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Population and systems genetics analyses of cortisol in pigs divergently selected for stress

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Kadarmideen HN, Janss LL. Population and systems genetics analyses of cortisol in pigs divergently selected for stress. *Physiol Genomics* 29: 57–65, 2007. First published November 28, 2006; doi:10.1152/physiolgenomics.00144.2006.—This study presents a systems genetic analysis on the physiology of cortisol in mice and pigs with an aim to show the potential of a comprehensive computational approach to quickly identify candidate genes and avoid a costly whole-genome quantitative trait locus (QTL) mapping. Population genetics analyses were performed on measurements of cortisol from a pig selection experiment. Expression QTL were mapped and gene networks were built using gene expressions for *Crhr1* (corticotrophin-releasing hormone receptor) gene and single nucleotide polymorphisms from public mouse data. Results from mouse data were used to infer potential candidate regulatory genes involved in pig cortisol regulation, using a comparative or translational systems genetics approach. The pig data used were from a 10-yr divergent genetic selection experiment, providing data on 417 individuals. Population genetics analysis showed that cortisol is highly genetically determined with heritabilities of 0.40–0.70. Furthermore, a major gene with an additive effect of 86 ng/ml is segregating. Genetical-genomics investigations revealed two trans-acting eQTL for *Crhr1* gene expression on chromosomes 2 and 13. Candidate gene search under trans-eQTL peaks yielded 63 genes for *Crhr1* expression phenotypes. Functional links for *Crhr1* genes with other genes/proteins in the gene network using mouse data were shown for the first 10 statistically significant genes involved. Results show translational or comparative systems genetics approaches reduce costs and time in large-scale genetics and "-omics" investigations. This is the first study to report a strong genetic basis for cortisol physiology using a systems approach.

heredity; expression quantitative trait locus

CORTISOL, THE TERMINAL PRODUCT of the hypothalamo-pituitary-adrenal axis (HPA) affects protein, carbohydrate, and fat metabolism and, hence, mechanisms of energy storage and release. Circulating cortisol levels are directly or indirectly related to many behavioral, physiological, and nutritional diseases/disorders in mammals. Cortisol's metabolic effects are, in general, opposite to those of insulin and tend to increase plasma glucose concentration, which leads to obesity and diabetes, the most devastating disease in human populations. Besides affecting carbohydrate metabolism, glucocorticoids also accelerate protein breakdown to be cleared by kidneys and affects heart function (13). In addition to the metabolic effects of cortisol, there is a strong association between cortisol levels and behavior in mammals. Cortisol secretion is individually variable, depending on the individual sensitivity to stress, which is well documented in the literature (6, 8, 11). However, inconsistent relationships were found between submissive and aggressive behavior and the cortisol levels in plasma or saliva in different species. For instance, low salivary cortisol in adolescent boys was associated with lack of self-control and increased aggressive behavior later in life (26). In monkeys, plasma cortisol was positively correlated to the amount of aggression observed. This corresponds to some finding in pigs: McGlone (24) found that urine from mildly stressed pigs applied to the skin of other pigs caused increased aggression in these animals. In another study, baseline adrenocortical activity was higher in subordinate than in dominant animals (9), and increased cortisol levels have even been described in middle-ranking sows (33). Thus, the effect of cortisol on the establishment of dominance status in pigs is still not well understood.

Although several physiological and nutritional experiments and observations were made on cortisol, the population genetics of this substance is rarely known in animals and humans. In particular, there is no information on the heredity of cortisol levels, on the possible segregation of monogenic factors or major genes affecting this trait, on DNA polymorphic sites [e.g., single nucleotide polymorphisms (SNPs)], or on genomic regions regulating the expression of cortisol-related genes. The aim of this study is to present a comprehensive systems approach to these genetic questions, therefore coined "systems genetics," using a combination of proprietary and public data sources: pig data from a selection experiment conducted at the Federal Institute of Technology (ETH) of Switzerland and public mouse data on gene expressions and SNPs. In this comprehensive systems genetics analysis we will investigate heritability of these traits (e.g., 17, 19), the possible segregation of monogenic hereditary factors in the pig pedigrees using Bayesian segregation analysis (e.g., 14, 15, 18), and potential candidate regulatory genes for cortisol-related gene expressions. For identification of candidate regulatory genes in pigs, we use mouse data in a "comparative or translational systems approach" by mapping expression quantitative trait loci or eQTL (e.g., 20) affecting the expression of corticotrophin-releasing hormone receptor 1 (*Crhr1*), by building gene networks, and by searching for candidate genes affecting cortisol physiology. The overall aim of this study is to show the potential of "in silico" systems genetics within and across species as a computational tool to reduce sets of candidate genes and avoid costly lab experiments such as whole genome quantitative trait locus (QTL) mapping studies.

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MATERIALS AND METHODS

Cortisol Selection Experiment

The animal experiment research station of the ETH, located at Chamau, Zug, conducts experiments on genetics, physiology, and nutritional characteristics of various animals. The experimental procedures of the farm followed the Swiss Law on Animal Protection and were approved by the Committee for the Permission of Animal Experiments of the Canton of Zug, Zug, Switzerland. The cortisol experiment was started in 1995s to study the possible differences of the HPA axis in two lines of domestic pigs (Swiss Landrace pigs) with striking differences in response to various stressors such as corticotrophin-releasing factor and/or vasopressin administration, physical exercise, and injection of insulin. These two lines were raised by a systematic selection from a single family according to a selection index including daily weight gain and back fat layer thickness (9).

During the urine sample collection period, the animals were housed individually in the metabolic cages for 48 h. Cages were equipped with a permanent nipple watering place, feeding dispenser, and steel grid floor with an underlying large funnel to collect feces and urine separately. Animals were fed ad libitum, and feed intake was measured for individual pigs. Urine samples were taken on the second day (after acclimatization for 24 h), and cortisol level was investigated. Urinary cortisol was measured using the Cortisol kit (catalog no. TKCO1 DPC, Los Angeles, CA) with Cobra 11 Auto Gamma instrument (Perkin Elmer, Schwerzenbach, Switzerland). Before and after the sample collection, pigs were housed in group pens in two stables (one litter per pen, which was between 8 and 14 animals). All stables were climate controlled for temperature and humidity. It was likely that water intake during the experiment (or just playing with the water nipple) influences the cortisol level in the urine; therefore, we also measured urinary creatinine levels. The average water consumption (in normal housing conditions) is ~1–2 l per animal and day for animals with 10–20 kg live weight and 3–4 l per animal and day for animals with 20–50 kg live weight. Urinary creatinine was measured using the Roche kit (no. 114892991-216) with Cobas Mira Roche S Instrument (Roche Diagnostic, Rotkreuz, Switzerland).

The dataset for population genetic analyses (to estimate polygenic heritabilities and correlations) consisted of 417 animals of both sexes from 25 sires and 62 sows. For segregation analyses, the dataset was further edited to remove those animals with both parents unknown and no progeny of their own. Such animals do not contribute to (segregation) genetic analyses but may help only in estimation of environmental effects. Cortisol data after this editing were available on 299 pigs, with pedigrees for these pigs containing a total of 484 animals. The data consisted generally of small full-sib groups nested within half-sib sire groups.

Analysis of Experimental Data

Estimation of heritabilities for cortisol. An individual animal model accounting for relationship among the animals was used. The generalized linear mixed model (e.g., 19) was fitted to cortisol and consisted of sex, year, and weight at measurement, and feed intake as "fixed systematic environmental effects" and animal's own genetic effect and residuals as "random" effects. In the statistical model, creatinine level was used as a covariate to account for the fact that it affects cortisol levels. Alternatively, a ratio of cortisol to creatinine was also analyzed as a different trait. Estimation of variance and covariance components of genetic and residual effects for cortisol and ratio of cortisol to creatinine was performed by a software package ASReml, which is based on residual maximum likelihood methods. Assumptions and implementation were the same as those described in Kadarmideen et al. (19).

Exploratory analysis for segregation of major gene for cortisol. An exploratory analysis looking at variance heterogeneity between sire families was performed for cortisol. A Bartlett's test was performed on the nine largest half-sib groups, testing for variance heterogeneity using R (http://www.r-project.org). The Agostino test for skewness and Shapiro-Wilk test for checking Normality within each sire group were also performed using R.

Bayesian segregation analysis of cortisol. Using all data and additional pedigree, a Bayesian segregation analysis (BSA) was performed using Monte Carlo Markov chain techniques called Gibbs sampling, for instance as recently described by Kadarmideen and Janss (18) for osteochondral diseases in pigs. The statistical model for BSA adjusted data for all known systematic environmental effects as was done for the estimation of heritabilities. This included adjustment of cortisol data for creatinine levels. This implementation used blocked sampling of genotypes of each sire and its final progeny to prevent poor mixing in larger families (15). For further validation of a Mendelian factor identified by BSA, transmission probabilities were estimated as exemplified in Kadarmideen and Janss (18). In the post-Gibbs analysis of the samples, an analysis of variance was used to check for equality of chains and the dependency of the samples kept within chain (convergence of chain). The marginal posterior means were used as point estimators of the parameters. The highest posterior density regions (HPDR) were determined for all model parameters, which includes the part $(1-\alpha)$ of the probability mass about the smallest possible region of sampled parameter values. Chains were run with 15,000 cycles burn in, then output every 50th sample until 65,000 cycles, with four repeated chains. We further examined distribution of residuals from the polygenic model as well as BSA mixture model to assess the fitness of model to data. Regressions of absolute residuals on predicted values were computed and skewness was tested by the Agostino test in R.

Comparative Systems Genomics of Cortisol

Transcriptome linkage and eQTL mapping on mouse genome. A genetical-genomics investigation was first conducted using microarray and linkage data from a BXD set of recombinant inbred (RI) mouse strains which are accessible on WebQTL platform (http://www.genenetwork.org/). Genetical-genomics (20) links the gene expression levels measured using microarrays with genetic markers on the linkage maps to identify genomic regions containing a QTL or mutation that affects/regulates gene expression phenotypes or abundance of gene transcripts (i.e., eQTLs). These eQTLs responsible for variation in gene expression could map within the gene itself (cis-acting eQTL or cis-eQTL) or map to some other location on the genome (in which case they are called trans-acting eQTLs or trans-eQTL). A whole genome scan included 19 autosomes and X chromosome for identifying these cis-eQTL and trans-eQTL. The population of RI strains used here has been extensively sequenced and is known to differ at ~2.0 million SNPs. Interval eQTL mapping was conducted for *Crhr1* gene using the INIA Brain mRNA M430 (Jan 2006) RMA database (Trait ID 1418810_at_A). Further details on source dataset are at http://www.genenetwork.org by querying Trait ID. The software and statistical methods used are described in Wang et al. (30) and Kadarmideen et al. (20).

Gene/Protein network analyses for Crhr1. In this section, we investigate possible functional interaction of *Crhr1* genes with other genes/proteins in mouse using STRING software (Search Tool for the Retrieval of Interacting Genes/Proteins; http://string.embl.de/). STRING produces edges or predicted functional links that consist of up to eight lines, with each line/color representing a particular type of evidence. This evidence comprises neighborhood, gene fusion, co-occurrence, co-expression, experimental evidence, databases, text mining, and homology. Each one of the evidence types was given a score, and a total score was computed based on all available evidence. As illustrated in Kadarmideen et al. (20), we might expect generalizability of gene network across species because effects of pathways are more replicable than exact gene effects. Furthermore, this comparative gene network analyses can be accelerated by other -omics

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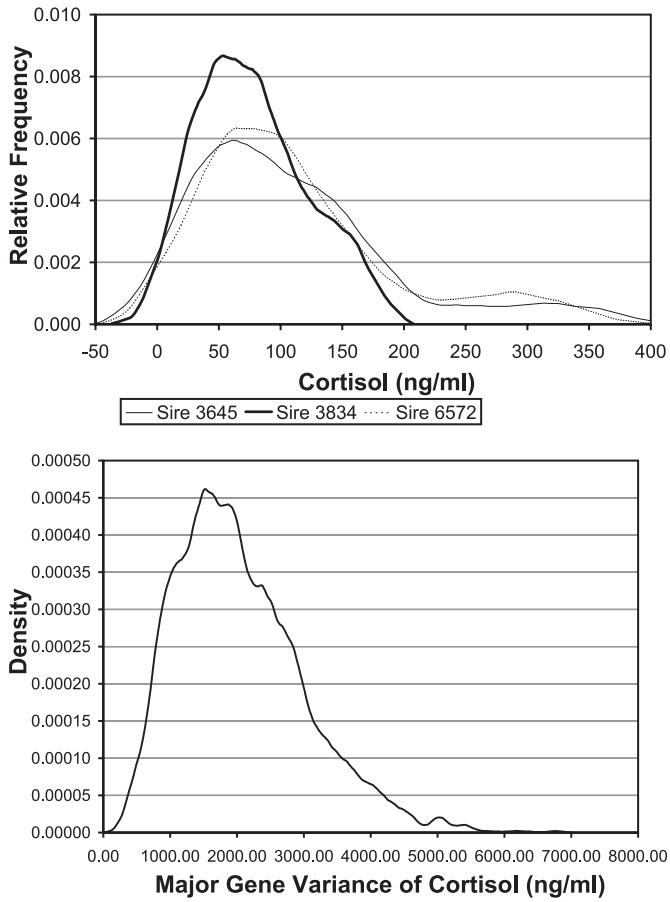

Fig. 1. *Top*: distribution of cortisol in progeny groups of three sires. *Bottom*: marginal posterior distributions of major gene variance for cortisol (ng/ml).

technology, such as genetical-genomics and proteomics, which will add extra evidence to validate the plausible gene network.

RESULTS

Heredity of Cortisol

The mean (SD) of cortisol was 120.68 (89.18) ng/ml. The weight, sex, and feed intake significantly ($P < 0.10$) affected cortisol levels. Regression coefficient (SE) for feed intake was −55.36 (22.60), meaning that for every kg increase in feed intake, the cortisol level decreases by 55.36 ng/ml. Compared with male pigs, female pigs have 40.22 ng/ml more cortisol. Each kg increase in weight was associated with 2.02 ng/ml increase in cortisol. The estimated effect of creatinine on cortisol levels was that there was an increase of 32.75 ng/ml cortisol for every mg/ml increase in creatinine. The variability of animals' genetic value as deviation from the population mean (so-called estimated breeding values or EBVs) was high: the minimum and maximum EBV for cortisol was −132.20 and 248.60 ng/ml and SD was 56.05 ng/ml. The estimated polygenic heritability h^2_p (SE) of cortisol was 0.40 (0.02); h^2_p nearly doubled to 0.70 (0.01) when cortisol levels were adjusted for creatinine levels. However, the h^2_p for the ratio of cortisol to creatinine was lower than that for cortisol levels alone (0.17 with SE of 0.01). The genetic and phenotypic correlations (with SE) between cortisol and this ratio were 0.28 (0.01) and 0.11 (0.01), respectively. This indicates that raw cortisol is quite different from analysis of the same trait as a function of creatinine. Analysis of residuals from this standard polygenic model for heritability showed a deviation from normality of the residuals with a skewness of 1.44 (highly significant $P < 0.0001$) and with a relationship between absolute residual and predicted value (regression of 0.58 and R^2 of 0.26), indicating that the polygenic model may not adequately fit the data.

Exploratory Analysis for the Presence of a Major Gene

For an exploratory analysis of the cortisol data, smoothed histograms were produced for the largest three sire groups as shown in Fig. 1, *top*. Figure 1 shows a right-skewness in two sire groups that is completely absent in 3rd sire group (sire ID 3834). For the sires showing right-skewness (thin and dotted lines) in Fig. 1, the distributions even tended to slight bimodality, suggesting that this is not ordinary data skewness but more likely a genuine mixture. Summary statistics for the nine largest sire groups for cortisol distribution is given in Table 1. This table indicates that SDs of cortisol in progeny groups deviate up to a factor of 2 between sires (which correspond to up to a factor of 4 in variances). Bartlett's test, testing for heterogeneity of variance was applied to these nine largest sire groups, resulted in a highly significant test statistic ($P < 0.0001$), indicating presence of heterogeneity between sires for cortisol. The coefficient of variation (CV) for different sires showed that CVs are far from constant and range from 40 to 75%; this indicates that in the high-variance sire groups, SD has increased relatively more than the mean and that differences in SD cannot be explained with a constant mean-SD relationship. This supports the notion that there are genuine differences between sires, which could be caused by the segregation of a major gene. Hence, based on the apparent presence of two types of sires (those that do and those that do not show a segregation into two groups), the most plausible major gene hypothesis would then be presence of a recessive cortisol-increasing major gene. Sires not showing the segregation of the extreme phenotype would then be homozygous noncarriers of the recessive allele; the others would be a mix of carriers and double carriers.

BSA

Parameter estimates for a major gene model including polygenic background genes and additive and dominance effect at

Table 1. *Summary statistics for progeny data from 9 largest sire groups*

Sire	*n*	Mean	SD	Agostino Skewness Test (*P* value)	Shapiro-Wilk Normality Test (*P* value)
1763	13	88.95	36.05	0.2018	0.0450
655	12	62.39	36.55	0.3916	0.1704
3834	37	76.93	42.51	0.3750	0.1016
1007	17	99.50	49.20	0.3354	0.1995
5030	15	110.63	65.34	0.1330	0.0190
7015	16	123.01	72.75	0.1922	0.0274
6572	33	112.22	76.28	0.0352	<0.0001
3645	51	108.87	79.60	0.0146	<0.0001
6592	21	114.18	85.69	0.0711	0.0016

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Table 2. *Summary of parameter estimates for segregation analysis of urinary cortisol (ng/ml) given as PSD and left and right bounds of the HPDR$_{95}$*

	Normal BSA			Recessive BSA Model		
	Mean	PSD	HPDR$_{95}$	Mean	PSD	HPDR$_{95}$
Error variance	1122	253.0	628.4–1622	1089	251.21	618.02–1599.3
Polygenic variance	1257	379.5	551.1–2013	1305	386.62	592.75–2088.48
Major gene variance	2045	961.9	421.6–3992	1980	888.10	430.06–3684.19
Polygenic heritability	0.52	0.12	0.283–0.747	0.54	0.12	0.301–0.771
Major gene heritability	0.44	0.10	0.250–0.621	0.49	0.01	0.35–0.64
Frequency of cortisol$^+$ allele	0.256	0.067	0.131–0.388	0.260	0.064	0.137–0.384
Additive effect (a)	85.30	7.204	70.86–99.17	86.91	5.89	75.55–98.48
Dominant effect (d)	96.90	12.85	−123.2–−72.87	−86.91	5.89	−98.40–−75.47
a-|d|	11.60	15.10	−42.78–16.72	0.00	0.00	0.00

The parameter major gene variance includes additive and dominance variance at the major locus. Frequency of cortisol$^+$ allele is a recessive allele increasing cortisol levels. PSD, posterior mean and standard deviation; HPDR$_{95}$, 95% highest posterior density regions.

the major locus are presented in Table 2. BSA results confirm presence of a recessive, cortisol-increasing major gene (see negative estimate for dominance), with a frequency of the recessive (increasing) allele of 0.256. The posterior distribution of major gene variance for cortisol is in Fig. 1, *bottom*. This major gene component is significant as the HPDR$_{95}$ for its variance does not include 0. Partial dominance can be rejected, hence simple complete dominance can be accepted, because the HPDR$_{95}$ for the difference between the additive effect and absolute dominance effect covers 0. A recessive BSA model forcing complete dominance (Table 2) estimated an additive effect of 86.9 [with posterior standard deviation (PSD) of 5.89] and with a frequency of the recessive allele of 0.260. For the mixed inheritance model the fit was clearly improved based on analysis of residuals: skewness in residuals was low (0.43, nonsignificant) and a relationship between absolute residual and predicted value was virtually absent (regression of 0.03 and R^2 of 0.01). This indicates that the mixture distribution as postulated in the segregation analysis is explaining well the nonnormality and the differences between the sires observed in the raw data.

For final validation of evidence for major genes for cortisol, Mendelian transmission probabilities were estimated, fixing hyperparameters to those of a model with complete dominance. PSD for transmission probabilities to inherit an A allele from AA, AB, and BB genotypes were 0.84 (0.01), 0.58 (0.01), and 0.31 (0.17), respectively. The left-right bounds of HPDR$_{95}$ for these corresponding probabilities were 0.703–0.978, 0.436–0.726, and 0.000–0.600. These estimates show significant differences between transmission probabilities (the HPDR$_{95}$ of the first and last transmission probability do not overlap), indicating that an environmental hypothesis for the inferred mixture distribution for cortisol can be rejected, and hence a genetic factor to be associated with this mixture can be accepted. Moreover, estimated transmission probabilities are close to Mendelian so that a simple autosomal recessive gene is the most plausible mode of inheritance for this genetic factor. Statistically, the HPDR$_{95}$ coverage for one of the three transmission probabilities is just excluding the expected Mendelian value, which also leaves some indication for a genetic factor other than simple Mendelian.

Systems Genetic Analysis of Cortisol

Transcriptome and eQTL mapping. Figure 2 shows results from whole genome (19 autosomes and an X chromosome) interval mapping of *Crhr1* expression phenotypes (Fig. 2, *top*) and the physical mapping of Chr 13 only (Fig. 2, *bottom*). Peaks in the heavy blue line [likelihood ratio statistic (LRS)] show locations of a putative eQTL, and the yellow histogram beneath it shows frequent peak location for bootstrap samples. Permutation-based significance thresholds (LRS) are given by horizontal dashed lines; the upper line corresponds to a genome-wide 5% significance threshold. A positive additive coefficient (green line) indicates that DBA/2J alleles increase trait values. In contrast, a negative additive coefficient (red line) indicates that C57BL/6J alleles increase trait values, in this case, expression of *Crhr1* gene. The whole genome eQTL linkage map (Fig. 2, *top*) shows two statistically significant eQTL regions, one on Chr 2 and the other on Chr 13 affecting expression of *Crhr1*. The eQTL on Chr 2 only reached a suggestive threshold (10.63), while eQTL on Chr 13 was above the suggestive threshold, but none were over the genome-wide significance threshold value of 17.10. The sequence site of *Crhr1* itself is shown by a triangle on Chr 11 at 103.99 Mb (Fig. 2). Hence, *Crhr1* gene is likely trans-regulated than cis-regulated. In Fig. 2, *top*, each point on the *x*-axis of LRS profile is a marker, the exact identity of which is readily seen in WebQTL. Table 3 provides results from the marker regression report and lists SNP marker loci that exceeded the 5% LRS value, together with their additive effect on expression of the *Crhr1* gene. Here, the additive effect is half the difference in the mean phenotype of all cases that are homozygous for one parental allele at a marker minus the mean of all cases that are homozygous for the other parental allele at this marker.

Candidate gene search. The physical map of Chr 13 where the eQTL peak was found to be >5% LRS is given at the bottom of the Fig. 2. This trans-eQTL region on Chr 13 can be viewed as candidate regions that contain regulatory genes for the *Crhr1* gene. This trans-eQTL peak with highest bootstrap frequency spans 3 Mb in length, ranging from 51 to 54 Mb. Marker loci flanking this eQTL peak are identified as rs6209128 at 51.33 Mb and rs8273881 at 54.00 Mb. With the search for genes within this physical region of 3 Mb, we identified 63 genes (names not shown). These results can be

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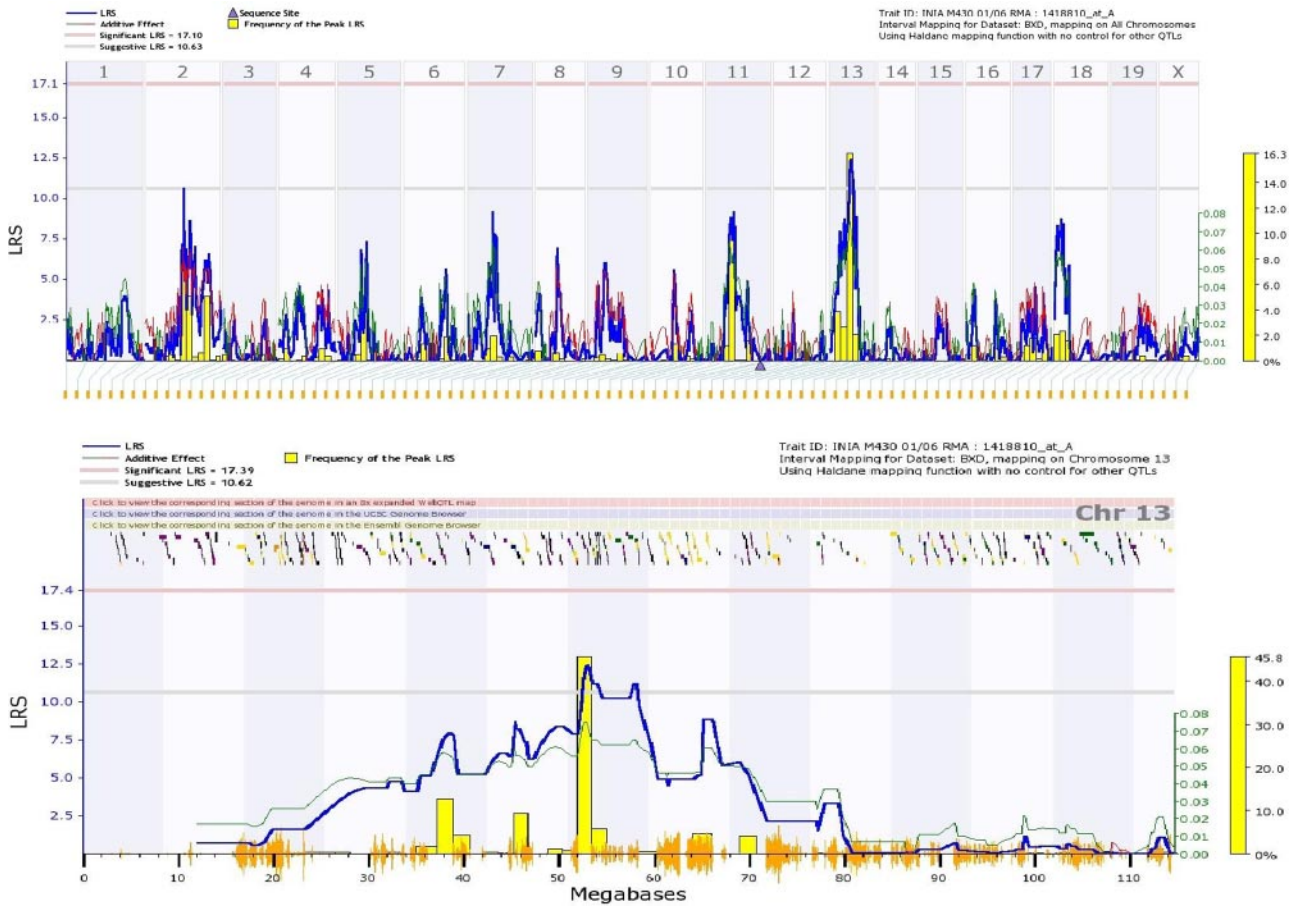

Fig. 2. *Top*: whole genome scan for expression quantitative trait loci (eQTLs) influencing gene expression of corticotrophin-releasing hormone receptor 1 (*Crhr1*) on Chr 11 at 103.99 Mb using Mouse BXD recombinant inbred line data. *Bottom*: physical maps showing eQTLs affecting expression of *Crhr1* (on chromosome 13). LRS, likelihood ratio statistic.

used for further inference on regulatory network and comparative eQTL mapping, including investigation on possible sequence variations and candidate gene identification, within this region.

Gene/Protein network for Crhr1. The results of the STRING analyses are given in the form of summary networks in Fig. 3. The exact names of genes and their strength of functional association (a score) with a *Crhr1* gene are given in Table 4. The predicted functional links for *Crhr1* gene (in red box of

Table 3. *Additive effect of SNPs that exceeded the 5% significance threshold value of LRS of 10.63, on the expression of Crhr 1 gene*

LRS	Chr	Marker Locus	Additive Effect
10.674	2	rs13476676	−0.062
10.674	2	rs3727022	−0.062
11.205	13	rs13481817	0.065
11.205	13	rs8273881	0.065
11.205	13	mCV22624058	0.065
11.205	13	rs4184167	0.065
11.205	13	gnf13.057.501	0.065

A positive additive effect indicates that DBA/2J alleles increase trait values. Negative additive effect indicates that C57BL/6J alleles increase trait values. SNP, single nucleotide polymorphism; LRS, likelihood ratio statistic; *Crhr 1,* corticotrophin-releasing hormone receptor 1 gene; Chr, chromosome.

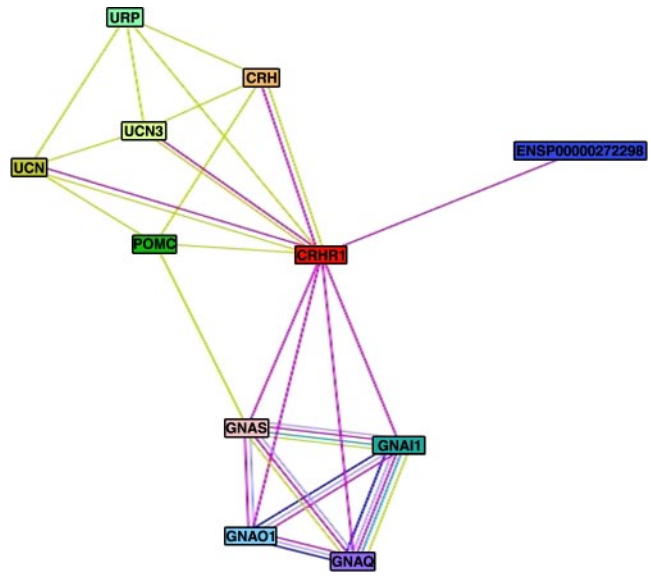

Fig. 3. Gene networks showing functional association of *Crhr1* with other genes (and proteins) produced using multiple pieces of evidence. Each line of evidence is colored as follows: neighborhood (dark green), gene fusion (red), co-occurrence (blue), co-expression (purple), experiments (deep pink), databases (sky blue), text mining (green), and homology (light blue).

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Table 4. *Predicted gene networks of Crhr1 genes, limited to first 10 genes with highest functional association score (from 0.000 to 1.000), based on several prediction methods*

First 10 Genes Involved in Network of *Crhr 1* in Humans	Type of Evidence	Score
1. CRH: Corticoliberin precursor (Corticotropin-releasing factor) (CRF) (Corticotropin releasing hormone)	Experiments Text mining	0.999
2. UCN: Urocortin precursor	Experiments Text mining	0.994
3. UCN3: Urocortin III precursor (UCN III) (Stresscopin)	Experiments Text mining	0.953
4. POMC: Corticotropin-lipotropin precursor (Pro-opiomelanocortin)	Text mining	0.932
5. URP: Urocortin II precursor (UCN II) (Stresscopin-related peptide) (Urocortin-related peptide)	Text mining	0.825
6. GNA11: Guanine nucleotide-binding protein G(i), alpha-1 subunit (Adenylate cyclase-inhibiting G alpha protein)	Experiments Text mining	0.747
7. GNAO1: Guanine nucleotide-binding protein G(O), alpha subunit 1	Experiments Text mining	0.747
8. ENSP00000272298: Calmodulin	Experiments Text mining	0.747
9. GNAQ: Guanine nucleotide-binding protein G(q), alpha subunit	Experiments Text mining	0.747
10. GNAS: Guanine nucleotide-binding protein G(S), alpha subunit (Adenylate cyclase-stimulating G alpha protein)	Experiments Text mining	0.747

Score reflects prediction methods used in forming networks; each gene in the network is given a functional association score based on the following evidence, each weighted according to importance. They are neighborhood, gene fusion, co-occurrence, co-expression, experiments, databases and text mining and homology. The maximum score is 1.000.

Fig. 3) with other genes could be a single or multiple lines (up to 8) with one color for each type of evidence that support that link. Each piece of evidence in Fig. 3 is indicated by colored lines as follows: neighborhood (dark green), gene fusion (red), co-occurrence (blue), co-expression (purple), experiments (deep pink), databases (sky blue), and text mining (green) and homology (light blue).

Among the first 10 genes (or their gene products) shown in the network, all genes except POMC and URP had evidence accumulated from both "text mining" (green line) and "experiments" (deep pink). Experimental evidence means that there was original experimental and biochemical evidence to prove that the gene or protein being interrogated (i.e., *Crhr1*) has functional association with the other genes in the network. Text-mining evidence means that genes in the network are jointly mentioned with interrogated gene (i.e., *Crhr1*) in published texts or abstracts. All evidence, including experimental and text mining, is supported by references to original sources in which it appeared and can be readily viewed in STRING databases. In this case, POMC and URP had only "text-mining" evidence. The POMC gene, however, had total score of 0.999 out of 1.00 with CRH (corticotrophin releasing hormone) and 0.932 with CRH receptor gene, *Crhr1*. The calmodulin gene has no link with any gene in the network except *Crhr1* with a score of 0.747. Another observation that is made from the network in Fig. 3 is that four genes, GNAI1, GNAO1, GNAQ, and GNAS, all have more evidence (as seen by 2–5 different colored lines linking these genes) showing that they are functionally closely related to each other. The evidence that relates *Crhr1* gene with each one of these four closely related genes has an equal score of 0.747 (Table 4). While this "network" analysis is an individual method on its own right to find functionally associated gene, intuitively, one can make a link between this proteomic information with eQTL information: that is, by using these gene networks, one can cross-validate detected regulatory loci or trans-eQTL peaks on chromosomes (Chr2 and Chr13 for *Crhr1*) and ascertain whether those eQTLs indeed correspond to physical regions of these regulators in Table 4 or Fig. 2 (that is, checking whether peaks in Fig. 2 correspond to any one of the regulatory gene network nodes from pathway analyses of these genes in Fig. 3).

Possible Role of Candidate Genes From Mouse for Human and Pig Cortisol Levels

In this section we conduct text mining to ascertain whether ten genes shown by STRING gene network analyses in mouse (Table 4) have any influence on cortisol gene effects in pigs (and human). First, the CRH or corticotrophin releasing factor (CRF) is the principal regulator of the stress response, which has receptor genes expressed in several organs including brains and heart (here we used *crhr1,* which is the type 1 CRH receptor). UCN I (urocortin I precursor), UCN II (urocortin I precursor, which is a stresscopin-related petide), and UCN III (stresscopin) were among the 10 genes. It has been shown that this gene group is a member of the CRH peptide family and is found in many discrete brain regions and that it influences feeding, anxiety, and auditory processing behaviors (28). Furthermore, these genes have been shown to modulate endocrine, autonomic, and behavioral responses to stress, as well as a range of peripheral (cardiovascular, gastrointestinal, and immune) activities (22). The stresscopin (and stresscopin-related peptide) genes encode specific ligands for the type 2 CRH receptor (*crhr2*), which mediates stress coping responses during the recovery phase of stress. Wardlaw (32) reported a relationship between POMC gene and human obesity. He stated that POMC-deficient patients and mice are obese despite having profound secondary adrenal insufficiency and that POMC neurons in the hypothalamus are important regulators of energy homeostasis. Calmodulin gene has been associated with stress as per our STRING analysis possibly due to its role in signal transduction and the synthesis and release of neurotransmitters, which are heavily involved in behavioral responses. The GNAS and GNAQ genes were also shown to be

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related to stress responses in human by Lu et al. (23) and Alfonso et al. (1), respectively. These foregoing experiments conducted on individual genes in the gene network indicate that it is worthwhile to investigate these genes also in pigs in a targeted genetical genomics/systems genetics experiments.

DISCUSSION

Population Genetics of Cortisol

The underlying idea of population genetic analyses of cortisol was that the interindividual variation in cortisol response to stress stimuli is of genetic origin. Specifically, the additive genetic values or EBVs reflect the genetically transmissible amount of cortisol from parental generations to offspring generations. The wide range of an animal's genetic values for cortisol and significant heritability (0.40–0.70) indicates that genetically best and worst animals for stress can be distinguished and can be managed and bred accordingly. Individual genetic variation and transmission (heritability) has been reported in humans (2, 26). For instance, Kupper et al. (21) reported a heritability of 32–34% for salivary cortisol level in human twin studies. However, there is a large genetic variation in children: heritability was highest (60%) for cortisol levels during the sample taken ~45 min after awakening (3). In animals, published estimates of urinary cortisol levels were not found; however, our heritability estimate of 0.40 or 0.70 is closer to human studies. Cortisol is known to be a physiological indicator of stress (5, 29); it affects growth and meat quality in animals (5, 10); and also for stress, heritable components have been reported in humans (7) and animals (4). The findings on heritability of cortisol in pigs stress the importance of cortisol in the breeding for improved welfare in pigs whenever direct measures are not available.

Segregation Analysis of Cortisol

The results of segregation analyses suggest that cortisol levels are determined by a mixture of genes with large and small effects. Multiple validation of evidence for major gene for cortisol involved three steps, namely, exploratory analysis, BSA, and estimation of Mendelian transmission probabilities. Furthermore, we also studied the distribution of residuals in the polygenic and major gene model, which indicates that the major gene model is adequate for these data and has normal residuals, but a polygenic model leaves skewed and nonnormal residuals. This shows that nonnormality observed in some sires can be explained well with a mixture distribution. These multiple validations of evidence confirmed that cortisol has multifactorial inheritance with a common major gene and that the genetic nature of this segregating factor is Mendelian, with transmission probabilities close to those for a simple biallelic autosomal Mendelian factor. This is the first study to report a major gene effect for cortisol in pigs and hence cannot be compared with literature. Even for other farm animals, not many QTL or major genes for behavioral traits, especially for stress, have been identified. In a study of cortisol as a stress hormone, Vasilyeva et al. (27) reported the effect of a major gene in inheritance of cortisol content in blood of domestic silver foxes (*Vulpes vulpes*) and indicated that breeding for domestic behavior is accompanied by selection of individuals homozygous with respect to recessive genes controlling the cortisol level. In humans, Feitosa et al. (8) estimated heritabilities of 38 and 31% for white and black samples, respectively. Overall, monogenic factors identified by this segregation analysis of the pig family may be useful in linkage studies to detect stress susceptibility loci or QTLs, not only in pigs but also in other animal models. In fact, Desautes et al. (6) found a highly significant QTL effect for cortisol level on chr 7, explaining 7–20% of the phenotypic variance. This genomic validation of existing QTL for cortisol in pigs also supports the phenotypic evidence for a major gene reported in our pig population.

Systems Genetics of Cortisol in Mouse

The systems genetics approach is a result of merger of systems biology with the study of genetic variation of gene expressions. In this investigation, we showed the potential of "comparative or translational systems genetics" in that the list of candidate regulatory genes affecting cortisol in mouse would form a basis for experiments specifically targeted to test their influence on cortisol levels in pigs.

The eQTL mapping showed two trans-eQTL regions that formed the basis for drawing a gene network to indicate that they contain regulatory genes of cortisol-related genes. A candidate gene search within these two trans-eQTL regions provided a list of major candidate regulator genes for *Crhr1* gene. This is useful and time-efficient in that, by comparative approaches, one can use these candidate genes and eQTL results from the mouse and apply them in pig or human eQTL mapping or directly target these regions for candidate gene searches in a different species. In fact, it has been shown that there is a clear conservation between mouse and human QTLs in terms of remarkable synteny between locations of diseases, disorders, and phenotype-specific QTLs detected in mouse crosses and human population studies (31). Hence it is acceptable that the trans-eQTLs that we found here for stress-related genes in mouse may be suitable candidate genes for human stress. In some circumstances, for e.g., in animal breeding or selection, these major candidate regulator genes (within eQTL regions) themselves could be used in marker- or gene-assisted selection programs, much like incorporating normal gene or markers that bracket a normal gene (20).

In addition to genetical-genomics and candidate gene analyses, we also conducted a gene network or functional association tests for the *Crhr1* gene product. Our STRING analyses predicted gene networks for the *Crhr1* gene, based on series of evidences that support functional association at the level of proteomes. As Kadarmideen et al. (20) suggested, we cross-checked the genes under eQTL peaks with those in the network to pick up a short list of candidate regulator genes under the eQTL peak. However, no direct match was found for the first 10 genes in the network versus a limited 1–4 Mb search within eQTL regions. It may be that these genes could be called differently or it could have had a direct hit, if either the network or length of search over the physical region was expanded. As a complementary approach to functional association or pathway analysis, candidate (regulatory) genes can be found in silico, in public databases dedicated to genetic map and sequence data collected in man, in model species, and in domestic animal species (as found in http://www.ensembl.org/ and http://www.ncbi.nlm.nih.gov/). For instance, Desautes et al. (6) identified *Cbg* gene encoding the carrier protein for

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cortisol in plasma (corticosteroid-binding globulin) as a candidate gene based on in silico investigation.

Validation of Candidate Genes in Human and Pigs

The basic concept of "comparative systems genetics" analyses in mice was to show the value of in silico or computational biology tools that molecular geneticists could use to target specific regions of chromosomes and use the short list of candidate regulator genes in an other (related) species such as pigs or humans. This would result in partially or fully skipping the costly and time-consuming whole genome and transcriptomics experiments in the species of interest (e.g., pigs or human). It was not our aim in this study to actually conduct microarray and linkage (genetical genomics) experiments in pigs to validate what we found in mice (Table 4) but to leave these important results for molecular geneticists to validate in further experiments. Although we did not conduct molecular validation of those candidate genes in pigs, several original experiments (found by text mining) show that they are indeed the candidate genes that are worthwhile to investigate also in pigs in a targeted genetical genomics/systems genetics experiments. The other advantage of systems genetics is in reducing costs and time in finding QTLs and/or eQTLs. For instance, interval QTL mapping or "genome scan" approaches (e.g., 2, 16) are used to identify major genes or QTLs for eventual utilization in selecting and breeding animals with favorable genotypes. Here we claim that this interval QTL mapping lies exactly between the quantitative genetics and systems genetics investigation, and we show that under certain situations, these (costly and time-consuming) QTL experiments may be made more efficient or skipped by (comparative) systems genetics data analyses in a closely related species, such as mouse or human.

To conclude, we presented systems genetics and genomics approaches, investigating the genetics and genomics of cortisol physiology. We found multiple pieces of evidence for important genetic mechanisms and large genetic determination of cortisol. Notably, we presented evidence for the segregation of monogenic Mendelian factors (major genes) affecting cortisol and located eQTL affecting expressions of associated genes. Various cortisol-related information on constructed gene networks, list of candidate genes etc., were proposed based on systems genetics methods. Results from such a (partly) in silico study provide important and focused input for further validation of the genetic architecture of cortisol physiology. We conclude that systems genetics, a result of combined systems biology and Mendelian genetics, has a great potential to provide novel insights into the biological processes underlying complex, multifactorial diseases and metabolic/physiological disorders. Furthermore, we showed the potential of translational or comparative systems genetics approaches in cutting costs and saving time in large-scale genetics and -omics investigations. We propose that our set of cortisol related genes selected from mouse data will be prime candidates to explain the observed large Mendelian factor segregating in the pig data.

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