning for implicating KIF11 in cell polarity was based on its involvement in establishing centriole polarity during mitosis. However, microtubule nucleation at the centriole has been shown

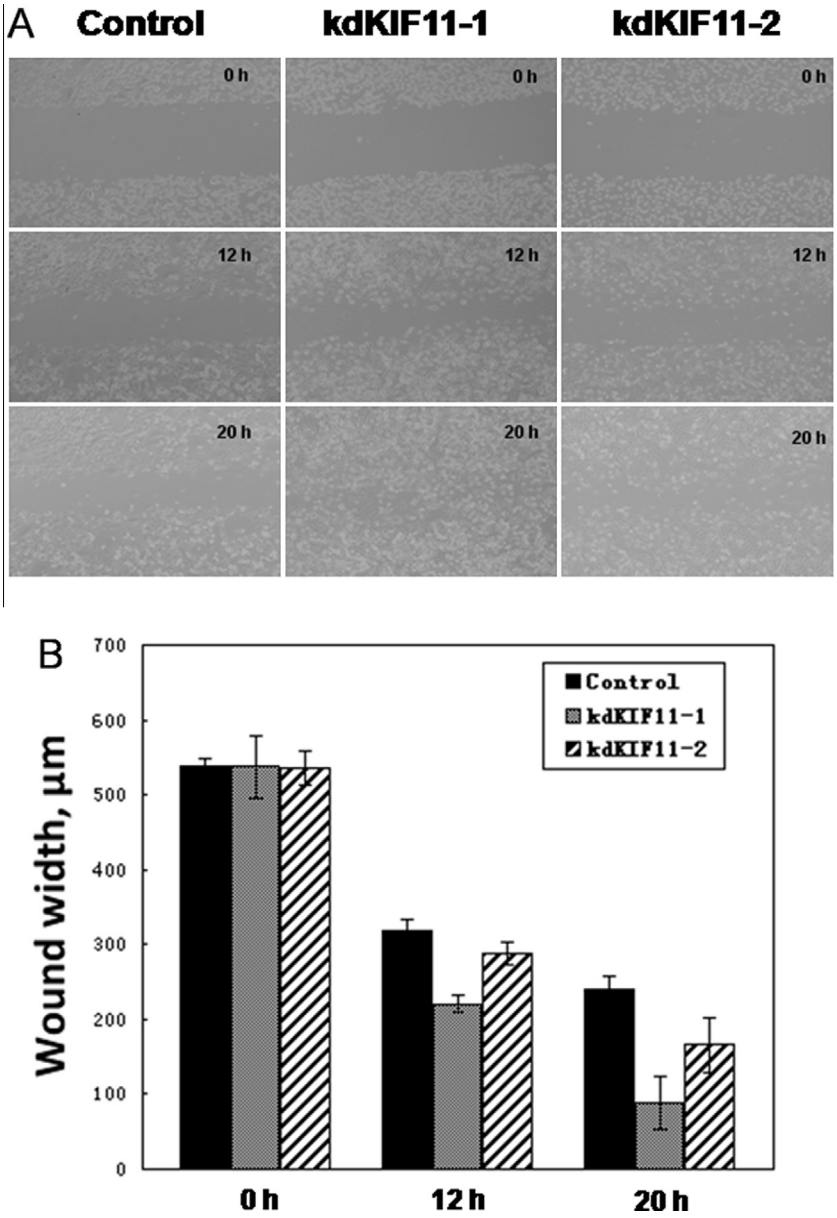

Fig. 3. KIF11 knockdown enhances wound healing-related migration. Cell motility was examined using a scratch wound healing assay. (A) Representative migration of control, kdKIF11-1 and kdKIF11-2 cell lines (100X). (B) Quantification of migration at the indicated time points. Note that KIF11 knockdown increased the distance migrated by MDA-MB-231(GFP-IMP1) human breast carcinoma cells at 12 and 20 h. $^{*}p < 0.05$.

to be the same at both leading and trailing edges [28]. Nevertheless, microtubules extending toward the leading edge are more stable than microtubules extending toward the trailing edge [28]. In Drosophila, kinesin-5/Kip1 has been reported to stabilize microtubules while tracking their plus ends during late anaphase [29], and in mammalian cells, increasing microtubule dynamics during mitosis reduces the sensitivity of cells to KIF11 inhibition [30], further suggesting that KIF11 itself can stabilize microtubules. Therefore, KIF11-mediated localization of centrioles to direct and stabilize microtubules in the direction of migration remains a possibility. Nevertheless, our results now link signal transducing molecules known to govern cell polarity, such as CDC42, directly to the microtubule apparatus through KIF11.

Our identification of KIF11 as a key downstream molecule is unique in that not only does our discovery link epidermal growth factor-mediated chemotactic signals directly to the microtubule apparatus in response to directional cues, but contrasts other motility-related molecules in that inhibition of KIF11 does not inhibit cell motility. This is an important distinction and indicates that KIF11 activity is critical and specific to the decision-making process in determining cell polarity. Most importantly, our results place known molecular mechanisms, such as microtubule force generation, delivery of signaling proteins and microtubule capture [6–12], independent and downstream of KIF11 activity.

EGF receptor inhibitors are already in clinical use for the treatment of breast cancer. Because chemokines, in addition to EGF, also play a role in metastasis, inhibition of a mechanism central to a variety of chemokines would be expected to exert more potent anti-metastatic effects than inhibition of EGF receptors alone. Our demonstration that epidermal growth factor-dependent chemotaxis is completely abolished by KIF11 knockdown suggests a central role for KIF11 in sensing chemotactic cues and establishing the direction of migration, and further suggests that KIF11 inhibitors could serve as potent anti-metastatic agents by eliminating

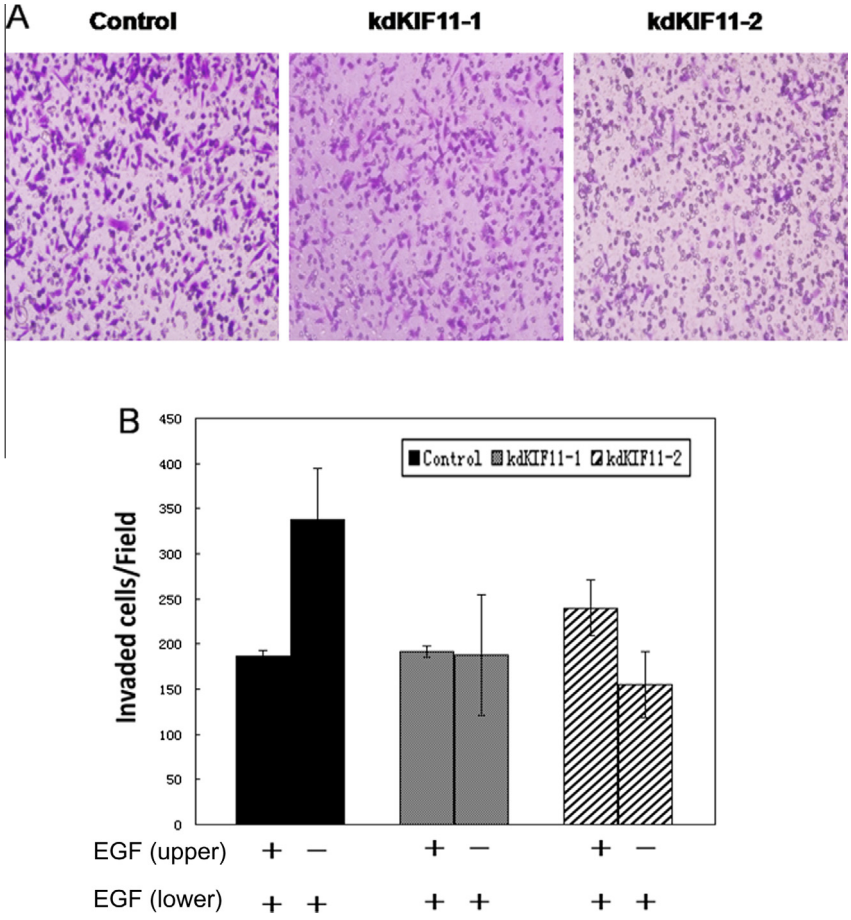

Fig. 4. KIF11 knockdown reduces EGF-mediated chemotaxis of human breast carcinoma cells. Effect of stable KIF11 knockdown in EGF-mediated chemotaxis was examined using a 6-h collagen-coated transwell migration assay ($n = 4$). (A) Representative fields of crystal violet-stained cells (200X). (B) the average number of cells counted per 20X-objective field is shown. Note that KIF11 knockdown decreased chemotaxis of human breast carcinoma cells by 50–56% compared to control. Also note that inclusion of EGF in the upper chamber decreased transwell migration for the control cell line only, indicating sensitivity to EGF-mediated directional cues, but not EGF-stimulated migration, was abolished by KIF11 knockdown. $^{*}p < 0.05$.

the response to directional cues for intravasation. KIF11 inhibitors could be used in the context of upcoming low-dose therapies, such as metronomic therapy, even for tumors resistant to the anti-mitotic effects of KIF11 inhibition.

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