DEREGULATION OF THE SERUM- AND GLUCOCORTICOID-INDUCIBLE KINASE SGK1 IN THE ENDOMETRIUM CAUSES REPRODUCTIVE FAILURE

Madhuri S Salker[1], Mark Christian[1], Jennifer H. Steel[1], Jaya Nautiyal[1], Stuart Lavery[1], Geoffrey Trew[1], Zoe Webster[2], Marwa Al-Sabbagh[1], Goverdhan Puchchakayala[3], Michael Föller[3], Christian Landles[1], Andrew M Sharkey[4], Siobhan Quenby[5], John D Aplin[6], Lesley Regan[1], Florian Lang[3] & Jan J Brosens[1,5]

[1]Institute of Reproductive and Developmental Biology, Imperial College London, Hammersmith Campus, London W12 0NN, UK. [2]Embryonic Stem Cell Facility, MRC Clinical Sciences Centre, Imperial College London, Hammersmith Campus, London W12 0NN, UK. [3]Institute of Physiology, University of Tübingen, Tübingen, Gmelinstr. 5, 72076, Tübingen, Germany. [4]Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK. [5]Division of Reproductive Health, Warwick Medical School, Clinical Sciences Research Laboratories, University Hospital, Coventry CV2 2DX, UK. [6]Maternal and Fetal Health Research Centre, University of Manchester, St Mary's Hospital, Oxford Road, Manchester M13 9WL, UK.

Address correspondence to: Jan Brosens, Division of Reproductive Health, Warwick Medical School, Clinical Sciences Research Laboratories, University Hospital, Coventry CV2 2DX, UK. Phone: +44-24 7696 8704; E-mail: J.J.Brosens@warwick.ac.uk

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: SGK1**,** serum- and glucocorticoid-inducible kinase 1; RPL, recurrent pregnancy loss; ENaC, epithelial sodium channel; HESC, human endometrial stromal cells; ROS, reactive oxygen species; IVF, *in vitro* fertilization; dpc, days post coitus.

Infertility and recurrent pregnancy loss (RPL) are prevalent but distinct causes of reproductive failure that often remain unexplained despite extensive investigations[1,2]. Analysis of mid-secretory endometrial samples revealed that SGK1, a kinase involved in epithelial ion transport and cell survival[3-6], 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 significance 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, fetal growth retardation and subsequent demise. Compared to WT animals, pregnancy-dependent induction of genes involved in oxidative stress defenses was grossly impaired in *Sgk1*$^{-/-}$ mice. Relative SGK1 deficiency was also a hallmark of decidualizing stromal cells from RPL subjects 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 to 4 days during the mid-secretory phase of the cycle, enables the embryo to contact and stably adhere to the endometrial luminal epithelium before invading the decidualizing stroma[7-9]. 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 pregnancy[10-12]. Failure of the endometrium to express a receptive phenotype is a major cause of conception delay and IVF

treatment failure. On the other hand, perturbations in the maternal decidual response inevitably lead to pregnancy complications, most prevalent of which is miscarriage[2].

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 stroma[13-15]. 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 their expression by inhibiting the ubiquitin ligase NEDD4-2[5,6]. SGK1 is also involved in proliferation and cell survival responses[3,4]. To determine if SGK1 regulates embryo implantation, we first examined its expression in mid-secretory endometrial samples from women with proven fertility and from subjects with either unexplained infertility or a history of RPL, defined here as 3 or more consecutive miscarriages (**Supplementary Table 1**). As previously reported[13], endometrial *SGK1* transcripts levels were higher in infertile 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, especially in the luminal and glandular epithelial compartments, whereas staining of stromal cells underlying the luminal epithelium appeared lower in RPL samples (**Fig. 1b & 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 & Supplementary Fig. 2**).

Sgk1 mRNA levels transiently decline in the luminal epithelium during the window of endometrial receptivity in mice[16]. Immunohistochemistry confirmed that this is paralleled by a marked reduction in phosphorylated SGK1 levels (**Supplementary Fig. 3**). To establish if sustained SGK1 activity in the luminal epithelium interferes with embryo implantation, the uterine lumen of C57BL/6 mice was injected 1.5 days post coitus (dpc) with an expression vector that encodes for a constitutively active SGK1 mutant (^{S422D}SGK1) or a control plasmid. A week later (8.5 dpc), 5 of 7 control animals were pregnant, each with 7 implantation sites

(**Fig. 2a**). In contrast, implantation sites were absent in animals expressing the constitutively active SGK1. Strikingly, ^{S422D}SGK1-expressing uteri weighed approximately half of those of non-pregnant 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 critical role of SGK1 in osmoregulation. In agreement, expression of ^{S422D}SGK1, which was confined to the luminal epithelium and some underlying glands, also markedly upregulated the alpha subunit of ENaC (α-ENaC; **Fig. 2b & 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 confers uterine receptivity during the window of implantation (4.5 dpc). As shown in Fig. 2c, expression of ^{S422D}SGK1 selectively abolished or attenuated the induction of *Lif*, *Hb-egf*, and *Hoxa10*[17-20]. The regulation of other implantation genes, perhaps more relevant for the postimplantation uterine response, such as *Ihh*, *Wnt4* and *Bmp2*[21-23], was not significantly affected (**Supplementary Fig. 5**). In addition to α-ENaC, constitutive SGK1 activity at 4.5 dpc also upregulated the expression of cystic fibrosis transmembrane conductance regulator (CFTR; **Supplementary Fig. 6**), a chloride channel implicated in IVF treatment failure[24].

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 with $Sgk1^{-/-}$ males, thereby negating the potential contribution of the offspring's genotype. While the number of implantation sites at 8.5 dpc was maintained in $Sgk1^{-/-}$ females, the average litter size was significantly reduced, indicating an excess of 30% spontaneous fetal loss (**Fig. 3a**). The implantation sites not only appeared invariably smaller in pregnant $Sgk1^{-/-}$ mice but there was also histological evidence of bleeding and immune

cell infiltration in approximately half of sites examined. This was not observed in pregnant WT females (**Fig. 3b & Supplementary Fig. 7**). Apoptosis, a physiological phenomenon at the feto-maternal interface[25,26], was focally more pronounced in pregnant *Sgk1*$^{-/-}$ 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[27,28]. Thus, to examine the role of SGK1 in human pregnancy failure, we decidualized primary human HESCs from 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. To provide insight into the functional relevance of these observations, we used small interfering RNA (siRNA) to silence SGK1 expression before differentiating primary HESC cultures. Knockdown was highly efficient (**Fig. 4b & 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 (PARP), an apoptosis marker (**Fig. 4b**). To investigate if lack of SGK1 sensitizes decidual cells to environmental stress signals, primary cultures transfected with non targeting or SGK1 siRNAs were differentiated for 72 h, loaded with a cell permeable oxidation-sensitive fluorescence probe (2'7'-dichlorofluorescein; DCF) and pulsed with hydrogen peroxide. As shown in **Fig. 4c**, 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 (ROS). 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 RPL subjects compared to controls, as was the induction of the mouse homologues in pregnant *Sgk*1$^{-/-}$ mice (**Fig. 4d and Supplementary Fig. 11**).

Decidualization of HESCs is dependent upon activation of the NADPH oxidase NOX-4/p22^{PHOX} complex and endogenous free radical production[29]. As SGK1 is essential for the induction of various ROS 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 & 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*, *TIMP3*, and *LEFTY2*[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 differentiation[34]. Again, this deregulated pattern of expression was recapitulated in decidualizing primary cultures from RPL subjects and, with the exception of *Igfbp1,* in pregnant *Sgk*1$^{-/-}$ mice. Notably, increased *Igfbp1* expression *in vivo* does not necessarily infer a heightened decidual reaction but may reflect a compensatory maternal response to a suboptimal or failing pregnancy[35]. In agreement, expression of *Prl8a2*, a mouse decidual marker gene related to human *PRL*[10], was significantly lower in *Sgk*1$^{-/-}$ animals (**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 signaling[36]. Like SGK1, endometrial LEFTY-A secretion is markedly reduced during the window of implantation and *in vivo* gene transfer leads to implantation failure in mice[37,38]. Analysis of timed endometrial biopsies confirmed higher *LEFTY2*

transcript levels in the infertile group but also revealed lower expression in RPL subjects when compared to fertile controls (**Supplementary Fig. 14**). 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. Conversely, we demonstrated that SGK1 deficiency in decidualizing stromal cells interferes with LEFTY-A expression (**Supplementary Fig. 13**). Thus, it appears 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 remodelling, influx of inflammatory cells, and dynamic changes in local perfusion and oxygen tension[12,39,40].

Acknowledgements

We are grateful to all the women who participated in this study. This work was further supported by funds to J.J.B. and M.C. from CONRAD/CICCR (CIG-08-122), the NIHR Biomedical Research Centre funding scheme and from the Genesis Research Trust (M.S.S) We are grateful to Malcolm Parker for his insightful suggestions.

Author contribution

M.C., F.L. and J.J.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.J.B. analyzed the data; and J.J.B. wrote the paper.

Figure legends

Figure 1 Deregulated SGK1 expression and activity is associated with infertility and RPL. (**a**) Timed mid-secretory endometrial biopsies from RPL subjects (*n=9*), fertile controls (FER; *n=9*), or women with unexplained infertility (INF; *n=9*) were analyzed for the expression of *SGK1* transcripts using RTQ-PCR. Transcript levels were normalized to the housekeeping gene *L19*. (Note logarithmic scale). (**b**) Immunostaining for phosphorylated SGK1 in timed mid-secretory endometrium of fertile women (FER) and subjects with RPL or unexplained infertility (INF). Additional images are presented in Supplementary Fig. 1. (Scale bar upper right panel: 100 μm). (**c**) Total protein lysates from snap-frozen endometrial samples (*n=12*) were subjected to Western blot analysis and immunoprobed with antibodies specific to pan and phosphorylated (p-) SGK1. 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 dpc) of a control plasmid (pcDNA3.1) or an expression vector that encodes the constitutively active mutant ^{S422D}SGK1. Five out of 7 control animals achieved pregnancy, all with 7 implantation sites each (middle panel). There were no implantation sites in any of the animals transfected with ^{S422D}SGK1 (*n=7*; right panel). Moreover, ^{S422D}SGK1-transfected uteri appear thin and small when compared to non-pregnant pcDNA3.1 transfected control mice (left panel). (**b**) Immunostaining of corresponding uterine tissue sections for total SGK1, phosphorylated (p-) SGK1 and α-ENaC. (Scale bar: 100 μm). (**c**) Increased SGK1 activity perturbs the expression of selective receptivity genes during the window of implantation in the mouse. Expression of leukemia inhibitory factor (*Lif*), heparin-binding EGF-like growth factor (*Hb-egf*), and homeobox A10 (*Hoxa10*) transcripts was determined in uterine horns 3 d after transfection (4.5 dpc) with a control plasmid (pcDNA3.1; *n=6*) or an expression vector that encodes the constitutively active mutant ^{S422D}SGK1 (*n=8*). In addition, expression levels were compared

to those in WT female animals (*n=4*) that were not mated. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are presented as means (± SEM).

Figure 3 Spontaneous pregnancy loss in *Sgk1*$^{-/-}$ pregnant mice. (a) Mean (± SD) number of implantation sites at 8.5 dpc and pups per litter in *Sgk1*$^{-/-}$ female mice crossed with WT males (*n=8*) and WT females with *Sgk1*$^{-/-}$ males (*n=8*). (**b**) Hematoxylin and eosin staining of implantation sites at 8.5 dpc in WT females crossed with *Sgk1*$^{-/-}$ males and *Sgk1*$^{-/-}$ female mice with WT males. Additional images are presented in Supplementary Fig. 3. The implantation sites in pregnant *Sgk1*$^{-/-}$ female mice were invariably smaller with evidence of early-onset fetal growth retardation and bleeding and inflammation at the decidual-placental interface (arrows). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4 Lack of SGK1 activity in decidualizing cells enhances susceptibility to oxidative cell death. (**a**) SGK1 mRNA expression in undifferentiated HESCs (0 d) or cultures decidualized with cAMP and MPA for 2–8 d. Primary cultures were established from women with RPL (*n=9*) and from control subjects (*n=11*). Horizontal bars indicate the median expression in each group. Note the logarithmic y-axis. (**b**) Primary HESC cultures were first transfected with either non targeting (NT) or SGK1 siRNA, then decidualized for 4 d, and harvested for RTQ-PCR or Western blot analysis. Total cell lysates were probed for SGK1 and cleaved PARP expression. β-ACTIN served as a loading control. (**c**) The oxidation status of decidualizing cells transfected with NT or SGK1 siRNA was determined prior and following treatment with 250 μM H_2O_2 using DCF. (**d**) RTQ-PCR analysis of cultures, treated as described in (b), demonstrated that SGK1 silencing impairs the expression of *GPX3* (upper panel). Expression of *GPX3* transcripts was also determined in undifferentiated and decidualizing primary HESC cultures established from RPL and control (CON) subjects (middle panel); as well as 8.5 dpc in uteri of pregnant *Sgk1*$^{-/-}$ female mice crossed with WT males (*n=6*) and WT females with *Sgk1*$^{-/-}$ males (*n=6*; lower panel). (**e**) Western blot analysis of total cell lysates of differentiating primary HESC cultures first transfected as indicated and

then decidualized with cAMP and MPA for 4 d. (**f**) Primary HESC cultures, transfected as indicated, were plated in 96-well plates and decidualized with cAMP and MPA for the indicated time-points. Cell viability, assessed by MTS assay, was normalized to the viability of undifferentiated cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

References

1. Evers, J.L. Female subfertility. *Lancet* **360**, 151-159 (2002).
2. Rai, R. & Regan, L. Recurrent miscarriage. *Lancet* **368**, 601-611 (2006).
3. Amato, R.*, et al.* Sgk1 activates MDM2-dependent p53 degradation and affects cell proliferation, survival, and differentiation. *J Mol Med* **87**, 1221-1239 (2009).
4. Brunet, A.*, et al.* Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol* **21**, 952-965 (2001).
5. Lang, F.*, et al.* (Patho)physiological significance of the serum- and glucocorticoid-inducible kinase isoforms. *Physiol Rev* **86**, 1151-1178 (2006).
6. Loffing, J., Flores, S.Y. & Staub, O. Sgk kinases and their role in epithelial transport. *Annu Rev Physiol* **68**, 461-490 (2006).
7. Dey, S.K.*, et al.* Molecular cues to implantation. *Endocr Rev* **25**, 341-373 (2004).
8. Horcajadas, J.A., Pellicer, A. & Simon, C. Wide genomic analysis of human endometrial receptivity: new times, new opportunities. *Hum Reprod Update* **13**, 77-86 (2007).
9. Wilcox, A.J., Baird, D.D. & Weinberg, C.R. Time of implantation of the conceptus and loss of pregnancy. *N Engl J Med* **340**, 1796-1799 (1999).
10. Alam, S.M.*, et al.* A uterine decidual cell cytokine ensures pregnancy-dependent adaptations to a physiological stressor. *Development* **134**, 407-415 (2007).
11. Gellersen, B., Brosens, I.A. & Brosens, J.J. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med* **25**, 445-453 (2007).
12. Leitao, B.*, et al.* Silencing of the JNK pathway maintains progesterone receptor activity in decidualizing human endometrial stromal cells exposed to oxidative stress signals. *FASEB J* **24**, 1541-151 (2010).
13. Feroze-Zaidi, F.*, et al.* Role and regulation of the serum- and glucocorticoid-regulated kinase 1 in fertile and infertile human endometrium. *Endocrinology* **148**, 5020-5029 (2007).
14. Jeong, J.W.*, et al.* Identification of murine uterine genes regulated in a ligand-dependent manner by the progesterone receptor. *Endocrinology* **146**, 3490-3505 (2005).
15. Talbi, S.*, et al.* Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* **147**, 1097-1121 (2006).
16. Campbell, E.A.*, et al.* Temporal expression profiling of the uterine luminal epithelium of the pseudo-pregnant mouse suggests receptivity to the fertilized egg is associated with complex transcriptional changes. *Hum Reprod* **21**, 2495-2513 (2006).
17. Satokata, I., Benson, G. & Maas, R. Sexually dimorphic sterility phenotypes in Hoxa10-deficient mice. *Nature* **374**, 460-463 (1995).
18. Stewart, C.L.*, et al.* Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359**, 76-79 (1992).
19. Wang, H. & Dey, S.K. Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet* **7**, 185-199 (2006).
20. Xie, H.*, et al.* Maternal heparin-binding-EGF deficiency limits pregnancy success in mice. *Proc Natl Acad Sci U S A* **104**, 18315-18320 (2007).
21. Franco, H.L.*, et al.* WNT4 is a key regulator of normal postnatal uterine development and progesterone signaling during embryo implantation and decidualization in the mouse. *FASEB J* **25**, 1176-1187 (2011).
22. Lee, K.*, et al.* Indian hedgehog is a major mediator of progesterone signaling in the mouse uterus. *Nat Genet* **38**, 1204-1209 (2006).
23. Lee, K.Y.*, et al.* Bmp2 is critical for the murine uterine decidual response. *Mol Cell Biol* **27**, 5468-5478 (2007).
24. Yang, J.Z.*, et al.* Abnormally enhanced cystic fibrosis transmembrane conductance regulator-mediated apoptosis in endometrial cells contributes to impaired embryo implantation in controlled ovarian hyperstimulation. *Fertil Steril* **95**, 2100-2106 (2011).

25. Joswig, A., Gabriel, H.D., Kibschull, M. & Winterhager, E. Apoptosis in uterine epithelium and decidua in response to implantation: evidence for two different pathways. *Reprod Biol Endocrinol* **1**, 44 (2003).
26. Yue, L.*, et al.* Cyclin G1 and cyclin G2 are expressed in the periimplantation mouse uterus in a cell-specific and progesterone-dependent manner: evidence for aberrant regulation with Hoxa-10 deficiency. *Endocrinology* **146**, 2424-2433 (2005).
27. Aghajanova, L.*, et al.* The protein kinase A pathway-regulated transcriptome of endometrial stromal fibroblasts reveals compromised differentiation and persistent proliferative potential in endometriosis. *Endocrinology* **151**, 1341-1355 (2010).
28. Salker, M.*, et al.* Natural selection of human embryos: impaired decidualization of the endometrium disables embryo-maternal interactieons and causes recurrent pregnant loss. *PLoS One* **5**(4):e10287(2010).
29. Al-Sabbagh, M.*, et al.* NADPH oxidase-derived reactive oxygen species mediate decidualization of human endometrial stromal cells in response to cyclic AMP signaling. *Endocrinology* **152**, 730-740 (2011).
30. Cloke, B.*, et al.* The androgen and progesterone receptors regulate distinct gene networks and cellular functions in decidualizing endometrium. *Endocrinology* **149**, 4462-4474 (2008).
31. Higuchi, T.*, et al.* Induction of tissue inhibitor of metalloproteinase 3 gene expression during in vitro decidualization of human endometrial stromal cells. *Endocrinology* **136**, 4973-4981 (1995).
32. Takano, M.*, et al.* Transcriptional cross talk between the forkhead transcription factor forkhead box O1A and the progesterone receptor coordinates cell cycle regulation and differentiation in human endometrial stromal cells. *Mol Endocrinol* **21**, 2334-2349 (2007).
33. Tang, M., Naidu, D., Hearing, P., Handwerger, S. & Tabibzadeh, S. LEFTY, a member of the transforming growth factor-beta superfamily, inhibits uterine stromal cell differentiation: a novel autocrine role. *Endocrinology* **151**, 1320-1330 (2010).
34. Fluhr, H., Krenzer, S. & Zygmunt, M. Different regulation of tissue inhibitors of metalloproteinases-1, -2 and -3 in human endometrial stromal cells during decidualization *in vitro*. *Reproductive Medicine and Biology* **7**, 169-175 (2008).
35. Crossey, P.A., Pillai, C.C. & Miell, J.P. Altered placental development and intrauterine growth restriction in IGF binding protein-1 transgenic mice. *J Clin Invest* **110**, 411-418 (2002).
36. Sakuma, R.*, et al.* Inhibition of Nodal signalling by Lefty mediated through interaction with common receptors and efficient diffusion. *Genes Cells* **7**, 401-412 (2002).
37. Tabibzadeh, S.*, et al.* Dysregulated expression of ebaf, a novel molecular defect in the endometria of patients with infertility. *J Clin Endocrinol Metab* **85**, 2526-2536 (2000).
38. Tang, M., Taylor, H.S. & Tabibzadeh, S. In vivo gene transfer of lefty leads to implantation failure in mice. *Hum Reprod* **20**, 1772-1778 (2005).
39. Jauniaux, E., Poston, L. & Burton, G.J. Placental-related diseases of pregnancy: Involvement of oxidative stress and implications in human evolution. *Hum Reprod Update* **12**, 747-755 (2006).
40. Jauniaux, E.*, et al.* Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. *Am J Pathol* **157**, 2111-2122 (2000).

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 as three or more consecutive pregnancy losses before 24 weeks gestation. The fertile controls consisted of healthy volunteers with proven fertility. All participants had regular cycles and 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 pre-ovulatory LH surge (LH+7 to LH+11). For the time-course experiments, additional biopsies were obtained from subjects with or without RPL. Women with pelvic endometriosis or intrauterine 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/F-12 containing 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) and 1% antibiotic-antimycotic solution[30]. We decidualized confluent monolayers in DMEM/F-12 containing 2% DCC-FBS/DMEM-F12 with 0.5 mM 8-bromo-cAMP (cAMP; Sigma) and 10^{-6} M medroxyprogesterone acetate (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, expanded and confluent cultures treated with cAMP and MPA for the indicated duration.

Animal experiments. All the experiments were carried out in accordance with the UK Home Office Project Licence (PPL70/6867). We examined SGK1 expression during the window of endometrial receptivity in pseudo-pregnant C57BL/6 female mice (6–8 weeks; Charles River Ltd), as previously described[16]. Briefly, female mice were caged with vasectomized males overnight and those plug positive for pseudo-pregnancy, designated 1 dpc, removed to a separate cage. Four pseudo-pregnant females were subsequently killed by cervical dislocation at each of the following times: 3.5 dpc (pre-receptive), 4 dpc (early receptive) and 5 dpc (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 it was designated as 1 dpc, mice were anaesthetized 1.5 dpc and subjected to laparotomy to expose the uterus. The HVJ-E vector system (GenomeONE-NEO ^{TM}, Ishihara Sangyo Kaisha Ltd) was found to be capable of delivering expression constructs in both luminal and glandular epithelium without discernable tissue toxicity. We used two groups of animals, a control group transfected with 10 μg of control cDNA (pcDNA3.1; Invitrogen) and a study group transfected with 10 μg ^{S422D}SGK1pIRES2eGFP, 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 recovery of the mice. We culled the animals on 4.5 or 8.5 dpc. 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 *Sgk*1$^{-/-}$ mice, we backcrossed heterozygous *Sgk*1$^{-/-}$ mice with 129/SvJ WT mice for two generations and then intercrossed to generate homozygous *Sgk1*$^{-/-}$ and WT littermates. We genotyped the animals using standard PCR methods. Implantation events were determined in 6-8 week old *Sgk1*$^{-/-}$ or WT female mice crossed with WT or *Sgk1*$^{-/-}$ males, respectively, to ensure that all implanting embryos were heterozygous (*Sgk1*$^{+/-}$). Pregnant animals were either sacrificed 8.5 dpc 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 hematoxylin and eosin staining of transverse tissue sections.

Statistical analysis. Data were analyzed with the statistical package Graphpad Prism (Graphpad software Inc). 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, RTQ-PCR, transient transfection, flow cytometry, cell proliferation assay and measurement of cellular oxidation status can be found in **Supplementary Methods**.