Tetraspanin CD9 regulates invasion during mouse embryo implantation

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Abstract

The expression of tetraspanin CD9 was found on blastocysts in mice and endometrium epithelial cells in human and bovine. However, it remains unknown how CD9 is involved in the precise dialogue between embryo and uterus during early pregnancy. This study was designed to investigate the functional roles of CD9 in the embryo implantation with monoclonal antibody against CD9 protein (anti-CD9 mAb) and antisense oligonucleotide against CD9 gene (AS-CD9). Our results showed that intrauterine injection of anti-CD9 mAb on day 4 of pregnancy significantly increased the number of embryos implanted (7·24±0·39 versus 4·04±0·38). *In vitro*, anti-CD9 mAb or AS-CD9 significantly enhanced embryo-outgrowth ability on the monolayer of uterus epithelial cells in a dose-dependent manner. However, the attachment of blastocysts to epithelial cells was unaffected. Furthermore, we found that anti-CD9 mAb or AS-CD9 stimulated matrix metalloproteinase 2 (MMP-2) production of blastocysts on Fibronectin. LY294002, a specific inhibitor of phosphoinositide 3-kinase, was able to counteract the effect of anti-CD9 mAb and AS-CD9 on outgrowth ability and production of MMP-2. Our results indicated that CD9 played a role of inhibiting embryo implantation. CD9 was able to impair embryo invasion and the production of MMP-2 through the phosphoinositide 3-kinase signaling pathway.

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Introduction

CD9, a member of the tetraspanin family, is a 24–27 kDa cell-surface protein with four predicted transmembrane domains. CD9 has been implicated in the regulation of cell-biological functions, including cell adhesion, motility, proliferation, differentiation, and signaling (Ikeyama *et al.* 1993, Anton *et al.* 1995, Aoyama *et al.* 1999, Clay *et al.* 2001). Regarding tumor progression, previous experimental and clinical studies have demonstrated that CD9 had a functional role as a tumor metastasis suppressor. CD9 transfection into Chinese hamster ovary, MHO10, ARH77, and MAC 10 cells, for example, inhibited cell motility (Ikeyama *et al.* 1993, Miyake *et al.* 2000). Many clinical studies have revealed that reduced CD9 expression is associated with a poor prognosis in various cancers, including non-small-cell lung cancers, breast cancers, and colon cancers (Huang *et al.* 1998, Funakoshi *et al.* 2003, Hashida *et al.* 2003). Although originally identified as a surface antigen on a pre-B cell line (Kersey *et al.* 1981), CD9 is also expressed on blastocysts in mice and endometrium epithelial cells in human and cattle (Le Naour *et al.* 2000, Park *et al.* 2000, Xiang & MacLaren 2002). Given the striking similarities between embryogenesis and the biology of cancer cells, especially in the process of the invasion, CD9 might be involved in embryo-invasive behaviors.

Embryo implantation is an important step in the establishment of pregnancy. Successful invasiveness or migration into the extracellular matrix environment is a fundamental property of embryo implantation. Matrix metalloproteinases (MMPs), one of the well-known families of molecule involved in embryo invasion, were expressed by trophoblast cells and endometrium during embryo implantation (Salamonsen 1999). *In vitro*, MMP-2 was secreted by human embryos (Graham *et al.* 1993, Puistola *et al.* 1989). Maximal enzyme concentrations occur at days 4 and 5 of culture, corresponding with the time of implantation *in vivo* (Turpeenniemi-Hujanen *et al.* 1992). Treatment with monoclonal antibodies to various tetraspanin proteins specifically stimulates production of MMP-2 and induced formation of long invasive protrusions of the MDA-MB-231 cells in the Matrigel assay (Sugiura & Berditchevski 1999). However, it is still not clearly known whether there are the similar effects of tetraspanin proteins on MMP-2 in the process of embryo implantation.

Attachment of epithelial cells to the underlying basement membrane via integrins activates the phosphoinositide 3-kinase (PI3K)/Akt/protein kinase B survival signal. However, upon detachment, these cells undergo anoikis (Lee & Juliano 2000). Constitutive activation of the PI3K signaling pathway, which is one of major regulators of cell survival, is found in various human

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cancers (Bin *et al.* 2002, Samuels *et al.* 2004). In addition, the role for PI3K-mediated MMP-2 activity in malignant gliomas and effects of CD9 on MDA-MB-231 cells have recently been demonstrated (Sugiura & Berditchevski 1999, Hess *et al.* 2003). Although significant progress has been made recently towards identifying key elements within the CD9 signaling network, relatively little is known about its involvement in embryo invasion.

In this study we analyzed the effects CD9 on embryo implantation by *in vivo* and *in vitro* experimental models. Our results indicated that CD9 played an important role in regulating embryo implantation and contributing to the production of MMP-2.

Materials and methods

Reagents

Rat anti-mouse CD9 (KMC8) monoclonal antibody (mAb) was purchased from BD Bioscience Pharmigen (catalog no. 553758). PI3K inhibitor LY294002 (10 μM) was from Sigma, and mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase kinase (MEK) inhibitor PD98059 (50 μM), Janus kinase 2 (JAK2) inhibitor AG490 (50 μM) and p38 inhibitor SB203580 (50 μM) were obtained from Calbiochem.

Animals

Kunming white strain mice (Cai *et al.* 2000, Liu *et al.* 2002; Experimental Animal Center, Institute of Genetic Science, Chinese Academy of Sciences, Beijing, People's Republic of China) were housed in the animal facility of the State Key Laboratory of Reproductive Biology. Adult female mice (25–30 g, 5–8 weeks old) were mated with males of the same strain at room temperature and with a constant photoperiod (light:dark cycle, 14 h/10 h). Food and water were freely available. Guidelines for the care and use of the animals were followed. Mice were killed by cervical dislocation after deep anaesthesia with isoflurane by inhalation. All experiments were approved by the local Animal Care Committee (Beijing).

Indirect immunofluorescence and laser scanning confocal microscopy

After being flushed, pre-implantation embryos were washed with Dulbecco's PBS (DPBS) containing $CaCl_2$ and $MgCl_2$ (1 mM each) and fixed in 4% paraformaldehyde (15 min, room temperature). Embryos were permeabilized with 0·1% Triton X-100 in DPBS for 4 min, incubated for 1·5 h in DPBS containing 10% goat serum at room temperature, and reacted with anti-CD9 mAb (1:100) at 4 °C overnight followed by secondary antibody (fluorescein isothiocyanate (FITC)-labeled anti-rat IgG) for 1 h at 37 °C. Nuclei were stained with 5 μg/ml propidium iodide (Sigma) for 5 min. Finally, the blastocysts were rinsed in DPBS to remove excess fluorescence agents, and viewed under laser scanning confocal microscope (Leica).

Treatment with antisense oligonucleotides

Phosphorothionated antisense oligonucleotide to mouse CD9 mRNA (5′-GAGCAGGTATTTGATGCA-3′) and control complementary phosphorothionated sense oligonucleotide (5′-TGCATCAAATACCTGCTC-3′) were synthesized. The efficacy of antisense oligonucleotide against CD9 gene (AS-CD9) has been described previously (Shallal & Kornbluth 2000). We modified three bases in order to match completely the mouse CD9 gene. After being washed and incubated as described by Shallal & Kornbluth (2000), blastocysts were transferred to 0·4% BSA (Sigma) in Ham' F-12 medium with antisense or sense CD9 oligonucleotide at a concentration of 0·2–20 μM and incubated for 72 h.

Intrauterine injection

Each female mouse was caged with one male and allowed to mate naturally overnight. Day 1 of pregnancy was designated as the next morning when a vaginal plug was formed. Pregnant mice on day 4 were injected with 1·5 μg anti-CD9 mAb into the lumen of one uterine horn adjacent to the ovary, while another horn was injected with 1·5 μg isotype rat IgG as a control. On day 7, the treated animals were killed to count the number of implanted embryos.

Preparation of the monolayer of uterine epithelial cells

On day 4 of pregnancy, preparation of a monolayer of uterine epithelial cells was carried out using the method reported by Zeng & Cao (1996). Harvested epithelial cells were placed in 24-well sterile plastic plates and cultured in the medium of Ham's F-12 medium supplemented with 10% fetal calf serum. After 24 h, the monolayer was rinsed three times in Ham's F-12 medium, and then co-culture medium was added containing Ham's F-12 medium supplement with 0·5% BSA.

Mouse embryo attachment and outgrowth assay

Blastocysts were obtained by flushing the uterine horns of day-4 pregnant mice with Hank's solution and rinsed three times in medium Ham's F-12 medium, then transferred in droplets of preheated medium Ham's F-12 medium supplemented with 0·5% BSA. For the CD9 block assay, the blastocysts were incubated with

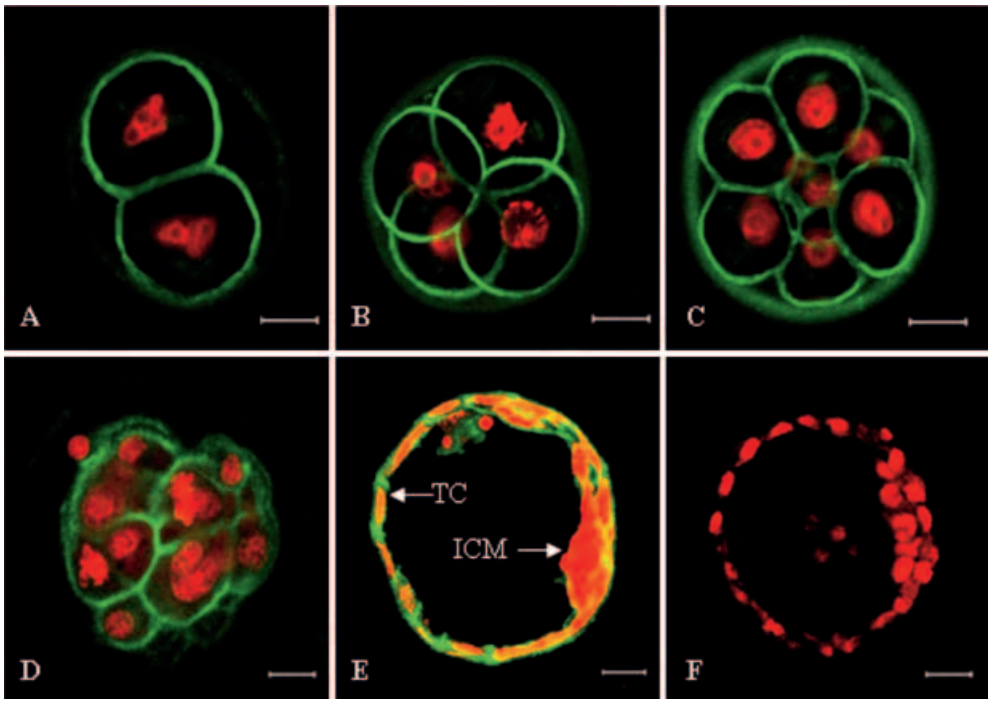


Figure 1 Localization of CD9 protein on mouse pre-implantation embryos. CD9 protein was localized immunohistologically using a FITC-conjugated secondary antibody (green). Nuclei were labeled with propidium iodide (red). (A) Two-cell embryo. (B) Four-cell embryo. (C) Eight-cell embryo. (D) Morula. (E) Blastocyst. (F) Immunofluorescence with isotype rat IgG. Note the positive signal for CD9 protein in the trophoblast cells of blastocysts and blastomeres of two-, four-, and eight-cell embryos and morulae. ICM, inner cell mass; TC, trophoblast cells. Scale bars, 20 μm.

anti-CD9 mAb at concentrations of 10–1000 ng/ml or AS-CD9 at concentrations of 0·2–20 μM. In control cultures, blastocysts were incubated with a purified isotype rat IgG or sense oligonucleotide against CD9 gene (S-CD9) at the same concentration as the treated blastocysts. To determine embryo attachment, the plate was shaken for 20 s with one rotation/s. If the blastocyst was found to stay at the same place, this blastocyst was designated as attachment; if not, it was designated as non-attachment. After attachment, blastocysts began to outgrow outwards. When primary giant trophoblast cells were visible around the attachment site of the attached blastocysts, we designated the blastocysts as outgrowth (Sherman & Atienza-Samols 1978). The outgrowth area, which is occupied by primary giant trophoblast cells, was recorded and measured by a digital camera. Each experiment was repeated three times.

Gelatin zymography

The culture medium (6 μl) at 48 h was mixed with 4 × sample buffer (8% SDS (w/v), 0·04% Bromophenol Blue (w/v), 40% glycerol (v/v) and 0·25 M Tris) and then subjected to electrophoresis in a 10% polyacrylamine gel containing 0·5 mg/ml gelatin (Sigma). The gel was washed in 2·5% Triton X-100 and 50 mM Tris/HCl, at pH 7·5 for 1 h to remove the SDS and incubated for 18 h in calcium assay buffer (50 mM Tris, 200 mM NaCl and 10 mM $CaCl_2$, pH 7·5) at 37 °C. After staining with 0·2% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid, the gel was destained with 10% acetic acid. The lytic bands were quantified by computer-aided densitometry. Each experiment was repeated three times.

Statistical analysis

All the results are shown as means ± S.E.M. All the data were analyzed using one-way analysis of variance (ANOVA) on SPSS software. The value of $P<0·05$ was considered to represent statistical significance.

Results

Expression of CD9 in mouse pre-implantation embryos

By using immunofluorescence staining, CD9 antigen was detected to express strongly on the surface of blastomeres of two-, four-, and eight-cell embryos and morulae, as well as on the trophoblast cells of blastocysts

(Fig. 1). This expression pattern suggested that CD9 might play a role in mouse embryo development and embryo implantation.

Anti-CD9 mAb increases the number of implanted embryo

Given the abundant presence of CD9 on trophoblast cells of blastocysts, we addressed the question as to whether the CD9 has the potential to affect embryo implantation. Thus we further tested the requirement for CD9 protein in the mouse embryo implantation by injecting the anti-CD9 mAb into the lumen of the uterus on day 4 of pregnancy (the 'implantation window'). As shown in Fig. 2, injection of the anti-CD9 mAb significantly increased the number of embryos developed to day 7 (the mean numbers of implanted embryos were $7{\cdot}24 \pm 0{\cdot}39$ versus $4{\cdot}02 \pm 0{\cdot}38$, $P<0{\cdot}01$). This result indicates that CD9 protein may have an effect on embryo implantation or development *in vivo*.

Anti-CD9 mAb and AS-CD9 promote mouse blastocyst spreading ability

Furthermore, we utilized a widely used blastocyst/uterine epithelial cell co-culture system to study the effects of CD9 on aspects of implantation. Indeed, the data accumulated in this *in vitro* model provided important insights into the molecular mechanism that might control embryo implantation. In the course of this study we investigated the effects of anti-CD9 mAb and AS-CD9 on blastocyst implantation by examining attachment, outgrowth, and the outgrowth area of blastocysts on uterine epithelial cells. Data were collected at different times (24, 48, and 72 h) after embryos hatched and transferred onto the monolayer of the uterine epithelial cells. Results showed that, compared with the control group, both anti-CD9 mAb and AS-CD9 had little effect on blastocyst attachment to uterine epithelial cells (data not shown). However, anti-CD9 mAb (0·1 and 1 μg/ml) and the AS-CD9 (2 and 20 μM) had significantly promoted the percentage of outgrowth at 48 h (Fig. 3B) and the blastocyst outgrowth area at 72 h (Fig. 3C). Taken together, these results indicate that CD9 may regulate embryo implantation, probably by inhibiting embryo invasion.

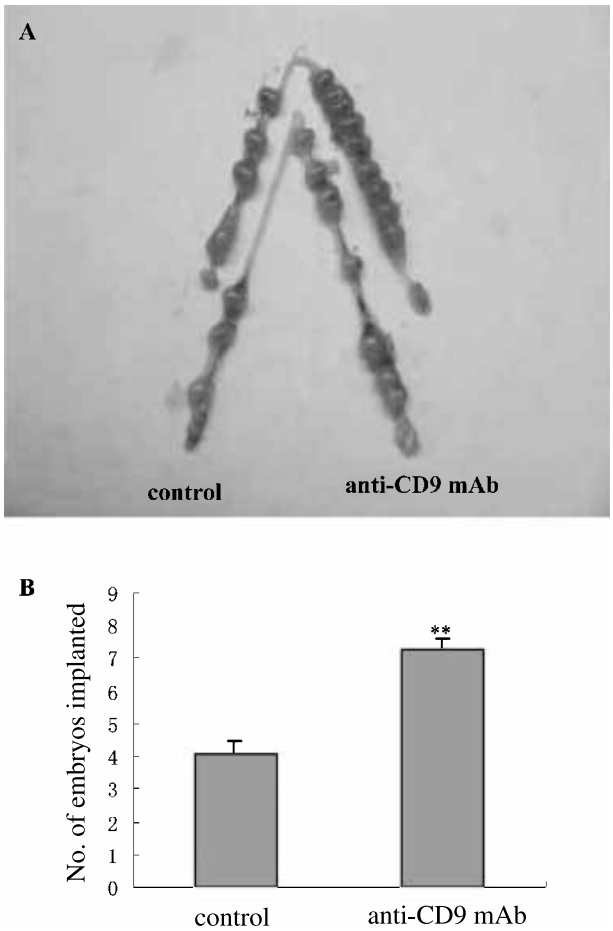
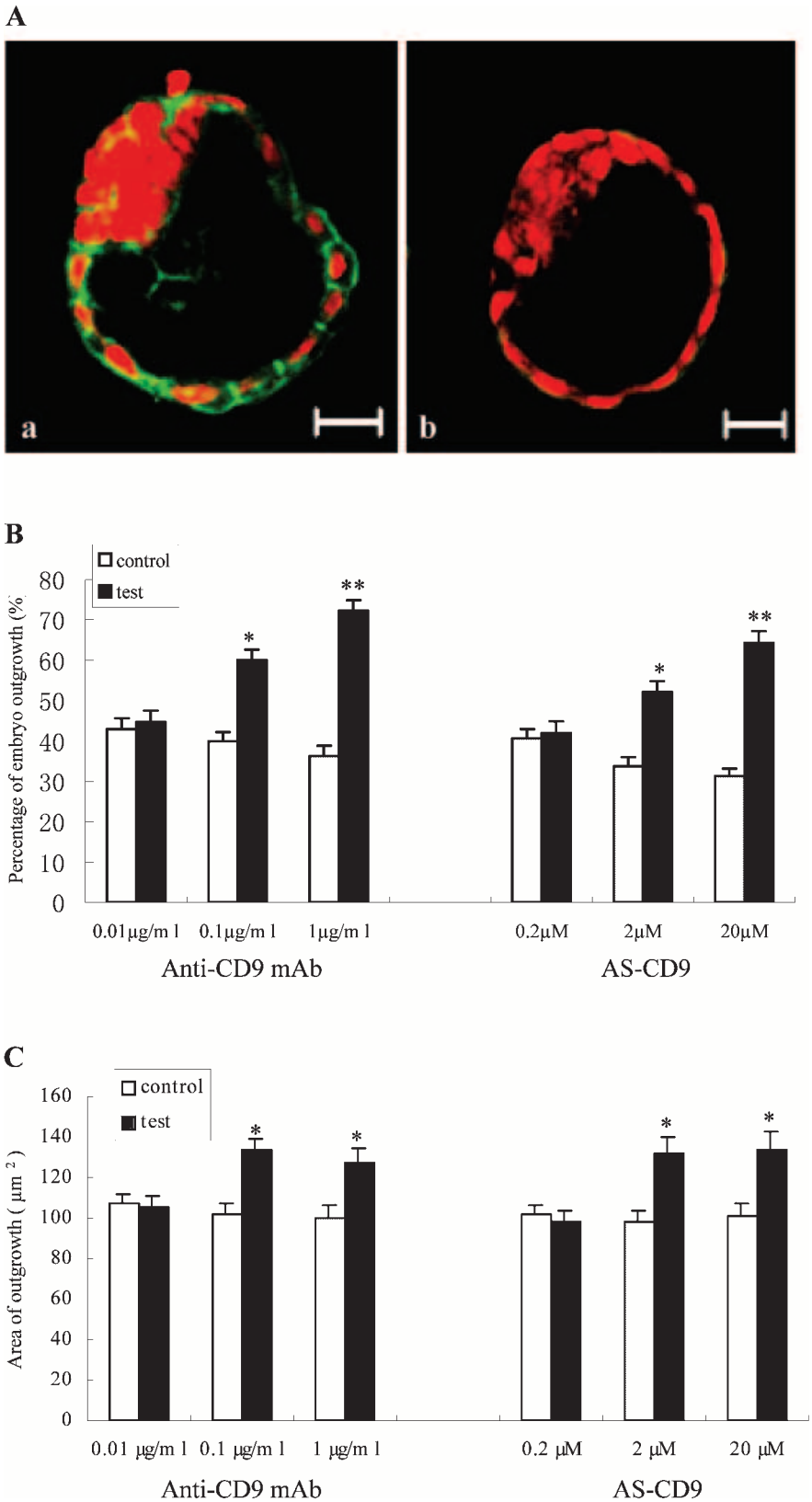


Figure 2 Effect of intrauterine injection of anti-CD9 mAb on embryo implantation in the mouse. The uterine horn of day-4 (14:00 h) mice was injected with 1·5 μg (3 μl) anti-CD9 mAb and isotype rat IgG in another uterine horn. Day-7 animals (n=50) were killed to count the number of embryos. Kunming strain mice yield nine or so offspring per litter at one time under physiological conditions (**$P<0{\cdot}01$ versus control).

Effects of anti-CD9 mAb and AS-CD9 on production of MMP-2

It is well established that MMP-2 may play an important role in controlling embryo invasion in the process of the embryo implantation. In particular, embryos *in vitro* secreted a large amount of MMP-2 onto fibronetin-coated Petri dishes. Given these results, we next wanted to know whether CD9 is able to regulate MMP-2

Figure 3 Effects of anti-CD9 mAb and AS-CD9 on percentage and area of outgrowth of the blastocyst. Hatched blastocysts were incubated on the monolayer of uterine epithelial cells in serum-free media supplemented with BSA plus 0·01, 0·1, or 1 μg/ml anti-CD9 mAb or 0·2, 2, or 20 μM antisense oligonucleotide against CD9, or the same concentrations of isotype rat IgG or S-CD9 as a control. (A) Efficacy of 2 μM AS-CD9 in downregulating the expression of CD9 on blastocysts after 24 h culture. (a) S-CD9; (b) AS-CD9. (B) After hatched blastocysts were incubated for 48 h, the percentage of blastocyst outgrowth was calculated. (C) After hatched blastocysts were incubated for 72 h, the area of blastocyst outgrowth was calculated (n=40 blastocysts per well). *$P<0{\cdot}05$ versus control; **$P<0{\cdot}01$ versus control.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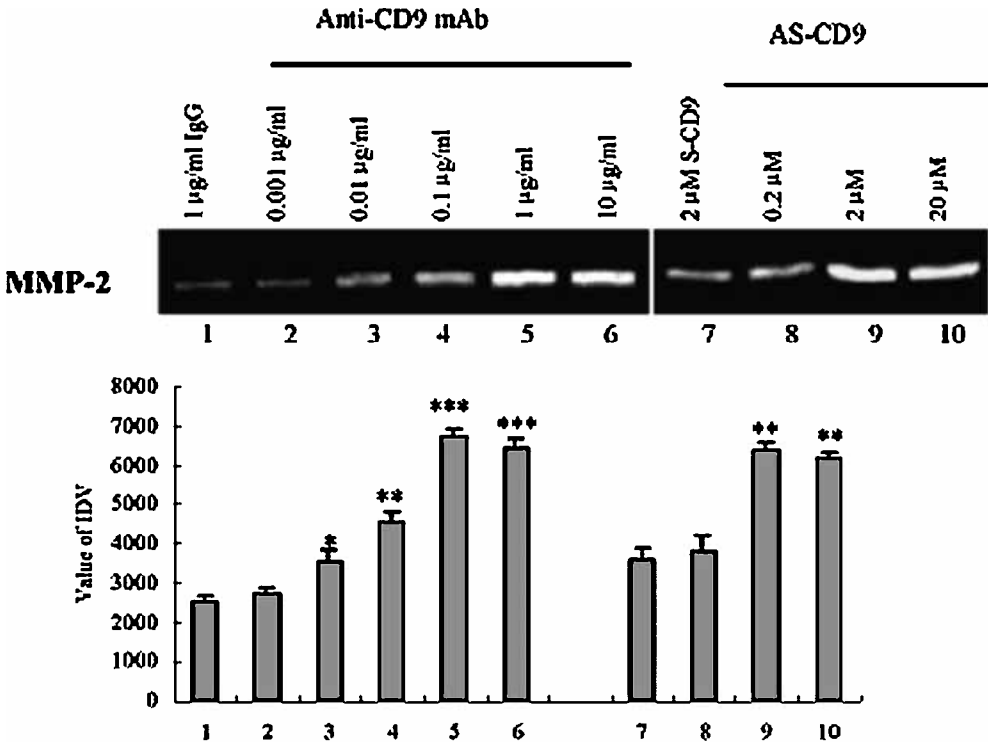

Figure 4 Anti-CD9 mAb and AS-CD9 induce production of MMP-2. Blastocysts were incubated for 72 h on petri dishes coated with FN in serum-free media supplemented with different concentrations of anti-CD9 mAb or AS-CD9 as shown. Controls were treated with 0·1 μg/ml isotype rat IgG (lane 1) or 2 μM S-CD9 (lane 7). Conditioned media were analyzed by gelatin zymography. Gelatinolytic activities of the samples were quantified by scanning densitometry using the AlphaImager V5·5 software package. IDV indicates a relative scanning value (n=50 blastocysts per well). *$P<0·05$, **$P<0·01$, ***$P<0·001$ versus control.

production. Hatched embryos were incubated with anti-CD9 mAb or AS-CD9 for 72 h on FN-coated Petri dishes, and the conditioned media were analyzed by gelatin zymography. As illustrated in Fig. 4, treatment with anti-CD9 mAb and AS-CD9 enhanced the production of MMP 2 by 2–3 fold.

Effects of pharmacological inhibitors on anti-CD9 mAb- or AS-CD9-induced blastocyst invasion ability

In an attempt to determine which signaling pathways were involved in the regulation of anti-CD9 mAb- and AS-CD9-induced blastocyst invasion, blastocysts were cultured on the monolayer of uterine epithelial cells in the presence of various pharmacological inhibitors, which specifically inhibit key regulators of different signaling pathways. The results of these experiments are shown in Fig. 5. Notably, a specific inhibitor to PI3K, LY294002, inhibited the CD9-mediated percentage of outgrowth and blastocyst outgrowth area. In contrast, similar treatments with MEK, p38, or JAK2 inhibitors had modest effects on CD9-mediated outgrowth ability of blastocysts. In addition, we investigated the effects of inhibitors such as PD98059, AG490, SB203580, and LY294002 on JAR cells (human trophoblastic cell line) and testified that they were working. Under normal culture conditions, LY294002 had little role in blastocyst outgrowth (data not shown). Collectively, these results demonstrated that CD9-mediated outgrowth ability of blastocysts involves the PI3K signaling pathway.

Next we examined the effects of different inhibitors on production of MMP-2. Of the various inhibitors tested, we found that only LY294002 (inhibitor of PI3K) had a negative effect on MMP-2 production induced by anti-CD9 mAb and AS-CD9 (Fig. 6). Thus we concluded that PI3K-dependent signaling pathway makes an essential contribution to the production of MMP-2 triggered by CD9 protein.

Discussion

Blastocyst adhesion and invasion into uterine endometrium are two important steps of embryo successful

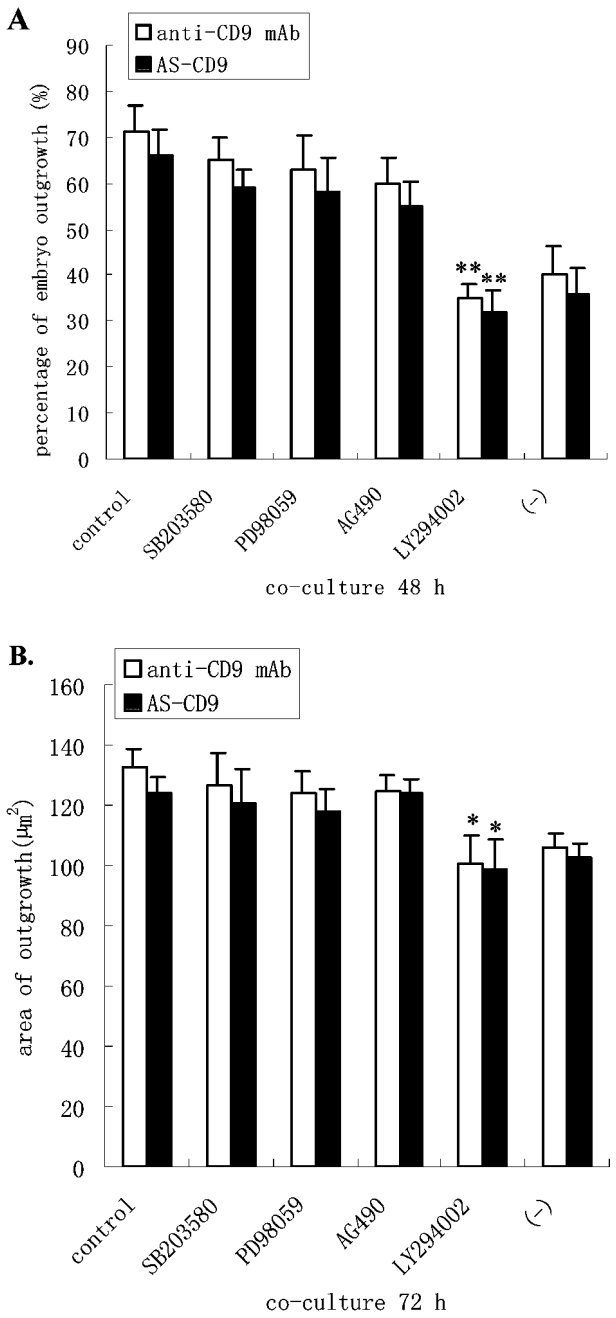

Figure 5 Effect of the pharmacological inhibitors on the anti-CD9 mAb- and AS-CD9-mediated spreading ability of blastocysts. The hatched blastocysts were incubated for 48 or 72 h on the monolayer of uterine epithelial cells in media containing various pharmacological inhibitors as shown. Control indicates anti-CD9 mAb or AS-CD9 only. (–) Indicates isotype rat IgG or S-CD9. Concentrations of anti-CD9 mAb and AS-CD9 were 0·1 μg/ml and 2 μM, respectively. (The concentration of LY294002 was 10 μM and the concentrations of PD98059, AG490 and SB203580 were 50 μM) (A) The effect on percentage of outgrowth. (B) The effect on outgrowth area. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ versus control.

implantation. To gain an insight into the cellular and molecular mechanisms that control this process, we investigated the attachment and outgrowth behavior of blastocysts *in vitro* on a monolayer of uterine epithelial cells, a widely used mimic of uterine conditions. In this study, for the first time we have demonstrated the expression profile of CD9 in pre-implantation embryos and the functional role that CD9 plays in embryo implantation in the mouse. Results showed that CD9 was expressed on blastomeres of two-, four-, and eight-cell and morula embryos and on trophoblast cells of blastocysts, and that CD9 is involved in the regulation of embryo invasion and MMP-2 production through the PI3K signaling pathway.

Although CD9 protein was expressed in endometrium epithelial cells and pre-implantation embryos, it played an inhibitory role in embryo implantation. At present, the ligands or mediators for CD9 have not been confirmed, but there is evidence to suggest that morphological or functional alteration of the CD9 molecule by anti-CD9 mAb can lead to significant biological changes in various cells, such as anti-CD9 mAb (KMC8) activating mouse macrophages; inducing cell aggregation in the mouse macrophage cell line J774 and causing hematopoietic cells to migrate (Forsyth 1991, Jennings *et al.* 1990, Kaji *et al.* 2001, Aoyama *et al.* 1999). We showed that expression of CD9 on blastocysts was too low to be detected after blastocysts were transferred to culture medium containing AS-CD9 for 24 h. At the same time, AS-CD9 and anti-CD9 mAb had similar effects on embryo outgrowth and MMP-2 production, so we assumed that anti-CD9 mAb played an important role in embryo implantation by blocking the CD9 extracellular domain, although the mechanism of which ligands contact with CD9 is not fully understood.

In addition, CD9 seems to act as a suppressor of metastasis because its transfection into melanoma cells reduces their metastatic potential and an inverse correlation between expression of CD9 and appearance of metastasis in melanoma, colon, lung, and breast cancers has been described (Gutierrez-Lopez *et al.* 2003, Higashiyama *et al.* 1995, Miyake *et al.* 1995, 1996, Mori *et al.* 1998). However, when $CD9^{-/-}$ or $CD9^{+/+}$ two-cell embryos were transferred into wild-type pseudopregnant females, the rate of embryos developing to term was not significantly different (42 and 24%, respectively; Miyado *et al.* 2000). There exist some discrepancies between their results and ours, which may be due to anti-CD9 mAb blocking simultaneously CD9 antigen in blastocyst and uterus epithelium. Although similarities between trophoblast invasion and tumor cell invasion have been investigated extensively, the factors which limit trophoblast invasion within the uterus and which cause these invading trophoblasts to revert into their quiescent, non-invasive state are largely unknown.

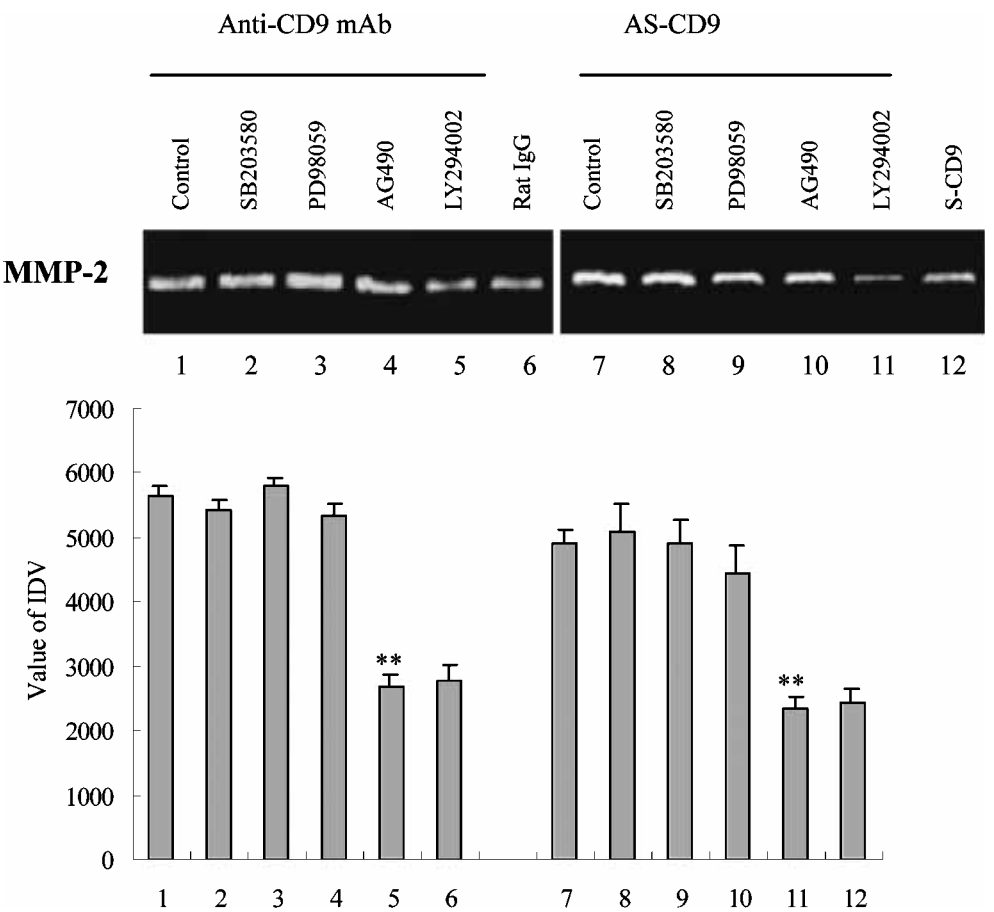

Figure 6 Effect of pharmacological inhibitors on anti-CD9 mAb- or AS-CD9-induced production of MMP-2. The blastocysts were incubated for 72 h on a petri dishes coated with FN in serum-free growth media supplemented with anti-CD9 mAb or AS-CD9 and different pharmacological inhibitors as shown. Concentrations of anti-CD9 mAb and AS-CD9 were 0·1 μg/ml and 2 μM, respectively. The concentrations of LY294002 was 10 μM, PD98059 and AG490, SB203580 were 50 μM). $^{**}P<0{\cdot}01$ versus control.

Given the expression of CD9 in the uterus epithelium and embryo and the effect of anti-CD9 mAb on embryo invasion, we assumed that this role might be related to avoidance of excessive trophoblast invasion during embryo implantation by blocking CD9 of blastocyst and uterus epithelium.

Several earlier studies have shown that the contribution of CD9 to migration or adhesion seems to be linked to signaling pathway components such as signal transduction and activators of transcription (STAT), PI3K, or MAPK in cancer cells (Hemler 1998, Wang *et al.* 2002). Sugiura & Berditchevski (1999) have reported that CD9 controlled invasive migration of tumor cells through two distinct PI3K-dependent mechanisms. As a part of the current study, we specifically addressed the question as to whether or not the CD9 molecule is also linked to the PI3K signaling pathway in the process of embryo implantation. In agreement with Sugiura & Berditchevski's reports in human breast cancer MDA-MB-231 cells, our data indicated that this link was possible. We found that LY29004, a specific inhibitor of PI3K, completely abolished the effect of anti-CD9 mAb- or AS-CD9-induced blastocyst outgrowth. These data illustrated a functional connection between CD9 and the PI3K-dependent pathway in embryo implantation.

Embryo implantation is a balanced process of extracellular matrix degradation and re-establishment. Although different groups of extracellular matrix-degrading enzymes may be potentially involved, previous reports indicated that MMPs have a major role in embryo degradation of the basement membrane of the uterine epithelium and invasion of the uterine stroma (Alexander *et al.* 1996, Behrendtsen *et al.* 1992). Among these MMPs, MMP-2 is a major enzyme secreted by *in vitro*-cultured embryos. In addition, production of MMP-2 is clearly regulated in both

cultured embryos and cancer tissue. However, surprisingly little is known about the signaling pathways that control this process. Our data indicated that both anti-CD9 mAb and AS-CD9 enhanced the production of MMP-2 of blastocysts cultured on FN. Notably, this role did not involve the activation of cAMP-dependent protein kinase, but instead required the activity of PI3K. These data suggested that CD9 modulated MMP-2 production of blastocysts cultured on FN via the PI3K signaling pathway. This result is in agreement with studies of MMP-2 in NIH3T3 and MDA-MB-231 cells (Sugiura & Berditchevski 1999, Choi *et al.* 2004).

Taken together, the results obtained in these four different functional assays showed that CD9 is a suppressor of embryo implantation and MMP-2 production in the mouse and that this inhibitory effect is mediated by the PI3K signaling pathway. In addition, the role of CD9 occurred at the embryo invasion stage, not the adhesion stage.

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