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Focal adhesion kinase PTK2 autophosphorylation is not required for the activation of sodium–hydrogen exchange by decreased cell volume in the preimplantation mouse embryo

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Summary

Recovery from decreased cell volume is accomplished by a regulated increase of intracellular osmolarity. The acute response is activation of inorganic ion transport into the cell, the main effector of which is the Na⁺/H⁺ exchanger NHE1. NHE1 is rapidly activated by a cell volume decrease in early embryos, but how this occurs is incompletely understood. Elucidating cell volume-regulatory mechanisms in early embryos is important, as it has been shown that their dysregulation results in preimplantation developmental arrest. The kinase JAK2 has a role in volume-mediated NHE1 activation in at least some cells, including 2-cell stage mouse embryos. However, while 2-cell embryos show partial inhibition of NHE1 when JAK2 activity is blocked, NHE1 activation in 1-cell embryos is JAK2-independent, implying a requirement for additional signalling mechanisms. As focal adhesion kinase (FAK aka PTK2) becomes phosphorylated and activated in some cell types in response to decreased cell volume, we sought to determine whether it was involved in NHE1 activation in the early mouse embryo. FAK activity requires initial autophosphorylation of a tyrosine residue, Y397. However, FAK Y397 phosphorylation levels were not increased in either 1- or 2-cell embryos after cell volume was decreased. Furthermore, the selective FAK inhibitor PF-562271 did not affect NHE1 activation at concentrations that essentially eliminated Y397 phosphorylation. Thus, autophosphorylation of FAK Y397 does not appear to be required for NHE1 activation induced by a decrease in cell volume in early mouse embryos.

Introduction

The regulation of cell volume is essential for cell viability (Hoffmann *et al.*, 2009). Mammalian cells respond to a decrease in volume by increasing intracellular osmolarity, with the acute response being the activation of inorganic ion import using transporters in the plasma membrane (Hoffmann *et al.*, 2009). Decreased cell volume activates Na⁺/H⁺ exchangers of the NHE (Slc9) family, mainly the ubiquitous NHE1 (Slc9a1 gene product) (Alexander & Grinstein, 2006; Donowitz *et al.*, 2013). Activation of Na⁺/H⁺ exchange not only imports Na⁺ but also results in an increase in intracellular pH (pHi) because of the coupled H⁺ efflux. This in turn causes the secondary activation of HCO₃⁻/Cl⁻ exchangers of the AE (Slc4) family (Romero *et al.*, 2013), which opposes the pHi increase and imports Cl⁻ (Humphreys *et al.*, 1995; Jiang *et al.*, 1997). The net result is the coupled import of Na⁺ and Cl⁻ to increase intracellular osmolarity without any net change in pHi. A second mechanism that can be involved in responding to decreased cell volume is the Na⁺,K⁺,2Cl⁻ cotransporter NKCC1 (Slc12a2) (Arroyo *et al.*, 2013; Rasmussen *et al.*, 2015). Like NHE1, NKCC1 can be quickly activated by a volume decrease. In most mammalian cell types, however, the NHE1-based mechanism is thought to constitute the principal acute response to cell volume decreases (Hoffmann *et al.*, 2009; Alexander & Grinstein, 2006).

The mechanisms linking cell volume sensing to cell volume regulation are incompletely understood (Alexander & Grinstein, 2006; Hoffmann *et al.*, 2009; Hoffmann & Pedersen, 2011; Rasmussen *et al.*, 2015). NHE1 is a key regulator of pHi and is activated directly by decreased cytoplasmic pH through protonation of the NHE1 protein (Aronson *et al.*, 1982). NHE1 can also be activated downstream of growth factor signalling by direct phosphorylation

(Malo & Fliegel, 2006; Sardet *et al.*, 1990). However, neither decreased pHi nor direct phosphorylation of NHE1 protein are implicated in its activation when induced by decreased cell volume (Alexander & Grinstein, 2006; Hoffmann *et al.*, 2009). Only one pathway has been clearly implicated in signal transduction between decreased cell volume and NHE1 activation. Janus kinase 2 (JAK2) was found to be phosphorylated and activated upon decreased cell volume in several cell types including 2-cell mouse embryos (Gatsios *et al.*, 1998; Zhou & Baltz, 2013; Xu *et al.*, 2017). Activated JAK2 then phosphorylates calmodulin (CaM) which in turn binds to and activates NHE1 to mediate cell volume recovery (Garnovskaya *et al.*, 2003). However, it appears unlikely that this non-canonical JAK2 signalling pathway is the sole mechanism of NHE1 activation by decreased cell volume, as complete inhibition of JAK2 fails to completely abolish NHE1 activation, at least in the cell types that have been examined thus far (Hoffmann *et al.*, 2009; Garnovskaya *et al.*, 2003; Zhou & Baltz, 2013).

In addition to JAK2, other kinases can be activated by cell shrinkage and therefore could have a role in activating NHE1. In several cell types, decreased cell volume has been shown to quickly stimulate phosphorylation of tyrosine residues on focal adhesion kinase (FAK aka PTK2). In general, the initial activation of FAK requires autophosphorylation of Y397. SRC then binds to the region of FAK that includes phosphorylated Y397, in turn phosphorylating other residues on FAK such as Y576/577, Y861, and Y925, leading to further activation of FAK (Schaller *et al.*, 1994; Schlaepfer & Hunter, 1996; Schlaepfer *et al.*, 2004; Arold, 2011; Rasmussen *et al.*, 2015). Upon cell shrinkage, FAK is rapidly phosphorylated on Y397 in many (although not all) cell types that have been investigated (Lunn & Rozengurt, 2004; Lunn *et al.*, 2007; Rasmussen *et al.*, 2015) and therefore FAK was an attractive candidate to contribute to activation of NHE1 in cell volume regulation, including in embryos.

Early preimplantation embryos of mammals are especially sensitive to osmotic stress and decreased cell volume. In the mouse, relatively minor dysregulation of cell volume control causes development to become irreversibly blocked at the 2-cell stage (Lawitts & Biggers, 1992; Hadi *et al.*, 2005; Baltz & Tartia, 2010; Baltz & Zhou, 2012). It was found that this embryonic arrest was likely to be due to the accumulation of inorganic ions via NHE1 needed to balance even physiologically normal external osmolarity (Zhou & Baltz, 2013; Zhou *et al.*, 2013; Xu *et al.*, 2017). Because of the crucial role that cell volume regulation plays in early embryogenesis, their mechanisms have been extensively investigated (Baltz & Tartia, 2010; Baltz & Zhou, 2012). We have shown that mouse embryos throughout preimplantation development activate NHE1 in response to decreased cell volume and that NHE1 activity is needed to maintain their cell volumes (Zhou & Baltz, 2013; Xu *et al.*, 2017).

NHE1 has several roles that, besides cell volume regulation as detailed above, mainly include regulating pHi by mediating recovery from intracellular acidosis. It has previously been established that NHE1 is present and active in pHi regulation in mouse embryos (Gibb *et al.*, 1997; Steeves *et al.*, 2001; Harding *et al.*, 2002; Siyanov & Baltz, 2013). NHE1 activity is elicited by decreased pHi throughout preimplantation development, exhibiting its highest activity at the 8-cell and morula stages (Siyanov & Baltz, 2013). By contrast, NHE1 activation by a cell volume decrease is most robust at the 2-cell stage with less activity at the 8-cell and morula stages (Steeves *et al.*, 2001; Siyanov & Baltz, 2013; Zhou & Baltz, 2013; Xu *et al.*, 2017).

NHE1 activation by decreased cell volume in mouse embryos, at least at the 1- and 2-cell stages, is entirely dependent on the action of tyrosine kinases, as their broad-spectrum inhibition by genistein completely prevents NHE1 activation (Zhou & Baltz, 2013; Xu *et al.*, 2017). JAK2 has been implicated as a tyrosine kinase mediating NHE1 activation at the 2-cell embryo stage (Zhou & Baltz, 2013); it is rapidly activated upon a cell volume decrease and rapidly inactivated when volume is restored (Xu *et al.*, 2017). At the 2-cell stage, much of NHE1 activation appears to be mediated by JAK2, but a significant proportion of NHE1 activation remains even when JAK2 is entirely inhibited (Zhou & Baltz, 2013; Xu *et al.*, 2017). By contrast, although NHE1 is also activated by a cell volume decrease at the 1-cell embryo stage (although to a lesser extent than at the 2-cell stage), its activation is almost entirely independent of JAK2; the JAK2-dependent portion only appears at the end of the 1-cell stage just before cleavage (Xu *et al.*, 2017). This led naturally to the hypothesis that the same JAK2-independent component was present at both the 1-cell and 2-cell stages, with a JAK2-dependent component added at the 2-cell stage. JAK2 phosphorylation by decreased cell volume may peak at the 2-cell stage, as 8-cell embryos did not show a significant increase in tyrosine phosphorylation in response to a cell volume decrease (Zhou & Baltz, 2013).

It has previously been shown that FAK (PTK2 protein) is present in mouse oocytes and fertilized eggs (McGinnis *et al.*, 2013; McGinnis & Kinsey, 2015). The aim of this study was to determine if FAK autophosphorylation was required for activation of NHE1 in response to a cell volume decrease. The early preimplantation mouse embryo is one of only a few cell types in which the signalling leading to osmotic NHE1 activation has been elucidated (Garnovskaya *et al.*, 2003; Hoffmann *et al.*, 2009; Zhou & Baltz, 2013) and the only one in which JAK2-dependent signalling has been shown to appear during development (Xu *et al.*, 2017). We focused on phosphorylation of FAK Y397 as it is the initial autophosphorylation event considered to be required for FAK activation. The results of this study did not support the hypothesis that FAK, at least its Y397 autophosphorylation, is involved in activation of NHE1 in mouse embryos.

Materials and methods

Chemicals and solutions

Chemicals were obtained from Sigma-Aldrich (Oakville, Ontario, Canada) unless otherwise specified. Components of culture medium were embryo-tested or cell culture-grade. The FAK inhibitor PF-562271 was manufactured by Selleck Chemicals (Houston, Texas). SNARF-1-AM (carboxysemaphthorhodafuor-1-acetoxymethyl ester) was purchased from Invitrogen Molecular Probes (Eugene, OR, USA). Stock solutions were prepared for nigericin in ethanol and for valinomycin, SNARF-1-AM, and PF-562271 in dimethyl sulfoxide and stored at -20°C .

The medium used with embryos were modified HEPES-buffered potassium-supplemented simplex-optimized medium (KSOM) mouse embryo medium (Lawitts & Biggers, 1993) designated mHEPES-KSOM which contains (in mM) 95 NaCl, 2.5 KCl, 0.35 KH_2PO_4 , 0.2 MgSO_4 , 10 Na lactate, 0.2 glucose, 0.2 Na pyruvate, 4 NaHCO_3 , 1.7 CaCl_2 , 0.01 tetrasodium EDTA, 0.03 streptomycin SO_4 , 0.16 penicillin G, and 21 HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)). Polyvinyl alcohol (PVA) was used instead of bovine serum albumin as the macromolecular

component. pH was adjusted to 7.4 with NaOH. For pHi measurements, PVA was omitted, 9 mM of Na lactate was replaced with NaCl, and NaHCO₃ was replaced with equimolar NaCl, resulting in bicarbonate-free mHEPES–KSOM (0bicHEPES–KSOM). Media were equilibrated with air and used at 37°C.

Collection of embryos

All mice were obtained from Charles River, St. Constant, QC, Canada). The Animal Care Committee (ACC) of the University of Ottawa approved all animal protocols. Euthanasia was by cervical dislocation by trained personnel. Because anaesthetic agents can interfere with membrane transporters, anaesthesia was not used, which is acceptable under the Canadian Council on Animal Care Guidelines on Euthanasia of Animals Used in Science, 2010 (Section 5.6) and was approved by the ACC.

Embryos were obtained from CF1 female mice 4–6 weeks of age. Females were superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin (eCG) followed by human chorionic gonadotropin (hCG, 5IU) 47 h later (both from Intervet, Kirkland, QC). Females were caged overnight with BDF1 (B6D2F1) males for mating. Embryos were flushed from excised oviducts with mHEPES–KSOM ~19–22 h post-hCG for 1-cell embryos and ~42–44 h for 2-cell embryos. A brief exposure to hyaluronidase (300 mg/ml) was used to free 1-cell embryos from the cumulus matrix. Embryos that were not of high quality (e.g. with uneven blastomeres, granular cytoplasm, or fragmentation) were uncommon and were discarded.

Each female mouse yields about 10–15 embryos. For experiments in which pHi was measured on 9–12 embryos simultaneously, embryos were obtained from 1–2 females. For western blots, in which each lane contained lysate from 60 embryos, embryos were pooled from females until the requisite number was obtained.

Intracellular pH measurements

pHi was measured with a quantitative imaging microscope (ISee Imaging Systems, Raleigh, NC) as previously described (Zhou & Baltz, 2013; Xu *et al.*, 2017). Embryos were loaded with the pH-sensitive fluorophore, SNARF-1 by incubating them for 30 min with 5 μM SNARF-1-AM. pHi was determined by fluorescence ratio imaging using emission wavelengths of 600 and 640 nm with excitation at 535 nm. Images were divided after background subtraction to obtain 640/600 ratiometric images, which are dependent mainly on pHi. Ratios were calibrated using the nigericin/high K⁺ method with valinomycin (Thomas *et al.*, 1979) as previously described (Baltz & Phillips, 1999). All measurements were done at 37°C in air. NHE1 activity was measured by monitoring the pHi of a group of embryos simultaneously, first for 10 min at 250 mOsM and then for 15 min at 350 mOsM, for in total, 25 min. Where plots of pHi *v.* time are presented, the osmolarity is indicated within boxes on the time axis. Where its presence is indicated, embryos were exposed to the inhibitor (PF-562271) for ~30 min before osmolarity was increased and continued to be present in the higher osmolarity medium as previously described (Xu *et al.*, 2017).

We have previously established and validated this method for measuring cell volume decrease-induced NHE1 activity in embryos (Zhou & Baltz, 2013; Xu *et al.*, 2017). Briefly, pHi was shown to increase in response to an increase in external osmolarity concomitant with the decrease in cell volume, and this increase was confirmed as being due to Na⁺/H⁺ exchange by

showing that it did not occur in the absence of external Na or in the presence of amiloride, a general inhibitor of Na⁺/H⁺ exchange mediated by NHE isoforms (Zhou & Baltz, 2013). The Na⁺/H⁺ exchanger activity was established as being due to NHE1 using the highly selective NHE1 inhibitor, cariporide, which completely blocked the pHi increase and impaired the ability of embryos to maintain their resting volumes (Zhou & Baltz, 2013). Finally, unfertilized eggs and preimplantation embryos have been shown to express the Slc9a1 mRNA that encodes NHE1 (Siyonov & Baltz, 2013). Therefore, the rate of pHi increase upon a cell volume decrease has been established to reflect NHE1 activity. The initial rate of increase of pHi was determined by a linear least square fit to the initial portion of the curve to provide a quantitative measure of NHE1 activity (Xu *et al.*, 2017).

Western blots

The primary antibodies used were as follows. For FAK phosphorylated on Y397, rabbit affinity-purified polyclonal raised against a peptide (ab81298, Abcam, Toronto, ON, Canada) was used at 1:15 000 dilution. For total FAK, the rabbit C-20 polyclonal directed against the FAK C-terminus (sc-558, Santa Cruz Biotechnology, Mississauga, ON, Canada) was used at 1:800 dilution. For GAPDH, rabbit FL-335 antibody against full-length GAPDH was used (sc-25778, Santa Cruz) at 1:500 dilution. The secondary antibody for all was HRP goat anti-rabbit IgG (1721019, Bio-Rad, Mississauga, ON, Canada) was used at 1:10 000 for Y397 and 1:5000 for FAK and GAPDH. All antibodies were blocked with 5% milk powder.

For western blots, embryos were isolated and then incubated in 250 mOsM medium for 30 min either in the presence of the FAK inhibitor PF-562271 or with vehicle (dimethyl sulfoxide) alone. The embryos were then transferred to 550 mOsM medium in the continued presence of the inhibitor or vehicle and incubated at 550 mOsM for 15 min. At the end of this incubation, the embryos (10 embryos per μl) were transferred onto the inside of the lid of a 0.2 ml microcentrifuge tube into an equal volume of 2 × lysis buffer (containing 5% SDS, 200 μM VO₄, and 80 μM phenylarsine oxide) and, after lysis, centrifuged into the tube and stored at –80°C. In total, 60 embryos were added to each tube.

Embryo lysates (60 embryos per lane) were separated by SDS PAGE (10% gels), transferred to nitrocellulose membranes, blocked for 1 h, and incubated with primary antibody overnight at 4°C. Blots were then washed and incubated with secondary antibody for 1 h at room temperature. Visualization was performed using an ECL Prime Detection Kit (Amersham, GE Healthcare UK Limited, Buckinghamshire, UK). Before incubation with primary antibodies, blots were cut at ~50 kDa. The upper portion was probed for Y397. After detection, these blots were stripped and re-probed for total FAK. The lower portion was probed for GAPDH.

Western blots band densities were quantified from scanned images of films with Image J (v1.51, NIH) using its standard algorithm (<https://imagej.nih.gov/ij/docs/menus/analyze.html#gels>). Relative band densities were normalized to the sum of the density on each western blot, which is arbitrarily set to 100. FAK Y397 phosphorylation was quantified by dividing each normalized band density to total FAK in the same lane.

Imposed cell volume decreases

Embryo cell volume decreases were imposed using hypertonic mHEPES–KSOM whose osmolarity was increased with the

trisaccharide D-(+)-raffinose. Osmolarity was measured with a vapour pressure osmometer (model 5520, Wescor, Logan, UT, USA). We have extensively used hypertonic raffinose solutions for decreasing cell volume in embryos, as raffinose is not transported into or metabolized by mammalian cells and this use has been extensively validated in embryos (Dawson & Baltz, 1997; Zhou & Baltz, 2013; Xu *et al.*, 2017). Briefly, a rapid cell volume decrease was imposed by changing the external osmolarity from the normal 250 mOsM of mHEPES-KSOM to a higher value, thus decreasing embryo cell volumes due to the increased external osmotic pressure. For pHi measurements, decreased cell volume was induced with 350 mOsM medium. We previously showed that the rate of pHi increase was robust at both 350 and 550 mOsM (Zhou *et al.*, 2013). For western blots, we had previously shown for JAK2 that phosphorylation was more easily detected at 550 than 350 mOsM (Zhou & Baltz, 2013). We therefore used 350 mOsM for pHi measurements to minimize cellular stress in live embryos during the measurements but 550 mOsM for western blots to minimize the number of embryos required per lane, as previously described and validated (Xu *et al.*, 2017). Embryos remain viable through such exposures, as 2-cell embryos will develop to blastocysts following a 15 min exposure to 550 mOsM medium comparable to unexposed embryos (our unpublished observation).

Data analysis

Plots were generated, and statistical analyses performed using Prism 7 (GraphPad Software, San Diego, CA, USA). Data are presented as the mean \pm standard error of the mean (SEM). Means were compared by unpaired *t*-test (two groups) or one-way analysis of variance (ANOVA) (>2 groups) followed by Tukey's multiple comparison test when the overall ANOVA indicated a significant difference. Difference of a mean to a theoretical value was tested by one-sample *t*-test. A *P*-value < 0.05 was considered significant, but actual calculated *P*-values are reported here when reported by Prism.

Results

We first determined whether maximally inhibiting FAK would affect the ability of 2-cell embryos to activate NHE1 in response to a cell volume decrease. These experiments relied on our previously validated assay that detects volume-mediated increases in NHE1 activity as a marked intracellular alkalinization when cell volume is decreased by introduction of hypertonic medium (Zhou & Baltz, 2013; Zhou *et al.*, 2013; Xu *et al.*, 2017). We used the selective FAK inhibitor PF-562271 at a high concentration that would be expected to completely block any FAK activity (Roberts *et al.*, 2008) and that was similar to concentrations previously used to inhibit FAK in other types of intact cells (Wiemer *et al.*, 2013; Yoon *et al.*, 2014). We chose PF-562271 because it was considered among the most selective for FAK and was being developed as an anti-cancer drug and therefore its selectivity had been extensively assessed (Roberts *et al.*, 2008; Wiemer *et al.*, 2013). PF-562271 is about 10-fold more selective for FAK over the closely related kinase PYK2 in *in vitro* assays; at higher concentrations it also exhibits inhibition of several cyclin-dependent kinases and GSK3 (Roberts *et al.*, 2008), which are, however, not implicated in cell volume regulation.

The addition of 10 μ M PF-562271 significantly decreased the intracellular alkalinization induced by a cell volume decrease after

introduction of hypertonic medium (350 mOsM 0bicHEPES-KSOM) in both 1- and 2-cell embryos (Fig. 1). In 1-cell embryos the inhibitor almost completely prevented the increase in intracellular pH, while in the 2-cell embryos it reduced the rate of intracellular alkalinization by \sim 50 % relative to the control.

We next sought to determine whether FAK was indeed activated by decreased cell volume in early mouse embryos. As the initial activation of FAK requires autophosphorylation of Y397 (Arold, 2011), we used an antibody that detects FAK phosphorylated on Y397. A band was detected at the expected size of \sim 125 kDa in both 1- and 2-cell embryos that were incubated in isotonic (250 mOsM) medium (Fig. 2A). The band detected likely represented a phosphorylated protein, as it was eliminated by the presence of PF-562271 (10 μ M). When the cell volume of embryos was decreased by exposure to 550 mOsM medium for 15 min, there was, however, no increase in Y397 phosphorylation at either stage (Fig. 2B). Therefore, it does not appear that FAK in early mouse embryos becomes further autophosphorylated and activated in response to decreased cell volume.

The observation that 10 μ M PF-562271 inhibited NHE1 activation upon a cell volume decrease in both 1-cell and 2-cell stage embryos (Fig. 1) but FAK Y397 phosphorylation was not increased were possibly contradictory. Pharmacological inhibitors generally can have off-target effects, particularly at higher concentrations (Karaman *et al.*, 2008). PF-562271 is reportedly selective for FAK, but at higher concentrations could inhibit a number of other tyrosine kinases (Roberts *et al.*, 2008) as discussed above. Therefore, we hypothesized that, if the inhibition of

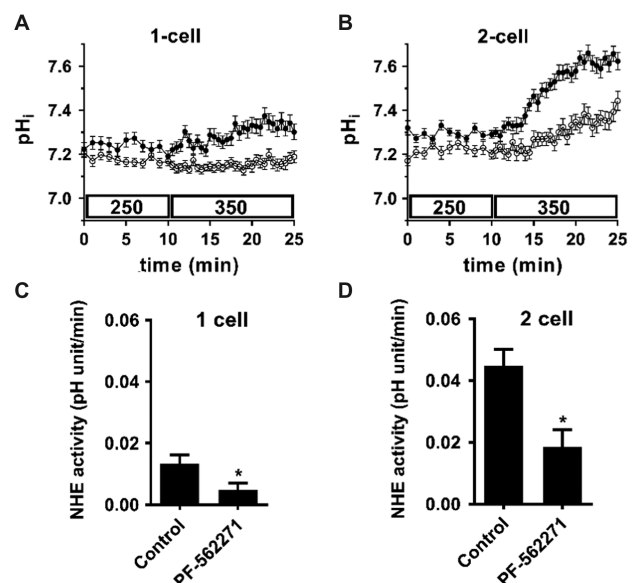


Figure 1. Inhibition of NHE1 activity stimulated by a decreased cell volume in both 1-cell and 2-cell embryos by the selective FAK inhibitor PF-562271. Representative examples of pHi as a function of time in 1-cell (A) or 2-cell (B) embryos in the presence of PF-562271 (10 μ M; open circles) or control (vehicle alone; 0.02% DMSO; filled circles). Each trace represents the mean pHi (\pm SEM) of 10 (A) or 11 (B) embryos measured simultaneously for each treatment. A decrease in cell volume was induced at $t=0$ min by replacing 250 mOsM with 350 mOsM medium. PF-562271 or vehicle was present from $t=0$ min. NHE1 activity was quantified as the mean rate of increase in pHi after introduction of 350 mOsM medium for $N=3$ independent repeats (C, D), examples of which are shown in (A) and (B). Each repeat had $N=9-12$ embryos within each treatment group. Means were compared within each embryo stage by *t*-test. **P*=0.019 in (C), *P*=0.025 in (D). These initial results were consistent with the hypothesis that FAK mediates NHE1 activation by decreased cell volume at the 1-cell stage and that this component persists into the 2-cell stage when an additional component mediated by JAK2 is added.

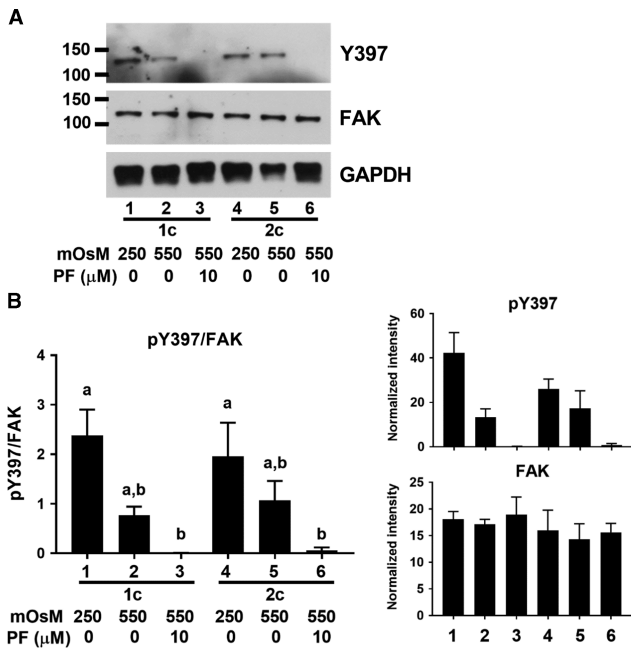


Figure 2. FAK Y397 phosphorylation in 1- and 2-cell embryos. (A) Representative example of western blot showing phosphorylated FAK Y397 and total FAK in 1-cell (lanes 1–3) and 2-cell (lanes 4–6) embryos at 250 mOsM, after 15 min at 550 mOsM, or with 10 μM PF-562271 as indicated by labels at bottom (60 embryos per lane). Vehicle (0.02% DMSO) was present in controls. GAPDH was also probed as indicated. (B) Quantification of Y397 phosphorylation. Y397 band intensities were normalized to total FAK in the same lane (left). Bars that do not share the same letter are significantly different by ANOVA with Tukey’s multiple comparison test (a v. b, $P < 0.05$; overall ANOVA $P = 0.005$). Mean band densities measured for Y397 and FAK are shown at right. $N = 3$ independent repeats, each with 60 embryos/lane.

NHE1 activity by a high concentration of PF-572271 was due to an off-target effect, the inhibitor might not affect NHE1 activation at lower concentrations in which it nonetheless could still block FAK Y397 phosphorylation. We therefore determined the lowest concentration of PF-562271 that could still inhibit the increase in alkalinization in 2-cell embryos after the introduction of hypertonic medium. This revealed that, while 5 μM PF-562271 significantly decreased the rate of intracellular alkalinization, up to 2.5 μM had no significant effect (Fig. 3), implying that NHE1 activation was not inhibited by PF-562271 at concentrations of 2.5 μM or less.

We then determined whether Y397 phosphorylation was inhibited by PF-562271 within this range. A pilot experiment indicated that both 2.5 and 5 μM PF-562271 eliminated Y397 phosphorylation as effectively as 10 μM (not shown), and so we tested lower concentrations. Y397 phosphorylation was almost completely eliminated by 0.1 μM PF-562271 and there were no detectable bands at 1 μM or 10 μM (Fig. 4A and B). Because we had found that 1 μM was well within the range in which there was no effect on NHE1 activation induced by decreased cell volume, we performed an independent set of measurements to confirm that this concentration completely eliminated Y397 phosphorylation (Fig. 4C and D). Therefore, NHE1 could still be maximally activated by a cell volume decrease at a concentration of PF-562271 (1 μM) that eliminated FAK autophosphorylation.

Discussion

It has been well established that NHE1 is activated by decreased cell volume and is generally required for cell volume homeostasis

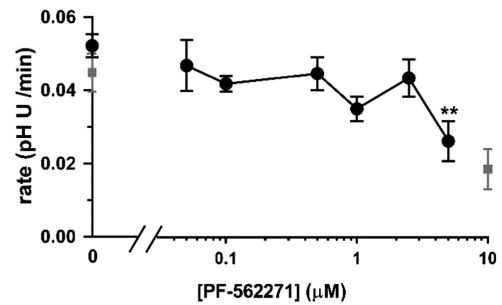


Figure 3. NHE1 activity in 2-cell embryos as a function of PF-562271 concentration. NHE1 activity induced by a cell volume decrease was measured as the mean rate of increase in pH after introduction of 350 mOsM medium, as in Fig. 1. There was no significant decrease in NHE1 activity at any concentration of PF-562271 (filled circles) compared with vehicle only (0 μM), except 5 μM (** $P < 0.01$ by ANOVA with Tukey’s multiple comparison test; overall ANOVA $P = 0.003$). Each point represents the mean (\pm SEM) of 5–7 independent repeats, except for 0 μM ($N = 23$ due to inclusion of controls each day). NHE1 activities measured at 0 and 10 μM PF-562271 from Fig. 1 are shown (grey squares) for comparison (not included in statistical analysis here).

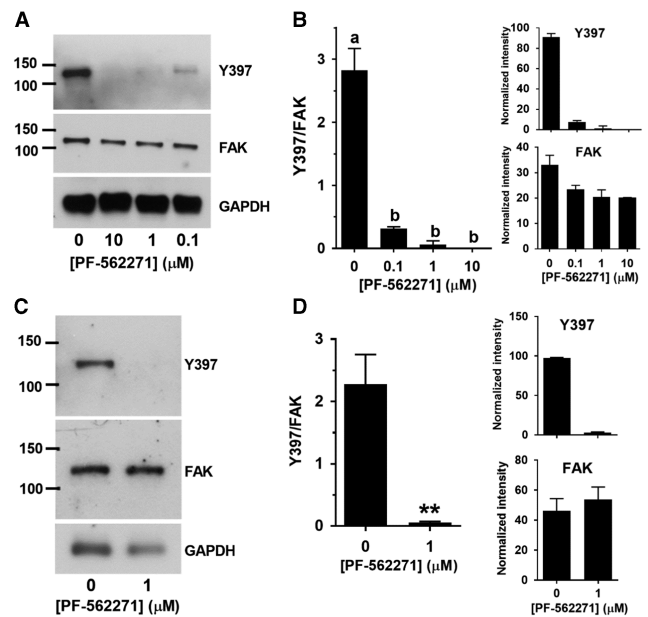


Figure 4. FAK Y397 phosphorylation in 2-cell embryos after 15 min at 550 mOsM at various concentrations of PF-562271. (A) Representative western blot of phosphorylated Y397, total FAK or GAPDH in 2-cell embryos at 0, 0.1, 1.0 and 10 μM PF-562271 (indicated at bottom; 50 2-cell embryos per lane). (B) Quantification of Y397 band intensity expressed as mean (\pm SEM) relative to total FAK (example in (A)), analyzed as described in Fig. 2 legend. All bands differed from control (0 = vehicle only) by ANOVA with Tukey multiple comparison test (a v. b, $P < 0.0001$; overall ANOVA $P < 0.0001$). $N = 3$ independent repeats. The mean inhibition at each concentration determined using Y397/FAK was 89%, 98%, and 100% for 0.1, 1.0 and 10 μM, respectively. The values for Y397/FAK were also tested by one-sample t -test for difference from 0. The values were significantly different from 0 for 0 and 0.1 μM PF-562271 ($P = 0.015$ and 0.010 , respectively) but were not significantly different from 0 for 1.0 or 10 μM ($P = 0.42$ and 1.0 , respectively). (C) Representative western blot (60 embryos per lane) of independent test of 0 v. 1 μM PF-562271 in 2-cell embryos. (D) Quantification of $N = 3$ separate repeats of western blots an example of which is shown in C. ** $P = 0.01$ by t -test.

in mammalian cells (Hoffmann *et al.*, 2009; Alexander & Grinstein, 2006) including in preimplantation embryos (Zhou & Baltz, 2013; Xu *et al.*, 2017). Furthermore, such NHE1 activation appears to be entirely dependent on the activity of tyrosine kinases as it is abolished by the broad-spectrum tyrosine kinase inhibitor, genistein (Krump *et al.*, 1997; Zhou & Baltz, 2013; Xu *et al.*, 2017). It has been shown in several types of mammalian

cells, including 2-cell embryos, that JAK2 accounts for a portion of this regulation (Garnovskaya *et al.*, 2003; Zhou & Baltz, 2013; Xu *et al.*, 2017) but a major component is JAK2-independent (Hoffmann *et al.*, 2009). Therefore, additional tyrosine kinase signalling is implicated but the kinase involved remains unidentified. The tyrosine kinase FAK (PTK2) had been proposed as a signalling mechanism involved as it has been shown to become phosphorylated and activated by decreased cell volume in a number of cell types (Lunn & Rozengurt, 2004; Lunn *et al.*, 2007; Rasmussen *et al.*, 2015).

The results reported here, however, do not support the hypothesis that the autophosphorylation of FAK on Y397 is likely to be involved in NHE1 activation in early mouse embryos. We detected FAK protein expression at both the 1-cell and 2-cell embryo stages, this is consistent with previous studies showing its expression in oocytes and early embryos (McGinnis *et al.*, 2011, 2013) but FAK autophosphorylation on Y397, considered to be an initial step in its activation, was not increased when cell volume was decreased at either stage. Furthermore, NHE1 activation was unaffected by concentrations of the FAK-selective inhibitor PF-562271 that rendered Y397 phosphorylation essentially undetectable by western blot.

We found that only the highest concentrations of PF-562271 that were tested (5 and 10 μM) inhibited NHE1 activation, which occurred well above the 0.1 and 1.0 μM that greatly diminished or abolished FAK Y397 phosphorylation in embryos (above) and other cells (Wendt & Schiemann, 2009; Yoon *et al.*, 2014) and well in excess of the reported IC₅₀ for PF-562271 of ~ 5 nM in intact cells (Roberts *et al.*, 2008). Therefore, we interpret the inhibition of NHE1 activation by high concentrations of PF-562271 as likely to be due to off-target inhibition of other tyrosine kinases, which has been reported to occur at high micromolar concentrations of the compound (Roberts *et al.*, 2008).

Had these results instead supported a role for FAK in the activation of NHE1 in embryos, additional experiments would have clearly been required in addition to using the single inhibitor, PF-562271. To establish a required role for FAK, additional selective inhibitors would have needed to be employed as well as independent techniques such as knockdown or dominant-negative inhibition of FAK. Indeed, this was the strategy we used to establish a role for JAK2 at the 2-cell stage, in which several different selective JAK2 inhibitors and expression of a dominant-negative JAK2 all produced similar results (Zhou & Baltz, 2013). However, given that complete elimination of FAK Y397 phosphorylation failed to have a detectable effect on NHE1 activation, an investigation of PF-562271 specificity at the concentrations used in embryos was not pursued.

So far, the only signalling mechanism convincingly implicated in transducing a signal from decreased cell volume to NHE1 activation in mammalian cells (including 2-cell mouse embryos) remains the JAK2-calmodulin signalling pathway (Garnovskaya *et al.*, 2003; Zhou & Baltz, 2013; Xu *et al.*, 2017). However, this mechanism only accounts for a portion of NHE1 activation and is not active in all cell types examined (Garnovskaya *et al.*, 2003; Hoffmann *et al.*, 2009; Xu *et al.*, 2017) including 1-cell mouse embryos (Xu *et al.*, 2017). While the observation that generally inhibiting tyrosine kinase activity with genistein completely eliminates NHE1 activation (Zhou & Baltz, 2013; Xu *et al.*, 2017) implicates at least one other tyrosine kinase in addition to JAK2, the identities of this putative tyrosine kinase and other components of the signalling pathway remain unknown.

Despite our findings here, FAK is present and is likely to have roles in early embryos, as it has been shown that FAK localized in the egg cortex becomes activated upon sperm–egg binding and fusion (McGinnis *et al.*, 2013). FAK may also still play some role in the overall cellular response to decreased cell volume or increased tonicity independent of NHE1. FAK becomes phosphorylated in a number of cell types upon a cell volume decrease. This has been reported to involve phosphorylation of Y397 that eventually returns to baseline as well as phosphorylation of other tyrosine residues (e.g., Y576/577, Y861) which are longer lived (Lunn & Rozengurt, 2004; Lunn *et al.*, 2007; Rasmussen *et al.*, 2015). It has been shown that longer lived FAK phosphorylation protects cells from the long-term effects of increased osmolarity by inducing anti-apoptotic pathways (Lunn *et al.*, 2007). Therefore, while FAK may not have a role in mediating the acute recovery from perturbed cell volume, its longer-term phosphorylation may be involved in survival under conditions of chronic hypertonic stress. This could potentially be of importance to early embryos, which are subjected to developmental arrest when cell volume regulation is perturbed (Baltz & Zhou, 2012), but this remains to be investigated.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. The ACC of the University of Ottawa approved all animal protocols, which conform to Canadian Council on Animal Care standards.

References

- Alexander RT and Grinstein S (2006) Na^+/H^+ exchangers and the regulation of volume. *Acta Physiol (Oxf)* **187**, 159–67.
- Arold ST (2011) How focal adhesion kinase achieves regulation by linking ligand binding, localization and action. *Curr Opin Struct Biol* **21**, 808–13.
- Aronson PS, Nee J and Suhm MA (1982) Modifier role of internal H^+ in activating the Na^+/H^+ exchanger in renal microvillus membrane vesicles. *Nature* **299**, 161–3.
- Arroyo JP, Kahle KT and Gamba G (2013) The SLC12 family of electroneutral cation-coupled chloride cotransporters. *Mol Aspects Med* **34**, 288–98.
- Baltz JM and Phillips KP (1999) Intracellular ion measurements in single eggs and embryos using ion-sensitive fluorophores. In *A Comparative Methods Approach to the Study of Oocytes and Embryos*, ed. JD Richter, Oxford University Press, New York and Oxford, pp. 39–82.
- Baltz JM and Tartia AP (2010) Cell volume regulation in oocytes and early embryos: connecting physiology to successful culture media. *Hum Reprod Update* **16**, 166–76.
- Baltz JM and Zhou C (2012) Cell volume regulation in mammalian oocytes and preimplantation embryos. *Mol Reprod Dev* **79**, 821–31.
- Dawson KM and Baltz JM (1997) Organic osmolytes and embryos: substrates of the Gly and beta transport systems protect mouse zygotes against the effects of raised osmolarity. *Biol Reprod* **56**, 1550–8.
- Donowitz M, Ming Tse C and Fuster D (2013) SLC9/NHE gene family, a plasma membrane and organellar family of Na^+/H^+ exchangers. *Mol Aspects Med* **34**, 236–51.
- Garnovskaya MN, Mukhin YV, Vlasova TM and Raymond JR (2003) Hypertonicity activates Na^+/H^+ exchange through Janus kinase 2 and calmodulin. *J Biol Chem* **278**, 16908–15.
- Gatsios P, Terstegen L, Schliess F, Häussinger D, Kerr IM, Heinrich PC and Graeve L (1998) Activation of the Janus kinase/signal transducer and activator of transcription pathway by osmotic shock. *J Biol Chem* **273**, 22962–8.

- Gibb CA, Poronnik P, Day ML and Cook DI (1997) Control of cytosolic pH in two-cell mouse embryos: roles of H⁺-lactate cotransport and Na⁺/H⁺ exchange. *Am J Physiol* **273**, C404–19.
- Hadi T, Hammer MA, Algire C, Richards T and Baltz JM (2005) Similar effects of osmolarity, glucose, and phosphate on cleavage past the 2-cell stage in mouse embryos from outbred and F1 hybrid females. *Biol Reprod* **72**, 179–87.
- Harding EA, Gibb CA, Johnson MH, Cook DI and Day ML (2002) Developmental changes in the management of acid loads during preimplantation mouse development. *Biol Reprod* **67**, 1419–29.
- Hoffmann EK and Pedersen SF (2011) Cell volume homeostatic mechanisms: effectors and signalling pathways. *Acta Physiol* **202**, 465–85.
- Hoffmann EK, Lambert IH and Pedersen SF (2009) Physiology of cell volume regulation in vertebrates. *Physiol Rev* **89**, 193–277.
- Humphreys BD, Jiang L, Chernova MN and Alper SL (1995) Hypertonic activation of AE2 anion exchanger in *Xenopus* oocytes via NHE-mediated intracellular alkalization. *Am J Physiol* **268**, C201–9.
- Jiang L, Chernova MN and Alper SL (1997) Secondary regulatory volume increase conferred on *Xenopus* oocytes by expression of AE2 anion exchanger. *Am J Physiol* **272**, C191–202.
- Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri P, Davis MI, Edeen PT, Faraoni R, Floyd M, Hunt JP, Lockhart DJ, Milanov ZV, Morrison MJ, Pallares G, Patel HK, Pritchard S, Wodicka LM and Zarrinkar PP (2008) A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol* **26**, 127–32.
- Krump E, Nikitas K and Grinstein S (1997) Induction of tyrosine phosphorylation and Na⁺/H⁺ exchanger activation during shrinkage of human neutrophils. *J Biol Chem* **272**, 17303–11.
- Lawitts JA and Biggers JD (1992) Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media. *Mol Reprod Dev* **31**, 189–94.
- Lawitts JA and Biggers JD (1993) Culture of preimplantation embryos. *Methods Enzymol* **225**, 153–64.
- Lunn JA and Rozengurt E (2004) Hyperosmotic stress induces rapid focal adhesion kinase phosphorylation at tyrosines 397 and 577: role of Src family kinases and Rho family GTPases. *J Biol Chem* **279**, 45266–78.
- Lunn JA, Jacamo R and Rozengurt E (2007) Preferential phosphorylation of focal adhesion kinase tyrosine 861 is critical for mediating an anti-apoptotic response to hyperosmotic stress. *J Biol Chem* **282**, 10370–9.
- Malo ME and Fliegel L (2006) Physiological role and regulation of the Na⁺/H⁺ exchanger. *Can J Physiol Pharmacol* **84**, 1081–95.
- McGinnis LK and Kinsey WH (2015) Role of focal adhesion kinase in oocyte–follicle communication. *Mol Reprod Dev* **82**, 90–102.
- McGinnis LK, Carroll DJ and Kinsey WH (2011) Protein tyrosine kinase signaling during oocyte maturation and fertilization. *Mol Reprod Dev* **78**, 831–45.
- McGinnis LK, Luo J and Kinsey WH (2013) Protein tyrosine kinase signaling in the mouse oocyte cortex during sperm–egg interactions and anaphase resumption. *Mol Reprod Dev* **80**, 260–72.
- Rasmussen LJ, Muller HS, Jorgensen B, Pedersen SF and Hoffmann EK (2015) Osmotic shrinkage elicits FAK- and Src phosphorylation and Src-dependent NKCC1 activation in NIH3T3 cells. *Am J Physiol Cell Physiol* **308**, C101–10.
- Roberts WG, Ung E, Whalen P, Cooper B, Hulford C, Autry C, Richter D, Emerson E, Lin J, Kath J, Coleman K, Yao L, Martinez-Alsina L, Lorenzen M, Berliner M, Luzzio M, Patel N, Schmitt E, LaGreca S, Jani J, Wessel M, Marr E, Griffor M and Vajdos F (2008) Antitumor activity and pharmacology of a selective focal adhesion kinase inhibitor, PF-562,271. *Cancer Res* **68**, 1935–44.
- Romero MF, Chen AP, Parker MD and Boron WF (2013) The SLC4 family of bicarbonate (HCO₃⁻) transporters. *Mol Aspects Med* **34**, 159–82.
- Sardet C, Counillon L, Franchi A and Pouyssegur J (1990) Growth factors induce phosphorylation of the Na⁺/H⁺ antiporter, glycoprotein of 110 kD. *Science* **247**, 723–6.
- Schaller MD, Hildebr JD, Shannon JD, Fox JW, Vines RR and Parsons JT (1994) Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* **14**, 1680–8.
- Schlaepfer DD and Hunter T (1996) Evidence for *in vivo* phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol Cell Biol* **16**, 5623–33.
- Schlaepfer DD, Mitra SK and Ilic D (2004) Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochim Biophys Acta Mol Cell Biol Lipids* **1692**, 77–102.
- Siyonov V and Baltz JM (2013) NHE1 is the sodium–hydrogen exchanger active in acute intracellular pH regulation in preimplantation mouse embryos. *Biol Reprod* **88**, 157.
- Steeves CL, Lane M, Bavister BD, Phillips KP and Baltz JM (2001) Differences in intracellular pH regulation by Na⁺/H⁺ antiporter among 2-cell mouse embryos derived from females of different strains. *Biol Reprod* **65**, 14–22.
- Thomas JA, Buchsbaum RN, Zimniak A and Racker E (1979) Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. *Biochemistry* **18**, 2210–18.
- Wendt MK and Schiemann WP (2009) Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-β signaling and metastasis. *Breast Cancer Res* **11**, R68.
- Wiemer AJ, Wernimont SA, Cung T.-D, Bennin DA, Beggs HE and Huttenlocher A (2013) The focal adhesion kinase inhibitor PF-562,271 impairs primary CD4⁺ cell activation. *Biochem Pharmacol* **86**, 770–81.
- Xu B, Zhou C, Meredith M and Baltz JM (2017) Acute cell volume regulation by Janus kinase 2-mediated sodium/hydrogen exchange activation develops at the late one-cell stage in mouse preimplantation embryos. *Biol Reprod* **96**, 542–50.
- Yoon H, Choi Y.-L, Song J.-Y, Do I, Kang SY, Ko Y.-H, Song S and Kim B.-G (2014) Targeted inhibition of FAK, PYK2 and BCL-XL synergistically enhances apoptosis in ovarian clear cell carcinoma cell lines. *PLoS One* **9**, e88587.
- Zhou C and Baltz JM (2013) JAK2 mediates the acute response to decreased cell volume in mouse preimplantation embryos by activating NHE1. *J Cell Physiol* **228**, 428–38.
- Zhou C, FitzHarris G, Alper SL and Baltz JM (2013) Na⁺/H⁺ exchange is inactivated during mouse oocyte meiosis, facilitating glycine accumulation that maintains embryo cell volume. *J Cell Physiol* **228**, 2042–53.