Transcript profiling of candidate genes in testis of pigs exhibiting large differences in androstenone levels

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Abstract

Background: Boar taint is an unpleasant odor and flavor of the meat and occurs in a high proportion of uncastrated male pigs. Androstenone, a steroid produced in testis and acting as a sex pheromone regulating reproductive function in female pigs, is one of the main compounds responsible for boar taint. The primary goal of the present investigation was to determine the differential gene expression of selected candidate genes related to levels of androstenone in pigs.

Results: Altogether 2560 boars from the Norwegian Landrace and Duroc populations were included in this study. Testicle samples from the 192 boars with most extreme high or low levels of androstenone in fat were used for RNA extraction, and 15 candidate genes were selected and analyzed by real-competitive PCR analysis. The genes Cytochrome P450 c17 (CYP17A1), Steroidogenic acute regulatory protein (STAR), Aldo-keto reductase family 1 member C4 (AKR1C4), Short-chain dehydrogenase/reductase family member 4 (DHRS4), Ferritin light polypeptide (FTL), Sulfotransferase family 2A, dehydroepiandrosterone-preferring member 1 (SULT2A1), Cytochrome P450 subfamily XI polypeptide 1 (CYP11A1), Cytochrome b5 (CYB5A), and 17-beta-Hydroxysteroid dehydrogenase IV (HSD17B4) were all found to be significantly (P < 0.05) up-regulated in high androstenone boars in both Duroc and Landrace. Furthermore, Cytochrome P450 c19A2 (CYP19A2) was down-regulated and progesterone receptor membrane component 1 (PGRMC1) was up-regulated in high-androstenone Duroc boars only, while CYP21 was significantly down-regulated (2.5) in high-androstenone Landrace only. The genes Nuclear Receptor co-activator 4 (NCOA4), Sphingomyelin phosphodiesterase 1 (SMPD1) and 3β-hydroxysteroid dehydrogenase (HSD3B) were not significantly differentially expressed in any breeds. Additionally, association studies were performed for the genes with one or more detected SNPs. Association between SNP and androstenone level was observed in CYB5A only, suggesting cis-regulation of the differential transcription in this gene.

Conclusion: A large pig material of highly extreme androstenone levels is investigated. The current study contributes to the knowledge about which genes that is differentially expressed regard to the levels of androstenone in pigs. Results in this paper suggest that several genes are important in the regulation of androstenone level in boars and warrant further evaluation of the above mentioned candidate genes, including analyses in different breeds, identification of causal mutations and possible gene interactions.

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on the levels of androstenone in carcass and heritability estimates are found to range from 0.25 to 0.87 [4,5].

Several studies have aimed at finding the enzymes or key regulatory proteins involved in regulation of androstenone concentrations (reviewed by Robic et al. [6], and Zamaratskaia and Squires [7]), although on a limited number of genes. The primary objective of this study was to test for differential expression in fifteen selected candidate genes involved in the regulation of androstenone levels in pigs. Some of the genes were selected as candidate genes for boar taint due to their biological function, while others were selected on the basis of an earlier microarray gene expression study [8]. The latter set of candidate genes were included in order to confirm earlier findings in another and extended animal material. Recent studies have demonstrated the effect of genetic variation on gene expression levels [9,10]. The individual variation in gene expression consists of two varieties; cis-acting which results from DNA variations of a gene that is directly influencing transcription level of that gene, and trans-acting which is due to alteration of other genetic variations. Therefore, we performed an association test examining the phenotypic effects of different alleles for some of the candidate genes (CYB5A, CYP11A1, HSD3B and NCOA4) displaying differential expression.

Results
Differential gene expression
A total of 12 out of 15 candidate genes were found to be differentially expressed between high/low androstenone groups at a significance level of P < 0.001 to P < 0.05 in one or both of the breeds Landrace and Duroc. All the genes significant differentially expressed (Table 1 and 2) were up-regulated in the high-androstenone boars compared to low-androstenone boars, except for CYP19A2 and CYP21 which were down-regulated in the high-androstenone boars. The genes Aldo-keto reductase family 1 member C4 (AKR1C4), CYB5A, Cytochrome P450 c17 (CYP17), CYP11A1, Short-chain dehydrogenase/reductase family member 4 (DHRS4), Ferritin light polypeptide (FTL), 17-beta-Hydroxysteroid dehydrogenase IV (HSD17B4), Steroidogenic acute regulatory protein (STAR) and Sulfotransferase family 2A dehydroepiandrosterone-preferring member 1 (SULT2A1) were all significantly up-regulated in high-androstenone Duroc and Landrace boars. Furthermore, Cytochrome P450 c19A2 (CYP19A2) was down-regulated and progesterone receptor membrane component 1 (PGRMC1) was up-regulated in high-androstenone Duroc boars only, while CYP21 was significantly down-regulated (2.5) in high-androstenone Landrace only. The genes NCOA4, Sphinomyrlin phosphodiesterase 1 (SMPD1) and HSD3B were not significantly differentially expressed in any breeds.

Allele-specific differential expression
Assays were designed for the investigation of allele-specific differential expression of one SNP within CYB5A and two SNPs within each of the genes CYP21 and HSD3B. Additionally, one assay was made to study differential expression of the CYB5A isoforms 1 and 2. Expression of the CYB5A isoform 2 was, however, not

| Gene                | Fold change | Log10 Fold change | Bias     | Std error | P value |
|---------------------|-------------|-------------------|----------|-----------|---------|
| AKR1C4              | 2.6         | 0.42              | 0.0050   | 0.11      | 0.0000  |
| CYB5A_8(5’UTR)      | 2.6         | 0.42              | 0.0034   | 0.17      | 0.0090  |
| CYB5A_iso1-2        | 2.4         | 0.37              | 0.0026   | 0.19      | 0.0210  |
| CYP11A1             | 3.1         | 0.50              | 0.0050   | 0.13      | 0.0000  |
| CYP17A1             | 2.9         | 0.46              | 0.0034   | 0.09      | 0.0000  |
| CYP19A2             | 0.8         | -0.10             | -0.0003  | 0.13      | 0.2120  |
| CYP21_exon9         | 0.1         | -0.91             | -0.0033  | 0.46      | 0.0200  |
| DHRS4               | 2.6         | 0.41              | 0.0043   | 0.11      | 0.0005  |
| FTL                 | 2.3         | 0.35              | 0.0036   | 0.11      | 0.0005  |
| HSD3B_exon2         | 1.2         | 0.06              | 0.0067   | 0.24      | 0.3810  |
| HSD3B_5’UTR         | 0.8         | -0.10             | 0.0023   | 0.20      | 0.3120  |
| HSD17B4             | 2.2         | 0.34              | 0.0034   | 0.12      | 0.0030  |
| NCOA4               | 1.3         | 0.11              | 0.0019   | 0.13      | 0.1970  |
| PGRMC1              | 1.2         | 0.08              | 0.0045   | 0.13      | 0.2700  |
| SMPD1               | 1.1         | 0.04              | 0.0032   | 0.09      | 0.3200  |
| STAR                | 13.5        | 1.13              | 0.0032   | 0.14      | 0.0000  |
| SULT2A1             | 3.0         | 0.48              | 0.0031   | 0.13      | 0.0002  |

*Fold changes are calculated relative to baseline, which is the group of low androstenone (LL) in this case, and are therefore indicating the times of up-regulation in high-androstenone group compared to the low-androstenone group. All genes are adjusted for the housekeeping gene HPRT.
detected in any of the samples. Also, no significant differential allele-specific expression between high and low androstenone animals (both breeds) was detected in any of the genes investigated (results not shown).

For the SNPs in \textit{CYB5A} (-8(5' UTR)), \textit{CYP21} (exon8 and exon9), as well as for the SNPs in \textit{HSD3B} (-15 (5' UTR)), the two alleles had nearly identical expression levels, with expression levels ratios in the range of 0.46-0.54. For the SNP located in \textit{HSD3B} exon2, on the other hand, the two alleles were expressed differentially (P < 0.05), although not with regard to the levels of androstenone. In Duroc, allele A had generally higher expression levels than allele G (average ratio 0.80), whereas in Landrace allele A had lower expression levels than allele G (average ratio 0.41). Notably, allele A is quite rare in both breeds. The allele frequencies used in the assays of allele-specific expression are shown in Table 3.

\textbf{Association studies}

Five SNPs were detected in the candidate genes \textit{CYB5A}, \textit{CYP11A1}, \textit{HSD3B} and \textit{NCOA4} (Table 4), and single SNP association studies were performed in both Landrace and Duroc breeds. The SNP located in position -8 of the \textit{CYB5A} gene was significantly associated to androstenone levels in Duroc (P < 0.01), although it was not reproducible within Landrace (P = 0.14). No other SNPs were significantly associated with androstenone in this study (Table 4). Association results including SNPs in some of the other genes investigated in this study have previously been presented in Moe et al. [11].

\textbf{Discussion}

In the present study, fifteen candidate genes potentially affecting androstenone levels in boars were selected, based on the biochemistry and physiology of the trait, and on results from a microarray study published by Moe et al. [8]. The main objective of the study was to determine whether the genes were differentially expressed in pigs with high and low androstenone levels. Variation in gene expression between different alleles in mammals [12] and genetic variation in single nucleotide polymorphisms (SNPs) within the candidate genes may also contribute to the androstenone variability. Therefore, investigation of allele-specific expression and association tests were also performed for the candidate genes in question.

The synthesis of 16-androstene steroids, including androstenone, occurs by the action of several enzymes and some of them have been found to be more important than others. Members of the Cytochrome P450 superfamily function as monooxygenases, utilizing electrons to catalyze the hydroxylation and cleavage of substrates. The formation of the 16-androstene steroids from pregnenolone is catalyzed by the andien-β synthase enzyme system [13]. Major enzymes in this system are \textit{CYP17A1} along with \textit{CYB5A} and the associated reductases [14]. Several studies have previously studied \textit{CYB5A} as a candidate gene for boar taint [15-17]. Levels of mRNA for total \textit{CYB5A} were found to be significantly correlated with levels of androstenone in fat [16]. These results are in accordance with our results, showing

| Table 2 Results from the rcPCR bootstrap statistics (×4000), Duroc. |
|-------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Gene                    | Fold change*   | Log10 Fold change | Bias     | Std error | P value         |
| AKR1C4                  | 1.6            | 0.21             | 0.0007   | 0.11      | 0.0270          |
| CYB5A_8(5'UTR)          | 2.0            | 0.31             | -0.0011  | 0.11      | 0.0040          |
| CYB5A_iso1-2            | 1.6            | 0.19             | 0.0030   | 0.15      | 0.0910          |
| CYP11A1                 | 2.3            | 0.37             | 0.0006   | 0.12      | 0.0010          |
| CYP17A1                 | 2.4            | 0.38             | 0.0004   | 0.11      | 0.0005          |
| CYP19A2                 | 0.6            | -0.22            | 0.0002   | 0.12      | 0.0360          |
| CYP21_exon8             | 0.9            | -0.04            | 0.0042   | 0.25      | 0.4380          |
| CYP21_exon9             | 1.0            | 0.01             | 0.1255   | 0.32      | 0.4780          |
| DHR54                   | 2.1            | 0.33             | 0.0011   | 0.11      | 0.0020          |
| FTL                     | 1.9            | 0.27             | 0.0008   | 0.11      | 0.0090          |
| HSD3B_exon2             | 0.8            | -0.11            | 0.0005   | 0.17      | 0.2490          |
| HSD3B_5UTR              | 1.0            | 0.02             | -0.0011  | 0.15      | 0.4460          |
| HSD17B4                 | 1.6            | 0.20             | 0.0002   | 0.11      | 0.0430          |
| NCOA4                   | 1.5            | 0.18             | -0.0018  | 0.11      | 0.0620          |
| PGRMC1                  | 1.6            | 0.21             | 0.0005   | 0.11      | 0.0330          |
| SMPD1                   | 1.1            | 0.03             | 0.0011   | 0.11      | 0.3940          |
| STAR                    | 4.7            | 0.68             | -0.0009  | 0.12      | 0.0000          |
| SULT2A1                 | 2.1            | 0.32             | -0.004   | 0.11      | 0.0030          |

*Fold changes are calculated relative to baseline, which is the group of low androstenone (LD) in this case, and are therefore indicating the times of up-regulation in high-androstenone group compared to the low-androstenone group. All genes are adjusted for the housekeeping gene HPRT.
significant up-regulation of total CYB5A (CYB5A_8 (5′UTR) in Tables 1 and 2) expression in the high androstenone animals in both breeds. Two variants, a soluble (isoform 1) and membrane bound (isoform 2) form, of CYB5A cDNA have been isolated in pigs [18], and later detected as a low molecular weight form (isoform 1) and a high molecular weight form (isoform 2) in porcine testis [16]. The CYB5A isoforms are derived from one mRNA via alternative splicing [19]. A study by Davis et al. [16] found that levels of the CYB5A isoform 1, but not levels of isoform 2, were correlated with both the rate of 16-androstene steroid synthesis and fat androstenone concentrations, indicating that increased levels of the isoform 1 of CYB5A are linked to a higher level of androstenone production in pig testis. In our study, the assay “CYB5A_iso1-2” (Table 1 and 2) was used to study differential expression between isoform 1 and 2. Results show that the expression of isoform 1 was significantly up-regulated (P < 0.05) in high-androstenone Landrace animals. The Duroc breed did not reach the defined level of significance, although it was rather close (P = 0.09). Expression levels of CYB5A isoform 1 seems to be slightly less up-regulated (although not significantly) compared with total CYB5A expression (Tables 1 and 2). CYB5A isoform 2 was not detected in any of the samples. Another purpose of the assay “CYB5A_-8 (5′UTR)” was to study differences in allele-specific expression of the SNP detected in the 5′UTR, 8 bp upstream of the translation start codon. The results revealed no significant differential allele-specific expression between the high and low androstenone groups. Furthermore, an association study on the same polymorphism in the entire material of Landrace and Duroc, i.e. 2560 boars altogether, were performed. Only homozygous G and heterozygous GT animals were detected in our populations, reflecting a low frequency of the T allele which is also seen in other populations [17,20]. The polymorphism was found to be significantly associated to androstenone levels in Duroc, but not in Landrace. In both breeds, however, the LS mean values of androstenone were lower in the heterozygous (GT) animals than in the homozygous G genotype (Table 4).

| Table 3 Frequency of alleles used in the assays of allele expression, Landrace and Duroc. |
| Gene, LANDRACE | Genotype | Frequency | Gene, DUROC | Genotype | Frequency |
|---------------|----------|-----------|-------------|----------|-----------|
| HSD3B_5UTR    | CC       | 7         | CC          |          | 4         |
|               | CT       | 34        | CT          |          | 31        |
|               | TT       | 55        | TT          |          | 60        |
| HSD3B_exon2   | AA       | 1         | AA          |          | 0         |
|               | AG       | 13        | AG          |          | 16        |
|               | GG       | 82        | GG          |          | 79        |
| CYP21_exon8   | CC       | 8         | CC          |          | 55        |
|               | CT       | 32        | CT          |          | 25        |
|               | TT       | 56        | TT          |          | 16        |
| CYP21_exon9   | CC       | 64        | CC          |          | 96        |
|               | CT       | 28        | CT          |          | 0         |
|               | TT       | 4         | TT          |          | 0         |
| CYB5A_-8(5UTR)| GG       | 94        | GG          |          | 89        |
|               | GT       | 2         | GT          |          | 6         |
|               | TT       | 0         | TT          |          | 0         |

Table 4 The associations between SNPs and levels of androstenone in Duroc and Landrace boars*  

| B | Gene | Genotype 1 | Genotype 1/2 | Genotype 2 | P value |
|---|------|------------|--------------|------------|---------|
| D | CYB5A_8(5U) | G(n = 902):3.45 (± 0.12) | G/T(n = 51): 2.56(± 0.38) | - | 0.01 |
| D | HSD3B_15(5U) | C (n = 67): 2.38 (± 0.45) | C/T(n = 293):2.70(± 0.20) | T (n = 409):3.25(± 0.12) | 0.11 |
| D | HSD3B_271-exon2 | T(n = 74): 2.82(± 0.11) | A/G(n = 74):2.82(± 0.11) | G (n = 64):3.03(± 0.28) | 0.68 |
| D | NCOA4_3 | A(n = 326):3.48 (± 0.23) | A/G(n = 413):3.49(± 0.21) | G(n = 180):3.67 (± 0.26) | 0.22 |
| L | CYB5A_8(5U) | G(n = 1278):1.18(± 0.04) | G/T(n = 25): 0.97 (± 0.15) | - | 0.14 |
| L | CYP11A1_150-exo1 | A(n = 321):1.05 (± 0.13) | A/G(n = 362):1.23(± 0.11) | G(n = 150):0.96 (± 0.20) | 0.39 |
| L | HSD3B_15(5U) | C(n = 74): 1.00(± 0.19) | C/T(n = 267):1.31(± 0.13) | T(n = 430): 1.02(± 0.11) | 0.16 |
| L | HSD3B_271-exon2 | A(n = 14): 1.00(± 0.21) | A/G(n = 94): 0.88(± 0.16) | G(n = 512): 1.11(± 0.29) | 0.64 |
| L | NCOA4_3 | A(n = 204):1.24(± 0.09) | A/G(n = 530):1.18(± 0.05) | G(n = 425): 1.10(± 0.06) | 0.36 |

*The number of boars is shown between parentheses after each genotype. The least square means are shown for all genotypes and estimated standard errors are shown between the parentheses. Dash is no genotype found. B = Breed, D = Duroc and L = Norwegian Landrace.
This trend is in accordance with the results of Lin et al. [15] reporting this SNP allele to be associated with a decrease in fat androstenone production in vivo, as well as CYB5A protein expression in vitro, in a variety of breeds. This is also confirmed by two other studies [17,20]. Both differential gene expression and association with androstenone might indicate a Cis-acting regulation of CYB5A expression in pigs. Furthermore, CYB5A is a protein widely involved in biological processes, being a component of electron transfer chains in a number of pathways [21]. For example, interactions between CYB5A and the FTL may affect levels of androstenone through the CYB5A/CYP450 electron transfer [22]. In this study, the FTL was highly up-regulated in both breeds (P < 0.01), which is in agreement with the study of Moe et al. [8]. The FTL gene provides instructions for making the ferritin light chain. Ferritin stores and releases iron in cells and plays a central role in numerous essential cellular functions (reviewed by Hentze and Kuhn [23]).

The major enzymes Cytochrome P450 c17 (CYP17) and CYB5A interacts in the andien-β synthase system [13], and since CYP17A1 also converts pregnenolone into precursors of the androgens and estrogens it is also a very potent candidate gene for androstenone production. However, no significant effects have so far been detected in association studies [24] or on the protein expression level [16]. In this study we did, however, find CYP17A1 cDNA levels to be significantly up-regulated in high androstenone boars of both Landrace and Duroc. No SNPs were detected within the CYP17A1 gene in our populations.

The key rate-limiting factor for the maintenance of steroid production is the continuous provision of the cholesterol substrate from the outer mitochondrial membrane to the enzymatic component in the inner membrane, which is mainly facilitated by STAR [25]. Next, the CYP11A1 enzyme, localized to the mitochondrial inner membrane, catalyzes the conversion of cholesterol to pregnenolone in the first and rate-limiting step in the synthesis of the steroid hormones [26]. This is a very important step in the production of androstene, and interestingly STAR and CYP11A1 are both highly up-regulated in high androstenone animals in both Landrace and Duroc. STAR was found as much as 13.5 times up-regulated in Landrace and 4.7 times up-regulated in Duroc. Highly differentiated expression of STAR was also seen in our previous microarray study [8]. STAR has previously shown increased gene expression during the time of sexual differentiation [27]. Regulation of STAR has, however, been suggested to be both on the post transcriptional level, in a developmental stage- and tissue-specific manner [28], and at transcription level [29]. No SNPs were detected in STAR in this study, and further studies are needed to reveal molecular basis for this variation. Also CYP11A1 was found up-regulated in the previous microarray study [8] and confirmed in this study. A SNP located in CYP11A1 exon 1 was not significantly associated with androstenone levels in Landrace boars (Table 4), while no data were obtained for the Duroc breed. In contrast, another polymorphism in CYP11A1 exon 1 has previously been found to be significantly associated with androstenone levels in Yorkshire boars [30], whilst not in a Large White and Meishan cross [31].

Furthermore, sulfotransferase family 2A dehydroepiandrosterone-prefering member 1 (SULT2A1) is a key enzyme in the testicular and hepatic metabolism of 5α-androstenone and responsible for sulfoconjugating the 16-androstenone steroids. Previous studies have indicated that increased levels of sulfoconjugated 16-androstenone steroids present in the systemic circulation are associated with reduction in the accumulation of 5α-androstenone in adipose tissue [32]. Additionally, testicular SULT2A1 activity was found to be negatively correlated with 5α-androstenone concentrations in fat, SULT2A1 enzyme activity was positively correlated with SULT2A1 protein level, and finally the gene expression level was positively correlated with increased protein level [33]. The findings in our study are, however, contradictory to this since we have an up-regulation of SULT2A1 gene expression level in high androstenone animals (both breeds).

The last steps in the formation of androgens and estrogens are catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes [34]. Previously, these enzymes have been assigned to porcine Leydig and Sertoli cells [35] and several porcine tissues have been shown to express HSD17B4 as a predominant dehydrogenase [36]. HSD17B4 has also been shown to inactivate estrogens very efficiently in several tissues because of its preference for steroid oxidation [37]. Our study, however, indicate that the HSD17B4 gene is rather up-regulated in testes in both high androstenone Duroc and Landrace boars. Due to this it is important to note that several roles of HSD17B4 are suggested [37]. A study done by Chen et al. [38] did not detect any differences in HSD17B gene expression between boars of high and low androstenone in a Landrace x Yorkshire crossbred. Five SNPs within HSD17B were detected and tested in this population by Moe et al. [11], but no significant associations were detected.

The gene expression of AKR1C4, which belongs to the cytosolic aldo-keto reductases that act as 3α- /3β-/17β-/ 20α-hydroxysteroid dehydrogenases (HSDs) in human [39], was also investigated. Significant up-regulation of the gene AKR1C4 was detected in high androstenone boars in both breeds, although it was more pronounced...
in Landrace (Tables 1 and 2). This is in accordance with results of Moe et al. [8]. All the isoforms AKR1C1-AKR1C4 have previously been found to convert active androgens and estrogens to their associated inactive metabolites, preventing excess of circulating steroid hormones and turning the steroids into substrates for conjugation reactions [39]. However, the role of AKR1C4 in regulation of androstenone level in testes needs to be clarified. Members of the dehydrogenase/reductase (SDR) family are other enzymes involved in the process of oxidation of 3β-hydroxysteroid precursors into ketosteroids. Several family members have previously been shown to be important in catalyzing an essential step in the biosynthesis of all classes of active steroid hormones [40]. The member DHR54 was found to be highly up-regulated in high androstenedione boars in both Landrace and Duroc [8], and this was confirmed in an extended animal material in this study (P < 0.005). Interestingly, the DHR54 was very recently shown to have a role in 3β-hydroxysteroid synthesis, and DHR54 was shown to be induced via PPARα activation [41]. PPARα has previously been shown to regulate various genes controlling gluconeogenesis, ketone body synthesis, heme synthesis and cholesterol metabolism [42].

Breed differences in levels of androstenone (e.g. Tajet et al. [5]), sequence variation, mRNA and protein levels have been found in several studies [43-45]. In this study we found breed differences in level of expression for the genes CYP19A2, PGRMC1 and CYP21. CYP19A2 was significantly down-regulated and PGRMC1 significantly up-regulated in high androstenedione Duroc boars, while none of them were differentially expressed in Landrace. Cytochrome P450 c19 (CYP19) encodes the enzyme aromatase, which catalyses the synthesis of estrogens from androgens. Unusually high levels of estrogens are secreted from the porcine testes [46] and pig is the only mammal known to express functionally distinct isoforms of the CYP19 gene [47]. Notably, our results for the isoform CYP19A2 are not supported by previous microarray results showing up-regulation in high androstenedione boars in both breeds [8]. Results in this study are based on more animals compared with the previous microarray study, and results in the current study might suggest that the significant results of differential expressions of CYP19A2 in Moe et al. [8] are false positives. Another explanation might be that other transcripts or isoforms (e.g. CYP19A1, CYP19A3) than CYP19A2 are picked up and quantified in one of the gene expression methods, although the oligo assay designed for CYP19A2 in the realPCR experiment is made specifically to distinguish between the isoforms. The results for the PGRMC1 gene were, however, in concordance with the results reported by Moe et al. [8]. PGRMC1 is suggested to have a role in binding heme and to catalyze steroids by cytochrome P450 enzymes, analogous to the roles played by CYB5A (reviewed by Cahill [48]). CYP21 is a member of the cytochrome P450 superfamily enzymes, which is a key enzyme for corticosteroidogenesis [49] and suggested to have arisen evolutionarily from the same gene as CYP17A1 [50]. From a physiological point of view, CYP21 leads to drastic fertility changes in human females [51]. CYP21 was significantly down regulated (P < 0.02) in high androstenedione Landrace in this study, although it is important to point out that expression levels were generally very low and the standard error high in both breeds (Tables 1 and 2). Gene expression of CYP21 in testes has previously not been studied in any species. A QTL for androstenone level of boars from a Large White/Meishan cross was detected in this region, and CYP21 was suggested as a positional candidate gene, although no polymorphisms were detected in the coding region and no association study performed [31]. An association study performed on the same populations as described in this paper detected seven SNPs within the CYP21 gene, although none of them were significantly associated with androstenedone in any of the breeds [11].

The candidate genes HSD3B, NCOA4 and SMPD1 were all chosen because they have relevant functions regarding production of androstenedone. HSD3B is an enzyme catalyzing the biosynthesis of steroids in testis [52], and the enzyme has also been shown to catalyze the initial step of the hepatic metabolism of androstenedione in pigs [53]. Recently, expression of the 3β-HSD protein was shown to be repressed in liver in pigs with high androstenedone, but not in testis [54]. Significantly reduced levels of mRNA expression in high androstenedione Landrace and Yorkshire boars were obtained in another study [38]. In this study we were, on the other hand, not able to detect significant differences in gene expression levels of HSD3B. Furthermore, no differences in allele expression were observed, as well as none significant associations with any of the SNPs investigated (Table 4). The association results are in concordance with a recent study by Cue et al. [45], obtaining no significant associations between the HSD3B SNPs, all located in the 5’UTR, and the androstenedone level in fat from several breeds.

Nuclear receptor co-activator 4 (NCOA4; often referred to as ARA70) is identified as an androgen receptor specific co-activator [55], and is suggested to have a role in the modulation of the sex hormone specificity in humans [56]. NCOA4 was shown to be significantly up-regulated in high androstenedione Duroc boars in the recent microarray study [8]. In this study, however, we were not able to confirm this result in an extended animal material, although results were close to significant (P = 0.06). One SNP from the NCOA4 3’UTR
region was genotyped in both populations but no significant association was observed (Table 4).

Sphingomyrlin phosphodiesterase 1 (SMPD1) is ubiquitous lysosomal hydrolase that cleaves sphingomyelin to ceramide, which again has been shown to inhibit CYP19 activity through induction of transcription factors [57]. SMPD1 was down-regulated in high androstenone Duroc animals in the microarray study [8]. This result was, however, not confirmed in the rPCR study performed by Moe et al. [8], and not either in the current study performed on extended animal material.

Previous studies have shown that differential expression of alleles is quite common in mammals and that such variation may contribute to phenotypic variability [12,58]. Interestingly, 54% of tested genes were found to have preferential expression of one allele in some individuals and almost half of them showed greater than four-fold difference between the two alleles [12]. Therefore, when possible, assays were designed to allow simultaneous transcript profiling of alleles in a heterozygous individual. Five SNPs in three genes were analyzed to see whether such differentially allelic expression is present, although no significant differences were obtained (results not shown).

Summarizing this study, the genes AKR1C4, CYB5A, CYP11A1, CYP17A1, CYP19A2, CYP21, DHR54, FTL, HSD17B4, SULT2A1, STAR and PGRMC1 were found to be differentially expressed in this study. HSD3B was not differentially expressed in this study, contradictory to results seen in the Yorkshire breed [38]. Association between SNP and androstenone level was observed in the CYB5A gene only, suggesting cis-regulation of differential transcription. The frequency of the favorable allele is, however, very low (see Table 4), which makes it less useful for selection purposes. SNP detection needs to be performed also for the other differentially expressed genes in this study to find potentially useful markers for selection against boar taint. Previous to selection against androstenone it is, however, important also to find the relationship between the candidate SNPs and other reproduction related traits. Two of the most up regulated genes in this study, STAR and CYP17A1, have for example previously been found to be elevated in preovulatory estrogenic follicles in pigs [59]. Results in this paper suggest that several genes are important in the regulation of androstenone level in boars and warrant further evaluation of the above mentioned candidate genes, including analyses in different breeds, identification of causal mutations and possible gene interactions.

Conclusion
The gene expression of fifteen candidate genes is investigated in a large pig material of highly extreme androstenone levels. The current study contributes to new knowledge about the genes and pathways involved in regulation of androstenone in pigs, as well as contributing to important confirmation of genes previously investigated. Results highly suggest that several genes are important in the regulation of androstenone level in boars. For some of the genes the results also indicate whether there are cis- or trans regulated differences in level of transcription.

Methods
Animals and Sampling
Samples and phenotypes from 1533 Landrace and 1027 Duroc boars were included in this study, and all of them were tested in NORSVIN’s (the Norwegian Pig Breeders Association) boar testing stations. The animals were reared on the standard commercial feed with an energy content of 14.9 MJ digestible energy, 17.8% raw protein, 5.6% fiber, 6% raw fat, 6% raw ash and 1.12% lysine, without food or water restrictions. Blood samples were collected from all boars at the boar testing stations up to two weeks before slaughter. All animals were cared for according to laws and internationally recognized guidelines and regulations controlling experiments with live animals in Norway (The Animal Protection Act of December 20th, 1974, and the Animal Protection Ordinance Concerning Experiments with Animals of January 15th, 1996); according to the rules given by Norwegian Animal Research Authority.

The boars were harvested during a period of 26 months and the Landrace and Duroc boars were on average 143 and 156 days at 100 kg live weight, respectively. They were slaughtered 15 days later on average. Samples were taken from testicles on the slaughter line, snap frozen in liquid N2 and thereafter stored at -80°C. Blood samples for plasma suspension and DNA extraction were taken three days before slaughter. For androstenone measurements, samples of subcutaneous adipose tissue were collected from the neck region and stored at -20°C. The length of glandula bulbo urethralis was measured at the slaughter line. All boars, 2560 altogether, were included in the association study performed for the SNPs detected in 3β-hydroxysteroid dehydrogenases (HSD3B), Cytochrome P450 subfamily XIA polypeptide 1 (CYP11A1), Cytochrome b5 (CYB5A), and nuclear receptor co-activator 4 (NCOA4), while the 192 boars with most extreme levels of androstenone were selected for gene- and allele expression studies. For gene- and allele expression 6 and 9% of the most extreme animals in Landrace and Duroc were selected, respectively.

Analyses of Androstenone
The levels of androstenone were analyzed at the hormone laboratory at the Norwegian School of Veterinary Sciences (NVH) by a modified time-resolved fluoroimmunoassay [60], using antibody produced by Andresen.
Average androstenone levels were 1.17 μg/g (SD = 1.10) and 3.22 μg/g (SD = 2.69) for the entire Landrace and Duroc populations, respectively.

The 192 most extreme high/low androstenone boars in both Landrace and Duroc were divided into four groups consisting of 48 individuals. The 48 high androstenone Landrace and 48 low androstenone Landrace boars had average androstenone values of 5.62 μg/g (SD = 1.74) and 0.16 μg/g (SD = 0.04), respectively. Likewise, the 48 high androstenone Duroc and 48 low androstenone Duroc boars had average androstenone values of 10.59 μg/g (SD = 2.47) and 0.39 μg/g (SD = 0.14), respectively.

Nucleic acid purification and cDNA synthesis
Total RNA was isolated from testes using the M48 (Qiagen) and treated with TURBO DNA-free™ (Ambion, Huntingdon, UK) for removal of contaminating DNA. RNA quality and concentration were determined using Nano 6000 Nano LabChip® Kit on 2100 BioAnalyzer (both from Agilent Technologies, USA) and Nanodrop, ND-1000 spectrophotometer (Nanodrop Technologies, DE, USA), respectively. First strand cDNA synthesis was conducted using SuperScript™-II Rnase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA). 0.5 μg of total RNA from each testicle sample was used as template.

DNA was used for the association study was isolated from porcine leukocytes using the MagAttract DNA Blood Midi M48 protocol on the Bio-Robot M48 (Qiagen, Hilden, Germany). Concentration and quality were measured on a Nanodrop, ND-1000 spectrophotometer (Nanodrop Technologies, DE, USA) and on a 1420 Victor plate reader (Turku, Finland) using PicoGreen fluorescence (Molecular Probes, OR, USA).

MassARRAY Assay Design
The porcine gene sequences used to create a multiplexed 19-assay panel for gene- and allele-specific expression analysis via real-competitive PCR (rcPCR) and MassARRAY were annotated with respect to exon/intron boundaries. Assays were designed such that one of the PCR primers spanned an exonic junction (to ensure binding specificity to cDNA) using MassARRAY QGE Assay Design software v1.0 (SEQUENOM, San Diego, USA) and for non-polymorphic loci from each transcript. Amplicon sequences from these designs were then used as templates for a second round of assay design to create a multiplex containing the additional polymorphic loci used for allele-specific expression analysis of SNPs located in Cytochrome P450 subfamily 21 (CYP21), HSD3B, CYB5A, and typing of CYB5A isoforms. These designs were created using the iQSNP module of the MassARRAY SNP Assay Designer software v3.0 (SEQUENOM, San Diego, USA). Primers and competitors from this design are shown in Additional files 1 and 2.

Gene expression analysis
Real-competitive (rc) PCR gene expression analysis was used to study differential gene- and allele expression [62]. The method is based on the MassARRAY methodology, using the Quantitative Gene Expression (QGE) iPLEX system (Sequenom, San Diego, CA). The competitor, a synthetic DNA molecule matching the sequence of the targeted cDNA region at all positions except for one single base, served as an internal standard for each transcript. A 10-fold dilution of competitor was initially used over a wide range of concentrations to determine an approximate equivalence point (equal co amplification of target cDNA and competitor), followed by a 7-fold dilution of competitor from $4.04 \times 10^{-11}$ to $1.43 \times 10^{-19}$ M (a molar concentration of $1.00 \times 10^{-18}$ is equivalent to 3 competitor molecules) to achieve more accurate measurements. The cDNA and competitor were co-amplified in the same PCR reaction with PCR conditions 95°C for 15 minutes, followed by 45 cycles of 95°C for 20 second, 56°C for 30 seconds and 72°C for 1 minute, and finally 72°C for 3 minutes. After a clean-up step to remove unincorporated nucleotides, the PCR products were used as templates for the primer extension reaction. The preparation of iPLEX reaction cocktail mix and PCR were performed as described in the Sequenom application guide http://www.sequenom.com/.

Parallel PCR-reactions were performed for all samples and each of the products was printed with 2 replicates on a SpectroCHIP. The primer extension reaction generates short oligonucleotides with distinct masses for competitor and cDNA-derived products, and MALDI-TOF mass spectrometry analysis of these DNA fragments generated signals which were quantified based on peak areas for each respective assay. To detect and confirm differentially expressed genes, hypoxanthine guanine phosphoribosyltransferase 1 (HPRT) were used as a reference transcript or ‘housekeeping gene’.

Allele-specific expression analysis
Differential allele-specific expression was tested for two SNPs within the transcripts of each of the genes CYP21 and HSD3B. The CYP21 SNPs were located in exon 8 and 9 and the HSD3B SNPs were located in the 5’UTR and in exon 2 (all assays shown in Additional files 1 and 2). For the candidate gene CYB5A one SNP in the 5’UTR was tested for differential expression. Additionally, an assay was designed for differential transcription profiling of the CYB5A isoforms 1 and 2.

Genotyping
SNPs were genotyped using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) assays. Multiplex assays for use in the Sequenom MassARRAY system were designed using MassARRAY Assay Design software v3.0 (Sequenom, San Diego, USA). Primers for the genotyping are shown
in Table 5. Genotyping was done by the iPLEX protocol using manufacturer’s instructions (for complete details see iPLEX Application Note, Sequenom, San Diego). The MassARRAY Typer software was used for automated genotype calling.

Statistical analysis

**Gene expression**

Gene expression was quantified using the MassARRAY QGE software v3.4 (SEQUENOM, San Diego, USA) and TITAN version (1.0-13) [63] that runs in the R statistical environment. Titration of competitor concentration was used to determine the competitor concentration at which cDNA and competitor amplify equally well (EC50). The relative amounts of cDNA and competitor at each titration point were estimated by MassARRAY QGE software (Sequenom, San Diego, USA), using the mass spectra obtained. According to the Sequenom terminology the mass spectra are referred to as allele frequencies based on the calculation of the peak area ratios between extension products within each assay. The sum of all allele frequencies is equal to 1.0 for each assay [64]. For ordinary gene expression there is one frequency for the cDNA and one for the competitor. When measuring allele-specific expression, there is one cDNA frequency per allele, and one for the competitor. For the assays designed for detection of differential allele expression, the expression levels of the alleles were summed before estimation of the total gene expression level of the gene (for the assays designed for estimation of allele expression, see below). The raw data from the Genotype Analyzer Software (Sequenom) was imported into R, where the data was preprocessed in order to remove bad data points (i.e. when no signal was detected for neither cDNA nor competitor), and the median frequency of the printing replicates were calculated. To identify differentially expressed genes, the preprocessed data were analyzed using TITAN. In TITAN, the frequencies were first transformed using a log transformation ($y = \log_{10}(f/1-f)$), in order to obtain a linear relationship between frequencies and the competitor concentration (log10 scale). After that, a linear model was fitted per gene using the log10 concentration of the competitor as x and high/low androstenone levels as covariates. For each treatment, the model is interpolated in order to find the concentration where the amounts of cDNA and competitor are identical. Log fold changes are calculated as the difference between high and low androstenone on the log scale. The housekeeping gene (HPRT) was used for normalization. In the analysis using TITAN, default values of linear least squares polynomial regression and 4000 bootstrap replicates were used. Based on the bootstrap replicates, confidence intervals and p-values for the fold changes were calculated. The threshold for significance was set at $P < 0.05$. Details about the TITAN software are available from http://www.well.ox.ac.uk/~tprice/titan.

**Allele-specific expression**

The assays for allele-specific expression of SNPs in CYB5A and HSD3B were designed to amplify transcripts of the two alleles as well as the competitor. The frequencies of the two alleles were summarized in order to get total cDNA and thus total gene expression as described above. The relative expression of allele 1 was determined as the average ratio $f_1/(f_1+f_2)$ across the whole titration range, where $f_1$ and $f_2$ are the frequencies of allele 1 and 2, respectively. We used the average ratio across all titration points, since it is reasonable to assume that this ratio is constant across the titration range. In order to find whether the allele expressions were differentially expressed, the general linear model (GLM) procedure of the Statistical Analysis System (SAS) Version 9.1.3 [65] was used. Treatment (high/low androstenone) and alleles were included in the analyses as fixed effects. Results were considered to be significant at $P < 0.05$.

**Association study**

Associations between androstenone and the four candidate genes HSD3B, CYP11A1, CYB5A and NCOA4 were evaluated using the GLM procedure of SAS Version 9.1 [65]. Models were fitted to identify other significant environmental and genetic effects apart from the genotypes, by elimination of non-significant effects. Levels of androstenone in fat were log-transformed to normalize the distribution of observed values. Analyses were carried out separately for the two populations using the following statistical model:

$$Y_{ijkl} = \mu + \text{sire}_i + \text{gene}_j + \text{hys}_{k} + \text{bulbo}_l + \epsilon_{ijkl}$$

where $Y_{ijkl}$ is ln(ppm levels of androstenone in adipose tissue) of animal $j$, offspring of sire $i$; gene $j$ is the fixed

| SNP         | SNP-Localization | Forward primer | Reverse primer |
|-------------|------------------|----------------|----------------|
| CYB5A_8UTR  | 8UTR             | CTCTGTTCCGGCTCATCTCTG | ATACCTCACGGCTTTTGTCGG |
| HSD3B_15UTR | 15UTR            | TCCCCAGTTTGTCTGCTTC | CCATCCACGGCTATGCTAAAC |
| HSD3B_271exon2 | 271, exon2      | TCATCCACACTGCTCTTATC | TTGACCTCATGACCGTCTC |
| CYP11A1_150exon1 | 150, exon1  | TGATCCTCCACTAAACCCC | ACCGTACAGGTTAATCCAGC |
| NCOA4_3UTR  | 3UTR             | TGCAGTCCCCAGTGCTATTAC | GTTCTAAATGGTTACTGGGG |
effect of the candidate gene genotype; hysk is the fixed effect of herd/year/season, and bulbo is the random effect of glandula bulbo urethralis. Sire was included as fixed effect in the model to ensure that the genotype effects were not confounded with selection in the sires. The length of glandula bulbo urethralis is taken into account because it is highly correlated with the level of sexual maturation in boars [66]. Least squares means were estimated for each genotype and overall F tests were used to determine level of significance. Back-transformed least-squares mean without further corrections are presented in Table 4, thus giving the medians in the original skewed distributions. Results were considered to be significant at P < 0.05. Standard errors are supplied in the Table 4.

Furthermore, a chi-square test was conducted to test the association between SNPs within candidate genes and compounded related to boar taint and reproduction. BMC Genetics 2009, 10:32.

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Authors’ contributions

EG conducted the molecular work, performed statistical analyses and drafted the manuscript. IB did statistical supervision and carried out the programming involved. MM was involved in the molecular work. PO carried out the assay design for gene- and allele expression analyses. SL was involved in planning the project, provided laboratory facilities and took part in writing the paper. All authors read and approved the final manuscript.

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