Characterization of tetraspanin protein CD81 in mouse spermatozoa and bovine gametes

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Abstract

Sperm–egg interaction and fusion represent a key moment of fertilization. In mammals, it is not possible without the interaction of the tetraspanin superfamily proteins including CD81. A detailed immunohistochemical localization of CD81 was monitored in bovine oocytes during different maturation stages, as well as during early embryogenesis. In addition, characterization of CD81 was carried out in bovine and mouse sperm. In bovine eggs, CD81 was detected on the plasma membrane of the germinal vesicle, metaphase I and metaphase II oocytes. During fertilization, accumulation of CD81 molecules in the perivitelline space of fertilized oocytes, which appeared as vesicles associated with plasma membrane, was observed. In majority of bull-ejaculated sperm and caput, corpus and cauda epididymal sperm, as well as mouse cauda epididymal sperm, CD81 was found on the plasma membrane covering the apical acrosome. Although the process of capacitation did not influence the localization of CD81, it was lost from the surface of the acrosome-reacted spermatozoa in bull, in contrast to mouse sperm where there was a relocalization of the CD81 protein during acrosome reaction across the equatorial segment and later over the whole sperm head. The presented results highlight conservative unifying aspects of CD81 expression between cattle and mouse, together with mouse-specific traits in sperm CD81 behaviour, which emphasizes certain species-specific mechanisms of fertilization to be considered.

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Introduction

Sperm–egg interaction and fusion represent a key moment of fertilization, and it is not possible without an interaction of proteins belonging to the tetraspanin superfamily. The general feature of tetraspanins is their ability to interact with each other and with other partners, building up a complex called the tetraspanin web, which participates in signalling, cell–cell and cell–extracellular matrix adhesion, as well as in processes such as cell migration/motility, viral infection, immune responses, tumour metastasis and haematopoietic stem cell differentiation (reviewed in [Charrin et al. 2014](#)). It is well known that the tetraspanin web is not a constant formation but a highly dynamic unit, in which activity is concentrated to tetraspanin-enriched microdomains, distinct from raft domains and located in the cell plasma membrane. However, interaction of tetraspanins is not restricted to these areas ([Boucheix & Rubinstein 2001](#), [Hemler 2003](#), [Levy & Shoham 2005](#)).

In the light of current knowledge, it is very complicated to understand the mechanism of tetraspanin web function. Therefore, the information about localisation and dynamics of individual molecules expected to participate in the web can provide a significant contribution to the complex comprehension of gamete interaction in mammals.

CD81 is a tetraspanin protein considered as a candidate molecule involved in mammalian fertilization due to its partnership with the primary egg fusion protein CD9 ([Ohnami et al. 2012](#)). Although CD9 expression on the egg plasma membrane has been shown to be indispensable for sperm–egg fusion in mice ([Kaji et al. 2000](#), [Le Naour et al. 2000](#), [Miyado et al. 2000](#)), the behaviour of CD81 in tetraspanin web formation during egg fertilization seems to be complementary to CD9 ([Ohnami et al. 2012](#)). This supposition is supported by findings in cattle ([Zhou et al. 2009](#)), where CD9 antibody treatment of zona pellucida (ZP)-free oocytes inhibited the penetration rate only partially. Regarding

the structure, CD81 is composed of four transmembrane regions: cytoplasmic amino and carboxy termini and two extracellular regions (Boucheix & Rubinstein 2001), thus sharing more than 45% of amino acid sequence homology with CD9 throughout the four transmembrane regions.

We would hereby like to present distribution of CD81 on ZP-intact and ZP-free cow oocytes in different maturation stages: from germinal vesicle (GV) through metaphase II (MII) to zygotes and four-cell embryos. Moreover, we propose that the CD81 tetraspanin web formation on bovine eggs involves the production of CD81-containing plasma membrane vesicles after fertilization. The expression of CD81 on bull sperm at different stages of maturation, such as the epididymal and ejaculated, capacitated and acrosome-reacted, was also investigated. In parallel, characterization of CD81 localization in mouse epididymal spermatozoa was performed, as the aspect of mouse male gamete seems to be missing in the literature to date. We deliver the evidence of CD81 expression on the plasma membrane of both bull- and mouse-intact sperm in the apical region of the sperm head, suggesting the primary function of this protein in the early stages of sperm–egg interaction before acrosome reaction (AR). The presented comparative studies should also help to identify conserved and species-specific molecular interactions that could highlight the key components involved in reproduction processes, such as the mechanism of fertilization.

Materials and methods

Animals

Inbred C57BL/6Ncrl mice were obtained from VELAZ s.r.o., Prague, Czech Republic, and housed in animal facilities of the Laboratory of Reproduction, Faculty of Science, Charles University, Prague, Czech Republic. Reporter transgenic male mice expressing enhanced green fluorescent protein (EGFP) in their sperm acrosome (C57BL/6Ncrl Acr3-EGFP) were generated in the Transgenic Unit of the Czech Centre for Phenogenomics, Institute of Molecular Genetics, CAS, Prague, Czech Republic; construct was kindly donated by Professor Masaru Okabe (Nakanishi *et al.* 1999). Food and water were supplied *ad libitum*. The male mice used for all experiments were of a reproductive age between 10 and 12 weeks. All animal procedures and experimental work were carried out in strict accordance with the Animal Scientific Procedure, Art 2010, and subjected to be reviewed by the local ethics committee (accreditation no. 247732008-10001).

Freshly ejaculated or frozen-thawed bovine sperm used in experiments were obtained from bulls (*Bos taurus*) of Slovak Breeding Services, Inc., Luzianky, Slovak Republic. Donors of sperm were Holstein bulls (age 4–6 years, with an average body condition score of 3.5). The bull epididymides and cow ovaries were obtained at a local slaughterhouse.

Bovine model

Collection of sperm from epididymis

The bull epididymis was dissected into three segments: caput, corpus and cauda. These tissue segments were used for the separation of epididymal spermatozoa. Each segment was cut into small pieces, incubated in 10 mL PBS for 15 min at 37°C and the cloudy suspension was centrifuged at 50g for 10 min to remove pieces of tissue debris. The pellets containing spermatozoa for immunofluorescence analysis were obtained after centrifugation at 200g for 10 min.

Capacitation and acrosome reaction

Freshly ejaculated spermatozoa were separated from seminal plasma via centrifugation at 200g for 10 min at room temperature. Washed spermatozoa were resuspended in a commercially supplied TL medium for bovine sperm cell capacitation (TL medium) (Minitube, Celadice, Slovak Republic) to a final concentration of 10^7 /mL and capacitated at 39°C with 5% CO₂ in a humidified atmosphere for 4 h. Acrosome reaction was induced in capacitated sperm by 10 µmol/L Calcium Ionophore A23 187 (Cal) (Sigma Aldrich) for 1 h at 39°C and 5% CO₂.

Immunolabelling of bovine sperm

An immunofluorescence assay was performed on epididymal, freshly ejaculated, frozen-thawed, capacitated and acrosome-reacted sperm. The pellets of cryo-conserved sperm were washed twice in PBS at 200g for 10 min at room temperature. After washing, the sperm suspension was smeared on slides and fixed by cold acetone–methanol (1:1) wet fixation and dried. All treatments were applied in a humid chamber to prevent the drying out of the cell smears. Sperm were blocked with Super Block Blocking Buffer (Thermo Scientific) for 1 h at 37°C and treated with the primary antibody anti-CD81 (H121; Santa Cruz Biotechnology) (1:100) for 1 h at 37°C in a humidified chamber. As a secondary antibody, goat anti-rabbit IgG-FITC was conjugated (1:200) (Vector Laboratories, Burlingame, CA, USA) for 30 min in the dark at room temperature. Nuclear DNA of both CD81-reactive and non-reactive sperm was stained by Vectashield mounting medium with DAPI (Vector Laboratories). The intactness of sperm acrosomes was assessed by Peanut agglutinin-TRITC (PNA-TRITC, Vector Laboratories). Immunostaining was evaluated under a Leica DM5500 B epifluorescence microscope at 400 and 1000× magnifications and the fluorescence images were recorded using a Leica DFC340 FX digital camera and processed using Leica Advanced Fluorescence software. Representative results are shown.

Isolation, maturation and in vitro fertilization of bovine oocytes

Cumulus–oocyte complexes (COC) were aspirated from follicles (2–8 mm) of ovaries obtained at a slaughterhouse and transported to the laboratory in physiological saline at 37°C. COC with an intact compact cumulus and homogenous

ooplasm in the GV or metaphase I (MI) stage were collected under a stereomicroscope Olympus SD30. Maturation and *in vitro* fertilization of bovine oocytes were performed according to [Makarevich & Markkula \(2002\)](#). Cumulus–oocyte complexes were matured in drops (500 µL) of medium 4.5 mL TCM-199 with GlutaMAX (Gibco, Invitrogen) with 10% foetal calf serum, 50 µL sodium pyruvate (27.5 mg/mL physiological saline), 10 µL PLUSET (mixture of FSH and LH 25 U/mL) (Minitube) and 5 µL gentamicin (40 mg/mL), for 18–20 h at 39°C in a humidified atmosphere with 5% CO₂. Cumulus cells were removed by 5-min vibration. Frozen spermatozoa thawed in TL medium were resuspended to a concentration of $1 \times 10^6/100 \mu\text{L}$ in IVF medium (Minitube). The matured oocytes (25) and sperm (0.5×10^6) were co-incubated in microdrops (100 µL) overlaid with Ultrapure mineral oil (USB Corporation, Cleveland, OH, USA) at 39°C with 5% CO₂ for 24 h. After fertilization, sperm were removed from presumptive zygotes by 1-min vibrations. Embryos were incubated for a further 48 h in ISM1 culture medium (East Port Life Sciences, Prague, Czech Republic) overlaid with mineral oil at 39°C and 5% CO₂.

Immunolabelling of bovine oocytes

GV and MI oocytes were washed in PVA–Dulbecco's PBS (1 mg/mL) and freed of cumulus cells by 5 min of vortexing in hyaluronidase type IV-S from bovine testes (150 U/mL) (Sigma Aldrich). Cumulus cells from MII oocytes were removed by 5 min of vibration. For preparation of zona pellucida-free oocytes, pronase (50–10 mg/mL) was applied under optical control. All oocytes, zygotes and embryos were fixed in paraformaldehyde in PBS (38 mg/mL) for 7–15 min at room temperature and incubated with the anti-CD81 polyclonal antibody (IgG-200 µg/mL). Goat anti-rabbit IgG-FITC conjugate was applied at a dilution of 1:300 in the dark for 30 min at room temperature as a secondary antibody. Negative controls were performed for all stages of the oocytes by omitting the primary antibody. After washing, the oocytes were moved to slides and mounted with Vectashield mounting medium with DAPI. Confocal images were obtained by a Carl Zeiss LSM 880 NLO confocal microscope at 400× magnification. Huygens Professional version 16.05 (Scientific Volume Imaging, The Netherlands, <http://svi.nl>) software was used for 3D visualization of the images. An open source software Fiji was used for other image processing.

Immunolabelling of bovine oocytes was performed at least three times with a minimum of 25 oocytes in each experimental group. Representative results are shown.

Mouse model

Capacitation

Sperm from the distal regions of the cauda epididymis were released into a 200 µL droplet of M2-fertilizing medium (Sigma Aldrich, M7167) under paraffin oil (P-Lab, Czech Republic, P14501) in a Petri dish and pre-tempered at 37°C in the presence of 5% CO₂. Released sperm were assessed for motility and viability under a light inverted microscope with

a thermostatically controlled stage at 37°C. Sperm stock was diluted to the required concentration ($5 \times 10^6/\text{mL}$) in 100 µL M2 medium under paraffin oil. Sperm were left freely to capacitate. Sperm samples were collected at 90 min or the incubation was continued by an induction of acrosome reaction. The freshly released epididymal sperm, which have not undergone capacitation, were used for the protein detection to monitor a protein status before capacitation.

Acrosome reaction

Spermatozoa from the distal regions of the cauda epididymis were capacitated for 90 min as described above followed up by a 90-min induction of AR by Calcium Ionophore (Cal, A 23187, Sigma Aldrich), which was added to the capacitated sperm M2 droplet at a final concentration of 5 µM. All the sperm samples were incubated at 37°C under 5% CO₂.

Monitoring of sperm quality and acrosome status

All the sperm samples were incubated at 37°C under 5% CO₂. Transgenic mouse line EGFP in the sperm acrosome enabled us to monitor the acrosomal status in living spermatozoa immediately by fluorescence microscopy.

Immunolabelling of mouse sperm

Immunofluorescent detection of CD81

Freshly released live sperm from cauda epididymis, and sperm after the acrosome reaction, were washed twice in 500 µL PBS and the supernatant was removed. The antibody detection further continued in a tube for all groups. Sperm were blocked with 10% BSA in PBS for 1 h and incubated with the primary antibody anti-CD81 (sc-31234; Santa Cruz Biotechnology) diluted 1:25 in PBS followed by washing in 500 µL PBS twice and incubation with Alexa Fluor 568 donkey anti-goat IgG (Molecular Probes) secondary antibody at a dilution of 1:300 in PBS for 1 h at room temperature, followed by a final washing in 500 µL PBS without fixation.

The samples were examined with an Olympus IX81 fluorescent microscope and photographed with Hamamatsu ORCA C4742-80-12AG, using Olympus Soft Imaging Solutions software (Laboratory Imaging Ltd, Prague, Czech Republic). Representative results are shown.

Immunofluorescent detection of dual staining of CD81 and oestrogen receptor β (ER β)

Live Acr-EGFP transgenic sperm ([Nakanishi et al. 1999](#)) with a green acrosome were used for a dual immunofluorescent labelling of CD81 and ER β . Moreover, dried sperm smears were used to compare the staining with a previously published data ([Sebkova et al. 2012](#)).

Freshly released cauda epididymis sperm were washed twice in PBS. The antibody detection further continued in a tube or sperm were smeared onto glass slides and air-dried. Sperm smears were fixed with 3.7% formaldehyde in PBS (pH 7.34) at room temperature for 10 min, followed by washing

Figure 1 Detection of CD81 on the surface of bull cauda epididymal spermatozoa. (A) Acrosome-intact; (B) acrosome-reacted. Fluorescence images correspond to their respective phase contrast. CD81 (green), PNA (red) and DNA (blue). Scale bar represents 10 µm.

in PBS. Sperm were blocked with 10% BSA in PBS for 1 h and incubated with primary antibody anti-CD81 (sc-31234; Santa Cruz Biotechnology) diluted 1:25 in PBS and primary antibody anti-ERβ H-150 (sc-8974; Santa Cruz Biotechnology) diluted 1:50 in PBS over night at 4°C, followed by Alexa Fluor 568 donkey anti-goat IgG and Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes) secondary antibodies 1:300 in PBS for 1 h at room temperature. In case of dual staining, both secondary antibodies were applied together.

After washing, the slides were mounted into Vectashield mounting medium with DAPI (Vector Laboratories). The samples were examined with an Olympus IX81 fluorescent microscope and photographed with Hamamatsu ORCA C4742-80-12AG, using Olympus Soft Imaging Solutions software (Laboratory Imaging Ltd). Representative results are shown.

Immunofluorescent detection of dual staining of CD81 and CD46

Freshly released cauda epididymis sperm were washed twice in PBS, smeared onto glass slides and air-dried. Sperm smears were fixed with 3.7% formaldehyde in PBS (pH 7.34) at room temperature for 10 min, followed by washing in PBS. Sperm were blocked with 10% BSA in PBS for 1 h and incubated with primary antibody anti-CD81 (sc-31234; Santa Cruz Biotechnology) diluted 1:25 in PBS and primary antibody anti-CD46 (HM-1118; Hycult Biotech, Uden, The Netherlands) diluted 1:50 in PBS over night at 4°C, followed by Alexa Fluor 568 donkey anti-goat IgG and Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes) secondary antibodies 1:300 in PBS

Figure 2 Localization of CD81 on ZP-intact bovine oocytes of germinal vesicle (GV) (line I), metaphase I (MI) (line II), metaphase II (MII) (line III), zygote (line IV) and four-cell embryo (line V). CD81 (green), DNA (blue). Scale bar represents 20 µm.

for 1 h at room temperature. In the case of dual staining, both secondary antibodies were applied together.

After washing, the slides were mounted into Vectashield mounting medium with DAPI (Vector Laboratories). The samples were examined with an Olympus IX81 fluorescent microscope and photographed with Hamamatsu ORCA C4742-80-12AG, using Olympus Soft Imaging Solutions software (Laboratory Imaging Ltd). Representative results are shown. For all sperm specimens, staining patterns were evaluated by scoring a minimum of 200 sperm for each assay and each experiment was repeated at least three times.

STED super-resolution microscopy

Freshly released sperm were used for STED imagery. Sperm were collected the same way as described previously with some differences. Sperm samples were always prepared onto a microcover glass (thickness 0.13–0.17 mm; Hirschmann). Moreover, after application of primary and secondary antibodies, sperm were incubated for 30 min with 2.5 mM PNA lectin (Molecular Probes, L-32458) in PBS. After washing, DAPI (0.85 µg/mL; Thermo Scientific) was added for 5 min and washed three times in PBS. At the end, sperm were washed once in distilled water and air-dried. Dry samples were covered with 90% glycerol with 5% anti-fade *N*-propyl gallate (Sigma Aldrich). Fluorescent images were collected with a Leica TCS SP8 STED 3X microscope using the software LAS X 64bit package with LAS AF SP8 Dye Finder, 3D visualization, deconvolution and colocalization module (Microscopy Centre, IMG AS, Prague, Czech Republic).

Figure 3 CD81 detection in mouse sperm from cauda epididymis. Freshly released sperm (line I), sperm during the acrosome reaction (lines II and III). CD81 (red), acrosin-EGFP (green). Scale bar represents 2 μ m.

Results

CD81 on bovine sperm

In our study, epididymal spermatozoa isolated from the caput, corpus and cauda of bull epididymis as well as freshly ejaculated and conserved in liquid nitrogen were examined. The presence of CD81 was already detected in the majority of sperm isolated from the caput of bull epididymis and the reaction remained observable on the sperm head surface from epididymal corpus as well as cauda. The fluorescent signal of CD81, which appeared as a thin line on the apical part of the intact sperm head, was consistent with sperm obtained from all parts of bull epididymis (Fig. 1A). In a proportion of the sperm population, a signal in the equatorial region was observed. The same pattern was detected in freshly ejaculated sperm. The process of capacitation did not influence the localization of CD81. In the sperm suspension induced for AR, the acrosome-reacted sperm showed no CD81 and no PNA staining in the acrosomal region of the sperm head (Fig. 1B).

CD81 on bovine oocytes

GV and MI bovine oocytes

With regard to cattle, there is no study available reporting the expression of CD81 on the surface of bovine oocytes. In our experiments, when the GV as well as metaphase

II oocytes isolated from cow ovarian follicles (2–8 mm in diameter) were inspected, the following results were achieved. Immunofluorescent analysis of GV (Fig. 2, line I and [Supplementary Video 1](#), see section on [supplementary data](#) given at the end of this article) and MI (Fig. 2, line II and [Supplementary Video 2](#)) cow oocytes revealed the CD81 pattern to be an interrupted signal or a brindle staining on the surface of the plasma membrane. No staining of the ZP was recognized.

Metaphase II bovine oocytes

Bovine oocytes matured in defined conditions *in vitro* to approach the metaphase II stage were also subjected to our examination. CD81 positively labelled the plasma membrane of MII oocytes (Fig. 2, line III and [Supplementary Video 3](#)) and there was negative staining of both the perivitelline space (PVS) and ZP. The MII stage gave the patchiest signal over the oolema, with visible CD81 clustering (Fig. 2, line III). The surface of cumulus oophorus cells was also positively stained when examined (not shown). Regardless the nuclear stage of oocytes, patches of CD81 on the plasma membrane of 50–70% of zona pellucida-free oocytes appeared.

Bovine zygotes and embryos

Based on the assumption that tetraspanin CD81 can participate in some of the steps of the fertilization process,

Figure 4 CD81 and ER β detection in freshly released cauda epididymal mouse sperm. CD81 (red), ER β (green). Both proteins have the same localization on the plasma membrane covering the apical acrosome. Scale bar represents 2 μ m.

Figure 5 CD81 and CD46 detection in freshly released cauda epididymal mouse sperm. CD81 (red), CD46 (green). Both proteins display different localization. CD81 is localized on the plasma membrane covering the apical acrosome. CD46 is localized on the acrosomal membranes. Scale bar represents 2 μ m.

we were interested in the change in the distribution of this protein after fertilization. An irregular fluorescent signal of CD81 remained on the plasma membrane, and moreover, we observed an accumulation of CD81 in PVS of zygote (Fig. 2, line IV and [Supplementary Video 4](#)) and two-cell embryo (not shown) and four-cell stage embryo (Fig. 2, line V and [Supplementary Video 5](#)) that appeared as dots/vesicles associated with plasma membrane and released during fertilization.

CD81 on mouse spermatozoa

In the intact living mouse sperm, CD81 is localized on the plasma membrane covering the apical acrosome (Fig. 3, line I). During the acrosome reaction, two patterns were detected in the sperm head; the positively labelled equatorial segment (Fig. 3, line II) and the whole sperm head (Fig. 3, line III). Intact sperm were detected using acrosin-EGFP signal. EGFP signal was absent when protein was localized over the equatorial segment or/and whole sperm head. Utilizing previously published information on ER β , being it expressed in the plasma membrane of acrosome-intact sperm ([Sebkova et al. 2012](#)) and CD46 in the acrosomal membranes ([Johnson et al. 2007](#)), we prepared two kinds of dual staining: first, protein CD81 with ER β (Fig. 4) and secondly CD81 with CD46 (Fig. 5). The ER β was detected on the apical plasma membrane of the mouse sperm head exactly at the same position as CD81, both on the fixed (Fig. 4) and live sperm without fixation (not shown). On the other hand, CD46 is typically present on the acrosomal membranes; this localization was thus different from CD81 (Fig. 5). Moreover, the accurate position of CD81 was investigated using STED super-resolution microscopy. This method also confirmed the presence of CD81 on the apical plasma membrane of the mouse sperm head (Fig. 6 and [Supplementary Fig. 1](#)).

Discussion

To date, previous analyses of CD81 tetraspanin aimed to characterize and inspect its role on oocytes ([Ziyyat et al. 2006](#), [Ohnami et al. 2012](#), [Barraud-Lange & Boucheix 2013](#)), and localization of CD81 was described to be on the egg plasma membrane in mice ([Takahashi et al. 2001](#)). [Ohnami et al. \(2012\)](#) confirmed

a distribution of CD81 in the perivitelline space close to ZP, and [Barraud-Lange and Boucheix \(2013\)](#) described its punctuate signal in ZP. On the other hand, [Tanigawa et al. \(2008\)](#), documented, in stained ovaries, a higher level of CD81 expression in the granulosa and cumulus cells in comparison to oocyte. Moreover, [Ohnami et al. \(2012\)](#) referred to CD81, together with CD9 tetraspanin, as part of extracellular oocyte components corresponding to the perivitelline space (CD9) and ZP (CD81) and proposed that CD9 is primarily produced by oocyte, whereas CD81 is predominantly produced in cumulus cells and subsequently localized to ZP. In our observation on bovine eggs, CD81 was expressed on the plasma membrane of GV, MI and MII oocytes as well as on cumulus cells consistent with mouse oocytes ([Tanigawa et al. 2008](#)). However, a completely different situation of CD81 was seen regarding to the presence of CD81 in ZP. We did not record any CD81-positive signal in the ZP of oocytes isolated from antral follicles, as well as oocytes matured to metaphase II *in vitro*. This observation is similar to the one observed in humans, where CD81 is localized on the plasma membrane

Figure 6 CD81 detection in freshly released cauda epididymal mouse sperm by STED super-resolution microscopy. CD81 (red), PNA (green) and DAPI (blue). The localization of CD81 is on the plasma membrane in the acrosomal cap area. Scale bar represents 1 μ m.

of ZP-free as well as ZP-intact human oocytes (GV, MI and MII) (Coskun *et al.* 2003, Ziyat *et al.* 2006). However, patchy staining of CD81, simultaneously with alpha6beta1 integrin and CD151, appeared on the oocyte surface after ZP was mechanically removed and they colocalized with CD81, but not with CD9, (Ziyat *et al.* 2006). The same patchy pattern was observed in our bovine eggs although ZP was removed enzymatically. In mouse fertilization assay, the redistribution of CD9, together with alpha6beta1 integrin from the site of gamete interactions gathering into small clusters after fusion, was also observed (Ziyat *et al.* 2006). In the future, we would like to inspect the behaviour of CD81 during these processes in mice as well as in cattle based on the fact that at a simultaneous expression of CD81 and CD9 on the human embryonic kidney 293 cell line, the majority of CD9 molecules form a complex with CD81 (Stipp *et al.* 2001). In cattle, the presumable cooperation of CD81 with CD9 in this manner is supported by Zhou *et al.* (2009), which documented the expression of CD9 on the plasma membrane of cow oocytes, and our presented findings confirmed the localization of CD81 on the same site. Based on a different expression of CD81 and CD9 on the oocyte in mice, a distinct model of cooperation, these tetraspanin partners may be proposed. Ohnami *et al.* (2012) showed that CD9 and CD81 expressed on mouse oocyte plasma membrane can act along each side upon fertilization, but their activity seems to be independent of each other. Taking all together, the indirect involvement of CD81 apart from CD9 and alpha6beta1 integrin (and other proteins) in the fusion can be expected, although the precise mechanism is not known.

Based on our observations, CD81 is expressed on the bovine oocyte plasma membrane and organized in membrane-derived vesicles, which is supported by an uneven, spotty signal of CD81 on the oocyte plasma membrane with facilitation and accumulation of CD81 molecules in the perivitelline space of *in vitro* produced zygotes and four-cell bovine embryos. This observation is in correlation with a release of CD9-positive vesicles derived from murine oocytes before fertilization (Miyado *et al.* 2008) as well as a presence of CD9 and CD81 in the extracellular parts of murine oocyte and exosomes (Ohnami *et al.* 2012). To further support this interpretation, tetraspanins are viewed as membrane proteins that cluster into plasma membrane microdomains and can be also enriched on membrane-bound extracellular vesicles (Escola *et al.* 1998, Hemler *et al.* 2003, Rubinstein *et al.* 2006) known to mediate intercellular communication between cells and tissues (reviewed in Machtinger *et al.* 2016). It is of relevance that exosomes derived from mouse and human mast cell lines, as well as primary bone marrow-derived mouse cells, contain functional mRNA (and small RNA, including microRNAs) that

can be translated after a transfer to other mouse or human cells (Valadi *et al.* 2007). Transfer of proteins by CD9-positive microvesicles from cauda epididymal fluid to live maturing sperm was confirmed by Caballero *et al.* (2013). Therefore, the interaction of vesicles with other cells, including spermatozoa, can modulate the protein spectrum of target cells and the role of CD81 accruing from its tetraspanin character in the gamete fusion machinery is suggested. Moreover, a highly dynamic movement was shown, particularly for CD9, which was embedded in small membrane clusters enriched by CD81 in human PC3 cells as a result of the frequent escape or trapping of CD9 (Espanel *et al.* 2008). While the extracellular components distributed outside the oocyte cell membrane also formed bilayers, highly dynamic interactions in the tetraspanin web may be proposed for these extracellular vesicles. Taken all together, CD81-positive vesicles derived from the oocyte plasma membrane can serve as a "vehicle" for the transfer of CD81 onto the surface of penetrated bull sperm that is consistent with the surface localization of CD81 on intact sperm head and its loss from the surface of the acrosome-reacted sperm.

Recently, a partner for the sperm protein IZUMO1, referred to as JUNO (folate receptor 4), which is a protein essential for gamete adhesion was described (Bianchi & Wright 2014). JUNO is localized on the plasma membrane of unfertilized oocytes, but within 1h after fertilization, it is undetectable at this site and it is relocated to egg plasma membrane-derived vesicles. These vesicles are released into the perivitelline space of fertilized eggs, which suggests a mechanism of polyspermy prevention. In the light of these findings, a similar role can also be considered for vesicles carrying CD81.

So far, the female germinal cells have been quite reasonably characterized in terms of CD81 expression and its possible function; however, little is known about the sperm. We hereby present the CD81 expression on the surface of bull sperm extracted from all the three epididymis parts such as caput, corpus and cauda as well as on freshly ejaculated sperm. We also accompany these results with mouse sperm characterization and compare the similarities and differences between bull and mouse species. Moreover, the CD81 localization during the process of *in vitro* capacitation and AR induced by Cal was documented. Based on our results, the presence of CD81 seems to be directly linked to intactness of the acrosome. Almost all spermatozoa, which possessed intact undetached acrosomes, were observed as CD81 positive. Recently, a similar localization was described for CD9, an assumptive partner involved in the tetraspanin web (Antalikova *et al.* 2015). We propose that the presence of the CD81 protein, along with CD9, on the surface of intact bull sperm and its loss from the acrosomal region after AR could be significant for the maintenance of protein

organization within the sperm membrane during the events foregoing the sperm–egg fusion.

In parallel, we also detected protein CD81 in the plasma membrane of the acrosomal cap of mouse acrosome-intact sperm. We used dual labelling of CD81 and CD46/ER β /Acr-EGFP proteins with a well-known localization to confirm the accurate protein position. Knowing that CD46 is expressed solely on the acrosomal membranes in acrosome-intact sperm (Johnson *et al.* 2007, Frolikova *et al.* 2011): using a dual labelling of CD81 and CD46, we confirmed the absence of CD81 in the membrane defining the acrosome. Additionally, dual labelling of CD81 and receptor ER β pointed at a colocalization of both signals. In view of the fact that ER β is expressed on the plasma membrane of acrosome-intact sperm (Sebkova *et al.* 2012), the expression of CD81 on the plasma membrane is highly probable, which was later ratified by STED super-resolution microscopy. Acr-EGFP transgenic sperm (Nakanishi *et al.* 1999), with a green acrosome, were used for further identification. The green signal of Acr-EGFP is only detected if the acrosome is completely intact. This is contrary to PNA, which still remains detectable in the acrosomal area at the early stages of the acrosome reaction. So, using Acr-EGFP transgenic sperm (Nakanishi *et al.* 1999) enabled us to accurately distinguish the acrosome-intact sperm from sperm in the initial stage of the acrosome reaction.

Contrary to bull sperm, mouse sperm CD81 relocates after the acrosome reaction. In mice, CD81 started relocating to the equatorial segment of the sperm head after the initialization of the acrosome reaction and subsequently it covered the whole sperm head in the later stages of the acrosome reaction. We hypothesize that the difference between the presence/absence of the CD81 relocation process in mouse/bull sperm may be determined by a different morphology of sperm head as well as due to differences in their reproduction strategies. Resembling interspecies dissimilarity in sperm protein behaviour has already been described for other proteins, e.g. CD46 (Frolikova *et al.* 2011).

Jin *et al.* (2011) published that the acrosome reaction of mouse sperm could be already initiated during the transferring of sperm through cumulus cells and before their contact with ZP. It is known that there are many interspecies variations in mammalian reproductive strategies. In the case of mouse and cattle, there are significant dissimilarities in sperm head morphology and egg ZP composition. Therefore, it may not be of surprise that there also exist differences in protein expression, localization and dynamics and it is also the case for CD81.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0304>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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