Original Research

Osteopontin (OPN)/Secreted Phosphoprotein 1 (SPP1) Binds Integrins to Activate Transport of Ions Across the Porcine Placenta

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Abstract

Background: Fetal-placental development depends on a continuous and efficient supply of nutrients from maternal blood that are acquired by exchange through the placenta. However, the placenta is a low permeability barrier, and effective transport of substances depends on specific transport mechanisms. Active transport requires that ions or nutrients be moved against an electrical and/or concentration gradient. In pigs, active transport of ions occurs across the chorioallantois placenta to produce an electrochemical gradient that changes throughout gestation. The aim of this study was to utilize Ussing chambers to detect regulation of ion transport across the porcine chorioallantois by a factor(s) within the uterine-placental environment of pigs. **Methods**: For the measurement of transchorioallantoic voltage potential as an index of ion transport across the placenta, pieces of chorioallantoic tissue from Day 60 of gestation were mounted into the cassettes of Ussing chambers, and treatments were added to the mucasal side of the tissue. Treatments included: (1) media incubated with Day 60 chorioallantois (placenta-conditioned media); (2) osteopontin/secreted phosphoprotein 1 (OPN/SPP1) purified from cow's milk; (3) placenta-conditioned media from which OPN/SPP1 was removed; and (4) recombinant rat OPN with an intact RGD integrin binding sequence or a mutated RAD sequence. Ouabain was added to both sides of the chamber. Immunofluorescence was utilized to localize beta 3 integrin, aquaporin 8 and OPN/SPP1 in porcine placental tissues, and OPN/SPP1 within porcine lung, kidney and small intestine. **Results**: Day 60 chorioallantoic membranes had greater transepithelial voltage in the presence of porcine placenta-conditioned media, indicating that a molecule(s) released from the placenta increased ion transport across the placenta. OPN/SPP1 purified from cow's milk increased ion transport across the placenta. When OPN/SPP1 was removed from placenta-conditioned media, ion transport across the placenta did not increase. Recombinant rat OPN/SPP1 with a mutated RGD sequence that does not bind integrins (RAD) did not increase ion transport across the placenta. Ouabain, an inhibiter of the sodium-potassium ion pump, ablated ion transport across the placenta. **Conclusions**: The present study documents a novel pericellular matrix role for OPN/SPP1 to bind integrins and increase ion transport across the porcine chorioallantoic placenta.

Keywords: pig; placenta; ion transport; ussing chambers; osteopontin; secreted phosphoprotein 1

1. Introduction

Fetal growth and development depends on a continuous and efficient supply of nutrients from maternal blood. Most nutrients required by the fetus are acquired by exchange through the placenta [1,2]. However, the placenta is a low permeability barrier, and the effective transport of nutrients and substrates for fetal growth depends less on intrinsic diffusion, and more on a host of specific transport mechanisms [1]. As such, the placenta resembles the intestinal mucosa or renal epithelium [2]. Active transport requires that ions or nutrients be moved against an electrical and/or concentration gradient.

In pigs, active transport of ions occurs across the chorioallantois to produce an electrochemical gradient (potential difference) that changes throughout gestation [3,4]. Isolated chorioallantoic membranes mounted in Ussing chambers produce an electrical potential at 20 days of gestation that increases 4-fold by days 45–67 [5], and is maintained through the 107th day of gestation [4]. This electrical potential across the porcine chorioallantois is likely maintained by a Na^+/K^+ ATPase pump. Na^+ levels in allantoic fluid are lower, and K^+ levels in allantoic fluid are higher than expected for equilibrium, indicating that these ions are actively transported [6,7]. Export of Na^+ from the cell provides a concentration gradient that drives active transport by several membrane proteins to import glucose, fructose, amino acids and other nutrients through the cell. As Na^+ flows back through cells down the concentration gradient formed by the Na^+/K^+ ATPase pump, ions, water, glucose, amino acids and other nutrients follow. The net result is increased allantoic fluid volume and total allantoic fluid nutrients, including amino acids [8], glucose, and fructose during pregnancy in pigs [7].

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Nutrients that pass from the uterus through the chorion can directly access allantoic vasculature for transport to the fetus, but nutrients can also diffuse across the allantoic mesenchyme for active transport across the allantoic epithelium into the allantoic sac. Allantoic fluid is rich in electrolytes, sugars, amino acids, and proteins, and serves as a nutrient reservoir. Because the allantoic epithelium is derived from the hindgut and is, therefore, absorptive in nature, these stored nutrients can be transported back across this epithelium to the allantoic vasculature for transport to the fetus. The mechanistic trigger(s) for these active transport events across the maternal and fetal sides of the chorioallantois is unknown. The regulation of transport may be affected by intrinsic mechanisms, in which intracellular proteins interact with transporters, or by extracellular influences such as effectors and their receptors in the chorionic and allantoic membranes [2].

Active transport requires that ions or nutrients be moved against an electrical and/or concentration gradient. Ussing chambers are a physiological tool to distinguish between active transport of ions, nutrients and drugs across epithelia and passive movement of ions through paracellular pathways [9]. This method utilizes short-term tissue culture that enables precise measurement of electrical and transport parameters of intact, polarized epithelia in a physiological context [9,10]. The aim of this study was to utilize these chambers to detect regulation of ion transport across the porcine chorioallantois placenta by a factor(s) within the uterine-placental environment of pigs. Osteopontin (OPN)/Secreted Phosphoprotein 1 (SPP1) is a secreted matricellular effector molecule perfectly localized to bind integrin receptors on chorion and allantoic epithelium to increase ion and nutrient transport, e.g., glucose, fructose and amino acids, across the pig placenta [11–15].

2. Materials and Methods

2.1 Animals, Tissue Collection, and Placental Tissue Incubation

Briefly, seven gilts (F1 crosses of Yorkshire × Landrace sows and Duroc × Hampshire boars) had free access to a corn-soybean meal-based diet (2.7 kg/day feed intake) beginning at 6 months of age until 8 weeks before breeding at 8 months of age [16]. Gilts were checked daily for estrus with fertile boars and bred at the onset of the second estrus and 12 h later. On Day 60 of gestation, gilts were anesthetized and then hysterectomized to obtain the placentae and endometria. Several 1–1.5 cm sections of intact uterine-placental interface from the middle of each horn were (1) embedded in OCT compound, frozen in liquid nitrogen, and stored at –80 °C; and (2) fixed in 4% paraformaldehyde and paraffin-embedded. In addition, portions of fresh placenta were (1) placed in 20 mL of cell culture media (DMEM/F12 medium supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin (PS) and 0.10 U/mL insulin; 20 g of tissue), and incubated at 25 °C for 2 h to obtain placenta-conditioned media; or (2) mounted into the cassettes of Ussing chambers (1 cm^2 of tissue) [17,18]. Lung, kidney and small intestinal tissues were obtained from a laboratory archive from 5 Day 13 cyclic gilts. Tissue sections (~1 cm thick) were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Laboratory, St. Louis, MO, USA).

2.2 Ussing Chambers for Measuring Transchorioallantoic Voltage Potential

For the measurement of transchorioallantoic voltage potential as an index of ion flux across the placenta [9], pieces of chorioallantoic placental tissue (1 cm^2) from Day 60 of gestation were mounted into the cassettes of Ussing chambers (Physiologic Instruments, San Diego, CA, USA) in 5 mL Krebs buffer. Both sides of the chambers contained the same volume of Krebs biocarbonate buffer (pH 7.4, 37 °C) and were gassed with 95% O$_2$ and 5% CO$_2$ continuously. Krebs bicarbonate buffer contained 119 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 20 mM Hepes, and 5 mM glucose [16–18]. The following was added to the mucosal side of the tissue: (1) placenta-conditioned media (placentae from 7 pigs, 0.2 mL placenta-conditioned media, incubation times of 0, 5, 10 and 20 min); or (2) OPN/SPP1 purified from cow's milk as previously described [13,19] (4 different placentae from 1 pig, concentrations of 0, 4, 8, 12 and 16 μg/mL, incubation time of 15 min); or (3) placenta-conditioned media in which OPN/SPP1 was removed (4 different placentae from 1 pig, 0.2 mL placenta-conditioned media lacking OPN/SPP1, incubation times of 0, 5, 10 and 20 min); or (4) recombinant rat OPN with an intact RGD (Arginine-Glycine-Aspartate) integrin binding sequence or a mutated RAD (Arginine-Alanine-Aspartate) sequence that does not bind OPN/SPP1 [15] (for RGD, 4 different placenta from 1 pig (20 μg/mL; for RAD, 4 different placentae from 1 pig, 20 μg/mL, incubation times of 0, 2, 5, 10, 12, 15 and 20 min). Ouabain (100 μM), an inhibiter of the sodium-potassium ion pump, was added to both the mucosal and serosal sides of the tissue for each experiment.

2.3 Immunoprecipitation and Western Blotting

Immunoprecipitation of OPN/SPP1 from placenta-conditioned media was performed by combining placenta-conditioned media with Protein A-Sepharose (Pierce, Rockford, IL, USA), 0.5% Triton X-100 in TBS, and a pool of two rabbit antibodies directed against human OPN/SPP1 (LF-123 and LF-124) [20] and a rabbit anti-bovine OPN/SPP1 IgG (generously provided by Dr. George J. Killian) [21], or irrelevant rabbit IgG. This mixture was rotated gently overnight at 4 °C, centrifuged briefly to pack down the beads with antibodies bound to OPN/SPP1, and the supernatant containing placenta-

conditioned media depleted, or not depleted (rabbit IgG), of OPN/SPP1 harvested [13]. The concentrations of protein in placenta-conditioned media subjected to immunoprecipitation with with rabbit IgG and placenta-conditioned media subjected to immunoprecipitation with antibodies specific for OPN/SPP1were determined using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as the standard. Western blotting was performed as previously described [22]. Briefly, proteins in placenta-conditioned media (20 μg) were denatured in Laemmli buffer, separated using 10% (total monomer) 1D-SDS-PAGE, and transferred to nitrocellulose. Blots were blocked overnight in TBST (20 mM Tris [pH 7.5], 137 mM NaCl, 0.05% Tween-20) containing 5% dried milk. Blots were washed 3 times for 5 min each in TBST and then incubated overnight, rocking at 4 °C in a cocktail containing rabbit polyclonal antibodies against recombinant human OPN/SPP1 (LF-123 and LF-124; 5 μg/mL) in TBST containing 2% dried milk. Blots were then washed 3 times for 10 min each in TBST and placed in goat anti-rabbit IgG-horseradish peroxidase conjugate (1/15,000 dilution KPL, Bethesda, MD, USA) for 1 h at room temperature while rocking. Blots were washed 3 times for 10 min each in TBST, and immunoreactive proteins were detected using enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, Rochester, NY, USA).

2.4 Immunofluorescence Analyses

For immunofluorescence staining, primary antibodies included rabbit anti-beta 3 integrin (ITGB3) IgG (AB1932; EMD Millipore, Burlington, MA, USA; 1/1000 dilution for OCT-embedded tissue sections), mouse anti-AQP8 IgG (Sigma Aldrich, St. Louis, MO, USA; 1/200 dilution for paraffin-embedded tissue sections), rabbit anti-OPN/SPP1 IgG (AB10910; EMD Millipore; 1/1000 dilution for OCT-embedded tissue sections, and 1/200 dilution for paraffin-embedded tissue sections, boiling citrate antigen retrieval for paraffin-embedded tissue sections), and mouse anti-E-cadherin monoclonal IgG (BD Biosciences; San Jose, CA, USA; 610182; 1/200 dilution for paraffin-embedded tissue sections, boiling citrate antigen retrieval). The secondary antibodies included goat anti-rabbit-Alexa Fluor 488-conjugated IgG, goat anti-rabbit Alexa Fluor 594-conjugated IgG, and goat anti-mouse-Alexa Fluor 594-conjugated IgG (Life Technologies, Grand Island, NY, USA; 1/250 dilution). In addition an FITC-conjugated lectin from Dolichos biflorus (Sigma-Aldrich; L9142-1MG; 1/200 dilution) was used to co-localize with OPN/SPP1 immunofluorescence.

Immunostaining of frozen tissue sections was performed to localize ITGB3 to placental areolae and OPN/SPP1 to allantoic epithelium as previously described [23]. Briefly, frozen sections (~10 μm) of intact uterus and placenta were cut with a cryostat (Hacker-BrightOTF, Hacker Instruments, Inc., Winnsboro, SC, USA) and

mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were fixed in –20 °C methanol for 10 min, permeabilized at room temperature with 0.3% Tween-20 in 0.02 M PBS (rinse solution), and blocked in 10% normal goat serum for 1 h at room temperature. Sections were then dipped in rinse solution at room temperature and incubated overnight at 4 °C with each primary antibody, and detected with fluorescein-conjugated secondary antibody (goat anti-rabbit-Alexa Fluor 488-conjugated IgG for detection of ITGB3) or (goat anti-rabbit Alexa Fluor 594-conjugated IgG and FITC-conjugated lectin from Dolichos biflorus) [24]. Slides were then overlaid with a cover-glass and Prolong antifade mounting reagent containing DAPI (Molecular Probes).

Immunostaining of paraffin-embedded tissue sections was performed to localize aquaporin 8 (AQP8) to placental areolae as previously described [25]. Briefly, sections (5 μm thick) were deparaffinized and rehydrated in an alcohol gradient. Antigen retrieval was performed using boiling citrate. Sections were then blocked in 10% normal goat serum for 1 h at room temperature. Antigen retrieval was performed using boiling citrate. Tissue sections were then blocked in 10% normal goat serum for 1 h at room temperature and incubated overnight at 4 °C with the primary antibody. Immunoreactive proteins were detected using goat anti mouse Alexa Fluor 594-conjugated secondary antibody for 1 h at room temperature, tissue sections were then washed three times for 5 min/wash in PBS, counterstained with Prolong Gold Antifade reagent containing DAPI, and coverslipped.

Dual immunofluorescence staining of OPN/SPP1 and E-cadherin proteins in paraffin-embedded tissue sections followed the same procedures as described for normal immunofluorescence staining except that the two primary antibodies were added simultaneously on the first day and the two secondary antibodies (goat anti-rabbit-Alexa Fluor 488-conjugated IgG and goat anti-mouse-Alexa Fluor 594-conjugated IgG) were added simultaneously on the second day [25]. Images were taken using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY, USA) interfaced with an Axioplan HR digital camera.

2.5 Statistics

All statistical analyses were performed using Graph-Pad Prism (GraphPad Software, La Jolla, CA, USA). Data for the effect of conditioned media and OPN/SPP1 on ion transport were subjected to one-way ANOVA followed by a post-hoc Tuckey analysis. For comparison of two treatment groups over time, a two-way ANOVA with Bonferroni multiple comparison test was used. All data are presented as mean $\pm$ standard errors of the mean (SEM) with significance set at $p < 0.05$.

3. Results

Day 60 chorioallantoic membranes were placed into Ussing chamber cassettes and 0.2 mL of placenta-conditioned media added to the mucosal side of the tissue. A statistically significant increase in the transepithelial voltage, as an index of ion transport across the placenta, was observed between 0 and 5 min after the addition of 0.2 mL placental solution to the "mucosal side" of the using chamber, and this increase was maintained through the remaining 15 min (Fig. 1). When 100 μM ouabain, an inhibiter of the sodium-potassium ion pump, was added to both the mucosal and serosal sides of the tissue, transepithelial voltage was nearly eliminated (data not shown in Fig. 1, Fig. 2A,B (Ref. [11]), but this is illustrated in Fig. 2C).
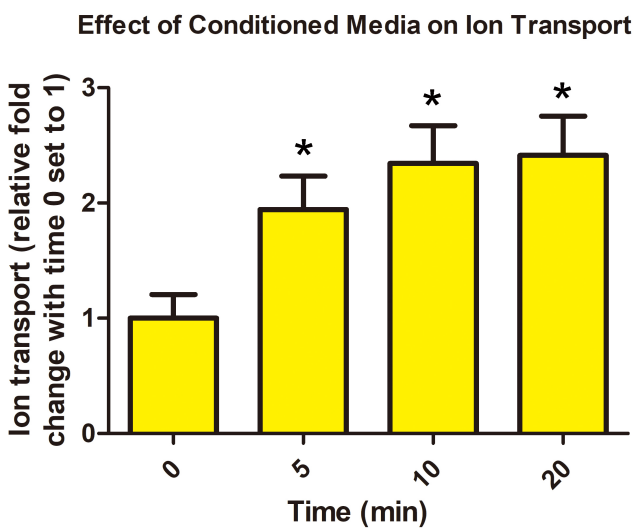
Effect of Conditioned Media on Ion Transport

Fig. 1. Porcine chorioallantois releases a factor(s) that increases ion transport across the porcine placenta. Addition of 0.2 mL of placenta-conditioned media to the mucosal side of the Ussing chamber increased transepithelial voltage across Day 60 chorioallantois by 10 min of incubation. Values are ± SEM, n = 7 gilts. In the absence of placental solution, ion transport was constant for 20 min.

Day 60 chorioallantoic membranes were placed into Ussing chamber cassettes and OPN/SPP1 purified from cow's milk was added to the mucosal side of the tissue. A statistically signifant increase in the transepithelial voltage was observed in response to treatment with 4 μg/mL bovine OPN/SPP1, and this increase was maintained with doses of 8, 12 and 16 μg/mL bovine OPN/SPP1 (Fig. 2A). Fig. 2B demonstrates Western blotting to detect OPN/SPP1 in placenta-conditioned media. Immunoreactive proteins of about 70, 45 and 25 kDA were detected in placenta-conditioned media immunoprecipitated with an irrelevant rabbit IgG, similar to previously reported for the pig using these antibodies [11], however these immunoreactive proteins were greatly decreased in placenta-conditioned

Osteopontin (OPN)/Secreted Phosphoprotein 1 (SPP1) Increases Ion Transport Across the Porcine Placenta
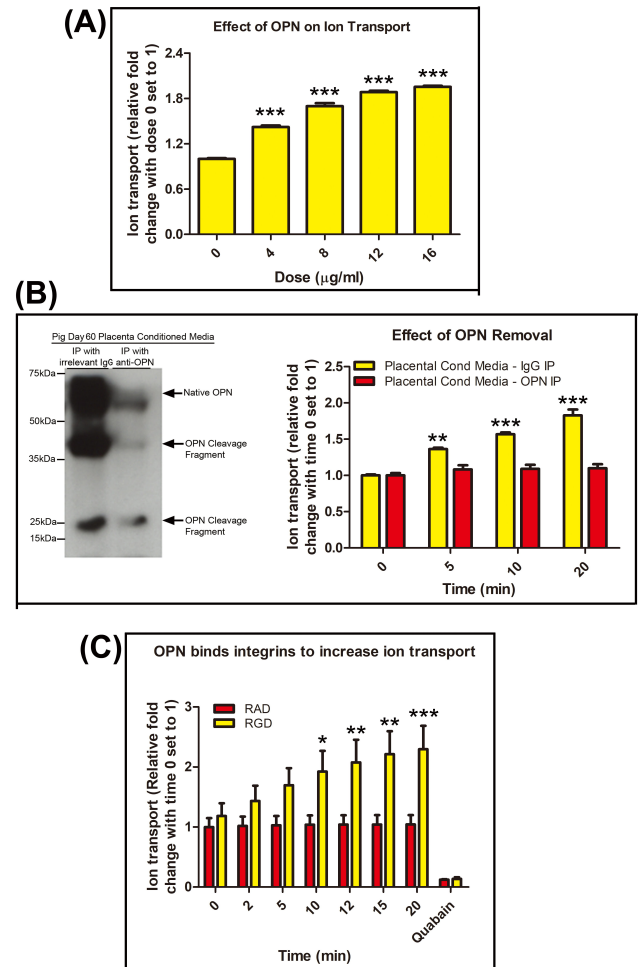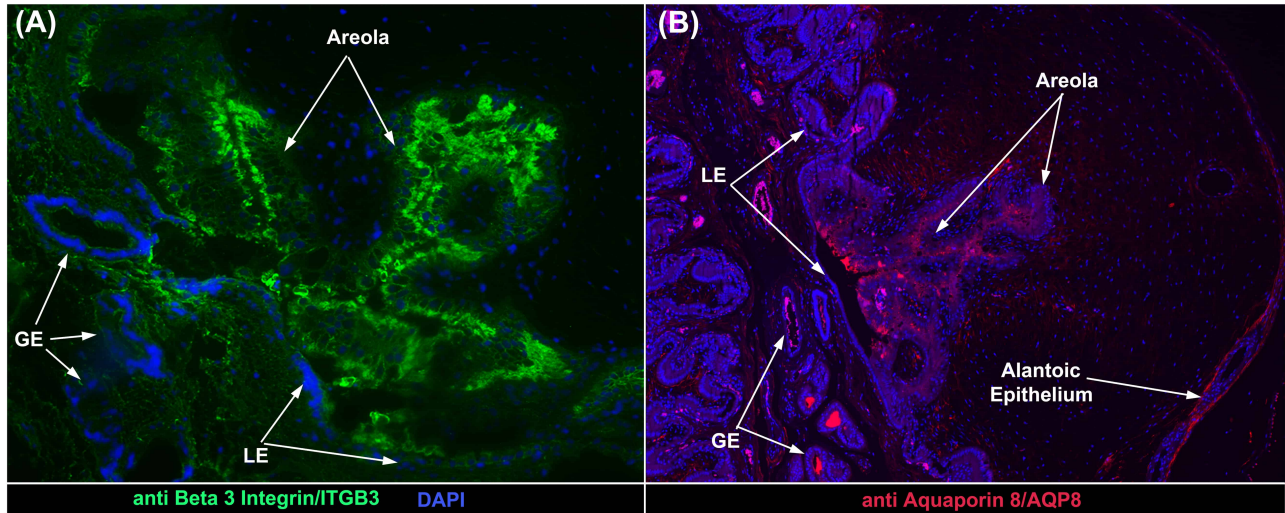

Fig. 2. Osteopontin (OPN)/Secreted Phosphoprotein 1 (SPP1) increases ion transport across the porcine placenta. (A) Effect of addition of OPN/SPP1 purified from bovine milk to the mucosal side of a Ussing chamber. OPN/SPP1 from bovine milk increased transepithelial voltage across Day 60 chorioallantois in a dose dependent manner. (B) Western blot demonstrating removal of OPN/SPP1 from placenta-conditioned media via immunoprecipitation. Rabbit IgG controls for these antibodies in porcine tissue have previously been published [11]. Addition of placenta-conditioned media with OPN/SPP1 removed (immunoprecipitation with anti-OPN) to the mucosal side of a Ussing chamber did not increase transepithelial voltage across Day 60 chorioallantois. (C) Effect of addition of recombinant rat OPN/SPP1 with an intact integrin binding RGD sequence and recombinant rat OPN/SPP1 with an integrin binding sequence mutated to RAD to the mucosal side of a Ussing chamber. Addition of OPN/SPP1 containing an RAD sequence did not increase transepithelial voltage across Day 60 chorioallantois. 100 μM of ouabain were added to both sides of the Ussing chamber to demonstrate changes in transepithelial voltage are dependent on an active sodium-potassium ion pump. Values are means ± SEM, n = 4 different placentae.

Beta 3 Integrin/ITGB3 and Aquaporin 8/AQP8 Protein in Areolae

Osteopontin (OPN)/Secreted Phosphoprotein 1 (SPP1) Protein in the Allantoic Epithelium
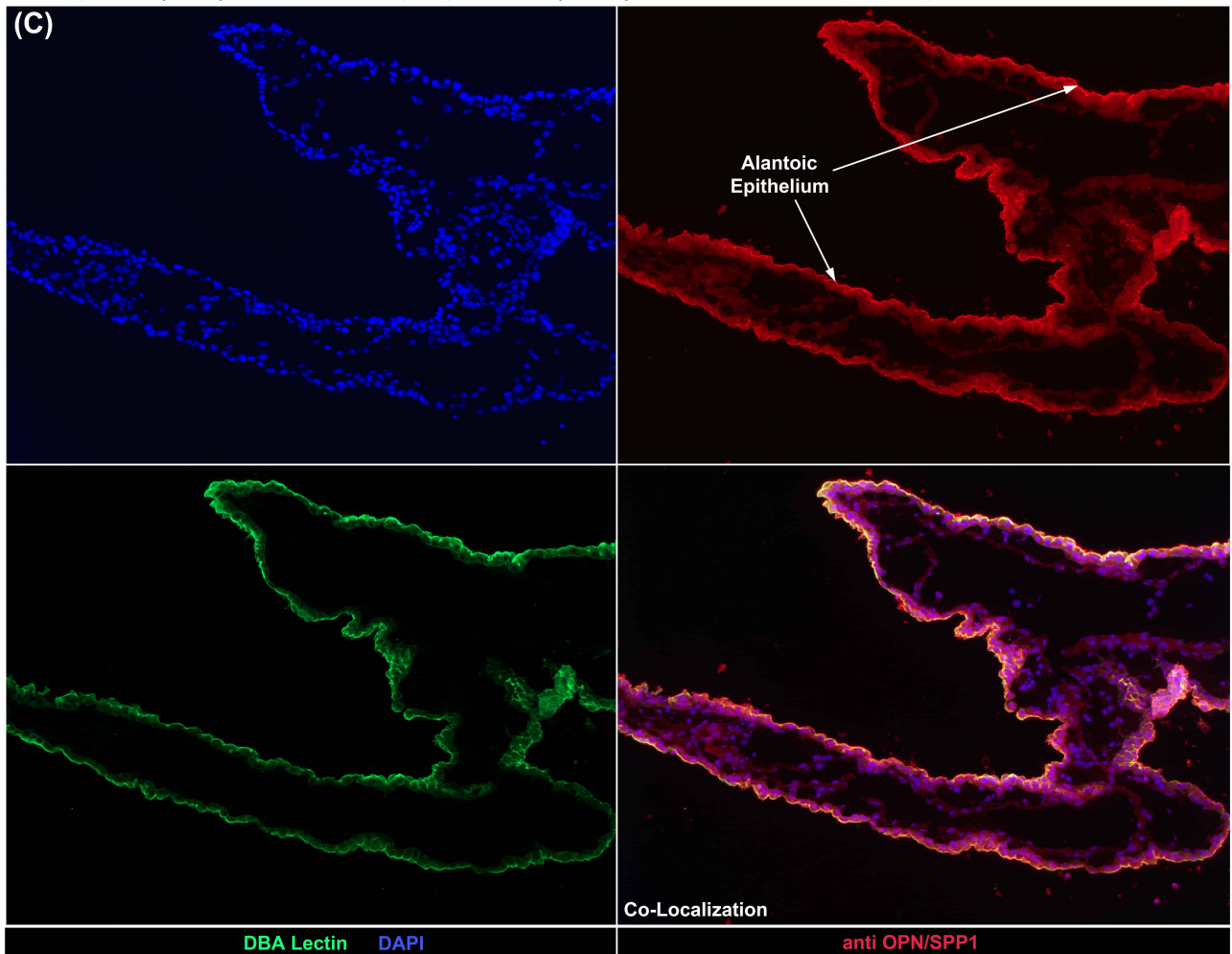

Fig. 3. Immunofluorescence (IF) staining for proteins in the porcine placenta. (A) IF localization of the beta 3 integrin subunit (ITGB3) to the tall columnar cells of a Day 60 chorionic areola. (B) IF localization of aquaporin 8 (AQP8) to the tall columnar cells of a Day 60 chorionic areola, as well as to the allantoic epithelium. (C) IF co-localization of OPN/SPP1 and DBA lectin to the apical surface of allantoic epithelial cells on Day 60 of gestation. LE, uterine luminal epithelium; GE, uterine glandular epithelium. Rabbit and mouse IgG controls are shown in Fig. 4. Width of fields is 895 μm.

media immunoprecipitated by anti-OPN/SPP1 IgGs, illustrating successful removal of OPN/SPP1 from placenta-conditioned media through immunoprecipitation. When Day 60 chorioallantoic membranes were placed into Ussing chamber cassettes and placenta-conditioned media in which OPN/SPP1 was removed was added to the mucosal side of the tissue, no increase in transepithelial voltage across the placenta was observed (Fig. 2B). Similar to results shown in Fig. 1, placenta-conditioned media containing OPN/SPP1 increased transepithelial voltage within 5 min of incubation. A further increase was observed at 10 min and this was maintained through 20 min of incubation (Fig. 2B). When Day 60 chorioallantoic membranes were placed into Ussing chamber cassettes and recombinant rat OPN with an intact RGD integrin binding sequence or a mutated RAD sequence that does not bind OPN/SPP1 were added to the mucosal side of the tissue, OPN/SPP1 with an RGD sequence significantly increased transepithelial voltage across the placenta between 0 and 10 min of incubation. A further increase was observed between 10 and 12 min, and again between 15 and 20 min of incubation (Fig. 2C). OPN/SPP1 with an RAD sequence did not increase transepithelial voltage as an index of ion transport across the placenta (Fig. 2C). Addition of ouabain, an inhibiter of the sodium-potassium ion pump, resulted in near elimination of transepithelial voltage (Fig. 2C).

Chorionic areolae are composed of specialized tall columnar epithelial cells that are not closely apposed to the uterine luminal epithelium (LE), and form a pouch at the openings of the mouths of uterine glands to receive the secretions of uterine glandular epithelium (GE). Areolae transport glandular secretions across the placenta and into the fetal-placental circulation [26]. ITGB3 can non-covalently link to the alpha v integrin subunit (ITGA3) to form an integrin receptor that binds to OPN/SPP1 and has been demonstrated to engage OPN/SPP1 on porcine uterine LE and conceptus trophectoderm cells [13,14]. Aquaporins (AQPs) are water-selective channels that function as pores for water transport through the plasma membrane [27]. Fig. 3A,B demonstrate expression of ITGB3 and AQP8 protein at the apical surface of the tall columnar cells of porcine areolae on Day 60 of gestation. In addition AQP8 is expressed at the apical surface of uterine GE and allantoic epithelial cells (Fig. 3B).

The allantoic epithelium lines the allantoic cavity and is a tissue conduit for transport of ions, water and nutrients from the allantois of the placenta to the allantoic cavity for storage in allantoic fluid [26]. Fig. 3C demonstrates the localization of OPN/SPP1 protein to the allantoic epithelium, and this expression is at the apical surface of the allantoic epithelium because OPN/SPP1 expression co-localizes with DBA lectin, which is only expressed at the apical surface of epithelial cells (Fig. 3C).
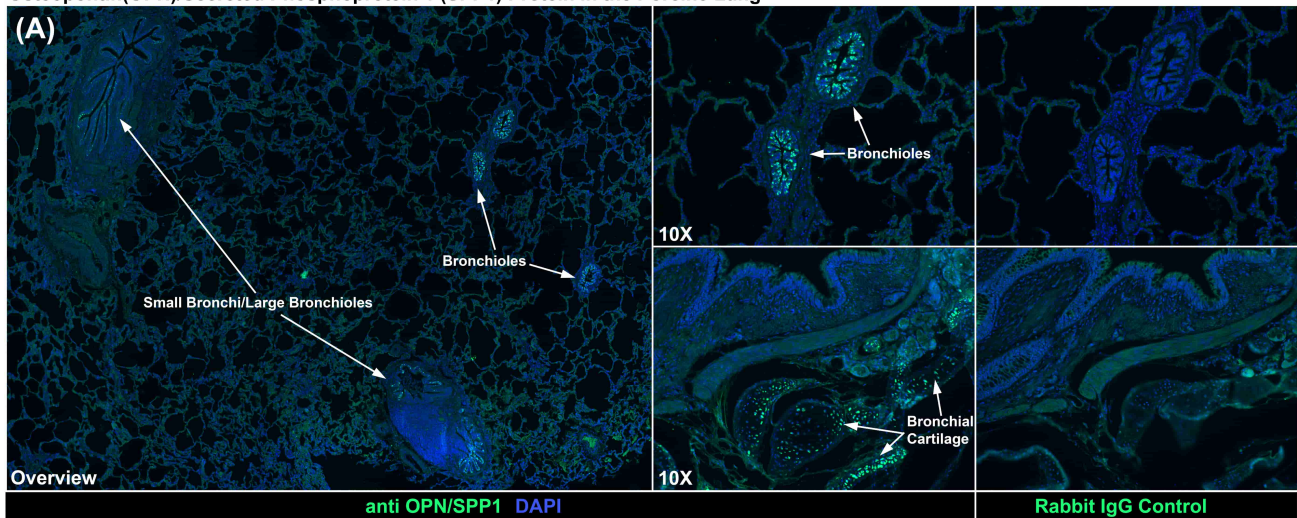
Results strongly suggest that OPN/SPP1 mediates ion transport across the porcine choriollantois. The porcine placenta, indeed all placentae, are highly adapted for transport of ions, water, nutrients, etc. across the tissue, and OPN/SPP1 is expressed by porcine uterine LE and GE [11,12]. Therefore we examined the cell-type specific expression of OPN/SPP1 in other tissues known to be adapted for transport including the lung, kidney and small intestine. Within the lung, OPN/SPP1 protein was localized to a small subset of bronchioles with a punctate pattern of immunostaining (Fig. 4A). In addition OPN/SPP1 protein was detected in the bronchial cartilage, which is to be expected, and suggests effective immunostaining (Fig. 4A). Within the kidney, OPN/SPP1 protein was localized to the collecting ducts of both the cortical medullary rays and the renal medulla (Fig. 4B). Within the small intestine OPN/SPP1 protein was localized to a subpopulation of goblet cells (Fig. 4C).
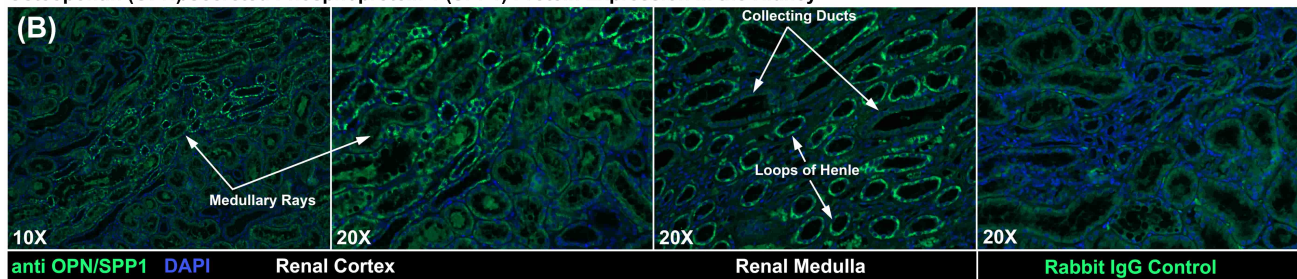
4. Discussion

Prevailing dogma asserts that OPN/SPP1 binds integrins to mediate attachment and migration of placental epithelia during implantation and placentation [28–31]. However, in the present study we have documented a novel pericellular matrix role for OPN/SPP1 as it binds integrins to increase ion transport across the porcine chorioallantoic placenta. Day 60 chorioallantoic membranes from pigs were placed in Ussing chambers and the transepithelial voltage potential was measured as an index of ion flux across the placenta. Addition of Day 60 placenta-conditioned media doubled the transepithelial voltage, indicating that a molecule(s) released from the pig placenta increases ion transport across the placenta. We tested the ability of OPN/SPP1 to affect ion transport. OPN/SPP1 purified from cow's milk increased ion transport, and maximum transepithelial voltage plateaued at levels similar to those for placenta-conditioned media. To confirm that OPN/SPP1 is the factor in placenta-conditioned media responsible for increasing ion transport, we removed OPN/SPP1 from placenta-conditioned media by immunoprecipitation using a mixture of antibodies developed against OPN/SPP1. We also performed immunoprecipitation using an irrelevant rabbit IgG. Placenta-conditioned media depletion of OPN/SPP1 did not stimulate ion transport across the placenta. To confirm that OPN/SPP1 binds integrins to mediate ion transport, Day 60 chorioallantoic membranes from pigs were placed into Ussing chambers, recombinant rat OPN with an intact RGD integrin binding sequence was added to the mucosal side of the chamber, and transepithelial voltage and current were measured. Addition of increasing amounts of rat OPN/SPP1 with an intact RGD dose-dependently increased ion transport across the placenta. When the same experiment was performed using recombinant rat OPN/SPP1 with a mutated RGD sequence that does not bind integrins (RAD), no increase in placental ion transport was observed.

Osteopontin(OPN)/Secreted Phosphoprotein 1 (SPP1) Protein in the Porcine Lung

(A)

Small Bronchi/Large Bronchioles

Bronchioles

Bronchioles

10X

Bronchial Cartilage

Overview

10X

anti OPN/SPP1 DAPI

Rabbit IgG Control

Osteopontin (OPN)/Secreted Phosphoprotein 1 (SPP1) Protein Expression in the Kidney

(B)

Collecting Ducts

Medullary Rays

Loops of Henle

10X

20X

20X

20X

anti OPN/SPP1 DAPI

Renal Cortex

Renal Medulla

Rabbit IgG Control

Osteopontin (OPN)/Secreted Phosphoprotein 1 (SPP1) Protein Expresion in the Small Intestine

(C)

Crypts

20X

40X

63X

63X

anti OPN/SPP1 anti E-Cadherin DAPI

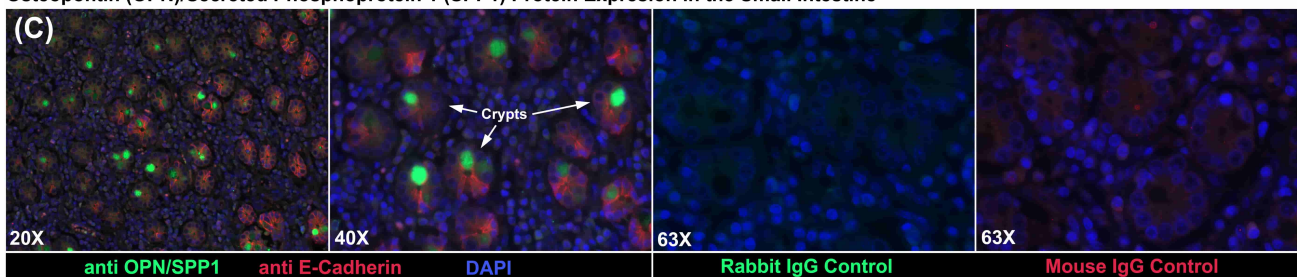Rabbit IgG Control

Mouse IgG Control

Fig. 4. Immunofluorescence (IF) staining for OPN/SPP1 protein. (A) IF localized OPN/SPP1 protein to some bronchioles and the cartilage of bronchi within the lung. (B) IF localized OPN/SPP1 protein to collecting ducts within the kidney. (C) IF localized OPN/SPP1 protein to some goblet cellswithin the small intestine. Rabbit and mouse IgG negative controls are shown. Width of fields for overview image is 4120 μm. Width of fields for microscopic images at 10X, 20X, 40X, and 63X is 895, 448, 224, and 142 μm, respectively.

OPN/SPP1 is highly expressed within the intrauterine environment of pregnant pigs [32]. During pregnancy, in pigs, OPN/SPP1 mRNA is induced initially by estrogens secreted by the conceptus (embryo and associated placental membranes) in discrete regions of the uterine LE juxtaposed to the conceptus during the apposition and adhesion phases of implantation. OPN/SPP1 mRNA expands to the entire uterine LE by Day 20 when firm adhesion of conceptus trophectoderm to uterine LE occurs [11,12], and OPN/SPP1 protein remains abundant along the apical surfaces of uterine LE and trophectoderm in all areas of direct contact throughout pregnancy. In addition, there are specialized cells of the chorionic areolae at the openings of the mouths of uterine glands. Indeed, the open space between the chorionic areolae and uterine GE is filled with secretions of the uterine GE collectively termed histotroph [33]. The areolae transport secretions of uterine GE such as macromolecules, particularly proteins, across the placenta. Total uterine OPN/SPP1 mRNA increases 20-fold between Days 25 and 85 of gestation in the uterine GE [11], which results in accumulation of OPN/SPP1 in allantoic fluid (unpublished results). Therefore, large amounts of soluble OPN/SPP1 are present at both the maternal and fetal surfaces of the chorioallantois. OPN/SPP1 expression in uterine GE during the later stages of pregnancy is similar in sheep [34], and a microarray study in rats showed
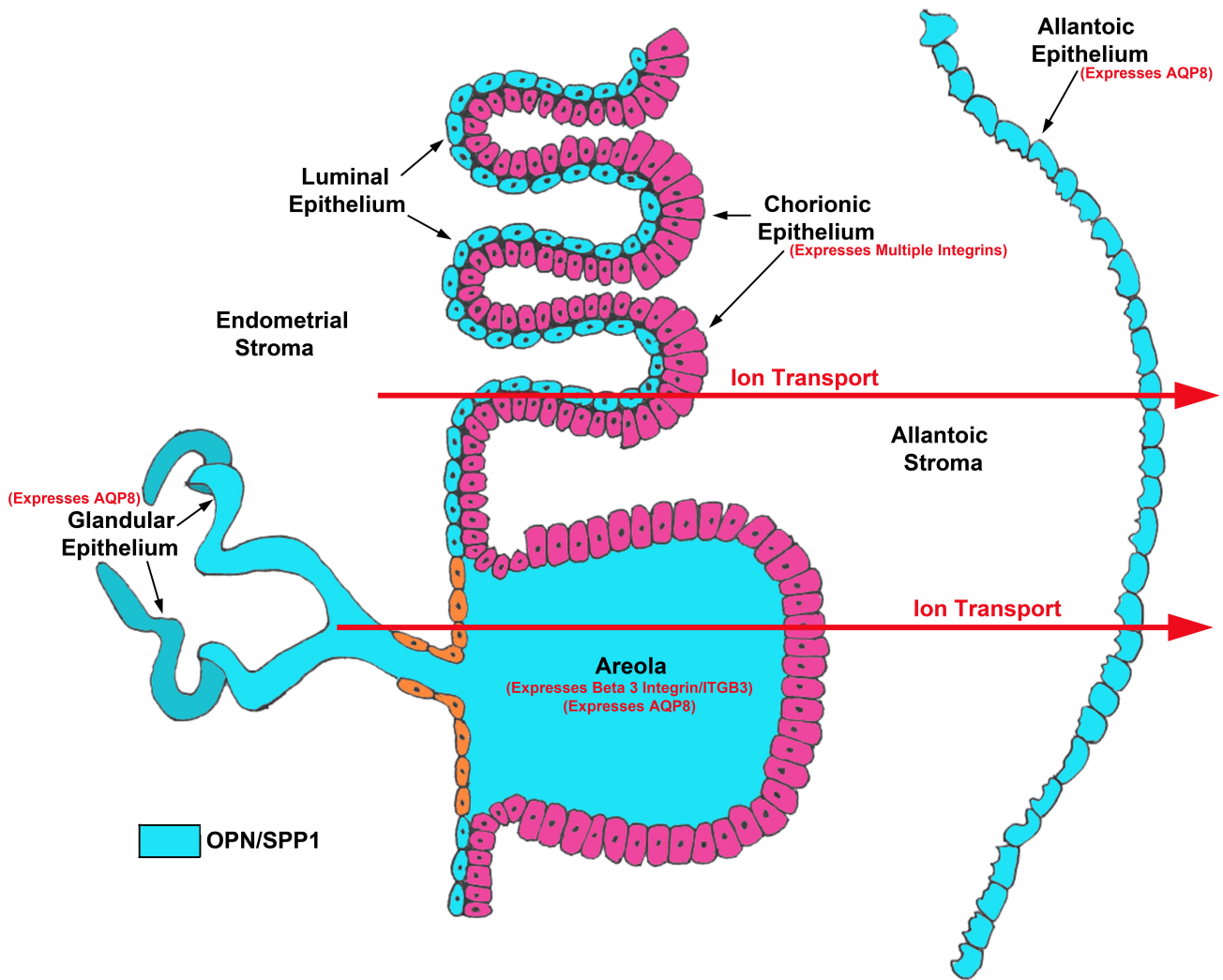
Fig. 5. Working model for OPN/SPP1 mediated ion transport across the porcine placenta. In the pig, for an ion to transport from the mucosal to the serosal side of the chorioallantois it must pass through the cytoplasm and basement membrane of the chorionic epithelial cells of the folded inter-areolar regions of placentation and areolae, across the remaining allantoic stroma, and through the basement membrane and cytoplasm of the allantoic epithelial cells [26]. OPN/SPP1 is highly expressed by both the uterine LE and GE of pigs which potentially supply secreted OPN/SPP1 protein to the to areolar and inter-areolar chorionic epithelium [11]. OPN/SPP1 has also been localized within the porcine allantoic stroma [15], and Fig. 3C demonstrates OPN/SPP1 expression in the allantoic epithelium. Multiple integrin subunits that could assemble into integrin receptors that bind OPN/SPP1 have been localized to the apical surface of inter-areolar chorionic epithelium [14], and Fig. 3A demonstrates beta 3 integrin (ITGB3) subunit expression in areolar chorionic epithelial cells. Transporters have been localized to key tissues within the chorion of pigs including SLC2A3 and SLC2A8 [24,41], and Fig. 3B demonstrates aquaporin 8 (AQP8) expression in the uterine GE, areolar chorionic epithelium and allantoic epithelium.

that OPN/SPP1 expression increased 60-fold between Day 0 of the estrous cycle and Day 20 of pregnancy [35], likely within the decidua because OPN is expressed by uterine natural killer cells of the mouse decidua [24]. In humans, OPN/SPP1 is expressed by the cytotrophoblasts of chorionic villi, and its $\alpha v \beta 3$ (ITGAV/ITGB3) receptor is present on the syncytiotrophoblasts [36,37]. Secretions of uterine GE in domestic animals, secretions of decidua in rodents and primates, and the trophoblasts of humans are critical to embryo/fetal growth and development [38–40]. We have evidence that an OPN/SPP1-based nutrient transport system is present in the pig chorioallantois, as illustrated in Fig. 5 (Ref. [11,14,15,24,26,41]).

Integrins are transmembrane proteins composed of α and β subunits that are non-covalently bound to each other. Their name reflects roles to integrate signals directed from the outside to the inside of cells and vice versa [42]. The N-terminal domain of integrins is located outside the cell and is involved in binding integrins to extracellular matrix (ECM) proteins primarily via their Arginine-Glycine-Aspartate (RGD) amino acid motif. The cytoplasmic tail of the β subunit allows the integrin receptor to interact with

the actin cytoskeleton and induce an abundance of different signaling molecules, collectively termed integrin adhesion complexes, which serve as signaling centers from which numerous intracellular pathways can regulate cell growth, proliferation, survival, adhesion, differentiation, migration, and gene expression [43,44]. There are multiple integrin receptors expressed on the conceptus trophectoderm and uterine LE of pigs, humans and other species [23,45–49] that serve as intermediaries between the placenta and uterus by interacting with ECM molecules like OPN/SPP1 [13–15,46,48,50].

Integrins mediate cell-cell and cell-ECM adhesion to regulate cell motility, proliferation and differentiation [42,47]. These events are often accompanied by changes in ion flow. Integrins form macromolecular complexes that localize ion channels to the plasma membrane [51,52]. Integrins regulate K^+ channels in erythroleukemic, neuroblastoma, and immune cells [53–55], and Ca^{2+} channels in endothelial, fibroblastic and vascular smooth muscle cells [51,56–60]. However, ion transport across epithelia in general, and chorion in particular, has not previously been linked to integrin activation. Further, although other integrin-binding ECM proteins, including fibronectin and vitronectin [53,55], affect ion transport, this is the first report of OPN/SPP1 in this role. Further studies are warranted to determine whether L-arginine, which is known to enhance placental ion transport [61] and placental global gene expression [62,63] in gestating gilts, may regulate the expression and function of OPN/SPP1 in the uterine GE and LE and other tissues.

5. Conclusions

We identified a here-to-fore unknown role of OPN/SPP1 and integrins at the uterine-placental interface of pregnancy in enhancing placental ion transport. We hypothesize that in pigs OPN/SPP1 is synthesized and secreted from uterine GE and LE, binds to integrins on the chorionic epithelium and activates ion transporters that alter the magnitude of and/or cellular localization of nutrient transporters and/or the activity of those transporters to increase nutrient transport across the chorionic and allantoic membranes to the placental vasculature and allantoic cavity for transfer to the embryo/fetus (Fig. 5). This novel finding of our current study may provide key insight into why OPN/SPP1 is highly expressed at sites of active nutrient transport in a variety of placentae including the uterine-placental interface of species with epitheliochorial and synepitheliochorial placentae, uterine decidua of rodents, and cytotrophoblasts of human chorionic villi.

Author Contributions

GW contributed funding to support the studies, performed the experiments to measure ion transport using Ussing chambers, contributed to the interpretation of data, and contributed to manuscript preparation. XL contributed extensively to animal husbandry including feeding, pen care, heat checking and breeding. HS contributed to immunohistochemical studies and manuscript prepartation. BAM contributed immunohistochemical studies. ACK contributed to immunohistochemical studies. FWB contributed funding to support the studies, contributed to the surgical procedures to procure placentae, contributed to the interpretation of data, and contributed to manuscript preparation. GAJ contributed funding to support the studies, contributed to animal husbandry and the surgical procedures to procure placentae, directed the studies, contributed to interpretation of data, and contributed to manuscript preparation.

Ethics Approval and Consent to Participate

This research protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University (Animal Use Protocol number 2015-0396).

Conflict of Interest

The authors declare no conflict of interest. GW is serving as the editorial board member of this journal. We declare that GW had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to BMA and GP.

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