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ORIGINAL ARTICLE *Reproductive biology*

Breast-cancer anti-estrogen resistance 4 (BCAR4) encodes a novel maternal-effect protein in bovine and is expressed in the oocyte of humans and other non-rodent mammals

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STUDY QUESTION: Does BCAR4 have a role in mammalian embryo development?

SUMMARY ANSWER: Expression, localization and functional data support that BCAR4 is a maternal-effect protein in non-rodent mammals.

WHAT IS KNOWN ALREADY: BCAR4 was previously identified as an oocyte-specific gene in cattle, and as a marker of certain breast tumors in humans.

STUDY DESIGN, SIZE, DURATION: Human oocytes were obtained from patients undergoing IVF, but had failed to mature after ovarian stimulation. Dog oocytes were obtained from ovariectomized bitches. Pig, horse and bovine ovaries were obtained from commercial slaughterhouses for extraction of immature oocyte–cumulus complexes. *In vivo* matured bovine matured oocytes were obtained after ovulation induction and ovulation inducing treatment of Montbeliard heifers.

MATERIALS, SETTING AND METHODS: Expression at the RNA level was analyzed by reverse transcription coupled to polymerase chain reaction. Western blot and immunolabeling coupled to confocal or electronic microscopy were used to analyze bovine protein expression and intracellular localization. For the functional approach, short-interfering RNA were microinjected into mature bovine oocytes, followed by IVF; cleavage and embryo development were recorded.

MAIN RESULTS AND THE ROLE OF CHANCE: The *BCAR4* gene is conserved in mammalian species from various orders and has been lost in rodents after divergence with lagomorphs. The transcript is expressed in the oocytes of humans and domestic species. We bring the first experimental evidence of the BCAR4 protein in mammals. In cattle, the protein is not detected in immature oocytes but starts to be synthesized during maturation, increases in the zygote and persists until the morula stage. The protein is detected throughout the cytoplasm in mature oocytes, concentrates in and around the pronuclei in the zygote, and appears to shuttle in and out of the nuclei starting in the 2-cell embryo; BCAR4 is also present at the junctions between blastomeres from 2-cell to morula. In our functional approach, targeting the BCAR4 transcript by small-interfering RNA significantly compromised development to the morula or/and blastocyst stages ($P < 0.05$, logistic regression).

LIMITATIONS, REASONS FOR CAUTION: As indicated above, protein expression and function were investigated in cattle and mostly *in vitro* matured oocytes were used.

WIDER IMPLICATIONS OF THE FINDINGS: This study provides a novel candidate gene whose mutation or deregulation may underlie certain cases of unexplained female infertility.

STUDY FUNDING/COMPETING INTEREST(S): This work was sponsored by grants from the French Ministry of Research (#03P409), Agence Nationale de la Recherche (#ANR-07-GANI-004-01) and Apisgene. No competing interests declared.

Key words: oocyte quality / embryo development / gene expression / animal model

Introduction

Despite improvements in assisted reproduction technologies, ~15% of couples remain unable to conceive a child without identified causes and are diagnosed with idiopathic infertility (Matzuk and Lamb, 2002). For a subset of them, fertilization does not appear affected, but fertilized eggs are unable to proceed through the early stages of preimplantation development. This observation incriminates oocyte quality, due to the essential role of maternal factors during this period when the embryo relies mostly on maternal transcripts and proteins. In this context, the so-called maternal-effect genes should draw particular attention. They are genes transcribed in the oocyte whose products (RNA or proteins) are not necessary for normal ovarian function and fertilization, but are indispensable for embryo development. It appears likely that mutations in some of these genes underlie certain cases of unexplained infertility in women. Some of these studies are mentioned below. Polymorphisms in the MATER (Maternal Antigen That Embryos Require) gene are being investigated in a cohort of women with reduced number of two pronuclei embryos and a poor embryo quality ((Gustofson *et al.*, 2005) and ClinicalTrial.gov identifier NTC00361816). Indeed, following initial identification of MATER as a human autoantigen associated with premature ovarian failure (Tong *et al.*, 2000b), the essential role of *Mater* in embryo development was demonstrated using knockout mice: oocytes from homozygous null females are ovulated and fertilized, but the resulting embryos fail to develop beyond the 2-cell stage (Tong *et al.*, 2000a). *Formin*-2 (*Fmn2*) has also been investigated as a candidate gene for unexplained infertility in woman. The Fmn2 protein is indispensable for the migration of the metaphase spindle and the formation of the first polar body (Leader *et al.*, 2002); fertilization of murine oocytes deficient in Fmn2 produced polyploid embryos resulting in pregnancy loss and subfertility in $Fmn2^{-/-}$ females. An additional study that was designed to characterize the human gene and to search for mutations in women displaying unexplained infertility and multiple failed IVF cycles could not identify a causative mutation among seven patients (Ryley *et al.*, 2005).

These two examples illustrate how transgenic mice have been a useful and elegant approach to identify and characterize maternal-effect genes in mammals (Li *et al.*, 2010). Many of these are conserved in the human genome. However, it should be kept in mind that the mouse should not be the sole model to uncover genes important for embryonic development. Indeed, mouse embryo preimplantation development displays very specific features. Major genome activation occurs as early as the 2-cell stage while it is delayed in most other species, up until the 4/8-cell transition in humans and 8/16-cell transition in bovine (Camous *et al.*, 1986; Braude *et al.*, 1988; Telford *et al.*, 1990). Thus, maternal factors are expected to support development through several cell cycles in these species. The timing of compaction, when the cells become polariszd and adherens junctions form between them, occurs also at different stages relative to embryo development. It occurs in 8-cell mouse embryos, whose blastomeres all have an outward facing apical domain, while 16- and 32-cell human and bovine embryos already display external and internal cells when compaction occurs (Krupinski *et al.*, 2011). Other similarities in oocyte maturation and embryo development between humans and bovine have been reviewed (Menezo and Herubel, 2002; Krisher, 2012). They include the chronology of meiotic maturation, embryo development and implantation, the organization and dynamics of the cytoskeleton and centrosome during oocyte maturation and fertilization, and the embryo metabolic requirements.

In order to discover novel putative maternal-effect genes in mammals, a library of bovine oocyte cDNAs was generated (Pennetier *et al.*, 2005). In this library, we have isolated several clones representing the *Breast Cancer Anti-estrogen Resistance 4* (*BCAR4*) gene, which we reported for the first time as an oocyte-preferential gene (Thelie *et al.*, 2007). The human gene had independently been identified in a screening for genes responsible for tamoxifen resistance in breast cancer cells (Meijer *et al.*, 2006); forced expression of BCAR4 in breast cancer cells induced estrogen-independent cell proliferation and rapid tumor growth. High BCAR4 mRNA levels were associated with tumor aggressiveness and a shorter metastasis-free survival (Godinho *et al.*, 2010). The objective of this study was to investigate whether *BCAR4* expression in oocytes was conserved among mammals including humans and, using the bovine as a model, to demonstrate that the gene actually encodes a protein; characterize its spatio-temporal pattern throughout *in vitro* preimplantation development; and investigate the consequences of microinjecting interfering RNA on embryo development. Altogether, our expression and functional data strongly support the hypothesis that *BCAR4* is a novel maternal-effect gene in mammals. In light of our data and its proliferative promotion properties in cancer cells, the role of BCAR4 in preimplantation development is discussed.

Materials and Methods

Chemicals and cell culture products were from Sigma (Saint Quentin Fallavier, France) unless otherwise indicated.

Bioinformatics sequence analysis

Transmembrane domains were predicted using Simple Modular Architecture Research Tool version 7 (Letunic *et al.*, 2012) and Phobius (Kall *et al.*, 2007). The presence of a signal peptide or nuclear export signal was searched using signalP 4.0 (Petersen *et al.*, 2011) and NET-NES (la Cour *et al.*, 2004), respectively.

Sequence alignment was generated with MUSCLE (Edgar, 2004) and curated manually, then with GBLOCKS (Castresana, 2000). An unrooted tree was obtained as the result of computation with PhyML methods (Dereeper *et al.*, 2008) with 100 bootstrap iterations.

Oocyte collection and embryo production

Human oocytes were obtained with informed consent from five patients undergoing an IVF protocol; they had failed to fully mature after hormonal stimulation. Dog oocytes were from ovariectomized animals; they were a gift from Dr Karine Reynaud, Ecole Nationale Vétérinaire d'Alfort, France. *In vivo* matured bovine oocytes were obtained by ovum pick-up from 6 to 15 mm follicles, from Montbeliard heifers after ovulation induction and ovulation inducing treatment.

Pig, horse and cow ovaries were obtained from commercial slaughterhouses. Immature oocyte–cumulus complexes (OCCs) were aspirated from antral follicles (3–8 mm follicles for bovine), selected following morphological criteria and washed several times in TCM199 Hepes medium. Some bovine OCCs were subjected to *in vitro* maturation in TCM199 supplemented with EGF, IGF-I, FGF, hCG, PMSG, insulin, transferrin and selenium, for 22 h at 39°C in humidified air with 5% of CO_2 (Donnay *et al.*, 2004). For inhibiting meiotic resumption, the oocytes were incubated in this medium supplemented with 25 μM roscovitin for 22 h, then transferred into the permissive medium for another 22 h. IVF was performed in Talp medium (Tyrode medium supplemented with bicarbonate, lactate, pyruvate, BSA and gentamycin) and 20 h later, presumed zygotes were denuded and transferred into droplets of modified synthetic oviduct fluid supplemented with 5% fetal calf serum (MP Biomedicals, Illkirsh, France) (Holm *et al.*, 1999). Embryos were cultured at 39°C in humidified atmosphere with 5% CO_2/5% O_2/90% N_2. For protein analysis, pools of 10–25 immature and mature oocytes with a polar body as well as zygotes (Day 1 post-IVF), 2-cell and 4-cell embryos (Day 2), morulae (Day 5), expanded blastocysts (Day 7) and hatched blastocysts (Day 9) were analyzed. Parthenotes were produced by activation using ionomycin and 6-dimethylaminopurine.

Small-interfering RNA microinjection

The small-interfering RNA (siRNA) design algorithm (BLOCK-iT™ RNAi Designer, Invitrogen, Marly-le-Roi, France) was used to design two distinct siRNAs species targeting the coding sequence of bovine BCAR4 mRNA. Genomic databases were interrogated to rule out homology to any other known gene. Two distinct couples of *in vivo* grade HPLC purified stealth RNAi were ordered and diluted to 25 μM with RNAse free water. The sense–antisense duplexes sequences were, respectively, AAACCAGGUCCACUGACUGUUAGCU-AGCUAACAGUCAGUGGACCUGGUUU (RNAi1) and GAGAAUUGCUCAGGAUCCUUGUAAG-CUUACAAGGAUCCUGAGCAAUUCUC (RNAi2). Groups of 50 denuded MII oocytes (DOs) were microinjected with ~2 pl of either a 25 μM solution of a stealth™ RNAi negative control duplex (Invitrogen) or a specific duplex RNAi species, using an inverted microscope equipped with micromanipulators. In parallel, non-microinjected groups of 50 OCCs and 50 DOs were incubated as controls. Oocytes then underwent IVF in the presence of additional mature OCCs. Thereafter, presumed zygotes originating from OCCs and from denuded (microinjected) oocytes were separated, and the latter were submitted to *in vitro* development, as described above. Cleavage, morulae, blastocysts and hatched blastocysts rates were recorded. For investigating BCAR4 mRNA and protein knockdown, respectively, individual embryos and pools of 10 embryos at both the 2-cell and 4-cell stages were collected, from five independent experiments (100 microinjected oocytes per group per replicate).

Protein analysis

Antibody generation and characterization

A keyhole limpet hemocyanin (KLH)-coupled peptide corresponding to aa 89–102 of bovine BCAR4 was used to immunize rabbits (Eurogentec, Angers, France). By western blot, the corresponding serum recognized a protein migrating at 18 kDa in mature oocytes, which was neither detected in immature oocytes nor in cumulus cells, nor with the pre-immune serum (not shown). Next the antibody was purified by peptide affinity. A band at 18 kDa was detected in mature oocytes, but not in cumulus cells. The specificity of the antibody was further confirmed by detection of the band in HEK293T cells transfected with the pSG5-BCAR4 expression plasmid but not the empty vector (not shown).

Western blot

To analyze protein secretion, maturation and fertilization media, either fresh or following incubation for 20–24 h with 100 OCCs, were concentrated and desalted using Vivaspin 500 μl columns. For detection in oocytes or embryos, pools of 10–25 structures were analyzed. After heat denaturation in Laemmli buffer, proteins were resolved on 15% SDS–PAGE gels and transferred onto nitrocellulose membranes. Blots were blocked with 5% milk and then probed with anti-BCAR4 antibody (0.34 μg/ml, i.e. 1/1000) or with anti-tubulin or anti-vinculin antibody overnight at 4°C. The membranes were then washed and incubated for 2 h at room temperature with the appropriate HRP-conjugated secondary antibodies (Molecular Probe, Cergy Pontoise, France). Revelation was performed using Western lightning chemiluminescence reagent plus kit (Perkin Elmer, Courtaboeuf, France). Quantification was done with the Image Scanner and ImageMaster 1D elite v4.10 software (GE Healthcare, Velizy, France).

Immunofluoresence

Oocytes and embryos were fixed in 4% paraformaldehyde for 30 min at room temperature. After permeabilization with PBS–triton 0.2% and blocking in 10% goat serum, they were incubated overnight at 4°C with both anti-BCAR4 antibody (1/1000) and mouse anti-LAMIN A/C antibody (Ozyme) (1/1000) or equivalent concentrations of rabbit and mouse IgG as a negative control. After washing, they were incubated for 2 h at room temperature with Alexa 488 conjugated goat anti-rabbit antibody and Alexa 594 conjugated goat anti-mouse antibody (Molecular Probe). Slides were mounted with Mowiol containing Hoechst 33258. Immunofluorescence was observed using an LSM 700 confocal microscope (Zeiss, le Pecq, France). Seven to 14 oocytes/embryos at each stage were observed.

Electron microscopy

Fixing and labeling of immature oocytes (as negative controls) and zygotes was as described above, except that the secondary antibody was an anti-rabbit antibody conjugated to 20 nm gold particles. After immunolabeling, oocytes and zygotes were washed with PBS and additionally fixed with 1% glutaraldehyde in PBS (2 h), washed with PBS and post-fixed by incubation for 1 h with 2% osmium tetroxide (Electron Microscopy Science, Hatfield, PA, USA). Samples were dehydrated in a graded series of ethanol solutions (from 50 to 100%) and propylene oxide, and embedded in Epon resin, which was allowed to polymerize for 24 h at 37°C followed by 48 h at 60°C. Ultra-thin sections (70 nm) were cut with a Leica Ultracut UCT ultramicrotome, placed on EM one-slot grids coated with Formvar film and stained with uranyl acetate (5% water solution, 20 min). The sections were then observed at 100 kV with a Jeol 1011 transmission electron microscope (JEOL, Tokyo, Japan) connected to a digital camera driven by

Digital Micrograph software (Gatan, Pleasanton, CA, USA) for image acquisition and analysis.

RNA purification, reverse transcription and polymerase chain reaction

RNA extraction and reverse transcription

After adding 1 pg of luciferase mRNA (Promega, Charbonnières-les-bains, France) per oocyte/embryo as an external standard, RNA was extracted from pooled oocytes or individual bovine embryos conserved in RNAlater (Ambion, Huntingdon, UK) using PicoPure RNA Isolation Kit (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed at 37°C using oligo(dT) primers (Promega) and Mouse Moloney Leukaemia Virus reverse transcriptase (Invitrogen).

Polymerase chain reaction and real-time polymerase chain reaction

Target cDNA were amplified using the iQ Sybrgreen Supermix (Bio-rad, Marnes-la-Coquette, France) and gene-specific primers (Table I) and quantified relative to exogenous luciferase as described in Thelie *et al.* (2007).

Statistical analysis

The percentages of cleaved embryos, morulae and blastocysts were analyzed with logistic regression models using the GLIMMIX procedure of the SAS® software (SAS Institute Inc., 2009) with a logit function. The models included the fixed effects of replicate and condition. The interaction between replicate and condition was tested and then removed from the model when non-significant. Results are presented as estimated means (least square means) + SEM. Estimated means were subsequently compared between conditions using a *t*-test. Comparison of mRNA or protein abundance between embryos originating from oocytes microinjected with control or specific RNAi was performed using the non-parametric Mann–Whitney test (GraphPad Prism® 5 software, La Jolla, CA, USA) and differences were considered statistically significant at the 95% confidence level ($P < 0.05$). Data are represented as means + SEM.

Results

Sequence analysis and phylogeny

Putative orthologs were previously reported in boreoeutherian mammals of various orders: primates, lagomorphs (rabbit and American pika), artiodactyls (cow), perissodactyls (horse) and scandantiae (common treeshrew) (Thelie *et al.*, 2007; Godinho *et al.*, 2011). In this study, we extended the search but focused on species for which we have some developmental data. By sequence alignment, we found putative orthologs in the syntenic region of two additional domestic species, dog (a carnivore) and pig, but not in the rodent Guinea pig. Sequence alignment is presented in Fig. 1A. Indeed, the sequence has significantly diverged between mammals, with 36–58% similarity between proteins of species from different orders (73% between cow and pig, which are both artiodactyls). The higher degree of similarity, in the middle part of the protein, corresponds to the putative transmembrane domains and the intermediary amino acids stretch.

An unrooted phylogenetic tree was built with mammalian BCAR4 proteins (Fig. 1B). It regrouped BCAR4 proteins from primates, rabbit and horse apart from BCAR4 from dog, bovine and pig. In search for amino acids that may have a particular role in certain species/orders, we ran a positive selection analysis, but failed to identify amino acids under positive selection.

The presence of putative transmembrane domains (Thelie *et al.*, 2007; Godinho *et al.*, 2011) and a signal peptide (Godinho *et al.*, 2011) in BCAR4 was previously reported. As these features are difficult to distinguish and previous tools were prone to generate false-positive signal peptides, the signalP 4.0 version was recently developed specifically to discriminate between signal peptides and N-terminal transmembrane helices (Petersen *et al.*, 2011). Using SignalP4.0, when transmembrane domains were considered, the program did not predict a signal peptide in any BCAR4 ortholog. Predicted transmembrane domains were found in the canine and porcine sequences (aa30–49 and 55–78, and aa 26–50 and 56–78, respectively), and confirmed for other species; these domains were also predicted by the SMART and Phobius tools. The latter predicted N- and C-terminal cytoplasmic regions with a short, 5–11 amino-acid extracellular part in all orthologous proteins. Finally, potential leucine-rich nuclear export signals were found using the NET-NES tool. Corresponding residues are shown in Fig. 1A.

Expression in mammalian oocytes

BCAR4 mRNA expression was investigated for the first time in human oocytes. By polymerase chain reaction (PCR), the transcript was readily detected in oocytes from all five patients, but absent from cumulus cells, conversely to the transcript for anti-Müllerian hormone (AMH) (Fig. 2). The ubiquitous transcript for ribosomal protein 19 (RPL19) was detected in both compartments.

We also collected oocytes from mare, bitch and gilt. Using species-specific primers (Table I), we amplified a fragment of expected size by RT–PCR (not shown). Identity with predicted BCAR4 was further confirmed by sequencing.

Altogether, our data show that oocyte expression of BCAR4 is conserved throughout distinct orders of non-rodent mammals.

Expression of BCAR4 protein during bovine oocyte maturation and preimplantation embryo development

So far, the human BCAR4 gene has been annotated as a non-protein-coding gene in several public genomic databases, and there is no report of protein expression in the literature. An antibody targeting a peptide within the C-terminal region of the bovine protein was generated and tested (see the Materials and Methods section). Expression of BCAR4 protein was analyzed by western blot during oocyte maturation and embryo development. BCAR4 was not detected in immature oocytes. Protein synthesis started between 10 and 15 h incubation, corresponding to transition between metaphase I and anaphase I when chromosomes are separating to the poles; it became more abundant after the oocytes had completed nuclear maturation (Fig. 3A). BCAR4 synthesis was inhibited when in the presence of roscovitin, an inhibitor of cyclin-dependent kinases, but resumed after the oocytes were transferred to a permissive medium, showing that meiosis resumption and maturation were required for expression (Fig. 3B). BCAR4 was also detected after *in vivo* maturation (Fig. 3A). Protein level increased sharply in zygotes, then remained stable throughout early development up to the 8-cell stage. It decreased in morulae, and was degraded in blastocysts (Fig. 3C). Interestingly, a

Table I Primers sequences.

Gene symbol	Species	Primer	Sequence (5′→3′)	Experiment
Luciferase	Firefly	Luciferase-SI Luciferase-ASI	TCATTCTTCGCCAAAAGCACTCTG AGCCCATATCCTTGTCGTATCCC	Real-time PCR
AURKA	Cow	AURKA-SI AURKA-ASI	TCGGGAGGACTTGGTTTCTT TGTGCTTGTGAAGGAACACG	Real-time PCR
BCAR4	Cow	BCAR4-SI BCAR4-ASI	GAAGGGTGTTTGCTGATTTCTGTTAAG CATTGTTGTTACCAGGGCGAAGG	Real-time PCR
	Pig	pBCAR4-SI pBCAR4-ASI	ACCCCTGCAGCTAAAACTCA TTAACATCCCAAAGATTTCCAG	PCR
	Dog	dBCAR4-SI dBCAR4-ASI	GGCGACTCAAGCTCATAGG GCCATGGCTGCTTCTACTTC	PCR
	Horse	hBCAR4-SI hBCAR4-ASI	GCCGGTACAAGGATGTAATCA AAAGACTCCGTACCGTCTCG	PCR
	Human	huBCAR4-SI huBCAR4-ASI	ATACAATGGCGTAATCATAGC AGACATTCAGAGCAAGACA	PCR
AMH	Human	AMH-SI AMH-ASI	CCTACACCTGGAGGAAGT CCTCGTCACAGTGACCTC	PCR
RPL19	Human	RPL19-SI RPL19-ASI	TGAGACCAATGAAATCGCCAATGC ATGGACCGTCACAGGCTTGC	PCR

similar BCAR4 level was detected in zygotes, in parthenotes, as well as in mature oocytes incubated for an additional 24 h period in the fertilization media without spermatozoa (Fig. 3D).

To investigate whether BCAR4 could be secreted by oocytes and embryos, the presence of the protein in the maturation or fertilization media was analyzed. We failed to detect the corresponding band by western blot, suggesting that the protein is not secreted (not shown).

Intracellular localization

In order to determine intracellular localization of the BCAR4 protein in bovine oocytes and embryos, we performed immunofluorescent staining followed by confocal microscopy analysis (Fig. 4). As expected from the western blot, no staining was detected in germinal vesicle (GV) stage oocytes. In metaphase II oocytes, labeling was readily detected throughout the ooplasm. The protein started to display a preferential spatial localization in pronuclear zygotes, as it was observed mostly within the pronuclei and in the surrounding cytoplasmic region (Fig. 4C). This was confirmed by pre-embedding immunogold labeling coupled to electron microscopy, followed by quantification of gold particles over successive sections (Fig. 5). In 2- and 4-cell embryos, BCAR4 was present at the junctions between blastomeres; it was also observed in the cytoplasmic area surrounding the nuclei, and within or excluded from the nuclei. Interestingly, in 2-cell embryos, both blastomeres displayed the same pattern (Fig. 4D and E), while the two patterns could be observed in distinct blastomeres of a 4-cell embryo (Fig. 4F). In morulae, roughly the same distribution was observed albeit the labeling was less intense (Fig. 4G). In expanded blastocysts, BCAR4 was not detected, as previously observed by western blotting (Fig. 4H). As a negative control, no staining was revealed using rabbit IgG as the primary antibody (not shown). Overall, in bovine embryos between the zygote and morula stages, BCAR4 protein displayed preferential localization in the cytoplasmic region surrounding the (pro)nuclei, in the (pro)nuclei of a subset of embryo or blastomeres and at the junction between blastomeres.

Functional analysis by siRNA microinjection

BCAR4 expression specifically in maturing oocytes and early-stage embryos strongly suggested a role as a maternal-effect factor. To investigate its functional importance during embryonic development, RNAi-mediated gene silencing was performed by microinjection into the cytoplasm of denuded mature bovine oocytes. Two RNAi targeting two distinct regions of the coding sequence were used. Following fertilization, the rates of cleavage and development to morula, expanded blastocysts at Day 7 and/or 8 and hatched blastocysts at Day 9 post-insemination, were evaluated. In a first set of experiments, in order to validate the microinjection procedure, cleavage (relative to the number of oocytes) and morulae and blastocysts rates (relative to the number of cleaved embryos) were compared between non-microinjected OCC or DO and negative control RNAi-injected DO (Fig. 6). As expected, due to the presence of the cumulus, cleavage (reflecting fertilization) rate was higher for OCC, whereas no significant difference was observed between the other two groups, showing that microinjection and negative control RNAi were not deleterious. In a second set of experiments, the effect of BCAR4 targeting RNAi versus negative control RNAi was investigated (Fig. 7). No difference in cleavage rate was observed between control and specific RNAi-microinjected groups (Fig. 7A), but development to morulae and expanded blastocysts was specifically altered in the presence of either BCAR4-targeting RNAi (Fig. 7B–E); the differences were significant ($P < 0.05$) except in one situation, i.e. for Day 7 blastocysts (Fig. 7C) originating from RNAi1-microinjected oocytes (with still a tendency, $P = 0.08$). Developmental rates were decreased by 20–30% for morulae, 30–50% for blastocysts and 55–65% for hatched blastocysts. The efficiency of the RNAi was controlled by assessing BCAR4 transcript and protein abundance by real-time PCR and western blotting, respectively (Fig. 8). The effect of RNAi was not observed in 2-cell (Fig. 8A and C) embryos, but appeared to be delayed to the 4-cell stage (Fig. 8B and D). At this stage, although the decrease in mRNA abundance was not significant, possibly due to the

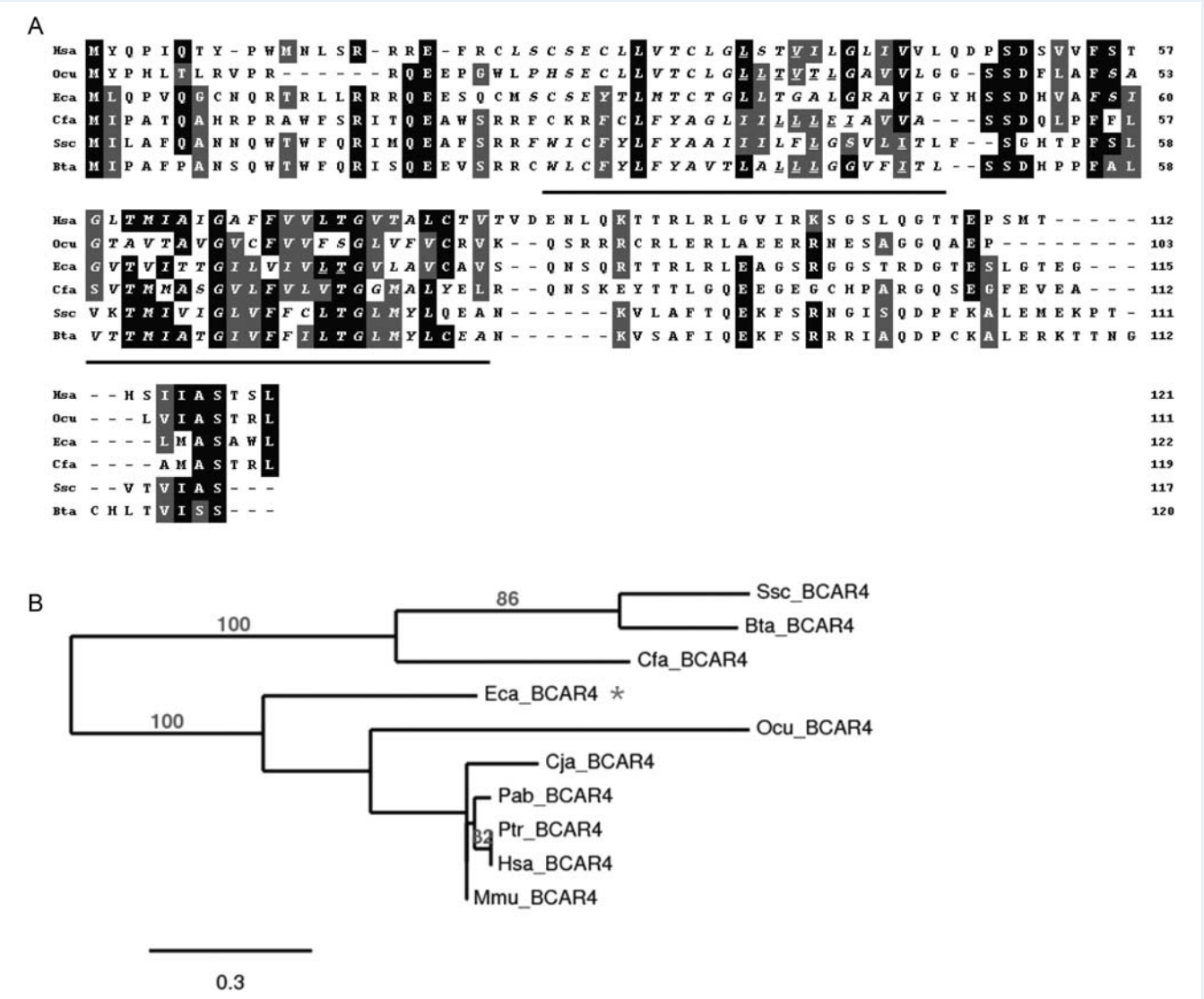

Figure 1 Evolution of BCAR4 in non-rodent mammals. (**A**) Alignment of putative protein sequences in humans (Hsa), rabbit (Ocu), horse (Eca), dog (Cfa), pig (Ssc) and cow (Bta). Amino acids conserved or similar in a majority of proteins are in black or gray boxes, respectively. Amino acids predicted in transmembrane domains are in italic, and indicated with a black line in cow. Amino acids in putative leucine-rich nuclear export signals are underlined. (**B**) Phylogenetic tree with bootstrap values >80%. Stars correspond to branches that are non-congruent with species tree.

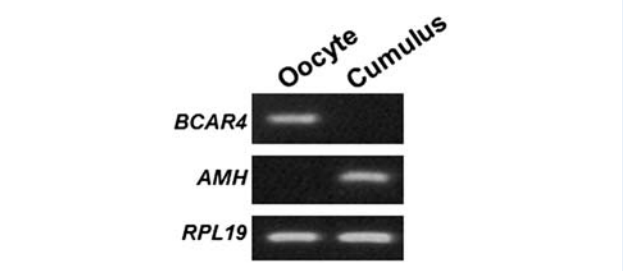

Figure 2 BCAR4 transcript is expressed in human oocytes. Detection of BCAR4 and AMH expression by RT–PCR in human oocytes and cumulus cells, respectively. RPL19 was detected in both cell types.

high variability in control embryos, protein expression was significantly decreased by 63 and 75%, respectively, by the two RNAi species.

Discussion

In this paper, we have demonstrated that *BCAR4* encodes a novel maternally expressed protein with maternal-effect properties, and is conserved in various non-rodent mammals. Our data are discussed in the context of its putative function during preimplantation embryo development.

Evolution

The human BCAR4 gene was discovered as a marker of a subset of breast cancer tumors (Meijer *et al.*, 2006). In parallel, we had

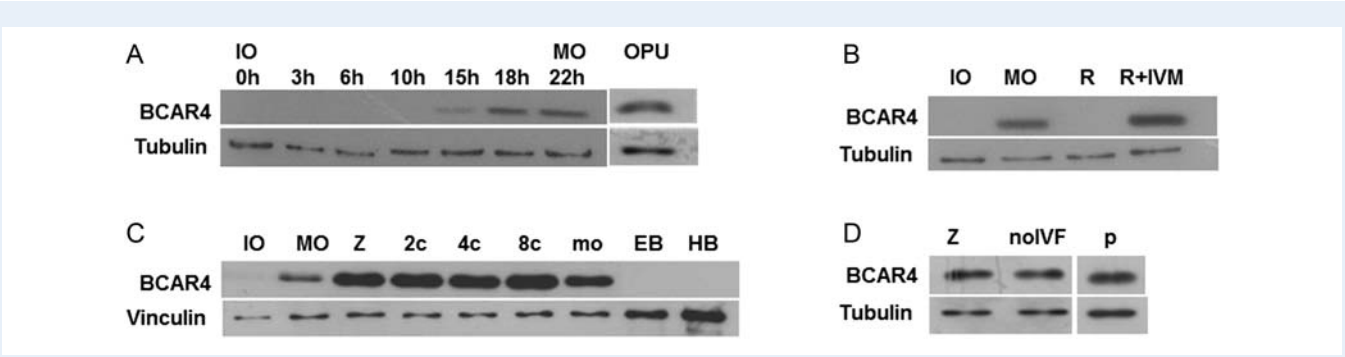

Figure 3 Expression of BCAR4 protein throughout bovine oocyte maturation and embryo preimplantation development. Western blot analysis of BCAR4 and Tubulin or Vinculin as a control. (**A**) Kinetics of protein synthesis in oocytes during *in vitro* maturation, in immature oocytes (IO), after 3, 6, 10, 15, 18 and 22 h (metaphase-II mature oocytes, MO) incubation in maturation medium, and in *in vivo* matured oocytes (OPU). (**B**) Effect of roscovitin. Expression in IO, after 22 h in maturation medium in the absence (MO) or presence (R) of roscovitin, and the latter followed by another 22 h in maturation medium (R + IVM). (**C**) Profile throughout development. Detection in IO, MO, zygotes (Z), 2-cell (2c), 4-cell (4c) and 8-cell (8c) embryos, morulae (mo), expanded (EB) and hatched (HB) blastocysts; (**D**) effect of fertilization. Detection in Z, mature oocytes incubated in fertilization medium without sperm (noIVF), and parthenotes (p).

discovered the bovine ortholog as an oocyte preferentially expressed gene (Thelie *et al.*, 2007). Although putative protein sequences were reported, experimental studies were limited to expression of the transcript and to this day, the human gene remains annotated as non-protein coding. However, due to the coding sequence being fairly short, included within a single exon, and without well-known consensus motifs, automated annotation tools are likely to have missed it. Here for the first time, we have actually demonstrated expression of the protein in bovine, suggesting that proteins exist in other species too.

Beyond humans and bovine, based on sequence alignment, several homologs have been reported in various orders of eutherian mammals: among primates, lagomorphs (rabbit and American pika), perissodactyls (horse) and scandantiae (common tree shrew) (Godinho *et al.*, 2011). Here we have discovered orthologs in the genomes of sus scrofa, a second artiodactyl and canis familiaris, a carnivore. The phylogenetic tree is incongruent to the species tree and reveals the major divergence of the horse protein relative to the cow, pig and dog orthologs, even though all four species belong to the Laurasiatheria superorder. The absence of the gene in mouse and rat demonstrates that the gene has been lost after the speciation between lagomorphs and rodents. Among these species, major genome activation occurs as early as the 2-cell stage in mouse and presumably rat, but one or several cell cycles later in the pig, horse, human, dog, cow and rabbit [rat: (Zernicka-Goetz, 1994), dog:(Bysted *et al.*, 2001), horse: (Brinsko *et al.*, 1995), rabbit: (Brunet-Simon *et al.*, 2001), human: (Braude *et al.*, 1988), pig, cow: (Camous *et al.*, 1986), for a review, see Telford *et al.* (1990)]. Thus, the presence of the *BCAR4* gene correlates with delayed embryonic genome activation in mammals. Considering the pro-proliferative properties of the human gene (Godinho *et al.*, 2010), it can be speculated that BCAR4 may be involved in cell division up until the embryo can transcribe its own factors.

Bioinformatics analysis of protein sequences did not identify precise functional domains nor related protein families. All orthologous protein sequences displayed two putative transmembrane domains, with predicted cytoplasmic N- and C-termini. The presence of a signal peptide is unlikely, suggesting that the protein is not truncated, in agreement with our failure to detect a protein of lower molecular weight in oocytes, embryos or culture medium by western blot. On the other hand, amino acids expected to participate into a nuclear export signal were identified in a leucine/isoleucine-rich homologous region. This is consistent with the protein subcellular localization in blastomeres.

Expression and intracellular localization of BCAR4: a comparison with other proteins of maternal or embryonic origin

The BCAR4 protein displayed interesting features in its expression and localizations patterns. While proteins encoded by oocyte-specific maternal-effect gene in mammals are usually already present in immature oocytes, such as JY1, NPM2, ZAR1, MATER or STELLA in mouse or bovine (Tong *et al.*, 2000b; Burns *et al.*, 2003; Payer *et al.*, 2003; Wu *et al.*, 2003; Bettegowda *et al.*, 2007; Lingenfelter *et al.*, 2011). BCAR4 started to be synthesized during late maturation and became more abundant in zygote, similar to the origin recognition complex 6 (ORC6) protein that is required for the first cleavage of mouse embryos (Murai *et al.*, 2010). This is consistent with a role for BCAR4 specifically during the first embryonic divisions. Interestingly, BCAR4 translation appeared to switch from a developmental control during maturation to a chronological control afterwards. Indeed, BCAR4 translation was inhibited in the presence of the meiotic inhibitor roscovitin, and this was reversible, as previously reported for several proteins (Vigneron *et al.*, 2003). Later, BCAR4 level was increased in fertilized zygotes, but also in parthenotes, and quite surprisingly in *in vitro* aged mature oocytes, showing that sperm penetration or egg activation is not required for stimulating BCAR4 translation. In this respect, BCAR4 is again reminiscent of, although not identical to, mouse ORC6. ORC6 was found to be translated during maturation and increased in 1-cell fertilized embryos (Murai *et al.*, 2010). The protein was also detected (if not increased) after parthenogenetic activation and after *in vitro* oocyte aging for 6 h. ORC6 detection in aged oocytes required protein synthesis, either *de*

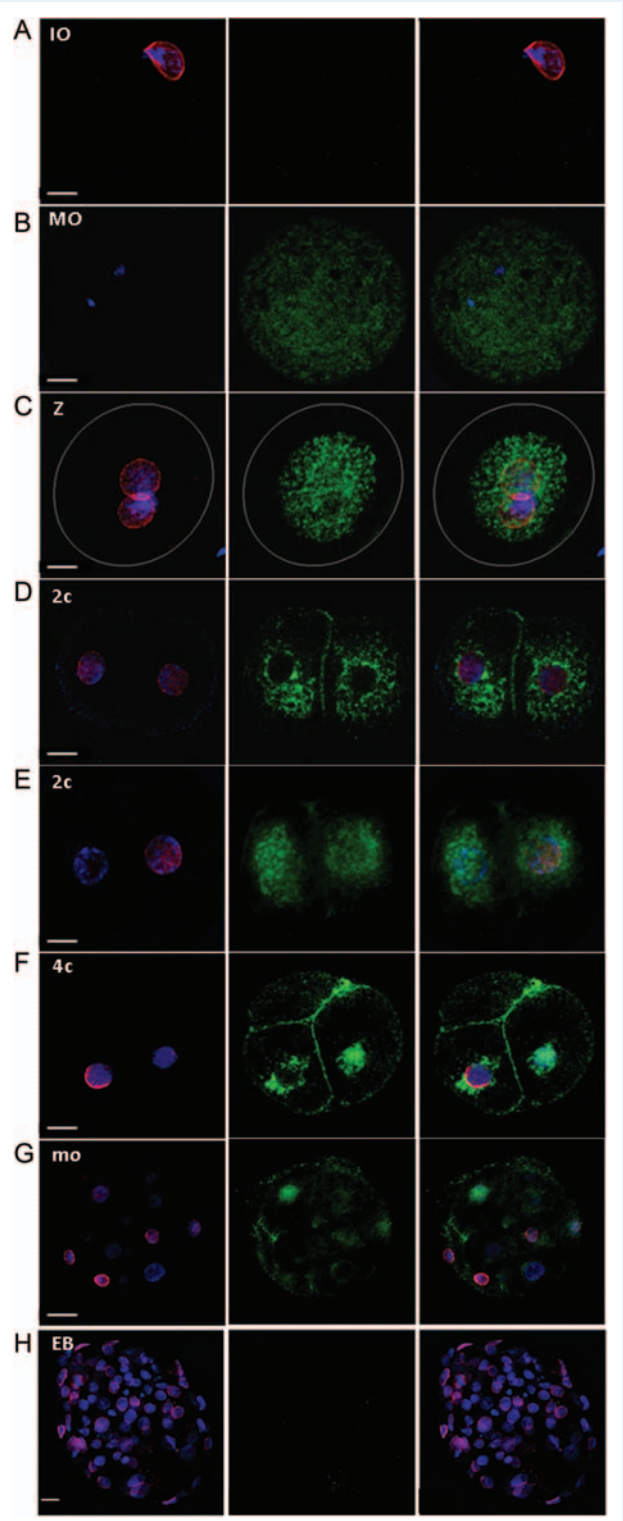

Figure 4 Spatial distribution of BCAR4 in bovine oocytes and preimplantation embryos by immunofluorescence and confocal microscopy. Chromatin in blue and lamins A/C in red (left panel), BCAR4 in green (middle panel) and merged images (right panel). Representative images of (**A**) immature oocytes (IO), (**B**) *in vitro* matured oocytes (MO), (**C**) zygotes (Z) (with the cytoplasmic membrane outlined), (**D** and **E**) distinct 2-cell (2c) embryos, (F) 4-cell (4c) embryos, (G) morulae (mo) and (H) expanded blastocyst (EB). Scale bar : 20 μm

novo synthesis of ORC6 itself or synthesis of proteins involved in ORC6 stabilization. Other oocyte-abundant proteins were reported to be synthesized after fertilization or after oocyte aging, but the reciprocal analysis was not run. For example, the mouse oocyte-specific oogenesin and Sam68 proteins were shown to increase between the ovulated oocyte and the 1-cell stage (Minami *et al.*, 2003; Paronetto *et al.*, 2008). Conversely, in pig, a proteomic approach detected four proteins showing a significant increase between MII and *in vitro* aged oocytes, i.e. AKR1B1, SLC25A6, PRDX2 and PCNA (Jiang *et al.*, 2011).

Regarding intracellular localization throughout maturation and development, BCAR4 displays a unique pattern, as we could not find another protein with the same spatio-temporal distribution. Nevertheless, it shares some features with several proteins important for preimplantation development. In zygotes, the protein is found in both pronuclei and their vicinity. The latter may reflect protein processing within the Golgi complex, for future addressing to the plasma membrane, although at this stage no membrane localization is observed yet. Indeed, in cleavage stage embryos, BCAR4 is present at the junction between blastomeres, reminiscent of mouse E-cadherin, the adherens junction protein which mediates compaction (Vestweber *et al.*, 1987; Ohsugi *et al.*, 1996; De Vries *et al.*, 2004). In bovine, E-cadherin localization has not been reported, but expression from the embryonic genome is important for blastocyst formation (Nganvongpanit *et al.*, 2006). Unlike mouse E-cadherin however, BCAR4 expression has started to decrease in morulae and is not reactivated afterwards. Thus, a hypothesis is that BCAR4 may be involved in blastomere adhesion before major activation of the embryonic genome and possibly up to compaction. Another possibility is that BCAR4 could be retained at the cell surface by interacting with an integral membrane protein, as is β-catenin by E-cadherin. β-Catenin is a structural component of adhesion receptors and functions as a transcriptional co-activator of specific target genes in the Wnt pathway, regulating cell fate and proliferation (Davidson, 2010). In bovine, as major activation of the embryonic genome is delayed, BCAR4 presence in the nucleus as early as the 2-cell stage is unlikely to reflect a role in transcription. Rather, it might be involved in DNA replication. Finally, BCAR4 shares similarities with the tight junction protein zona occludens 2 (ZO2). In hamster, ZO2 was detected throughout the cytoplasm of unfertilized eggs, in the (pro)nuclei of embryos, and it was present at the membrane between adjacent blastomeres from the 2-cell stage onwards (Wang *et al.*, 2011). ZO2 displayed a different pattern in mouse, with preferential accumulation near the spindle in MII oocytes, and then a BCAR4-similar localization in (pro)nuclei and surrounding cytoplasmic region of zygotes and 2-cell embryos, nuclear localization in 4- and 8-cell embryos, before switching to blastomere junction only in post-compaction 16-cell embryos and later stages (Sheth *et al.*, 2008). Overall, several proteins in rodents share localization features with BCAR4. But knowledge on how these proteins are located in embryos from non-rodent species is currently lacking and might be helpful in understanding BCAR4 function.

Hypotheses on mediators of BCAR4 action in oocytes and embryos

In BCAR4-expressing cells derived from the human breast carcinoma cell line ZR-75-1, v-erb-b2 erythroblastic leukemia viral oncogene

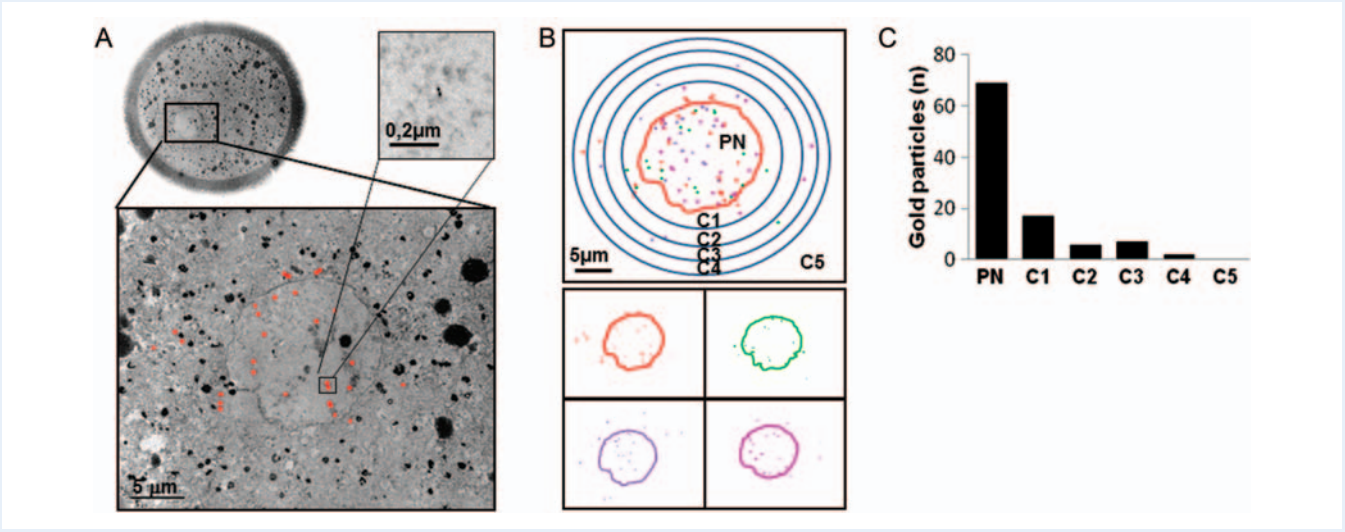

Figure 5 Intracellular localization of BCAR4 in bovine zygotes by immunogold staining coupled to electron microscopy. (**A**) Localization of gold particles in one section. Top left: section of the full oocyte; bottom: close-up onto a region showing the pronucleus and surrounding cytoplasm. Red dots highlight gold particles. Top right: close-up showing two gold particles. (**B**) Section. PN, pronucleus; c1–c5, cytoplasmic zones; c1–c4 have an equal area. (**C**) Counting of gold particles in the pronucleus and regions c1–c5 over four sections of one zygote.

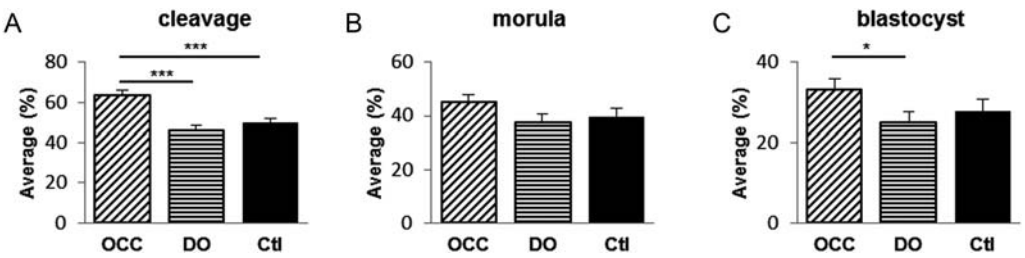

Figure 6 Validation of the siRNA microinjection procedure. Rate of development to (**A**) 2-cell, (**B**) morula, (**C**) blastocyst rate on Day 8, after fertilization of non-microinjected OCCs and DO, or DO microinjected with a negative control RNAi (Ctl). Data from 11 replicates of 50 oocytes per group. Asterisks denote statistical significance (* for $P < 0.05$, *** for $P < 0.001$).

homolog 2 (ERBB2), ERBB3 and downstream components of the ERBB pathway were activated by phosphorylation (Godinho *et al.*, 2010; van Agthoven *et al.*, 2012). We searched whether this pathway could be a target of BCAR4 in oocytes and embryos. The transcripts are not or hardly detected in mouse oocytes to 2-cell embryos, based on Unigene expression profiles, RT–PCR (Brown *et al.*, 2004) or microarrays in the Gene Expression Omnibus database (records# GDS578 (Hamatani *et al.*, 2004); GDS2300 (Su *et al.*, 2007); GDS592 (Su *et al.*, 2004); GDS2387 (Potireddy *et al.*, 2006). Yet, microinjecting antisense oligonucleotides to Erbb2 or recombinant protein into mouse oocytes was recently reported to inhibit or promote maturation, respectively, suggesting that the gene is not only expressed but involved in maturation (Zheng *et al.*, 2012). In bovine, the transcript appears scarce in oocytes based on our own microarray experiment (unpublished results), while ERBB3 was detected until the 2-cell stage by RT–PCR (Yoshida *et al.*, 1998). Finally, ERBB2 was detected in human oocytes (GDS3256 record Kocabas *et al.*, 2006). Besides these dispersed and somewhat conflicting data, it has to be kept in mind that, until the MET (maternal to embryo transition), the proteins might be present even if the transcripts are not. Better knowledge of ERBB2 and ERBB3 protein expression at these stages is necessary to know whether they could mediate BCAR4 function.

Alternatively, the same signalling pathway could be activated by BCAR4 through the epidermal growth factor receptor (EGFR). EGFR contribution in ZR-75-1 cells could not be investigated due to low expression and cross-reactivity of the antibody (Godinho *et al.*, 2010; van Agthoven *et al.*, 2012). In mouse oocytes and pre-MET embryos, the transcript was detected by RT–qPCR (Brown *et al.*, 2004) and microarray and its relative level in oocytes was high compared with most tissues (records# GDS578 (Hamatani *et al.*, 2004); GDS813 (Zeng *et al.*, 2004); GDS2300 (Su *et al.*, 2007); GDS592 (Su *et al.*, 2004). It also appears to be expressed in humans (record# GDS3256; Kocabas *et al.*, 2006).

Finally, Erbb4 transcript was detected in mouse 1- and 2-cell embryos but undetected from the 8-cell stage onwards (Brown

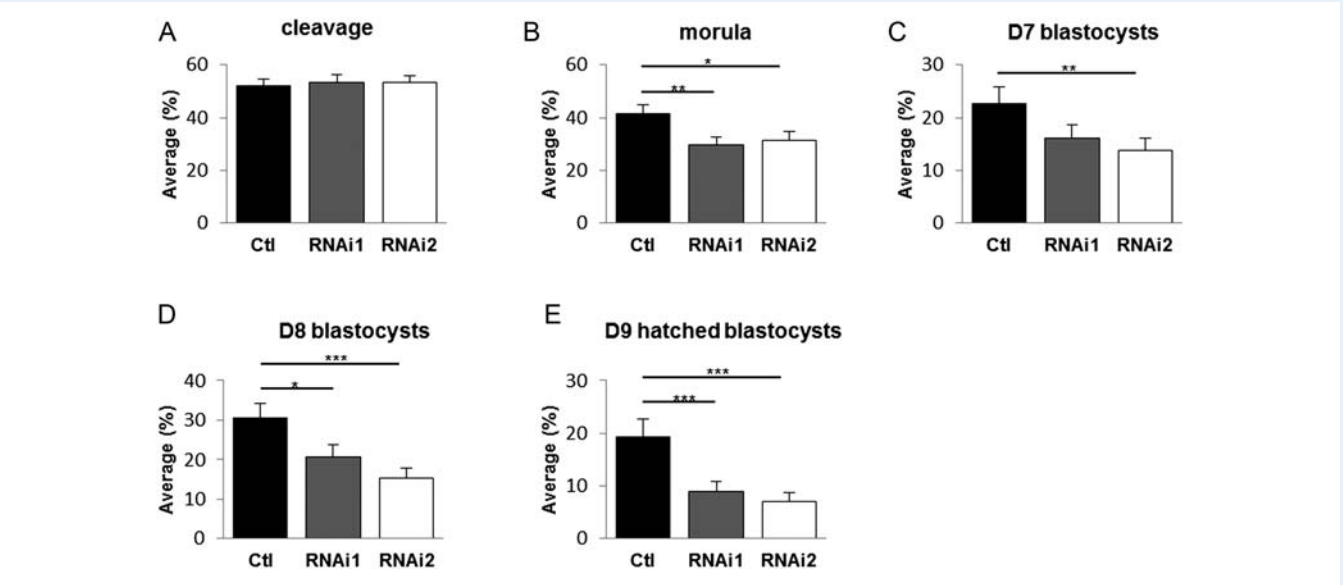

Figure 7 Effect of BCAR4-targeting RNAi onto bovine embryo development. Rates of (A) cleavage, (B) morula, (C) blastocysts on day 7, (D) blastocysts on day 8, (E) hatched blastocysts on day 9, after fertilization of oocytes microinjected with the negative control RNAi (Ctl), or a specific RNAi (RNAi1, RNAi2). Data from 8 replicates of 50 oocytes per group. Asterisks denote statistical significance (* for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$).

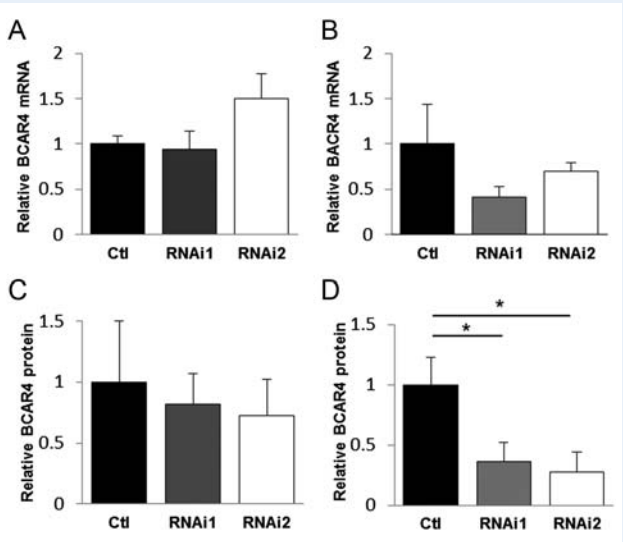

Figure 8 Effect of RNAi microinjection onto bovine BCAR4 mRNA and protein expression. (**A** and **B**) Analysis of mRNA abundance relative to AURKA by real-time PCR, and (**C** and **D**) protein abundance relative to Tubulin by western blot in (A and C) 2-cell embryos and (B and D) 4-cell embryos. mRNA and proteins were examined in embryos obtained after fertilization of oocytes microinjected with the negative control RNAi (Ctl), or a specific RNAi (RNAi1, RNAi2). Data from four to five replicates. The asterisk denotes statistical significance ($P < 0.05$).

et al., 2004). It was also detected by microarray in human oocytes (GDS3256; Kocabas et al., 2006). It appears as another putative mediator of BCAR4 effect in early development.

Conclusion

In this study, we have brought the first experimental evidence of the BCAR4 protein in mammals and characterized its expression in oocyte maturation and early embryo development. In agreement with its spatio-temporal pattern of expression, it shows a maternal-effect in bovine. Future studies should be oriented toward elucidating molecular mechanisms underlying this action. In parallel, we plan to investigate whether altered expression of the gene could underlie certain cases of human infertility.

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Authors' roles

L.A. did western blots, immunofluorescence analysis, IVF and RNA interference experiments together with C.Pe., and wrote the manuscript. P.P. collected bovine ovaries, did RNA purification and RT–PCR on all species. S.U. participated into western blot, immunofluorescence and electron microscopic analyses with R.U. N.L. validated the antibody and analyzed the protein profile during embryo development by western blot. V.C. collected human oocytes. D.R. supervised the human IVF lab and obtained patients consent. S.F. did the statistical analysis. C.G.-J. collected bovine OPU oocytes. C.Po. brought insight throughout the study. R.D.-T. did the bioinformatics and phylogeny

analysis, coordinated and supervised the work and wrote the manuscript.

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Conflict of interest

The authors indicate that there are no conflicts of interest and that they have no professional and financial affiliations that may be perceived as having biased the presentation.

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