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Identification of the pregnancy-associated glycoprotein family (PAGs) and some aspects of placenta development in the European moose (*Alces alces* L.)

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ABSTRACT

This study describes the identification and a broad-based characterization of the pregnancy-associated glycoprotein (*PAG*) genes expressed in the synepitheliochorial placenta of the *Alces alces* (Aa; N = 51). We used: (1) both size measurements (cm) of various Aa embryos/fetuses (crown-rump length) and placentomes (PLCs); (2) PCR, Southern and sequencing; (3) Western-blot for total placental glycoproteins; (4) deglycosylation of total cotyledonary proteins; and (5) double heterologous IHC for cellular immune-localization of the PAGs as pregnancy advanced (50–200 days *post coitum*). The crown-rump length and PLC size measurements permitted a novel pattern estimation of various pregnancy stages in wild Aa. The PLC number varied (5–21) and was the greatest at the mid and late stages of gestation in females bearing singletons or twins. The genomic existence of the identified PAG-like family was named *AaPAG-L*. Amplicon profiles of the *AaPAG-L* varied in the number and length (118–2000 bp). Southern with porcine cDNA probes confirmed specificity and revealed dominant *AaPAG-L* amplicons in males and females. Nucleotide sequences of the *AaPAG-L* amplicons shared 86.27% homology with the bovine *PAG1* (*bPAG1*) gene. Amino acid AaPAG sequences revealed *in silico* 88.23% to 100% homology with the bPAG1 precursor. Western-blots revealed a dominant mature 55 kDa AaPAG fraction, and the major ~48 kDa glycosylated form that was deglycosylated to ~44 kDa. The AaPAG-Ls was immuno-localized to mono- and bi-nucleated trophectodermal cells (TRD–chorionic epithelium), where signal intensity resembled intense TRD proliferation within developing PLCs as pregnancy advanced. This is the first study identifying the AaPAG-L family in the largest representative among the Cervidae.

1. Introduction

The genera of the moose and the Eurasian elk (*Alces alces*–Aa) are taxonomically the largest extant mammalian two sister subspecies among the deer family (Cervidae). Both subspecies have been classified in the international Red List of Threatened Species (www.iucnredlist.org),

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within the "least concern" category because they are still widespread and relatively abundant, despite fairly intense hunting in Russia, Scandinavia, and some other Asiatic areas. With the paucity of *post mortem* tissues for detailed study, and also many difficulties of serial sampling from wild animals, little scientific attention has been directed toward the placental development and identification of various secretory proteins involved in the maintenance/regulation of single or multiple gestations (until term 235–250 days *post coitum*; dpc).

The pregnancy-associated glycoproteins (PAGs) family is known as a large group of conserved genes encoding multiple secretory chorionic products, classified into two subfamilies of placental aspartic proteinases (EC 3.4.23), catalytically active and potentially inactive [1,2]. Among the identified complementary DNAs (cDNAs) of the *PAGs* (deposited in the GenBank/NCBI), at least 75 diversified cDNAs have been cloned in domesticated taxa only, including cattle, sheep, pig, horse, goat, cat, mouse, and water buffalo [1,3,4]. Only in a few wild taxa, a lower number of catalytically diversified *PAG* cDNAs (32) have been cloned: in the zebra [5], white-tailed deer (wtd) [6], American bison [7], wapiti [8], and giraffe [9]. Thus, in the wild species, this lower number of various *PAG* cDNAs is caused by many difficulties associated with the proper conditions for placenta collection required for high quality of total RNA isolation, allowing for effective cloning of full-length cDNA, including coding (ORF) and noncoding regions (5′UTR and 3′UTR).

Genomic analyses indicated the exonic-intronic structure (nine exons and eight introns, A–H) and specific promoter with a unique duplicated sequence within the bovine *PAG* (*bPAG1*) and porcine (*pPAG2*) cloned genes only [10,11]. Thus, most of the previous studies of the *PAG* gDNA templates were focused on domestic but not wild species.

Within each placenta type in various eutherians, different embryo-originated cells constitute the outer chorionic layer that forms a very precise interface with the uterus [12,13], where placental expression profiles of multiple *PAGs* are cell and pregnancy stage dependent [1,2]. Various distinct *PAG* transcripts have been identified in the pre-placental trophoblast (TR) during the peri-implantation period, whereas some other *PAGs* have been identified in the trophectoderm (TRD–chorionic epithelium) as the outer embryonic layer during the post-placental period until pregnancy term. So far, temporary specific placental *PAG* mRNA expression has been identified in: the cattle and sheep [14–16], the pig [17–19], the horse [5], the goat [20,21], the European bison [22], the white-tail deer [6], and some camelids, including the alpaca, dromedary, and Bactrian camel [23].

In domestic ruminants, the transcribed PAGs are comprised of two evolutionarily distinct groups. The first group, the 'modern' PAGs, is exclusively transcribed in specialized and moderately invasive TR/TRD, known as binucleate cells (BNCs). The second grouping, known as the 'ancient' PAGs, is transcribed in both cell types, mononucleate cells (MNCs) and BNC [14,15,24]. Also, immunoreactive PAGs were localized within various TRD cells (MNC/BNC) throughout placenta development in some taxa: cattle [25], pig, European bison, alpaca, and both camels [26–29]. Both cell types (MNC and BNC), producing multiple and diversified secretory PAGs, release their secretory granules into the maternal blood [1].

Protein studies allowed the identification of several distinct NH_2-terminal micro-sequences of native PAG isoforms (35–76 kDa) purified from the placenta of some domesticated species, including cows, goats, sheep [14,30–32], zebu [33], water buffalo [34], and wild American and European bison [35,36]. Also, *in vitro* studies revealed multiple secretory PAG isoforms produced by chorionic explants of domestic and wild species [22,37]. Other types of *in vitro* studies indicated the potential physiological importance of the PAGs, as embryo-originated signaling ligands interacting with different maternal gonadotropin receptors (gonadal and extra-gonadal) in cyclic pigs and cows [38] or pregnant pigs [39], and thus involved in the regulation of pregnancy maintenance.

The native and recombinant PAGs [40,41] have been applied for prenatal tests (radioimmunoassay and ELISA) to diagnose early pregnancy and to monitor fetus mortality, based on varying PAG concentrations in peripheral blood or milk of various domestic [1,42–47] or wild ruminants [1,48,49]. Moreover, PAG tests are useful for identification of fetal sex, single, twin, or multiple gestations, as well as to forecast miscarriages after embryo transfer and to detect pathological pregnancies [50,51].

Therefore, the objectives of our study were to identify the expected existence of the PAGs in Aa: (1) in the genome; (2) expression within the placental proteome as pregnancy advanced: including (2a) determination of glycosylation profiles, and (2b) cellular localization during subepitheliochorial/cotyledonary placenta development.

2. Materials and methods

2.1. Animals and tissue harvesting

Only wild Aa animals (N = 51); 40 males and 11 females during single: 50, 81, 103, 115, and 190 dpc or twin gestation: 72, 115, 175, and 200 (n = 2) dpc were used. All animals were bagged (September–March, 2008–2014) at the experimental hunting area of the Russian Research Institute of Game Management and Fur Farming, Kirov Region, in the east European part of the Russian Federation (58:3 N; 50:4 E). The annual quota of Aa to be shot is from 60 to 85 individuals on the territory of a scientific hunting area (64,000 hectares). All samples were culled with the appropriate required principles of ethics, traditions, and rules of hunting. Every Aa was bagged according to official local agreements/permissions for animal hunting for scientific purposes given by the Department of Conservation and Use of Wildlife of the Kirov Region, according to Federal Hunting Law (signed by the President of Russia on 24.07.2009), Federal Hunting Rules (signed by the Minister of Natural Resources and Ecology of Russia; 16.11.2010), and Hunting Realization Parameters (confirmed by the Governor of the Kirov Region; 14.12.2012).

Various tissue samples (skin, vas deferens, testes, and placentomes [PLCs]) were harvested *post mortem* from Aa of different ages (0.5–7.5 years). The start point of the dpc counting was generally used as September 10th, when the greatest activity of Aa rutting and pairing in the Kirov