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Immunohistochemical distribution of the tetraspanin CD9 in normal porcine tissues

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Abstract The tetra-membrane-spanning protein, CD9 is a 24–27 kDa cell surface glycoprotein expressed in a wide variety of human cells being involved in a variety of cell processes, including signaling, adhesion, motility, fertilization and tumor cells metastasis. By means of a polyclonal antibody (N1) raised against recombinant swine CD9 protein, we studied the immunohistochemical expression of CD9 on different normal swine tissues. Immunochemistry shows that swine CD9 was distribute in a similar form than in human tissues, being present on epithelial cells of lung, liver, kidney, skin, tonsil, testis (epididymo), gut mucosa, uterus and mama. Furthermore, polyclonal antibody against swine CD9 reacts with white matter from cerebrum and cerebellum, peripheral nerves fibers and Hassal corpuscle from thymus and ovum. Platelets react strongly with our antibody, but monocytes and neutrophils react lightly. These results suggest that CD9 antigen should play a similar functional role in swine and human and therefore studies on CD9 on swine as an animal model would allow new knowledge about its role in adhesion, fertilization and tumor metastasis among other important biomedical processes.

Keywords Pig · CD9 · Tetraspanin · Immunohistochemistry

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Abbreviations

BSA	Bovine serum albumin
DIG	Digoxigenin
DMSO	Dimethylsulphoxide
EGFR	Epidermal growth factor receptor
MT1-MMP	Membrane type-1 matrix metalloproteinase
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PRP	Porcine platelet-rich plasma
SDS	Sodium dodecylsulfate
TGF	Transforming growth factor

Introduction

CD9 is a 24-kDa member of the tetraspanin or transmembrane-4 (TM4) superfamily of surface molecules (33 human members) that cross the cellular membrane four times, having both NH_2 and COOH termini inside the cell, exhibits two extracellular loops and short cytoplasmic domains [1, 2]. CD9 is widely present on the surface of normal and malignant animal cells [3–6]. Originally, CD9 was identified as a surface antigen on a wide variety of lymphohemopoietic cells [7], however it is also widely expressed in non-lymphohemopoietic tissues including tissues of the central and peripheral nervous system [3, 8, 9]. In fact, CD9 molecule was absent on primitive hematopoietic progenitors and has been proposed as a tool for enrichment of hematopoietic stem cells [10, 11].

CD9 molecule has been involved in a variety of biological functions including cellular growth and development, activation, adhesion, and motility [12–14]. It is well known that CD9 is involved in platelet aggregation together with FCyRII receptor and $a_{II}\beta_3$ integrin [15]. It is also

known that CD9 participates in adhesive and migratory events via integrins of the β_1 family, especially $a_4\beta_1$ and $\alpha_5\beta_1$, with which it seems to be non-covalently associated in various non hematopoietic cells lines [16, 17]. Furthermore, it has been also known that CD9 can associate with other proteins, like EFGR, or the membrane metalloproteinase MT1-MMP [18, 19], and that CD9 may exhibit its effect as a molecular organizer by modulating the composition of adhesive complexes important in facilitating cell adhesion and matrix assembly [18, 20].

CD9 is also related to cell differentiation, having been observed a drastic reduction in its expression in some metastatic cancers as lung, breast, colon, bladder pancreatic cancer and squamous cell carcinoma, as well as malignant melanoma, when they are compared with the primary cells [21–23]. In fact, the CD9 expression level has been inversely correlated with invasion and metastasis [5, 24], or with the prognosis of patients with different types of cancer [25, 26]. Other recent results suggest that CD9 might have an important role that attenuates EGFR signaling affecting EGF-induced signaling in cancer cells [27]. These results provide insight into the role of CD9 in the presentation of TGF-α in epithelial and carcinoma cells, whose physiology is driven by ligand-induced EGFR activation [28].

On the other hand, CD9 also plays an important role in the gamete membrane interactions that regulate the fertilization in mice. For example, oocytes from CD9 knockout mice were rarely fertilized although both the ovulation and the maturation were normal [29]. Additionally, reduced sperm binding was found in zona-pellucida free mouse oocytes treated with anti-CD9 monoclonal antibodies [30–33]. Recently, has been shown [34] that CD9 is also expressed on pig ovarian tissues, oocytes and spermatozoa and that the fertilization can be blocked by anti-CD9 antibodies.

In domestic animals, CD9 has also been found to be a putative cellular receptor for distemper virus in canine specie [35] and for the immunodeficiency virus in feline species [36].

In a previous investigation we have cloned and sequenced the porcine CD9 gene, and studied the expression pattern of the porcine CD9 mRNA [37] showing that, like its homologs in other species, CD9 transcripts are ubiquitously expressed, although with heterogeneous levels depending on cells and tissues.

However, except for the localization of the CD9 pig protein on oocytes and stem cell populations, scarce information is available for swine CD9 protein expression on cells and tissues, although swine, beside to be a major food-animal group for humans, constitutes an animal model in biomedical research due to the anatomical and physiological similarities with humans [38]. For this, it is an important task in porcine immunology research to study more precisely the expression and distribution of CD9 on swine cells and tissues.

On the other hand, although, as an exception, it has been used a cross reactive anti-human CD9 monoclonal antibody to localize CD9 in porcine oocytes [34], porcine CD9 molecule is not usually recognized by human antibodies against it [39]. Therefore, to detect the porcine CD9 it is more effective to produce specific anti-porcine CD9 antibodies.

With the aim of defining the detailed expression of the porcine CD9 protein, we first produced a polyclonal antibody (N1) against a recombinant protein of the porcine CD9 molecule and then we used it to test its immunochemical distribution in a broad variety of healthy swine cells and tissues.

Materials and methods

Tissues and cells

All tissues (skin, gut, kidney, liver, pancreas, brain -cerebral and cerebellum cortex-, tonsil, ovary, mama, testis -epididymo-, peripheral nerve, thymus, spleen and lymph node) were obtained fresh from the local abattoir from 6 to 8 months old swine. Peripheral blood mononuclear cells (PBMCs) and granulocytes were isolated from pig blood by density gradient centrifugation on Ficoll-Paque (Pharmacia). Porcine platelet-rich plasma (PRP) was obtained by low centrifugation of blood anticoagulated with trisodium citrate. Platelets were pelleted from PRP by centrifugation at 2,200*g* for 7 min and washed three times with PBS containing 5 mM EDTA.

Recombinant CD9 protein (rpCD9)

cDNA sequence covering nucleotides 426–674 that encodes the third TM domain and most part of the second extracellular domain, EC2, of the porcine CD9 protein [see 37] was amplified by PCR from plasmid template. Primers used for amplification contained restriction sites (indicated in bold) enabling ligation into the expression vector PGEX-4T-1 (Amersham Biosciencies) following digestion of the PCR product and the vector with *Eco*RI. Primers were as follows: F426-*Eco*RI 5′-GGA*GAATCC*CACAAGGAT CAGGTGATCAAA-3′ (sense) and R674-*Eco*RI 5′-GGC *GAATTC*GATGTGTAACTTGTTTTGGAA-3′ (antisense). A 249 bp PCR product was ligated into the expression vector pET28b and used to transform *Escherichia coli* strain BL21 (DE3) (Novagen). Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. Cells were collected by centrifugation at 4,000*g*, 20 min, 4°C, and suspended in

PBS buffer, then sonicated 15 times at intervals of 60 s on ice followed by incubation in lyses buffer 1% Triton X-100, 1 mg/ml lysozyme) for 1 h, 4°C. After centrifugation at 1,000*g*, 30 min, 4°C, an about 8 kDa recombinant EC2-CD9 protein was purified from the supernatant by affinity chromatography using a Glutatione Sepharose 4B Matrix (Amersham Biosciences) according to the manufacturer's instructions. Purified recombinant protein was dialyzed against PBS containing 5 M urea and adjusted to a final concentration of 1 mg/ml. Purity of the recombinant protein was assessed by SDS-PAGE, followed by Coomassie blue staining. Protein identification was carried out by MALDI-TOF mass spectrometry analysis of the tryptic peptides.

Polyclonal antibody production

New Zealand rabbits, obtained from the animal facilities at University of Córdoba, Spain, were intradermally immunized with rpEC2-CD9 (50 mg) in an equal volume of complete Freund's adjuvant, and then were boosted five times with an emulsion of recombinant protein and incomplete Freund's adjuvant at 1-week intervals. Two week after the last booster, rabbit serum was collected and titers of antibodies specific for rpEC2-CD9 assayed using an antibody capture ELISA.

Western blot

Purified porcine rpEC2-CD9 was mixed with the sample buffer and run on a 5–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The separated protein were transferred to an Immobilon membrane (Millipore, Bedford, MA, USA) using a semi-dry transfer cell (Millipore). Immobilon free binding sites were blocked with PBS-2% BSA. Strips were then cut and incubated with the polyclonal antibody for 2 h at room temperature, followed by 1 h incubation with a peroxidase-conjugated rabbit anti-mouse Ig (Sigma). Peroxidase activity was visualized using the ECL detection method (Amersham).

Immunohistochemistry

All tissue specimens were fixed in Bouin liquid for 16 h. Tissues were dehydrated in ascending concentrations of ethanol and xylene and embedded in paraffin. Sections of 5 μm were prepared from selected tissue blocks and consecutive sections were placed on slides coated with Vectabound (Vector Laboratories, Inc.). The tissue slides were kept at 55°C for 45 min in an oven to improve the adherence of sections to glass.

The sections were deparaffinized and rehydrated in xylene and descending concentrations of ethanol, respectively. Endogenous peroxidase activity was inhibited by treatment with 3% hydrogen peroxidase in distilled water for 30 min at room temperature. After washing with PBS, the sections were incubated with normal goat serum (1:10 dilution in PBS) (Vector) for 30 min at room temperature. After removing the serum, polyclonal antibody (1/3,000 dilution in PBS) or normal rabbit serum (as negative control) were added for 18 h at 4°C in a wet chamber. The sections were washed in PBS and incubated with biotinylated anti-mouse Ig (Dako) diluted 1/50 in PBS for 30 min at room temperature. After washing again in PBS, tissue sections were covered with avidin–biotin peroxidase complex (Sigma) diluted 1/50 with PBS for 1 h in a wet chamber at room temperature, washed and then developed with 3, 3′-diaminobenzidine (Sigma) (5 μg in 10 ml PBS). Sections were counterstained with Mayer hematoxylin and mounted with Eukitt. The sections of lymph node were covered with biotin–streptavidin–alkaline phosphatase complex (Biogenex). The chromogen substrate for alkaline phosphatase was naphthol and fast red (20% w/v). After incubation, slides were washed and mounted in aqueous medium of Shandon Immuno-Mount (Sigma).

Results

Purification of recombinant CD9 protein (rpCD9)

A swine recombinant CD9 protein (rpCD9) encoded by cDNA sequence covering nucleotides 426–674 that includes the third TM domain and most part of the second extracellular domain, EC2, was purified after the expression of the cDNA PCR sequence into pGEX-4T-1 expression vector. Figure 1a shows three fractions of the purified rpCD9 protein showing a molecular weight of

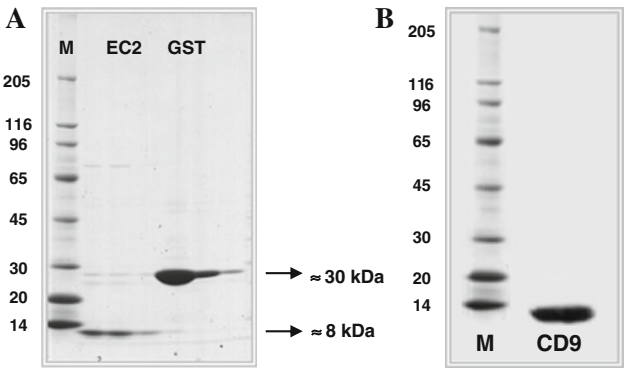

Fig. 1 **a** Electrophoresis of purified swine CD9 recombinant (8 kDa) and GST (30 kDa) proteins. **b** Immunoblotting of rpCD9 by N1 antibody

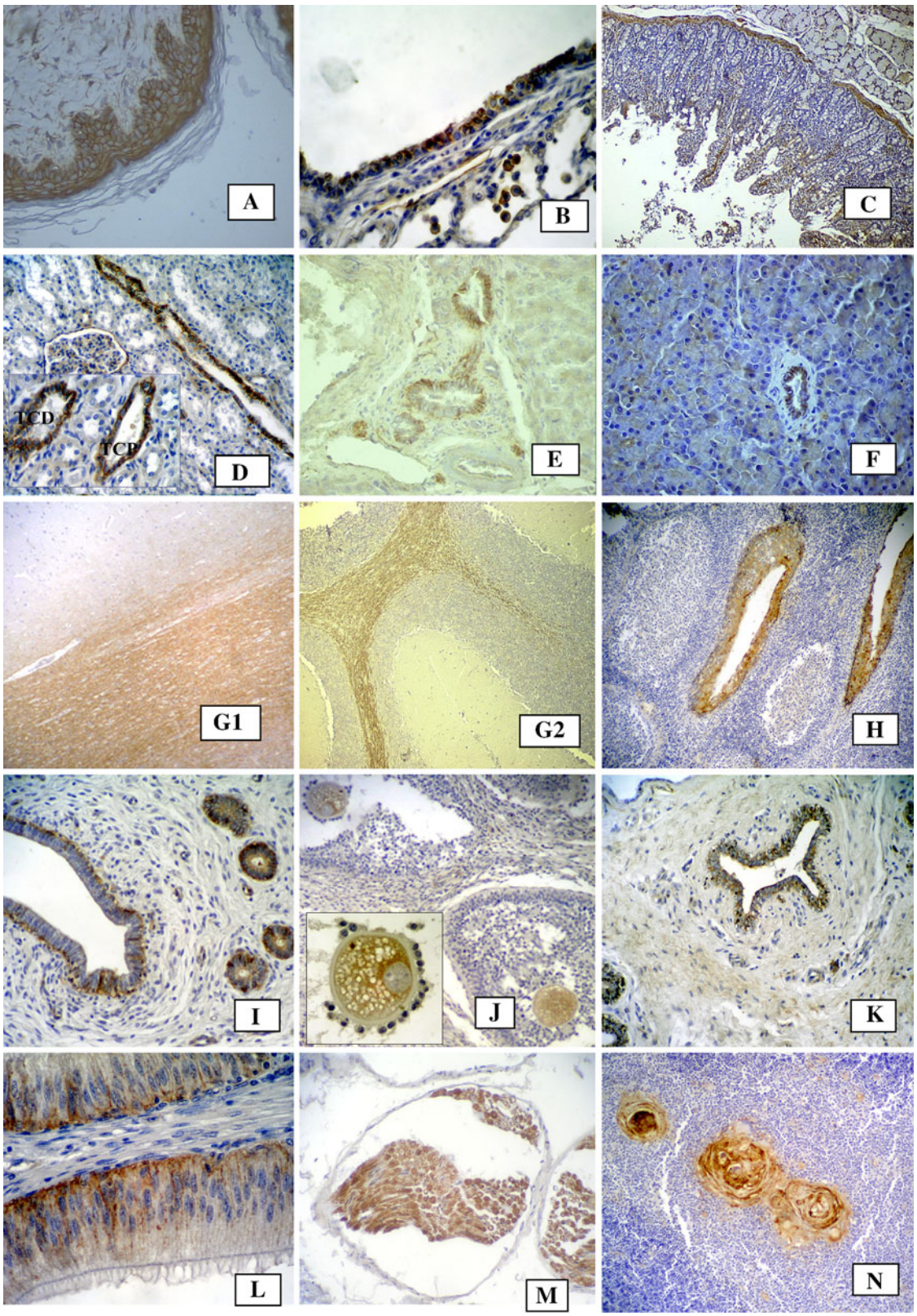

Fig. 2 Immunohistochemical staining of formalin-fixed, paraffin-embedded sections of different porcine tissues with N1 polyclonal antibody. **a** Skin (×20), **b** lung (×40), **c** gut (×10), **d** kidney (×20) and detail of tubules (×40), **e** liver (×40), **f** pancreas (×20), **g1** cerebron (×4), **g2** cerebellum (×10), **h** tonsila (×10), **i** uterus (×20), **j** ovary (×10) and ovum detail (×40), **k** mama (×20), **l** testis, epididymo (×40), **m** peripheral nerve (×40), **n** thymus (×20)

about 8 kDa, as well as the glutathione-*S*-transferase (GST) tail (about 30 kDa) released from the fusion protein after thrombin digestion. After rabbit immunization with the purified 8 kDa rpCD9 protein the N1 polyclonal antibody was obtained. Figure 1b shows an immunoblotting of the N1 antibody detecting the rpCD9 protein.

Tissue distribution of the swine CD9 molecule

The precise localization of the protein coded by *CD9* gene was studied by immunohistochemistry with N1 polyclonal antibody obtained using a porcine CD9 recombinant protein. The antibody was raised against a recombinant protein corresponding to a 243 amino acid polypeptide fragment located in the EC2 extracellular domain of the porcine CD9 molecule, the most variable domain among the species compared [37]. The reactivity of this polyclonal antibody was tested on a broad panel of healthy porcine cell and tissues: PBMCs, granulocytes, platelets, skin, gut, kidney, liver, pancreas, brain -cerebral and cerebellum cortex-, tonsil, ovary, mama, testis -epididymo-, peripheral nerve, thymus, spleen and lymph node. The representative results of this immunohistochemical analyses are shown in Fig. 2.

In skin, the anti-CD9 polyclonal produced an intense staining of the he epidermis, especially in the membrane of all keratinocytes. However it did not stain the dermis (Fig. 2a).

In lung, the reaction was positive within the parenchyma, in the smooth muscle associated with bronchi, in epithelium of bronchi and bronchioles, in blood vessels, and in alveolar pneumocytes. Macrophages inside alveolar sacs also showed some staining (Fig. 2b).

In gut, CD9 was detected on the crypts and on the bases of the villi. Smooth muscle was also stained. Staining of some intraepithelial lymphocytes was also observed (Fig. 2c).

In kidney, the anti-CD9 strongly stained renal corpuscules and epithelial cells of collecting ducts, detecting a weaker staining on glomerular endothelium and Bowman capsule (Fig. 2d).

In liver, the endothelium in hepatic sinusoids was weakly stained. The cell membranes of hepatocytes also showed a weak staining (Fig. 2e).

In pancreas, epithelial cells of excretory conducts are stained but acinar cells and pancreatic islets were either unstained or lightly stained for CD9 (Fig. 2f).

In brain, cerebral and cerebellum cortex, the anti-CD9 polyclonal stained the white matter. However, the cytoplasm of the cells gray matter showed a strong diffuse staining, and the Purkinje cells were not stained (Fig. 2g).

In tonsil, squamous epithelium, germinal centers, and high endothelial venules (HEVs) were stained by polyclonal ant-CD9. A strong staining for CD9 was observed on cell membranes throughout the epithelium (Fig. 2h).

In uterus, the epithelial cells covering both the endometrio and the endometrial glandules were stained. Reaction was only observed in membrane at the basal zones of the cellules (Fig. 2i).

In ovary, the cytoplasm of oocytes were strongly stained (Fig. 2j).

In mama, two clearly differentiate zones were detected: the epithelial cells of the glandule conducts were strongly stained, and the epidermis of the nipple showed a weakly staining (Fig. 2k).

In testis, the epididymo epithelial cells were strongly stained, however this stain was not homogeneous, being concentrated on the basal zone (Fig. 2l).

In peripheral nerves, the fibbers were stained within transverse and longitudinal sections of skeletal muscle. The perineurium of peripheral nerve fascicles was strongly positive for CD9 (Fig. 2m).

In thymus, the Hassall's corpuscles were stained by N1 antibody, and some infiltrate macrophages were also lightly stained (Fig. 2n).

In spleen and lymph node, a negative staining was obtained (data not shown).

In peripheral blood the polyclonal against CD9 stained platelets (Fig. 3a) and several other hemopoietic cell

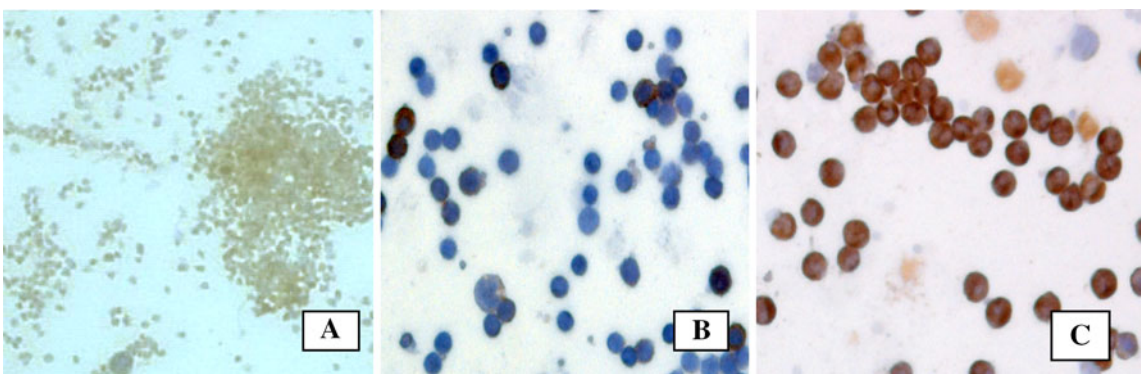

Fig. 3 Immunohistochemical staining of blood cells with N1 polyclonal antibody. **a** Platelets (×40), **b** PBMC (×40), **c** granulocytes (×40)

Table 1 Comparisons between immunohistochemical distribution of CD9 in porcine and human normal tissues

Tissues	Swine CD9	Human CD9
Skin		
Stratum basale	?	++++[a]
Stratum spinosum	?	+++++[a]
Stratum granulosum	+++++	+++++[a]
Gut		
Epithelium	+++++	+++[a, b]
Lamina propria	?	+++++[a]
IELs	?	+[a]
Connective tissue		
Fibroblasts	+++++	++++[a]
Lung	+++++	++++[a, b]
Kidney		
Glomeruli	++	++[a]
Proximal/distal tubules	?	+[a, b]
Collecting ducts	++++	+++++[a, b]
Liver		
Hepatocytes	++	+[a]
Bile ducts	?	++++[a]
Sinusoids	++	+[a]
Pancreas		
Acini	−	−[a], +[b]
Ducts	++	−[a] +[b]
Cerebral cortex		
Vasculature	−	++++[a]
Gray matter	−	+[a]
White matter	+++++	+++++[a]
Cerebellum cortex		
Vasculature	−	nt
Gray matter	−	nt
White matter	+++++	nt
Spleen		
White pulp	−	+[a]
Red pulp	−	+++[a]
Central arteries	−	+++++[a]
Tonsil		
Squamous epithelium	+++++	+++++[a]
Germinal center	?	+++[a]
HEVs	?	++++[a]
Circulatory		
Arteries	nt	++++[a]
Veins	nt	++++[a]
Capillaries	nt	+++[a]
Perivascular smooth muscle	nt	+++++[a]
Peripheral blood		
Red blood cells	−	−[a]
Lymphocytes	−	∓[a]
Neutrophils	∓	−[a]
Monocytes	∓	+[a]
Platelets	+++++	+++++[a]
Bone marrow MNCs		
Megakaryocytes	nt	+++++[a]
Skeletal muscle		++[a, b]
Peripheral nerve		
Perineurium	+++++	+++++[a]
Endoneurium	?	−[a]
Schwann cells	?	++++[a]
Cardiac muscle	nt	−
Adrenal		
Cortex	nt	++[a]
Thyroid		
Follicular cells	nt	++[a]
Parafollicular cells	nt	++[a]
Ovary		
Ovum surface	+++++	+++++[b]
Mammary gland	++	nt
Uterus	++	nt
Testis	−	+[b]
Epididymo	+++	nt
Lymph node	−	nt

Level of staining was scored from + (weakly positive) to ++++ (strongly positive)

nt non tested, *MNCs* mononuclear cells, *IELs* intraepithelial lymphocytes, *HEVs* high endothelial venules

[a] Human data are from Sincock et al. [40] and [b] Nakamura et al. [41]

types, including neutrophils, macrophages and monocytes (Fig. 3b) and granulocytes (Fig. 3c).

Discussion

A specific polyclonal antibody against swine CD9 molecule have been produced and used to study the expression of CD9 molecule in a broad group of swine cells and tissues. A summary of differences and coincidences on the expression of swine and human CD9 can be observed in Table 1 where data obtained by Sincock et al. [40] and Nakamura et al. [41] were compared with our own data. The following conclusions can be remarked from our study:

The peripheral blood cells distribution of CD9, as described in the present study, agrees with previous studies in humans [8, 40, 42].

CD9 expression on swine ovum cytoplasm is in according to data about the importance of CD9 in fertilization of mice and swine offered by Miyado et al. [43]

and Li et al. [34], respectively, and supports that CD9 might have an important role in the fusion of sperm and oocytes.

Expression of CD9 in the human brain [44] has also been confirmed in swine with the positive stain of the white matter of cerebellum and cerebrum cortex.

The general distribution of CD9 in epithelial cells supports that this molecule is involved in multiple cellular interactions and possibly in intercellular adhesion.

The expression of CD9 by gut epithelium was restricted to the bases of the villi. Therefore, CD9 function may be limited to interactions with specific extracellular matrix components in the crypt microenvironment.

The widespread expression of these antigens throughout connective tissue probably reflects their interaction with components of the extracellular matrix such as collagen, laminin and fibronectin.

Discrepancies have found especially in spleen, pancreas and some peripheral blood cells like neutrophils may arise because different anti-CD9 antibodies may not necessarily be equivalent in their recognition of CD9. For example, the glycosylation state of CD9 in different cell types may vary, thus modifying antigen recognition by certain antibodies, especially if these are monoclonal antibodies [40, 41].

In summary, this study has shown by the first time the tissue distribution of swine CD9. Evidence about the similar distribution of CD9 between human and swine cells and tissues have been presented. All these evidences open the possibility to use swine as animal model in studying human biological processes, as adhesion, motility, fertilization or tumor metastasis, in which CD9 molecule is or could be involved.

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