The Na\(^+\)/H\(^+\) exchanger NHE1 is required for directional migration stimulated via PDGFR-α in the primary cilium

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We previously demonstrated that the primary cilium coordinates platelet-derived growth factor (PDGF) receptor (PDGFR) α-mediated migration in growth-arrested fibroblasts. In this study, we investigate the functional relationship between ciliary PDGFR-α and the Na\(^+\)/H\(^+\) exchanger NHE1 in directional cell migration. NHE1 messenger RNA and protein levels are up-regulated in NIH3T3 cells and mouse embryonic fibroblasts (MEFs) during growth arrest, which is concomitant with ciliogenesis. NHE1 up-regulation is unaffected in Tg737orpk MEFs, which have no or very short primary cilia. In growth-arrested NIH3T3 cells, NHE1 is activated by the specific PDGFR-α ligand PDGF-AA. In wound-healing assays on growth-arrested NIH3T3 cells and wild-type MEFs, NHE1 inhibition by 5′-(N-ethyl-N-isopropyl)amiloride potently reduces PDGF-AA-mediated directional migration. These effects are strongly attenuated in interphase NIH3T3 cells, which are devoid of primary cilia, and in Tg737orpk MEFs. PDGF-AA failed to stimulate migration in NHE1-null fibroblasts. In conclusion, stimulation of directional migration in response to ciliary PDGFR-α signals is specifically dependent on NHE1 activity, indicating that NHE1 activation is a critical event in the physiological response to PDGFR-α stimulation.

Introduction

The process of cell migration is pivotal to many fundamental physiological processes, including embryonic and fetal development, immune responses, wound healing, and tissue homeostasis. Consequently, altered function of components of the migratory machinery is associated with severe pathophysiological conditions, including developmental defects, chronic inflammatory diseases, and cancer metastasis (Ridley et al., 2003; Vicente-Manzanares et al., 2005; Schwab et al., 2007).

Cell migration is a complex multistep process involving extensive cytoskeletal rearrangement and the concerted action of multiple ion transport proteins and membrane receptors. These processes result in the formation of lamellipodia and other protrusive structures and in local changes of cell–matrix interactions and cell volume, which collectively enable the cell to move forward (Ridley et al., 2003; Vicente-Manzanares et al., 2005; Schwab et al., 2007). Directional migration relies on the ability of the cell to sense and react to chemosensory stimuli (Wu, 2005) such as PDGF (Heldin and Westermark, 1999; Jechlinger et al., 2006). Notably, exposure to PDGF activates the ubiquitous plasma membrane Na\(^+\)/H\(^+\) exchanger NHE1 (Cassel et al., 1983; Ma et al., 1994; Yan et al., 2001), which is a central player in the regulation of proliferation, survival, and migration (Putney et al., 2002; Pedersen, 2006; Stock and Schwab, 2006). We and others have demonstrated an essential role for NHE1 in cell migration and invasion in many cell types, including a variety of cancer cell types (Lagana et al., 2000; Denker and Barber, 2002; Stock

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Abbreviations used in this paper: ANOVA, analysis of variance; EIPA, 5′-(N-ethyl-N-isopropyl)amiloride; gas, growth arrest specific; MEF, mouse embryonic fibroblast; PDGFR, PDGF receptor; pHi, intracellular pH; WT, wild type.

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et al., 2005, 2007; Cardone et al., 2005b; Stuwe et al., 2007; Hayashi et al., 2008). In migrating cells such as fibroblasts or epithelial cells, NHE1 localizes predominantly to the leading edge (Denker et al., 2000; Lagana et al., 2000; Cardone et al., 2005a; Stock et al., 2007), and findings in fibroblasts indicate that NHE1 activity and attachment to the ERM (ezrin/radixin/moesin) family of plasma membrane F-actin linker proteins are required for cell polarity and migration (Denker and Barber, 2002). This is of substantial interest in the context of human cancers, in which metastatic capacity is linked to increased expression of NHE1 (Cardone et al., 2005b). However, the possible link between PDGF signaling and NHE1 in migration/chemotaxis has not previously been addressed.

PDGF exists as homo- or heterodimers of PDGF-A, -B, -C, and -D chains. Although PDGF-BB activates both homo- and heterodimers of PDGF receptor (PDGFR) α and PDGFR-β, PDGFR-AA is specific for the PDGFR-α homodimer (Heldin and Westermark, 1999). Both PDGFR-AA and PDGFR-BB have been shown to stimulate migration in various cell types (Shure et al., 1992; Hayashi et al., 1995; Yu et al., 2001). Recently, we showed that PDGFR-α signaling is coordinated by the primary cilium in mammalian fibroblasts (Schneider et al., 2005). PDGFR-α, which is encoded by a growth arrest–specific (gas) gene (Lih et al., 1996), is targeted to the primary cilium, where ligand-dependent activation of the receptor and of the ERK1/2 and protein kinase B/Akt pathways is initiated (Schneider et al., 2005; unpublished data). The primary cilium is an essential sensory organelle in most growth-arrested mammalian cells and coordinates a series of critical signal transduction pathways in development and tissue homeostasis, which, in addition to PDGFR-α signaling, include the Hedgehog and Wnt pathways (Christensen et al., 2007). In NIH3T3 fibroblasts, we previously showed that primary cilia are virtually absent in nonconfluent cycling interphase cells but grow on almost all confluent quiescent growth-arrested cells within 24 h of serum starvation (Schneider et al., 2005). Similarly, serum-starved wild-type (WT) mouse embryonic fibroblasts (MEFs) grow normal primary cilia that coordinate PDGFR-α–mediated signal transduction and directional cell migration, whereas defects in assembly of the primary cilium in Tg737orpk MEFs block these events (Schneider et al., 2005; unpublished data). These results indicate that the primary cilium is part of the positioning machinery that coordinates cell polarity, which is essential for wound healing and developmental processes (Schneider and Haugh, 2006).

In this study, we investigated the interactions between the primary cilium and NHE1 in PDGFR-α–dependent directional cell migration. We demonstrate that NHE1 mRNA and protein levels are increased during growth arrest independent of the formation of the primary cilium. In contrast, NHE1 activity is strongly attenuated by serum deprivation of NIH3T3 fibroblasts, which is coincident with the time course of primary cilia appearance (Schneider et al., 2005). NHE1 activity is partly restored by stimulation with PDGF-AA, which also stimulates directional cell migration (unpublished data). Inhibition of NHE1 activity by S'-ethyl-N-isopropyl) amiloride (EIPA) potently inhibits PDGF-AA–mediated directional cell migration in growth-arrested WT MEFs in which primary cilia are present, whereas migration in growth-arrested Tg737orpk mutant MEFs is unaffected by either PDGF-AA or EIPA. Furthermore, PDGF-AA fails to stimulate migration in NHE1-deficient fibroblasts. We conclude that in fibroblasts, PDGFR-α signaling initiated in the primary cilium controls directional migration responses via up-regulation of NHE1 activity, indicating that NHE1 activation is a critical event in the physiological response to activation of PDGFR-α.

Results

Expression and localization of NHE1 in NIH3T3 fibroblasts and in WT and Tg737orpk MEFs

We first asked whether NHE1 is up-regulated during growth arrest in NIH3T3 fibroblasts (i.e., concomitant with formation of the primary cilium). NIH3T3 cells were serum starved for 0, 12, and 48 h, and mRNA and protein levels were measured by quantitative PCR using specific NHE1 TaqMan probes and Western blotting, respectively. As shown in Fig. 1 A (top), the NHE1 mRNA level was up-regulated about twofold after both 12 and 48 h of serum starvation. The protein level increased more slowly and was nearly tripled after 48 h of serum starvation (Fig. 1 A, bottom). NHE1 up-regulation was reversible upon reentry into the cell cycle (Fig. 1 A).

To test whether NHE1 up-regulation during growth arrest depends on the formation of the primary cilium, we performed similar experiments in WT MEFs, which, as previously reported, grow cilia at quiescence, and in Tg737orpk mutant MEFs, which form no or only very short primary cilia (Fig. 1 B, a and b; Schneider et al., 2005). Similar to the findings in NIH3T3 cells, the NHE1 protein level was nearly tripled at time 48 h after serum starvation in both WT and Tg737orpk MEFs (Fig. 1 C). Collectively, these data indicate that NHE1 is a gas protein but that, in contrast to the up-regulation of PDGFR-α during growth arrest (Schneider et al., 2005), up-regulation of NHE1 is independent of assembly of the primary cilium. In growth-arrested WT and Tg737orpk MEFs, NHE1 localized to the plasma membrane, in particular to lamellipodia-like structures at the edges of the cells and intracellularly, in perinuclear membranes and in a cytoplasmic vesicular-like pattern (Fig. 1 B, c and d). A similar pattern of localization was observed in NIH3T3 cells (unpublished data).

The NHE1 localization pattern was not detectably different in WT and Tg737orpk MEFs after 1 h of exposure to 50 ng/ml PDGF-AA (n = 3; unpublished data). Collectively, these results demonstrate that NHE1 expression is up-regulated concomitant with formation of the primary cilium and that neither expression nor localization of NHE1 are compromised in Tg737orpk MEFs.

NHE1 activity in NIH3T3 cells

Because NHE1 activity is very tightly regulated (Pedersen, 2006), an up-regulation of NHE1 expression does not mean that NHE1 activity is also up-regulated. To address this point, NHE1 activity was determined in NIH3T3 fibroblasts using an NH4Cl prepulse, which is a classical protocol for evaluating intracellular pH (pH) recovery after an acid load (Boron, 2004). In this protocol, the cells initially rapidly alkalinize as NH4+ diffuses...
Migration of fibroblasts with primary cilia: growth-arrested NIH3T3 cells and WT MEFs

Having established that NHE1 is up-regulated concomitantly with formation of the primary cilium and stimulated by ciliary PDGFR-α signaling, we next addressed the possible functional relationship between ciliary PDGFR-α signaling and NHE1 activity in control of directional fibroblast migration. To this end, we performed wound-healing assays and determined two parameters: (1) the translocation of the cell during the course of the experiment, i.e., the distance covered by the cell over 5 h, and (2) the distance covered by the cell specifically in the direction perpendicular to the wound, which is a measure of the directionality of movement and allows the calculation of the velocity of movement into the wound. A summary of the translocation data for NIH3T3 cells and MEFs can be found in Table I, and details and original traces are given in Figs. 3 and 5 for growth-arrested and interphase NIH3T3 cells and in Figs. 4 and 6 for WT and Tg737 orpk MEFs, respectively.

We first determined the effect of EIPA on migration of two types of fibroblasts with primary cilia: growth-arrested and serum-starved NIH3T3 cells (Fig. 2, C and D). This effect was abolished by 5 µM EIPA, demonstrating that stimulation of ciliary PDGFR-α partially restored NHE1 activity in growth-arrested fibroblasts (Fig. 2, C and D).
NIH3T3 cells and growth-arrested WT MEFs. Fig. 3 A shows trajectories of growth-arrested NIH3T3 cells in the absence and presence of 50 ng/ml PDGF-AA normalized to common starting points. The radii of the red circles represent the mean distances covered within the experimental periods of 5 h. Fig. 3 B provides a statistical summary of these experiments. In growth-arrested NIH3T3 cells, translocation was 21 ± 3.6 µm and was reduced by ~50% by EIPA to 11 ± 1.8 µm. In the presence of PDGF-AA, translocation was increased to 45 ± 5.7 µm, and under these conditions, translocation was reduced by more than two thirds by EIPA to 13 ± 1.5 µm (Fig. 3 B).

Next, we assessed the role of NHE1 specifically in directional migration of the growth-arrested NIH3T3 cells in the presence of PDGF-AA. The directionality of movement was estimated by plotting the mean distance covered by the cells in the direction perpendicular to the wound edge (Fig. 3 C, the x direction) as a function of time. The slope of this curve, obtained by linear regression, corresponds to the velocity of directional movement into the wound. The slope of the corresponding plots for the movement parallel to the wound edge (Fig. 3 C, the y direction) was always close to zero (not depicted), indicating that movement in this direction was random and essentially non-progressing so that directional movement occurred only perpendicular to the wound edge. The directional migration data for growth-arrested, PDGF-AA–treated NIH3T3 cells are summarized in Fig. 3 D. As seen (Fig. 3 D, legend), the cells moved into the wound at a velocity of 0.104 ± 0.004 µm/min, whereas in the presence of 10 µM EIPA, this velocity was reduced by ~75% to 0.028 ± 0.001 µm/min.

To ascertain that the effect of EIPA on PDGF-AA–stimulated migration was not caused by an inhibition of PDGFR-α activation, we monitored Tyr754 phosphorylation of PDGFR-α, corresponding to receptor activation, after a 5-min exposure to 50 ng/ml PDGF-AA in the absence of EIPA and after 1 and 6 h of exposure to 5 µM EIPA (Fig. 3 E). As seen, the level of PDGFR-α expression and the ability of PDGF-AA to induce Tyr754 phosphorylation of PDGFR-α were unaffected by the presence of EIPA. Furthermore, neither EIPA (Fig. S1 A) nor siRNA-mediated knockdown of NHE1 (Fig. S1 B) significantly affected the ability of fibroblasts to form primary cilia. These results show that the inhibitory effect of EIPA on PDGF-AA–induced cell migration is not caused by an effect of cilia formation and that EIPA exerts its effect on NHE1 downstream from activation of PDGFR-α.

Finally, confirming that the effect of EIPA on cell migration reflected inhibition of NHE1, siRNA-mediated knockdown of NHE1 inhibited cell migration (Fig. S1 C).

Next, we performed similar experiments on WT MEFs. Fig. 4 A shows the trajectories of growth-arrested WT MEFs in the presence of PDGF-AA and in the absence and presence of EIPA, and Fig. 4 B provides statistical summaries (see also
Table I. Summary of translocation data

| Cell type       | Primary cilium       | PDGF-AA | EIPA | Translocation μm |
|-----------------|----------------------|---------|------|------------------|
| NIH3T3          | No (interphase)      | −       | −    | 60 ± 8.7 (17)    |
| NIH3T3          | No (interphase)      | −       | +    | 30 ± 3.0 (25)    |
| NIH3T3          | No (interphase)      | +       | −    | 60 ± 5.4 (26)    |
| NIH3T3          | No (interphase)      | +       | +    | 33 ± 2.7 (25)    |
| NIH3T3          | Yes                  | −       | +    | 21 ± 3.6 (23)    |
| NIH3T3          | Yes                  | −       | +    | 11 ± 1.8 (23)    |
| NIH3T3          | Yes                  | +       | −    | 45 ± 5.7 (25)    |
| NIH3T3          | Yes                  | +       | +    | 13 ± 1.5 (26)    |
| WT MEF          | Yes                  | −       | −    | 45 ± 4.2 (53)    |
| WT MEF          | Yes                  | −       | +    | 27 ± 4.4 (22)    |
| WT MEF          | Yes                  | +       | −    | 111 ± 5.4 (23)   |
| WT MEF          | Yes                  | +       | +    | 15 ± 1.8 (20)    |
| Tg737orpk MEF   | No or very short     | −       | −    | 75 ± 5.7 (21)    |
| Tg737orpk MEF   | No or very short     | −       | +    | 63 ± 5.7 (19)    |
| Tg737orpk MEF   | No or very short     | +       | −    | 87 ± 7.8 (34)    |
| Tg737orpk MEF   | No or very short     | +       | +    | 57 ± 5.7 (30)    |

The table summarizes the translocation data obtained for NIH3T3 cells, WT MEFs, and Tg737orpk MEFs in 5-h wound-healing assays as a function of (a) the presence or absence of a primary cilium, (b) the presence or absence of 50 ng/ml PDGF-AA, and (c) the presence or absence of 10 µM of the NHE1 inhibitor EIPA. The absence or presence of primary cilia was assessed by visual inspection and has also been previously described (Schneider et al., 2005). Translocation data are shown as mean ± SEM, with the number of cells shown in parenthesis.

Videos 1–3. Exposure to PDGF-AA nearly tripled the translocation of WT MEFs from 45 ± 4.2 µm to 111 ± 5.4 µm. Similar to what was seen in the growth-arrested NIH3T3 cells, the effect of EIPA was substantially greater in the presence of PDGF-AA, but in WT MEFs, this effect was even more pronounced than in NIH3T3 cells. Inhibition of NHE1 by 10 µM EIPA had no statistically significant effect on translocation in WT MEFs in the absence of PDGF-AA but nearly abolished translocation in the PDGF-AA–treated WT MEFs (Fig. 4 B).

Fig. 4 C shows WT MEFs migrating into the wound and illustrates the x and y axes used to estimate directionality, and Fig. 4 D summarizes the directionality data. The growth-arrested, PDGF-AA–treated WT MEFs traveled at a rate of 0.301 ± 0.003 µm/min in the direction perpendicular to the wound. Upon inhibition of NHE1, this directional migration of WT MEFs was reduced by 90% to 0.030 ± 0.004 µm/min (Fig. 4 D). Collectively, these data indicate that NHE1 activity plays a pivotal role in mediating directional migration in response to PDGF-α signaling.

Migration of fibroblasts deficient in primary cilia: interphase NIH3T3 cells and Tg737orpk mutant MEFs

To evaluate whether the role of NHE1 in cell migration depends on signaling via the primary cilium, we next performed wound-healing assays on two types of fibroblasts deficient in primary cilia: interphase NIH3T3 cells, which lack primary cilia, and Tg737orpk mutant MEFs, which have no or only very short primary cilia and exhibit impaired PDGF-α expression (Schneider et al., 2005) and decreased directional migration in response to chemotactic stimuli (unpublished data). As seen in Fig. 5 (A and B), the translocation of interphase NIH3T3 cells was 60.0 ± 8.7 µm (17 cells in three experiments) under control conditions. Inhibition of NHE1 by 10 µM EIPA reduced translocation by ∼50% to 30.0 ± 3.0 µm (25 cells in three experiments). Importantly, in the interphase NIH3T3 cells, stimulation with PDGF-AA did not increase translocation, and NHE1 inhibition similarly reduced translocation after PDGF-AA stimulation by ∼50% (Table I). This indicated that the role of NHE1 in translocation was reduced in interphase NIH3T3 cells compared with growth-arrested, PDGF-AA–treated cells, in which EIPA inhibited translocation by ∼70% (compare with Fig. 5 B and Table I). Therefore, we next addressed whether this was also the case for directional migration. As seen in Fig. 5 C, in interphase NIH3T3 cells, migration into the wound proceeded at a velocity of 0.173 ± 0.002 µm/min under control conditions and was reduced by ∼55% to 0.077 ± 0.001 µm/min in the presence of EIPA compared with 75% in the growth-arrested cells (compare with Fig. 3 D). In other words, inhibition of NHE1 was less efficient in reducing both translocation and directional migration in interphase NIH3T3 cells than in growth-arrested, PDGF-AA–treated NIH3T3 cells.

To test the hypothesis that this difference reflected the presence (in growth-arrested NIH3T3 cells) versus absence (in interphase NIH3T3 cells) of a primary cilium, we next performed wound-healing assays on the primary cilia–deficient Tg737orpk mutant MEFs (Fig. 6). Tg737orpk MEFs exhibited a higher translocation in the absence of PDGF-AA than did WT MEFs (compare Fig. 6 B with Fig. 4 B). This is in accordance with our previous findings and indicates that the primary cilium may restrain excessive cell migration (unpublished data).

In another study (unpublished data), we have shown that translocation of Tg737orpk MEFs was unaffected by PDGF-AA (Fig. 6 B). Importantly, translocation of Tg737orpk MEFs was not significantly affected by NHE1 inhibition by 10 µM EIPA, neither in the presence nor in the absence of PDGF-AA (Fig. 6, A and B). With respect to directional migration, the velocity of migration of Tg737orpk MEFs into the wound was 0.31 ± 0.01 µm/min in the absence of EIPA and reduced by only 28% to 0.23 ± 0.01 µm/min in the presence of EIPA (Fig. 6 C). Compared with the 90% reduction in the WT MEFs (Fig. 4 D),...
Figure 3. Effects of PDGF-AA and EIPA in wound-healing assays on growth-arrested NIH3T3 fibroblasts. (A) Trajectories (normalized to common starting points) of growth-arrested NIH3T3 cells with and without 50 ng/ml PDGF-AA or 10 µM EIPA, as indicated. The radii of the red circles illustrate the mean translocation of the cells within a 5-h time period (n = 17–25). (B) Translocation (in micrometers and calculated as the distance between the position of the cell center at the beginning and at the end of the experiment) in the absence or presence of PDGF-AA or EIPA as indicated. (A and B) Data are individual trajectories (A) or means ± SEM (B) of 23–26 cells in three independent experiments. Data were analyzed using parametric or nonparametric ANOVA, and the level of significance is shown (*, P < 0.05; **, P < 0.01; ***, P < 0.001). (C) Images of the left side of the wound in a confluent monolayer of growth-arrested NIH3T3 cells (serum starved for 24 h) at time 0 and 4 h after the wound was made. The arrows indicate the x and y directions of movement, which were used for estimating directional migration. (D) Velocity of migration perpendicular to the wound (x direction) in PDGF-AA–stimulated, growth-arrested NIH3T3 cells in the absence (open circles) or presence (closed circles) of EIPA. Directionality of movement was estimated by plotting the mean of the distance covered by each cell in the x direction as a function of time. The slope of this line is the velocity in x, in micrometers/minute, which is taken as a measure of directionality. In PDGF-AA–stimulated, growth-arrested cells, the velocity of movement into the wound is 0.104 ± 0.004 µm/min, and in the presence of 10 µM EIPA, this value is reduced to 0.028 ± 0.001 µm/min. Data shown are means ± SEM of 23–26 cells in three independent experiments. (E) NIH3T3 cells were serum starved for 24 h to induce growth arrest, exposed to 5 µM EIPA for 0, 1, or 6 h as indicated, and treated with 50 ng/ml PDGF-AA for 5 min as indicated (+). Cells were lysed, and Western blots of total and Tyr754-phosphorylated PDGFR-α were performed as described in Materials and methods. β-Actin was used as a loading control. Data shown are representative of three independent experiments.
this demonstrates that the inhibitory effect of EIPA is strongly blunted in the cilia-deficient Tg737orpk MEFs. Collectively, these data indicate that NHE1 plays a major role in directional migration, in a manner highly dependent on the presence of the primary cilium and specifically on ciliary PDGFR-α signaling. NHE1 also plays a role, albeit a lesser role, in the migration of interphase cells lacking a primary cilium, suggesting that several pathways regulate NHE1 during cell migration.

Migration of NHE1-null and hNHE1-expressing PS120 fibroblasts

Although EIPA is a widely used and relatively specific inhibitor of NHE1 activity, we wished to further test the involvement of NHE1 using a more specific approach. To this end, we performed 5-h wound-healing assays similar to those performed on NIH3T3 cells and MEFs on growth-arrested NHE1-null PS120 fibroblasts (PSNEO) and on growth-arrested PS120 fibroblasts stably expressing NHE1 (PS120-NHE1; Denker et al., 2000). In the growth-arrested state, these cells migrated relatively slowly in wound-healing assays. As seen in Fig. 7 A, the mean translocation of the NHE1-expressing cells was 5.8 ± 0.60 µm (n = 52 cells in six independent experiments) and 8.9 ± 0.71 µm (n = 63 cells in six independent experiments) in the absence and presence of PDGF-AA stimulation, respectively (i.e., exposure to PDGF-AA–stimulated translocation; P < 0.01). In the NHE1-null cells, the mean translocation in the 5-h wound-healing

Figure 4. Effects of PDGF-AA and EIPA in wound-healing assays on growth-arrested WT MEFs. (A) Trajectories (normalized to common starting points) of growth-arrested WT MEFs in the presence of 50 ng/ml PDGF-AA and in the absence or presence of 10 µM EIPA, as indicated. The radii of the red circles illustrate the mean translocation of the cells within a 5-h time period. (B) Translocation (in micrometers) of growth-arrested WT MEFs in the absence or presence of 50 ng/ml PDGF-AA and 10 µM EIPA, as indicated, calculated as described in the legend for Fig. 3. (A and B) Data shown are individual trajectories (A) or means ± SEM (B) of 20–53 cells in three to seven independent experiments. Data were analyzed using parametric or nonparametric ANOVA, and the level of significance is shown (***, P < 0.001). (C) Images of the left side of the wound in a confluent monolayer of growth-arrested WT MEFs at time 0 and 4 h after the wound was made. The arrows indicate the x and y directions of movement, which were used for estimating directional migration. (D) Velocity of migration perpendicular to the wound (x direction) in PDGF-AA–stimulated, growth-arrested WT MEFs in the absence (open circles) or presence (closed circles) of EIPA. Directionality of movement was estimated by plotting the mean of the distance covered by each cell in the x direction as a function of time. The slope of this line yields the velocity in the x direction (in micrometers/minute), which is a measure of directionality. The velocity in growth-arrested, PDGF-AA–treated WT MEFs was 0.301 ± 0.003 µm/min in the absence and 0.030 ± 0.004 µm/min in the presence of 10 µM EIPA. Data shown are means ± SEM of 20–53 cells in three to seven independent experiments. The velocity in the presence of EIPA is significantly different from that in the absence of EIPA (P < 0.0001). Ctrl., control.
reduced number of primary cilia in the NHE1-null cells (Fig. 7 C).

Therefore, the extent to which these factors contribute to the lack of PDGF-AA-mediated stimulation of migration in NHE1-null cells requires further investigation. Importantly, this is not a concern for the EIPA-treated cells because EIPA affected neither the ability of cells to form primary cilia (Fig. S1 A) nor the activation of PDGFR-α by PDGF-AA (Fig. 3 E).

Discussion

We have previously shown that the primary cilium plays a major role in PDGFR-α-mediated regulation of cell growth
control (Schneider et al., 2005), cell migration, and chemosensory responses (unpublished data). In this study, we demonstrate that the expression but not the activity of the ubiquitous Na⁺/H⁺ exchanger NHE1 is up-regulated on quiescence in serum-free medium, identifying NHE1 as a gas protein. Moreover, we show that NHE1 activity is required for control of directional cell migration by ciliary PDGFR-α signaling, indicating that NHE1 activation is a critical event in the physiological response to activation of PDGFR-α.

The marked, reversible up-regulation of NHE1 protein levels upon growth arrest observed in fibroblasts appears to occur at the transcriptional level because NHE1 mRNA levels were increased to a similar extent. A similar level of up-regulation of NHE1 expression upon serum deprivation was previously reported in kidney epithelial cells (Carraro-Lacroix et al., 2006). Up-regulation of NHE1 expression during growth arrest in Tg737orpk MEFs occurs at a level comparable with that in WT MEFs, indicating that up-regulation proceeds through mechanisms that are independent of ciliary assembly. This is in sharp contrast to the up-regulation of PDGFR-α during growth arrest, which is blocked in Tg737orpk cells (Schneider et al., 2005). In a previous study in NIH3T3 cells stably expressing a fragment of the NHE1 promoter, it was reported that the addition of serum (from a serum level of 0.5 to 10%) increased NHE1 promoter activity (Besson et al., 1998). This could indicate that the growth arrest–induced increase in mRNA and protein levels observed in this study reflects increased mRNA stability. However, inhibition of extracellular signal-regulated kinase activity, which is a well-known consequence of serum deprivation in NIH3T3 cells (Schneider et al., 2005), was reported to stimulate NHE1 promoter activity (Besson et al., 1998), which appears to be in accordance with the present finding that NHE1 expression is increased during growth arrest.

The promoter region of NHE1 contains binding sites for several transcription factors, which are known or likely known to be regulated by serum deprivation, including AP-1 and cAMP response element–binding protein (Miller et al., 1991); however, the potential roles of these proteins in the up-regulation of NHE1 observed in this study remain to be tested.

In contrast to the up-regulation of NHE1 expression in growth-arrested cells, NHE1 activity in NIH3T3 cells was strongly reduced during growth arrest, a finding which is in agreement with studies on other cell types (Reshkin et al., 2000; Lehoux et al., 2001). Thus, in spite of increased expression, NHE1 activity was lower in quiescent cells, presumably reflecting a very tight control of NHE1 activity under these conditions. Recent work has demonstrated a permissive role for an NHE1-mediated increase in pHi, in cell cycle progression, apparently through regulation of cyclin D expression and Cdc2 kinase activity and, thus,

Figure 6. Effects of PDGF-AA and EIPA on translocation of growth-arrested Tg737orpk MEFs in wound-healing assays. [A] Trajectories (normalized to common starting points) of growth-arrested Tg737orpk MEFs in the presence of 50 ng/ml PDGF-AA and in the absence or presence of 10 μM EIPA, as indicated. The radii of the red circles illustrate the mean translocation of the cells within a 5-h time period. [B] Translocation (in micrometers) of growth-arrested Tg737orpk MEFs in the absence or presence of 50 ng/ml PDGF-AA and 10 μM EIPA, as indicated, calculated as described in the legend for Fig. 3. [A and B] Data shown are individual trajectories (A) or means ± SEM (B) of 19–34 cells in two to four independent experiments. [C] Velocity of migration perpendicular to the wound of Tg737orpk MEFs in the absence (open circles) or presence (closed circles) of EIPA. Data shown are means ± SEM of 19–34 cells in two to four independent experiments. The velocity in the presence of EIPA is significantly different from that in the absence of EIPA (P < 0.0001). Ctrl., control.
G2/M entry and transition (Putney and Barber, 2003). It is likely that up-regulation of NHE1 expression, but tight repression of its activity, serves to prepare the cell such that it can elicit a rapid intracellular alkalization at the appropriate time for reentry into the cell cycle upon delivery of the relevant signal. Thus, the dual control of NHE1 at the expression and posttranscriptional levels may serve as a form of coincidence detection, ensuring that these processes are tightly regulated both by cell cycle stage and by the availability of the relevant ligand. A similar mechanism likely pertains to the control of other NHE1-dependent processes (a case in point being the directional migration process addressed in this study).

Finally, although our findings clearly show that the increase in NHE1 expression was not dependent on the formation of the primary cilium, the close temporal correlation between these two important consequences of growth arrest warrants future investigations.

Exposure of growth-arrested NIH3T3 cells to the specific PDGFR-α ligand PDGF-AA partially restored NHE1 activity, which is measured as the global, cytoplasmic NHE1-dependent pH$_i$ recovery after acidification. Although activation of NHE1 by nonisoform-discriminating PDGFR ligands has been demonstrated in several cell types (Cassel et al., 1983; Ma et al., 1994; Yan et al., 2001), this is, to our knowledge, the first study to show that a specific PDGFR-α ligand activates NHE1.

Consistent with the marked decrease in NHE1 activity in quiescent cells, migration was strongly reduced in growth-arrested NIH3T3 cells. In addition to stimulating NHE1 activity, PDGF-AA markedly stimulated directional cell migration in growth-arrested NIH3T3 cells in a manner fully dependent on NHE1 activity. In growth-arrested WT MEFs, which, similar to growth-arrested NIH3T3 cells, have a primary cilium, both the net translocation and the directionality of PDGF-AA–mediated migration were similarly dependent on NHE1 activity. In congruence with the involvement of NHE1, PDGF-AA had no effect on translocation in NHE1-null fibroblasts. In the NHE1-null fibroblasts, reduced PDGFR-α activation may also have contributed to this effect because these cells have problems forming primary cilia as compared with their NHE1-expressing counterparts. As neither EIPA nor acute siRNA-mediated NHE1 knockdown affected cilia formation in NIH3T3 cells, this clearly does not represent a role for NHE1 in cilia formation but appears to be an anomaly reflecting long-term genetic alterations in the PSNEO cells, which was not further addressed in this study (Putney and Barber, 2004).

In marked contrast, in Tg737orpk MEFs, which are deficient in primary cilium and, thus, in PDGFR-α expression (Schneider et al., 2005), migration was unaffected by PDGF-AA and only very modestly affected by EIPA, further supporting the notion of a PDGFR-α–NHE1 axis being required for directional migration. Because PDGF-AA activates only the homodimeric PDGFR-α receptor, which is virtually only localized in the primary cilium, whereas NHE1 is activated at the leading edge of the cell, this poses the question of how activation of NHE1 may act at a distance. NHE1 in both WT and Tg737orpk MEFs localized to the cell surface in the leading edge, which is in agreement with previous studies (Lagana et al., 2000; Denker and Barber, 2002; Stock et al., 2005, 2007; Frantz et al., 2007; Stuwe et al., 2007), but also to intracellular vesicle-like structures. It remains a possibility that when PDGF-AA is added to

Figure 7. Migration, PDGFR-α activation, and cilia formation in NHE1-null and NHE1-expressing cells. (A) PS120 (expressing NHE1) and PSNEO (deficient for NHE1) cells were serum starved for 48 h to induce growth arrest, and migration was analyzed in 4-h wound-healing assays in the absence and presence of 50 ng/ml PDGF-AA, as indicated. The graph shows the mean translocation ± SEM of 45–64 cells in four to six independent experiments. Ctrl., control. (B) PS120 and PSNEO (PS120NEO) cells were serum starved for 48 h, exposed to 50 ng/ml PDGF-AA, and lysed, and Western blots of total and Tyr754-phosphorylated PDGFR-α were performed as described in Materials and methods. β-Actin was used as a loading control. As seen, PDGFR-α expression was similar in the two cell types, whereas PDGFR-AA–induced phosphorylation of PDGFR-α was reduced, although not abolished, in the PSNEO cells. Data shown are representative of three independent experiments for each cell line. (C) PS120 and PSNEO cells were serum starved for 48 h to induce growth arrest, paraformaldehyde fixed, and labeled for acetylated α-tubulin to visualize primary cilium. Approximately 300 randomly chosen cells from each experiment were analyzed for the presence or absence of primary cilia, and the results shown are the mean ± SEM of the percentage of cells with cilia in six independent sets of paired experiments. (A and C) Data were analyzed using parametric or nonparametric ANOVA, and the level of significance is shown (*, P < 0.05).
the medium, NHE1 may be transported to the lamellipodial plasma membrane at a higher level in quiescent WT cells, presumably by vesicular exocytosis. However, our initial observations have not convincingly shown an increase in overall plasma membrane localization of NHE1 after PDGF-AA treatment.

Several mechanisms may underlie the linkage between ciliary PDGFR-α signaling and NHE1 activation. One possible pathway is through ERK1/2 and its effector p90RSK, which activates NHE1 by direct phosphorylation on Ser703 of its C-terminal tail, after several types of stimuli (Takahashi et al., 1999; Cuello et al., 2007). Consistent with such a mechanism, we have previously shown that ERK1/2 is activated in NIH3T3 cells and WT MEFs but not in Tg737<sup>+/−</sup> MEFS in response to PDGFR-α activation in the primary cilium (Schneider et al., 2005). The PI3K–Akt pathway is also activated in MEFS downstream of PDGFR-α activation (unpublished data) and could also contribute to NHE1 stimulation, e.g., by direct phosphorylation of NHE1 (Ser<sup>648</sup> of human NHE1 being a high-probability consensus site for phosphorylation by Akt1) and/or by inducing actin and microtubule cytoskeletal rearrangements.

In contrast to the almost complete ablation of PDGF-AA–mediated migration by EIPA in cells with primary cilia (i.e., growth-arrested NIH3T3 cells and WT MEFS), migration of cells lacking cilia (i.e., interphase NIH3T3 cells and Tg737<sup>+/−</sup> MEFS) had a substantial EIPA sensitivity (Banizs et al., 2007). This only partial role for NHE1 in pHi regulation contrasts with the fact that during growth arrest when cell migration was previously demonstrated (Schwab et al., 2005). The rate of cell migration was previously found to be ∼40% lower in NHE1-null compared with NHE1-expressing PS120 fibroblasts (Denker and Barber, 2002), which is comparable with the difference between growth-arrested and interphase NIH3T3 cells seen in this study. The difference between interphase and growth-arrested cells presumably reflects the fact that although NHE1 expression is up-regulated in growth-arrested cells, NHE1 activity is essentially absent (shown in Fig. 2 for NIH3T3 cells). Collectively, these data indicate that the relative role of NHE1 in directional cell migration is cell cycle state and cell type dependent and that other ion transport proteins can fulfill roles similar to that of NHE1 under some conditions. A major candidate is Na<sup>+</sup>–HCO<sub>3</sub>− cotransport, for which a role in cell migration was previously demonstrated (Schwab et al., 2005).

A recent study showed that Na<sup>+</sup>–dependent HCO<sub>3</sub>− transport appeared more important than NHE1 in pH regulation in both WT and Tg737<sup>+/−</sup> choroid plexus epithelia in the mouse (Banizs et al., 2007). This only partial role for NHE1 in pH regulation contrasts with the fact that during growth arrest when primary cilia were present, both NIH3T3 cells and WT MEFS were fully dependent on NHE1 activity for PDGF-AA–mediated migration. Notably, NHE1 played a greater role in global pH recovery after acidification in interphase NIH3T3 cells than in growth-arrested, PDGF-AA–treated NIH3T3 cells (Fig. 2), but the relative contribution of NHE1 to both translocation and directional migration was greater in the latter. In other words, there is no strong correlation between the effect of NHE1 on global pH, and its role in directional migration. These data reinforce the possibility that the role of NHE1 in directional migration in response to ciliary PDGFR-α signaling involves a local signaling circuit at the leading edge of the migrating cells and does not reflect the contribution of the transporter to global pH recovery.

We can envision a sequences as follows: when PDGF-AA is added, (a) important ciliary PDGFR-α signaling components are activated, (b) the activation of downstream signaling components (e.g., ERK1/2 or Akt) activates NHE1 in the cell membrane for ion transport or alternatively promotes increased lamellipodial localization of NHE1, and (c) at the leading edge, NHE1, in turn, is pivotal for the F-actin organization required for cell migration. The effect of NHE1 on F-actin organization may be mediated via effects on Cdc42 activity (Frantz et al., 2007), on protein kinase activity (Pedersen et al., 2007a), or as a consequence of direct pH sensitivity of F-actin regulatory proteins such as cofilin or talin (Srivastava et al., 2007). Another possible pathway involves the interaction of NHE1 with ERM proteins, which has been found to play a role in cell migration (Denker and Barber, 2002) as well as in Akt activation after some (Wu et al., 2004) albeit not all (Rasmussen et al., 2008) stimuli.

In a few experiments, we observed by immunofluorescence microscopy analysis a weak localization of NHE1 to primary cilia of NIH3T3 cells (unpublished data), although, in most cases, we could not detect increased NHE1 labeling in the cilium. NHE1 contains two unique ciliary targeting peptide motifs, including an N-terminal sequence motif (R144FP), which is similar to that required for ciliary targeting of the transient receptor potential ion channel, and polycystin-2 (R6VXP; Geng et al., 2006). Although speculative at this point, it is thus possible that NHE1 has dual functions, i.e., in the primary cilium, where PDGFR-α–mediated signal transduction is initiated, and in the plasma membrane, where NHE1 is activated at the leading edge of migrating cells.

Thus, we have shown in this study that NHE1 activity is specifically required for PDGF-AA–mediated stimulation of fibroblast migration in a manner downstream from activation of PDGFR-α and not reflecting a role for NHE1 in formation of the primary cilium. Although a role for leading-edge NHE1 activity in cell polarity and directional migration has been demonstrated in a variety of cell types (Lagana et al., 2000; Denker and Barber, 2002; Patel and Barber, 2005; Stock et al., 2005, 2007; Frantz et al., 2007; Stuwe et al., 2007), this study is, to our knowledge, the first to demonstrate a pivotal role of NHE1 in the response to chemical stimuli detected and transmitted through the primary cilium. These findings support the notion that the primary cilium functions as a cellular positioning detector in directional cell migration. Therefore, the interplay between the primary cilium and NHE1 may be critical to the physiological response to PDGFR-α signaling in development processes and tissue homeostasis.

Materials and methods

Cell culture and reagents

Experiments were performed on NIH3T3 fibroblasts, WT and Tg737<sup>+/−</sup> mutant MEFS, NHE1-null PS120 fibroblasts (denoted PSNEO), and PS120 fibroblasts stably expressing NHE1 (provided by D. Barber, University of California, San Francisco, San Francisco, CA; Denker et al., 2000). Tg737 encodes the protein polariz/IFT88, which is part of the intraflagellar transport...
protein complex responsible for assembly and maintenance of the primary cilium (Rosenbaum and Witman, 2002), and 5′-GAGACCTGACATCCTGCTGTT-3′, 2 µM reverse primer 5′-GTGAT-GCTGGCAAAATTCCTC-3′, 2 µM NHE1 TaqMan probe 5′-TGCGCGCTAC- GTGTCCTCTATCACCT-3′, 30 nM reference dye (Agilent Technologies Inc.), and Brilliant QPCR master mix (Agilent Technologies Inc.). 18S was used as a reference gene (18S master mix: 2 µM forward primer 5′-TITTAATATTACCGATGAGGAA-3′, 2 µM reverse primer 5′-GACATCTGG- AGGGCAAGT-3′, 2 µM NHE1 TaqMan probe 5′-TACCCCGGTGTCGAC-3′, 30 nM reference dye, and Brilliant QPCR master mix). Real-time PCR was performed using a real-time thermal cycler (Agilent Technologies, Inc.; 95°C for 10 min, 40 cycles of 95°C for 30 s, and 58°C for 1 min), and data were analyzed with Max4000 software (Agilent Technologies, Inc.).

SDS-PAGE and Western blot analysis
Cells grown in Petri dishes were washed with ice-cold PBS, and RNA was purified using the RNeasy Mini kit (QIAGEN). RNA was converted to cDNA by reverse transcription using Superscript RT II (Invitrogen) and 250 µg/µl random primers (Invitrogen) according to the manufacturer’s instructions. Real-time PCR set up was performed as triplets of each individual sample. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature followed by permeabilization with 1% triton X-100. Cells were washed in PBS with 2% BSA for 30 min and incubated with the following primary antibodies at room temperature for 2 h: anti-acetylated α-tubulin (1:5,000 mouse antibody; Sigma-Aldrich), anti-PDGFRα (Santa Cruz Biotechnology, Inc.), and anti-NHE1 (Xb-17; both rabbit antibodies were used at 1:500). Cells were washed in PBS and incubated with DAPI (Invitrogen) and Alexa Fluor 568-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (both at 1:1,000; Invitrogen) for 1 h. Preparations were mounted in N-propargylgallate at 2% wt/vol in PBS/glycerin and were visualized at room temperature. For standard epifluorescence imaging, cells were visualized on a microscope (Eclipse E600; Nikon) with EPL3 filters, a 100× 1.25 NA objective, and a cooled charged-device camera (MagnaFire; Optronics). For confocal imaging, cells were visualized using a 40× 1.25 NA Plan-Apochromat objective and the 488-nm Ar/Kr laser line of a microscope (DMR/LE; Leica) with a confocal laser-scanning unit (TSC NT; Leica). Optical slice thickness was 1 µm, and pinhole size was 1 airy disc. Images shown are frame averaged and presented in RGB pseudocolor. No corrections for labeling were made in the absence of a control (untreated). Digital images were processed (overlays and brightness/contrast adjustment only) using Photoshop CS2 version 9.0.2 (Adobe).

Real-time PCR
Cells were grown in Petri dishes and washed with ice-cold PBS, and RNA was purified using the RNeasy Mini kit (QIAGEN). RNA was converted to cDNA by reverse transcription using Superscript RT II (Invitrogen) and 250 µg/µl random primers (Invitrogen) according to the manufacturer’s instructions. Real-time PCR set up was performed as triplets of each individual sample. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature followed by permeabilization with 1% triton X-100. Cells were washed in PBS with 2% BSA for 30 min and incubated with the following primary antibodies at room temperature for 2 h: anti-acetylated α-tubulin (1:5,000 mouse antibody; Sigma-Aldrich), anti-PDGFRα (Santa Cruz Biotechnology, Inc.), and anti-NHE1 (Xb-17; both rabbit antibodies were used at 1:500). Cells were washed in PBS and incubated with DAPI (Invitrogen) and Alexa Fluor 568-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (both at 1:1,000; Invitrogen) for 1 h. Preparations were mounted in N-propargylgallate at 2% wt/vol in PBS/glycerin and were visualized at room temperature. For standard epifluorescence imaging, cells were visualized on a microscope (Eclipse E600; Nikon) with EPL3 filters, a 100× 1.25 NA objective, and a cooled charged-device camera (MagnaFire; Optronics). For confocal imaging, cells were visualized using a 40× 1.25 NA Plan-Apochromat objective and the 488-nm Ar/Kr laser line of a microscope (DMR/LE; Leica) with a confocal laser-scanning unit (TSC NT; Leica). Optical slice thickness was 1 µm, and pinhole size was 1 airy disc. Images shown are frame averaged and presented in RGB pseudocolor. No corrections for labeling were made in the absence of a control (untreated). Digital images were processed (overlays and brightness/contrast adjustment only) using Photoshop CS2 version 9.0.2 (Adobe).

Measurements of pH
pH was monitored at 37°C essentially as previously described (Pedersen et al., 2007b). In brief, NIH3T3 cells or WT or Tg/3′-sMef were seeded on glass coverslips 24–48 h before experiments. Confluency at the time of experiments was 80–95%. Cells were loaded with 1.2 µM BCECF-AM in Ringer’s solution (150 mM NaCl, 1 mM Na2HPO4, 1 mM CaCl2, 10 mM Hepes, and 5 mM glucose, pH 7.4), and mounted in the cuvette of a spectrofluorometer (Ratiomaster; PTI). Emission was detected at 525 nm after excitation at 445 and 495 nm (NIH3T3 fibroblasts) or 445 and 500 nm (MEFs). The 445:495 nm ratio was calculated after background subtraction, and correlation to pH was performed using the 7-point nigerin/High K+ technique, essentially as previously described (Boyarsky et al., 1988). The high K+ Ringer solution was similar to the standard Ringer solution, except that 140 mM NaCl was replaced with KCl. The ability of the cells to recover after an acid load was evaluated by the NH4Cl prepulse technique: cells were exposed to 10 mM NH4Cl in Ringer’s solution for 5 min followed by NH4Cl removal. To induce intracellular acidification, the recovery from which is a measure of the capacity for 37°C/C02 and 95% humidity. Cells were examined at either 70% confluency and contributions from directed migration induced by the wound-healing condition and is a measure of sustained migration (Schwab et al., 2006). The directionality of movement was estimated by plotting the mean of the number of pixels within the cell outlines. We used two parameters to quantify translocation between the position of the cell center at the beginning and at the end of the experiment for the observed group of cells. Translocation summarizes and contributes from directed migration induced by the wound-healing condition and is a measure of sustained migration (Schwab et al., 2006). The directionality of movement was estimated by plotting the mean of the number of pixels within the cell outlines. We used two parameters to quantify translocation between the position of the cell center at the beginning and at the end of the experiment for the observed group of cells. Translocation summarizes
plot is the velocity in the x direction (in micrometers/minute) and is a measure of directionality in our experimental setup. The corresponding plots for directionality in the y direction (parallel to the wound) were always close to zero, indicating that in this experimental setup, directional movement is measured only perpendicular to the wound.

siRNA-mediated knockdown of NHE1
NHE1 knockdown in NIH3T3 cells was performed using SMARTpool siRNA (Thermo Fisher Scientific) against mouse NHE1 [NHE1] or scrambled oligonucleotides of comparable GC content (mock), both at 50 nM, and FECT transfection agent (Thermo Fisher Scientific) according to the manufacturer’s instructions. The day after siRNA treatment, the cells were serum starved as described in Cell culture and reagents and were used for experiments 48 or 72 h after siRNA treatment as indicated. Cilia counting experiments were always accompanied by parallel Western blots against NHE1 to verify knockdown.

Statistics
All experiments were repeated 3–11 times, and data are presented as representative individual experiments or as mean values ± SEM. The data were tested for significance using analysis of variance (ANOVA) or Kruskal Wallis test (nonparametric ANOVA). The level of significance was set at P < 0.05 except where otherwise stated.

Online supplemental material
Fig. S1 shows the effects of EIPA and NHE1 knockdown in NIH3T3 fibroblasts. Video 1 shows the migration of growth-arrested WT MEFs in a wound-healing assay in the absence of EIPA and PDGF-AA. Videos 2 and 3 show the migration of PDGF-AA–stimulated, growth-arrested WT MEFs in a wound-healing assay in the absence or presence of EIPA, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200806019/DC1.

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