Small-conductance calcium-activated K\(^+\) channels 3 (SK3) regulate blastocyst hatching by control of intracellular calcium concentration

Yong-Chao Lu\(^1\), Guo-Lian Ding\(^1\), Jing Yang\(^1\), Yan-Ling Zhang\(^1,2\), Shuai Shi\(^3\), Run-Ju Zhang\(^1\), Dan Zhang\(^1\), Jie-Xue Pan\(^1\), Jian-Zhong Sheng\(^3\), and He-Feng Huang\(^1,2,4,*\)

\(^1\)Department of Reproductive Endocrinology, Women’s Hospital, School of Medicine, Zhejiang University, 1 Xueshi Road, Hangzhou, Zhejiang 310006, China \(^2\)The Key Laboratory of Reproductive Genetics, Ministry of Education, China \(^3\)Department of Pathology & Pathophysiology, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310058, China \(^4\)The Key Laboratory of Women’s Reproductive Health of Zhejiang Province, Hangzhou, Zhejiang, China

*Correspondence address. Tel: +86-571-87010523; Fax: +86-571-87061878; E-mail: huanghefg@hotmail.com

Submitted on July 28, 2011; resubmitted on December 9, 2011; accepted on February 6, 2012

BACKGROUND: The present study was designed to investigate the expression of small-conductance calcium-activated K\(^+\) channels 3 (SK3) in preimplantation embryos and to explore their role in the underlying mechanism of blastocyst hatching.

METHODS: Human preimplantation embryos were donated by patients who achieved successful pregnancy with in vitro fertilization. Mouse preimplantation embryos in different stages were collected and cultured with or without siRNA cell injection. The expression of SK3 was examined by RT–PCR, quantitative real-time PCR, western blot and immunofluorescence. Functional expression of SK3 was investigated using the patch-clamp technique. [Ca\(^{2+}\)]\(_i\) was measured by fluorescent imaging. Embryos were cultured in vitro to investigate the effect of SK3 knockdown or apamin, an SK3 inhibitor, on blastocyst hatching and F-actin formation.

RESULTS: In human blastocysts, the level of SK3 expression was significantly lower in blastocysts that failed to hatch than in blastocysts that hatched successfully. In mouse embryos, SK3 mRNA and protein were not found in zygotes, but were detected from the 2-cell stage onward, with the highest levels observed in blastocysts. SK3 was predominately located in the trophectoderm cell membrane of expanded blastocysts. SK3 knockdown in trophectoderm cells not only suppressed the SK3 current, but also reduced [Ca\(^{2+}\)]\(_i\) elevation and membrane potential hyperpolarization induced by thapsigargin. Although the formation of expanded blastocysts was not affected, blastocyst hatching and F-actin formation were significantly inhibited after SK3 knockdown in trophectoderm cells.

CONCLUSIONS: SK3-mediated [Ca\(^{2+}\)]\(_i\) elevation and membrane potential hyperpolarization in trophectoderm cells are important for blastocyst hatching, and defects in SK3 expression may contribute to infertility.

Key words: small-conductance calcium-activated K\(^+\) channel 3 / blastocyst hatching / motility / membrane potential

Introduction

In mammalian preimplantation embryonic development the cleavage stages lead to the first differentiation process which results in the creation of a blastocyst (Telford et al., 1990). The blastocyst contains two physiologically distinct cell types: trophectoderm, which develops into the placenta, and the inner cell mass, which is composed of relatively undifferentiated cells that will give rise to the embryo (Cross, 2000). During the formation of the blastocyst, the mammalian embryo remains enclosed in the zona pellucida (ZP), which is an acellular matrix, and is involved in regulating sperm–egg interaction and protecting the embryo during preimplantation development (Wassarman et al., 2005). Prior to implantation, the blastocyst emerges out of the ZP by a critically important process called hatching, which is followed by the attachment, adhesion and invasion of the blastocyst into the receptive maternal uterine endometrium (Seshagiri et al., 2009). Any dysregulation of the hatching process causes implantation failure leading to infertility (Petersen et al., 2005). Around the time of
hatching, blastocyst development and implantation is governed by an extremely complicated but cooperative interplay of various cellular factors and biomolecules, which regulate the organization of the cytoskeleton of blastocysts responsible for the morphological transition from spherical to tubular and filamentous forms (Mattson et al., 1990; Simon et al., 1996; Seshagiri et al., 2002). Trophoderm cells in the expanding blastocyst exhibit pericellular distribution of actin that later forms continuous actin-rich lateral borders and stress fibers along their basal surface. Thus, actin-based modifications in trophoderm cells appear to prepare them to shape the blastocyst to hatch from the ZP (Bazer et al., 2009). However, information on this developmentally critical phenomenon is scarce. It is necessary to further investigate the cellular and molecular mechanisms of blastocyst hatching.

The small-conductance calcium-activated K+ (SK) channels are one subfamily of calcium-activated potassium channels (KCa), including SK1 (KCa2.1, KCNN1), SK2 (KCa2.2, KCNN2) and SK3 (KCa2.3, KCNN3) (Stocker, 2004). They have high sensitivity and responsiveness to intracellular calcium concentration ([Ca2+]i) changes, and can be activated by relatively low [Ca2+]i (<500 nM) (Kohler et al., 1996). Chemical compounds, such as apamin, can inhibit the activity of SK channels, and therefore, have been widely used in SK channel studies (Stocker et al., 1999). The SK3 channel, in particular, is expressed in a number of adult tissues, including the brain, smooth muscle and blood vessel endothelia (Bond et al., 2005; Brown et al., 2007). The SK3 channel has several functional characters that may contribute to cell migration: (i) cyclical activation and inhibition, following intracellular Ca2+ oscillations and/or cell shape changes; (ii) the SK3 channel, an activated-membrane molecule, routinely adopts a polarized cell location (Barfod et al., 2007; Liebau et al., 2007) and (iii) the activity of the SK3 channel is sensitive to variations of the [Ca2+]i often associated with cell motility/migration (Chen et al., 2004). Other previous reports indicate that SK3 is involved in cell motility by enhancing membrane hyperpolarization, although the exact mechanisms remain unknown (Chen et al., 2004; Potier et al., 2006). Previous studies showed that a Ca2+-activated K+ channel in human oocytes could be activated by fertilization (Gianaroli et al., 1994; Dale et al., 1996). This suggests that a member of the KCa family is expressed in human oocytes and functions on fertilization. However, SK expression and its possible involvement in preimplantation embryo development have not been studied.

In the process of blastocyst hatching, hydrostatic (mechanical) pressure exerted by the increasing expansion of the blastocyst causes initial formation of a nick in the ZP. Subsequently, the blastocyst gradually egresses out of the zona through a hole pierced in the ZP by zonalytic protease (Lopata and Hay, 1989; Seshagiri et al., 1999). Movement of the blastocyst plays an important role in blastocyst hatching. As SK3 channels mediate cell motility by elevating [Ca2+]i to activate pathways to promote F-actin assembly for cell motility (Chen et al., 2004; Potier et al., 2006; Balijnymam et al., 2010), the alteration of SK3 expression in the preimplantation embryo might be associated with blastocyst hatching.

The present research was designed to discover if SK3 expression is associated with blastocyst hatching, and to explore its role in the underlying mechanism.

Materials and Methods

Patients and embryo samples

Human preimplantation embryos were donated by patients who had a successful pregnancy from an in vitro fertilization program at Women’s Hospital, School of Medicine, Zhejiang University. Donation was voluntary and informed consent was given. This research was approved by the Ethics Committee for Research on Human Subjects of Zhejiang University. All the donated embryos were fertilized successfully by ICSI methods in order to exclude the effect of sperm that adhere to the ZP.

Animals

The care and use procedures for the ICR mice were in accordance with the Institutional Guide for Laboratory Animals established by the Animal Care and Use Committee (ACUC), and were approved by the ACUC of the School of Medicine, Zhejiang University. The mice were housed under a 12/12-h light/dark cycle at 25 ± 0.5 and 50–60% humidity, and were fed ad libitum with a standard diet and water.

Embryo recovery and culture

Female ICR mice (8-week-old) were superovulated by intraperitoneal injections of 10 IU pregnant mares’ serum gonadotrophin (PMSG, Sigma, St. Louis, MO, USA), followed by 10 IU human chorionic gonadotrophin (hCG, Sigma) at 48 h post-PMSG. Females were caged with ICR males (10-week-old) immediately following hCG injection. Embryos at different stages were obtained by sacrificing the mice at the times stated after hCG injection: zygote 18 h, 2-cell 44 h, 4-cell 56 h, 8-cell 68 h, morula 80 h, blastocyst 92 h. Blastocysts were collected by flushing the uterus with human tubal fluid HEPES (HTF-HEPES, Irvine Scientific, Irvine, CA, USA) medium; embryos at other stages were collected by flushing the oviducts with HTF-HEPES from the infundibular end. The embryos were transferred to HTF (Irvine Scientific) medium and cultured under 5% CO2 at 37°C.

RT–PCR and quantitative real-time PCR

cDNA of preimplantation embryos was synthesized from 100 embryos for each stage with Cell-to-cDNA II kit (Ambion, Naugatuck, CT, USA). RT–PCR was performed by using the iCycler (Bio-Rad, Foster City, CA, USA). Quantitative real-time PCR was performed by using the ABI Prism 7900HT (Applied Biosystems, Carlsbad, CA, USA). The specific primers were provided by Sangon, Shanghai, China. The full list of primer sequences for RT–PCR (mouse SK1, SK2 and SK3) and quantitative real-time PCR (mouse SK3 and GAPDH, human SK3 and GAPDH) is shown in Table I.

Western blot analysis

One hundred embryos were pooled in each group with sample buffer (15% Glycerol, 0.125 M Tris-Cl pH 6.8, 5 mM EDTA, 2% SDS, 0.1% Bromophenol Blue, 1% β-mercaptoethanol) and lysed. As a positive control, mouse brain tissues were lysed with RIPA buffer followed by centrifugation at 15 000g for 30 min at 4°C to get the lysates. All the lysates were denatured at 95°C for 5 min before being loaded to 10% SDS–PAGE. Blots were incubated with anti-SK3 primary antibody (1:100; AB 5350, Chemicon, Temecula, CA, USA), anti-β-actin (1:500; sc-130656, Santa Cruz Biotechnology, CA, USA), anti-HBOA (1:300; sc-25379, Santa Cruz Biotechnology), anti-TBP (1:500; sc-34863, Santa Cruz Biotechnology). Enhanced chemiluminescence was visualized by film development.

Downloaded from https://academic.oup.com/humrep/article-abstract/27/5/1421/698790 by guest on 20 November 2018
Involvement of SK3 in blastocyst hatching

Immunofluorescent staining

The embryos were fixed with 4% paraformaldehyde for 1 h and then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 30 min. After incubation with 10% goat serum for 1 h to block the non-specific antigen, they were incubated with the polyclonal rabbit anti-SK3 primary antibody (1:100 dilution) (AB 53580, Chemicon, CA, USA) or rabbit anti-F-actin primary antibody (1:500 dilution) (MAI-80729, Thermo, USA) at 4°C overnight, washed with PBS, and then incubated with fluorescein isothiocyanate-conjugated secondary antibody at room temperature for 45 min followed by nuclear staining with 4',6-diamidino-2-phenylindole. The fluorescence was observed under a fluorescence microscope (Olympus, Japan). The intensities of F-actin in embryo were quantified using Image-J software (National Institutes of Health, USA). The background was selected randomly and its intensity was also obtained by the software. The intensities of F-actin were normalized to that of the background to calculate the gray values of F-actin in embryos.

$[Ca^{2+}]_{i}$ measurements

Blastocysts were manipulated on the stage of an inverted microscope mounted a perfusion chamber. They were transferred onto a glass cover slip and allowed to stick. The ZP was removed using acid Tyrode’s solution, and the embryos were extensively washed in fresh culture medium. $[Ca^{2+}]_{i}$ imaging experiments were performed as described earlier (Gackiere et al., 2006). $[Ca^{2+}]_{i}$ was measured by Fluorescence Microscope Photometry Systems (FMPS) (Nikon, Tokyo Japan). Bath medium used for the experiments was Hank’s balanced salt solution (HBSS) containing 142 mM NaCl, 5.6 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 0.34 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 10 mM HEPES and 5.6 mM glucose. The cells were loaded with 2 μM Fura-2/AM prepared in HBSS for 45 min at room temperature and subsequently washed three times with the same dye-free solution. The coverslip was then transferred onto a perfusion chamber on an inverted microscope equipped for fluorescence. Fluorescence was alternatively excited at 340 and 380 nm and was captured after filtration through a long-pass filter (510 nm). Acquisition and analysis were performed with the Image-pro plus 6.0 software (Media Cybernetics, USA). The $[Ca^{2+}]_{i}$ was derived from the ratio of the fluorescence intensities for each of the excitation wavelengths (F340/F380) and from the equation of Grynkiewicz et al. (1985). All recordings were carried out at room temperature. The cells were continuously perfused with the HBSS and chemicals were added via the perfusion system.

Whole-cell patch-clamp recording

Before the patch-clamp recording experiment, the trophectoderm cells of the blastocyst were grown on glass coverslips. Bath medium used for the experiments was HBSS as described above. Patch-clamp recordings were performed in the whole-cell configuration by using a conventional whole-cell configuration with an RK-300 patch-clamp amplifier (Bio-Logic, Claix, France), which was driven by Pulse 8.30 software (HEKA Elektronik, Lambrecht, Germany) as described earlier (Mariot et al., 2002). Membrane potentials were recorded with the current-clamp method and digitized at 20 kHz using an ITC16 computer interface (Instrutech Corp., Long Island, NY, USA) low-pass filtered at 3 kHz and stored on-line on the hard-drive of the computer. Patch pipettes were made from borosilicate glass (Vitrex, Modulohm A/S, Herlev, Denmark) on a micropipette puller (HEKA, Germany) to a resistance of 5 MΩ after being filled with pipette solution. The pipette solution contained 140 mM K-glutamate, 1 mM EGTA, 1 mM MgCl$_2$, 5 mM HEPES. All patch-clamp recordings were performed at room temperature (22°C).

Simultaneous recording of membrane potential and $[Ca^{2+}]_{i}$ of the trophectoderm cells

The trophectoderm cells on the coverslip were loaded with 2 μM Fura-2/AM, and then transferred onto a chamber on the stage of the microscope equipped with the RK-300 patch-clamp amplifier and FMPS as described above for simultaneous recordings.

siRNA cell injection

Scrambled RNA (Control RNA; sc-44230, Santa Cruz Biotechnology), SK3 siRNA (sc-37034, Santa Cruz Biotechnology) or histone acetyltransferase (HBOA) siRNA (sc-35531, Santa Cruz Biotechnology) was dissolved in RNase-free water. At the 2-cell stage, each blastomere of 2-cell embryos was injected with 50 fmol of RNA solution using the micromanipulation system (Nikon, Japan). Forty-eight hours after injection, the expression of SK3 was validated at the blastocyst stage using quantitative real-time PCR.

Statistical analysis

Each experiment was repeated at least three times. Data are presented as the means ± standard error (SEM). Student’s unpaired t-test was used for comparison between two groups. One-way analysis of variance (ANOVA) was used for comparison among three or more groups. A probability of $P < 0.05$ was considered statistically significant.

| Table I Nucleotide sequences of primers used for RT–PCR and quantitative real-time PCR (qPCR) (SYBR Green). |
|-----------------------------------------------|------------------------------------------------|
| Target RNA | Forward (5’–3’) | Reverse (5’–3’) | Product size (bp) |
| Mouse SK1 (for RT–PCR) | TGCTGCGGGAACATGCTGGG | CAGCCGGTTGGCTACTGTCG | 335 |
| Mouse SK2 (for RT–PCR) | TTGAGCTCTGGCGCCGGGAA | CTCAAACAGGAGGGCCGGGAT | 324 |
| Mouse SK3 (for RT–PCR) | CCCGGCTCTTCTCCGCTTT | GAAGTGGGGCCCTGAGCC | 554 |
| Mouse SK8 (for qPCR) | ATCCTGCCGGCGCCGAGGTCA | TACCGGTCGGAAAGGAGTGGAG | 101 |
| Mouse GAPDH (for qPCR) | CCCCGAGAAGGACTGAGGAAAGAG | GCCCCCTCTGTATTATGGGGTGTC | 107 |
| Human SK3 (for qPCR) | GAGCCACCTCTCTTTGGGACGG | GGCGCACTGCCTGGCTAAA | 187 |
| Human GAPDH (for qPCR) | TGGCCCCTCGGGGAACACTGT | ACCTGCCCACAGCCTGGG | 93 |
Results

The expression and localization of SK3 in preimplantation embryos

As shown in Fig. 1A, we found that SK3 is expressed in human blastocysts. Moreover, the levels of SK3 expression in unincubated blastocysts were much lower than those in hatched blastocysts, suggesting that the SK3 channel may play an important role in human blastocyst hatching. In the mouse, SK3 mRNA, but not SK1 and SK2 mRNA, was detected in all preimplantation stage embryos, except the zygote (Fig. 1B). Quantitative real-time PCR and western blot analyses showed that the expression of SK3 mRNA and protein appeared from the 2-cell stage onward, after activation of the embryonic genome, and their levels gradually increased during preimplantation embryo development, with the highest level observed in blastocysts (Fig. 1C and D). Immunofluorescence analysis showed that SK3 protein was absent in the membrane as well as the cytoplasm of zygotes. From the 2-cell stage to the morula stage, SK3 proteins were all expressed in the cytoplasm but not in the membrane of embryos. In contrast, at the expanded blastocyst stage, SK3 protein was predominately located at the trophectoderm cell membrane (Fig. 1E).

SK3 knockdown by SK3 siRNA injection

Quantitative real-time PCR results showed that SK3 mRNA expression sharply decreased by 80% of control in trophectoderm cells after SK3 siRNA injection (Fig. 2A). To value the expression of SK3 protein, western blot analysis was also used to detect the level of SK3 protein in blastocysts after SK3 siRNA injection. The results showed that the expression of SK3 protein was significantly suppressed after SK3 siRNA injection (Fig. 2B). However, the injection with scrambled RNA did not affect the expression of SK3 and β-actin that is used as the interference. In order to investigate whether activation of RNA-induced silencing complex (RISC) signaling pathway affected blastocyst hatching, we used another siRNA targeting for the mRNA of HBOA, a house-keeping protein, to examine blastocyst hatching (Eisenberg and Levanon, 2003). The TATA-binding protein (TBP) was used as the internal reference for nucleoprotein. The results showed that HBOA knockdown did not affect blastocyst hatching, indicating that SK3 knockdown by the siRNA injection is specific (Fig. 2C–E).

Ca2+ -induced activation of SK3 channels hyperpolarizes mouse trophectoderm cell membrane potential

Using techniques of patch-clamp in whole-cell configuration, we detected a voltage-dependent current in trophectoderm cells that was significantly reduced by treatment of cells with SK3 siRNA (Fig. 3A). This result confirms SK3-mediated K+ current in trophectoderm cells. Thapsigargin (TG) can inhibit the endoplasmic reticulum SERCA Ca2+ pump, causing calcium mobilization from intracellular stores followed by store-operated calcium entry in trophectoderm cells (Stryma et al., 2000). Combined current-clamp and calcium-imaging experiments, the TG-induced increase in [Ca2+]i was accompanied by a large hyperpolarization of membrane potential (Fig. 3B). SK3 knockdown caused a significantly reduced [Ca2+]i elevation and membrane potential hyperpolarization in trophectoderm cells induced by TG (Fig. 3B–D). When membrane potential was clamped at resting potential (−48 mV) to investigate whether SK3 is increasing [Ca2+]i by raising the driving force for Ca2+ entry, we found that there was no significant difference in the [Ca2+]i elevation induced by TG between scrambled RNA-treated and SK3 siRNA-treated trophectoderm cells (Fig. 3E and F). Furthermore, we found that TG at 80 nM could enhance the voltage-dependent current in trophectoderm cells. This enhanced current induced by TG was blocked by 100 nM apamin, a specific SK inhibitor, indicating that SK is involved (Fig. 3G). On the other hand, an application of TG induced membrane potential hyperpolarization, which was also reversed in the presence of apamin (Fig. 3H).

Involvement of SK3 in blastocyst hatching

SK3 knockdown in 2-cell mouse preimplantation embryo had no effect on the formation of expanded blastocysts (Fig. 4A and B). However, the rate of blastocyst hatching significantly decreased after SK3 knockdown (Fig. 4C and D). We also found that treatment of embryos with apamin, before hatching, for 12 h significantly reduced blastocyst hatching (Fig. 4E and F). In addition, SK3 knockdown sharply reduced the formation of cytoskeleton protein F-actin, which is known to be involved in cell motility and considered a marker for blastocyst hatching (Fig. 4G and H).

Discussion

In the present study, we found that SK3 was expressed in human blastocysts, and that less was expressed in blastocysts that failed to hatch, consistent with the idea that the SK3 channel may play an important role in human blastocyst hatching. In the mouse embryos express SK3 mRNA and protein during early development and SK3 channels in trophectoderm cells of the blastocyst mediated intracellular Ca2+ elevation via hyperpolarizing membrane potential. Moreover, down-regulation of SK3 induced changes in blastocyst morphology and reduced blastocyst hatching via regulating the assembly of F-actin, an intracellular contractile component.

In order to elucidate the function of SK3 in preimplantation embryo development, we used mouse embryos for further investigation. In mouse, we found that mRNA for the SK3 gene expressed after activation of the embryonic genome from the 2-cell stage onward, with the highest levels in the blastocyst stage, suggesting that fertilization activated SK3 expression and biological function during early embryonic stages. However, no expression of SK1 and SK2 transcript could be observed at the same stage, suggesting that SK3 is the only SK subfamily member involved in the functions of [Ca2+ ]i during preimplantation embryo development. In our study, SK3 protein could also be detected from the 2-cell stage onward, and appeared to localize to the cytoplasm of blastomeres. Interestingly, during the late blastocyst stage, SK3 protein expressed both in membrane and cytoplasm. Many reports have suggested the possibility that SK3 protein forms the heteromeric channel with the other SK channel subunits SK1 and SK2 (Ishii et al., 1997; Monaghan et al., 2004). In our study, SK1 and SK2 channels were not detected in the preimplantation embryo, suggesting...
that SK3 proteins, may form SK homomeric channels and be involved in cell migration.

Using the patch-clamp electrophysiological technique, we detected the functional expression of the SK3 channel on the apical membrane of trophectodermal cells. Immunofluorescence analysis confirmed the expression and location of SK3. The increased conductance in trophectoderm cells in response to an increase in [Ca$^{2+}$]$_i$ was due to SK3 channel activation, which was responsible for the outward K$^+$ current.

Figure 1 Expression of SK3 in preimplantation embryos. (A) Expression of SK3 in hatched and unhatched human blastocysts. * indicates $P < 0.05$ (by unpaired $t$-test, $n = 3$) when compared with hatched blastocysts. (B) Expression of SK1, SK2 and SK3 mRNA in mouse preimplantation embryos. M, Marker; z, zygote; 2, 2-cell embryo; 4, 4-cell embryo; 8, 8-cell embryo; m, morula; b, blastocyst; n, minus cDNA template negative control. (C) Quantitative real-time PCR results showing the levels of SK3 transcripts relative to GAPDH expressed during mouse early embryo development. *indicates $P < 0.05$, *** indicates $P < 0.001$ (One-way ANOVA) when compared with that in the 2-cell embryo stage. (D) Western blot results showing the expression of SK3 protein in mouse embryos during preimplantation development. The positive control is mouse brain tissue. (E) Immunofluorescent results showing the location of SK3 protein in mouse embryos during preimplantation development. Scale bar: 50 μm.
and hyperpolarization induced by an increase in \([\text{Ca}^{2+}]_i\). The properties of the detected channels in the present study are consistent with the activities of SK3, confirming the expression and function of a \(\text{Ca}^{2+}\)-activated \(\text{K}^+\) channel, SK3, in trophectodermal cells of the blastocyst.

The involvement of SK3 in cell membrane potential has been shown in the breast cancer cell (Potier et al., 2006). It has been reported that SK3 was up-regulated in a melanoma cancer cell line (Chantome et al., 2009), which led to plasma membrane hyperpolarization and enhanced cell motility. The studies on cancerous mammary epithelial cell line MDA-MB-435s also found that SK3 regulates resting membrane potential as in the central nervous system and smooth muscle (Herrera et al., 2003; Taylor et al., 2003). It was found that \(\text{K}^+\) efflux could lead to water efflux and causes localized cell volume decreases that allow retraction of the rear end of migrating cells (Schwab et al., 1999). Using SK3-specific siRNA, we showed that the SK3 channel appears to play an important role in trophectoderm cell membrane potential responses to changes in \([\text{Ca}^{2+}]_i\). It has been reported that \(\text{K}^+\) channels may be involved in cell cycle progression of cells (Villalonga et al., 2007). Here, we found that blastocyst hatching was arrested after SK3 knockdown in vitro, although embryo cleavage and even blastocyst formation were not affected. Previous studies found that blastocyst hatching in most mammalian species is characterized by shedding of the ZP (Massip and Mulnard, 1980; Seshagiri and Hearn, 1993; O’Sullivan et al., 2001; Sathananthan et al., 2003). The phenomenon of blastocyst hatching is believed to be regulated by (i) dynamic cellular components such as actin-based trophectodermal projections (TEPs) and (ii) a variety of autocrine and paracrine molecules such as growth factors, cytokines and proteases (Gonzales et al., 1996). During the period of the blastocyst hatching, the fully expanded blastocyst exerts a mechanical pressure on the ZP forcing a hole to form that is enlarged by protease. TEPs have been found to function as cargo carriers for the endogenous, embryo-derived zona lysins and act to deliver them to the ZP causing its lysis (Khalifa et al., 1992; Seshagiri et al., 2009). However, the intracellular mechanism of trophectoderm cell motility is unknown. Many of the key proteins involved in cell motility, including the actomyosin cytoskeleton (Lauffenburger and Horwitz, 1996), can be regulated by intracellular free \(\text{Ca}^{2+}\). Also, studies showed that \(\text{KCa}\) channels localize to caveolae close to the cytoskeleton to form an actin–\(\text{KCa}\) channel–caveolin microdomain complex in smooth muscle (Brainard et al., 2005). Then, in SK3-expressing trophectoderm cells, \([\text{Ca}^{2+}]_i\) may be elevated using the electrochemical driving force generated by \(\text{K}^+\) efflux through SK3 channels to increase \(\text{Ca}^{2+}\) entry through plasma membrane voltage independent \(\text{Ca}^{2+}\) channels (Hoenderop et al., 1999; Tsavaler et al., 2001; Sheng et al., 2009). Increased \(\text{Ca}^{2+}\) entry may stimulate intracellular \(\text{Ca}^{2+}\) release from intracellular \(\text{Ca}^{2+}\) store by calcium-induced calcium release pathway (Sheng et al., 2009). We found that the cytoskeleton F-actin assembly...
Figure 3 Functional expression of SK3 and involvement in membrane potential of trophectoderm cells of mouse blastocyst. (A) Typical traces obtained using the patch-clamp technique in whole-cell configuration showing the involvement of SK3 in voltage-dependent currents. (B) A simultaneous recording of the [Ca\textsuperscript{2+}]\textsubscript{i} (a) and membrane potential (b) induced by TG in trophectoderm cells after scrambled RNA injection. (C) A simultaneous recording of the [Ca\textsuperscript{2+}]\textsubscript{i} (c) and membrane potential (d) induced by TG in trophectoderm cells after SK3 siRNA injection. (D) Summary of the results from (B) and (C). * indicates P < 0.05 (by unpaired t-test, n = 3) when compared with scrambled RNA injection. (E) Recording of the [Ca\textsuperscript{2+}]\textsubscript{i} induced by TG as membrane potential clamped at resting potential (~48 mV) by voltage clamp in trophectoderm cells after scrambled RNA or SK3 siRNA injection. (F) Summary of the results from E. ns indicates P > 0.05 (by unpaired t-test, n = 6) when compared with scrambled RNA injection. (G and H) Typical tracings obtained using the patch-clamp technique in whole-cell configuration showing that apamin, an SK3 inhibitor, blocked both the K\textsuperscript{+} currents (H) and the membrane hyperpolarization (G) induced by TG. TG, thapsigargin.
was decreased by SK3 knockdown to suggest that SK3 may enhance F-actin assembly by elevating $[Ca^{2+}]_i$. Based on this finding, because the generation and control of cytosolic calcium is a fundamental mechanism for cell motility (Baljinnyam et al., 2010), it is evident that SK3 is critical for trophectoderm cell motility in blastocyst hatching.

**Figure 4** Effect of SK3 on hatching and F-actin formation of mouse blastocysts in vitro. (A) Embryo development after 3 days of culture following scrambled RNA or SK3 siRNA injection. Scale bar: 50 μm. (B) Summary of the results from (A). ns indicates $P > 0.05$ (by unpaired t-test, $n = 3$) when compared with scrambled RNA injection. (C) Blastocyst hatching after 4 days of culture following scrambled RNA or SK3 siRNA injection. Scale bar: 50 μm. (D) Summary of the results from (C). ** indicates $P < 0.01$ (by unpaired t-test, $n = 3$) when compared with scrambled RNA injection. (E) Blastocyst hatching after 4 days of culture without or with apamin. Scale bar: 50 μm. (F) Summary of the results from (E). ** indicates $P < 0.01$ (by unpaired t-test, $n = 4$) when compared with control. (G) F-actin immunofluorescent stained expanded blastocyst. Scale bar: 50 μm. (H) Summary of the results from (G). ** indicates $P < 0.01$ (by Unpaired t-test, $n = 3$) when compared with scrambled RNA injection.
In conclusion, as well as providing new evidence of enhanced SK3 expression in blastocyst trophoblast cells at the mRNA and protein levels, our findings also contribute substantial evidence about the mechanisms by which SK3 modulates blastocyst trophectoderm cell motility. These observations suggest that the SK3 channel may become a novel therapeutic target and/or new molecular marker of infertility. The precise mechanism of the functional SK3 channel effective in blastocyst hatching needs to be further explored.

**Authors’ roles**

Y.-C.L.: contributed to the conception and design of the present research, and acquisition data, analysis and interpretation of data; to drafting the article and revising it critically for important intellectual content; and to final approval of the version to be published. G.-L.D.: contributed to the acquisition, analysis and interpretation of data; to drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. J.Y.: contributed to the acquisition of data and final approval of the version to be published. Y.-L.Z.: contributed to revision of the paper critically for important intellectual content and final approval of the version to be published. S.S., R.-J.Z., D.Z., J.-X.P.: contributed to the acquisition of data and final approval of the version to be published. J.-Z.S.: contributed to the analysis and interpretation of data; to revision of the paper critically for important intellectual content; and to final approval of the version to be published. H.-F.H.: contributed to revision of the paper critically for important intellectual content and final approval of the version to be published.

**Funding**

This work was supported by National Basic Research Program of China (No. 2012CB944901 and No. 2011CB944502), the National Natural Science Foundation of China (No. 30670789 and No. 30901604) and the Charity Project from Department of Science and Technology of Zhejiang province (No. 2010C33167).

**Conflict of interest**

None declared.

**References**


