Deregulation of the serum- and glucocorticoid-inducible kinase SGK1 in the endometrium causes reproductive failure

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Infertility and recurrent pregnancy loss (RPL) are prevalent but distinct causes of reproductive failure that often remain unexplained despite extensive investigations1,2. Analysis of midsecretory endometrial samples revealed that SGK1, a kinase involved in epithelial ion transport and cell survival3–6, is upregulated in unexplained infertility, most prominently in the luminal epithelium, but downregulated in the endometrium of women suffering from RPL. To determine the functional importance of these observations, we first expressed a constitutively active SGK1 mutant in the luminal epithelium of the mouse uterus. This prevented expression of certain endometrial receptivity genes, perturbed uterine fluid handling and abolished embryo implantation. By contrast, implantation was unhindered in Sgk1−/− mice, but pregnancy was often complicated by bleeding at the decidual-placental interface and fetal growth retardation and subsequent demise. Compared to wild-type mice, Sgk1+/− mice had gross impairment of pregnancy-dependent induction of genes involved in oxidative stress defenses. Relative SGK1 deficiency was also a hallmark of decidualizing stromal cells from human subjects with RPL and sensitized these cells to oxidative cell death. Thus, depending on the cellular compartment, deregulated SGK1 activity in cycling endometrium interferes with embryo implantation, leading to infertility, or predisposes to pregnancy complications by rendering the feto-maternal interface vulnerable to oxidative damage.

The sustained rise in postovulatory circulating progesterone levels renders the endometrium transiently receptive to embryo implantation. This ‘window of implantation’, confined to 2–4 d during the midsecretory phase of the human menstrual cycle, enables the embryo to contact and stably adhere to the endometrial luminal epithelium before invading the decidualizing stroma7–9. Decidualization, which denotes the transformation of stromal fibroblasts into specialized secretory decidual cells, is indispensable for pregnancy, as it governs local immune responses, controls trophoblast invasion and protects the conceptus against a variety of physiological stressors associated with pregnancy10–12. Failure of the endometrium to express a receptive phenotype is a major cause of conception delay and in vitro fertilization treatment failure. In contrast, perturbations in the maternal deciduol response inevitably lead to pregnancy complications, the most prevalent of which is miscarriage2.

SGK1, a serine-threonine protein kinase homologous to AKT, is rapidly induced in response to a rise in progesterone levels in both human and mouse endometrium, first in epithelial cells and then in the decidualizing stroma13–15. SGK1 is a key regulator of sodium transport in mammalian epithelia, most prominently through its ability to directly activate epithelial sodium channel (ENaC) and to enhance ENaC expression by inhibiting the ubiquitin ligase NEDD4-2 (refs 5,6). SGK1 is also involved in proliferation and cell survival responses3,4. To determine whether SGK1 regulates embryo implantation, we first examined its expression in midsecretory endometrial samples from women with proven fertility and from subjects with either unexplained infertility or a history of RPL, defined here as three or more consecutive miscarriages (Supplementary Table 1). As previously reported13, endometrial SGK1 transcript levels were higher in fertile women than in fertile controls (Fig. 1a). However, expression was lower in RPL subjects, not only when compared to infertile women but also to fertile controls (Fig. 1a). Immunohistochemistry indicated that phosphorylated SGK1 levels, reflecting activated kinase, are higher in the endometrium of infertile subjects compared to controls and subjects with RPL, especially in the luminal and glandular epithelial compartments, whereas staining of stromal cells underlying the luminal epithelium appeared lower in RPL samples (Fig. 1b and Supplementary Fig. 1). The difference in total and phosphorylated endometrial SGK1 levels between the infertile and RPL subjects was further exemplified by western blot analysis (Fig. 1c and Supplementary Fig. 2).

Sgk1 mRNA levels transiently decline in the luminal epithelium during the window of endometrial receptivity in mice16. Immunohistochemistry confirmed that this is paralleled by a marked

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reduction in phosphorylated SGK1 levels in mice (Supplementary Fig. 3). To determine whether sustained SGK1 activity in the luminal epithelium interferes with embryo implantation, we injected the uterine lumen of C57BL/6 mice 1.5 days post coitus (d.p.c.) with an expression vector that encodes a constitutively active SGK1 mutant (SGK-S422D) or a control plasmid. A week later (8.5 d.p.c.), five of seven control mice were pregnant, each with seven implantation sites (Fig. 2a). In contrast, implantation sites were absent in mice expressing the constitutively active SGK1. Strikingly, SGK-S422D–expressing uteri weighed approximately half compared to those of nonpregnant control mice, and morphometric analysis of tissue sections revealed markedly reduced glandular area and decreased distance between luminal epithelial cells (Supplementary Fig. 4a), observations in keeping with the crucial role of SGK1 in osmoregulation. In agreement, expression of SGK-S422D, which was confined to the luminal epithelium and some underlying glands, also markedly upregulated the α subunit of ENaC (α-ENaC; Fig. 2b and Supplementary Fig. 4b). The complete failure of implantation in response to increased endometrial SGK1 activity prompted us to examine the induction of a panel of genes that confer uterine receptivity during the window of implantation (4.5 d.p.c.). Expression of SGK-S422D selectively abolished or attenuated the induction of Lif, Hbegf and Hoxa10 (Fig. 2c)17–20. The regulation of other implantation genes, perhaps more relevant for the postimplantation uterine response, such as Ihh, Wnt4 and Bmp2 (refs. 21–23), was not significantly affected (Supplementary Fig. 5). In addition to α-ENaC, constitutive SGK1 activity at 4.5 d.p.c. also upregulated the expression of cystic fibrosis transmembrane conductance regulator (Supplementary Fig. 6), a chloride channel implicated in in vitro fertilization treatment failure24.

Our observations indicate that transient loss of SGK1 activity in the luminal epithelium is essential to render the endometrium receptive to implantation. Accordingly, SGK1 should be dispensable for implantation but perhaps not for the subsequent decidual response and placenta formation. To test this hypothesis, we first determined the number of implantation sites and pups per litter in Sgk1−/− female mice crossed with wild-type (WT) males and in WT females crossed with Sgk1−/− males, thereby negating the potential contribution of the offspring’s genotype. Whereas the number of implantation sites at 8.5 d.p.c. was maintained in Sgk1−/− females, the average litter size was significantly reduced, indicating an excess of 30% spontaneous fetal loss (Fig. 3a). Not only did the implantation sites appear invariably smaller in pregnant Sgk1−/− mice but also there was histological

Figure 1 Deregulated SGK1 expression and activity is associated with infertility and RPL. (a) Timed midsecretory endometrial biopsies from subjects with RPL (n = 9), fertile controls (Fer; n = 9) or women with unexplained infertility (Inf; n = 9) were analyzed for the expression of SGK1 transcripts using quantitative RT-PCR (qRT-PCR). Transcript levels were normalized to the housekeeping gene L19. AU, arbitrary units. (b) Immunostaining for phosphorylated SGK1 (p-SGK1) in timed midsecretory endometrium of fertile women and subjects with RPL or unexplained infertility. Additional images are presented in Supplementary Figure 1. Scale bar, 100 μm. (c) Total protein lysates from snap-frozen endometrial samples (n = 12) subjected to western blot analysis and immunoprobed with antibodies specific to total SGK1 and pSGK1. β-actin was used as a loading control. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 2 Increased SGK1 activity in the luminal epithelium blocks embryo implantation. (a) Gross uterine morphology 7 d after transfection (8.5 d.p.c.) of a control plasmid (pcDNA3.1) or an expression vector that encodes the constitutively active mutant SGK-S422D. Five out of seven control mice achieved pregnancy, all with seven implantation sites each (middle panel). There were no implantation sites in any of the mice transfected with SGK-S422D (n = 7). (b) Immunostaining of corresponding uterine tissue sections for total SGK1, p-SGK1 and α-ENaC. Scale bars: a, 1 cm; b, 100 μm. (c) Expression of leukemia inhibitory factor (Lif), heparin-binding EGF-like growth factor (Hbegf), and homeobox A10 (Hoxa10) transcripts, as determined in uterine horns 3 d after transfection (4.5 d.p.c.) with a control plasmid (pcDNA3.1; n = 6) or an expression vector that encodes the constitutively active mutant SGK-S422D (n = 8). In addition, expression levels were compared to those in wild-type (WT) female mice (n = 4) that were not mated. *P < 0.05; **P < 0.01. Data are presented as means ± s.e.m.
This was not observed in pregnant WT females. Apoptosis, a physiological phenomenon at the feto-maternal interface\textsuperscript{25,26}, was focally more pronounced in pregnant Sgk\textsuperscript{1/−} mice (Supplementary Fig. 8). Thus, lack of SGK1 in the decidua of pregnant mice seems to trigger a series of pathological events that are akin to human miscarriage, including uterine bleeding, early-onset growth restriction and fetal demise.

Early human pregnancy events cannot be studied directly, although the decidual response is recapitulated upon differentiation of primary human endometrial stromal cells (HESCs). Furthermore, there is evidence that an aberrant decidual response, associated with reproductive disorders such as endometriosis and RPL, is maintained in culture\textsuperscript{27,28}. Thus, to examine the role of SGK1 in human pregnancy failure, we decidualized primary human HESCs from subjects with RPL and control subjects over a time course lasting 8 d (Supplementary Table 1 and Supplementary Fig. 9). SGK1 transcript levels in both groups were comparable in undifferentiated HESCs and increased upon decidualization in a time-dependent manner (Fig. 4a). However, this increase was markedly blunted in the RPL group and, by 4 d of differentiation, almost 258% lower compared
to control cultures (Fig. 4a). To provide insight into the functional relevance of these observations, we used siRNA to silence SGK1 expression before differentiating primary HESCs. Knockdown was highly efficient (Fig. 4b and Supplementary Fig. 10), although we consistently noted a degree of cell death upon SGK1 silencing in decidualizing but not undifferentiated cells. This was confirmed by immunoprobing of total cell lysates for cleaved poly(ADP-ribose) polymerase-1, an apoptosis marker (Fig. 4b). To determine whether lack of SGK1 sensitizes decidual cells to environmental stress signals, we differentiated primary cultures transfected with nontargeting or SGK1-targeting siRNAs for 72 h, loaded them with a cell-permeable, oxidation-sensitive fluorescence probe (2′,7′-dichlorofluorescein) and pulsed with hydrogen peroxide. The basal oxidation status in decidualizing cells was considerably higher upon SGK1 knockdown, and the cells were less able to scavenge exogenous reactive oxygen species (Fig. 4c). SGK1 knockdown in decidualizing cells perturbed the induction of several major free-radical–scavenging molecules, including glutathione peroxidase 3 (Fig. 4d), superoxide dismutase 2, thioredoxin, peroxiredoxin-2 and glutaredoxin-1 (Supplementary Fig. 11). Expression of these scavengers was also significantly lower in decidualizing cultures from subjects with RPL compared to controls, as was the induction of the mouse homologs in pregnant Sgk1−/− mice (Fig. 4d and Supplementary Fig. 11).

Decidualization of HESCs is dependent upon activation of the NADPH oxidase NOX–p22PHOX complex and endogenous free radical production59. As SGK1 is essential for the induction of various reactive oxygen species scavengers in decidual cells, we inferred that unopposed endogenous pro-oxidant activity would be the primary cause of loss of cell viability upon knockdown of this kinase. Overexpression of catalase, an enzyme that decomposes hydrogen peroxide to water and oxygen, rescued decidualizing HESCs from oxidative cell death caused by SGK1 knockdown (Fig. 4e and Supplementary Fig. 12) but also induced a proliferative response (Fig. 4f). We assessed the decidual response by monitoring the expression of three highly induced marker genes, IGFBP1 (encoding insulin-like growth factor binding protein 1), TIMP3 (encoding TIMP metalloproteinase inhibitor 3) and LEFTY2 (left-right determination factor 2)30–33. In primary cultures, SGK1 knockdown did not negate but deregulated the induction of these markers (Supplementary Fig. 13), characterized by lower expression of LEFTY2 and IGFBP1 but increased levels of TIMP3, a gene transiently induced upon HESC differentiation34. Again, this deregulated pattern of expression was recapitulated in decidualizing primary cultures from subjects with RPL and, with the exception of Igfbp1, in pregnant Sgk1−/− mice (Supplementary Fig. 13). Notably, increased Igfbp1 expression in vivo does not necessarily imply a heightened decidual reaction but may reflect a compensatory maternal response to a suboptimal or failing pregnancy35. In agreement, expression of PrlBa2, a mouse decidua marker gene related to human PRL (encoding prolactin)36, was substantially lower in Sgk1−/− mice (Supplementary Fig. 13).

Unexplained infertility has also been linked to impaired endometrial expression of LEFTY2, encoding LEFTY-A, a member of the transforming growth factor-β superfamily that antagonizes Nodal signaling37. Like SGK1, endometrial LEFTY-A secretion is markedly reduced during the window of implantation and in vivo gene transfer leads to implantation failure in mice38. Analysis of timed endometrial biopsies confirmed higher LEFTY2 transcript levels in the infertile group but also revealed lower expression in subjects with RPL when compared to fertile controls (Supplementary Fig. 14a). Furthermore, treatment of Ishikawa cells, a widely used endometrial epithelial cell line, with recombinant LEFTY-A not only rapidly increased SGK1 transcript and protein levels but also enhanced phosphorylation of this kinase (Supplementary Fig. 14). Conversely, we found that SGK1 deficiency in decidualizing stromal cells interferes with LEFTY-A expression (Supplementary Fig. 13). Thus, it seems likely that perturbation of a broader regulatory circuit in the endometrium, involving LEFTY-A and SGK1, underpins reproductive failure.

In summary, deregulation of a single kinase in two distinct cellular compartments of the endometrium, the luminal epithelium and underlying stroma, is intricately linked to subsequent implantation failure and miscarriage, respectively. The mechanisms underpinning these reproductive phenotypes appear different, in keeping with the multifaceted function of SGK1. Our data show that continuous SGK1 activity in endometrial surface epithelium selectively disrupts the expression of implantation genes and perturbs the local fluid environment, leading to complete infertility. In pregnancy, however, endometrial SGK1 activity safeguards the decidual-placental interface against oxidative stress signals generated in response to the intense tissue remodeling, influx of inflammatory cells and dynamic changes in local perfusion and oxygen tension12,39,40.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS
M.C., F.L. and J.I.B. designed the research; M.S.S., J.H.S., J.N., Z.W., M.A.-S., G.P., M.F. and C.L. carried out the research; S.L., G.T., S.Q., L.R. and J.J.B. phenotyped the subjects and provided samples; M.S.S., A.M.S., J.D.A., M.C., F.L. and J.I.B. analyzed the data; and J.J.B. wrote the paper.

COMPETING FINANCIAL INTERESTS
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ONLINE METHODS

Human subject and sample collection. The study was approved by Hammersmith and Queen Charlotte’s & Chelsea Research Ethics Committee (1997/5065). We obtained written informed consent from all subjects before endometrial sampling. Women suffering from infertility and RPL were investigated according to the standard clinical protocol. Unexplained infertility was defined as conception delay of 24 months or more after excluding anovulation, tubal blockage, pelvic adhesions and endometriosis, or impaired semen quality, and RPL was defined as three or more consecutive pregnancy losses before 24 weeks of gestation. The fertile controls consisted of healthy volunteers with proven fertility. All participants had regular menstrual cycles and had monitored daily urinary luteinizing hormone (LH) levels using an ovulation prediction kit (Assure Ovulation Predictor). Endometrial biopsies were timed between 7 and 11 d after the preovulatory luteinizing hormone surge. For the time-course experiments, additional biopsies were obtained from subjects with or without RPL. Women with pelvic endometriosis or intra-uterine pathology, such as adhesions or polyps, were excluded from this study. The demographic details of the study and control groups are summarized in Supplementary Table 1.

Primary human endometrial stromal cell culture. We cultured HESCs isolated from endometrial tissues in maintenance medium of DMEM containing 10% dextran-coated charcoal-treated FBS (DCC-FBS) and 1% antibiotic-antimycotic solution. We decidualized confluent monolayers in DMEM containing 2% (vol/vol) DCC-FBS in DMEM with 0.5 mM 8-bromo-cAMP (cAMP; Sigma) and 1 × 10^-6 M MPA (Sigma) to induce a differentiated phenotype. Primary cultures were also treated with recombinant human LEFTY-A (R&D Systems). For time-course experiments, the cells were passaged once and expanded, and confluent cultures were treated with cAMP and MPA.

Mouse experiments. Mice were housed in specific pathogen-free conditions at the Central Biological Services, Hammersmith Campus, Imperial College, London, UK. Procedures were approved by the local ethical review committee and performed under UK Home Office Project License (PPL70/6867). We examined SGK1 expression during the window of endometrial receptivity in pseudopregnant C57BL/6 female mice (6–8 weeks old; Charles River), as previously described. Briefly, female mice were caged with vasectomized males overnight, and those plug positive for pseudopregnancy, designated 1 d.p.c., were removed to a separate cage. Four pseudopregnant females were subsequently killed by cervical dislocation at each of the following times: 3.5 d.p.c. (prereceptive), 4 d.p.c. (early receptive) and 5 d.p.c. (refractory).

For in vivo gene transfer studies, we conducted timed matings by placing C57BL/6 female mice with fertile males. The day when a vaginal plug was apparent was designated as 1 d.p.c., and mice were anesthetized 1.5 d.p.c. and subjected to laparotomy to expose the uterus. The HVJ-E vector system (GenomeONE-NEO; Ishihara Sangyo Kaisha) was found to be capable of delivering expression constructs in both luminal and glandular epithelium without discernable tissue toxicity. We used two groups of mice: a control group transfected with 10 µg of control cDNA (pcDNA3.1; Invitrogen) and a study group transfected with 10 µg SGK-S422DpIRES2eGFP, an expression vector that encodes both eGFP and a constitutively active SGK1 mutant. Briefly, both uterine horns of control and study mice were injected with an equal volume of transfection mix (100 µl) without clamping the cervix. The incision was then closed to allow for the mice to recover. We culled the mice on 4.5 or 8.5 d.p.c. The uteri were either fixed in formalin or snap-frozen and stored at −80 °C for further analysis.

To examine implantation sites and determine the litter size in Sgk1−/− mice, we backcrossed heterozygous Sgk1−/+ mice with 129/SvJ WT mice for two generations and then intercrossed to generate homozygous Sgk1−/− and WT littermates. We genotyped the mice with standard PCR methods. Implantation events were determined in 6- to 8-week-old Sgk1−/+ or WT female mice crossed with WT or Sgk1−/+ males, respectively, to ensure that all implanting embryos were heterozygous (Sgk1−/+). Pregnant mice were killed 8.5 d.p.c. to assess the number of implantation sites, or the pregnancy was allowed to continue and the number of pups per litter counted. The gross morphology of the implantation sites was assessed by H&E staining of transverse tissue sections.

Statistical analyses. Data were analyzed with the statistical package GraphPad Prism (GraphPad Software). Statistical analysis was performed using Student’s t test or Mann-Whitney U test when appropriate. In some cases, logarithmic transformations were used. Statistical significance was assumed when P < 0.05.

Additional methods. Details of additional cell line work, western blot analysis, confocal microscopy, immunohistochemistry, morphometric analysis, qRT-PCR, transient transfection, flow cytometry, cell proliferation assay and measurement of cellular oxidation status can be found in the Supplementary Methods.