

Monoallelic expression of *NNAT* gene in Nelore steers skeletal muscle

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ABSTRACT: The *NNAT* gene plays a role on adipogenesis, brain development and insulin secretion. *NNAT* is described as paternal imprinted in bovine fetal tissue, but it is still underexplored in adult tissues. In order to improve the understanding of *NNAT* expression, muscle from Nelore was used to assess its allelic expression. Genomic DNA from 38 steers was genotyped for identification of heterozygous individuals with known allele origin. Total mRNA from muscle was extracted and sequenced by HiScanSQ, and afterwards the reads of each allele were counted for each animal. A total of 8 reciprocal heterozygous had more than 10 reads for the chosen SNP. All animals showed monoallelic expression, being 5 with paternal allele expressed, while 7 had the G allele expressed. These results demonstrate that *NNAT* may not have the paternal imprint conserved in adult bovine muscle, and it suggests a G allele-dependent expression.

Keywords: *Bos indicus*; imprint; Neuronatin

Introduction

Imprinted genes have been target of many studies, mainly in human and mouse, and lately in bovines due to the importance of understanding the epigenetics mechanisms underlying important phenotypes, and the possibility of applying it in animal breeding programs in the future. The neuronatin (*NNAT*) gene is an example of the difference in available knowledge between humans/mouse and bovine. This gene has an imprinted expression profile well described for humans and mouse, however few information available for bovine. *NNAT* (ID: 353114) is located in BTA13 in bovine and codifies a proteolipid with unclear functions, even for humans and mouse. *NNAT* has been described as acting in many tissues as adipose, pancreas and brain. The main role is described in process like adipocyte differentiation and insulin secretion (Chu & Tsai, 2005; Suh et al., 2005; Joe et al., 2008; Li et al., 2010), in addition to neurons differentiation (Joseph et al. 1994; Evans et al., 2005). In diabetic rodents, the *NNAT* expression was increased in aortic endothelial cells (Mzhavia et al., 2008), but reduced and glucose-dependent in pancreatic β cells (Chu & Tsai, 2005; Joe et al., 2008; Selman et al., 2009). In humans, one SNP of *NNAT* was associated with obesity in children and adults (Vrang et al., 2010). *NNAT* is well conserved in humans and bovines with 3 exons and 2 introns, which results in 2 different transcripts (Dou &

Joseph, 1996). The difference between transcripts α and β is that the last one do not have the exon 2 (Dou & Joseph, 1996), and this probably eliminate a transmembrane domain (Joseph et al. 1994). Differently, mouse has 4 types of transcripts (Kagitani et al., 1997). The *NNAT* gene is expressed in many fetal and adult bovine tissues, including skeletal muscle (Zaitoun & Khatib 2006). The expression pattern is described as monoallelic paternal in different tissues and developmental stage, furthermore it seems to be conserved in other species (human, mouse, pig and cattle). The expression of the paternal monoallelic *NNAT* gene in cattle was first described in blastocysts by Ruddock et al. (2004) and by Zaitoun & Khatib (2006) in several fetal tissues, including fetal muscle, as well as in adult endometrial tissue. Both authors noticed the paternal allele expression of both gene isoforms, Ruddock et al. (2004) also described that α is the predominant isoform expressed during embryo early-stage, while the β isoform during late stage of embryo development. In pigs, Cheng et al. (2007) demonstrated this pattern in muscle and other tissues in 12 months old animals. Evans et al. (2001) showed that both isoforms are paternally expressed in human fetal brain, and Kagitani et al. (1997) showed that the four isoforms are paternally expressed in mouse embryos. To improve the understanding of imprint status of *NNAT* gene in cattle, allelic expression was analyzed for the first time in adult muscle tissue.

Materials and Methods

Animals, DNA extraction and genotyping. A total of 38 Nelore steers, produced as described in Tizioto et al. (2012), with available data from next generation RNA sequencing and with genomic DNA genotyped by BovineHD BeadChip, 770K (Illumina, San Diego, CA) was used in this study. The genotyping allowed for identification of heterozygous steers and determine the allele parental origin. The SNP (G/A, rs109895497) located in exon 3 of the *NNAT* gene, also available in this chip, and common to both transcripts, was selected to have its allelic expression evaluated.

RNA extraction and RNA-Seq data. Muscle samples were collected from *Longissimus dorsi* between the 12th and 13th ribs for RNA extraction. The samples were stored in liquid nitrogen and kept at -80 °C until processing. The tissues were pulverized in liquid nitrogen for RNA extrac-

tion with Trizol (Invitrogen). RNA samples were quantified using NanoDrop® and subjected to electrophoresis on 1% agarose gel stained with GelRed to assess the integrity of RNA. RNA libraries were prepared according to the Illumina protocol. Sequencing was performed on the equipment HiScanSQ (Illumina, San Diego, CA). The protocol *pair-end reads* with the TruSeq™ SBS – HS (50 cycles) sequencing kit was used, recommended for sequencing 100 bp of ribbons in only one direction.

Allele-Specific expression Study. RNA-Seq data obtained were analyzed using bioinformatics tools through the GALAXY Platform (Giardine et al, 2005; Blankenberg et al, 2010b, Goecks et al, 2010). The sequence data were converted to ASCII quality format. FASTQ Groomer version 1.0.4 (Blankenberg et al., 2010a) was used to convert the data to Sanger format and then the reads were visualized using the FastQC program (www.bioinformatics.bbsrc.ac.uk/projects/). The FAST Quality Trimmer program was used to trim low quality ends of reads (Blankenberg et al., 2010a). The reads were mapped to the reference genome (*Bos Taurus* UMD3.1 assembly) using TopHat v.2.0.9 software (Trapnell et al., 2009). In-house software based on the python module pySAM enable us assess the frequency of each nucleotide of the SNP location in all mapping files simultaneously.

Results and Discussion.

Among the 38 animals with RNA-Seq data available, 14 were heterozygous, and in three of them were not possible to determine the parental origin of the allele. Read counts for each allele was performed for all 38 animals, but only 8 met necessary requirements to evaluate the effect of the parental origin of allelic expression, i.e., the individuals must be homozygous with heterozygous father, and have at least 10 mapped reads (Table 1). The samples were divided into two groups according to the parental origin of the alleles (4 GpAm e 4 GmAp; p=paternal, m=maternal). Eight animals showed almost exclusive monoallelic expression as described in the literature. However, according to the parental imprint pattern of this gene previously described by other authors (Ruddock et al., 2004; Zaitoun & Khatib 2006), expression was expected in only the G allele in GpAm group and the A allele in GmAp group. Four animals in GpAm group expressed the paternal allele G as expected, but only 1 animal in GmAp group expressed the paternal allele (A), while others expressed maternal allele G. This animal which expressed the paternal allele A has the same father as one of the animals which expressed maternal allele G, suggesting that we can exclude the family effect in this case. These results demonstrate this gene may not have a paternal imprint pattern conserved among tissues and developmental stages in cattle as were predicted, however it could have a G allele-dependent expression since 87.5% of studied animals here showed almost exclusively expression of the G allele. This can be related due to linkage disequilibrium to either promoter SNP or variation in regulatory factors, considering that only one animal

showed expression of the A allele. This is the first report of allelic expression analysis of this gene in adult bovine skeletal. In other studies, the imprint pattern was observed in the *NNAT* blastocyst (Ruddock et al.), fetal muscle and other tissues (Zaitoun and Khatib, 2006), but in adulthood, only endometrial tissue was studied (Zaitoun and Khatib, 2006). Thus, this gene seems to show an allelic expression conserved behavior among fetal tissues in bovine, however it could not be inferred for adults, although being monoallelically expressed.

Table 1: Number of reads counted for each allele in each sample.

Sam- ple*	Father	Reference allele	Number of reads					Geno- type**
			A	C	G	T	N	
M1	1	G	1	0	10	0	0	GpAm
M2	2	G	1	0	29	0	0	GpAm
M3	3	G	0	0	28	0	0	GpAm
M4	3	G	0	0	11	0	0	GpAm
M5	4	G	15	0	1	0	0	GmAp
M6	6	G	0	0	29	0	0	GmAp
M7	5	G	0	0	14	0	0	GmAp
M8	4	G	0	0	15	0	0	GmAp

*GpAm = G allele inherited from father and A allele from mother; GmAp = G allele inherited from mother and A allele from father.

Conclusions

The *NNAT* gene was shown to be monoallelically expressed in adult bovine skeletal muscle when the SNP rs109895497 was used, however this pattern does not seem to be paternal-dependent. This gene still needs further investigation, since it plays an important role in adipogenesis process, which is of great interest for animal breeding programs.

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