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POST-TRANSCRIPTIONAL REGULATION OF POLYMORPHIC *PROTEASE-ACTIVATED RECEPTOR-2 (PAR-2)* GENE DISPLAYING CORRELATION WITH SKIN PIGMENTATION IN CATTLE

A. W. Nizamani [1, 2], O. Fumière[4], L. Forestier[2,3], S. Presciuttini[5], H. Leveziel[3,1] Z. A. Nizamani[6] and A. Oulmouden [2,3,]

[1] Department of Animal Breeding and Genetics, Sindh agriculture University Tandojam, Pakistan
[2] Université de Limoges, UMR1061 Génétique Moléculaire Animale, F-87000, Limoges France
[3]INRA, UMR1061 Génétique Moléculaire Animale, F-87000, Limoges France
[4] Département Qualité des Productions agricoles, Centre Wallon de Recherches agronomiques Chaussée de Namur, 24 B-5030 Gembloux (Belgium)
[5] Università di Pisa, Dipartimento di Scienze Fisiologiche, Via San Zeno 31, 56123 Pisa, Italy
[6] Department of Veterinary Pathology, Sindh Agriculture University Tandojam, Pakistan
Corresponding author E-mail:zaheer5feb@yahoo.com

ABSTRACT

Protease-activated receptor-2 (PAR-2) is a trypsin-activated member of the PAR receptor family. Following cleavage, the newly created N-terminus acts as a tethered receptor-activating ligand.*PAR-2* is expressed in keratinocytes and enhances the phagocytosis rate of keratinocytes, which leads to increased melanosome transfer and skin pigmentation. Here, we investigated to see whether the bovine *bPAR-2* plays a role in pigmentation of cattle skins resulting in shades ranging from dark-black to white. We have identifiedtwo *bPAR-2*natural variants located in the tethered ligand for the first time. Surprisingly, none of these seems to affect the receptor activity in relation to skin pigmentation as suggested by tethered ligand structure-activity relationships studies. Furthermore, we also showed for the first timethat*bPAR-2*is regulated at a post-transcriptional level in pigmentation-skin dependent manner. Modulation of PAR-2 activity enhances or decreases melanosome transfer from melanocytes to keratinocytes which results in a variation in skin pigmentation. In this study, we found that *bPAR-2* was subjected to post-transcriptional regulation in pigmentation-skin dependent manner and that two variants affecting its tethered ligand did not affect skin pigmentation. Understanding the regulation of PAR-2 expression and the activity of its tethered ligand is important, because PAR-2 is involved in a key step of skin pigmentation and plays a role in lightening or darkening of the skin.

Key words: Cattle, PAR-2, skin pigmentation, post-transcriptional regulation.

INTRODUCTION

Mammalian skin and hair pigmentation is a highly complex process which is under the coordinated action of over 127 genes that encode proteins involved in different biological aspects of pigmentation including development, distribution, anddifferentiation of melanocytes along withtheir regulation by various physiological factors (Bennett andLamoreux,2003). Melanocytes in the skin are found in two distinct populations, those residing at the dermal–epidermal junction, which give rise to skin colour, and those residing in hair follicles, which produce hair colour(Yamaguchi and Hearing, 2006).Althoughthese two melanocyte populations are regulated by shared signalling pathways, hair and skin melanocytes are regulated quite independently (Van Raamsdonk *et al.*,2009). Some furred mammals, including the mouse, do not exhibit extensive skin pigmentation despite of their pigmented hairs. On other hand, both melanization of the skin (black, white, pale) and hair (black, red, blonde) may also occur together in other mammals including humans. Although black hairs commonly coexist with black skin, a large fraction of human population has dark hair and white skin. Theconstitutive skin pigmentation is regulated by a number of factors, including the relative amounts of eumelanin (brown-black pigment) and pheomelanin (red-yellow pigment) synthesized within melanosomes, melanocyte dendricity, transport of melanosomes to dendrites, and the rate of melanosome transfer to the surrounding keratinocytes (Abdel-Malek and Kadekaro,2006).It is also reported that autophagy plays a role in skin colour in humans by regulating melanosome degradation in keratinocytes and thereby contributes to the ethnic diversity of skin color (Murase *et al*., 2013). Role of Tyrosinase, which is a central and rate-limiting enzyme in melanin biosynthesis, has also been described (Bae-Harboe and Park, 2012). Several studies suggest a pivotal role of the *protease-activated receptor-2 (PAR-2)* genein the regulation of human skin pigmentation. *PAR-2* is expressed by keratinocytes and it is involved in the melanosome transfer from melanocytes to neighbour keratinocytes (Seiberg *et al.,* 2000a). The significance of *PAR-2* was

substantiated by the finding that modulation of its activity affected skin pigmentation by altering melanosome transfer (Seiberg *et al.,* 2000b). Subsequent studies demonstrated that the irradiation of the human skin subjected to UVR up-regulated *PAR-2* expression, thus providing evidence for the association of *PAR-2* with the up-regulation of human skin pigmentation *in vivo*(Scott *et al.,* 2001). Moreover, in a recent study, Macelignan was found to inhibit melanosome transfer, in vitro, by down-regulating PAR-2, thereby reducing keratinocyte phagocytosis and PGE2 secretion, which in turn lead to inhibition of dendrite formation in B16F10 melanoma cells (Choi *et al.,* 2011).

PAR-2 (Nystedt *et al.,* 1994) is a seven transmembrane domain G-protein-coupled receptor which is activated by the trypsin proteolytic cleavage or mast cell tryptase (Déry *et al.,* 1998). After proteolytic cleavage, the newly exposed NH_2 terminus acts as a tethered peptide ligand, which binds to the receptor and activates it (Nystedt *et al.*1995; Molino *et al.,* 1997).The PAR-2, expressed in intact cells, displays selective trypsincleavage at the activation site (Al-Aniand Hollenberg, 2003). Trypsin fails to activate a mutant PAR-2 receptor in which the cleavage site has beenchanged by site-directed mutagenesis (Nystedt *et al.,* 1994). In other respects, synthetic peptides corresponding to the tethered ligand (SLIGRL in mouse, SLIGKV in human) can activate PAR-2 independently of receptor cleavage (Nystedt *et al.,* 1995; Böhm *et al.,* 1996). Moreover, the amino terminal tethered ligand sequence $^{37}S^{38}L$ plays a major role in the activation of PAR-2 (Al-Ani *et al.,* 2004). Furthermore, Lin *et al.* (2008) showed that a tetra peptide LIGR can also induce skin pigmentation. Their works suggested that LIGR is a more specific regulator of PAR-2 induced pigmentation relative to SLIGRL. To our knowledge, only one mutation within extracellular loop 2, which affects the PAR-2 activation, has been reported. However, the impact of this mutation on human pigmentation has not been studied (Compton *et al.,* 2000). In mouse, whose skin is not pigmented, no natural variant has beenreported thus far.

Given the central role of *PAR-2*in melanosome transfer, the disruption of this process either by mutations affecting the coding region or by transcriptional and/or post-transcriptional processes, would be expected to have severe consequences for skin pigmentation. Based upon the above information, we have used various cattle breedswith different patterns of skin and hair color to see whether *PAR-2* is involved in their skin pigmentation, as has been reported in human beings. Moreover, ifso, whether it is regulated in a similar manner. We have first screened the coding region of the gene for finding possible polymorphisms, and have thenstudied its expression at both mRNA and protein levels.

We report here, for the first time, the identification of two natural *PAR-2* alleles affecting the tethered ligand of the receptor. Furthermore, we show that post-transcriptional regulation of this polymorphic *PAR-2* is correlated with skin pigmentation in cattle.

MATERIALS AND METHODS

Animals: The animals used in the study belonged to standardized homogenous breeds with respect to coat color and skin pigmentation. The breeds included; Charolaise, Prim'Holstein, Limousine, Parthenaise, Blonde d'Aquitaine, Salers, Maine-Anjou (French breeds), Romagnola, Marchigiana, Chianina, Piemontese, Italian brown, Pezzata Rossa, Italian Friesian (Italian breeds) and Belgium Blue (Belgian breed).

Skin RNA Extraction and cDNA Synthesis: To investigate the expression of *bPAR-2* gene, we used skin samples from animals which were unrelated up to third generation and belongedto five French cattle breeds exhibiting different coat colors and skin pigmentation (Figure 1). The breeds used were; Prim' Holstein (dark-black skin and hair), Parthenaise (light-black skin and red hair), Salers (reddish-brown skin and hair), Limousine (golden-red skin and hair), Charolaise (white skin and hair). Skin samples (25 cm^2) were obtained from a slaughterhouse in Limoges, France and several breed associations or UPRA (Union pour la Promotion des Races Animals). Total skin RNAs were extracted and cDNAs were synthesized using 1 µg of total RNA as is described previously (Guibert *et al.,* 2004). The cDNAs were prepared from three individualsof each French cattle breed and stored at -20°C until use.

Full-length open reading frame cloning: PCR-amplification was used to obtain the full-length open reading frame (ORF) of *bPAR-2* using cDNAs of each cattle breed as described above. Primers (forward primer, 5'-ATGCGAAGC CCGAGCGCGGCGTGGCTGCTC-3'; reverse primer, 5'-TCAGTAGGAGCCTTTGACACT GGTTGAACT-3') were designed on the basis of available cDNA sequence of bovine *PAR-2* (NM_001046283). PCR amplification was carried out in 25 µl reaction volume containing 10 pmol of each primer and 12.5 µl of 2X working concentration PCR Master Mix (Abgene France, Courtaboeuf, France) under the following cycling conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles (95°C for 30 s, 65°C for 30 s, 72°C for 2 min) and one cycle (72°C for 5min). PCR products were gel purified (QIA quick gel extraction Kit, Qiagen, France) and either directly sequenced or cloned into pCR-XL-TOPO vector (Invitrogen SARL) and then sequenced (ABI Prism 310 DNA Genetic Analyzer, Perkin-Elmer France, Courtaboeuf, France).

***bPAR-2* expression:** The expression of *bPAR-2* gene was first examined by RT-PCR using 1.5 µl of cDNA from skins of each breed as described above. The primers were designed from coding exon 1 (forward primer, 5'-ATGCGA AGCCCGAGCGCGGCGTGGCTGCTC-3') and exon 2 (reverse primer, 5'-CATGTAAAT CACAGCGGGGTGCTTCTTCTT-3') to avoid the amplification from genomic DNA. PCR cycling conditions were same as described above except for the annealing step performed at 61°C.

Real-time PCR amplification: Samples from three unrelated animals per breed (15 samples in total) were tested. Three independent cDNA syntheses were performed on each skin sample (45 cDNAs in total) after a treatment of the all RNA samples with DNase (Sigma). cDNAs were prepared from purified RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions.

Measurements of mRNA levels were performed with Real-time PCRusing an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems). Briefly 5µl of each 10^{-2} dilution of cDNA was amplified in triplicate in 1X SYBR Green PCR Master Mix (Applied Bio systems France, Courtaboeuf, France) using 7.5 pmol of forward primer (5'-CACCGTCCCAGGAAACAAGT-3'and 7.5 pmol of reverse primer (5'-CCAGCGACCACAGGTGAGTT-3') in a 17.5 µl. Amplification conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60 °C for 1 min. The Ct value (starting cycle for amplification) was calculated by fixing a threshold in the linear portion ofeach amplification. The bovine GAPDH and TFIID house keeping genes were used to normalize the samples. Forward 5'-GTGAAGCAGGCGTCAGAGG-3' and reverse 5'-TTGAAGTCGCAGGAGACAACC-3' primers were used to amplify GAPDH cDNA. Forward 5'-CGTGCCCGAAATGCTGA-3' and reverse 5'-TTACTCTTGGCTCCTGTGCA-3' primers were used to amplify TFIID cDNA.

SDS PAGE and western blotting: Skin samples, either fresh or stored at -80°C, were prepared from the same animals as described above to obtain protein extracts. First, adipose tissue was removed and hairs were shaved. Then, skin was cut into small pieces and lysed in Extraction Buffer (Tris-HCl20 mM, EDTA2 mM, NaN_3 1mM, and protease inhibitor cocktail Cat #: 1697498, Roche). The samples were homogenized by using Ultra-Turrax® T25 basic (IKA-WERKE). Protein concentrations were analyzed by Bio-Rad Protein Assay Kit. The lysateswere separated using electrophoresis with 10% SDS-polyacrylamide gels. Gels were electro blotted on Nitrocellulose membrane (Amersham Biomacia Biotech, Aylesbury, UK) and analyzed by BM chemiluminescence Blotting Substrate (POD) western blotting (Roche). Mouse monoclonal PAR-2 antibody (SC-13504) raised against the human synthetic peptide (^{37}SLIGKVDGTSHVTG^{50}) was used at a 1: 1000 dilution to detect bovine bPAR-2. Goat anti-mouse IgG-HRP conjugated secondary antibody was used at dilutions of 1: 2000. Both antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Enzymatic deglycosylation assay: Fifty micrograms of total protein extract of each bovine skin, were deglycosylated using 1 U of PNGase F (Roche) (16h, 37°C) in a denaturing buffer [β-mercaptoethanol (5% v/v), SDS (2% v / v), Triton X-100 (0.1% v / v), bromophenol blue (0.02% w/v)].

PCR-RFLP genotyping: PCR amplifications were performed in a volume of 50 µl with the following primer set (forward primer: 5'-CTCAGTCGTTTCGCTTCTGC-3'; reverse primer: 5'-GATCGTGTAGACAACT GGAAGAAAG-3') from Eurogentec (Seraing, Belgium). The reaction mix contained about 100 ng of genomic DNA, 0.2 µM of each primer, 0.2 mMdNTPs (Invitrogen, Merelbeke, Belgium), PCR buffer 1x, 1.5 mM $MgCl_2$, 2 units of Taq polymerase (Invitrogen, Merelbeke, Belgium). The PCR thermal program was: a denaturation of the DNA for 2 minutes at 94°C, 35 cycles with 3 steps per cycle (94°C for 30 seconds, 61°C for 30 seconds and 72°C for 1 minute) and a final extension for 3 minutes at 72°C. A part of PCR products (15 µl) was analysed by electrophoresis on a 2.5 % agarose gel so as to determine the size of PCR products. A second part of PCR products was digested by the restriction enzymes *Hind* III (Roche Diagnostics GmbH, Penzberg, Germany) and *Nla*III (New England Bio Labs Inc., Ipswich, UK). Digestions were performed in a volume of 20 µl. The digestion mixture having *Hind* III enzyme contained 10 µl of PCR products, Buffer 1x, 10 units of enzyme and additional water to obtain a final volume of 20 µl. The digestion mixture having *Nla* III enzyme contained 10 µl of PCR products, Buffer 1x, 7.5 units of enzyme and additional water to obtain a final volume of 20 µl. The two digestion mixtures were incubated at 37°C for 1 h 30 min. The digestion products were analysed by electrophoresis on a 2.5 % agarose gel.

Statistical analysis: Genotype counts and calculation of allele frequencies were performed both at the single breed level and on the total samples. Adherence to Hardy Weinberg equilibrium (HWE) within samples was evaluated by the "exact test" implemented in the program ARLEQUIN 3.1 (Excoffier and Schneider, 2005). This program was also used to perform the exact test of population differentiation and to calculate heterozygosities and F_{ST}.

RESULTS

Bovine *bPAR-2* open reading frame: The 1188 bp-long*bPAR-2*ORF was obtained by PCR amplification of cDNA prepared from RNA of Prim' Holstein black-skin (Figure 1). The deduced bPAR-2 receptor sequencesare 395 amino acids long (supplementary material, Figure S1) exhibiting all characteristics previously reported in mouse (Nystedt *et al.*, 1994) and human (Nystedt *et al.*, 1995) counterparts including seven transmembrane domains and the extracellular N-terminal containing the tethered ligand that binds to and activates the cleaved receptor. Four of the first six residues of the tethered ligand are identical and the fifth and the sixth are similarly conserved. Furthermore, the second and third potential N-glycosylation sites are also conserved in the three species. In addition, bPAR-2 possesses an additional N-glycosylation site on N-terminal (supplementary material, Figure S1).

Screening for polymorphisms: To identify possible mutations causing variation of skin pigmentation in cattle, we sequenced the entire *bPAR-2* coding region of five breeds and identified three alleles, called *bPAR-2*, *bpar-2.1* and *bpar-2.2*. The *bPAR-2* allele was considered as wild type, since it exhibits conserved amino acids at the tethered ligand as previously reportedin humans. The *bpar-2.1*allele results from base substitution (C106T) of 106 bp from the ATG start codon, leading to an amino acid change from Leucine to Phenylalanine at position 36 (Figure 2). The third allele *bpar-2.2* is a substitution mutation of Thymine at position 111 to Guanine (T111G) resulting in amino acid change from Isoleucine to Methionine at position 37. Interestingly, the identified variants (Figure 2) correspond to the second and third positions of the hexapeptide tethered ligand that activates the receptor following cleavage.

Genetic variation of the *bPAR-2* among cattle breeds: We genotyped 293 individuals from 14 different European cattle breeds, using the two restriction sites associated with *bpar-2.1* and *bpar-2.2* and their absence in *bPAR-2*(Figure 2).

The *bPAR-2*gene is highly polymorphic in cattle (supplementary material, Table S1), with the three allelessegregating in most breeds and overall heterozygosity being about 40%.The less frequent *bpar-2.2* allele (frequency = 0.05 on average) isabsent insome breeds, though this result may be due to smaller sample size in some cases.There is no evidence of departure fromHardy-Weinberg equilibrium in any of the breeds after Bonferroni correction. Variation of allele frequency among breeds shows amoderate level of genetic differentiation (F_{ST} = 12.3%). Some breedsare clearly outliers (e.g., Chianina and Blond d'Aquitaine), but themajority of the breeds are indistinguishable from one another on thebasis of allele frequencies. The exact test of population differentiation reveals significant heterogeneity of four breeds only, when compared to some of the others. In sum, there is no evidence ofany association between a given allele and skin pigmentation.

Steady-state of *bPAR-2*mRNA: Skin expression of *bPAR-2* was first examined by RT-PCR using bovine skin RNA prepared from 5 bovine breeds (Figure 1).We found that *bPAR-2* was expressed at similar levels, as visualized by the band intensities, in all skin samples regardless of their pigmentation (Figure 3). To get more insight into transcript levels of *bPAR-2* gene according to the skin pigmentation of the selected bovine breeds, we used Real-time PCR amplifications. Although the results of Real Time PCR quantitation (Figure 4) showed a 2 to 3 fold higher gene expression than the control Prim' Holstein dark-black skin area, but the increase was not statistically significant (Figure 4). Furthermore, it is not correlated with skin pigmentation. Taken together, these results show that skin pigmentation in cattle breeds isnot correlated with *bPAR-2* mRNA levels as previously reported for skin color variations in humans (Babiarz-Magee *et al.*, 2004).

Steady-state of bPAR-2 protein in Prim' Holstein dark-black skin area: Since human black skin correlates with high *PAR-2* expression at both mRNA and protein levels, we also investigated the steady-state of bPAR-2 protein in dark-black skin area of the Prim' Holstein breed. Western blot analysis using anti-PAR-2monoclonal antibody reveals (Figure5A) a band of predicted molecular weight (43 kDa) that can be detected from 5µg of total protein extract. A second band of 55 kDa was consistently obtained from loadings of more than 5µg of skin protein extracts which may possibly represent an N-glycosylation form of bPAR-2. Therefore, we incubated the skin extracts with N-glycosidase PNGase F to remove such complex sugars. Results obtained are shown in the Figure 5B.Both treated and untreated samples show the same electrophoresis profile suggesting that the 55 kDa band is not an N-glycoslated form of bPAR-2. As*PAR-2* is widely expressed in mouse and humans (Nystedt *et al*., 1994, 1995), we wondered if the two bands could also be detected in other bovine tissues (liver, kidney, lung and heart). Interestingly, a unique band with the predicted molecular weight (43 kDa) was detected at 25 µg of protein extracts of kidneys and liver (Figure 5C). Our observation, that these two organs exhibit high levels expression of bPAR-2 is in agreement with the previous studies as assessed by Northern-blot analysis of mRNA from many tissues in human (Nystedt *et al.*, 1995). Thus, the 55 kDa protein exhibiting a skin specific expression is not an N-glycosylated form of the bPAR-2 receptor.

Taken together, these results showed that the used monoclonal antibody recognizedabovine bPAR-2 core protein both qualitatively and quantitatively (Figure

5) at the expected molecular weight size of 43 kDa in the skin and in others bovine tissues.

bPAR-2 protein levels correlate with skin pigmentation in cattle: To evaluate the steady-state of the bPAR-2 protein according to the skin pigmentation, we performed the western blots on skin (Figure 6) samples. Typical results obtained for three individuals of each breed are shown in Figure 6. A band of 55 kDa was detected in all cases, regardless the pigmentation of the sample, although it was less abundant in Limousine and Partheaise breeds. Interestingly, the highest expression of bPAR-2 (43 kDa) was observed in the dark-black of Prim' Holstein' skin. In the skin of other breeds, bPAR-2 decreased significantly (Figure 6), in a skin pigmentation-dependent manner from Parthenaise (light-black) to Limousine (red-light skin) and remained undetectable in the skin of Charolais (white skin). These results show that bovine bPAR-2 is expressed in skin pigmentation-dependent manner in cattle. Taken together, these results suggest a post-transcriptional regulation of *bPAR-2* according to the skin pigmentation in cattle.

Fig. 1. Cattle Breeds' coat colours (A) and pigmentation of skin and hair samples subjected to this study (B). Prim' Holstein (dark-black skin and hair with white spotting); Parthenaise (light-black skin and red hair); Salers (reddish-brown skin and hair); Limousine (golden-red skin and hair); Charolaise (white skin and hair)

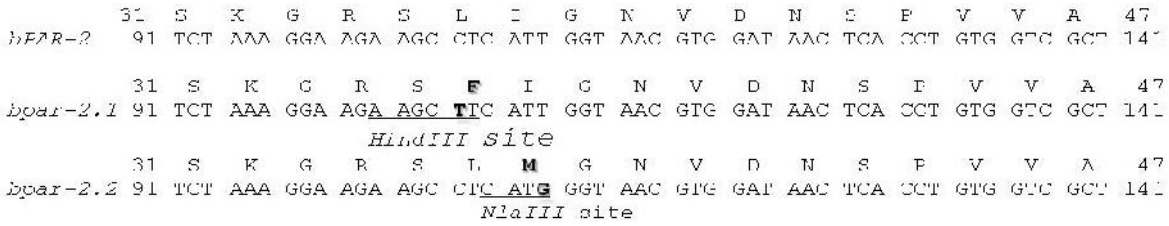

Fig.2. The three mutations identified (C106T and T111G) and, *Hind*III and *Nla*III restriction sites associated with *bpar2.1* and *bpar2.2* alleles are presented. The tethered ligand (SLIGNV) is underlined

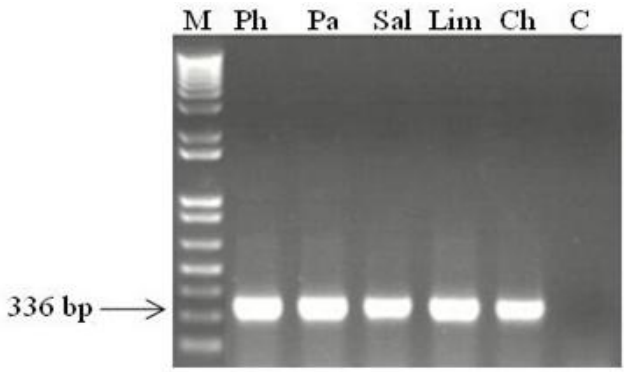

Fig.3. *bPAR-2* expression evidenced by RT-PCR. Ph: Prim'Holstein; Pa: Parthenaise; Sal: Salers; Lim: Limousine; Ch: Charolaise; M: molecular weight (100 bp DNA ladder); C: control (PCR essay without cDNA template)

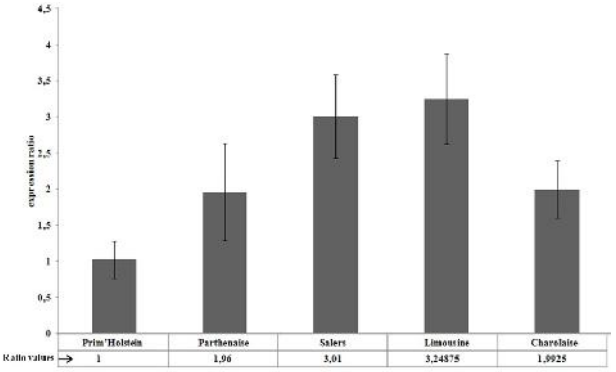

Fig.4. *bPAR-2* mRNA levels determined by Real-time PCR amplifications in five breeds exhibiting various skin pigmentation. The dark-black skin area of Prim'Holstein was used as a reference. Standard deviation bars represent variation between three animals tested of each breed.

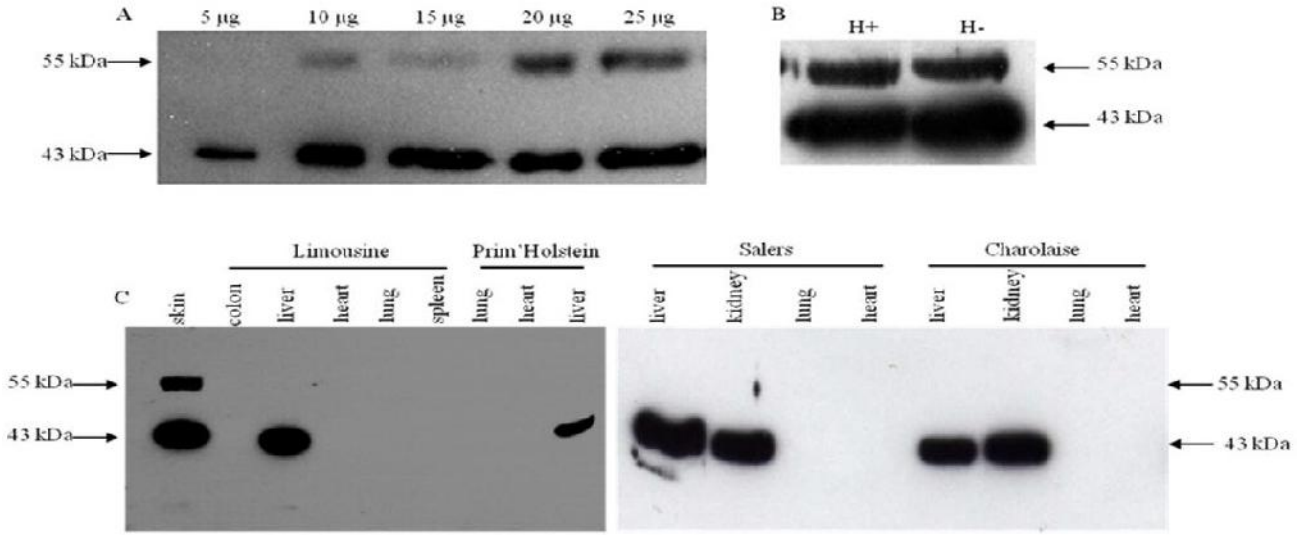

Fig.5. Western blot analysis of bPAR-2 expression. Different amount of proteins from Prim'Holstein black-skin area, were tested A); 30 µg of the sample were tested with (H+) or without (H-) PNGase F treatment B); bPAR-2 expression in various tissues (25 µg of proteins) of four cattle breeds C). Skin samples (in C) used as control was from Prim'holstein dark-black skin area.

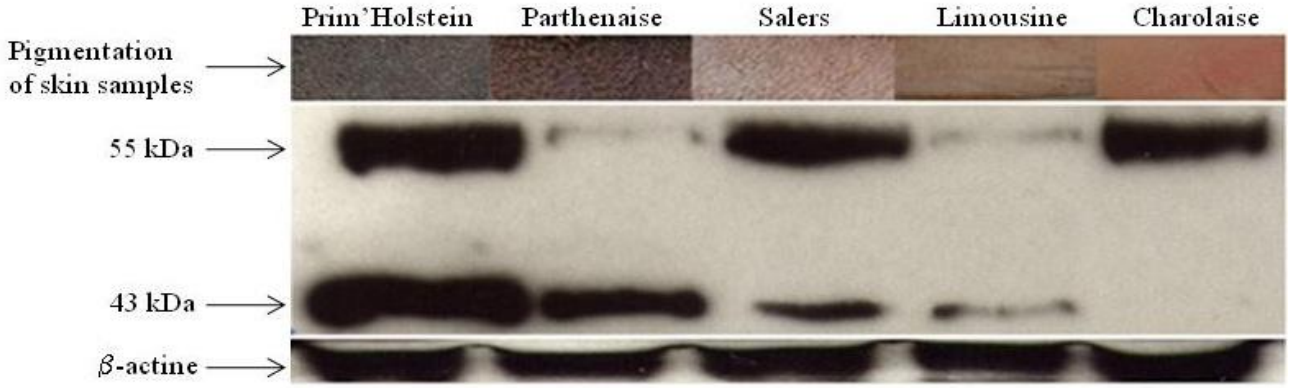

Fig.6. Western blot studies of bPAR-2 expression in cattle breeds with different skin pigmentation from dark-black skin (Prim'Holstein) to creamy white skin of Charolaise. -actin is shown as a loading control

Supplementary material
Supplementary table legend

Table S1.Genotype and allelic frequencies of *bPAR-2* alleles in 14 European Cattle Breeds.

Supplementary figure caption
Figure S1. Nucleotides and deduced amino acid sequences of *bPAR-2*. The solid lines indicate the seven predicted transmembrane domains (TMD) in the amino acid sequence. The arrow points to the proposed protease cleavage site. The tethered ligand (SLIGNV) and potential N-glycosylation sites N-X-S/T (where X is any amino acid except proline) are underlined.

Supplementary material - the following online material is available for this article:

- Table S1 – <Nucleotides and deduced amino acid sequences of *bPAR-2*>
- Figure S1 – <Genotype and allelic frequencies of *bPAR-2* alleles in 14 European Cattle Breeds.>

This material is available as part of the online article from http://www.scielo.br/gmb".
http://www.ncbi.nlm.nih.gov/pubmed/19064814

Fig. S1. Nucleotide and deduced amino acid sequences of *bPAR-2*. The solid lines indicate the seven predicted transmembrane domains (TMD) in the amino acid sequence. The arrow points to the proposed protease cleavage site. The tethered ligand (SLIGNV) and potential N-glycosylation sites N-X-S/T (where X is any amino acid except proline) are underlined.

M R S P S A A W L L G G V L LL A A 18
ATGCGAAGCCCGAGCGCGGCGTGGCTG CTC GGGGGCGTC CTC CTGCTGGCGGCC 54

▼ Cleavage site

S G S C **N[23] R T** V P G **N[29] K S** K G R **S L** 36
TCT GGC TCC TGC AAC CGC ACC GTC CCA GGA AAC AAG TCT AAA GGA AGA **AGC CTC** 108

I G N V D N S P V V A G R G V T V K 54
ATT GGT AAC GTG GAT AAC TCA CCT GTG GTC GCT GGC AGA GGC GTG ACA GTG AAA 162

P G F S V D E F S T S V L T G K L T 72
CCA GGC TTT TCC GTG GAT GAG TTT TCT ACC TCC GTC CTC ACT GGA AAG CTA ACT 216

T V F L P **V V Y T I V F V V G L P S** 90
ACTGTCTTTCTTCCAGTTGTC TAC ACGATCGTA TTC GTGGTTGGTTTGCCA AGC 270

TMD I

N G M A L W V F L F R T K K K H P **A** 108
AAT GGC ATG GCC CTG TGG GTC TTT CTT TTC CGA ACA AAG AAG AAG CAC CCC GCT 324

V I Y M A N L A L A D L L S V T WF 126
GTG ATT TAC ATG GCC AAC CTG GCC TTG GCC GAC CTC CTG TCG GTT ACG TGG TTC 378

TMD II

P L K I A Y H I H G N N W I Y G E S 144
CCT TTG AAG ATC GCT TAT CAC ATC CAT GGC AAC AAC TGG ATT TAC GGG GAG TCT 432

L C K **V L I G F F Y G N M Y C S I L** 162
CTTTGCAAGGTG CTC ATT GGCTTTTTCTACGGCAACATGTACTGCTCCATC CTC 486

TMD III

F M T C L S V Q R Y W V I V N P M V 180
TTC ATG ACC TGC CTT AGC GTG CAG AGA TAC TGG GTC ATC GTG AAC CCC ATG GTG 540

H P K K Q A N I **A I G V S L G I W L** 198
CAC CCC AAG AAG CAG GCA AAC ATT GCC ATC GGC GTC TCT CTG GGA ATA TGG CTG 594

TMD IV

L I L L L T I P L Y V V K Q T S Y I 216
CTC ATT CTG CTG CTT ACC ATC CCC TTG TAC GTC GTG AAG CAG ACC TCC TAC ATC 648

R A L **N[220] I T** T C H D V L P E E V L V 234
CGG GCC CTT AAC ATC ACG ACC TGT CAC GAC GTT TTG CCG GAG GAG GTG CTG GTG 702

G D M F N Y F L S **L A I G V F L F P** 252
GGA GAC ATG TTC AAT TAC TTC CTG TCT CTG GCC ATC GGA GTC TTT CTA TTC CCA 756

TMD V

A F L T A S A Y V L M I R T L Q S S 270
GCC TTC CTC ACG GCC TCG GCC TAT GTG CTC ATG ATC CGG ACC CTC CAG TCT TCT 810

A M D E S S G K K R R R A I K **L I V** 288
GCC ATG GAT GAG AGC TCA GGA AAG AAG AGG CGG AGG GCC ATC AAG CTC ATT GTC 864

T V L A M Y L I C F T P S N L L L V 306
ACC GTC CTG GCC ATG TAC CTG ATC TGC TTC ACT CCC AGT AAC CTT CTG CTC GTG 918

TMD VI

V H Y F L I K T R G Q S H V Y A L **Y** 324
GTG CAC TAC TTC CTG ATT AAG ACC CGG GGC CAG AGT CAC GTC TAC GCC CTG TAC 972
I V A L C L S T L N S C I D P F V Y 342
ATCGTGGCC CTC TGC CTC TCCACC CTC AACAGCTGCATCGAC CCC TTTGTC TAT 1026

TMD VII

Y F I S Q D F R D H A K N A L L C R 360
TAC TTC ATT TCA CAG GAC TTC AGG GAT CAC GCC AAG AAC GCC CTT CTC TGC CGG 1080
S V R T V K R M Q V S L S S K K F S 378
AGC GTC CGT ACT GTA AAG CGG ATG CAG GTA TCC CTC TCG TCA AAG AAA TTC TCG 1134
G K S S S Y S S S S T S V K G S Y * 395
GGG AAA TCC AGC TCT TAC TCT TCA AGT TCA ACC AGT GTC AAA GGC TCC TAC TGA 1188

Table S1. Genotype and allelic frequencies of *bPAR-2* alleles in 14 European Cattle Breeds.

Breed	Genotype Counts							Allele frequencies			H_{exp}
	bPAR-2/ bPAR-2	***bPAR-2/ bpar-2.1***	***bPAR-2/ bpar-2.2***	***bpar-2.1/ bpar-2.1***	***bpar-2.1/ bpar-2.2***	***bpar-2.2/ bpar-2.2***	**Total**	***bPAR-2***	***Bpar 2.1***	***Bpar 2.2***	
Belgian Blanc Bleu	-	11	1	10	-	-	22	0.27	0.70	0.02	0.44
Blonde d'Aquitaine	7	8	2	3	-	-	20	0.60	0.35	0.05	0.53
Chianina	1	3	4	1	12	1	22	0.20	0.39	0.41	0.66
Italian Brown	1	1	-	19	1	-	22	0.07	0.91	0.02	0.17
Italian Friesian	1	10	-	11	-	-	22	0.27	0.73	0.00	0.41
Limousine	1	9	1	8	1	-	20	0.30	0.65	0.05	0.50
MaineAnjou	-	6	-	14	-	-	20	0.15	0.85	0.00	0.26
Maremmana	-	2	-	20	-	-	22	0.05	0.95	0.00	0.09
Marchigiana	2	8	-	9	3	-	22	0.27	0.66	0.07	0.50
Parthenaise	-	5	-	10	-	-	15	0.17	0.83	0.00	0.29
Piemontese	1	10	1	10	-	-	22	0.30	0.68	0.02	0.46
Pezzata Rossa Italiana	1	7	-	14	-	-	22	0.20	0.80	0.00	0.33
Romagnola	6	9	-	7	-	-	22	0.48	0.52	0.00	0.51
Salers	3	5	3	9	-	-	20	0.35	0.58	0.08	0.56
Total	24	94	12	145	17	1	293	0.26	0.68	0.05	0.41

DISCUSSION

In this study, we investigated the pigmentary role of *protease-activated receptor-2* in cattle skins with various degrees of pheomelanic and/or eumelanic pigmentation. We first focused on study ofpolymorphism of the bovine *protease-activated receptor-2* (*bPAR-2)* coding region and then investigated the *bPAR-2* gene expression at both mRNA and protein levels. We report for the first time the identification of twonatural variants at the tethered ligand and a post-transcriptional regulation of *bPAR-2*.

The polymorphic variations described here affect the *bPAR-2* tethered ligand (^{35}SLIGNV^{40}) at amino acid 36 and 37 (position 2 and 3 of the tethered ligand, respectively), where Leu and Ile (wild type *bPAR-2* allele), Phe (*bpar-2.1* allele) and Met (*bpar-2.2* allele) can be found. We confirmed that the ^{36}Leu to ^{36}Ph (*bpar-2.1* allele) and ^{37}Ile to ^{37}Met (*bpar-2.2* allele) mutations were true by direct DNA sequencing and restriction fragment length analysis. Then, we genotyped the three alleles in 293 individualsfrom 14 European cattle breeds. Surprisingly, there was no evidence of association of thispolymorphism with skin pigmentation. The majority of the breeds, including some with black or white skin, segregated all three alleles. In addition, the overall level of genetic differentiation was similarto that generally observed in phenotypically neutral loci. The maximumfrequency of the *bPAR-2* allele was found in the Blonde d'Aquitaine, with white skin, whereas the maximum frequencies of *bpar-2.1* and*bpar-2.2* alleles were found in Chianina and Maremmana, both with blackskin.

Protease-activated receptors belong to G-protein-coupled receptors family. Among the four receptors i.ePAR-1 to PAR-4 (Macfarlane *et al.*, 2001), only PAR-2 is shown to be involved in skin pigmentation. Otherwise, no other natural variant affecting the tethered ligand was reported for any of these PARs family members. Thus, in the absence of natural variants, considerable work has been done to explore the structure-activity relationships for the synthetic PAR-activated peptides based on the distinct tethered ligand sequences. Interestingly, Maryanoff *et al.* (2001) reported

that the substitution of Leu at position 2 (SL----) by Phedidnot affect PAR-2 activation and they showed that at position 3 (SLI---) Ile was optimal, although various other amino acids were tolerated. Furthermore, the PAR-1 tethered peptide (SFLLRN) with F amino acid at position 2 activates PAR-2 instead of its natural SLIGNV tethered ligand (Hollenberg *et al.*, 1997). Thus, it is likely that the *bpar-2.1* allele, which encodes the same amino acid (F) at position 2, does not affect bPAR-2 activation.

Since *bpar-2.1* and *bpar-2.2* segregate in all cattle breeds with variable eumelanic and pheomelanic skin pigmentation, we suggest that *bpar-2.1* and *bpar-2.2* alleles do not affect the bPAR-2 receptor function and thus they are not causative mutations for differentskin pigmentation in cattle.Furthermore, we showed that the expression of *bPAR-2* mRNA,as assessed both by RT-PCR (figure 3) and Real-time PCR amplifications (Figure 4),does not differ significantly between skins of cattle breeds with variable skin pigmentation.

To get more insight about the potential role of *bPAR-2* in cattle skin pigmentation, we extended our studies to look for differential *bPAR-2* expression at the protein level by western blotting. Of considerable interest was the finding that the *bPAR-2*expression is correlated with skin pigmentation at the protein level in pigmentationskin-dependent manner (Figure 6). Indeed, whatever the skin pigmentation, we did not observe significant differences of bPAR-2 expressionamong others tissues expressingbPAR-2(as assessed by western blotting, Figure 5C).

In human skin, *PAR-2* expression level correlated with the skin colour phenotype (Babiarz-Magee *et al.*, 2004), modulatedpigmentation in response to ultraviolet light (Scott *et al.*, 2001) and was down-regulated in both the depigmented skin of vitiligo (Morett*et al.*, 2009) and the hypopigmented palmoplantar epidermis (Yamaguchi *et al.*, 2008); in all cases, *PAR-2* expression seems to be regulated at the transcriptional level. Interestingly, a possible post-transcriptional regulation was also suggested for human *PAR-2* to explain the differences observed between *PAR-2* mRNA (as demonstrated by RT-PCR) and protein (as demonstrated by immunostaining) in pigmented basal cell epithelioma (Sakuraba*et al.*, 2004). Whatever the type of *PAR-2* regulation, combining current knowledge in human skin pigmentationwith our findings in cattle (present work) strongly suggests that variation of*PAR-2* expression in the skin is closely related to the steady state of melanogenesis. Thus, we propose that high and lowexpression of *PAR-2*is the hallmark characteristic of high and low pigmented skins respectively. At this stage of the study we have no further evidence that could explain the unexpected post-transcriptional regulation of *bPAR-2* in pigmentation-skin dependent manner in cattle.

Conclusions: In sum, the main result of this study was the discovery ofnatural variants of tethered ligand of *PAR-2* and more interestingly its post-transcriptional regulation in a skin-pigmentation dependent manner. Considering the present study and previously reported data in humans, it seems clear that the *protease-activated receptor-2* (*PAR-2*) plays a pivotal role in regulating skin pigmentation. However, further studies are needed to further elucidate this process and its physiological regulation.

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