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Original Paper

Activation of SGK1 in Endometrial Epithelial Cells in Response to PI3K/AKT Inhibition Impairs Embryo Implantation

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Key Words
Serum/Glucocorticoid Regulated Kinase 1 • Epithelial Na^+ channel (ENaC) • Nedd4-2 • Implantation failure

Abstract
Background: Serum & Glucocorticoid Regulated Kinase 1 (SGK1) plays a fundamental role in ion and solute transport processes in epithelia. In the endometrium, down-regulation of SGK1 during the window of receptivity facilitates embryo implantation whereas expression of a constitutively active mutant in the murine uterus blocks implantation. ***Methods/Results:*** Here, we report that treatment of endometrial epithelial cells with specific inhibitors of the phosphoinositide 3-kinase (PI3K)/AKT activity pathway results in reciprocal activation of SGK1. Flushing of the uterine lumen of mice with a cell permeable, substrate competitive phosphatidylinositol analogue that inhibits AKT activation (AKT inhibitor III) resulted in Sgk1 phosphorylation, down-regulation of the E3 ubiquitin-protein ligase Nedd4-2, and increased expression of epithelial Na^+ channels (ENaC). Furthermore, exposure of the uterine lumen to AKT inhibitor III prior to embryo transfer induced a spectrum of early pregnancy defects, ranging from implantation failure to aberrant spacing of implantation sites. ***Conclusion:*** Taken together, our data indicate that the balanced activities of two related serine/threonine kinases, AKT and SGK1, critically govern the implantation process.

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Introduction

Successful implantation is dependent on the endometrium transiently expressing a receptive phenotype that enables a competent blastocyst to embed in an optimal uterine environment [1-4]. During this brief period, widely referred to as the 'window of implantation', the endometrium expresses an evolutionarily conserved repertoire of genes that enables a series of key events to take place, starting with absorption of uterine fluid, luminal 'closure' and apposition of the blastocyst on the endometrial surface epithelium, stable adherence to the apical surface of luminal epithelial cells, penetration through the luminal epithelium and its basal lamina, and finally, invasion of the decidualizing (differentiating) stroma [5].

The serine/threonine protein kinase Serum & Glucocorticoid Regulated Kinase 1 (SGK1) is rapidly induced in the endometrium in response to the ovulatory rise in progesterone levels [6-8]. It is first expressed in the luminal epithelial cells and then in the decidualizing stroma. SGK1 is a powerful stimulator of the sodium-potassium ATPase and various carriers and ion channels, including epithelial Na^+ channels (ENaC) [9, 10]. SGK1 expression and activity are, however, transiently down-regulated during the window of receptivity in both humans and mice [11]. Furthermore, overexpression of a constitutively active SGK1 mutant in the luminal epithelium of the mouse uterus deregulated the expression of endometrial receptivity genes, perturbed uterine fluid handling, and completely abolished embryo implantation [12]. These observations indicated that manipulation of SGK1 expression or activity could serve as a strategy to control fertility.

SGK1 is homologous to AKT, a serine/threonine kinase that relays growth factor signaling downstream of phosphoinositide 3-kinase (PI3K)[13]. AKT shares a large number of target proteins with SGK1 [12], including ENaC, which is activated at implantation in response to embryo-derived serine proteases [14]. Furthermore, AKT and SGK1 are both implicated in proliferation and cell survival responses through, amongst other mechanisms, targeting and inactivating the forkhead box transcription factors FOXO1 and FOXO3a [13, 15]. We previously reported that induction of SGK1 in differentiating endometrial cells is reciprocated by a decrease in AKT activity, which suggests the presence of a homeostatic mechanism that tightly balances the activities of these kinases in endometrial cells [6].

In this study, we explored if pharmacological inhibition of the PI3K/AKT pathway is sufficient to re-establish SGK1 activity during the window of receptivity and block embryo implantation.

Materials and Methods

Cell culture

Human epithelial endometrial cells (Ishikawa cells) [16] were maintained in Dulbecco's modified Eagle's medium/F12 (Invitrogen, Germany) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-Glutamine and 100 U ml^{-1} penicillin/streptomycin (Invitrogen) and incubated at 37°C in a humid atmosphere maintained at 5% (v/v) CO_2, and routinely tested for mycoplasma infection. Where indicated, the cells were treated with the AKT inhibitors II, III, IV, V, VI, VII, VIII, IX, X (Merck Chemicals, Darmstadt, Germany) and the AKT1/2/3 inhibitory and control peptides from Imgenex (*NBP2-31228-2mg;*San Diego, CA, USA).

AKT inhibition in vivo

All animal experiments were conducted in accordance with the Animals (Scientific Procedures) Act under Project Licences granted by the Home Office (PPL70/6867), United Kingdom. Prior to the experiments mice had free access to food and water ad libitum, and were kept under constant humidity (55 ± 10%), temperature (22 ± 2°C) and 12 hours light-dark cycle conditions. C57BL/6 mice were used for *in vivo* testing. Briefly, non-pregnant mice were anaesthetized, subjected to laparotomy to expose the uterus, and the infundibulum injected with 20 µM AKT inhibitor (inhibitor III and V) dissolved in 50 µl PBS or an

equal amount of vehicle control (dimethyl sulfoxide, DMSO). Mice were sacrificed 24 hours later and the uterine horns either fixed in formalin or snap-frozen and stored at -80°C.

To assess the effect of AKT inhibition on implantation, embryo transfer experiments were carried out with recipient pseudopregnant female mice mated with sterile males 2.5 days prior to surgery. At laparotomy, the uterine horns were flushed once with 50 µl solution containing either 20 µM AKT inhibitor III or vehicle (DMSO) control 10 min prior to embryo transfer. Between 8-10 cultured blastocysts were transferred to a single treated uterine horn. The uteri were harvested 4-7 days following surgery, implantation sites counted, and tissues fixed in formalin or snap-frozen for further analysis.

Immunohistochemistry

Paraffin embedded and formalin fixed tissue sections were deparaffinized, rehydrated in graded concentrations of ethanol, and endogenous peroxidase activity blocked by immersion of the slides for 30 min in a freshly prepared solution of 2 ml 30% hydrogen peroxide diluted in 200 ml PBS. The slides were then washed in PBS, pre-incubated in 1.5% non-immune goat serum in PBS for 30 min at room temperature, and incubated overnight at 4 °C with a rabbit polyclonal primary antibody directed against phospho-SGK1 (1:1000 dilution; Merck) or anti-epithelial Sodium Channel alpha α-ENaC (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The primary antibody was omitted as a negative control. The staining was visualized using the Vectastain Elite ABC rabbit IgG kit (Vector Laboratories, Burlingame, CA, USA).

Patch clamp

Patch clamp experiments were performed at room temperature in voltage-clamp, fast-whole-cell mode as described previously [17]. Ishikawa cells were continuously superfused through a flow system inserted into the dish [18]. The bath was grounded *via* a bridge filled with NaCl Ringer solution. Borosilicate glass pipettes (2-4 MOhm tip resistance; GC 150 TF-10, Clark Medical Instruments, Pangbourne, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). Currents were elicited by 200 ms square wave voltage pulses from -140 to +40 mV in 20 mV steps delivered from a holding potential of -50 mV with an acquisition frequency of 10 and 3 kHz low-pass filtered [14]. The liquid junction potential ΔE between the pipette and the bath solutions and between the salt bridge and the bath solutions was estimated as described previously [14]. Data were corrected for the estimated ΔE values.

Ishikawa cells were superfused with a bath solution containing: 145 mM Na-gluconate, 2.7 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5.5 mM glucose, 10 mM HEPES/NaOH, pH 7.4. The patch clamp pipettes were filled with: 135 mM K-gluconate, 6 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES/KOH, pH 7.2 [14].

Cell viability

Ishikawa cells were seeded onto 96-well plates, allowed to attach overnight, and incubated with various inhibitors at different concentrations. Cell viability was determined by using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA), according to manufacturer's instructions at the indicated time points.

Western Blot Analysis

Ishikawa cells were seeded at approximately 80% confluency and serum-starved overnight. Tissue or whole-cell lysates were homogenised with RIPA buffer and quantified using the Bradford assay. Whole-cell extracts or tissue lysates were immunoblotted (30 µg) as previously described [19]. The following primary antibodies were used: anti Total-SGK1 , anti-phospho-SGK1 (Ser255/Thr256), anti NEDD4-2, anti-Total-AKT, anti-Phospho-AKT (Ser473), cleaved PARP from Cell Signalling (Leiden, the Netherlands) and anti-αENaC antibody from Sigma-Aldrich (Taufkirchen, Germany). The primary antibodies were used at 1:1000 except for the antibodies to β-actin (Abcam, Cambridge, UK), which was diluted 1:100,000.

Statistical analysis

Data are expressed as arithmetic means ± standard error of means (SEM). Data were tested for significance using Students *t* Test or chi-square test as appropriate. $P < 0.05$ were considered statistically significant.

Results

AKT inhibition enhances SGK1 activity in endometrial cells

We previously observed that induction of SGK1 in differentiating endometrial cells is reciprocated by a decrease in AKT activity [6]. Based on this observation, we postulated that a homeostatic mechanism exists in endometrial cells that balances the activities of SGK1 and AKT, two closely related kinases [20]. If correct, inhibition of the PI3K/AKT pathway could constitute a strategy to enhance SGK1 activity, thereby preventing embryo implantation. To test this hypothesis, we screened 9 commercially available PI3K/AKT inhibitors (Fig. 1 & Table 1) for their effects on AKT and SGK1 kinase activities in Ishikawa cells, a well-characterized endometrial cell line representative of receptive endometrium [12]. Based on time-course and dose-response experiments, two AKT inhibitors (inhibitors III and V) were shown to produce the most pronounced induction of SGK1 activity (Fig. 2A, B). The modes of action of these compounds are distinct, with inhibitor III being a

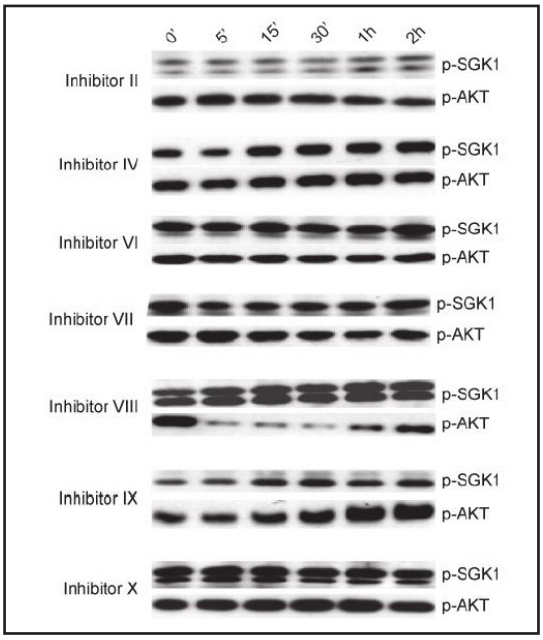

Fig 1. Modulation of SGK1 activity by different PI3K/AKT inhibitors. Ishikawa cells, cultured at 80% confluency, were treated with various PI3K/AKT inhibitors (at 20 μM, except for inhibitor IV, which was used at 1 μM concentration) for a total of 2 hours. AKT and SGK1 activities were assessed using Phospho-specific antibodies.

Table 1. AKT inhibitors

AKT INHIBITOR	INHIBITS	CELL PERMEABLE	COMMENTS
AKT Inhibitor	AKT, PI-3 K	Yes	PI analog, prevents PIP_3 formation and binding to AKT (IC_{50} = 5.0 μM). For PI-3 K (IC_{50} = 83 μM).
AKT Inhibitor II (SH-5)	AKT, PI-3 K	Yes	PI analog, prevents PIP_3 formation and binding to AKT. Phosphonate analog, metabolically more stable than AKT inhibitor.
AKT Inhibitor III (SH-6)	AKT, PI-3 K	Yes	PI analog, prevents PIP_3 formation and binding to AKT. Phosphonate analog, metabolically more stable than AKT inhibitor.
AKT Inhibitor IV	AKT	Yes	ATP-competitive inhibitor of a kinase upstream of AKT but downstream of PI-3 K.
AKT Inhibitor V, Triciribine (API-2, NSC 154020, TCN)	AKT	Yes	Inhibits the cellular phosphorylation/activation of AKT1/2/3 by targeting an AKT effector molecule other than PI-3 K or PDK1. Has shown efficacy *in vivo.*
AKT Inhibitor VI, *AKT-in*	AKT	No	Peptide that binds to AKT-PH domain and interferes with the AKT-phosphoinositide interaction.
AKT Inhibitor VII, TAT-*AKT-in*	AKT	Yes	Peptide that binds to AKT-PH domain and interferes with the AKT-phosphoinositide interaction.
AKT Inhibitor VIII, Isozyme-Selective, AKTi-1/2	AKT	Yes	A cell-permeable quinoxaline compound that potently and selectively inhibits AKT1/AKT2 activity.
AKT Inhibitor IX, API-59CJ-OMe	AKT	Yes	Induces apoptosis in human endometrial cancer cells (RL95-2 and Ishikawa) that exhibit elevated AKT activity (effective concentration = 12-24 μM), but not on cells with low AKT activity.
AKT Inhibitor X	AKT	Yes	Inhibits the phosphorylation of AKT and its in vitro kinase activity with minimal effect on PI 3-K, PDK1 and SGK1.

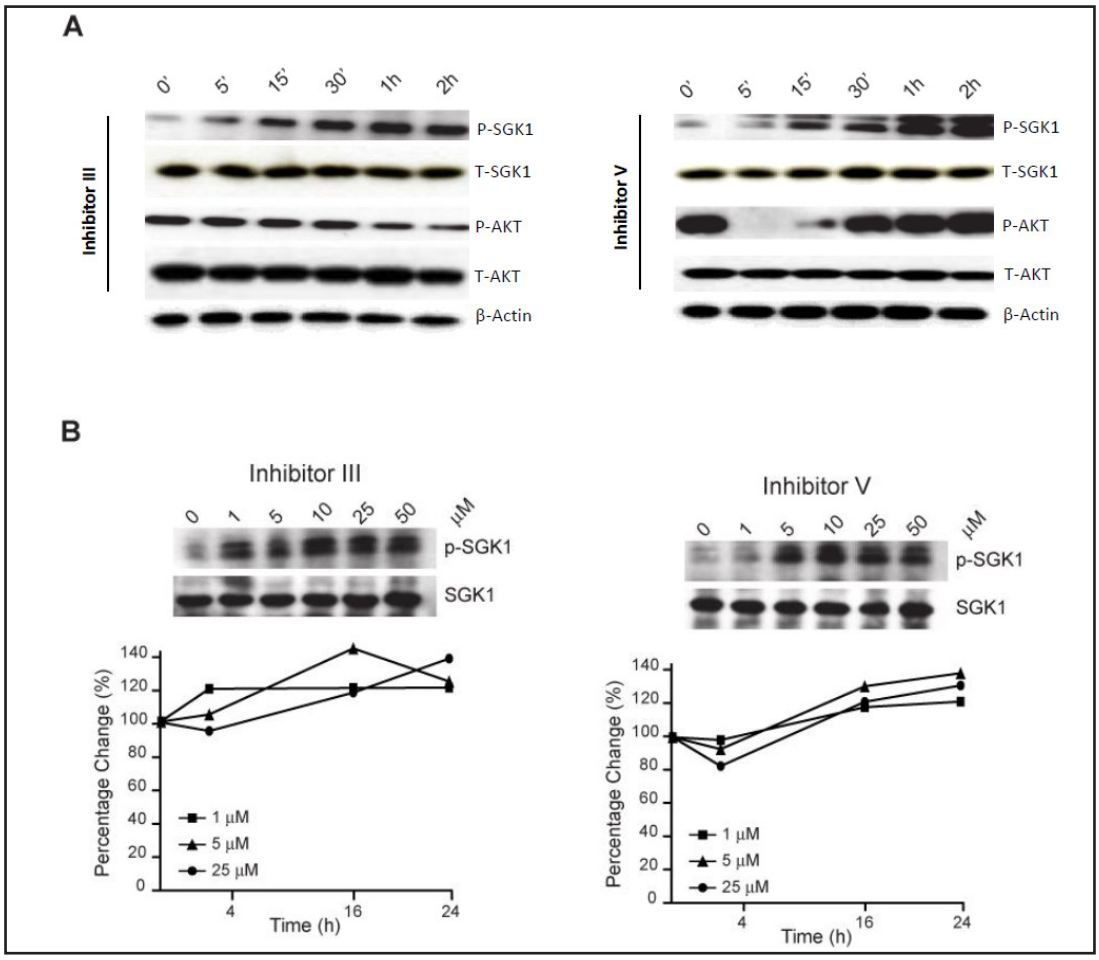

Fig 2. Modulation of SGK1 activity by inhibitor III and V. (*A*) Ishikawa cells, cultured at 80% confluency, were treated with various PI3K/AKT inhibitor III or V for a total of 2 hours. AKT and SGK1 activities were assessed using Total or Phospho-specific antibodies. β-Actin served as a loading control. (*B*) Ishikawa cells were treated with PI3K/AKT inhibitor III or V for a total of 2 hours at various concentrations (1-50μM). AKT and SGK1 activities were assessed using Total or Phospho-specific antibodies. β-Actin served as a loading control. Further, cell viability was measured using MTS using different concentrations of the inhibitor over a time-course lasting 24 hours.

phosphoinositide (PI) analogue that prevents phosphatidylinositol (3,4,5)-trisphosphate (PIP3) formation whereas inhibitor V inhibits AKT activity independently of PI3K or PDK.

Both inhibitor III and inhibitor V strongly induced SGK1 phosphorylation with minimal cytotoxicity at 1-25 μM concentration in Ishikawa cells. Cell viability was measured by MTS assay (Fig. 2B). We extended our search for ways of targeting AKT activity that are not based on small inhibitory molecules. A cell permeable AKT 1/2/3 inhibitory peptide was tested in our cell system (Fig. 3). This inhibitory peptide binds to the pleckstrin homology domain of AKT, thereby preventing its activation [21]. A control peptide was used to account for possible non-specific effects. The AKT-inhibitor peptide, but not the control peptide, was highly effective at enhancing SGK1 activity in Ishikawa cells (Fig. 3). Moreover, this peptide had no discernible cytotoxic effects in this cell line (Fig. 3). Thus, we identified and validated three compounds, two small molecules and a cell permeable peptide, capable of enhancing endometrial SGK1 activity indirectly by targeting AKT. Importantly, these compounds target AKT activity through distinct mechanisms, which raises the possibility that they may have differential effects *in vivo*.

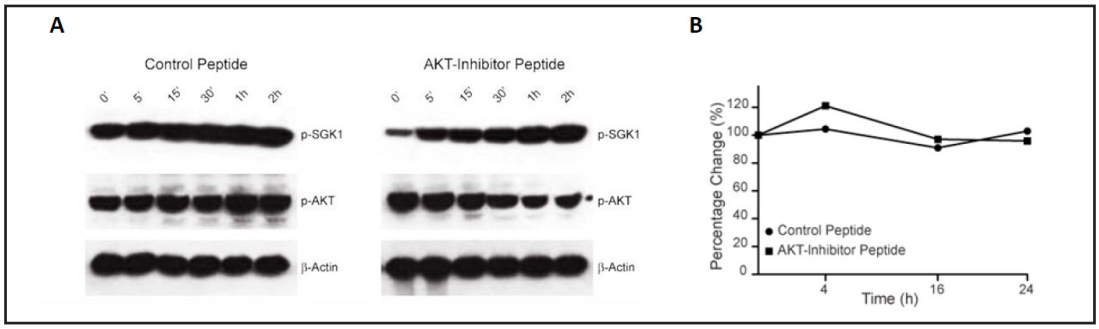

Fig 3. Modulation of SGK1 activity by AKT-inhibitor peptide. Ishikawa cells, cultured at 80% confluency, were treated with an AKT inhibitor peptide or a control peptide for a total of 2 hours. (*A*) AKT and SGK1 activities were assessed using Phospho-specific antibodies. (*B*) Cell viability was measured using MTS over a time-course lasting 24 hours.

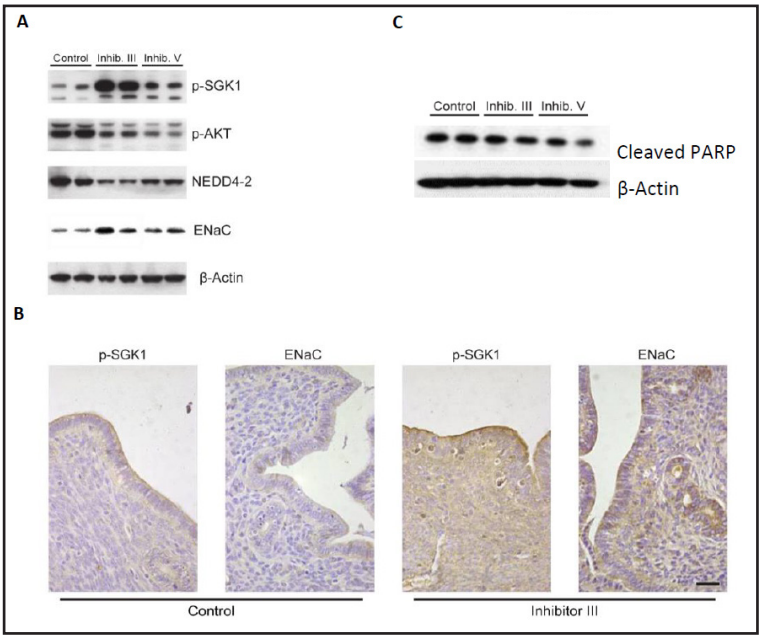

Fig. 4. AKT inhibition enhances endometrial Sgk1 activity in the mouse uterus. (*A*) Total protein lysates from non-pregnant uteri were extracted 24 hours after the lumen was flushed once with 20 μM AKT inhibitor III, inhibitor V, or an equal volume of DMSO (control) dissolved in 50 μl PBS, subjected to Western blot analysis, and immunoprobed with an NEDD4-2, α-ENaC and phospho-specific SGK1 and AKT antibodies. β-Actin served as a loading control. (*B*) Tissue distribution of phospho(p)-SGK1 and α-ENaC expression were analyzed by immunohistochemistry in uteri treated with inhibitor III or vehicle control. (*C*) Total cell lysates were probed for cleaved poly(ADP-ribose) polymerase-1 (Cleaved-PARP) levels. β-Actin served as a loading control.

AKT inhibition enhances endometrial SGK1 activity in vivo

To test if AKT inhibition enhances SGK1 activity in uterine luminal epithelial cells *in vivo*, the uterine infundibulum of non-pregnant mice was cannulated and the AKT inhibitor III or V (20 μM), dissolved in PBS, flushed through the uterine lumen. In control animals, an equal amount of vehicle control (DMSO) was injected. The injected fluid was allowed to run freely through the uterine lumen. Mice were sacrificed 24 hours later and the uterine horns fixed or snap-frozen. Western blot analysis demonstrated that a single flush of the uterine lumen with AKT inhibitors III or V was sufficient to up-regulate phospho-Sgk1 levels in a sustained fashion (Fig. 4A). As expected, induction of phospho-Sgk1 upon PI3K/AKT inhibition resulted in down-regulation of Nedd4-2 and up-regulation of cellular ENaC levels.

Although inhibitor V was more effective in attenuating AKT activity, induction of phospho-Sgk1 levels was much more pronounced with inhibitor III. Moreover, Western blot analysis and immunohistochemistry confirmed that treatment with this compound resulted in a downregulation of Nedd4-2 and a reciprocal induction of ENaC expression, respectively (Fig. 4B). Thus, this compound was further investigated for its contraceptive potential.

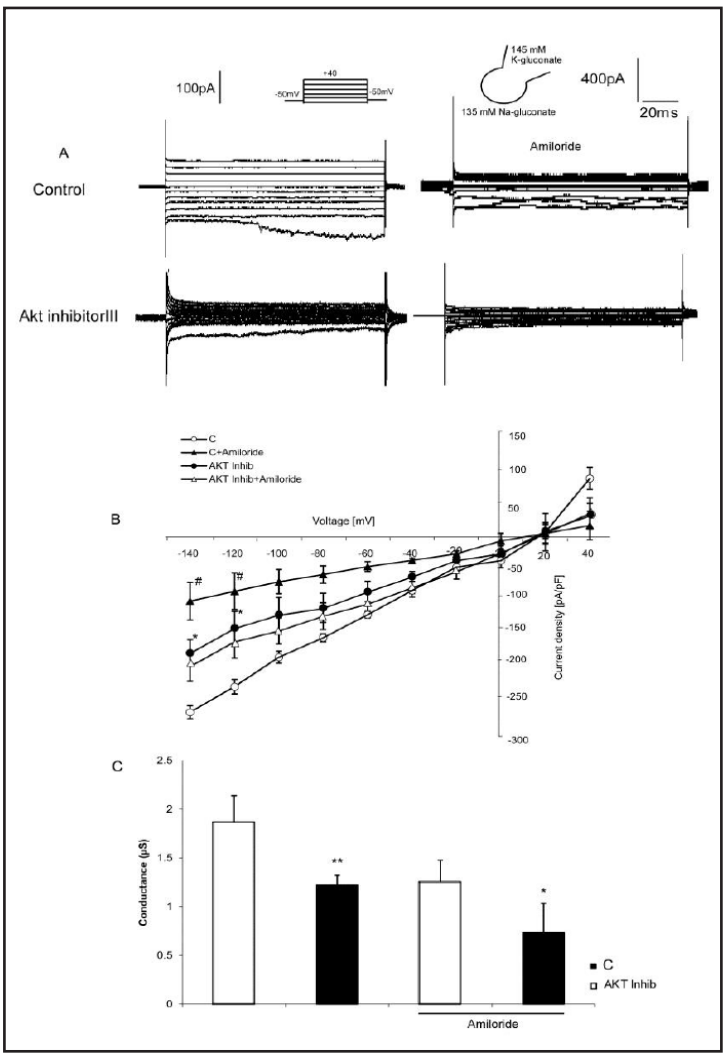

Fig. 5. Effect of AKT inhibitor III on amiloride-sensitive Na^+ currents in Ishikawa cells. (*A*) Original tracings of whole cell currents in untreated (upper panels) and AKT III inhibitor (20 µM, 2 hours) treated Ishikawa cells without (left panels) and with (right panels) presence of amiloride (50 µM). Arithmetic means (± SEM, n = 6-7) of whole cell currents as a function of potential difference across the cell membrane (*B*) untreated (white circle), treated with AKT III inhibitor (20 µM, 2 hours; black circle) or in the presence of 50 µM amiloride alone (black triangle) or AKT III inhibitor and amiloride (white triangle). (*C*) Arithmetic means (± SEM, n = 6-7) of inward conductance of the cell membrane calculated by linear fit of I/V-curves from -80 mV to -140 mV in untreated (white bar) and treated with AKT III inhibitor (20 µM, 2 hours) (black bar) Ishikawa cells in the absence and presence of amiloride (50 µM). * or # $P < 0.05$, ** $P < 0.01$ indicates statistically significant difference to AKT III inhibitor in the absence (*) or the presence (#) of amiloride (two-tailed unpaired Student's *t*-test).

There was no histological evidence of damage to the uterus in response to the procedure or compound or cell death (Cleaved PARP) (Fig. 4C).

AKT inhibition decreases endometrial ENaC activity

To validate whether increased ENaC expression was associated with enhanced activity, we performed whole-cell patch clamp in order to measure direct channel activation. Amiloride sensitive Na^+-currents in Ishikawa cells were determined utilizing whole cell Patch clamp. As illustrated in Fig. 5, the amiloride-sensitive whole cell currents were significantly decreased by AKT inhibitor III treatment (20 µM). The effect of AKT inhibitor III on the whole cell current was paralleled by the respective effect on inward conductance calculated from the individual I-V relations by linear regression of inward current between -80 and -140 mV. Taken together, these experiments showed a decrease in current density in Ishikawa cells after application of AKT inhibitor III, suggesting functional expression of ENaC in endometrial Ishikawa cells.

AKT inhibition disrupts embryo implantation

We reasoned that the effect of flushing the uterus with the compound would be restricted in time. To assess the effect of AKT inhibition on embryo implantation, we opted for an *in vivo* embryo transfer approach. Briefly, uteri of recipient pseudopregnant female mice mated with sterile males 2.5 days prior to surgery were flushed once with 20 µM AKT inhibitor III or vehicle control 10 minutes prior to embryo transfer. Between 8-10 cultured blastocysts were

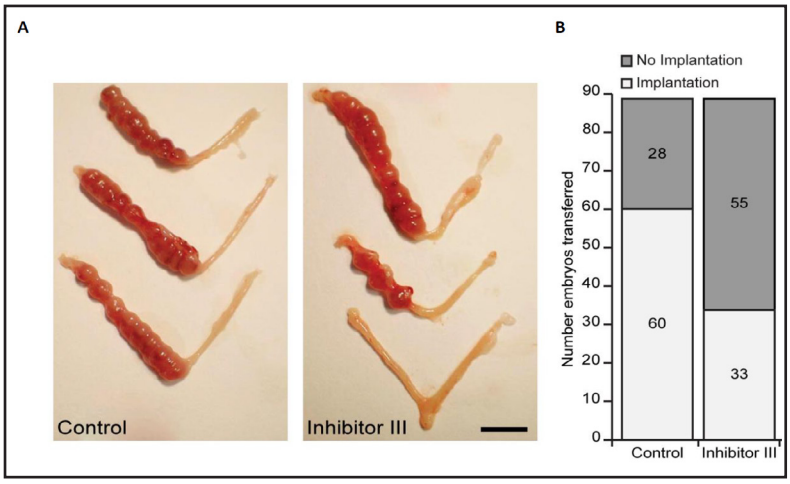

Fig. 6. AKT inhibition impairs embryo implantation *in vivo.* (*A*) Gross morphology of pregnant uteri flushed once with 20 µM AKT inhibitor III or vehicle control 10 minutes prior to embryo transfer (n = 88 in each group). Scale bar 1cm. (*B*) Graph showing reduction in the number of implantation sites in AKT inhibitor III treated animals (left panel), as summarized in the right panel (Chi-square test: $P < 0.0001$).

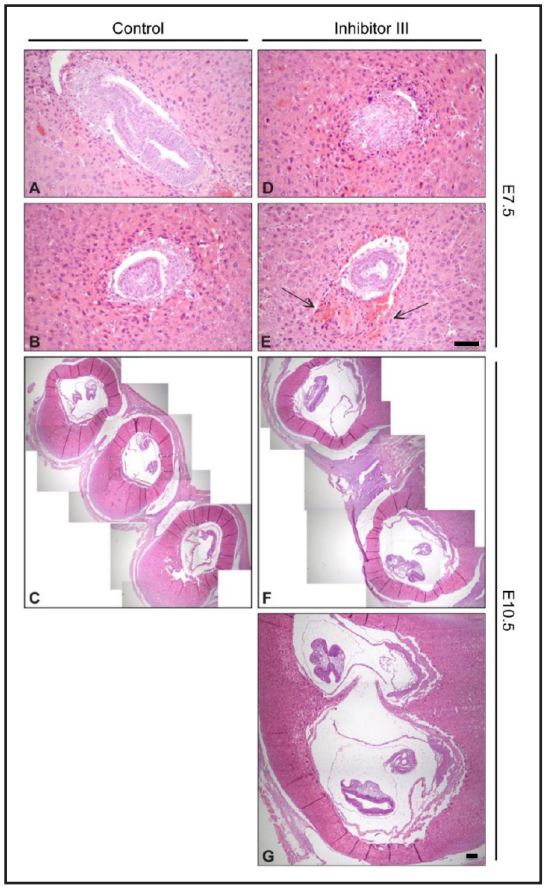

Fig. 7. AKT inhibition prior to implantation results in implantation defects. Histological assessment of implantation sites upon flushing of the uterus with vehicle control (panels on the left) or AKT inhibitor III (panels on the right) prior to embryo transfer. Analysis at embryonic day 7.5 (E7.5) revealed several abnormalities, including reduction or absence of the extra-embryonic space (top right) and focal bleeding at the implantation site (arrows). Moreover, embryo spacing was often disrupted and several twin implantation sites were observed at embryonic day 10.5 (E10.5). Scale bar 100 µm.

transferred to a single uterine horn. The uteri were harvested 4 or 7 days following surgery, implantation sites counted, and tissues fixed or snap-frozen for further analysis. Of a total of 88 embryos transferred in each group, 60 (68%) implanted in control mice compared to 33 (37.5%) treated with the AKT inhibitor III ($P < 0.0001$; Fig. 6). Moreover, analysis of the implantation sites 7.5 or 10.5 dpc revealed several abnormalities in treated animals, including focal bleeding, aberrant spacing of embryos and twin implantation sites (Fig. 7), rendering it unlikely that many would have survived pregnancy.

Discussion

We previously reported that endometrial SGK1 expression is enhanced in women experiencing unexplained infertility [6, 22, 23]. We also demonstrated that an increase in SGK1 activity is sufficient to abolish implantation and to shrink the entire uterus, reflected at the cellular level by a marked decrease in glandular area and epithelial cell width [22]. Cyclic regulation of the fluid environment in the uterine lumen is essential for key reproductive events, including sperm transport, embryo development and transport, as well as implantation [24]. Estrogens cause fluid secretion whereas progesterone promotes fluid absorption, mediated by amiloride-sensitive ENaC expressed on the apical surface of luminal

and glandular epithelial cells [10, 20, 25]. Thereby, a sustained increase in SGK1 activity, which in addition to ENaC activates a variety of other ion channels, could lead to implantation failure by causing premature uterine 'closure', characterised by complete apposition of the epithelium of the opposing uterine walls [14].

This scenario fits well with the observation that SGK1 activation using an AKT inhibitor prior to embryo transfer was less effective in preventing implantation. Other mechanisms may, however, be equally important. For example, it appears likely that the reorganization of the actin cytoskeleton that underpin the morphological changes induced by SGK1 will have a profound effect on expression [26] and signal transduction of adhesion molecules that control embryo-maternal interactions [27-29]. Moreover, implantation, at least in rodents, involves apoptosis of uterine epithelial cells in response to signals derived from an implanting blastocyst, a process referred to as displacement penetration [30]. Thus, continuous SGK1 activity during the implantation window could possibly render the luminal epithelium resistant to these embryo-derived pro-apoptotic signals.

As aforementioned, inhibition of the PI3K/AKT pathway was sufficient to induce SGK1 activity in endometrial cells, to reduce implantation *in vivo*, and to disrupt embryo spacing and early placental-decidual interactions. These observations provide proof of principle that SGK1 can be targeted for contraceptive purposes, although arguably not all of these pathological events may be attributable to increased SGK1 phosphorylation, NEDD4-2 down-regulation, and ENaC activation. The data were surprising, as acute inhibition of PI3K/AKT pathway results in a sustained SGK1 activation, maintained *in vivo* for at least 24 hours. At present the mechanisms that underpin these phenomena remain to be defined. Nevertheless, the data indicate that the AKT/SGK1 homeostat in endometrial cells is a promising target for fertility control.

The unique role of uterine SGK1 in regulating early implantation events and subsequent fetal survival renders it an attractive therapeutic target [22]. As alluded to, the endometrial AKT /SGK1 homeostat could be exploited for contraceptive purposes. It is also possible that SGK1 activity in different endometrial compartments is controlled by specific phosphatases that could be inhibited by small molecules, thereby rendering the endometrium refractory to implantation. Conversely, SGK1 inhibitors, such as heterocyclic indazole derivates, are currently being developed for a variety of diseases, ranging from diabetes, obesity and metabolic syndrome to kidney inflammatory disorders [31]. Based on our observations, selective SGK1 inhibitors could be useful in reproductive medicine in more than one way. For example, flushing of the uterine cavity with an inhibitor prior to embryo transfer could enhance the pregnancy rate after IVF treatment, especially in patients suffering from persistent implantation failure. Systemic and prolonged exposure, however, is likely to compromise decidual function, an effect that could be exploited for medical termination of pregnancy.

In summary, the present study revealed that pharmacological inhibition of AKT impacts on SGK1 activity during the window of implantation. Our findings point towards a promising new non-hormonal strategy to either facilitate or prevent embryo implantation.

Acknowledgments

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Disclosure Statement

The authors of this manuscript declare that they have no conflicts of interests.

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