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Genetic variation of the porcine *NR5A1* is associated with meat color

Andreas Görres[1] · **Siriluck Ponsuksili**[1] · **Klaus Wimmers**[1] · **Eduard Muráni**[1]

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Abstract Because of the central role of Steroidogenic factor 1 in the regulation of the development and function of steroidogenic tissues, including the adrenal gland, we chose the encoding gene *NR5A1* as a candidate for stress response, meat quality and carcass composition in the domestic pig. To identify polymorphisms of the porcine *NR5A1* we comparatively sequenced the coding, untranslated and regulatory regions in four commercial pig lines. Single nucleotide polymorphisms could be found in the 3′ UTR and in an intronic enhancer, whereas no polymorphisms were detected in the proximal promoter and coding region. A subset of the detected polymorphisms was genotyped in Piétrain x (German Large White x German Landrace) and German Landrace pigs. For the same animals, carcass composition traits, meat quality characteristics and parameters of adrenal function were recorded. Associations with meat color were found for two of the discovered SNPs in Piétrain x (German Large White x German Landrace) and German Landrace pigs but no connections to parameters of adrenal function could be established. We conclude that *NR5A1* variations influence meat color in a hypothalamus-pituitary-adrenal axis independent manner and that further regulatory regions need to be analyzed for genetic variations to understand the discovered effects.

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✉ Eduard Muráni
murani@fbn-dummerstorf.de

[1] Leibniz Institute for Farm Animal Biology (FBN), Institute for Genome Biology, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

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Introduction

Stress hormones, including catecholamins and glucocorticoids whose release is regulated by the sympathoadrenal system and the hypothalamus-pituitary-adrenal axis (HPA axis) respectively, help the farm animals to cope with the various stressors they are exposed to. Glucocorticoid hormones (for example in the pig cortisol) mobilize energy for the stress response by increasing gluconeogenesis, proteolysis, and lipolysis (Djurhuus et al. 2002; Simmons et al. 1984; Khani and Tayek 2001) and accordingly influence economically relevant traits, for example meat quality, carcass composition and growth (Yoshioka et al. 2005; Foury et al. 2005; Hambrecht et al. 2005; Hyun et al. 1998). Genetic background has been demonstrated to be a major cause for variability in HPA axis functionality (Désautés et al. 2002; Kadarmideen and Janss 2007). Genetic selection that has led to major improvements in phenotypic traits like lean growth has been shown to be accompanied by a reduction of HPA activity (Weiler et al. 1998; Foury et al. 2009). However, recently concerns were raised that reduced cortisol secretion may impair robustness (e.g., disease resistance, piglet survival) of livestock, and it was suggested that breeding for increased HPA axis function could be accomplished without reducing desirable traits of animals (Mormède et al. 2011). Therefore, better knowledge about genetic variability of HPA axis key regulators is necessary for balancing robustness and productivity traits by genetic selection.

The steroidogenic factor 1 (SF1), encoded by the *NR5A1* (nuclear receptor subfamily 5, group A, member 1) gene, is a constitutively active orphan nuclear receptor which plays a central role in the regulation of endocrine function within both the

hypothalamus-pituitary-gonadal axis and the hypothalamus-pituitary-adrenal axis. SF1 was first discovered due to its binding to the promoter region of P450 steroid hydroxylase genes (Rice et al. 1991; Lala et al. 1992), making it a candidate as a shared regulator of the coordinate expression of genes responsible for the synthesis of glucocorticoids, mineralocorticoids, and sex steroids. With the enzyme 3β-HSD and the transport protein StAR, additional elements of steroid synthesis have been reported to be under the transcriptional regulation of SF1 (Leers-Sucheta et al. 1997; Sugawara et al. 1997; Caron et al. 1997). The expression pattern of *NR5A1* in adult tissues generally correlates with its role in steroid biosynthesis: its transcript has been detected in all zones of adrenal cortex, leydig cells in testes and granulosa cells in ovaries, preceding aromatase expression (Ikeda et al. 1993, 1994). In addition to this, expression could be found in the sertoli cells in testes, theca cells of ovaries, gonadotropes of pituitary, and ventromedial region of the hypothalamus (VMH) (Ingraham et al. 1994; Shinoda et al. 1995).

Knock-out experiments in mice demonstrate a major role of *NR5A1* for the organogenesis of gonads and adrenal glands (Luo et al. 1994; Sadovsky et al. 1995). Complete loss of *NR5A1* function leads to death in the immediate perinatal period from adrenocortical insufficiency. All affected mice show male-to-female sex reversal, agenesis of adrenal glands and gonads. Furthermore, knock-out mice lack the VMH and show defects in splenic parenchyma. Heterozygous knock-out mice are viable and show a reduced phenotype, including reduced size of adrenal glands and an impairment of stress induced corticosterone secretion, thereby demonstrating dosage dependency of SF1 function. Several mutations of human *NR5A1* with a wide variety of phenotypes have been reported. Those include cases of male-to-female sex reversal, adrenal insufficiency, ovarian defects, and impaired development of sex organs, as cryptorchidism in the male and ovarian insufficiency in the female (reviewed by Ferraz-de-Souza et al. 2011).

Genetic polymorphisms of the porcine *NR5A1* have not been characterized yet. However, reported cases of polymorphisms of the human *NR5A1* suggest that even heterozygous or mild mutations might cause significant changes in phenotypic traits. In this study, we investigated *NR5A1* as a candidate gene modulating HPA axis activity and consequently economically relevant traits. Genetic variations of *NR5A1* were identified by re-sequencing the corresponding cDNA and regulatory regions and tested for associations with phenotypic variation in both parameters of HPA axis activity and traits of meat quality and carcass composition.

Materials and methods

Animals

For association studies, two breeds were analyzed: German Landrace (DL; 43 females and 159 barrows) and a Piétrain x (German Large White x German Landrace) crossbreed (PiF1; 223 females and 257 barrows). The animals were slaughtered at the experimental slaughter facility of the FBN Dummerstorf after reaching a bodyweight of around 115 kg (corresponding to an mean age of 176 days for PiF1 and 167 days for DL, respectively).

Phenotypic data were collected according to the German performance test directives (ZDS 2007). Conductivity, pH value and color of *M. longissimus dorsi* were measured between the 13th and 14th rib using Star-series equipment (R. Matthaeus, Klausa, Germany). Measurement of conductivity as well as pH value was performed 45 min *post mortem* and repeated after 24 h. Impedance was measured 24 h *post mortem* with a Meat Check device (Sigma Electronics, Erfurt, Germany). A Minolta CR 300 chromameter (Minolta, Ahrensburg, Germany) was used for measurement of the CIE-Lab color values L*, a* and b*. Drip loss of *M. longissimus dorsi* was calculated using the bag-method as described elsewhere (Honikel 1998). For determination of carcass lean content, a Fat-O-Meater device was used (SFK Technology, Werne, Germany). In addition to the already mentioned traits, loin eye area, fat area, average back fat thickness, and carcass length were recorded.

For DNA isolation liver samples were collected. In addition to the 202 DL and 480 PiF1 pigs used for association studies, liver samples from DL (n=35), purebred German Large White (DE; n=24), Piétrain (Pi; n=26) and Duroc (Du; n=21) pigs were collected for allele frequency estimation. A subgroup of the liver samples used for association studies and allele frequency estimation was used for re-sequencing of *NR5A1* (two animals of each DL, DE, Pi, and Du; eight animals of PiF1).

For morphometric analyses and RNA extraction left adrenal glands were quickly dissected. After weighting, the middle portion was excised and frozen in isopentane. The remaining tissue was frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

A sample of 50 ml trunk blood was collected from each animal during exsanguination and mixed with 1 ml 0.5 M EDTA. Plasma was prepared and stored at −80 °C until further use. Total plasma cortisol level was measured in duplicate using enzyme-linked immunosorbent assay (DRG, Marburg, Germany) according to manufacturer's recommendations.

Animal care and tissue collection processes followed the guidelines of the German Law of Animal Protection, and the experimental protocol was approved by the Animal Care Committee of the Leibniz Institute for Farm Animal Biology (FBN, Dummerstorf, Germany).

DNA isolation, RNA isolation and cDNA synthesis

Genomic DNA (202 DL and 480 PiF1 animals) was isolated using phenol-chloroform extraction. DNA quantity and purity

was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop, Peqlab, Germany).

RNA from adrenal gland tissue was isolated from six PiF1 animals with extreme values of adrenal gland function and eight animals of the breeds DL, DE, Pi, and Du (two animals of each breed) using TRI Reagent (Sigma, Taufkirchen, Germany). Samples were DNaseI (Roche, Mannheim, Germany) treated and RNA was cleaned using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany). Samples were tested on quantity and purity using a spectrophotometer. Integrity of RNA was tested with gel electrophoresis on a 1 % denaturing agarose gel.

For synthesizing first-strand cDNA, 1.5 μg RNA were reverse transcribed in a reaction mixture containing 500 ng random hexamers (Promega, Mannheim, Germany) and 500 ng oligo(dt)11VN primer. SuperScript III MMLV reverse transcriptase was used according to manufacturer's recommendations (Invitrogen, Karlsruhe, Germany).

Adrenal gland morphometry

For morphometric analysis adrenal glands of DL pigs (n=202) were used; 12 μm thick cross-sections were prepared with a cryostate (2800 Frigocut, Reichert-Jung) at −20 °C and mounted on microscopic slides (Polysine Slides, Thermo Scientific, Menzel-Gläser, Gerhard Menzel GmbH, Braunschweig, Germany). Until staining, the specimens were kept at −20 °C. After their fixation in 4 % paraformaldehyd, the sections were stained with Mayer's Hemalum solution (AppliChem GmbH, Darmstadt, Germany), counterstained with 0.1 % Eosin B, and dehydrated in an ascending ethanol series. Photographic images of two representative sections of each gland were taken with a photo camera (VisiCam 3.0, VWR) connected to a binocular. Area measurements of the complete adrenal cross-section and of the medullary part were performed using the VisiCam software (VisiCam Image Analyzer 6.2.3.1, VWR International, LLC).

In silico prediction of regulatory elements

Conservation of published transcription factor binding sites was tested using ClustalW2 (http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=clustalw2). For the comparative sequence analysis and prediction of potential transcription factor binding sites conserved in pig, human and mouse, Mulan and multiTF online tools were used (http://mulan.dcode.org/). Potential binding sites for vertebrate miRNA were detected using microinspector v1.5 (http://bioinfo.uni-plovdiv.bg/microinspector/).

Detection of polymorphisms

Complementary DNA sequence of the porcine *NR5A1* was obtained by assembling porcine expressed sequence tags retrieved from public databases using the NCBI nucleotide blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the human *NR5A1* (Accession Number NM_174403). The resulting cDNA sequence was compared with genomic sequence established by Mikawa et al. (Accession Number AP009124).

Primers for amplification and re-sequencing of eight ~750 bp fragments covering the coding sequence, the untranslated region, potential intronic fetal adrenal enhancer (FAdE), and 500 bp of the promoter region were designed (Table 1). The fragments were amplified in a standard PCR reaction using genomic DNA or cDNA as template. PCR was performed with 1 x PCR buffer containing 1.5 mM $MgCl_2$, 0.2 μM of each primer, 50 μM of each dNTP, and 0.5 U Supra Therm Taq Polymerase (GeneCraft, Münster, Germany). Cycling conditions consisted of initial denaturation at 95 °C for 3 min, followed by 40 cycles with 15 s denaturation at 95 °C, 1 min annealing at the primer specific Ta and 1 min extension at 72 °C; and a final primer extension at 72 °C for 5 min. PCR products were purified and sequenced using Big Dye Terminator Cycle sequencing kit V3.1 (Applied Biosystems, Darmstadt, Germany) on an ABI 3130 Genetic Analyzer.

Three of the identified SNPs were genotyped in the 682 test subjects of the association study (202 DL and 480 PiF1) and the 106 animals used for allele frequency estimation described

Table 1 Primer used for re-sequencing of the porcine *NR5A1*

Primer	Region	Template	Primer sequence (5′–3′)
F1	5′ UTR/CDS	cDNA	CCACCATCCAGCGTGAGAG
R1	5′ UTR/CDS	cDNA	GGATAGAGGTAGCCAGCCAG TG
F2	CDS	cDNA	TGGCCGCTGGTCCACCTAC
R2	CDS	cDNA	GCACCAGGCCATGCAGCAG
F3	CDS/3′ UTR	cDNA	TGCTGGAGTGAGCTGCTAGTG
R3	CDS/3′ UTR	cDNA	TCACCCACCTTCCCAAACAC
F4	3′ UTR	gDNA	GAGATGCCCCGAAACAACC
R4	3′ UTR	gDNA	TCCTCTAGGCTCCACCAGTCC
F5	3′ UTR	gDNA	GGAGGGCTTCCAGAATTTCC
R5	3′ UTR	gDNA	CCGTATTCCCACCCACAGAG
F6	3′ UTR	gDNA	TTGGTGCTGGGTCGTTATTTC
R6	3′ UTR	gDNA	ACCCATTCAGGAGGTTCTGC
F7	Intron	gDNA	TACCTGCGTCCATGCTCCTC
R7	Intron	gDNA	CCAGTCTGCCACAAATCTTCC
F8	Intron	gDNA	GGAAGATTTGTGGCAGACTGG
R8	Intron	gDNA	CAGCCCCTCTCCACCATAAAG
F9	5′ flanking	gDNA	CACACGGGAGGCATTCATTC
R9	5′ flanking	gDNA	GAACTCCTTCACGCCACAGC

in more detail in section Animals. SNP c.*748 G>T and c.*1296 A>G were genotyped using the RFLP method. To amplify polymorphic DNA regions PCR was performed as described above. Primers are shown in Table 2. Ten μl of amplification product were digested overnight with 5 U of *Mbo*II to genotype SNP c.*748 G>T or 2.5 U of *Hin*6I to genotype SNP c.*1296 A>G according to manufacturer's recommendations (Fermentas, St. Leon-Rot, Germany). Digestion products were evaluated on 2 % ethidium bromide-stained agarose gels.

SNP c.871-3864G>C was genotyped using SSCP analysis. The polymorphic region was amplified as described above with primers shown in Table 2. Amplification product was loaded onto a 12 % nondenaturing polyacrylamid gel (acrylamid to bisacrylamid ratio was 49:1) and separated at 400 V for 5 h. DNA signals were visualized by silver staining.

Statistical analysis

For each analyzed SNP, allele frequency was calculated and genotype distribution was tested for Hardy-Weinberg equilibrium in the breeds DL, DE, Du, and Pi using a web application (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

Associations between genotypic and phenotypic variation in the two test populations were analyzed using a mixed model (PROC Mixed, SAS v. 9.2; SAS Inc., Cary, USA). Fixed effects included genotype, sex, and *RYR1* genotype (for meat quality and carcass composition of PiF1 pigs). Sire and slaughter date (for cortisol level and meat quality) were considered as random effects. Slaughter order (for cortisol level) and body weight (for adrenal weight, histological traits, and carcass composition) were applied as covariates. *T*-test was used to compare least square mean values of the genotypes. *P*-values were adjusted using the Tukey-Kramer correction.

To estimate allele substitution effects, fixed effect of genotype was replaced by a variable indicating the number of derived alleles (0,1,2) of the tested SNP in the models described above.

Results

Re-sequencing of cDNA from eight pigs belonging to different breeds (DL, DE, Pi, Du) and from six pigs (PiF1) with extreme values of adrenal gland function confirmed that *NR5A1* transcript expressed in porcine adrenal glands consists of seven exons homologous to the seven exons of human SF1-encoding transcript. Alignment of the cDNA sequences obtained by re-sequencing along with the sequence obtained by EST-assembly and the genomic sequence provided by Mikawa et al. 2007 (AP009124) did not result in the detection of genetic polymorphisms in the coding sequence. Subsequent analysis of the promoter region, the 5′ UTR and the 3′ UTR based on genomic DNA lead to the detection of eight single nucleotide polymorphisms (SNP) in the 3′ UTR (Fig. 1). Comparative sequencing of porcine FAdE region (299131141 to 299131788, Sscrofa 10.2), homologous to an intronic regulator that has been shown to activate murine *NR5A1* in fetal adrenal glands (Zubair et al. 2006), allowed the detection of five single nucleotide polymorphisms. Information about all detected SNPs, including their position on chromosome 1 and dbSNP identifier, is summarized in Online resource 1.

In silico analysis of the 3′ UTR sequence shows that five of the SNPs lie within potential miRNA binding sites (Fig. 1). The SNPs c.*748 G>T, c.*858 G>A, c.*1004 C>T, c.*1015 G>A, c.*1030 T>C, and c.*1249 C>T were in linkage disequilibrium in the 14 pigs used for re-sequencing. Since the low allele frequency of c.*219 C>T would reduce statistical power of subsequent association studies, further investigations were restricted to the SNPs c.*748 G>T and c.*1296 A>G. Allele frequencies for those SNP are shown in Table 3. Genotypes of both SNP were in Hardy-Weinberg equilibrium. Since the polymorphism at locus c.*748 G>T does not segregate in the DL, it was genotyped only in the PiF1 population, whereas c.*1296 A>G was genotyped in the PiF1 population as well as in the DL population.

None of the identified FAdE SNPs affected the regulatory motives previously established by Zubair et al. (2006) in murine and human FAdE (Fig. 1). Three of the SNP — c.871-3759 C>T, c.871-3594 A>G and c.871-3589 C>T — seem to segregate in the analyzed breed with low frequency. Our analysis suggests that c.871-4032 G>T is in incomplete linkage disequilibrium with c.871-3864 G>C. To avoid genotyping of SNP with overlapping information content, only the SNP at locus c.871-3864 G>C was selected for further analysis. Allele frequency in four analyzed breeds is shown in

Table 2 Primer used for genotyping polymorphisms of the porcine *NR5A1*

Primer	SNP	Region	Template	Primer sequence (5′–3′)
SF1	c.*748 G>T	3′ UTR	gDNA	CCCTCCCTCAACCTTCTACCC
SR1	c.*748 G>T	3′ UTR	gDNA	CCCCTGCCCGTCCACATAC
SF2	c.*1296 A>G	3′ UTR	gDNA	GTGGGTGGGAATACGGTGAC
SR2	c.*1296 A>G	3′ UTR	gDNA	GGAGGAGTAGGGACTGAGATGG
SF3	c.871-3864 G>C	Intron	gDNA	GGCAGCCTCTCTGGACAG
SR3	c.871-3864 G>C	Intron	gDNA	GCCCAGGACTGACATGGT

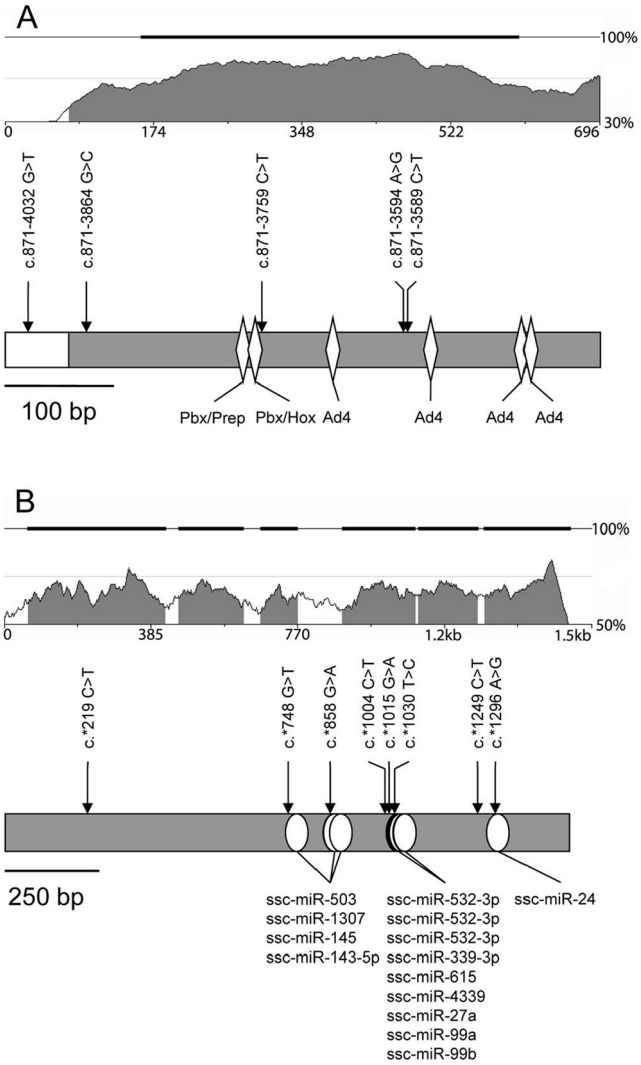

Fig. 1 Position of porcine FAdE (**a**) and 3′ UTR (**b**) SNPs in relation to predicted regulatory motives and evolutionarily conserved sequence. Plots of evolutionary conservation were created by aligning the sequences of pig, human, and cow with Mulan online software. Transcription factor binding motives of FAdE were taken from Zubair et al. (2006). miRNA binding elements were predicted using the microinspector tool searching for vertebrate miRNA. Only miRNA binding sites interfering with identified SNPs are shown

Table 3. Genotype proportions did not significantly deviate from Hardy-Weinberg equilibrium.

Polymorphisms of 3′ UTR and FAdE were tested for association with parameters of adrenal gland function, meat quality, and carcass composition. Results of the association study are summarized in Tables 4, 5, and 6 for parameters of adrenal gland function and meat color. Associations with the other tested meat quality traits and parameters of carcass composition are summarized in Online resource 2. Estimated allele substitution effects for all traits are summarized in Online resource 3.

Associations between any of the genotyped SNP with adrenal gland weight, plasma cortisol level or adrenal cortex/medulla ratio could not be found (Tables 4, 5 and 6). Whereas no consistent associations with carcass composition or meat quality traits could be found for c.*748 G>T (Table 4, Online resource 2 and 3), c.*1296 A>G showed significant association with meat color ($p<0.05$) in both test groups (Table 5). Wild type PiF1 pigs possessing two A-alleles show a lighter meat color than carriers of the G-allele ($p<0.05$). In DL however, the effect of the G-allele is less consistent. Animals homozygous for G at the c.*1296 A>G locus have a darker meat color than heterozygous animals, but differences between animals homozygous for the A-allele and carriers of one or two G-alleles do not reach significant level.

Similarly to the UTR SNP at locus c.*1296 A>G, significant associations with meat color in PiF1 crossbreed animals could be found for c.871-3864 G>C (Table 6). Presence of a single C-allele is associated with a lighter meat color than found in animals with two G-alleles. The effect of c.871-3864 G>C was not visible in DL.

The significant association between c.871-3864 G>C and meat color measured with an OPTO-Star device (MCOPTO) could be shown in a consistent way for lightness value L*. Furthermore, c.871-3864 G>C is significantly associated with b*, indicating an increase of the yellow color component in the presence of one or two C-alleles.

Table 3 Allele frequency of genotyped polymorphisms of the porcine *NR5A1*

SNP	Allele	DL[1] (n=35)[2] HWE[3]	DE (n=24) HWE	Pi (n=26) HWE	Du (n=21) HWE
c.*748 G>T	T	0.000	0.021	0.135	0.000
		–	p=1.000	p=0.371	–
c.*1296 A>G	G	0.662	0.292	0.635	0.000
		p=1.000	p=0.127	p=0.681	–
c.871-3864 G>C	C	0.100	0.292	0.596	0.000
		p=0.282	p=0.127	p=0.218	–

[1] *DL* German Landrace, *DE* German Large White, *Pi* Piétrain, *Du* Duroc

[2] *n*: number of animals

[3] *HWE* exact test of Hardy-Weinberg-equilibrium

Table 4 Associations between SNP c.*748 G>T and parameters of HPA axis activity and meat color

c.*748 G>T	Breed[2]	Genotype least square means[3] (LSM) (SE/*n*)[4]			*P*-value
Trait[1]		*GG*	*GT*	*TT*	
Plasma cortisol [ng/ml]	PiF1	92.9 (1.9/390)	93.0 (3.7/84)	120.7 (30.6/1)	0.663
Adrenal weight [g]	PiF1	2.28 (0.03/284)	2.26 (0.05/58)	2.55 (0.30/1)	0.631
MCOPTO	PiF1	68.2 (0.4/325)	67.3 (0.8/83)	68.8 (6.1/1)	0.513
MCML	PiF1	48.3 (0.2/325)	48.9 (0.3/83)	44.3 (2.8/1)	0.120
MCMA	PiF1	7.34 (0.13/325)	7.41 (0.17/83)	8.27 (0.98/1)	0.576
MCMB	PiF1	1.70 (0.08/325)	1.86 (0.12/83)	0.97 (0.91/1)	0.278

[1] *C/M* cortex area/medulla area ratio of adrenal glands, *MCOPTO* muscle color measured with an OPTO-Star device, *MCML* muscle color Minolta L*, *MCMA* muscle color Minolta a* (redness), *MCMB* muscle color Minolta b* (yellowness)

[2] *PiF1* Piétrain x (German Large White x German Landrace)

[3] Least square means with different superscripts [a,b]; [c,d]; [e,f] in the same row differ at $p<0.01$, $p<0.05$ or $p<0.1$ respectively

[4] *SE* standard error, *n* number of animals

Discussion

Murine *NR5A1* consists of seven exons (1, 3C, 4, 5, 6A, 7, and 8). By differential splicing and alternative promoter usage, three additional gene products (embryonal long terminal repeat-binding protein 1–3; ELP1-ELP3) are produced. Murine gene structure of *NR5A1* is conserved in human, although alternative promoter usage remains unclear (Wong et al. 1996).

Coding sequence of the porcine *NR5A1* elucidated in this work is identical to the genomic sequence provided by Mikawa et al. 2007 (accession number AP009124); however, here for the first time the untranslated exon 1 was verified. In human and mouse, first exon of *NR5A1* transcript encoding

Table 5 Associations between SNP c.*1296 A>G and parameters of HPA axis activity and meat color

c.*1296 A>G	Breed[2]	Genotype least square means[3] (LSM) (SE/*n*)[4]			*P*-value
Trait[1]		*AA*	*AG*	*GG*	
Plasma cortisol [ng/ml]	PiF1	92.8 (3.6/83)	92.8 (2.2/253)	93.2 (2.8/139)	0.993
Adrenal weight [g]	PiF1	2.26 (0.05/67)	2.26 (0.03/177)	2.32 (0.04/99)	0.320
MCOPTO	PiF1	66.1 (0.8/66)[c]	68.4 (0.5/215)[d]	68.6 (0.6/128)[d]	0.018
MCML	PiF1	48.9 (0.4/66)	48.4 (0.2/215)	48.3 (0.3/128)	0.313
MCMA	PiF1	7.42 (0.17/66)	7.31 (0.13/215)	7.40 (0.15/128)	0.593
MCMB	PiF1	1.91 (0.13/66)	1.68 (0.09/215)	1.71 (0.10/128)	0.202
Plasma cortisol [ng/ml]	DL	86.3 (8.0/22)	91.5 (5.5/86)	92.6 (5.5/92)	0.721
Adrenal weight [g]	DL	2.38 (0.09/21)	2.31 (0.06/84)	2.32 (0.06/92)	0.697
C/M	DL	4.12 (0.26/22)	3.86 (0.17/86)	3.86 (0.16/92)	0.568
Cortex area [mm^2]	DL	34.0 (2.2/22)	33.3 (1.6/86)	33.9 (1.6/92)	0.849
MCOPTO	DL	72.5 (0.9/22)	71.4 (0.6/87)[c]	73.0 (0.6/93)[d]	0.034
MCML	DL	48.6 (0.6/22)	48.7 (0.3/87)	48.0 (0.3/93)	0.151
MCMA	DL	6.79 (0.29/22)	6.83 (0.21/87)	6.85 (0.21/93)	0.972
MCMB	DL	1.00 (0.20/22)	1.12 (0.12/87)	1.11 (0.12/93)	0.838

[1] *C/M* cortex area/medulla area ratio of adrenal glands, *MCOPTO* muscle color measured with an OPTO-Star device, *MCML* muscle color Minolta L*, *MCMA* muscle color Minolta a* (redness), *MCMB* muscle color Minolta b* (yellowness)

[2] *PiF1* Piétrain x (German Large White x German Landrace), *DL* German Landrace

[3] Least square means with different superscripts [a,b]; [c,d]; [e,f] in the same row differ at $p<0.01$, $p<0.05$ or $p<0.1$ respectively

[4] *SE* standard error, *n* number of animals

Table 6 Associations between SNP c.871-3864 G>C and parameters of HPA axis activity and meat color

c.871-3864 G>C	Breed[2]	Genotype least square means[3] (LSM) (SE/*n*)[4]			*P*-value
Trait[1]		*GG*	*GC*	*CC*	
Plasma cortisol [ng/ml]	PiF1	92.2 (2.0/346)	96.9 (3.1/117)	94.5 (13.9/5)	0.386
Adrenal weight [g]	PiF1	2.27 (0.03/252)	2.27 (0.04/82)	2.55 (0.18/3)	0.295
MCOPTO	PiF1	68.6 (0.5/286)[c]	66.7 (0.7/111)[d]	66.0 (2.8/5)	0.026
MCML	PiF1	48.2 (0.2/286)[c]	49.0 (0.3/111)[d]	49.5 (1.3/5)	0.040
MCMA	PiF1	7.33 (0.13/286)	7.43 (0.15/111)	7.31 (0.48/5)	0.705
MCMB	PiF1	1.66 (0.08/286)[c]	1.93 (0.10/111)[d]	2.06 (0.42/5)	0.034
Plasma cortisol [ng/ml]	DL	91.6 (5.0/175)	91.3 (7.7/24)	–	0.964
Adrenal weight [g]	DL	2.31 (0.05/172)	2.37 (0.08/24)	–	0.480
C/M	DL	3.83 (0.14/174)	4.19 (0.24/25)	–	0.122
Cortex area [mm^2]	DL	33.6 (1.5/174)	34.2 (2.1/25)	–	0.709
MCOPTO	DL	72.3 (0.6/176)	71.8 (0.9/25)	–	0.572
MCML	DL	48.3 (0.3/176)	48.6 (0.6/25)	–	0.596
MCMA	DL	6.85 (0.19/176)	6.77 (0.28/25)	–	0.736
MCMB	DL	1.11 (0.10/176)	1.01 (0.19/25)	–	0.584

[1] *C/M* cortex area/medulla area ratio of adrenal glands, *MCOPTO* muscle color measured with an OPTO-Star device, *MCML* muscle color Minolta L*, *MCMA* muscle color Minolta a* (redness), *MCMB* muscle color Minolta b* (yellowness)

[2] *PiF1* Piétrain x (German Large White x German Landrace), *DL* German Landrace

[3] Least square means with different superscripts [a,b]; [c,d]; [e,f] in the same row differ at $p<0.01$, $p<0.05$ or $p<0.1$ respectively

[4] *SE* standard error, *n* number of animals

SF1 (mouse exon 1) consists solely of 5′ UTR, whereas translation starts within exon two (mouse exon 3C). However, the alternative *NR5A1* transcripts encoding ELP use transcription start points downstream of exon one (mouse exon 2, 3A, and 3B; Ninomiya et al. 1995). In this work, a structure of the SF1 transcription unit homologous to that found in human, including an untranslated first exon (AccNr. AP009124: 456840–457020) and a shorter second exon (AccNr. AP009124: 45277–452893), was predicted and confirmed in porcine adrenal glands. Upstream sequence of this first exon (corresponding to mouse exon 1) contains conserved promoter elements, such as a CAAT-box, an E-box, and a SP1 binding site, whose biological importance has been shown in mice (Nomura et al. 1995; Woodson et al. 1997).

None of the SNPs in the untranslated region or the fetal adrenal enhancer genotyped in this study showed any associations with parameters of HPA axis function. Apart from its important role in HPA axis function *NR5A1* is a key regulator of different biological processes including sex-hormone synthesis, brain organization and function, and possibly even immune system (Ramayya et al. 1997). Some of those processes have been suggested to involve stress-related traits independent from adrenal gland function, e.g., regulation of aggressive behavior and anxiety like behavior (Grgurevic et al. 2008; Büdefeld et al. 2011). However, which of those processes might be affected by genetic variation of the *NR5A1* gene in the pig warrants further research. While the adrenal phenotype in heterozygous knock-out mice is more noticeable than the gonadal phenotype, the vast majority of known human *NR5A1* polymorphisms are mainly characterized by their gonadal phenotype in heterozygous individuals (Hoivik et al. 2010). This implies that different networks regulated by *NR5A1* have different, species specific sensitivities for *NR5A1*.

Since the analyzed *NR5A1* SNP might affect economically important traits via different networks than the HPA axis, we were interested to see if they are associated with meat quality and carcass composition. Indeed, one of the 3′ UTR SNPs and one of the enhancer (FAdE) SNPs showed an association with meat color. Pale meat color might be a sign of PSE (pale, soft, exudative) meat, whereas dark meat color is an indicator of DFD (dark, firm, dry) meat. Both conditions are thought to be stress related, however, the changes of meat color associated with the different *NR5A1* genotypes did not reach values used as indicators for PSE meat (MCOPTO<60) or DFD meat (MCOPTO>80; ZDS 2007). Furthermore, no associations with traits of water holding capacity or pH value have been found, which are additional parameters related to DFD and PSE meat.

Meat color is both influenced by chemical and by structural characteristics of muscle fibers. In a pH value dependent manner, myoglobin is present as oxygenated, deoxygenated or oxidized molecule, each condition being characterized by a

different color (Karamucki et al. 2013). Loss of myoglobin might occur when myoglobin leaves muscle together with intracellular fluid (Swatland 2004). However, no associations between *NR5A1* polymorphisms and pH value or drip loss have been found in the present study (Online Resource 2 and 3).

On a physical level, muscle color is influenced by the scattering of light, which might occur on precipitated sacroplasmatic proteins (Swatland 2004). Previous studies have linked pH value independent effects of genetic polymorphisms on meat color with a modulation of proteolysis in muscle (Muráni et al. 2006).

Refraction of light when passing several myofibrils also contributes to light scattering. Refraction value of myofibers might be influenced by the *NR5A1* SNP; however, this was not tested in this study.

Previous studies implicated androgen receptor signaling pathway in muscle fiber distribution (Altuwaijri et al. 2004). Since no connections between *NR5A1* polymorphisms and cortisol level were found in this study, androgens might convey the effect of the detected polymorphisms on meat color instead. Like other steroid hormones, sex steroids are synthesized out of cholesterol due to the action of several SF1 regulated P450 enzymes. Presence and lyase activity of P450c17 are of striking importance for the production of androgens instead of mineralocorticoids or glucocorticoids (reviewed by Miller 2002). 17,20 lyase activity of P450c17 is enhanced by the allosteric factor cytochrome b5 which is, just like P450c17 itself, under the transcriptional control of SF1 (Hanley et al. 2001; Huang et al. 2005).

The analyzed SNPs do not affect previously identified transcription factor binding sites of the FAdE. However, *in silico* analysis pointed to the 3′ UTR of the porcine *NR5A1* as a potential target site of several miRNA. Some of the potential miRNA binding sites (ssc-miR-503 for c.*748 G>T and ssc-miR-24 for c.*1296 A>G) overlap with the analyzed polymorphisms. Although ssc-miR-24 is expressed in most porcine tissues (Kim et al. 2008), its expression in primary organs of *NR5A1* transcription — adrenal glands, gonads and the VMH — has not been established yet.

Since the full complement of regulatory regions of *NR5A1* is not discovered yet, the mutation responsible for the association with meat color might have escaped detection. Thus, the analyzed SNP might be in incomplete linkage disequilibrium with the causative mutation, which would explain the lack of consistency among the analyzed breeds.

In this study no evidence for the involvement of *NR5A1* polymorphisms in stress sensitivity or robustness traits of pigs could be found. Nonetheless, the repeatedly found associations with meat color suggest that genetic variants of *NR5A1* might be of interest for molecular breeding to improve pork quality. The influence of meat color on sensory assessment of meat by the consumer is well established (Moeller et al. 2010). This warrants further studies toward identification of the causal variants and elucidation of the underlying molecular and physiological functions.

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