Human Reproduction, Vol.27, No.12 pp. 3540–3551, 2012
Advanced Access publication on September 20, 2012 doi:10.1093/humrep/des330

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

Administration of calcitonin promotes blastocyst implantation in mice by up-regulating integrin β3 expression in endometrial epithelial cells

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Submitted on December 7, 2011; resubmitted on June 27, 2012; accepted on August 14, 2012

STUDY QUESTION: Does exogenous calcitonin improve the efficiency of implantation in mice by increasing uterine receptivity?

SUMMARY ANSWER: The administration of calcitonin could improve the efficiency of implantation by increasing the expression of several receptivity-related genes in endometrial epithelial cells (EECs).

WHAT IS KNOWN ALREADY: Calcitonin is one of the biomarkers of uterine receptivity, which is transiently produced in the uterine epithelia during the period of implantation both in humans and mouse.

STUDY DESIGN, SIZE, DURATION: Hormone-replaced mice were used for *in vivo* experiments. To evaluate the effect of calcitonin on uterine receptivity, the expression of endometrial genes was analyzed 36 h after i.p. injection of 0.5 IU calcitonin in a treatment group versus saline in the control. To evaluate the effect of calcitonin on implantation efficiency *in vivo*, two groups received 0.5 IU or 2 IU calcitonin (i.p.) 24 h before embryo transfer, and a control group received saline (i.p.) ($n = 18$ mice per group). Implantation sites were counted 7 days after embryo transfer. The RL95-2 human endometrial carcinoma cell line was used to study the mechanisms underlying the effect of calcitonin on gene expression in the endometria. Using an *in vitro* model of endometrium–trophoblast interaction, established with RL95-2 cells and JAR (human choriocarcinoma cell line) trophoblast, endometrial receptivity was evaluated by comparing attachment and outgrowth of JAR spheroids in control and treatment groups.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Uterine receptivity in ovariectomized mice was induced by injection of estradiol and progesterone. Expression of eight genes in murine endometrium and RL95-2 cells was analyzed by real-time RT–PCR, western blot, immunohistochemical analysis, flow cytometry and enzyme-linked immunosorbent assay. We tested the effects of a protein kinase C inhibitor, matrigel and an antibody against integrin αvβ3 using RL95-2 cells and performed attachment and outgrowth assays using the *in vitro* model of endometrium–trophoblast interaction. Implantation efficiency was evaluated by counting the implantation sites after embryo transfer.

MAIN RESULTS AND THE ROLE OF CHANCE: Calcitonin up-regulated αvβ3 in RL95 cells, which in turn resulted in increased levels of the leukemia inhibitory factor (LIF) and heparin binding-epidermal growth factor (HB-EGF) mRNA (both $P < 0.01$ versus control) and protein (both $P < 0.05$ versus control). The attachment and expansion of JAR spheroids was promoted by pretreatment of EECs with calcitonin ($P < 0.05$ versus control) together with significantly increased expression of αvβ3, LIF and HB-EGF. Moreover, the injection of calcitonin in the preimplantation phase increased the total number of implantation sites in treatment groups (55 in control versus 78 and 85 in 0.5 and 2 IU groups, respectively). Compared with the control group (3.11 ± 2.14), the average number of implantation sites in the 2 IU calcitonin treatment group increased (4.72 ± 1.87, $P = 0.022$).

LIMITATIONS, REASONS FOR CAUTION: Experiments were performed in mice and human cell lines but not in primary cultures of human endometrial cells.

WIDER IMPLICATIONS OF THE FINDINGS: The findings presented here have important implications, in that calcitonin administration (currently used for treatment of hypercalcemia or osteoporosis) may have clinical benefits in assisted reproduction programs, by facilitating endometrial receptivity and embryo implantation. However, further studies are required to confirm these findings.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by National Science Foundation of China (No. 81170619). There are no financial or commercial conflicts in this study.

Key words: calcitonin / endometrial receptivity / implantation / integrin β3

Introduction

Infertility is a growing problem among couples of reproductive age. In the last three decades, more and more married women have sought pregnancy assistance due to infertility (Mosher and Pratt, 1991; de Mouzon *et al*., 2010). Although assisted reproduction techniques (ART) have been widely used in clinical treatment of infertility resulting from benign gynecological disorders or primary unexplained infertility, the pregnancy rates are still unsatisfactory (de Mouzon *et al*., 2010). Failure of embryo implantation is one of the main reasons for the unsatisfactory outcomes. The implantation efficiency in ART remains relatively low, which is the major obstacle for the success of IVF and embryo transfer/ICSI treatments (Salamonsen *et al*., 2002; Dekel *et al*., 2010).

An embryo at the blastocyst stage and a receptive endometrium are required for successful implantation. In addition to the quality of embryo, implantation failure is mainly associated with inadequate endometrial receptivity (Sharkey and Smith, 2003; Haouzi *et al*., 2011). Uterine receptivity plays a key role in the establishment of successful pregnancies. The impairment of uterine receptivity may contribute to the subfertility in some benign gynecological diseases and limit ART success (Donaghay and Lessey, 2007; Cakmak and Taylor, 2011). However, so far there is no suitable approach to effectively improve endometrial receptivity in the clinical treatment of infertile patients. Available methods used for the treatment of infertile patients with benign gynecological diseases include medical therapies and surgical excision by laparoscopy or hysteroscopy, usually targeting the diseases themselves (Strandell *et al*., 2001; Littman *et al*., 2005). On the other hand, some methods have been introduced to treat failure of conception despite the repeated transfer of apparently good quality embryos in human IVF procedures, including systemic administration of progesterone (Nosarka *et al*., 2005), leukemia inhibitory factor (LIF) (Brinsden *et al*., 2009), heparin (Stern *et al*., 2003), aspirin (Pakkila *et al*., 2005) and prednisone (Galdo *et al*., 2005). Unfortunately, with the exception of luteal phase support by progesterone administration, none of the treatments mentioned above had significant beneficial effects on implantation rate or endometrial receptivity (Stephenson and Fluker, 2000; Nosarka *et al*., 2005; Brinsden *et al*., 2009; Cakmak and Taylor, 2011). Therefore, finding new approaches to facilitate endometrial receptivity is currently a major issue in ART.

Many components that are associated with implantation have been deciphered in recent years. In response to ovarian steroids secreted during the implantation window, the expression of many hormones, cytokines and growth factors in the endometrium is significantly increased. A complex interplay of these effectors mediates morphological and physiological alterations of endometrium that allows the reception of a blastocyst and supports implantation (Carson *et al*., 2000; Dey *et al*., 2004). The identification of these effectors has provided not only information about the molecular mechanisms underlying implantation but also a means to investigate the causes of implantation failure. Some of these effectors have the potential to be utilized for developing novel means to improve uterine receptivity (Cakmak and Taylor, 2011). Among these factors, calcitonin is transiently produced in the uterine epithelia during the period of implantation both in human and mouse (Kumar *et al*., 1998; Zhu *et al*., 1998b; Zaidi *et al*., 2002). Attenuation of calcitonin expression by intrauterine administration of antisense oligodeoxynucleotides against calcitonin mRNA during the preimplantation phase significantly decreases embryo implantation rates in rats (Zhu *et al*., 1998a). In a model of endometrium–trophoblast interaction, calcitonin promoted the outgrowth of trophoblasts on human endometrial epithelial cell (EEC) monolayer by modulating the EECs (Li *et al*., 2008). These findings not only suggest that calcitonin is one of the biomarkers of uterine receptivity (Cavagna and Mantese, 2003) but also imply that exogenous calcitonin, widely used in the clinical treatment of disorders of bone and mineral metabolism, may play a role in promoting the efficiency of embryo implantation. However, so far it is unclear whether calcitonin could be used as an adjuvant for IVF and embryo transfer programs, since the mechanisms underlying the effect of calcitonin on receptivity of the uterus have not been fully elucidated.

The effects of calcitonin are mediated by an increase in intracellular Ca^{2+} concentration in many types of cells (Li *et al*., 2002; Zaidi *et al*., 2002). Ca^{2+} is essential for the activation of protein kinase C (PKC) which regulates many cellular responses (Spitaler and Cantrell, 2004). The calcitonin-Ca^{2+}-PKC signaling pathway is involved in trophoblast–EEC interactions (Li *et al*., 2008). Calcitonin also modulates the expression of certain genes in the endometrium, including down-regulating the E-cadherin expression in rodent uterine epithelium (Li, *et al*., 2002) and inducing the tTGase expression in human EECs (Li *et al*., 2006). Therefore, we postulated that calcitonin may facilitate uterine receptivity by regulating the expression of a set of genes that control the cellular functions in the process of implantation.

The purpose of the present study was to explore whether the administration of calcitonin could improve the efficiency of implantation, and investigate the underlying mechanisms. Eight genes associated with endometrial receptivity were chosen for analysis of the effect of calcitonin on endometrial receptivity: integrin αv (*Itgav/ITGAV*), integrin β3 (*Itgb3/ITGB3*), mucin 1 (*Muc1/MUC1*), epidermal growth factor (*Egf/EGF*), heparin-binding EGF (*Hbegf/HBEGF*), transforming growth factor-β1 (*Tgfb1/TGFB1*), *Lif/LIF* and homeobox A10 (*Hoxa10/HOXA10*). Our data showed that calcitonin could increase the adhesion and expansion of trophoblast spheroids on EECs by directly up-regulating the expression of integrin β3 and indirectly up-regulating

the expression of heparin binding-epidermal growth factor (HB-EGF) and LIF in EECs. The administration of calcitonin facilitated the implantation efficiency after embryo transfer in mice.

Materials and Methods

Animals and cell lines

BALB/c mice were purchased from the Center of Medical Experimental Animals of Hubei Province (Wuhan, China) for studies approved by the Animal Care and Use Committee of Tongji Medical College. The human endometrial carcinoma cell line RL95-2 (TCHu198) and human choriocarcinoma cell line JAR (TCHu156) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured according to their guidelines.

Reagents and antibodies

Calcitonin used for *in vitro* experiments was purchased from Sigma-Aldrich (St. Louis, MO, USA). Calcitonin used for *in vivo* experiments was purchased from Novartis Pharma Schweiz AG (Switzerland). Progesterone, 17β-Estradiol (E_2) and calphostin C (PKC inhibitor) were purchased from Sigma. Mouse-anti-human αvβ3 antibody (LM 609), a blocking antibody, was purchased from Chemicon (Temecula, CA, USA) and used to detect αvβ3 on RL95-2 cells and block αvβ3 on RL95-2 cells. Rabbit-anti-mouse/human β3, goat-anti-mouse HB-EGF and anti-mouse LIF antibodies, and isotype controls were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A LIF enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Quantikine, Minneapolis, MN, USA). HB-EGF ELISA kit was purchased from Abcam (Cambridge, MA, USA).

Animal experiments and treatment protocol

Three-week-old virgin female BALB/c mice were ovariectomized and housed under a 12h/12 h light/dark cycle. To induce endometrial receptivity, mice were treated as previously described (Curtis *et al.*, 1999; Curtis Hewitt *et al.*, 2002). Briefly, after a period of 2 weeks, to clear endogenous ovarian hormones, the ovariectomized mice received a daily s.c. injection of 100 ng E_2 in 100 μl sesame oil from Day 1 to Day 3. On Days 4 and 5, no hormones were administered. In the morning of Days 6, 7 and 8, the mice received a s.c. injection of 1 mg progesterone and 6.7 ng E_2 in 100 μl sesame oil. The mice were then randomly divided into two groups. For treatment with calcitonin, the mice received an i.p. injection of calcitonin in saline (0.5 IU per mouse) in the afternoon of Day 7. The dosage of calcitonin is determined according to that for adult mice (50 IU/60 kg). The control group received an equal volume of saline. In embryo-transfer experiments, a dosage of 2 IU calcitonin per mouse was also used.

Cell treatment

To detect gene expression at the mRNA level, RL95-2 cells were cultured for 24 h in the absence or presence of calcitonin (100 nM, or as indicated). In some experiments, calphostin C was added to the culture (100 nM) 1 h before calcitonin. Matrigel (BD Biosciences, catalog number: 356234) was diluted with serum-free medium and used for coating culture plates. When indicated, RL95-2 cells were cultured for 24 h in matrigel-coated plates in the absence or presence of αvβ3 blocking antibody (20 μg/ml). To detect gene expression at the protein level, RL95-2 cells were cultured in the absence or presence of calcitonin (100 nM) for 48 h with or without calphostin C (100 nM).

Analysis of gene expression by real-time RT–PCR

To assay gene expression in the endometrium, the mice were killed 36 h after the injection of calcitonin and the uterine endometrial tissues were collected. For the assay of gene expression in RL95-2 cells, the cells were cultured for the indicated time under the indicated conditions. Total RNA was extracted from endometrial tissues and RL95-2 cells with TRIzol reagent (Invitrogen) according to manufacturer's instructions. Moloney murine leukemia virus reverse transcriptase (TOYOBO) was used for cDNA synthesis, which was then amplified with SYBR qPCR Mix (TOYOBO) in duplicate. The primers were designed with the Oligo Primer Analysis 4.0 software and the sequences were analysed in BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The primer sequences are shown in Table I. For sample analysis, the threshold was set based on the exponential phase of products, and the $2^{-\Delta\Delta CT}$ method was performed to analyze the data, as described previously (Livak and Schmittgen, 2001). The expression level of each gene was normalized to β-actin mRNA and expressed as *n*-fold difference relative to the control (calibrator).

Immunohistochemical analysis

The whole uteri, without removing the endometrium, were fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin-embedded tissues were sectioned to a thickness of 5 μm, and sections were incubated with primary antibody (anti-β3 or anti-HB-EGF antibody) or isotype antibody IgG overnight at 4°C. After washing with phosphate-buffered saline (PBS), all sections were incubated with biotin-conjugated secondary antibody and then avidin–peroxidase (Sigma). The brown color was developed with diaminobenzidine (DAB Kit) (Amresco). Staining intensity of cells was evaluated under a microscope and graded (0, absent; 1, weak; 2, moderate; 3, strong) in a blinded fashion by two examiners. Staining intensity of tissue sections was assessed using a semi-quantitative immunohistochemical scoring system, HSCORE. The HSCORE was calculated using the following equation: HSCORE $= \sum P_i(i+1)$, where i is the staining intensity of cells and P_i is the percentage of epithelial cells at each level of intensity (Ceydeli *et al.*, 2006; Ruan *et al.*, 2006).

Western blot analysis

Proteins were prepared from frozen uterine tissue by homogenization and from RL95-2 cells by lysis. Proteins and pre-stained molecular weight markers were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transfer onto nitrocellulose membranes. The membranes were blocked in TBST (Tris-buffered saline with 0.5% Triton X-100) containing 5% non-fat milk, and then incubated with anti-β3, anti-LIF or anti-β-actin antibody overnight at 4°C followed by 1 h incubation with the appropriate peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence according to the manufacturer's protocol (ECL kit, Thermo). To evaluate the expression of LIF in tissues, the bands were scanned using a GS-800 scanning densitometer (BioRad). The intensity of each protein band was quantified with BioRad Quantity One software analysis system. The relative level of LIF was expressed as the intensity ratio of LIF to β-actin, which was calculated by the formula: LIF/β-actin = intensity of LIF band/intensity of β-actin.

Flow cytometric analysis

RL95-2 cells were washed with 1× Hank's Balanced Salt Solution and incubated with fluorescein isothiocyanate-conjugated mouse-anti-human αvβ3 for 30 min at 4°C. A negative control (for background fluorescence) was included in which primary antibody was replaced by isotype control

Table I Primer sequences (all 5′–3′) used in real-time RT–PCR.

Genes	GeneBank accession no	Forward primer	Reverse primer
Mouse			
Itgav	NM_008402	GGCACAAAGACCGTTGAGTA	GCCACTTGGTCCGAAATGAG
Itgb3	NM_016780	TGCTCCAGAGTCTATTGAGTTCC	GAGAAAGACAGGTCCATCAAGTAG
Muc1	NM_013605	GTGCCGCCGAAAGAGCTA	GCCATTACCTGCCGAAAC
Egf	NM_010113	CCAAACGCCGAAGACTTATCC	TGATCCTCAAACACGGCTAGAGA
Hbegf	NM_010415	CTTGGCTGTGGTGGCTGTA	TAGCCACGCCCAACTTCA
Tgfb1	NM_011577	TGACGTCACTGGAGTTGTACGG	GGTTCATGTCATGGATGGTGC
Lif	NM_008501	CCCATCACCCCTGTAAAT	GTTAGGCGCACATAGCTT
Hoxa10	NM_008263	CCTGCCGCGAACTCCTTTT	GGCGCTTCATTACGCTTGC
Actb	NM_007393	TGACAGACTACCTCATGAAGATCC	TCGAAGTCTAGAGCAACATAGCAC
Human			
ITGAV	NM_002210	CTCGGGACTCCTGCTACCTC	AAGAAACATCCGGGAAGACG
ITGB3	NM_000212	CATCCTGGTGGTCCTGCTCT	GCCTCTTTATACAGTGGGTTGTT
MUC1	NM_002456	AAGCAGCCTCTCGATATAACCT	GGTACTCGCTCATAGGATGGT
EGF	NM_001963	TGGATGTGCTTGATAAGCGG	ACCATGTCCTTTCCAGTGTGT
HBEGF	NM_001945	CAAGGAGGAGCACGGGAAAAG	CCCATGACACCTCTCTCCA
TGFB1	NM_000660	CTAATGGTGGAAACCCACAACG	TATCGCCAGGAATTGTTGCTG
LIF	NM_002309	CCCATCACCCCTGTCAACG	GGGCCACATAGCTTGTCCA
HOXA10	NM_018951	CTGAGGTCAATGGTGCAAAGGA	TTTGCCAACCTGCATGTCCA
ACTB	NM_001101	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

IgG. After washing, the cells were used for flow cytometric analysis. Parameters were acquired on a Calibur fluorescence-activated cell sorter (BD Biosciences) and analyzed with Cell Quest software (BD Biosciences). Percentage staining was defined as the percentage of cells in the gate (M1) which was set to exclude ~99% of isotype control cells. The αvβ3 expression index was calculated using the formula: mean fluorescence × percentage of $\alpha v\beta 3^+$ cells (Vellon *et al.*, 2005).

ELISA assay

To detect the release of LIF and HB-EGF, RL95-2 cells were washed twice with PBS and cultured for an additional 2 days in the culture medium without serum (basal medium). The cells were then washed with PBS, and cultured for 48 h in basal medium in the absence or presence of calcitonin (100 nM) and calphostin C (100 nM). The conditioned media were collected, lyophilized and stored at −80°C for ELISA assay. LIF and HB-EGF were detected with an ELISA kit. The lowest detectable concentration of each cytokine by ELISA was 8 pg/ml (LIF) and 20 pg/ml (HB-EGF) as reported by the manufacturer. The results were expressed as pg/ml/mg protein.

Spheroid adhesion assay

The attachment of JAR spheroids to the RL95-2 cell monolayer was quantified by adhesion assay as previously described (Aboussahoud *et al.*, 2010; Liu *et al.*, 2011a) with modifications. Briefly, RL95-2 cells were seeded into 24-well plates, 5×10^5 cells per well, and cultured for 24 h to form a monolayer in the absence or presence of calcitonin (100 nM) and calphostin C (100 nM). Trophoblast spheroids were generated by shaking the trypsinized JAR cells on a gyratory shaker at 110 rpm for 24 h. JAR spheroids ($n = 30$) were delivered to each well with a confluent monolayer of RL95-2 cells in quadruplicate, and incubated various times at 37°C in a humidified atmosphere with 5% CO_2. When indicated, αvβ3 blocking antibody was added to the plates (20 μg/ml) 1 h before the delivery of JAR spheroids. Non-adherent spheroids were removed after centrifugation (with the cell spheroid surface facing down) at 12 *g* for 5 min. Adhered spheroids were counted under a microscope and adhesion rates were calculated by the formula: number of adhered spheroids/number of total spheroids delivered.

Assay of trophoblast outgrowth on EEC monolayer

RL95-2 cells were seeded into 96-well plates, 3×10^5 cells per well, and cultured for 24 h to form monolayer. The plates were un-coated or coated with matrigel as indicated. Then trophoblast spheroids were transferred to the monolayer (3–5 spheroids/well). Spheroids were photographed after 1 and 24 h co-culture. The length (L) and width (W) of spheroids were measured. The size of spheroids was calculated by the formula: $(L + W)/2$. The expansion of spheroids was determined on the basis of the spheroid size 1 h after co-culture (Li *et al.*, 2008). The fold expansion of spheroids was calculated by the formula: fold expansion = size at 24 h/size at 1 h. To delineate the margins of trophoblast spheroids 24 h after co-culture, the cells were stained with 10 μM of CellTracker™ Green CMFDA (Invitrogen) for 30 min before photographing under a fluorescent microscope. The fluorescence of JAR cells appeared dimmer than that of RL95-2 cells as a result of more extensive spreading after culture, and this clear contrast in staining enabled an accurate demarcation of the boundary of trophoblast outgrowth on the EEC monolayer. Scion Image software system (Scion Corporation, Frederick, MD, USA) was employed to measure the size of spreading trophoblast spheroids.

Embryo transfer and *in vivo* implantation test

Six- to eight-week-old virgin female BALB/c mice were used as embryo donors. The mice underwent ovulation induction by i.p. injection of 10 IU pregnant mare's serum gonadotrophin (Sigma, USA), followed by i.p. injection of 10 IU hCG (Pregnyl, Organon, The Netherlands) 48 h later. The mice were then mated with fertile males and successful matings were identified by inspecting vaginal plugs. The day of the vaginal plug presented was designated as 0.5 dpc (days post coitum). Embryos were collected on 3.5 dpc. The ovariectomized mice with receptive uteri induced by treatment with E_2 and progesterone were used as recipients. The mice were randomly divided into three groups (18 mice in each group) and received i.p. injection of 0.5 IU calcitonin, 2 IU calcitonin or saline (control) on Day 7. Six hours after hormone injection on Day 8, embryos were transferred to recipients (8 embryos per mouse) as previously described (Nagy *et al.*, 2003). Briefly, 3.5-dpc embryos were transferred into the right uterine lumen of recipients using IVF pasteur pipets (Humagen, Origio Inc., Denmark) through a hole which was made a few millimeters down from the utero-tubal junction. Then, daily injection of progesterone (1 mg per injection) was continued. The uteri were collected 7 days after embryo transfer, and inspected by eye for implantation sites. Nodules (apparent implantation sites) were dissected and analyzed for the presence of embryos under a dissecting scope. The embryos were identified according to the illustrations in a laboratory manual (Nagy *et al.*, 2003).

Statistical analysis

Results were expressed as the mean ± SD. Statistical analysis was performed on raw data by Student's *t*-test and one-way analysis of variance after testing for normal distribution (Skewness and Kurtosis test) and homoscedasticity (Levene test) of variance using Statistical Package for the Social Sciences 16.0 (SPSS, Chicago, IL, USA). In cases where data did not show a normal distribution or homoscedasticity of variance, values were log transformed prior to further statistical analysis. Mann–Whitney rank sum testing was used to test the differences in the number of implantation sites. A P value < 0.05 was considered statistically significant.

Results

Administration of calcitonin up-regulates the expression of integrin β3, HB-EGF and LIF in murine endometrium

As shown in Fig. 1A, the administration of calcitonin increased mRNA levels of *Itgb3*, *Hbegf* and *Lif* in endometrial tissues but did not significantly influence the expression of *Itgav*, *Muc1*, *Egf*, *Tgfb1* and *Hoxa10*. The increase in LIF in the endometrium was further confirmed by western blot: LIF in the endometrium of the calcitonin group was significantly higher than that of the control group (Fig. 1B). The increase in integrin β3 and HB-EGF in the endometrium was further confirmed by immunohistochemistry. Integrin β3 (Fig. 1C) and HB-EGF (Fig. 1D) were predominantly localized in uterine luminal epithelium and glandular epithelium. The staining intensity of integrin β3 in the calcitonin group was significantly higher than that in the control group (Fig. 1C). The calcitonin group also showed higher staining intensity of HB-EGF than the control group (Fig. 1D). Taken together, these results suggested that several types of molecules related to endometrial receptivity were increased in the endometrium by the administration of calcitonin.

Calcitonin up-regulates the expression of integrin β3 in EECs

We next investigated whether calcitonin could directly regulate gene expression in EECs. Calcitonin increased the mRNA levels of *ITGB3* in RL95-2 cells in a dose-dependent manner. The mRNA levels of *ITGB3* significantly increased when the concentration of calcitonin reached 10 nM but other genes were not influenced by calcitonin at even higher concentration (Fig. 2A). Since calcitonin had a maximum effect on gene expression at 100 nM, this concentration was utilized in following *in vitro* experiments. Moreover, calphostin C, an inhibitor of PKC, completely abrogated the effect of calcitonin on the expression of integrin β3 at both the mRNA and protein levels (Fig. 2B), suggesting that calcitonin increased the expression of integrin β3 through the PKC pathway.

We then further analyzed the membrane expression of integrin αvβ3 in RL95-2 cells after treatment with calcitonin. Flow cytometric analysis showed that the expression of integrin αvβ3 on RL95-2 cells was increased by calcitonin (Fig. 2C and D). The effect of calcitonin on αvβ3 was also abrogated by calphostin C. Taken together, these results suggested that direct stimulation of EECs by calcitonin mainly up-regulated integrin β3 in the cells, resulting in an increase in integrin αvβ3 on the surface of the cells.

Calcitonin augments the up-regulation of HB-EGF and LIF in EECs by ECM molecules

The above results showed that calcitonin only increased the expression of integrin β3 in EECs when it directly stimulated EECs *in vitro*, whereas the administration of calcitonin increased not only integrin β3 but also HB-EGF and LIF in murine endometrium *in vivo*. Since the increased expression of integrin β3 resulted in the increase of αvβ3 integrin on EECs, we wondered whether αvβ3 might be involved in the up-regulation of HB-EGF and LIF. Integrin αvβ3 is a receptor for many molecules in extracellular matrix (ECM), and therefore we investigated whether the expression of HB-EGF and LIF could be up-regulated in EECs in response to ECM molecules. To test this, we cultured the cells in the plates pre-coated with matrigel. In the presence of matrigel, *HBEGF* and *LIF* mRNA levels were increased (Fig. 3A). The effect of matrigel was abrogated by αvβ3 functional blocking antibody, suggesting that ECM molecules could up-regulate the expression of HB-EGF and LIF by stimulating integrin αvβ3.

We then further investigated whether calcitonin could indirectly stimulate HB-EGF and LIF in EEC by increasing integrin αvβ3. The results showed that pretreatment of the cells with calcitonin significantly increased the expression of HB-EGF and LIF in EECs in response to ECM molecules (Fig. 3B and C). Calphostin C significantly suppressed the effect of calcitonin. Taken together, these results suggested that ECM molecules could up-regulate the expression of HB-EGF and LIF, and that calcitonin augmented the effect of ECM molecules by up-regulating the expression of αvβ3.

Calcitonin modulates EECs to promote the attachment of trophoblast spheroids

We next asked whether the increased expression of αvβ3 by calcitonin might augment the adhesion of trophoblast spheroids

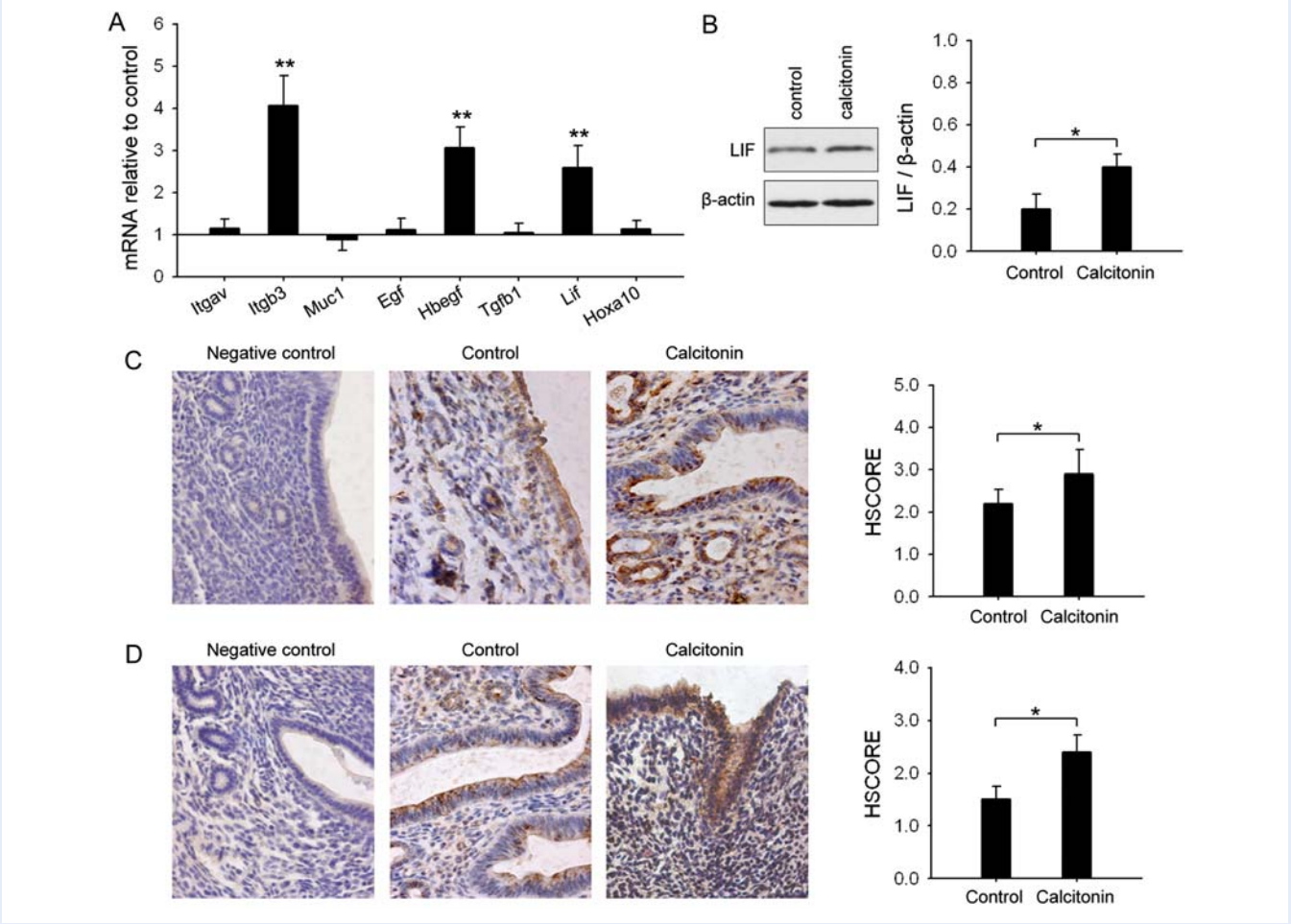

Figure 1 Effect of exogenous calcitonin on gene expression in the endometrial tissue of mice during implantation window. (**A**) Real-time RT–PCR analysis of eight receptivity-related genes in endometrial tissues. The mRNA level of each gene in the calcitonin group was shown as the fold of that in the control group. (**B**) Western blot analysis of LIF protein in the endometrium without and with calcitonin injection (left). The ratio of LIF to β-actin was calculated after densitometric analysis of western blots (right). (**C** and **D**) Immunohistochemical staining of endometrial integrin β3 (C) and HB-EGF (D) in the mice without (control) and with calcitonin injection. Sections incubated with isotype antibody immunoglobulin (Ig) G served as a negative control. Magnification: ×400 (left). The immunohistochemical staining intensity was measured using the HSCORE scoring system (right) as described in the section Materials and methods. Data are pooled from three independent experiments with a total of six samples in each group, *P* values, $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the control.

to EECs. A RL95-2 cell monolayer and JAR trophoblast spheroids were used as an *in vitro* model of blastocyst attachment. After 30-min co-incubation, 48.2 ± 4.5% of spheroids were attached to the endometrial cell monolayer. More than 80% of the spheroids were attached after 60-min or longer co-incubation (Fig. 4A). We then chose a 30-min co-incubation to investigate whether calcitonin-treated EECs may allow more efficient attachment of JAR spheroids, and showed that the pretreatment of RL95-2 cells with calcitonin significantly increased the attachment of JAR spheroids to EEC monolayer and calphostin C inhibited the effect of calcitonin (Fig. 4B). Blocking αvβ3 with antibody suppressed the attachment of JAR spheroids to EEC monolayer and also abrogated the effect of calcitonin (Fig. 4B). These data indicated that pretreatment of EECs with calcitonin allows more efficient attachment of trophoblast spheroid.

Calcitonin modulates EECs to promote the expansion of trophoblast spheroid

Using the model of endometrium–trophoblast interaction, we next investigated whether the expansion of trophoblast spheroids on an EEC monolayer could be augmented by the treatment of EECs with calcitonin and/or ECM molecules. Spheroids were attached to RL95-2 cell monolayer after 1-h co-culture and began to flatten and spread on the EEC monolayer. CellTracker staining of spheroid-EEC co-culture showed that the brightly stained RL95-2 EECs were displaced by the dimly stained JAR trophoblast cells after 24-h co-culture (Fig. 5A).

In the absence of matrigel, the expansion of trophoblast was increased when EECs were cultured with calcitonin before and/or after the transfer of trophoblast spheroid to EEC monolayer

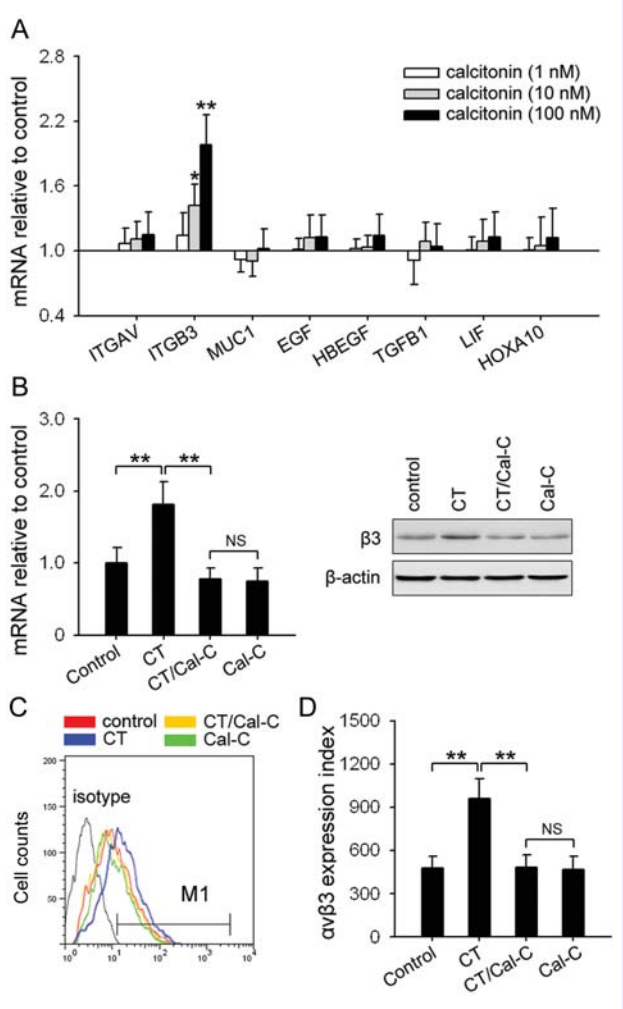

Figure 2 Calcitonin up-regulates the expression of integrin β3 in human endometrial epithelial cells. (**A**) The effect of calcitonin (CT) at different concentrations on the expression of eight endometrial receptivity-related genes in RL95-2 cells. The mRNA level of each gene in the calcitonin group was shown as the fold of that in the control group. (**B**) The effect of calcitonin and/or calphostin C (Cal-C, protein kinase inhibitor) on integrin β3 expression. The expression of integrin β3 was detected by real-time RT–PCR and western blot. The expression of integrin β3 in the control group was designated as 1. (**C** and **D**) Flow cytometric analysis of the membrane expression of integrin αvβ3. RL95-2 cells were treated as described in (B). αvβ3 on the cells was analyzed by flow cytometry (C). Cells incubated with isotype antibody IgG served as a negative control. (D). Data are pooled from three independent experiments with a total of six samples in each group. *P* values, $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the control.

(Fig. 5A and B). The effect of calcitonin was abrogated by calphostin C (Fig. 5C), suggesting that calcitonin could modulate EECs to promote trophoblast outgrowth on the EEC monolayer. In line with the up-regulation of HB-EGF and LIF in EECs by ECM molecules, the culture of EECs in the presence of matrigel significantly increased the expansion of trophoblast on EEC monolayer (Fig. 5A and B). In the presence of matrigel, the treatment of EECs with calcitonin further promoted the expansion of trophoblast on the monolayer (Fig. 5A and B), suggesting that the up-regulation of αvβ3 integrin by calcitonin could augment the effect of ECM molecules. Calphostin C abrogated the effect of calcitonin but did not influence the effect of matrigel, suggesting that the PKC pathway mediated the effect of calcitonin but was not involved in the effect of ECM molecules. Moreover, the presence of calcitonin, either before or after the transfer of trophoblast spheroids, could increase the expansion of trophoblast, whereas the continuous presence of calcitonin was more effective. Importantly, the continuous presence of both matrigel and calcitonin was most effective for the augmentation of trophoblast expansion. The spheroid size was increased more than 100% compared with the control group in the absence of matrigel and calcitonin. Spheroid size did not significantly expand on an EEC monolayer in the presence of αvβ3 antibody, whether the EECs were treated with calcitonin or not (data not shown).

Administration of calcitonin promotes implantation of blastocyst *in vivo*

The above results showed that calcitonin in combination with ECM molecules enabled EECs more receptive for trophoblasts to adhere and expand. We next wondered whether the administration of calcitonin could effectively promote implantation of blastocyst *in vivo*. Implantation sites were inspected 7 days after embryo transfer (Fig. 6A). The total number of implantation sites in both treatment groups was higher than in the control group (78 in 0.5 IU group and 85 in 2 IU group versus 55 in the control group) (Fig. 6B). Compared with the control group (3.06 ± 2.13), the average number of implantation sites in the 2 IU treatment group was increased (4.72 ± 1.87, $P = 0.022$) (Fig. 6C). These results suggested that calcitonin could promote implantation after embryo transfer, and that the higher dosage of exogenous calcitonin was more effective.

Discussion

Our findings in this study suggest that the administration of calcitonin could improve endometrial receptivity and promote implantation efficiency. In this study we induced the receptivity of the uterus in ovariectomized mice with progesterone and E_2, corresponding to the estrous cycle of mice (Wang and Dey, 2006). Using this model, we demonstrated that a single injection of calcitonin immediately before the putative implantation window could effectively promote the implantation in the mice.

Embryo implantation is a complex process including apposition and attachment of the blastocyst to the receptive endometrium and invasion of the trophoblast cells of the conceptus into the endometrium (Aplin, 2006; Singh *et al.*, 2010). A study using a mouse model found that calcitonin might have some effects on development of preimplantation embryos (Wang *et al.*, 1998) but some studies suggest that EECs are the main target of calcitonin in the process of implantation (Cavagna and Mantese, 2003; Li *et al.*, 2008). Our data from the *in vitro* model system provided direct evidence that calcitonin could indeed render EECs more receptive to allow trophoblast spheroid adhesion and invasion. Moreover, the pretreatment of RL95-2 cells with calcitonin promoted spheroid outgrowth in the absence of calcitonin, further confirming that EECs appear to be the main target of calcitonin.

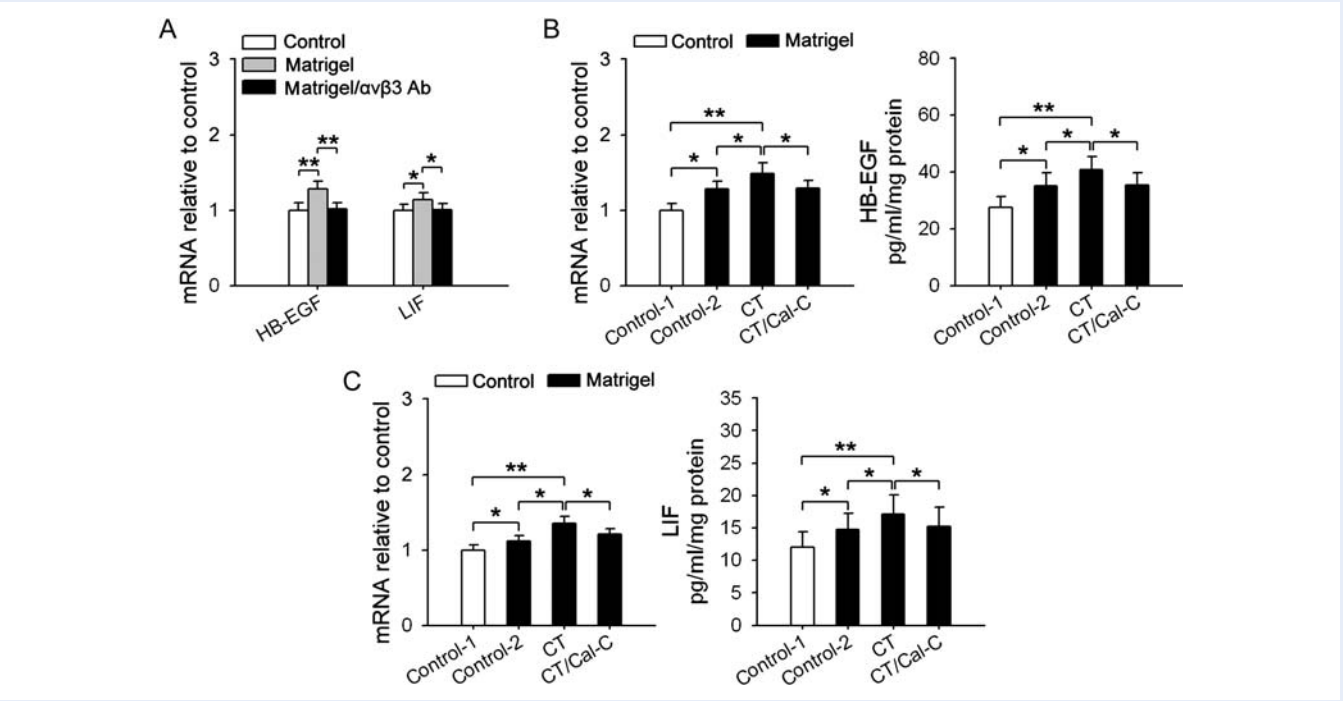

Figure 3 Calcitonin augments the up-regulation of *HB-EGF* and *LIF* genes in human EECs by ECM molecules. (**A**) Effect of ECM molecules on the expression of *HB-EGF* and *LIF* genes. RL95-2 cells were cultured in the absence or presence of matrigel and αvβ3 blocking antibody. The expression of *HBEGF* and *LIF* genes was detected by real-time RT–PCR. (**B** and **C**) Effect of calcitonin on HB-EGF and LIF in the presence of ECM molecules. RL95-2 cells were cultured in the absence or presence of matrigel, calcitonin (CT) and calphostin C (Cal-C). Unstimulated cells were also used as the control (control-1). HB-EGF (B) and LIF (C) mRNA levels were detected by real-time RT–PCR (left) and enzyme-linked immunosorbent assay (right) was used for secretion of protein. Data are pooled from three independent experiments with a total of six samples in each group. *P* values, $^{*}P < 0.05$; $^{**}P < 0.01$ versus control.

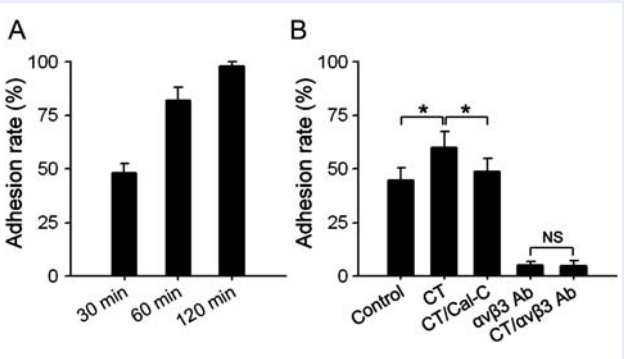

Figure 4 Effect of calcitonin on the attachment of trophoblast spheroids to EEC monolayer. (**A**) Attachment of JAR spheroids (human) to the RL95-2 monolayer. The adhesion rates of the spheroids were calculated. (**B**) Attachment of JAR spheroids to pre-treated RL95-2 monolayer. JAR spheroids were delivered onto RL95-2 EEC monolayers, which were treated with calcitonin in the absence or presence of calphostin C, and incubated in the absence or presence of anti-αvβ3 antibody. The adhesion rates of the spheroids were calculated. Data are pooled from three independent experiments with a total of six samples in each group. *P* values, $^{*}P < 0.05$ versus control.

In the present study, we analyzed the effect of calcitonin on the expression of eight receptivity-related genes in RL95-2 cells and murine endometrium. Among these genes, the expression of integrin β3 in RL95-2 cells was increased by calcitonin, which is consistent with a previous report that calcitonin selectively increased integrin β3 expression in a prostate cell line (Thomas *et al*., 2007). Integrin β3 in the endometrium has been shown to play important roles in endometrial receptivity (Boroujerdnia and Nikbakht, 2008; Li *et al*., 2010). In our experiments, exogenous calcitonin significantly increased β3 in both murine endometrium and RL95-2 cells, which might be an important mechanism underlying the improvement of endometrial receptivity. Moreover, our data showed that calcitonin increased the expression of αvβ3 in RL95-2 cells by increasing integrin β3, which was also supported by the finding that the expression of αvβ3 is limited by the level of integrin β3 subunit (Lessey *et al*., 2002). Integrin αvβ3 is one of the best characterized biomarkers of uterine receptivity (Lessey and Castelbaum, 2002; Kaneko *et al*., 2011). A significant decrease in endometrial αvβ3 has been found in women with luteal deficiency, endometriosis, hydrosalpinges and unexplained infertility (Lessey and Castelbaum, 2002; Cavagna and Mantese, 2003; Donaghay and Lessey, 2007) and the decrease in αvβ3 is associated with a lower pregnancy rate (Lessey, 2002; Tei *et al*., 2003). Therefore, the increase in αvβ3 by calcitonin should favor implantation in

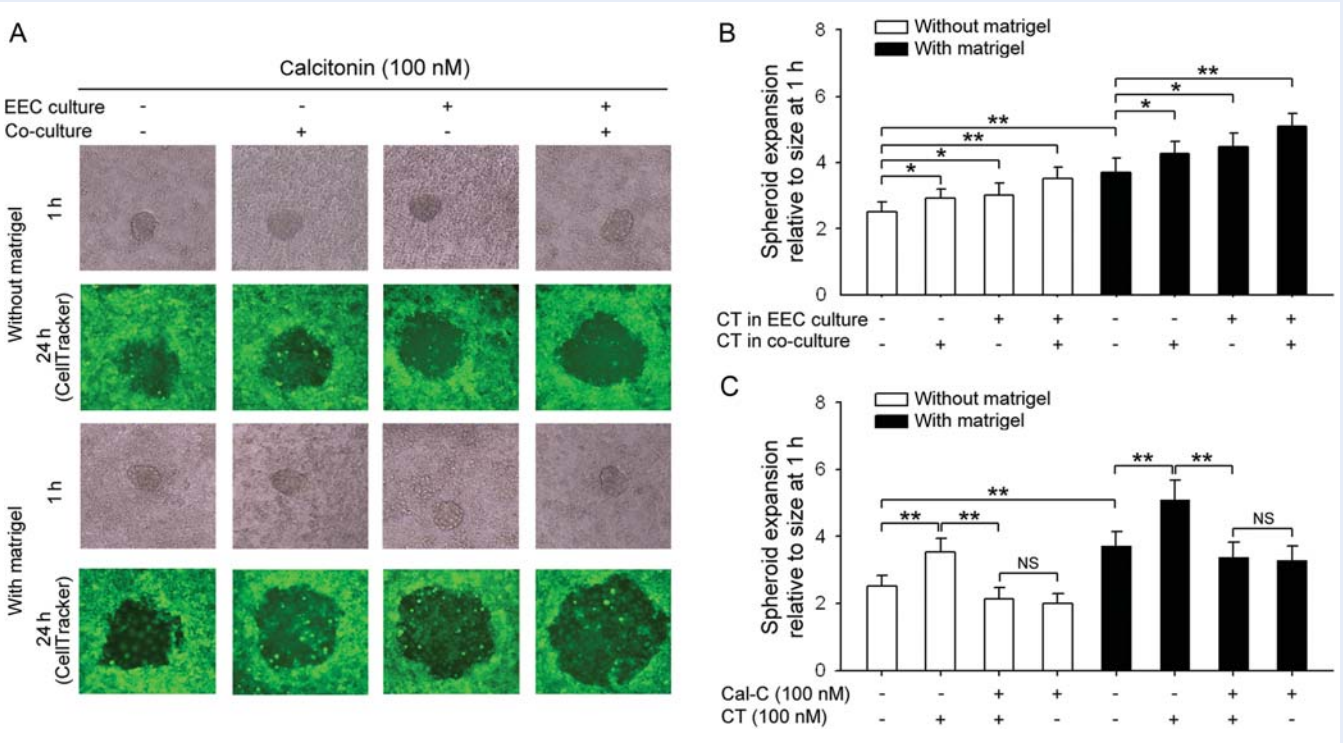

Figure 5 Effect of calcitonin on trophoblast spheroid outgrowth on EEC monolayer. (**A** and **B**) Outgrowth of trophoblast spheroids (human) on EEC monolayer. RL95-2 cells were cultured in un-coated or matrigel-coated plates to form a monolayer in the absence or presence of calcitonin. Then, JAR spheroids were added onto the EEC monolayer, and co-cultured in the absence or presence of calcitonin. The spheroids were photographed at 1 and 24 h after co-culture. Representative photographs are shown (A). Data are presented as spheroid expansion relative to their original size at 1 h (B). (**C**) Calphostin C abrogates the effect of calcitonin. RL95-2 cells were cultured in un-coated or matrigel-coated plates to form monolayer in the absence or presence of calcitonin and/or calphostin C. Then, JAR spheroids were added onto EEC monolayer, and co-cultured under the same conditions. Spheroid expansion relative to their original size at 1 h was calculated. Data are pooled from three independent experiments with a total of six samples in each group (B and C). *P* values, $*P < 0.05$, $**P < 0.01$ versus control.

subfertile patients. Given that αvβ3 has been studied as a predictor of IVF success (Thomas *et al*., 2003; Casals *et al*., 2010), the administration of calcitonin might be a usable approach to promote endometrial receptivity in IVF and embryo transfer programs.

A biological function like endometrial receptivity can rarely be attributed to a single type of molecule but rather it arises from a complex interaction among multiple types of molecules (Rashid *et al*., 2011). Our data showed that calcitonin not only increased the expression of integrin β3 by direct stimulation but also indirectly stimulated HB-EGF and LIF in EECs by up-regulating αvβ3 expression. All of these molecules have previously been identified as contributing to the development of endometrial receptivity and facilitating the process of embryo implantation (Das *et al*., 1994; Lim and Dey, 2009; Srinivasan *et al*., 2009; Aghajanova, 2010; Dimitriadis *et al*., 2010). Integrin αvβ3 serves as a receptor for several ECM molecules, including fibronectin, vitronectin and laminin (Smith and Cheresh, 1990; Dey *et al*., 2004). The interaction of αvβ3 and its ligand could induce intracellular signal transduction (Akiyama, 1996) and regulate gene expression in cells. In our experiment, matrigel, a gelatinous protein mixture containing several ECM molecules (Hughes *et al*., 2010), was used to stimulate RL95-2 cells. Our data showed that ECM molecules were required for the regulatory effect of calcitonin on the expression of HB-EGF and LIF in RL95-2 cells, which is consistent with the regulatory effect of calcitonin on these genes in murine endometrium. Interestingly, previous studies demonstrated that HB-EGF could stimulate epithelial expression of LIF and integrin β3 (Lessey *et al*., 2002), and that normal LIF signaling was required for the induction of HB-EGF during early implantation (Song *et al*., 2000). Based on our data and others, we propose that calcitonin may actually induce a positive-feedback loop to increase the production of these molecules. The putative positive-feedback loop might be the reason for the improvement in endometrial receptivity by a single injection of calcitonin in the preimplantation period.

Calcitonin regulates the functions of EECs through calcium mobilization and PKC activation (Li *et al*., 2002, 2006, 2008). The activation of PKC has been found to facilitate embryo implantation in a rat delayed implantation model (Johnson, 1996). Our study showed that activation of PKC is critical for the modulatory effect of calcitonin on receptivity of endometrial epithelium. In the presence of a PKC inhibitor, calcitonin failed to increase the expression of integrin β3 subunit as well as αvβ3 integrin. Inhibiting PKC did not influence the regulatory effect of ECM molecules on the expression of HB-EGF and LIF in RL95-2 cells but abrogated the promoting effect of calcitonin on the expression of these genes based on the stimulation of ECM molecules. These data further elucidated the mechanism underlying the crucial role of PKC in the process of implantation.

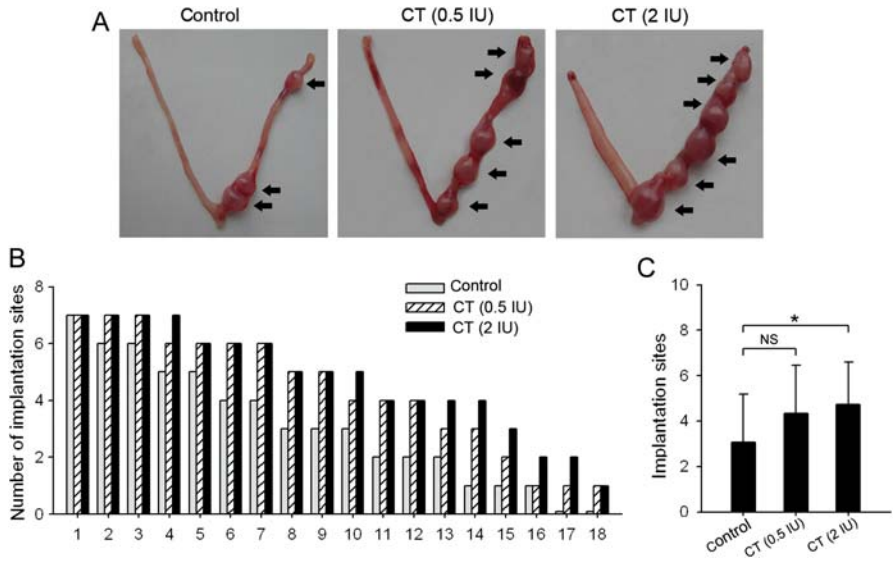

Figure 6 Effect of the administration of calcitonin on implantation of mouse blastocyst. The uterine receptivity of the ovariectomized virginal mice was induced as described in the section Materials and methods. The mice (18 mice in each group) received i.p. injection of 0.5 IU calcitonin, 2 IU calcitonin or saline (control) before donor embryos were transferred. The uteri were collected on Day 15 for inspection. (**A**) Representative uteri are shown. Implantation sites are indicated by arrows. (**B**) The number of implantation sites in each of the 18 mice in the three groups is shown. (**C**) An average number of implantation sites was calculated. *P* values, $^*P < 0.05$ versus control.

In recent years, ART protocols continue to evolve with the aim of achieving a higher pregnancy rate and minimizing the risk for multiple pregnancy (Cakmak and Taylor, 2011). The transfer of fewer embryos in an IVF/ICSI cycle is thought to be better for this purpose (Mastenbroek *et al*., 2011) and embryos of high quality together with a more receptive endometrium are required in this situation. Our data revealed that the administration of calcitonin, a drug used clinically for the treatment of hypercalcemia or osteoporosis, could effectively increase uterine receptivity by modulating the expression of several receptivity-related genes, and facilitate the efficiency of implantation after embryo transfer. Our findings have important implications in that, after further research, calcitonin might prove to be effective in facilitating endometrial receptivity and embryo implantation in ART programs.

The ovariectomized mouse model used here should have resulted in animals with highly similar serum and intrauterine hormone levels. However, the number of implantation sites may also have been influenced by the quality of the embryos transferred, the technical success of each transfer and the level of steroid hormones, which were not checked in our study, and these factors may be a limitation of our study.

Acknowledgements

We thank Prof. Zuo-Hua Feng for critical review of the manuscript, Prof. Sheng-Hong Liu for reviewing histology data and Prof. Zeng-Zeng Wang for supervising statistical methods.

Authors' roles

T.X. designed and performed the experiments, analyzed and interpreted the data and wrote the manuscript. Y.Z. performed the experiments with cell lines. D.H. and J.M. carried out the real-time RT–PCR analysis of gene expression in murine endometrial tissues and human EECs. R.W. and X.Y. carried out immunohistochemical analysis and flow cytometry analysis. J.A. performed the experiments with animals and participated in critical discussions. K.Q. performed the statistical analysis and contributed to the interpretation of data. H.Z. designed the study, did the data analysis and interpretation and wrote the manuscript. All authors have approved the final version of the manuscript.

Funding

This work was supported by National Science Foundation of China (No. 81170619).

Conflict of interest

None declared.

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