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ERas is constitutively expressed in full term placenta of pregnant cows

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ABSTRACT

ERas is a new gene recently found in mouse embryonic stem (ES) cells and localized on the X chromosome. It plays a role in mouse ES cell survival and is constitutively active without any mutations. It was also found to be responsible for the maintenance of quiescence of the hepatic stellate cells (HSCs), liver-resident mesenchymal stem cells, the activation of which results in liver fibrosis. This gene was not present in human ES cells. ERas was found to be activated in a significant population of human gastric cancer, where ERAS may play a crucial role in gastric cancer cell survival and metastases to liver via down-regulation of E-cadherin. ERas gene has been found to be expressed both in ES cells and adult tissues of cynomolgus monkey. Cynomolgus ERAS did not promote cell proliferation or induce tumor formation. ERAS was also detected in normal and neoplastic urothelium of the urinary bladder in cattle, where bovine ERAS formed a constitutive complex with platelet derived growth factor β receptor (PDGFβR) resulting in the activation of AKT signaling. Here, molecular and morphological findings of ERAS in the full term placenta of pregnant cows have been investigated for the first time. ERAS was studied by reverse transcriptase PCR (RT-PCR). Alignment of the sequence detects a 100% identity with all transcript variant bovine ERas mRNAs, present in the GenBank database (http://www.ncbi.nlm.nih.gov). Furthermore, ERAS was detected by Western blot and investigated by real time PCR that revealed an amount of ERAS more than ERAS found in normal bovine urothelium but less than ERAS present in the liver. Immunohistochemical examination revealed the presence of ERAS protein both at the level of plasma membrane and in cytoplasm of epithelial cells lining caruncular crypts and in trophoblasts of villi. An evident ERAS immunoreactivity was also seen throughout the chorionic and uterine gland epithelium. Although this is not a functional study and further investigations will be warranted, it is conceivable that ERAS may have pleiotropic effects in the placenta, some of which, like normal urothelial cells, might lead to activation of AKT pathway. We speculate that ERAS may play a key role in cellular processes such as cell differentiation and movement. Accordingly, we believe it may be an important factor involved in trophoblast invasiveness via AKT signaling pathway. Therefore, ERas gene is a functional gene which contributes to homeostasis of bovine placenta.

1. Introduction

RAS proteins are small GTPases that comprise several GTP-binding proteins including Harvey Ras (HRAS), Kirsten Ras (KRAS) and neuroblastoma Ras (NRAS) [1]. GTPases cycle between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate-bound conformations (Ras-GDP and Ras-GTP, respectively) [2].

At the beginnings of this century, a new Ras gene has been found in mouse embryonic stem (ES) cells and named ERas that appears to be localized on the X chromosome [3]. It encodes a protein of 227 amino acids showing 43%, 46% and 47% identity to HRAS, KRAS and NRAS respectively and promotes in vitro proliferation and tumorigenicity of murine ES cells by producing a constitutively active RAS protein [3].

ERas expression has also been found in hepatic stellate cells

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(HSCs), known to be liver-resident mesenchymal stem cells [4]. It has been suggested that ERas expression is important to maintain quiescence of these cells. Furthermore, ERas gene expression has also been detected both in ES cells and in adult tissues of cynomolgus, an Asian long-tailed macaque (*Macaca fascicularis*) [5].

ERas gene is not expressed in human ES cells since only a truncated Eras gene product was identified in these cells [6]. It is believed that ERas could be a pseudogene in humans [7,8]. However, human ERAS appears to be linked to certain human cancer [8]. Indeed, it has been shown that ERas is activated in a significant population of gastric cancer. A good correlation between ERAS and lymph node metastases was also shown; accordingly, it has been suggested that ERAS may play a crucial role in metastases to liver via down-regulation of E-cadherin [9,10]. ERas mRNA was also found in colorectal, pancreatic and breast carcinoma cell lines [11]. Furthermore, it has been suggested that ERas/phosphatidylinositol 3′-kinase (PI3K) pathway may promote transforming activity in ERas-overexpressing colon cancer [12] and neuroblastoma cells as well [13].

It has been suggested that bovine ERas orthologue could be identified with the v-Ha-ras 3 gene, believed to be a cattle pseudogene without any introns [6,14]. However, it has recently been demonstrated that ERas is constitutively expressed in normal urothelial cells of the urinary bladder of cattle; furthermore, ERAS over-expression was shown to occur in bovine urothelial cancer cells associated with papillomavirus infection. ERas is a functional gene of bovine adult urothelial cells in which it forms a constitutive complex with platelet derived growth factor β receptor (PDGFβR) [15]. We showed that ERAS is involved in Akt signaling, known to play a key role in cellular processes such as growth, movement and differentiation. We demonstrated that ERAS is overexpressed in urothelial cancer cells of cattle [15]. Accordingly, ERAS appears to be related to physiological events and contribute to tumorigenesis.

The placenta is among rapidly evolving tissues in mammals. Placenta evolution is a dynamic process during which the organ acquires novel genetic material, which allows mammalian placentas to be quite diverse in morphology and function [16]. Although expression of only a very limited number of genes is known to truly occur in the placenta, it is generally believed that genes involved in placental development and function are characterized by their temporal expression and are, for the most part, also involved in the development and function of other organs [16].

Pregnancy can be considered as 'pseudo-malignant' process characterized by cancer-like capabilities of the implanting and invading trophoblast [17,18]. The normal trophoblast cells express functional tumor-associated genes, which are essential prerequisites for a malignant phenotype. Consequently, trophoblast cells provide a molecular expression profile of striking similarity to that of tumor cells [17], which warrants to investigate the expression of oncogenes in the placenta. It is well-known that components crucial to tumor cell migration and invasion are shared by trophoblast cells [17].

We have an ongoing research project aiming to study the molecular expression of not well-known placental genes and morphological detection of their products [19] that could be important for physiological events in some farm animals.

Regarding transforming oncogene ERas, there is no information about its potential expression and/or localization in the placenta. We hypothesized that, like adult urothelial cells of healthy cattle [15], ERas could be a functional gene also of bovine placenta and we speculate that its expression, in a physiological context, may be important for key processes such as implantation, placentation and reproduction.

Here we describe some molecular and morphological findings about ERas expression in pregnant cows' term placenta.

2. Materials and methods

2.1. Animals

Placentomes from ten at term pregnant cows were collected immediately after normal delivery. They were longitudinally cut into two halves, with one of them being immediately fixed in 10% neutral buffered formalin for light microscopy. The contralateral half was frozen at −80 °C for biomolecular analyses.

2.2. Reverse transcriptase (RT)-PCR

Total RNA was extracted from full term placentas of pregnant cows and normal urothelium samples of the bovine urinary bladder known to express a constitutive ERAS [15] by RNeasy Mini Kit (Qiagen TM, ME, DE). 1 μg of the total RNA was used to generate the single strand of cDNA by the QuantiTect Reverse Transcription Kit (Qiagen TM, ME, DE), in according to the manufacturer's instructions. PCR was performed with a specific primer set designed by the Primer3 online tool for ERas. The following primers were used: ERas forward 5′- CCATGGCACAGCCAACAAAG -3′, reverse 5′-CACCACCACTGCCTTGTACT -3'. Conditions for PCR were: 94 °C for 5 min, 40 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30s.

2.3. Sequence analysis

PCR products, obtained by RT-PCR, were purified by Qiaquick PCR purification Kit (Qiagen TM, ME, DE) and bidirectionally sequenced using a BigDye_Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) following manufacturer's recommendations. Sequences were dye-terminator removed by DyeEx_2.0 spin kit (Qiagen TM, ME, DE) and run on a 3500 Genetic Analyzer (Applied Biosystems, CA, USA). Electropherograms were analyzed using Sequencing analysis v5.2 and sequence scanner v1.0 softwares (Applied Biosystems, CA, USA). The sequences obtained were analyzed by BLAST program.

2.4. Real time PCR

To perform real time PCR analysis, total RNA from liver, bladder and placenta bovine tissues was produced as previously described [19]. A single strand of cDNA was generated as above reported. Real time PCR was carried on a Bio Rad CFX Connect™ Real Time PCR Detection System (Bio Rad Hercules, CA, USA) using iTAq Universal SYBR® Green Supermix (Bio Rad Hercules, CA, USA). Each reaction was set in triplicate and the primers used for ERas were the same of RT-PCR. The thermal profile for the PCR was 95 °C for 10 min, 40 cycles of 94 °C for 15 s, 56 °C for 30 s, followed by melting curve. The relative quantification (RQ) was calculated by using CFX Manager™ software, based on the equation $RQ = 2^{-\Delta\Delta Cq}$, where Cq is the quantification cycle to detect fluorescence. Cq data were normalized to the reference β-actin gene (forward: 5′- TAGCACAGGCCTCTCGCCTTCGT-3′, reverse 5′-GCACATGCCGGAGCCGTTGT-3′).

2.5. Statistical analysis

Results are presented as means ± SE. The ERAS expression was assessed by one-way ANOVA, followed by Tukey's test for multiple comparisons of means using GraphPad PRISM software version 5 (GraphPad Software, San Diego, CA). Means were considered significantly different if $P < 0.05$.

2.6. Western blot

Samples of full term placentas from pregnant cows and normal urothelial tissue known to express a constitutive ERAS as positive control [15] were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 400 mM NaCl, 1 mM EDTA, 2 mM PMSF, 1.7 mg/ml Aprotinin, 50 mM NaF, and 1 mM sodium orthovanadate). They were clarified by centrifugation, separated by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, UK, RPN303D). Membranes were blocked with TBST (TBS and 0.1% Tween 20) containing 5% no fat dry milk for 1 h at room temperature, being subsequently incubated overnight at 4 °C with an anti-ERas (OriGene Technologies, Inc, MD,USA) and anti-actin (Santa Cruz Biotechnology, TX, USA) primary antibodies. Membranes were washed three times with TBST, incubated for 1 h at room temperature with goat anti-rabbit (Bio-Rad, CA,USA) and goat anti-mouse (Bio-Rad, CA, USA) HRP-conjugated secondary antibodies diluted at 1:2000 in TBST, and washed three times with TBST. Immunoreactive bands were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, TX, USA) and ChemiDoc XRS Plus (Bio-Rad, CA, USA). Images were acquired with Image Lab Software version 2.0.1 (Bio-Rad, CA, USA).

2.7. Co-immunoprecipitation studies

Samples of full term placentas from pregnant cows and bovine normal urothelial tissues as positive control [15] were lysed in immunoprecipitation buffer (50 mM Tris—HCl, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM ethylenediaminetetraacetic acid, 10 mM sodium butyrate) with protease inhibitors cocktails (Roche, BS, CH). Equal amounts of protein for each samples (800 μg) were precleared with protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, TX, USA) for 2 h at 4C°. The supernatants were obtained by centrifugation (2000 rpm for 10 min at 4 °C) and mixed with 1 μg of the related primary antibodies (ERas or Akt) or matched with immunoglobulin as a negative control rocking at 4 °C overnight. Fresh protein A/G PLUS-Agarose was then added, followed by rocking at 4 °C for1 h. The reaction mixtures were centrifuged at 2000 rpm for 10 min at 4 °C, the supernatant was then discarded, and the pellet was washed by immunoprecipitation buffer for three times. The pellets were resuspended in 30 μL of Laemmli sample buffer, heated at 98 °C for 5 min, resolved on 10% SDS-PAGE and transferred to PVDF membrane. The blots were probed with anti-ERas antibody (OriGene Technologies, Inc, MD,USA) and anti-Akt antibody (Cell Signaling Technology, MA, USA) followed by stripping and probing with an anti-PDGFβR antibody (Santa Cruz Biotechnology, TX, USA).

2.8. Immunohistochemistry

Tissues fixed in 10% neutral buffered formalin were progressively alcohol dehydrated and paraffin embedded. Five micron sections from placental tissues were immunolabeled using the avidin-biotin-peroxidase complex (ABC) method with the Vectastain ABC kit (Vector Laboratories, Inc. CA, USA). Tissue sections were deparaffinized in alcohol decreasing solutions and endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 in methanol for 20 min. Antigen retrieval was performed by pretreating with microwave heating (twice for 5 min each at 700 W) in EDTA, pH 8.0. The slides were washed three times with phosphate buffered saline (PBS, pH 7.4, 0.01 M), then incubated for 1 h at room temperature with normal goat serum (Santa Cruz Biotechnology, TX, USA) diluted at 20% in PBS. The excess serum was gently drained and a polyclonal rabbit anti-ERas primary antibody (OriGene Technologies, Inc. MD, USA) diluted at 1:50 in PBS was applied overnight at +4 °C in a humid chamber. The slides were washed three times with PBS, then incubated for 30 min with a goat anti-rabbit biotinylated secondary antibody (Vector Laboratories Inc. CA, USA) diluted at 1:200 in PBS. Sections were washed three times with PBS and then incubated with Vectastain ABC reagent (Vector Laboratories Inc. CA, USA) for 30 min in a humid chamber at room temperature. Color development was obtained by treatment with 3, 3′-diaminobenzidine (Vector Laboratories Inc. CA, USA) for 2–10 min. Sections were counterstained with Mayer's hematoxylin. Species- and isotype-matched immunoglobulin (IgG) replaced the primary antibody in the negative controls at the same concentration.

3. Results

RT-PCR analysis resulted in the amplification of specific fragments of ~150 bp both in full term placenta and normal urothelium samples (Fig. 1). The amplicons were directly sequenced and compared with reference sequences available in the GenBank database by BLAST program. The fragment amplified and sequenced results to have 100% identity with all transcript variants of bovine ERas mRNA, present in the GenBank database (http://www.ncbi.nlm.nih.gov) (Fig. 1). It was also evaluated the expression of ERas mRNA in the placenta. The tissue expression profile showed that the ERAS transcripts were more expressed, in a statistically significant manner, in the placenta rather than in the bladder urothelium ($p < 0.001$ *vs.* placenta). On the contrary, placenta ERAS was less expressed, in a statistically significant manner, than in liver samples (Fig. 2). To evaluate ERAS protein expression, we performed a western blot analysis. Data revealed that in all samples a ~25-kDa band was recognized (Fig. 3).

Since ERAS interacts with AKT [3] and in an attempt to better understand the potential role of ERAS in a physiological context, we performed co-immunoprecipitation studies. AKT was revealed by western blot in the ERAS immunoprecipitates and ERAS was also detected in the AKT immunoprecipitates. In both immunoprecipitates we showed the presence of PDGFβR by western blot (Fig. 4).

An evident immunoreactivity for ERAS was detected in the placenta samples. Indeed, a strong immunolabeling signal was seen at the level of plasma membrane and in the cytoplasm of the epithelial cells lining caruncular crypts and trophoblast cells of villi (Fig. 5A). No immunoreactivity was seen in placenta sections after replacing the primary antibody with purified rabbit immunoglobulins (Fig. 5B). Furthermore, ERAS was remarkably immunolabeled in the plasma membrane and in the cytoplasm of the chorionic epithelium of the interplacentomal region. A marked immunoreactivity was also seen in the epithelium of uterine glands (Fig. 6A). No immunoreactivity was shown when the primary anti-ERAS antibody was replaced by species- and isotype-matched immunoglobulins (Fig. 6B). A very strong immunoreactivity for ERAS was shown in bovine urothelial cancer cells used as positive control. Also in these cells, both membranous and cytoplasmic patterns of immunolabeling signal were detected (Supplementary Fig. 1).

4. Discussion

A wide functional and morphological diversity among animal species is one of the features of the placentas and interspecific comparison of the placental gene offers an opportunity to identify molecular mechanisms contributing to both conserved and divergent placental functions.

Here, we report that ERas gene, a new member of Ras family, is constitutively expressed in bovine placental tissues. To our knowledge, this is the first study showing molecular and morphological findings about the expression of ERAS in full term

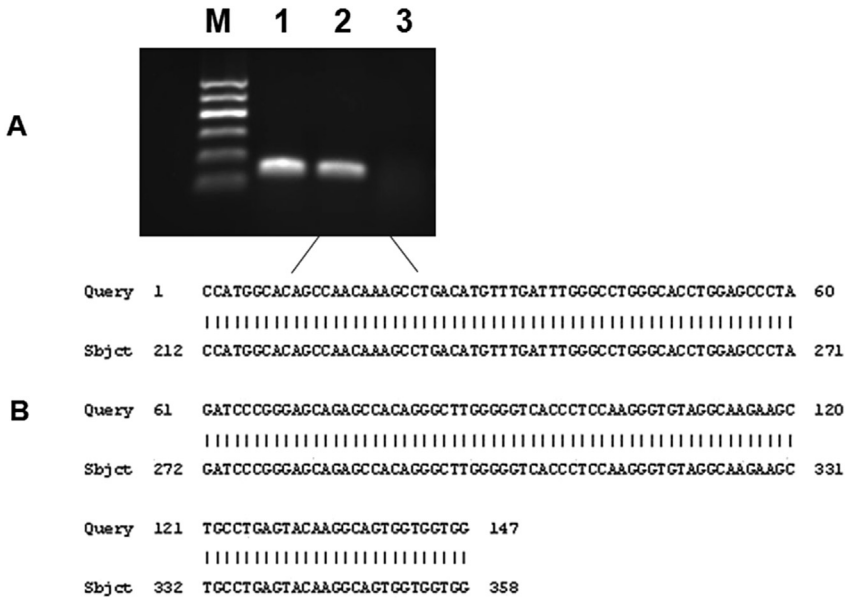

Fig. 1. Expression of ERAS cDNA in bovine placenta. (A) Total RNA prepared from bovine placenta and normal urothelium samples of the urinary bladder was analyzed by RT-PCR. Urothelium sample was utilized as a positive control. As a negative control, RNA without reverse transcription was subjected to PCR analysis. M: DNA marker ladder, Lane 1: bovine normal urothelium, Lane 2: bovine placenta; Lane 3: negative control. (B) Alignment of the sequences detects a 100% identity with all transcript variants of Bos taurus ERas mRNAs, present in the GenBank database (http://www.ncbi.nlm.nih.gov).

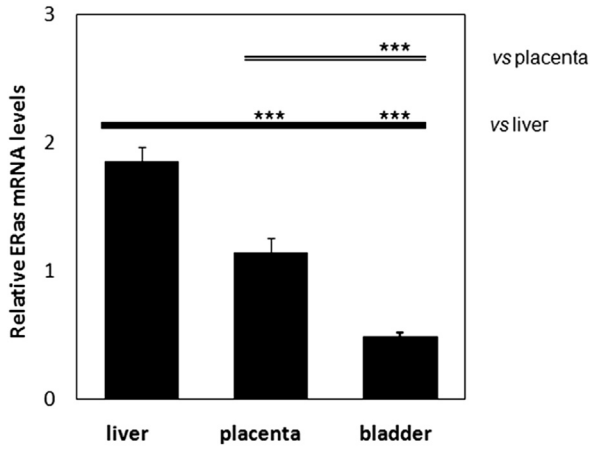

Fig. 2. Representative RT-qPCR shows liver, placenta, and bladder ERas mRNA levels. Data are expressed as mean and standard error of mean (S.E.M.) of three separate experiments, each performed in triplicate.
***p < 0.001 *vs* liver; ***$p < 0.001$*vs.* placenta performed in triplicate. ***$p < 0.001$*vs.* liver.
(▬); ***$p < 0.001$*vs.* placenta (═).

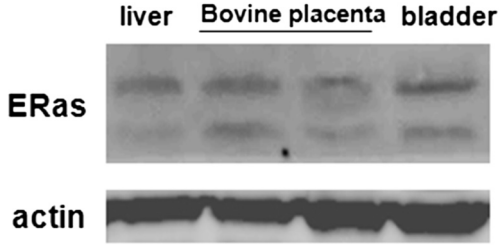

Fig. 3. An evident ~25-kDa band was shown by Western blot analysis for ERAS in lysates containing 100 μg of protein from bovine placenta; normal liver and urothelium of the urinary bladder were used as control. Actin protein levels were detected to ensure equal protein loading.

placenta of pregnant cows.

Real time PCR revealed that full term placentas contain ERAS transcripts more than normal urothelium but less than liver. From a morphological point of view, immunohistochemical findings detected both a membranous and cytoplasmic patterns of immunoreactivity for ERAS protein. These morphological findings support the notion that this compartmentalized localization of ERAS protein may be due to its different functional status. Indeed, it has been shown that ERas undergoes post-translational modifications, *e.g.* farnesylation and palmitoylation thus sharing the same post-translational modifications of H-Ras and N-Ras [20,21]. Plasma membrane localization of ERAS is achieved after palmitoylation of its C-terminus cysteines. This localization has been shown to be critical for its functionality whilst intracytoplasmic localization of ERAS can reflect an absence of palmitoylation [22]. Our morphological findings support proposed reports demonstrating that ERAS cycle between endomembranes, i.e. Golgi apparatus, and the plasma membrane via reversible and dynamic palmitoylation-depalmitoylation reactions [21]. It can be speculated that compartmentalization influences ERAS signaling. Accordingly proteomic analyses of compartmentalized Ras-specific responses have recently shown that Ras signaling depends on differential trafficking and organellar localization [22].

ERAS appears to play a role in numerous physiopathological mechanisms. The expression of ERAS probably coordinates cell proliferation as well as cell differentiation and appears to be involved in cell lineage specification during mouse early embryonic development [23]. Furthermore, it has been shown that ERAS plays a crucial role to maintain quiescence in Ito cells [4] and in reprogramming somatic cells via AKT signaling [24,25]. ERAS disruption caused by a mutation regulates tumor resistance in induced pluripotent stem cell (iPSCs) of naked mole-rat (NMR, *Heterocephalus glaber*), the longest-lived rodent species that exhibits extraordinary resistance to cancer [26].

We believe that, like cynomolgus ERAS, bovine ERAS neither promotes abnormal cell proliferation nor induces tumor formation in vivo but it may play a role in some physiological mechanism(s).

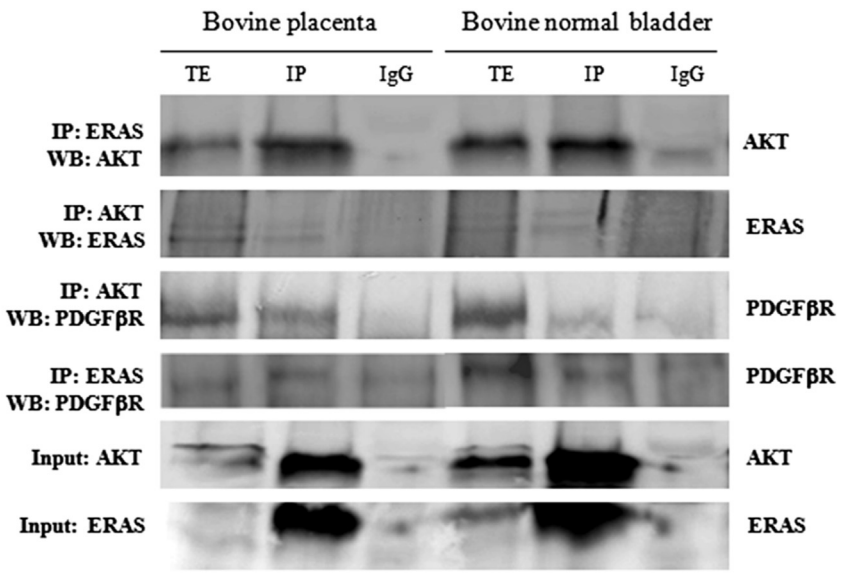

Fig. 4. Co-immunoprecipitation (IP) and western blot analyses showed a placental ternary complex composed of ERAS/AKT/PDGFβR. Normal bladder tissues were used as a positive control. Protein A/G beads with rabbit IgG were used as a negative control. TE = total extract.

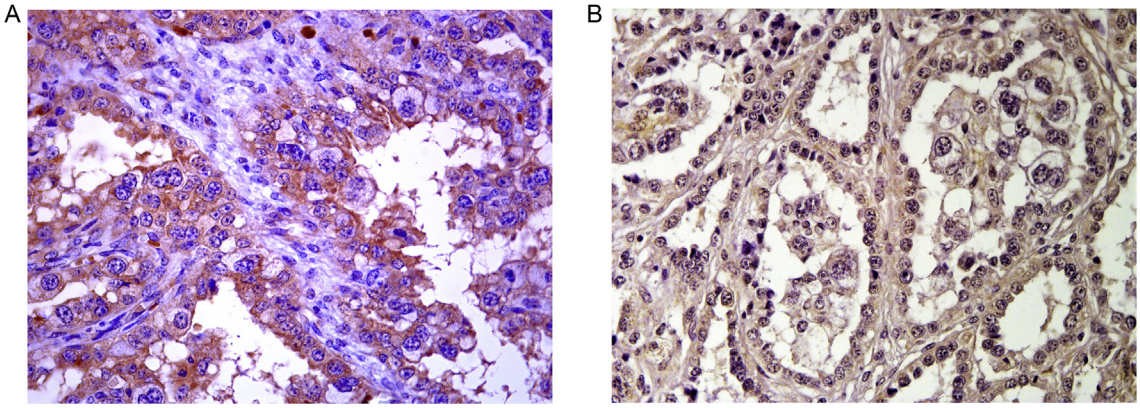

Fig. 5. A - An evident immunoreactivity for ERAS was detected at the level of plasma membrane and in the cytoplasm of the epithelial cells lining caruncular crypts and trophoblast cells of villi. Mag. X 40. B - No immunoreactivity was seen in negative controls. Species- and isotype-matched immunoglobulins replaced the primary antibody. Mag. X 40.

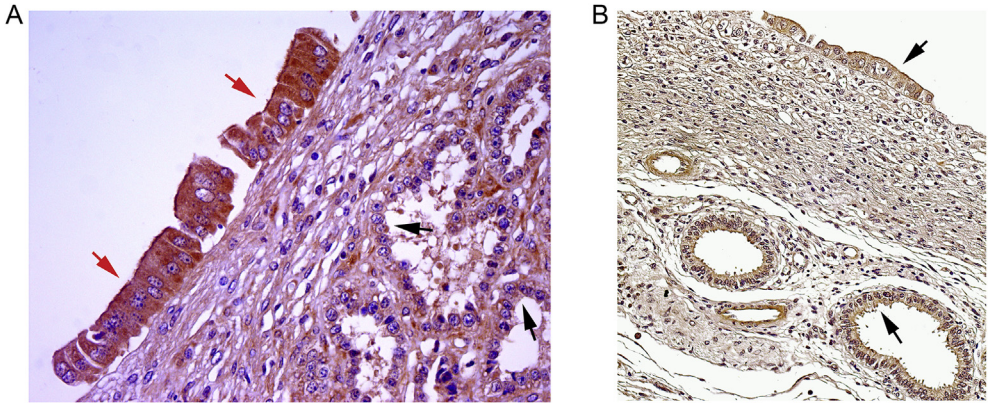

Fig. 6. A - ERAS was immunolabeled in the plasma membrane and in the cytoplasm of the chorionic epithelium of the interplacentomal region (red arrows) and endometrial glands (black arrows). Mag. X 40. B - No immunoreactivity of the chorionic and glandular epithelium (arrows) was detected when the primary anti-ERAS antibody was replaced by purified rabbit immunoglobulins. Mag. X 20.

Indeed, ERAS has also been detected in normal bovine urothelial cells, which allows us to suggest that ERas gene may be expressed in a variety of adult normal tissues of cattle.

The role of ERas as placental gene is completely unknown. There

are examples in which the genome has evolved in concert with placental development resulting in species-specific placental genes [15]; however, most of the genes essentially involved in placental development are not expressed exclusively in the placenta [27]. It has been suggested that most of the genes evolved to regulate placental development are identical to ones used in other organ system [28] and placenta uses genes for the same function as in other tissues and organs [16].

We did not perform any functional studies about possible role of ERAS in placental tissues. However, co-immunoprecipitation and western blot studies revealed the presence of a ternary complex in the bovine placenta composed of ERAS, PDGFβR and AKT. We suggest that in normal bovine placenta, like normal urothelial cells [15], ERAS has a physiological role interacting with PDGFβR which, in turn, activates AKT signaling. Accordingly, ERAS may be involved in key cellular processes of placenta, including innate and adaptive immunity. Thus it could play an important role in an appropriate regulation of cell homeostasis in the placentome, which is essential to maintain and complete gestation [29].

Our findings strengthen previous reports showing that PDGF can activate Ras/PI3K/AKT pathway [30]. The protein kinase AKT appears to be a ubiquitous response to activation of PI3K and is at a crossroad of many important signalings critical to cell proliferation and metabolism.

It will be needed to gain insights into the physiological role of ERAS in a variety of bovine tissues. Understanding molecular mechanisms contributing to normal organ development and function warrants future studies.

5. Conclusion

This study demonstrated that a ternary complex composed of ERAS, PDGFβR and AKT takes place in the bovine placenta at term which may play a crucial role in placental function and organogenesis since it is well known that PDGFβ ligand and its receptor, PDGFβR, have a central role in embryonic and placental development and angiogenesis [31,32].

In addition, the study of the control of oncogenes in placental cells, in the physiological context, is very important to elucidate their potential role in controlling trophoblast invasion critically involved in successful pregnancy outcome. Finally, in vivo studies of tumor-associated genes known to be physiologically expressed by trophoblast cells [17,18] may contribute to reveal a basis potentially useful for cancer.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2017.07.047.

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