Extracellular Ca^{2+} -sensing receptor expression and hormonal regulation in rat uterus during the peri-implantation period

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Abstract

The extracellular Ca^{2+} -sensing receptor (CaR) is a member of the superfamily of G protein-coupled receptors (GPCRs). It is an important mediator of a wide range of Ca^{2+} -dependent physiological responses in various tissues. In reproductive tissues it has been reported to play a significant role in promoting or maintaining placentation. Meanwhile, another Ca^{2+} regulated gene stanniocalcin-1 (STC-1) has been documented to be involved in decidualization and uterine remodelling. The phenomenon that CaR mediates STC-1's transcription responding to extracellular calcium in fish urges us to suppose that CaR, like STC-1, may also play a role in implantation and decidualization. To resolve this conjecture, we have examined the expression and hormonal regulation of the CaR gene in rat uterus during peri-implantation period.

CaR mRNA was expressed at a moderate level in the luminal epithelium of the early stage of pregnancy (from day 1 to day 3). From day 2–3 it began to be expressed more strongly in the stromal cells immediately underneath the luminal epithelium, but decreased to a basal level on day 4. From day 6 to day 9 continuously, both CaR mRNA and protein were highly expressed in the primary decidua. Expression of CaR mRNA and protein in these cells was also observed when a delayed implantation was terminated by estrogen treatment to allow the embryo implantation. In contrast, only basal level expression of the molecules was detected in the cells of animals subjected to a normal-delayed implantation or the pseudopregnant condition.

Embryo transplantation experiment confirmed that CaR expression at the implantation site was induced by the implanting blastocyst. Consistent with the normal pregnant process, CaR mRNA and protein in the cells were also induced by an artificial decidualization procedure. Further experiments demonstrated that treatment of the ovariectomized rat with estrogen or/and progesterone stimulated a high level expression of CaR mRNA in the uterine epithelial and glandular epithelium. In conclusion, CaR was specifically induced during the processes of implantation and subsequent decidualization and may play a role in these processes.

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Introduction

The extracellular Ca^{2+} -sensing receptor (CaR) is a member of the superfamily of G protein-coupled receptors (GPCRs) (Brown & MacLeod 2001). It was originally identified in parathyroid chief cells (Brown *et al.* 1993). Analysis of mutations in the human and mouse CaR gene has established that CaR functions in the control of Ca^{2+} homeostasis (Ho *et al.* 1995, Hendy *et al.* 2000). Indeed, a major physiological role of the CaR is to correct small changes in extracellular Ca^{2+} concentration by regulating parathyroid hormone secretion (Brown & MacLeod 2001). Except in kidney (Riccardi *et al.* 1995) and intestine (Chattopadhyay *et al.* 1998) where it regulates calcium absorption, CaR is also expressed in other tissues involved in systemic calcium homeostasis, including neurons and

their nerve terminals (Ruat *et al.* 1996), pancreas (Bruce *et al.* 1999), breast (Cheng *et al.* 1998a), ovary (McNeil *et al.* 1998a), endocrine cells (Ray *et al.* 1997) and connective tissue cells (McNeil *et al.* 1998b). Given its widespread cellular distribution, this receptor has been regarded as an important mediator of a wide range of Ca^{2+} -dependent physiological responses, possibly including the regulation of secretion, gene expression, cell proliferation, differentiation and apoptosis (Brown & MacLeod 2001).

Implantation involves complex and progressively intimate interactions between the blastocyst and the uterine epithelium. Calcium regulation at the endometrial–embryo interface is thought to be a potential important physiological response during this process (Li *et al.* 2002, Wang *et al.* 2003, Luu *et al.* 2004). However, the molecules and

pathways involved in the calcium homeostasis regulation are far away from being well elaborated. In rodent uterus during early pregnancy, it has been reported that stannocalcin-1 (STC-1), which is one of the calcium regulation polypeptides, is expressed uniquely in mouse mesometrial stromal cells and sequestered by decidualizing anti-mesometrial cells, suggesting that STC-1 may play a role in decidualization and uterine tissue remodelling (Stasko *et al.* 2001). Our unpublished data (L-J Xiao, X-X Song, Y-C Li, J-X Yuan, Z-Y Hu & Y-X Liu) in rat further confirmed that STC-1 was regulated during the onset of implantation and may be involved in the processes of implantation and decidualization.

In spite of this, we know very little about the receptor for STC-1. The discovery that CaR mediates STC-1 secretion responding to extracellular calcium fluctuation in fish (Radman *et al.* 2002) stimulated us to propose that CaR may mediate STC-1 calcium regulation during implantation.

In the first trimester and term placenta, CaR was detected in the extravillous cytotrophoblasts. It is well known that cytotrophoblasts play an important role in the process of embryo invasion and maintenance of placental immune privilege, therefore, CaR represents a possible target by which the maternal extracellular Ca^{2+} concentration could promote or maintain placentation (Bradbury *et al.* 1998, Bradbury *et al.* 2002). Based on all of the above background information, we wonder if the CaR is expressed and plays a role in the uterus during mammalian implantation and decidualization. To confirm this hypothesis, we have investigated the expression of both CaR mRNA and protein in rat uterus during estrous cycle and early pregnancy by Northern blotting, *in situ* hybridization and immunohistochemistry. Furthermore, we have designed a various experimental animal models, such as pseudopregnancy, delayed implantation, artificial decidualization, embryo transplantation and ovariectomized animals with the controlled-steroid treatment to confirm the hypothesis that CaR regulates the processes of implantation and decidualization.

Materials and Methods

Animals and treatments

Mature rats (Sprague-Dawley strain) were caged in a controlled environment with a 14 h light: 10 h dark cycle. The estrous cycle was determined by inspection of vaginal smears. Adult females were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy respectively (day 1 = day of vaginal sperm positive). Pregnancy on days 1–5 was confirmed by flushing embryos from the reproductive tracts. The implantation sites on days 6–7 were identified by intravenous injection of 1% Chicago blue in 0.85% sodium chloride (Sigma).

Hormonal treatments were initiated 2 weeks after the mature female rats were ovariectomized. The ovariectomized rats were treated with an injection of

estradiol-17 β (250 ng/rat, Sigma), progesterone (4 mg/rat, Sigma), or a combination of the same doses of progesterone and estradiol-17 β , controls received sesame oil only. The rats were sacrificed to collect uteri 6 h and 24 h after the hormonal treatment. All the steroids were dissolved in sesame oil and injected s.c.

To induce delayed implantation, the pregnant rats on day 4 of pregnancy were treated with progesterone (5 mg/rat, s.c.) and then ovariectomized at 0830–0900 h. Progesterone (5 mg/rat) was injected to maintain the delayed implantation from days 5–7. On day 8, one random part of the progesterone-primed delayed-implantation rats was additionally given estrogen (250 ng/rat) to terminate the delayed implantation. The left part was remained for injecting progesterone. The rats were sacrificed to collect uteri 24 h after the hormonal treatment. The implantation sites were identified by tail intravenous injection of trypan blue solution (Sigma). The delayed-implantation was confirmed by flushing hatched blastocysts from the uterus.

Artificial decidualization

Mature female rats were ovariectomized. Artificial decidualization was induced according to the procedures described by Kennedy and Ross (1997). The uteri were collected at 24 h, 48 h and 72 h after injection of the sesame oil.

Embryo transfer

Embryo transfer was done as previously described by Ertzeid and Storeng (2001). Pseudopregnant female rats on day 4.5 (00:00 h on Day 4) of pseudopregnancy were anesthetized by intraperitoneal injection of ketamine, the lateral uterine horns were exposed via lateral incisions. The embryos to be transferred were collected from day 4.5 of pregnant donors. The fine tip of the glass pipette was inserted into a hole made previously by insertion of a 23-gauge needle through the uterine wall at the oviductal end. The anti-lateral uterine horns were served as controls. Thereafter, the uterine horns were placed back into the abdominal cavity and the skin incision was stitched. On day 6, the recipients were killed 5 min after tail intravenous injection of trypan blue solution.

Northern blot analysis

micrograms of total RNA was transferred to the Zeta-Probe Blotting Membrane (Bio-Rad, Dercules, CA) with vacuum transfer apparatus for 2 h. After crosslinking, the membrane was pre-hybridized for 1 h at 68 °C and then hybridized overnight at 68 °C in hybridization buffer (50% deionized formamide, 5 × SSC (where 1 × SSC: NaCl 8.765 g, $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$ 4.41 g to 1 l with distilled water), 0.1% sodium dodecyl sarcosinate, 0.02% SDS, 2% blocking reagent, 2% dextran sulphate) with Dig-labeled (Digoxin-labeled) antisense cRNA probes. The membrane was first rinsed with solution containing

2 × SSC and 0.1% sodium dodecyl sulfate (SDS) for 30 min at room temperature. Second washing was performed in solution containing 0.2 × SSC and 0.1% SDS for 30 min at 68 °C. After short washing in Buffer I (100 mM Tris-HCl, 150 mM NaCl, pH 7.5), the non-specific binding was blocked by incubating the membrane in Buffer II (1% blocking reagent in Buffer I) at room temperature for 1 h. After that, the membrane was incubated in sheep anti-DIG antibody conjugated with alkaline phosphatase at room temperature for 2 h (1: 5000, Boehringer Mannheim) followed by CDP-Star incubation for 10 min. The membrane was then exposed to X-ray film and developed in dark.

In situ hybridization

A 423 bp rat CaR cDNA fragment was reverse-transcribed and amplified with the total RNA from rat uterus using forward primer 5'-GCAGGCTCCTCAGCAATAAGA and reverse primer 5'-GGAAATACTCAGGCATAGCAATCAG designed according to rat CaR cDNA sequence (711–1133 bp, Genbank accession number U20289). The PCR fragment for rat CaR was recovered from the agarose gel and cloned into T-vector (ProMega Corp. 2800 Woods Mollow Rd, Madison, WI, USA). The cloned rat CaR fragment was further verified by sequencing. Dig-labeled antisense or sense cRNA probes were transcribed *in vitro* using a DIG RNA labeling kit (T7 for sense, SP6 for antisense; Boehringer Mannheim, Mannheim, Germany).

Uteri were cut into 4–6 mm pieces and flash frozen in liquid nitrogen. Frozen sections (8 µm) were mounted on 3-aminopropyltriethoxy-silane (Sigma) coated slides and fixed in 4% paraformaldehyde solution in PBS. The sections were twice washed and in PBS treated in 0.2 M HCl for 10 min, then digested with 0.1 µg/ml proteinase K at room temperature for 10–15 min and incubated in 0.2% (w/v) glycine in PBS twice, 5 min duration each incubation. Postfixation was performed in 4% paraformaldehyde for 2 min. After acetylation in freshly prepared 0.25% (w/v) acetic anhydride in 0.1 mol/l triethanolamine (pH 8.0) for 10 min, the sections were washed in 2 × SSC (1 × SSC is 0.15 mol/l sodium chloride, 0.015 mol/l sodium citrate) twice for 5 min each, dehydrated in serial dilutions of ethanol and air dried. Following the prehybridization in hybridization buffer (4 × SSC, 50% (v/v) formamide, 5% (w/v) dextran sulfate, 1 × Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA, 0.25 mg/ml yeast tRNA) at 25 °C for 2–3 h, the sections were hybridized in hybridization buffer with 1–5 µg/ml DIG-labelled antisense or sense RNA probe for rat CaR at 55 °C for 16 h. After hybridization, the sections were washed in 4 × SSC at room temperature for 10 min and digested in 10 µg/ml RNase A (Boehringer Mannheim) in 0.01 mol/l Tris-HCl and 0.5 mol/l NaCl (pH 8.0) at 37 °C for 30 min. The sections were washed sequentially in 4 × SSC, 1 × SSC and 0.5 × SSC twice for 10 min each at 42 °C. After non-specific binding was blocked in 0.5% (w/v)

block mix (Boehringer Mannheim), the sections were incubated overnight in sheep anti-DIG antibody conjugated with alkaline phosphatase at 4 °C (1: 4000, Boehringer Mannheim). The signal was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Endogenous alkaline phosphatase activity was inhibited with levamisole (Sigma).

Immunohistochemistry

Rat uteri were immediately fixed in 10% Neutral Buffered Formalin solution (Beijing Chemical Reagents Co. Beijing, China) and embedded in paraffin. Sections (5 µm) were cut, deparaffinized and rehydrated. Antigen retrieval was performed by incubating the sections in 0.01 mol/l citrate buffer (pH 6.0) at 95 °C for 15 min and by cooling naturally at room temperature for 20 min. Non-specific binding was blocked in 10% (v/v) normal horse serum in PBS for 1 h. The sections were incubated with rabbit anti-CaR in 10% (v/v) horse serum (1: 100) for 12 h at 4 °C (Affinity BioReagents, Golden, CO). The sections were then incubated with biotinylated secondary antibody followed by an avidin-alkaline phosphatase complex and Vector Red according to the manufacturer's protocol (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA). Vector Red was visualized as a red colour. Endogenous alkaline phosphatase activity was inhibited with levamisole (Sigma). Moreover, rabbit anti-CaR was replaced with normal rabbit IgG as a negative control. The sections were counterstained with hematoxylin and mounted.

Microscopic assessment

Samples from three individual animals at least for each group were analysed. Experiments were repeated at least three times, and one representative from at least three similar results was presented. The mounted sections were examined using a Nikon microscope.

Results

Northern blot of CaR mRNA expression during process of implantation

As the first step in examining a possible role of CaR in reproductive tissues, we checked the expression of CaR mRNA in the implantation uterus by northern blot. As shown in Figure 1, CaR expression was detected in all the uteri examined. The expression of CaR mRNA in the implantation site on day 6, in the estrogen activated delayed implantation uterus and in the uterus on day 8 of pregnancy are much higher than that in the uteri of day 5 of pregnancy, day 6 of pseudopregnancy and the delaying implantation uterus, suggesting that CaR in pregnant uterus is temporal-spatially regulated.

Figure 1 Northern Blot of CaR mRNA in peri-implantation uterus, D5: day 5 of early pregnancy, D6m: implantation site of day 6 of early pregnancy, D6n: inter-implantation site of day 6 of early pregnancy, Stim: estrogen activated delayed implantation uterus, Delay: delaying implantation uterus, PD6: day 6 of pseudopregnancy, D8: day 8 of early pregnancy.

CaR mRNA during early pregnancy and pseudopregnancy

In order to look at the possible role of CaR in the uterus, we characterized the developmental expression of the CaR gene in normal pregnant and pseudopregnant rats. The results showed that CaR mRNA was mainly localized in the luminal epithelium on day 1 of pregnancy and pseudopregnancy (Fig. 2A). On day 2 and 3, additional signals of the messenger were detected in the stromal cells immediately underneath the luminal epithelium (Fig. 2B, 2D). Interestingly, the CaR mRNA expression in these cells decreased sharply on day 4 and remained at a basal level until day 6 (Fig. 2C, 2E), the time blastocysts begin to implant into the endometrium, CaR mRNA was again detected intermediately in the mesenteric stromal cells underneath the luminal epithelium and more strongly in the anti-mesenteric stroma surrounding the blastocyst. Only basal levels of expression could be detected in the interimplantation site (Fig. 2F, 2H). From days 7 to 8, CaR expression further increased and was strongly detected in the embryos and decidualized cells (Fig. 2G, 2I). On day 9, expression of CaR mRNA extended to the secondary decidualization zone (Fig. 2J).

To test if there was a correspondence between the levels of CaR mRNA and protein, we performed immunohistochemistry using an antibody specific to the CaR protein. CaR protein was barely detected in the uterus on day 1 of pregnancy and pseudopregnancy. An intermediate level of CaR protein appeared in the luminal and glandular epithelium from day 2 and remained at the same level through day 5 of pregnancy (Fig. 3A, 3B, 3C). On day 6 of pregnancy, CaR expression was mainly observed in the implanting blastocyst and the subluminal stromal cells at the implantation site, while the expression pattern in the inter-implantation site and day 6 of pseudopregnancy was similar to that observed between days 2 and 5 (Fig. 3E, 3D, 3F). From days 7 to 9, CaR protein was strongly detected in the embryos and the decidualized cells (Fig. 3G, 3H). These results suggest that CaR expression in the stromal cells in the receptive status of the uterus was induced by implanting blastocysts, while in epithelial cells during day 1 through day 5, the expression of CaR seems to be regulated by some non-embryonic factors.

Figure 2 *In situ* hybridization of CaR mRNA in rat uterus. CaR mRNA was only detected intermediately in the luminal epithelium on day 1 of pregnancy (A), besides in the glandular epithelium, CaR signal was also strongly detected in the stromal cells immediately underneath the luminal epithelium on day 2 of pregnancy (B) and on day 2 of pseudopregnancy, similar expression was seen (D). Only a basal level of CaR mRNA was detected on day 5 of pregnancy (C). On day 6, CaR mRNA was intermediately detected in the mesenteric stromal cells immediately underneath the luminal epithelium and strongly in the stroma surrounding the blastocyst (F), only basal level of CaR mRNA was detected in the interimplantation site uterus (H) and in day 6 of pseudopregnancy uterus (E). CaR mRNA was strongly detected in the embryos and decidualized cells on days 7, 8 and 9 of pregnancy (G, I and J). Arrow: implanting blastocyst. Magnification $\times 40$.

Figure 3 Immunohistochemistry of CaR protein in rat uterus. CaR protein was intermediate detected in the luminal and glandular epithelium on day 2 (A), day 5 (C) of pregnancy, day 2 (B), day 6 (D) of pseudopregnancy. On day 6, CaR protein was mainly observed in the implanting blastocyst and subluminal stromal cells at the implantation site (E), while the expression pattern in the inter-implantational site was similar to that of days 2 and 5 (F). CaR protein was strongly detected in the embryos and the decidua cells on days 8 and 9 of pregnancy (G, H). Magnification $\times 40$.

CaR expression during delayed implantation

In the rodent, delayed implantation is an artificial condition induced by ovariectomy before the preimplantation estrogen secretion on the morning of day 4, resulting in blastocyst dormancy and the inhibition of implantation. This condition of the blastocyst dormancy can be maintained for an extended period by daily progesterone (P4) treatment. Once exposed to estrogen together with P4, the dormant blastocysts was activated, resulting in the initiation of implantation. CaR mRNA and protein expression were at a basal level in the uterus under the delayed implantation (Fig. 4A, 4B). After the delayed implantation was terminated by the estrogen treatment and the embryo implantation occurred, the CaR mRNA and protein was very strongly induced in the stromal cells surrounding the implanting blastocyst. A low level of CaR protein was also detected in the implanting blastocyst (Fig. 4C, D). This result suggests that the activated blastocysts specifically induce CaR expression in the adjacent stromal cells.

CaR expression in implantation site of transplanted uterus

Embryo transplantation is another good model to study the interaction between the implanting embryo and the recipient uterus. To further confirm that CaR expression in the uterus was induced specifically by the embryo implantation, we transplanted the early blastocysts into the synchronous pseudopregnant recipient uterus. As shown in Fig 5, the expression of both CaR mRNA and protein in the implantation site in the transplanted uterus (Fig. 5A, 5E) was similar to their expression in the early pregnancy (Fig. 5B, 5F), while in the non-transplanted control uterus, no obvious CaR expression was detected (Fig. 5C, 5D). This result shows a high similarity to those in the early pregnancy and the delayed implantation model, once again confirming that CaR expression in the receptive uterus was specifically induced by the implanting blastocysts.

CaR expression under artificial decidualization

To confirm CaR expression in decidualizing stromal cells is specifically correlated to the process of decidualization, we checked expression of the CaR in the uterus of artificial decidualization. CaR mRNA was strongly detected in the whole decidualized stromal cells 24 h after the artificial decidualization by injecting sesame oil into the uterine lumen (Fig. 6A), the signal remained strong and expanded outward 48 h later (Fig. 6C). 72 h later, a strong mRNA signal was detected in the decidualized stromal cells adjacent to the myometrium, while the signal was much weaker inward (Fig. 6E). In the un-injected control horn, however, only an intermediate to low level of CaR expression was detected in the luminal and glandular epithelium (Fig. 6G).

CaR protein was intermediately detected in the decidualized cells 24 h after injection of the stimulated oil (Fig. 6B), the immunostaining gradually increased in a time-dependent manner (Fig. 6D, 6F). Only a basal level of CaR protein was observed in the un-injected control horn (Fig. 6H). These results strongly matched those in the decidualized tissue of the early pregnancy, showing that CaR expression temporal-spatially involves in the progress of decidualization during implantation.

CaR expression during estrous cycle

Because early embryonic development and embryo implantation is a hormone regulated physiological progress, we examined CaR expression throughout the estrous cycle to see if it is regulated by hormones. The results showed high levels of mRNA in the luminal epithelium at estrus and proestrus (Fig. 7A, 7G), whereas at diestrus and metestrus, a strong signal was also detected in the glandular epithelium (Fig. 7C, 7E). Low level of CaR protein was observed in the diestrus uterus (Fig. 7F), intermediate levels were detected in the luminal epithelium at

Figure 4 CaR expression under delayed implantation. Only a low level of CaR mRNA (A) and protein (B) were expressed in the control uterus. CaR mRNA (C) and protein (D) expression were significantly induced in the stromal cells underneath the luminal epithelium at the implantation site once delayed implantation was terminated by estrogen treatment and embryo implantation. Arrow: implanting blastocyst. Magnification $\times 40$.

estrus and proestrous (Fig. 7B, 7H), while a strong signal was detected in both luminal and glandular epithelium at metestrus (Fig. 7D). These results imply that CaR expression may be regulated by the ovarian steroid hormones.

Hormonal regulation of CaR expression

In mouse and rat, the processes of implantation and early pregnancy are delicately regulated by the ovarian steroid hormones. To further study the relationship between CaR expression and the steroid hormones, we looked at the CaR expression in the ovariectomized uterus. Only basal level of hybridization signal was detected in the glandular epithelium of the ovariectomized rat (Fig. 8G, 9G). Six hours after hormone treatment (estrogen, progesterone or estrogen plus progesterone) however, a strong expression of CaR mRNA was induced in the luminal and glandular epithelium (Fig. 8A, 8C, 8E). The induced expression continued until 24 h after estrogen or estrogen plus progesterone treatment (Fig. 9A, 9E), while the expression of CaR mRNA decreased to the control level 24 h after progesterone treatment (Fig. 9C).

On the protein level, no immunostaining signal was observed in the ovariectomized rat uterus (Fig. 8H, 9H). Estrogen alone or estrogen plus progesterone induced strong CaR expression 6 h after estrogen alone or estrogen plus progesterone (Fig. 8B, 8F); while only a weak signal was detected in the luminal and the glandular epithelium 24 h after the operation (Fig. 9B, 9F). Progesterone

alone has no change in the expression pattern 6 h after the treatment (Fig. 8B), while by 24 h, an intermediate expression of CaR protein in the luminal and glandular epithelium was induced (Fig. 9B). These results confirm that CaR expression is indeed regulated by estrogen and progesterone. Progesterone seems to induce the CaR expression transiently, while estrogen plays a persistent up-regulatory role in the gene expression.

Discussion

Implantation is an elaborated physiological process which requires complex interactions between the developing embryo and the uterus (Paria *et al.* 2002). However, the precise nature of the molecular mechanism remains unclear. We have demonstrated in this study that the expression of both CaR mRNA and protein in the rat uterus increased transiently at 5.5 day of pregnancy or a delayed implantation was terminated by estrogen treatment and the embryos implanted. These results together with those from the embryo transplantation experiment suggest that CaR expression at the implantation sites is specifically induced by either a blastocyst or an artificial decidualization stimulus under estrogen dominance, thus CaR seems to be a new signal mediator between embryo and uterus during implantation.

In the present study we have clearly demonstrated that the expression of CaR was switched from the luminal epithelium to stromal cells on days 1 to 3 of pregnancy and its expression diminished on day 4, but was again induced

Figure 5 CaR expression in implantation site of transplanted uterus, intermediate CaR mRNA and protein was seen in the stromal cells surrounding the blastocyst in transplanted uterus (A, E), similar to that in day 6 uterus of early pregnancy (B, F); No obvious CaR expression was seen in control uterus (C, D). Magnification $\times 40$.

by the implanting blastocyst. All these changes could be due to changing levels of estrogen and progesterone levels in the uterus during the preimplantation period. In the ovariectomized rat it clearly showed that only basal levels of CaR mRNA were detected in the glandular epithelium. Estrogen significantly induced CaR expression in the luminal and glandular epithelium within 6 h and the positive regulation continued up to 24 h. Additionally, the intermediate level of CaR expression in the uterus on day 1 of pregnancy and pseudopregnancy matched with the higher level of maternal estrogen. Progesterone also up-regulated CaR mRNA expression within 6 h, however, the up-regulation disappeared by 24 h. The regulation pattern of progesterone agrees with the result from the early pre-implantation pregnancy, we could observed a strong CaR mRNA expression on day 2 of pregnancy when progesterone concentration increased remarkably (Bridges 1984), however, on day 4, when the progesterone and estrogen levels should be still at higher levels, we could only detect basal levels of CaR mRNA expression. The molecular mechanism beneath this and the significance of the

expression pattern are still unclear and worthy to be studied further.

It's well known that in adult rodent uterus, estrogen stimulates proliferation of epithelial cells, while progesterone leads to stromal cell proliferation (Tan *et al.* 1999). The location of CaR in the epithelial cells on day 1 had switched to the stromal cells from day 2, reflecting that the CaR is regulated by progesterone and plays a role in regulation of the uterine stromal cell proliferation by preparing the uterus for the embryo implantation.

During implantation, after the blastocyst attaches to the endometrium, the uterus undergoes severe changes to accommodate and protect the developing conceptus. In particular, the stromal endometrial cells proliferate and differentiate to form the decidual tissue (decidualization). It is possible that the implanting blastocyst induces the CaR expression in the surrounding stromal cells during the initiation of attachment reaction. However, its extensive expression in stromal cells on days 7–9 seems to be the results of uterine decidualization, which also occurs in the artificial decidua. It is well known that the decidual reaction can be also induced experimentally by an intraluminal

Figure 6 *In situ* hybridization in left panels and immunohistochemistry in right panels of CaR expression under artificial decidualization. Only intermediate level of CaR mRNA (G) in luminal and glandular epithelium and basal level of CaR protein (H) were detected in the control uterus. CaR mRNA and protein expression 24 h after the artificial decidualization (A, B), 48 h after the artificial decidualization (C, D), 72 h later after the artificial decidualization (E, F), we can see strong signal in the decidualized cells. Magnification $\times 40$.

injection of a small amount of oil into the uteri of pseudo-pregnant rat. In the present study, both CaR mRNA and protein were highly expressed in the decidual cells on days 7–9 of the artificially induced decidual cells, we could specifically observed the expression of CaR mRNA in the decidualizing cells in the uterus at the late stage. The results show that CaR is involved in the decidualization progress, although we are not sure of the consequence between the CaR expression and the stromal cell decidualization. This happens to other implantation related molecules such as Basigin (Xiao *et al.* 2002), in these cases some embryo derived factors and/or hormones may regulate the expression of the molecules in the decidual cells.

It is increasingly recognized that CaR plays a key role in signal transduction by modulating the ERK1 and ERK2 cascade in different cell lines (Hobson *et al.* 2003). CaR regulation of parathyroid hormone-related peptide (PTHrP) release in H-500 cells involves activation of PKC as well as ERK1/2, p38 MAPK, and JNK pathways (Tfelt-Hansen *et al.* 2003). CaR acts as a sensor by relaying the meticulous changes in extracellular Ca^{2+} to intracellular components, leading to a variety of intracellular responses,

Figure 7 CaR expression during estrous cycle. *In situ* hybridization in left panels and immunohistochemistry in right panels. A strong level of mRNA signal in the luminal epithelium at estrus and proestrus (A, G), whereas at diestrus and metestrus, strong signal was also detected in the glandular epithelium (C, E). No CaR protein was seen at diestrus uterus (F), intermediate level of protein signal was detected in luminal epithelium at estrus and proestrus (B, H), while at metestrus (D), strong signal was seen in both luminal and glandular epithelium. Magnification $\times 40$.

including activation of phospholipases, generation of inositol trisphosphate (IP_3) and diacylglycerol, increases in intracellular Ca^{2+} , changes in protein phosphorylation, activation of ion channels, regulation of hormone secretion, modulation of nuclear transcriptional activity and differential gene expression (Yamaguchi *et al.* 2000, Uhlen *et al.* 2000). In spite of this being known, the molecular mechanism by which CaR fulfills its function during implantation and decidualization remains unresolved definitively. In murine bone marrow-derived stromal cell line, CaR was detected from both the mRNA and the protein level. Stromal cells support the formation of osteoclasts from their progenitors as well as the growth of hematopoietic stem cells, they also have the capacity to differentiate into bone-forming osteoblasts. CaR was suggested to participate in bone turnover by stimulating the proliferation and migration of such cells to sites of bone resorption and, thereafter, initiating bone formation after differentiation into osteoblasts (Yamaguchi *et al.* 1998). It was also reported that ovarian surface epithelial cell proliferation, in response to an increase in extracellular

Figure 8 CaR expression in the uterus of ovariectomized rats 6 hrs after hormonal regulation. *In situ* hybridization in left panels and immunohistochemistry in right panels. Only basal levels of CaR mRNA and protein signal were seen in the uterus of ovariectomized rat (G, H). While protein was strongly induced (C, D) in the uterus after progesterone treatment. CaR mRNA was strongly induced while protein only increased weakly in the luminal and glandular epithelium after estrogen alone (A, B) or estrogen addition progesterone treatments (E, F). Magnification $\times 40$.

calcium was mediated by G-protein-coupled CaR (Hobson *et al.* 2000). These reports suggested to us that CaR may play a similar regulatory role, as in the bone stromal cells and the ovarian surface epithelial cells, in stromal cell proliferation and differentiation during decidualization.

In summary, we have clearly demonstrated in this study for the first time that CaR is spatio-temporally expressed and localized in the mammalian uterus of early pregnancy under different conditions in close relation to the processes of implantation and decidualization. Estrogen and progesterone regulate CaR expression, that may be via different mechanisms. The strong CaR expression at the implantation site and decidual cells in rat uterus suggests that CaR might be important for blastocyst implantation and decidualization.

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Figure 9 CaR expression in the uterus of ovariectomized rats 24 hrs after hormonal treatment. *In situ* hybridization in left and immunohistochemistry in right. Only basal levels of CaR mRNA and protein signal were detected in the uterus of the ovariectomized rat (G, H). No mRNA change was observed (C) while the protein was strongly induced (D) in the uterus after progesterone treatment. CaR mRNA was strongly induced while protein only increased weakly in the luminal and glandular epithelium after treatment of estrogen alone (A, B) or estrogen plus progesterone (E, F). Magnification $\times 40$.

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