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Expression and imprinting analysis of the *NESP55* gene in pigs

Maria Oczkowicz [a,*], Agata Piestrzyńska-Kajtoch [b], Katarzyna Ropka-Molik [a], Barbara Rejduch [b], Robert Eckert [c]

[a] Laboratory of Genomics, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice, Poland
[b] Department of Cytogenetics and Molecular Genetics of Animals, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice, Poland
[c] Department of Animal Genetics and Breeding, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice, Poland

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ABSTRACT

Most imprinted genes play important roles in a mammalian development. One of them is *GNAS* complex *locus* which codes for several imprinted or biallelically expressed transcripts. The function of some of them are well understood (for example GSα-guanine nucleotide binding, α -stimulating protein is essential element of cell signaling), whereas the others are little known. The function of *NESP55* (Neuroendocrine secretory protein 55) remains elusive, although there are suggestions about its role in brain development. Imprinted genes are currently being studied as potential candidate genes for quantitative trait loci (QTLs) in farm animals. In our study, we analyzed tissue distribution of *NESP55* mRNA in pigs and established imprinting status of this gene in the brain stem, muscle, kidney and liver at several developmental stages. *NESP55* mRNA was most abundant in central nervous system (CNS) and pituitary. Substantial expression was also noticed in the kidney, testis and muscle. Moreover, we identified a 12-nucleotides deletion within the coding region of *NESP55* (accession number ss#342570450) which was used in imprinting analysis. The deletion was very rare in the analyzed populations and present only in heterozygous form. The imprinting analysis showed that *NESP55* is maternally expressed in young and adult pigs, similar to what was obtained in humans, mice and cattle.

NESP55 is a part of a complex of genes, so called *GNAS* complex *locus*. Due to alternative splicing a number of different transcription variants are produced within this *locus*. To date, seven reference sequences – alternative transcripts of porcine *GNAS* gene has been deposited to the NCBI GenBank, however as much as twenty-two are predicted in vega ensemble database (http://vega.sanger.ac.uk/Sus_scrofa/Gene). In human, mice and cattle *GNAS* complex *locus* was shown to be imprinted (Hayward et al., 1998a; Hayward et al., 1998b; Kelsey et al., 1999; Peters et al., 1999; Khatib, 2004; Sikora et al., 2011), which means that only one of the parental allele is expressed in the offspring at least at some developmental stages. In pigs, to our knowledge no experiment confirming imprinting status of *GNAS* has been performed so far. Bischoff et al. (2009) confirmed the imprinting status of several imprinted genes in porcine fetal tissues by comparing expression in parthenogenetic and biparental fetuses, however they failed on *GNAS* because of multiple alternative transcripts (Bischoff et al., 2009).

Imprinting status within *GNAS locus* are different for alternative transcripts. Biallelic transcripts are produced from exons 2–12, paternally expressed transcripts use alternative 5' exons (Gnasxl and ex1A), while promoter for maternally expressed *NESP55* transcript lies approximately 15 kb from first exon of Gnasxl (reviewed by Plagge et al., 2008). Moreover, some of the *GNAS* transcripts are imprinted in a tissue specific manner – for example transcript for GSα is biallelically expressed in most tissues, but paternally imprinted in renal cortex of mice (Yu et al., 1998).

Biallelic transcripts of *GNAS* codes for GSα-guanine nucleotide binding, α -stimulating protein. The GSα is one of the subunits of G-protein – crucial element of cell signaling. Knockout mice lacking GSα in homozygotic state do not survive (Yu et al., 1998; Chen et al., 2005; Germain-Lee et al., 2005, reviewed by Plagge et al., 2008), while heterozygotic mice display a number of severe abnormalities including obesity (Cattanach et al., 2000; Yu et al., 2000). In humans, mutations within fragment of *GNAS* complex *locus* coding for this protein lead to severe endocrinologic diseases like Mc-Cune-Albright Syndrome, pseudohyperthyroidism and pituitary tumors (McCune 1936; Albright et al., 1937; Albright et al., 1942; Lyons et al., 1990).

Other proteins such as XLαS -NH2-terminal variant of GSα and Alex are produced on the basis of paternally expressed transcripts in mice and humans. Interestingly, these two proteins are oncoded by the same transcript, but use distinct ORFs (Klemke et al., 2001). The function of Alex remains unclear, while XLαS seems to control

* Corresponding author. Tel.: +48 666081109.
E-mail address: majawrzeska@o2.pl (M. Oczkowicz).

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the same signaling pathways as GSα. Moreover, there are suggestions that paternally expressed protein XLαS acts oppositely to biallelic protein GSα in glucose and lipid metabolism, accordingly to parental conflict hypothesis (Trivers, 1974; Yu et al., 2000; Chen et al., 2005).

Neuroendocrine secretory protein 55 (NESP55) was originally described as a chromogranin-like protein in secretory vesicles of adrenal chromaffin cells. It was supposed that proteolytic processing of this protein may yield smaller physiologically active peptides (Ischia et al., 1997; Fischer-Colbrie et al., 2002). Despite intensive investigations, the function of NESP55 protein remains unclear to date. Surprisingly, *Nesp55*-deficient mice develop normally without obvious metabolic abnormalities and are fertile. The only difference was in the reaction of *Nesp55*-deficient mice to the new environment. Mice lacking *Nesp55* were more active when placed into the novel environment (Plagge et al., 2005). At present, the function of Nesp55 as a marker for neuroendocrinologic tumor development is being tested. It was shown that Nesp55 may be a useful marker for the prediction of the primary site of metastatic well-differentiated neuroendocrine tumors (Srivastava and Hornick, 2009). Moreover, NESP55 is involved in the secretory pathways of prolactinomas and GH adenomas (Gupta et al., 2011).

In addition, antisense transcript to *Nesp55 (Nespas or Gnasas*) have been identified, in mice and humans (Hayward and Bonthron, 2000; Wroe et al., 2000). *Nespas* is paternally expressed – opposite to sense transcript and is supposed to play a role in a control of imprinting of the *Gnas* complex *locus* (Hayward and Bonthron, 2000). Recently, it has been shown that *Nespas* is associated with silencing of overlapping protein-coding *Nesp55*, independently of DNA methylation (Williamson et al., 2011).

Imprinted genes are thought to be good candidate genes for economically important traits in farm animals. A growing numbers of experiments indicate that there are many more imprinted *QTLs* – (Quantitative Trait locus) in porcine genome than previously suggested (de Koning et al., 2000; Thomsen et al., 2004; Boysen et al., 2010). In pigs, several *QTLs* has been identified within the telomeric region of *SSC17* – the region where GNAS complex *locus* is located (Thomsen et al., 2004; Fan et al., 2008; Ponsuksili et al., 2008; Stratil et al., 2008). Thomsen et. al. (2004) observed imprinted *QTL* for body mass of piglets and growth of piglets, while Fan et al. (2008) identified *QTL* for meat color. Furthermore, expression *QTL (eQTL)* positively correlated with drip loss was identified for *GNAS* in pigs (Ponsuksili et al., 2008). Knowledge about the imprinting status of genes which potentially affect economically important traits is crucial before they can be used in Marker Assisted Selection (MAS).

Although imprinting status of many genes seems to be largely conserved among mammals, there are a number of genes which are differentially expressed in various mammals, various tissues of the same species or even at the different developmental stages (Khatib, 2007; Hou et al., 2010).

The aims of our study were to detect new polymorphisms within *NESP55* gene and to establish its imprinting status in pigs. We identified a 12-bp deletion within the coding sequence of porcine *NESP55* gene. Moreover, we analyzed its tissue distribution and imprinting status in brain stem, muscle, liver and kidney of pigs at two developmental stages of young and adult pigs.

1. Results

The PCR-SSCP method was employed for screening 180 animals whose tissues had been collected for previous experiments (Rejduch et al., 2010). Three pairs of primers were designed to cover the whole coding region of the *NESP55,* however only one polymorphism was detected – 12 bp deletion (accession number ss#342570450) at the position 783 of reference sequence nr GenBank: (NM_001130215.1). The deleted fragment is one of the two repeats of the motif (GAGCCCGAGACC) and results in an in-frame loss of four amino acids (EPET) in positions 133–136 of polypeptide. The mutation was present in heterozygous form in 7 gilts whose father was homozygous without deletion. The tissues of these gilts were further used for imprinting analysis.

We established the frequency of this mutation by screening 452 animals of different breeds using electrophoresis in poliacrylamid gel. No mutated animals were present in 134 samples of Polish Landrace and in 80 samples of Pietrain. Among 238 Large White animals only 6 animals were heterozygous, and no homozygotes with mutation were observed. All animals with the deletion did not display any abnormalities.

The RT-PCR analysis showed that *NESP55* is strongly expressed in central nervous system (CNS) (brainstem, cortex, midbrain) and pituitary. Substantial abundance of *NESP55* mRNA was also present in kidney, muscle and testis and was very weak or undetected in lungs, fat, heart, stomach, liver, spleen and ovary expression (Fig. 1). Nonetheless, when we reverse transcribed RNA isolated from the liver with gene specific primers, we obtained weak RT-PCR product. Beta actin was used as an endogenous control and product of similar intensity was obtained for all samples (Fig. 1).

Twelve nucleotide deletion within the coding region of the *NESP55* gene (ss#342570450) was present in heterozygous form in 7 animals slaughtered at 60, 90, 180 and 210 days of age (doa) (Rejduch et al., 2010). Muscle tissue for expression studies was

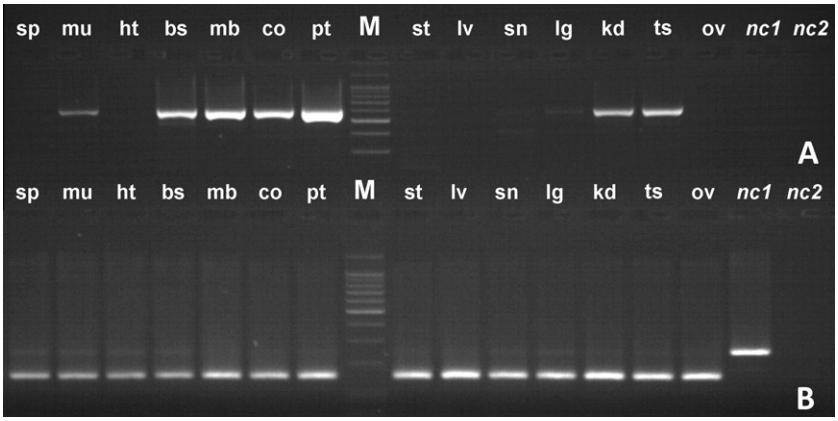

Fig. 1. Tissue distribution of *NESP55* mRNA in adult pigs: sp-backfat, mu-muscle, ht-heart, bs-brainstem, mb-midbrain, co-cortex, pt-pituitary, M-marker (100 bp), st-stomach, lv-liver, sn-spleen, lg-lung, kd-kidney, ts-testis, ov-ovary, nc1-DNA, nc2-water.