Zygote **10** (November), pp 317–326. © 2002 Cambridge University Press
DOI: 10.1017/S0967199402004057 Printed in the United Kingdom

Localisation of the hyaluronan receptor CD44 in porcine cumulus cells during *in vivo* and *in vitro* maturation

Masaki Yokoo[1,2], *Paisan Tienthai*[1,2,3], *Naoko Kimura*[1,2], *Koji Niwa*[3], *Eimei Sato*[2] *and Heriberto Rodriguez-Martinez*[1]
Swedish University of Agricultural Sciences, Uppsala, Sweden, Tohoku University, Sendai, Japan and Okayama University, Okayama, Japan

Date submitted: 5.5.02. Date accepted: 25.7.02

Summary

Polyspermy is fairly common during porcine *in vitro* fertilisation (IVF), perhaps due to incomplete *in vitro* oocyte maturation (IVM). Porcine cumulus cells (CCs) layered around the oocyte produce large amounts of extracellular hyaluronan (HA) when forming an expanding cell cloud during the last phase of oocyte maturation. The specific actions of HA are mediated via HA-binding proteins (HABPs), such as CD44, which act as receptors. In this study using immunocytochemistry and western blotting we investigated the localisation of CD44 in CCs obtained from *in vivo*-matured pig cumulus–oocyte complexes (COCs) and compared it with that in CCs from immature COCs and of COCs subjected to IVM and IVF procedures. Immunolabelling of CD44 was absent or very weak in CCs from immature COCs but strongly present on the surface of the CCs obtained from *in vivo*, displaying a similar localisation in the *in vitro*-matured COCs. In the latter, the labelling decreased but did not disappear in CCs 4 h after sperm co-incubation during IVF. Immunoblotting detected bands of between 73 and 88 kDa, corresponding to CD44, in the protein extract from *in vivo* CCs collected immediately prior to, or following spontaneous ovulation. The *in vitro*-matured CCs, however, presented bands ranging from 81 kDa to 88 kDa. Also, the bands found in the *in vivo*-matured CCs showed a larger variation of intensity and migration among animals than did the batches of *in vitro*-matured CCs. No CD44 band was detected on aliquots of the frozen-thawed boar spermatozoa used for IVF. The results clearly demonstrate that the specific HA receptor CD44 is present in expanding CCs of *in vivo*-matured pig COCs, in relation to increasing amounts of inter-CC HA. The subtle differences in molecular weight and migration ability observed between *in vivo* and *in vitro* samples may relate to differences in glycosylation and thus explain differences in HA-binding ability, of consequence for optimising *in vitro* culture conditions.

Keywords: CD44, Cumulus cells, Hyaluronan (HA), Oocyte maturation, Pig

All correspondence to: Dr Paisan Tienthai, Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences (SLU), Box 7039, SE-750 07 Uppsala, Sweden. Tel: +46 18 672323. Fax: +46 18 673545. e-mail: paisan.tienthai@og.slu.se
[1]Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences (SLU), SE-750 07 Uppsala, Sweden.
[2]Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, Sendai 981–8555, Japan.
[3]Division of Animal Science and Technology, Faculty of Agriculture, Okayama University, Okayama 700–8530, Japan.

Introduction

The cumulus–oocyte complex (COC) is a structural unit of the ovarian follicle and consists of several layers of compacted cumulus cells (CCs) closely surrounding the oocyte (Moor *et al*., 1980). In response to the pre-ovulatory gonadotropin surge, oocytes resume meiosis and the CCs are induced to synthesise hyaluronan (HA) which, deposited in the intercellular spaces, leads to the characteristic pre-ovulatory expansion of COCs (Salustri *et al*., 1992; Mattioli, 1994). In addition, CCs are very important for the completion of oocyte cytoplasmic maturation, a prerequisite to acquiring the capacity to support male pronuclear formation, counteract

polyspermic fertilisation and facilitate early embryonic development (Chain *et al*., 1994; Yamauchi & Nagai, 1999).

HA is a non-sulfated glycosaminoglycan that is found in extracellular matrices, at the cell surface and inside the cells and is related to several molecular events during cell behaviour (Toole, 2001). The localisation of HA is strongly expressed in the CC layer during *in vitro* maturation (IVM) of rodent oocytes (Salustri *et al*., 1992). A high concentration of HA is also found in the porcine follicular fluid (Saito *et al*., 2000) and in the oviductal fluid (Tienthai *et al*., 2000). HA has also been found to be beneficial during *in vitro* fertilisation (IVF) and early embryo development in pigs. For instance, addition of exogenous HA to fertilisation medium has been reported to increase the rate of monospermic penetration (Suzuki *et al*., 2000). Exogenous HA has been found *in vitro* to support early pig embryo development to the blastocyst stage (Miyoshi *et al*., 1999). The effects of HA are mediated by a multitude of HA-binding proteins (HABPs) which interact with HA within the extracellular matrix or at the plasma membrane level. At the plasma membrane level HABPs act as cell surface receptors, as is the case with the HA receptors CD44 and RHAMM (Knudson & Knudson, 1993).

The main cell surface receptor for extracellular matrix HA is thought to be CD44 (Aruffo *et al*., 1990). The interaction between the cell surface receptor CD44 and HA has been proposed to mediate a variety of cellular functions, including cell migration (Thomas *et al*., 1992), cell–matrix adhesion (Carter & Wayner, 1988) and cell–cell adhesion (St John *et al*., 1990). CD44 is expressed in a wide variety of cell types such as lymphocytes and epithelial cells (Alho & Underhill, 1989; Lesley *et al*., 1993). It is also expressed in *in vitro*-matured human CCs (Ohta *et al*., 1999) and preimplantation embryos in mammals (Campbell *et al*., 1995). Recently CD44 mRNA was also detected in porcine CCs (Kimura *et al*., 2002).

Polyspermy rarely occurs *in vivo*, but it is an unsolved problem during IVF in pigs. To disclose a possible role for HA-modulating sperm capacitation and interactions during *in vivo* fertilisation (for a review see Rodriguez-Martinez, 2000) requires increasing our knowledge of the location of its receptor in target cells. Given the present scarcity of data concerning CD44 localisation in *in vivo*-matured oocytes, we have attempted in this study to determine whether the HA receptor CD44 is expressed in the COCs of the pig during oocyte maturation *in vivo* compared with standard IVM, using immunocytochemistry and western blotting.

Materials and methods

Collection of *in vivo*-matured cumulus–oocyte complexes

Multiparous cross-bred (Swedish Yorkshire × Swedish Landrace) sows (n = 4, parity 2–5) were recruited for experiments from a commercial farm on the day of weaning, and individually penned at the Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. The sows received water *ad libitum* and standard ratio, according to Swedish standards (Simonsson, 1994). A fertile boar was always penned in the vicinity and the sows were checked twice daily by experienced personnel for spontaneous, behavioural oestrus. At the onset of oestrus, the sows were scanned every 4 h by transrectal ultrasonography (Mburu *et al*., 1995). The time of ovulation was defined as 2 h before the first signs of a significant reduction in the number of pre-ovulatory follicles or when pre-ovulatory follicles could no longer be seen. At that point the sows were killed and the ovaries and oviducts promptly removed. The COCs were collected by aspiration from the thus defined peri-ovulatory follicles or by flushing the oviductal ampulla. Between 20 and 25 COCs were collected per animal. Three to five COCs from each animal were used in immunocytochemistry and the rest in immunoblotting. In addition, ovarian tissue was fixed for immunohistochemistry, as described below. The experimental design was reviewed and approved by the local Ethics Committee for Experimentation with Animals in Sweden.

Collection of immature, *in vitro*-matured and *in vitro*-fertilised cumulus–oocyte complexes

For collection of immature COCs, ovaries were collected from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory within 1.0–1.5 h in 0.9% (w/v) NaCl containing 75 µg/ml potassium penicillin G (Meiji Seika, Tokyo, Japan) and 50 µg/ml streptomycin sulfate (Meiji Seika) at 37–39 °C. COCs were aspirated from antral follicles (2–6 mm in diameter) with an 18 gauge needle fixed to a 10 ml disposable syringe.

For collection of *in vitro*-matured COCs, COCs were washed three times with the maturation tissue cultured medium-199 (TCM-199 with Earle's salt and L-glutamine; Gibco, Grand Island, NY), either supplemented with 10% (v/v) porcine follicular fluid (pFF) and 10 IU/ml pregnant male serum gonadotropin (PMSG) (Serotropin; Teikoku-Zoki, Tokyo, Japan) or without supplementation. The TCM-199 contained 2.2 mg/ml sodium bicarbonate (Sigma, St Louis, MO), 0.1 mg/ml sodium pyruvate (Sigma), 10 mg/ml bovine

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serum albumin (BSA; Sigma), 100 IU/ml penicillin (Meiji Seika) and 100 µg/ml streptomycin (Meiji Seika). Fifty COCs with uniform ooplasm and compact CC mass were transferred to a 500 µl drop of either medium which had previously been covered with warm paraffin oil in a polystyrene culture dish (35 mm × 10 mm; Becton Dickinson, Franklin Lakes, NJ) and equilibrated in an atmosphere of 5% CO_2 in air for about 12 h, and cultured for 44 h at 39 °C under the same atmospheric conditions.

For collection of *in vitro*-fertilised COCs, cultured COCs were washed three times with modified Tris-buffered medium (mTBM), as described by Abeydeera & Day (1997), containing 2.5 mM caffeine (Sigma) and 0.4% (w/v) BSA (Sigma). Fifteen COCs were placed in a 50 µl drop of mTBM covered with warm paraffin oil in a culture dish (35 mm × 10 mm; Becton Dickinson), as above. The dishes were kept in the same incubator (5% CO_2 in air at 39 °C) for about 30 min until the spermatozoa were added for fertilisation. Spermatozoa were prepared as described by Wang *et al.* (1991), except that mTBM rather than TCM-199 was used as fertilisation medium and Dulbecco's phosphate-buffered saline (Dulbecco's PBS; Sigma) was used for washing medium. Briefly, two 0.1 ml pellets of frozen ejaculated spermatozoa obtained from boars of proved fertility were thawed in 10 ml of Dulbecco's PBS containing 0.1% (w/v) BSA (Sigma) at 37 °C. Immediately after thawing, the suspensions were washed three times in the same medium by centrifugation at 550 *g* for 5 min, three times. The sperm pellet was then re-suspended with fertilisation medium to give a concentration of 6×10^6/ml spermatozoa and pre-incubated at 39 °C under 5% CO_2 in air for 10–15 min. After pre-incubation, the spermatozoa were checked for motility and 50 µl of the sperm suspension was added to the *in vitro* fertilising drop with COCs. The COCs were co-incubated with spermatozoa for 4 h under 5% CO_2 in air at 39 °C.

About 200 COCs at different conditions (i.e. immature, and *in vitro* fertilised and matured) were used for immunoblotting analyses and 20 for fluorescent immunolocalisation.

CD44 immunolocalisation

In vitro-matured COCs (n = 24) collected from periovulatory follicles and/or the oviductal ampulla following spontaneous ovulation were fixed overnight in a 1% (v/v) solution of paraformaldehyde in 0.15 M PBS (pH 7.35) and conventionally embedded in paraffin. Also, ovarian tissue samples holding antral follicles were fixed and processed in the same manner. Sections 4 µm thick were cut and mounted on poly-L-lysine-coated microscope slides. After incubating at 40 °C for 4 h, the sections were deparaffinised and rehydrated. The sections were rinsed in Tris-HCl buffer (pH 7.4), coated sequentially for 15 min with avidin and biotin (Vector Laboratories, Burlingame, CA) followed with 5 min inter- and post-rinsing with Tris-HCl. Endogenous peroxide activity was eliminated by incubation with 3% (v/v) H_2HO_2 in methanol for 20 min. All sections were rinsed in Tris-HCl and incubated in normal horse serum at a dilution of 1:10 for 30 min at room temperature. After this step the sections without rinsing were incubated with the primary monoclonal antibody (anti-porcine CD44, PORC24V, VMRD, Pullman, WA) diluted with Tris-HCl at 1:100 at 4 °C overnight. The sections were rinsed in Tris-HCl for 5 min before applying a secondary biotinylated horse anti-mouse antibody at a dilution of 1:500 for 30 min at room temperature. After rinsing, the sections were incubated with Vectastain-Elite ABC-mouse reagent (Vector Laboratories) at a dilution of 1:100 for 30 min at room temperature and rinsed again. Immunostaining was visualised using 0.1% (v/v) diaminobenzidine (DAB) tetrahydrochloride (DAKO, Carpinteria, CA) in Tris-HCl mixed with 3% (v/v) H_2O_2 in Tris-HCl, pH 7.6, at room temperature. The sections thus processed were counterstained with Mayer's haematoxylin. The primary antibody was replaced with mouse IgG1 monoclonal antibodies (DAKO) and the immature CCs and follicular cells within the follicle were used as the negative controls. All sections were examined and photomicrographs taken (Nikon microphot-FXA microscope, Tokyo, Japan).

Immature, *in vitro* COCs matured in TCM-199 with pFF and PMSG, and *in vitro*-fertilised COCs (n = 80) were fixed in 2% (v/v) formaldehyde in PBS-PVP (pH 7.4), i.e. PBS-minus (Nissui, Tokyo, Japan) containing 3 mg/ml polyvinylpyrrolidone (PVP, Sigma), 100 IU/ml penicillin (Meiji Seika) and 100 µg/ml streptomycin (Meiji Seika) at 4 °C for 30 min. Thereafter they were washed three times with PBS-BSA, i.e. PBS-minus (Nissui) containing 3 mg/ml BSA (Sigma), 100 IU/ml penicillin (Meiji Seika) and 100 µg/ml streptomycin (Meiji Seika). The samples were permeabilised in 0.25% (v/v) Tween 20 in PBS-PBA at room temperature for 2 min and washed three times in PBS-BSA; thereafter they were stored in PBS-BSA containing 0.02% (w/v) sodium azide at 4 °C for 0–7 days. Fixed COCs were incubated in the primary monoclonal antibody (anti-porcine CD44, PORC24A, VMRD) diluted 1:50 in PBS-BSA at 37 °C for 24 h. After washing for 30 min in PBS-BSA-T, i.e. PBS-BSA containing 0.5% (v/v) Triton X-100 for permeabilisation, these samples were exposed to fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma) diluted 1:200 in PBS-BSA at 37 °C for 1 h 30 min. To detect nuclear localisation, the stained samples were incubated in 10 µg/ml propidium iodide (PI) at 37 °C for 1 h. The samples were washed in PBS-BSA-T and mounted under a coverslip with PBS-BSA. Whole

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mounted specimens were placed on an Axioplan Zeiss microscope (Carl Zeiss, Jena, Germany) and the staining of CD44 examined using a Bio-Rad MRC-1024 confocal laser scanning microscope unit (Bio-Rad Laboratories, Hercules, CA).

Protein extraction from cumulus cells and spermatozoa for western blot analysis

Two hundred COCs collected from immature, IVM and IVF conditions and 80 *in vivo* COCs from four sows were treated with 0.03% (w/v) hyaluronidase (Sigma) in Dulbecco's PBS (Nissui) for 15 min at 37 °C. The CCs were separated from COCs using a 30 µm Nylon mesh filter and centrifuged at 500 *g*. The separate CCs were washed three times in Dulbecco's PBS and stirred in extraction buffer consisting of 50 mM Tris (pH 7.5), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 M 6 amino-*n*-caproic acid, 5 mM benzamidine HCl and 1% (v/v) 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate or CHAPS (all chemicals from Sigma) at 4 °C for 1 h. The samples were centrifuged at 15 000 *g* for 30 min and the supernatants removed and stored at –20 °C.

A 0.1 ml pellet of the frozen boar spermatozoa was thawed in 1 ml of warm Dulbecco's PBS (Nissui) and spermatozoa were washed three times in Dulbecco's PBS by centrifugation at 10 000 *g* for 5 min. Thereafter proteins were extracted as described above.

CD44 western blotting

Extracted proteins of CCs at different conditions and spermatozoa (20 µg/lane) were separated by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under non-reducing conditions and electroblotted in a semi-dry blotting apparatus as described by Hirano & Watanabe (1990). Electroblotting was performed for 90 min at 0.8 mA/cm constant current and the membranes were blocked with 2% (w/v) skim milk in 0.05% (v/v) Tween 20 in PBS (PBS-T) overnight at 4 °C. After washing three times with PBS-T the membranes were incubated with anti-porcine CD44 (PORC24, VMRD) diluted in PBS-T 1:1000 for 1 h at room temperature and then reacted with secondary antibody (horseradish peroxidase-labelled anti-mouse IgG; Sigma) diluted in PBS-T 1:80 000. Following washing three times with PBS-T, the peroxidase activity was visualised using the electrochemiluminescence (ECL) western blotting detection system according to the manufacturer's instructions (Amersham Biotech, Arlington Heights, IL).

Results

Immunochemical localisation of CD44 in cumulus cells

CD44 was detected in CCS of mature COCs either using an ABC method (*in vivo* matured specimens; Fig. 1) or by indirect immunoflourescence (*in vitro* matured and *in vitro* fertilised specimens; Fig. 2). CD44-positive staining with the ABC method was present clearly on the surface of mature CCs and the cytoplasm of some CCs during maturation *in vivo*. However, immunolabelling was not detected on either immature COCs or on any other follicle cell components or antral follicles in the porcine ovary (Fig. 1*a*), the zona pellucida or the ooplasm of the *in vivo*-matured oocyte (Fig. 1c). Using indirect immunofluorescence (*in vitro* incubation) there was absent to very weak immunostaining in the CCs of the immature COCs (Fig. 2*a*), whereas strong immunolabelling was present in the CCs surrounding the IVM oocytes (Fig. 2*b*). Immunostaining of CD44 was intense on the cell membrane and in the cytoplasm of the IVM-CCs (Fig. 2*b*). No staining was present on the zona pellucida, the ooplasm, or the plasma membrane of spermatozoa (Fig. 2*c*) interspersed with the CCs. The immunostaining seemed to decrease in intensity after sperm co-incubation (IVF; compare Fig. 2*b* and *c*).

CD44 western blot analyses

In contrast to immature COCs (lane 1), the CCs matured *in vivo* (collected either just prior to expected spontaneous ovulation (lanes 2–4) or from the oviductal ampulla, lane 5) showed bands of CD44 HABP ranging from 73 kDa to 88 kDa (Fig. 3). The CCs of *in vitro*-matured COCs, collected from offal ovaries and cultured in TCM-199 without pFF and PMSG, did not show any band (Fig. 3, lane 6). Inclusion of pFF and PMSG in the culture medium revealed that *in vitro*-matured COCs did present bands with strong intensity and a narrower range than for *in vivo* CCs, consistently between 81 kDa and 88 kDa (Fig. 3, lane 7). A similar band, albeit weaker, was still present in COCs undergoing IVF (spermatozoa present among CCs; Fig. 3, lane 8). It is noteworthy that the bands found among the *in vivo* samples showed a higher variation in intensity and range between batches (animals), every band migrating more rapidly and the molecular weight being somewhat different from that determined in the *in vitro* samples (batches). The CD44 band was absent in the lane for frozen-thawed boar spermatozoa (Fig. 3, lane 9).

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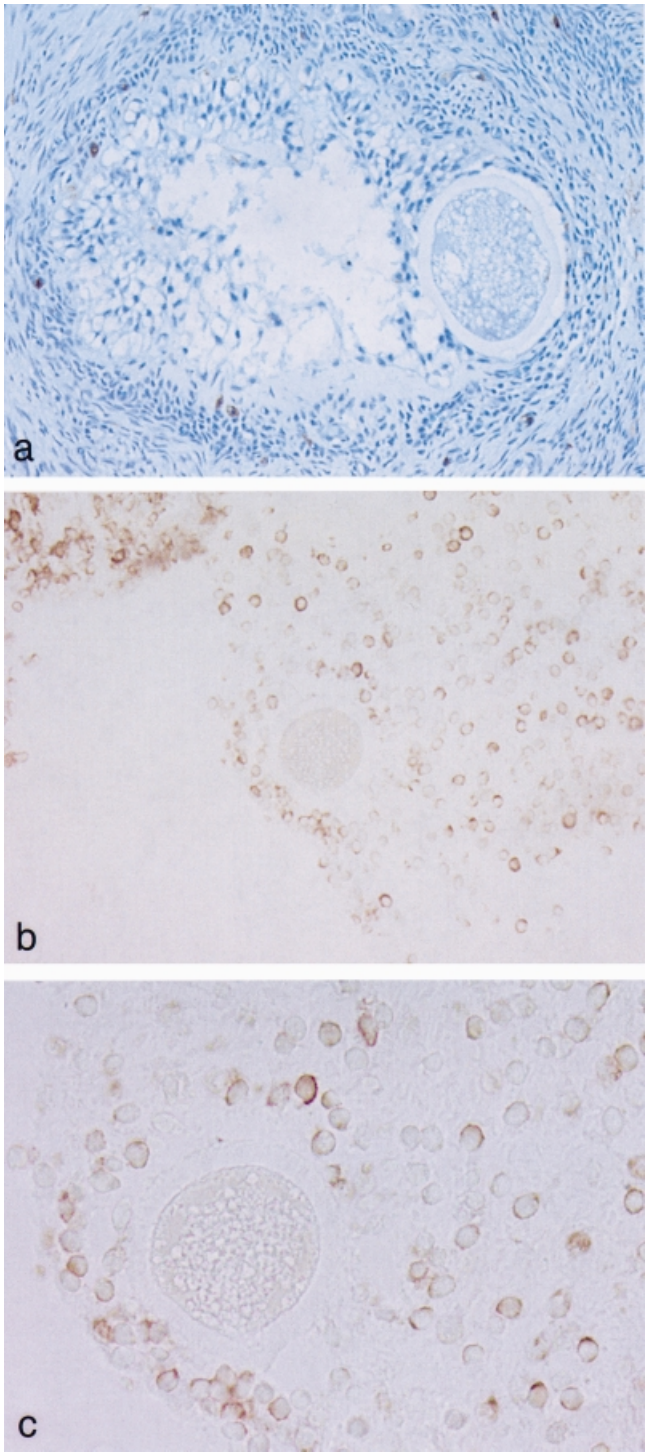

Figure 1 Immunolocalisation of CD44 in porcine COCs using an ABC method (Brown staining). (*a*) Immature COCs and follicular well cells in antral follicles. (*b*), (*c*) Strong CD44 immunolabelling on the surface of CCs during COC expansion (COCs aspirated from fully grown follicles). Magnification ×100 (*a*, *b*), ×200 (*c*).

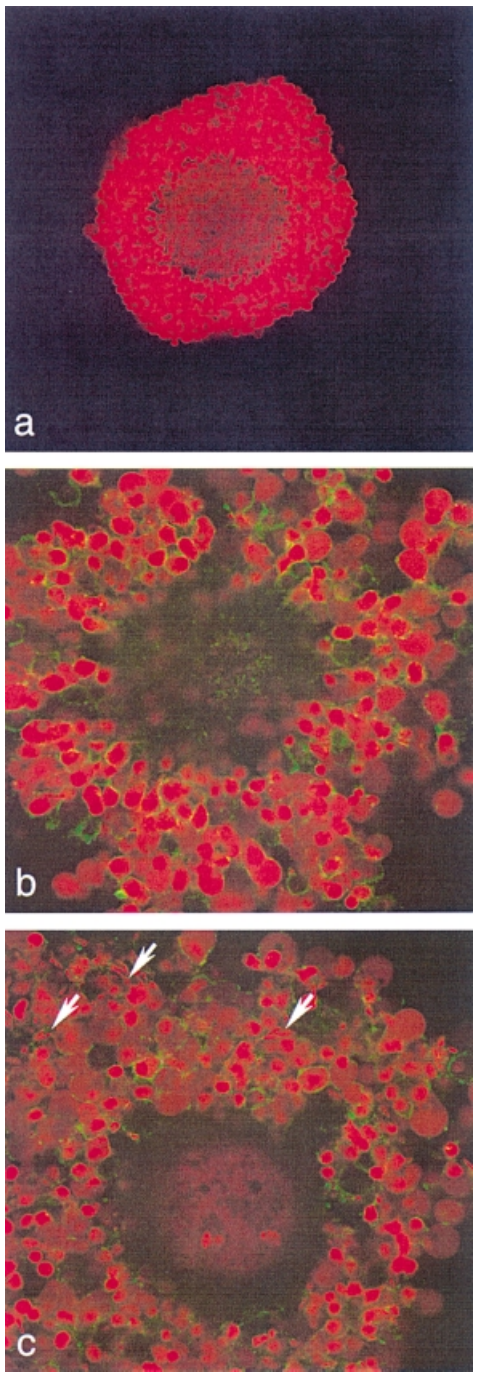

Figure 2 Immunofluorescence of CD44 (stained in green) of *in vitro*-cultured porcine COCs. (*a*) Immature COC; (*b*) COCs during expansion after completed IVM (culture including pFF and PMSG); and (*c*) COCs during IVF (spermatozoa added 4 h previously, arrows). Magnification ×200 (*a*), ×400 (*b*, *c*). Red fluorescence corresponds to PI nuclear loading.

Discussion

In this study, immunostaining for the HA receptor CD44 was clearly present on the surface of porcine CCs during cumulus expansion *in vivo*, while CD44 immunostaining was absent in antral follicles. CD44

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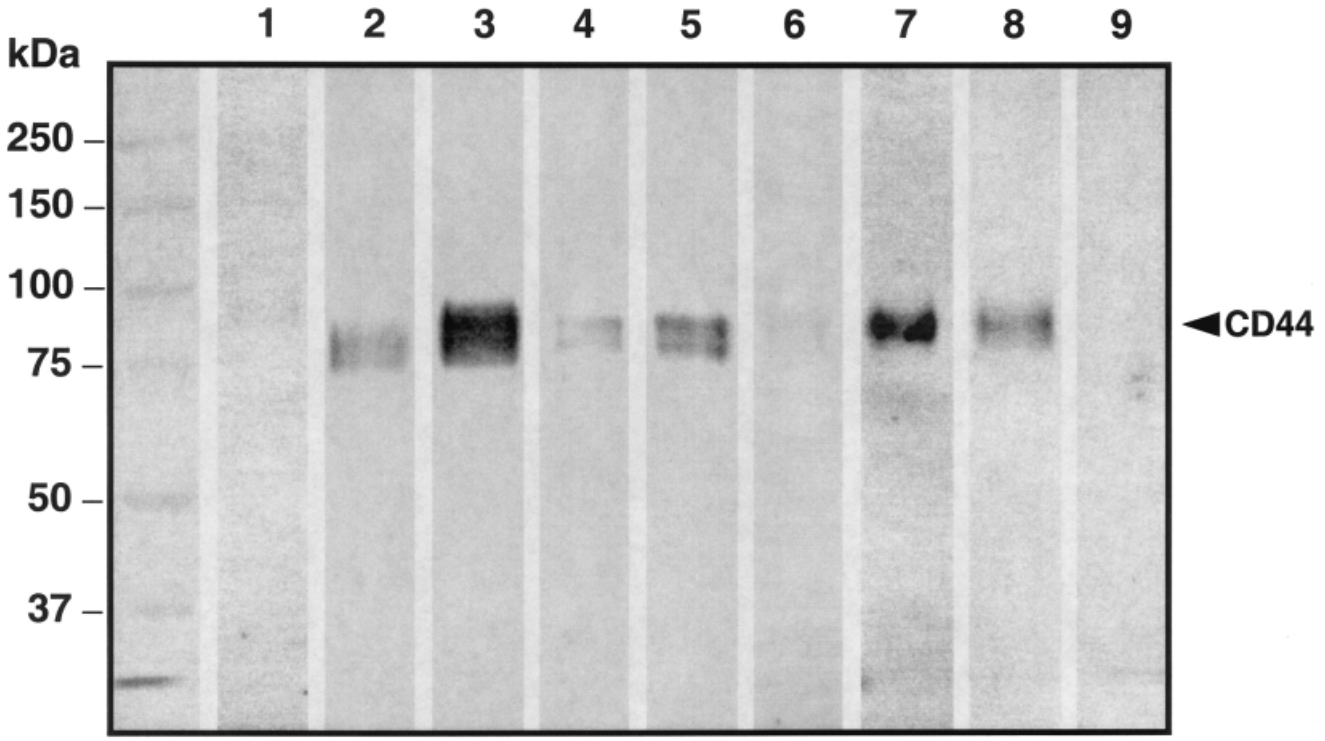

Figure 3 Western blot analysis for CD44 HABP expression of porcine cumulus cells and frozen-thawed spermatozoa. Lane 1, immature COCs; lanes 2–4, *in vivo*-matured COCs (aspirated from fully grown follicles, just before spontaneous ovulation); lane 5, *in vivo*-matured COCs (flushed from the oviductal ampulla immediately after spontaneous ovulation); lane 6, *in vitro*-matured COCs cultured in TCM-199 without pFF and PMSG; lane 7, *in vitro*-matured COCs cultured in TCM-199 with pFF and PMSG; lane 8, cumulus cells with spermatozoa in *in vitro*-fertilised COCs; lane 9, frozen-thawed boar spermatozoa.

immunostaining was also absent (or very weak) in immature COCs collected for *in vitro* culture, reaching maximum intensity by the end of the IVM procedure concomitantly with maximal CC expansion, to decrease thereafter during sperm co-incubation (IVF), thus confirming our previous findings (Kimura *et al*., 2002) and in agreement with other species (Ohta *et al*., 1999). The present study also determined immunoreactive bands of 73–88 kDa molecular weight among the extracted proteins of *in vivo*-matured CCs, which was different from the narrower range of 81–88 kDa bands for *in vitro*-matured CCs. In a previous study we confirmed that out of 13 bands of HABPs found in porcine COCs during *in vitro* culture the protein band of about 85–90 kDa, the specific band for CD44 (Yokoo *et al*., 2001), was the strongest. To the best of our knowledge, no reports have been published to demonstrate the molecular weight of CD44 of *in vivo* porcine CCs.

Although pig oocytes matured *in vitro* can be penetrated by spermatozoa under appropriate conditions, this is associated with low rates of pronuclear formation and a high incidence of polyspermy (Hunter, 2000). This implies that conditions *in vitro* are clearly inferior to those *in vivo* for reasons that are not yet fully disclosed. The cellular relationship between the oocyte and somatic follicular cells, especially the CCs, is fundamental to oocyte maturation, particularly during the 36–40 h prior to spontaneous ovulation (Moore *et al*., 1990). Culture of oocytes in a chemically defined medium has shown that biological factors such as gonadotropins and CCs affected the final steps of both nuclear and cytoplasmic maturation (Sun *et al*., 2001). Several follicular factors are associated with the modulation of oocyte maturation, such as gap junctions (Racowsky & Baldwin, 1989), cyclic adenosine monophosphate (cAMP) (Dekel & Beers, 1980), calcium (Goren *et al*., 1990) and growth factors (Carneiro *et al*., 2001).

It is known that CD44 is a transmembrane protein consisting of extracellular and cytoplasmic domains linked through transmembrane segments in the cell membrane of a variety of cells (Bajorath, 2000). The extracellular domain of CD44 contains the necessary motifs for recognising HA (Lesley & Hyman, 1998) and this recognition provides a physiologically important switch between its adhesive and signalling functions (Aruffo, 1996). For instance, HA can reduce the incidence of apoptotic CCs via CD44 (Kaneko *et al*., 2000).

Accumulation of HA, produced by the CCs, promotes detachment of the COCs from the follicle wall in preparation for ovulation (Saito *et al*., 2000). The synthesis of HA by CCs appears to be controlled at the

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level of transcription of mRNA for hyaluronan synthase 2 (HAS2) (Fulop *et al*., 1997). We have recently suggested that the degree of cumulus expansion is related to the level of HAS2 mRNA and also to the expression of CD44 mRNA (Kimura *et al*., 2002). Furthermore, the addition of follicle-stimulating hormone (FSH), PMSG or pFF to the culture medium has been shown to stimulate cumulus expansion (Nagyova *et al*., 1999; Kimura *et al*., 2002). These findings indicate that gonadotropins modulate the synthesis of HA/CD44 in CCs during the expansion process. The present findings, with CD44 immunolabelling clearly present during cumulus expansion *in vivo* but present *in vitro* only when exogenous gonadotropins were added to the culture maturation medium, provide evidence for this assumption in the pig, thus suggesting, albeit indirectly, a role for CD44 in the modulation of final *in vivo* oocyte maturation in this species.

CD44 exists in a number of isoforms of different molecular size from 80 to 250 kDa, with the most common form of CD44, the so-called CD44 standard (CD44s), being about 85–90 kDa (Lesley & Hyman, 1998). Therefore, the bands of *in vitro*- and *in vivo*-matured CCs observed by us in the present study are likely in the range of CD44s.

In the present study, however, a fairly consistent CD44 band of 81–88 kDa appeared in all samples of CC expansion after IVM and during IVF. In the *in vivo*-expanded cumuli we observed that the CD44 bands of these *in vivo*-collected samples migrated more rapidly than did those of the *in vitro* samples and the range of their molecular weights appeared to be somewhat different (73–88 kDa). The amino acid sequence of CD44s predicts a polypeptide of <40 kDa, which contrasts with its apparent size on gel electrophoresis (80–95 kDa). This difference is, apparently, the result of extensive glycosylation of the extracellular domain (Borland *et al*., 1998). Glycosylation has been implicated in the regulation and function of CD44-mediated cell binding for HA (Bartolazzi *et al*., 1996). Increased levels of *N*- and *O*-linked glycosylation of the CD44 receptor, as can be found in inducible forms of the receptor, often lead to a decreased ability to bind HA (Bajorath, 2000). The inducible or variant form of CD44 in mouse T cells has been reported to be more heavily glycosylated and to have less ability to bind HA than does the active or standard form (i.e. CD44s) (Lesley *et al*., 1995). Although in the present study we have not measured the degree of glycosylation, the differences in the migration and molecular weight of CD44 seen between the *in vitro*- and *in vivo*-matured COCs may represent differences in glycosylation, which may, in turn, relate to differences in HA-binding capacity. As the present results demonstrate, the CD44 band for *in vivo* CCs starts from 73 kDa while the *in vitro* CCs present a band starting at 81 kDa.

Considering all the above, it is tempting to speculate that the *in vivo* CD44 bands represent a form of CD44s which is less glycosylated than those *in vitro*, with CD44s responding to a certain setup of tissue culture conditions, which has not proved as successful as *in vivo*, in terms of monospermic fertilisation, among other markers. If this is the case, and since the *in vivo* CCs represent the physiological situation, the first-named may be better able to bind HA to the CCs, expand and, eventually, provide the best basis for oocyte maturation.

Volumetric expansion of the COC correlates, at least in the pig, with the outcome of fertilisation. The rate of embryo development of *in vitro*-matured and -fertilised COCs is significantly lower than that observed *in vivo* (Sun *et al*., 2001), calling for a better and deeper understanding of the latter in order to optimise existing *in vitro* technologies (Hunter & Rodriguez-Martinez, 2002). Whether insufficient functioning of the HA-CD44 pathway occurs during *in vitro* maturation in relation to impaired oocyte maturation remains to be proven, but it is a tempting hypothesis. This sub-optimal nature of the *in vitro* maturation of porcine oocytes might explain, for instance, the lower ability of these oocytes to counteract polyspermy. As determined recently, there are ultrastructrural differences in the morphology of the zona pellucida of *in vivo*-fertilised versus *in vitro*-fertilised pig oocytes with respect to the zona reaction (Funahashi *et al*., 2000, 2001). These differences have been explained by incomplete oocyte maturation or a lack of oviductal influence in the *in vitro*-matured oocytes, as opposed to their *in vivo* counterparts.

Interactions between HA and CD44 have been reported to be involved in the processes of cell migration and cell locomotion, processes involving complex interaction between the cytoskeleton, cell surface receptors and matrix components during embryonic development or tumour metastasis (Sherman *et al*., 1994). Expansion of the cumulus is a primitive expression of cell separation in preparation for ovulation and fertilisation. In the preliminary stages of fertilisation, spermatozoa encounter the cumulus cloud which is apparently dispersed or penetrated by the action of the sperm membrane protein PH-20 which is present in human, macaque and equine spermatozoa (Sabeur *et al*., 1998; Cherr *et al*., 1999; Meyers & Rosenberger, 1999). At least in the spermatozoa of the macaque, the PH-20 molecule plays a dual role, first by depolymerising the cumulus intercellular matrix, through its hyaluronidase activity (involving peptides 1 and 3), thus facilitating sperm penetration through the CCs towards the zona pellucida (Yudin *et al*., 2001). Further, by providing a putative HA-binding site built up by the peptide 2 region (amino acids 205–225) of the molecule, PH-20 appears involved in cell signalling

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changes, such as an increase in $[Ca^{2+}]_i$ (Viens *et al*., 2001). The present study did not reveal any CD44 immunoreactivity in frozen-thawed boar spermatozoa by either immunofluorescence or western blotting. We do not know either whether boar spermatozoa possess PH-20 which, as reported above for other mammals, might be an alternative interacting molecule for HA.

Although there is no conclusive evidence that a HA-CD44 pathway plays a role in oocyte maturation, there are reports that degradation products of HA induce the phosphorylation of the CD44 receptor, leading to activation of a cytoplasmic kinase cascade, such as Raf-1 kinase, mitogen-activated protein kinase (MAPK) kinase and extracellular signal-regulated kinase (Slevin *et al*., 1998). The MAPK cascade plays an essential role in M-phase promoting factor activation and stabilisation during oocyte maturation (Kosako *et al*., 1994; Sato *et al*., 2001).

In conclusion, the specific HA receptor CD44, with molecular sizes similar to that of CD44s, is present in expanding CCs of *in vivo*-matured pig COCs, its expression increasing in direct relation to the degree of cumulus expansion. These temporal changes in localisation and expression of CD44 in the COCs were also seen *in vitro*, calling for the presence of an autocrine pathway involving HA-CD44. Further studies need to be performed to disclose both the possible importance of this pathway with regard to the regulation of oocyte maturation and whether the observed minor variation in molecular weight of the CD44 between *in vivo* and *in vitro* samples is an expression of the suboptimality of current *in vitro* procedures to achieve normal fertilisation rates of pig oocytes.

Acknowledgements

The skilled technical support of Åsa Jansson is acknowledged. Funding has been provided by FORMAS, formerly the Swedish Council for Forestry and Agricultural Research (SJFR), as well as the Swedish Foundation for International Co-operation in Research and Higher Education (STINT) through the SLU-Japan Programme on Reproductive Biotechnology, Uppsala, Sweden, the Programme for Promotion of Basic Research Activities for Innovative Biosciences and 'Research for the Future' Programme, the Japan Society for the Promotion of Science (JSPS-RFTF97L00904), Japan. The authors thank the Thai Government and Chulalongkorn University for providing a study leave grant to P.T.

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