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Transcriptome analyses identify five transcription factors differentially expressed in the hypothalamus of post- versus prepubertal Brahman heifers[1]

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ABSTRACT: Puberty onset is a developmental process influenced by genetic determinants, environment, and nutrition. Mutations and regulatory gene networks constitute the molecular basis for the genetic determinants of puberty onset. The emerging knowledge of these genetic determinants presents opportunities for innovation in the breeding of early pubertal cattle. This paper presents new data on hypothalamic gene expression related to puberty in *Bos indicus* (Brahman) in age- and weight-matched heifers. Six postpubertal heifers were compared with 6 prepubertal heifers using whole-genome RNA sequencing methodology for quantification of global gene expression in the hypothalamus. Five transcription factors (TF) with potential regulatory roles in the hypothalamus were identified in this experiment: *E2F8*, *NFAT5*, *SIX5*, *ZBTB38*, and *ZNF605*. These TF genes were significantly differentially expressed in the hypothalamus of postpubertal versus prepubertal heifers and were also identified as significant according to the applied regulatory impact factor metric ($P < 0.05$). Two of these 5 TF, *ZBTB38* and *ZNF605*, were zinc fingers, belonging to a gene family previously reported to have a central regulatory role in mammalian puberty. The *SIX5* gene belongs to the family of homologues of Drosophila sine oculis (*SIX*) genes implicated in transcriptional regulation of gonadotrope gene expression. Tumor-related genes such as *E2F8* and *NFAT5* are known to affect basic cellular processes that are relevant in both cancer and developmental processes. Mutations in *NFAT5* were associated with puberty in humans. Mutations in these TF, together with other genetic determinants previously discovered, could be used in genomic selection to predict the genetic merit of cattle (i.e., the likelihood of the offspring presenting earlier than average puberty for Brahman). Knowledge of key mutations involved in genetic traits is an advantage for genomic prediction because it can increase its accuracy.

Key words: *Bos indicus*, gene expression, hypothalamus, puberty, transcription factors

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INTRODUCTION

Puberty is a developmental process regulated by interacting genes. Gene interactions associated with puberty are described as a regulatory gene network composed of functional modules (Ojeda et al., 2006). The hypothalamus plays a pivotal role in the central control of reproduction in mammals. The hypothalamic release of pulsatile GnRH is considered the trigger for mammalian puberty because it initiates the

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release of LH and FSH, hormones required for gonadal activity and gametogenesis. In turn, pulsatile GnRH release is regulated by a gene network, interacting signals and pathways relevant to achieving puberty (Ojeda et al., 2010). Transcriptional regulation and epigenetic mechanisms are known to influence puberty progression (Lomniczi et al., 2015a,c). For example, the acetylation of *KiSS1* promoter region can contribute to its expression and influence GnRH release (Tomikawa et al., 2012). The existence of these complex *trans*-synaptic pathways overlaid by regulation of gene expression makes the task of understanding which genes regulate puberty particularly challenging. Phenotypes controlled by multiple genes, such as age at puberty, are, by definition, quantitative traits and can be targeted in breeding programs (Hill, 2010). Breeding cattle for younger age at puberty is desirable in *Bos indicus* cattle, which are generally older at puberty when compared with *Bos taurus* (Laster et al., 1976; Abeygunawardena and Dematawewa, 2004; Brito et al., 2004; Johnston et al., 2009). Efforts to breed cattle for early puberty are already part of breeding programs (MacGregor and Casey, 1999; Cammack et al., 2009; Johnston, 2014). Knowledge of specific genes, mutations, and gene networks can be used to enhance breeding programs that use genomic selection (Snelling et al., 2013; Fortes et al., 2014; Pérez-Enciso et al., 2015). In this context, we collected data on hypothalamic gene expression of postpubertal versus prepubertal *B. indicus* heifers to construct a co-expression gene network. We have hypothesized that it is possible to identify differentially expressed genes in the comparison between postpubertal and prepubertal heifers and to predict interactions between these genes and their regulators.

MATERIALS AND METHODS

Animals and Samples

Management, handling, and euthanasia of animals were approved by the Animal Ethics Committee of The University of Queensland, Production and Companion Animal group (certificate number QAAFI/279/12). Twelve young Brahman heifers with clear phenotypic characteristics of *B. indicus* cattle were sourced in October 2012 from 2 commercial herds in Queensland, Australia. They were unrelated heifers born during the wet season of 2011/2012 (<250 kg BW). Heifers were managed at the Gatton Campus beef cattle facilities of the University of Queensland (Gatton, QLD, Australia), where they grazed together in a pasture system.

The aim was to collect samples for comparison of 6 postpubertal heifers, at the luteal phase, with 6 prepubertal heifers, which had not experienced a luteal phase, that were of similar age and weight. Heifers were examined every 2 wk for observation of pubertal development, from October 2012 to May 2013. Rectal palpation was performed and ovarian activity was observed using ultrasonography (HS-2000 (VET); Honda Electronics Co., Ltd., Aichi, Japan). Pubertal status was defined by the first presence of a corpus luteum (**CL**) observed with ultrasound. Timing of tissue harvest was based on date of first CL observation. Heifers were euthanized 23 d, on average, after observation of the first CL. When a CL was observed, the postpubertal heifer was identified and then paired with a prepubertal heifer, which was randomly chosen from the remaining heifers and processed on the same day. The heifers were weighed and body condition was scored (5-point scale) prior to tissue harvest. Heifers were euthanized in pairs by stunning with a nonpenetrating captive bolt followed by exsanguination for the postpubertal heifers on the luteal phase of their second estrus cycle. The nonpenetrating captive bolt methodology was used in preference to a penetrating bolt as it served to protect the integrity of the hypothalamus tissue, as previously noted (Cánovas et al., 2014a).

The presence of a CL was confirmed on the ovary at tissue harvest, and a blood sample was collected for progesterone analysis. Progesterone concentrations in plasma extracts were measured by RIA at the Animal Endocrinology Laboratory of the University of Queensland (Brisbane, QLD, Australia). Progesterone concentrations in hexane extracts of the plasma samples were measured by RIA as described by Curlewis et al. (1985) except that progesterone antiserum C-9817 (Bioquest Ltd., North Ryde, NSW, Australia) was used. Extraction efficiency was 75% and the values reported herein were not corrected for these losses. The sensitivity of the assay was 0.1 ng/mL and the intra- and interassay CV was 5.0%.

After euthanasia, hypothalamic tissue harvest was approximately 1 cm^3 of tissue ranging from the preoptic region to the arcuate nucleus, verified by anatomical landmarks such as the crossing of the optic chiasm and the mammillary bodies as previously described (Cánovas et al., 2014a). Samples were preserved by snap freezing in liquid nitrogen and kept at −80°C until RNA extraction. In total, 12 hypothalami (from 6 postpubertal and 6 prepubertal heifers) were separately processed for RNA extraction and sequencing.

Ribonucleic Acid Extraction and Sequencing

Prior to RNA extraction, hypothalamic tissue was pulverized under liquid nitrogen and homogenized to form a uniform sample representative of the whole organ. Total RNA was isolated from 25 mg of the homoge-

nized samples from postpubertal and prepubertal heifers using a RNeasy mini kit (QIAGEN Pty Ltd., Melbourne, VIC, Australia) combined with Trizol and its recommended methodologies (Life Technologies Inc., Los Angeles, CA). Total RNA was resuspended in ribonuclease-free ultrapure water and stored at −80°C until further use. Ribonucleic acid concentrations were measured by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) with an optimal 260:280 nm ratio between 1.8 and 2.1. Intact 28S and 18S rRNA subunit integrity was assessed with an Agilent Bioanalyzer (RNA integrity number 6.9 or above for all samples; Agilent Technologies, Santa Clara, CA); traces did not show DNA presence. From total RNA, we used the TruSeq RNA sample preparation kit (Illumina Inc., San Diego, CA) for library preparation. The kit protocol includes mRNA purification using poly-T oligo-attached magnetic beads. Purified mRNA was then fragmented and converted to cDNA, which was double stranded, ligated to adapters, and amplified with PCR to create the libraries (all performed per kit protocol following the manufacturer's instructions). Libraries were multiplex, 6 libraries per lane, and paired-end sequenced with an Illumina HiSeq 2000 analyzer (Illumina Inc.). Sequence fragments were mapped to the annotated bovine reference genome (UMD3.1, release annotation 77; ftp://ftp.ensembl.org/pub/release-77/genbank/bos_taurus/; accessed 15 May 2015) using the CLC Genomics workbench software (CLC bio, Aarhus, Denmark) with its default parameters for alignment, quality control, and calculation of gene expression levels. The algorithm underpinning the CLC software is ERANGE (Mortazavi et al., 2008). In short, for the assembly procedure, the sequences were mapped to the reference genome accounting for a maximum of 2 gaps or mismatches in each sequence. Quality control analysis was performed using procedures described by Cánovas et al. (2013) with the CLC Genomics workbench software. This tool assesses sequence quality indicators based on the FastQC project (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; accessed 15 May 2015). Quality was measured taking into account sequence-read lengths and base coverage, nucleotide contributions, and base ambiguities and quality scores as emitted by the base caller and overrepresented sequences (Cánovas et al., 2014b). All the samples analyzed passed all the QC parameters having the same length (100 bp); 100% coverage in all bases; 25% of A, T, G and C nucleotide contributions; 50% GC on base content; and less than 0.1% overrepresented sequences, indicating a very good quality. Data was normalized by calculating the "reads per kilo base per million mapped reads" (**RPKM**) for each gene (Mortazavi et al., 2008). To select expressed genes, a threshold of RPKM ≥ 0.2 was used (Wickramasinghe et al., 2012). Only genes with average RPKM ≥ 0.2 in at least 1 tissue were considered expressed and had their data used in subsequent analyses (Wickramasinghe et al., 2012; Cánovas et al., 2014a).

Differential Gene Expression

Genes differentially expressed (DEx) in the hypothalamus of postpubertal versus prepubertal heifers were identified with analyses of the RPKM values. Base-2 logarithmic transformation of the RPKM values was performed to avoid bias DE, especially for genes with fewer reads (Bullard et al., 2010). Base-2 log-transformed RPKM values were then normalized using mixed models, which fit the library as a fixed effect and the random effects of gene and gene × animal × tissue interaction (Reverter et al., 2004; Cánovas et al., 2014a). It was possible to fit tissue as a random interaction with gene and animal because this study is part of a larger experiment where 5 tissue samples were evaluated per animal (hypothalamus, pituitary, ovaries, uterus, and liver). Similarly, the library effect was not confounded with animal as there were 5 libraries per animal (1 for each tissue). Solutions to these mixed models were estimated using VCE6 software (ftp://ftp.tzv.fal.de/pub/vce6/; accessed 15 June 2015). Normalized expression values for each gene in each sample were estimated from linear combinations of the mixed model solutions for library, gene, animal, and tissue. Normalized expression values were subjected to a t test to compare the average expression in postpubertal versus prepubertal heifers and identify DEx genes using as a threshold for significance of $P < 0.05$. This P-value threshold was considered in the context of the harsh normalization performed and the subsequent analyses that used the DEx gene list as a starting point for further scrutiny.

Gene Network Prediction and Key Regulators

To construct a co-expression gene network, normalized gene expression values were used as input to the partial correlation and information theory (**PCIT**) algorithm of Reverter and Chan (2008). A partial correlation between 2 genes is the correlation between this particular pair of genes that is independent of a third gene. In brief, PCIT is a data-driven approach that explores all correlations between possible triplets of genes before determining which pairwise correlations are significant. First, we estimated correlations for all expressed transcription factors (**TF**) and all DEx genes. Then, correlations deemed significant formed the connections between genes in the network, which was further pruned to contain only DEx genes and top-ranking TF from the regulatory impact

factor (**RIF**) metric described below (Reverter and Chan, 2008). The gene network was then visualized and analyzed using Cytoscape (Shannon et al., 2003).

To identify regulatory elements in our data set, we used the AnimalTFDB bovine database (http://bioinfo.life.hust.edu.cn/AnimalTFDB/download_index?tr = Bos_taurus; accessed 15 June 2015), which includes classification and annotation of genomewide TF, transcription cofactors, and chromatin remodeling factors. A RIF metric (Reverter et al., 2010) was applied to identify key regulators among hypothalamic expressed TF. We explored the RIF as 2 alternative measures, RIF1 and RIF2, calculated from the number of DEx genes and the predicted interactions between TF and target DEx genes as previously described (Hudson et al., 2009; Reverter et al., 2010). The 2 alternative measures of the RIF explored were computed as follows:

$$\mathrm{RIF1}i = \left(1/n_{dex}\right)\sum_{j=1}^{j=n_{dex}} \hat{a}_j \times \hat{d}_j \times \mathrm{DW}_{ij}^2 = \left(1/n_{dex}\right)\sum_{j=1}^{j=n_{dex}} \mathrm{PIF}_j \times \mathrm{DW}_{ij}^2$$

and

$$\mathrm{RIF2}i = \left(1/n_{dex}\right)\sum_{j=1}^{j=n_{dex}} \left[\left(e1_j \times r1_{ij}\right)^2 - \left(e2_j \times r2_{ij}\right)^2\right],$$

in which n_{dex} is the number of DEx genes; $\hat{a}_j$ is the estimated average expression of the jth DEx gene, averaged across the 2 conditions being contrasted (after puberty and before puberty); $\hat{d}_j$ is the differential expression of the jth DEx gene; and **DW** is the differential wiring (or connectivity) between the ith TF and the jth DEx gene and computed from the difference between $r1_{ij}$ and $r2_{ij}$, the co-expression correlation between the ith TF and the jth DEx gene in conditions 1 (after puberty) and 2 (before puberty):

$$\mathrm{DW}_{ij} = r1_{ij} - r2_{ij}.$$

The calculation of RIF1 uses the concept of phenotype impact factor defined for each DEx gene and computed from the product of its average expression and its differential expression (Reverter et al., 2010).

In essence, the first metric, RIF1, captures TF showing differential connectivity to DEx genes between the 2 pubertal states (i.e., qualitative changes in predicted TF–target gene interactions). The alternative RIF2 focuses on TF showing evidence as predictors of change in abundance of DEx genes between postpubertal and prepubertal heifers (i.e., quantitative changes in predicted TF–target gene interactions). Both RIF1 and RIF2 identified key TF from the DEx genes. For comparisons between both RIF1 and RIF2 and across data sets, RIF measures were transformed to a z-score by subtracting the mean and dividing by the SD. Using a nominal $P < 0.05$, a TF was deemed as a key TF if either of the 2 RIF scores was higher than 1.96 SD.

Functional Enrichment Analyses

Three lists of genes associated with puberty in *B. indicus* heifer emerged from abovementioned analyses: 1) DEx genes and regulatory elements, 2) genes and TF that formed the predicted network, and 3) top-ranking TF. These lists of genes were used as target gene lists (one at the time) to compare with a background gene list formed by all genes expressed in the hypothalamus. The target vs. background lists comparison was performed by uploading these lists into DAVID, used to perform functional enrichment analyses (Huang et al., 2009). With DAVID, genes were annotated in terms of their known function, gene ontologies, and pathways. The output of DAVID analyses are overrepresented pathways or ontologies associated with each target gene list. Significant results after Benjamini–Hochberg correction for multiple testing are reported.

RESULTS

At tissue harvest, average serum concentration of progesterone was 0.4 ± 0.2 ng/mL for prepubertal heifers and 2.0 ± 0.7 ng/mL for postpubertal heifers. This range of progesterone levels could be considered normal for the luteal phase of pubertal *B. indicus* heifers, which are lower than that observed for cows or *B. taurus* cattle (Sartori and Barros, 2011). The observation of 2 consecutive progesterone values higher than 1 ng/mL has been used as a criterion for puberty achievement in heifers (Lopez et al., 2006; Shirley et al., 2006). No significant difference in BW or BCS between postpubertal and prepubertal heifers was observed at euthanasia. Body weight averages were 363 (SD 38.62) and 338 kg (SD 54.17; $P = 0.38$), and BCS (scale 1–5) averages were 3.75 (SD 0.41) and 3.5 (SD 0.44; $P = 0.18$).

The number of genes expressed in the hypothalamus was 14,671 (genes with RPKM values greater than 0.2, on average, in hypothalamus samples). Of note, the number of genes deemed expressed and its splice variants would vary with the use of newer and increasingly annotated versions of the bovine reference genome, which should be available in the foreseeable future (FAANG Consortium et al., 2015). To confirm "typical" hypothalamic gene expression from this list of expressed genes is challenging. A recent study of female cattle tissues found that less than 1% of annotated genes presented a restricted tissue-specific expression (McGettigan et al., 2016). The 10 most hypothalamic-specific genes, according to McGettigan et al. (2016), were expressed in all our samples (RPKM > 0.2 in each

Table 1. In the comparison between post- and prepubertal heifers, 5 transcription factors (TF) were significant in 2 analyses: genes differentially expressed (DEx) and regulatory impact factor (RIF) metrics (RIF1 and RIF2; *P*-value < 0.05)

TF	ENSB[1] tag	Description	RIF1	RIF2	DEx[2]	FC[3]	*P* (DEx)
E2F8	ENSBTAG00000017446	E2F transcription factor 8	3.10	0.84	2.25	1.44	0.01
NFAT5	ENSBTAG00000013412	Nuclear factor of activated T-cells 5, tonicity-responsive	−0.38	−2.00	−0.37	0.75	0.04
SIX5	ENSBTAG00000013346	SIX homeobox 5	−0.19	1.96	0.28	3.82	0.05
ZBTB38	ENSBTAG00000040061	Zinc finger and BTB domain containing 38	0.49	−2.31	−0.15	0.91	0.03
ZNF605	ENSBTAG00000005240	Zinc finger protein 605	−2.44	0.34	−0.29	0.56	0.03

[1]ENSB = http://www.ensembl.org; assessed 15 May 2015.

[2]The DEx values represent the difference in expression values between post- and prepubertal heifers average gene expression values (normalized base-2 log transformed "reads per kilo base per million mapped reads" [RPKM] values).

[3]FC = fold change. This represents the fold change in gene expression (normalized base-2 log transformed RPKM values) between post- and prepubertal heifers.

sample) and did not differ between postpubertal and prepubertal heifers. These 10 genes, including the most specific myelin proteolipid protein, might be considered constitutively expressed in the hypothalamus because they could be confirmed by others and were independent of developmental status in our samples ($P > 0.05$).

Just over 2% of expressed genes were DEx in the postpubertal heifer versus prepubertal heifer comparison. Of the 392 DEx genes, 173 were downregulated and 219 were upregulated after puberty (Supplemental Table S1; see the online version of the article at http://journalofanimalscience.org). This result represented the difference in gene expression between heifers at their second luteal phase (after puberty) and heifers that never experienced a luteinic phase (before puberty). Therefore, gene expression differences in postpubertal versus prepubertal heifers could be influenced by the presence versus absence of progesterone feedback to the hypothalamus and therefore reflect physiological changes due to progesterone signaling as well as those that are involved in bringing about puberty onset. Among the DEx genes, 29 were TF, 7 were transcription cofactors, and 4 were chromatin remodeling factors. These elements were further investigated in network analyses, taking into account the RIF metrics obtained for TF (see below).

The RIF metric was applied to all 1,085 TF expressed in the hypothalamus, DEx or not. These analyses identified 111 significant TF ($P < 0.05$). This TF list was significantly enriched for 2 pathways: prostate cancer ($P = 3.4 \times 10^{-4}$) and pathways in cancer ($P = 1.1 \times 10{-}3$). Of the 111 top-ranking TF, 29 were genes that code for TF of the zinc finger family: 15 zinc finger (**ZNF**) genes and 14 others, including uncharacterized proteins with zinc finger core characteristics, as per AnimalTFDB (Supplemental Table S2; see the online version of the article at http://journalofanimalscience.org). Five TF were significant according to both RIF and DEx analyses (Table 1).

A co-expression network was predicted from RNA sequencing data, illustrating the complex nature of hypothalamic activity associated with postpubertal versus prepubertal differences. First, we analyzed the network formed by correlations between all expressed TF and DEx genes. Then, we focused on the network that contained only DEx genes, DEx regulatory elements, and top-ranking TF from the RIF analyses (Fig. 1).

The genes in the above network were not significantly enriched for particular functional pathways when interrogated using DAVID software. The functional gene annotation analysis nevertheless detected 6 genes in the network that participate in the cross-talk between adiponectin, leptin, and insulin signaling pathways: *TNFRSF1A*, *RELA*, *NFKB1*, *PRKAG2*, *CPT1C*, and *LEPR* (Supplemental Fig. S1; see the online version of the article at http://journalofanimalscience.org). All of these genes were upregulated after puberty with the exception of *CPT1C*.

DISCUSSION

Of the 111 hypothalamus-expressed TF that were ranked most highly in the RIF analysis, 29 were genes from the zinc finger family: 15 ZNF genes and 14 others, including uncharacterized proteins. This result seems to agree with reported findings regarding the central role of zinc finger family members in the regulation of puberty onset (Lomniczi et al., 2015b). Given the tested contrast, the identified zinc finger molecules might play a role in the hypothalamic response to progesterone feedback in heifers. Specifically, it is tempting to hypothesize that *ZBTB38* and *ZNF605* are downregulated after puberty as a results of either pubertal development as a whole or simply as a result of progesterone feedback. In either case, the role for these 2 high-ranking and DEx zinc fingers in heifer reproductive biology should be further investigated.

The current study provides new evidence for physiological mechanisms associated with *B. indicus* puberty.

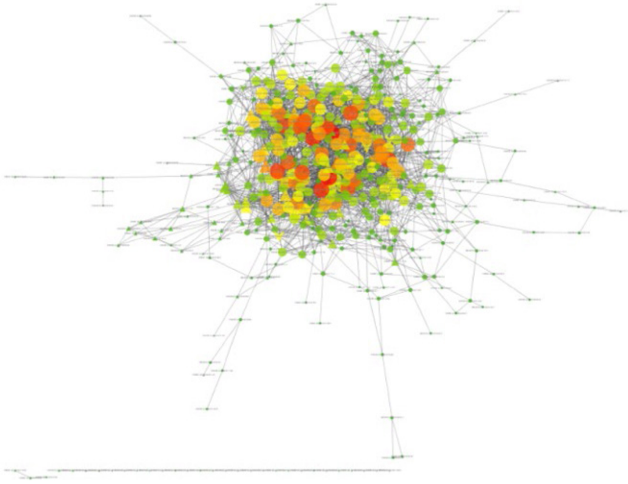

Figure 1. Co-expression network in the hypothalamus of post- and prepubertal *Bos indicus* heifers. Predicted interactions between differentially expressed genes and top-ranking transcription factors. The color gradient illustrates network degrees, from low (green) to medium (yellow) and high (red), based on the number of predicted interactions for each gene.

The studied heifers formed a contrast between hypothalamus samples under progesterone influence after puberty (second estrus cycle) and hypothalamus samples from heifers that had never experienced a luteal phase (before puberty). Given this contrast, it is likely that DEx genes could be influenced by puberty onset and the presence versus absence of progesterone feedback in the hypothalamus. Progesterone feedback is an important step in pubertal development and peripubertal heifers tend to experience short luteal phases in the first cycle (Atkins et al., 2013). Other contrasts should be explored in future experiments for detailing gene expression profiles at additional time points. Future experiments could compare samples from postpubertal heifers at all phases of the estrus cycle, compare gene expression from one estrous phase to another, and compare each phase with prepubertal gene expression. These other contrasts would allow researchers to differentiate progesterone influence on gene expression from postpubertal versus prepubertal differences in the absence of progesterone influence.

The only other RNA sequencing study, to our knowledge, of heifer puberty investigated a contrast similar to the one presented here in terms of progesterone influence. Cánovas et al. (2014a) reported 275 DEx genes in the hypothalamus of prepubertal Brangus (three-eighths Brahman × five-eighths Angus) heifers with low levels of serum progesterone (0.5 ± 0.3 ng/mL) compared with postpubertal heifers with higher levels corresponding to CL presence at euthanasia (7.1 ± 1.0 ng/mL). However, the prepubertal heifers in that study were much younger and smaller than the age-matched heifers in the current study. Comparing the Brangus heifer study with the current results of Brahman heifers, only 2 genes were DEx in both breeds: *BARX2* and *VAX1*. Breed differences, including *B. taurus* and *B. indicus* genetics, and experimental design (euthanasia for postpubertal and prepubertal heifers was not matched in the Brangus experiment) could contribute to these contrasting results. However, encouraging confirmation in terms of key TF emerged from both studies because 43 of 111 (approximately 39%) top-ranking TF in Brahman were also deemed regulators in the Brangus network: *BSX*, *DLX1*, *DLX5*, *DMRT2*, *E2F3*, *E2F7*, *EGR3*, *EGR4*, *ETS1*, *ETV6*, *FOXA1*, *FOXA2*, *HAND2*, *HOXD9*, *INSM1*, *IRX2*, *LHX5*, *LHX9*, *MESP2*, *NFAT5*, *NFKB1*, *NR1H4*, *NR5A2*, *OVOL1*, *OVOL2*, *PAX3*, *PAX7*, *POU4F2*, *POU4F3*, *PPARG*, *PROP1*, *RAX*, *SHOX*, *SIX3*, *SOX5*, *SP5*, *TAL1*, *TCF21*, *TFCP2L1*, *TSC22D3*, *TTF1*, *USF2*, and *WT1*. Note that *NFAT5* is 1 of the 5 DEx and top-ranking TF. Understanding the specific roles of these potential regulators of heifer puberty will be our next challenge. For some of these genes, or at least gene families, previous reports provides clues as to their function in the context of puberty mechanisms. This is the case for *PPARG* and *PROP1*, genes of the family of homologues of Drosophila sine oculis (***SIX***) genes and genes of the family of the adenovirus E2 promoter binding factor (**E2F**), as well as some tumor-related genes, all alleged to participate in the cross-talk upstream of GnRH release, as discussed in the following paragraphs.

Previous work derived gene networks for cattle puberty from genomewide association studies using polymorphism co-association to predict gene interactions (Fortes et al., 2010, 2011). Some of the key regulators identified in those studies and postulated here were the same: *PROP1* and *PPARG*. Despite being previously identified as key regulators of heifer puberty and having significant RIF scores (Supplemental Table S1; see the online version of the article at http://journalofanimalscience.org), these TF were not central nodes in the co-expression network reported here because they were connected to only 7 or fewer genes. Central nodes in the network were connected to up to 54 genes.

Genes from the SIX family determine DNA binding specificity, mediate protein–protein interactions, and were implicated in developmental processes and in the maintenance of differentiated tissue states (Boucher et al., 2000). In this study, *SIX5* was DEx and a top-ranking TF. In Cánovas et al. (2014a), *SIX6* was deemed a key regulator. In both studies, *SIX3* was identified as a potential regulator of heifer puberty. Although *SIX5* did not affect fertility in knockout mice (Klesert et al., 2000), it is possible that members of the SIX family contribute to pubertal development. Knockdown and knockout experiments of *SIX6* support roles for *SIX3* and *SIX6* in transcriptional regulation of gonadotrope gene expression and *SIX3* and *SIX6* have been shown to functionally compensate for each other (Xie et al.,

2015). Compensatory roles and our results might suggest that *SIX5*, a top TF in Brahmans, is more important in a *B. indicus* background and could compensate for *SIX6* detected only in Brangus. Meanwhile, *SIX3* may play a role in both *B. indicus* and *B. taurus* cattle.

The cross-talk between adiponectin, leptin, and insulin pathways in the hypothalamus has long been discussed as an underlying mechanism that links nutritional status with puberty (Coope et al., 2008; Cardoso et al., 2015). In the current study, we found 6 DEx genes, *TNFRSF1A*, *RELA*, *NFKB1*, *PRKAG2*, *CPT1C*, and *LEPR*, known to belong to all 3 pathways. It is likely that these genes are important factors in energy homeostasis, in the context of heifer puberty. Out of the 6, only *NFKB1* had been identified by a previous study (Cánovas et al., 2014a). It is noteworthy that these genes are also known to modulate *NPY* neurons postulated to affect GnRH release in association with leptin signaling in heifers (Cardoso et al., 2014).

Alleles of *SIX6* and *LIN28B* associated with increased age at puberty in girls were also associated with taller adult height (Perry et al., 2014). A confirmed mutation in *PLAG1* associated with age at puberty in heifers follows the same pattern: the allele associated with late puberty increases height and weight (Karim et al., 2011; Littlejohn et al., 2012; Nishimura et al., 2012; Fortes et al., 2013; Utsunomiya et al., 2013; Saatchi et al., 2014). It is conceivable that *SIX5*, *SIX6*, and *SIX3* may present another example of genes that link growth and energy homeostasis to timing of puberty in cattle. Genes of the SIX family might act similarly to leptin and adiponectin, providing a permissive link between energy homeostasis and GnRH release (Amstalden et al., 2014; Cardoso et al., 2015). Elucidation of *SIX* pathways and how it may fit into the cross-talk upstream of GnRH release requires further investigation.

Certain tumor-related genes are thought to be puberty-delaying genes, transcriptional repressors central to the regulatory network that controls puberty onset (Roth et al., 2007; Ojeda et al., 2010). Genes from the E2F family might fall into this "tumor-related" category of important TF for puberty. They are known to affect cell cycle and progression, basic processes that are relevant in both cancer and developmental processes (Christensen et al., 2005). The *E2F8* and *NFAT5* genes may fall into this category, as these rank within the 5 DEx and top-ranking TF and have been shown to play roles in cancer development (Jauliac et al., 2002; Chen et al., 2009). However, from our data, *E2F8* had higher levels of expression after puberty whereas *NFAT5* had higher levels before puberty, so it is likely that their effects on puberty are in opposite directions. First described as a tonicity-responsive TF crucial to kidney function, *NFAT5* is also expressed in brain and testicular tissues; however, the roles it may play in those tissues are largely unknown (Trama et al., 2000; Lopez-Rodriguez et al., 2004). It is worth noting that *NFAT5* is the only one of the 5 DEx and top-ranking TF that was identified by the previous Brangus heifer hypothalamus study (Cánovas et al., 2014a). Furthermore, mutations in *NFAT5* were associated with age at menarche (puberty) in women (Chen et al., 2012).

Knowledge of key regulators, specific genes, mutations, gene networks, and pathways can be used to enhance genomic approaches for selective breeding (Snelling et al., 2013; Fortes et al., 2014). In this context, data presented herein regarding hypothalamic gene expression of postpubertal versus prepubertal *B. indicus* heifers could have practical implications. The regulators for which this study proposes a potential role in puberty should be mined for mutations that could be tested for their effect on age at puberty in cattle. Mutations in these regulators, together with other genetic determinants discovered in previous genomewide association studies, could form the basis for DNA diagnostic tools predicting early puberty onset. Knowledge of biologically relevant mutations is an advantage for genomic selection, increasing the accuracy and supporting prediction of phenotypes across breed (Snelling et al., 2013; Pérez-Enciso et al., 2015).

LITERATURE CITED

Abeygunawardena, H., and C. M. Dematawewa. 2004. Pre-pubertal and postpartum anestrus in tropical Zebu cattle. Anim. Reprod. Sci. 82–83:373–387. doi:10.1016/j.anireprosci.2004.05.006

Amstalden, M., R. C. Cardoso, B. R. Alves, and G. L. Williams. 2014. Reproduction Symposium: Hypothalamic neuropeptides and the nutritional programming of puberty in heifers. J. Anim. Sci. 92:3211–3222. doi:10.2527/jas.2014-7808

Atkins, J. A., K. G. Pohler, and M. F. Smith. 2013. Physiology and endocrinology of puberty in heifers. Vet. Clin. North Am. Food Anim. Pract. 29:479–492. doi:10.1016/j.cvfa.2013.07.008

Boucher, C. A., C. L. Winchester, G. M. Hamilton, A. D. Winter, K. J. Johnson, and M. E. S. Bailey. 2000. Structure, mapping and expression of the human gene encoding the homeodomain protein, SIX2. Gene 247:145–151. doi:10.1016/S0378-1119(00)00105-0

Brito, L. F., A. E. Silva, M. M. Unanian, M. A. Dode, R. T. Barbosa, and J. P. Kastelic. 2004. Sexual development in early- and late-maturing *Bos indicus* and *Bos indicus* × *Bos taurus* crossbred bulls in Brazil. Theriogenology 62:1198–1217. doi:10.1016/j.theriogenology.2004.01.006

Bullard, J. H., E. Purdom, K. D. Hansen, and S. Dudoit. 2010. Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. BMC Bioinf. 11:94. doi:10.1186/1471-2105-11-94

Cammack, K. M., M. G. Thomas, and R. M. Enns. 2009. Review: Reproductive traits and their heritabilities in beef cattle. Prof. Anim. Sci. 25:517–528.

Cánovas, A., A. Reverter, K. L. DeAtley, R. L. Ashley, M. L. Colgrave, M. R. S. Fortes, A. Islas-Trejo, S. Lehnert, L. Porto-Neto, G. Rincon, G. A. Silver, W. M. Snelling, J. F. Medrano, and M. G. Thomas. 2014a. Multi-tissue omics analyses reveal molecular regulatory networks for puberty in composite beef cattle. PLoS One 9:e102551. doi:10.1371/journal.pone.0102551

Cánovas, A., G. Rincón, C. Bevilacqua, A. Islas-Trejo, P. Brenaut, R. C. Hovey, M. Boutinaud, C. Morgenthaler, M. K. VanKlompenberg, P. Martin, and J. F. Medrano. 2014b. Comparison of five different RNA sources to examine the lactating bovine mammary gland transcriptome using RNA-sequencing. Sci. Rep. 4:5297.

Cánovas, A., G. Rincón, A. Islas-Trejo, R. Jimenez-Flores, A. Laubscher, and J. F. Medrano. 2013. RNA sequencing to study gene expression and single nucleotide polymorphism variation associated with citrate content in cow milk. J. Dairy Sci. 96:2637–2648. doi:10.3168/jds.2012-6213

Cardoso, R. C., B. R. C. Alves, L. D. Prezotto, J. F. Thorson, L. O. Tedeschi, D. H. Keisler, M. Amstalden, and G. L. Williams. 2014. Reciprocal changes in leptin and NPY during nutritional acceleration of puberty in heifers. J. Endocrinol. 223:289–298. doi:10.1530/JOE-14-0504

Cardoso, R. C., B. R. C. Alves, S. M. Sharpton, G. L. Williams, and M. Amstalden. 2015. Nutritional programming of accelerated puberty in heifers: Involvement of pro-opiomelanocortin neurones in the arcuate nucleus. J. Neuroendocrinol. 27:647–657. doi:10.1111/jne.12291

Chen, C. T. L., L. Fernandez-Rhodes, R. G. Brzyski, C. S. Carlson, Z. Chen, G. Heiss, K. E. North, N. F. Woods, A. Rajkovic, C. Kooperberg, and N. Franceschini. 2012. Replication of loci influencing ages at menarche and menopause in Hispanic women: The Womens Health Initiative SHARe Study. Hum. Mol. Genet. 21:1419–1432. doi:10.1093/hmg/ddr570

Chen, H.-Z., S.-Y. Tsai, and G. Leone. 2009. Emerging roles of E2Fs in cancer: An exit from cell cycle control. Nat. Rev. Cancer 9:785–797. doi:10.1038/nrc2696

Christensen, J., P. Cloos, U. Toftegaard, D. Klinkenberg, A. P. Bracken, E. Trinh, M. Heeran, L. Di Stefano, and K. Helin. 2005. Characterization of E2F8, a novel E2F-like cell-cycle regulated repressor of E2F-activated transcription. Nucleic Acids Res. 33:5458–5470. doi:10.1093/nar/gki855

Coope, A., M. Milanski, E. P. Araujo, M. Tambascia, M. J. Saad, B. Geloneze, and L. A. Velloso. 2008. AdipoR1 mediates the anorexigenic and insulin/leptin-like actions of adiponectin in the hypothalamus. FEBS Lett. 582:1471–1476. doi:10.1016/j.febslet.2008.03.037

Curlewis, J. D., M. Axelson, and G. M. Stone. 1985. Identification of the major steroids in ovarian and adrenal venous plasma of the brush-tail possum (*Trichosurus vulpecula*) and changes in the peripheral plasma levels of estradiol and progesterone during the reproductive cycle. J. Endocrinol. 105:53–62. doi:10.1677/joe.0.1050053

FAANG Consortium, L. Andersson, A. L. Archibald, C. D. Bottema, R. Brauning, S. C. Burgess, D. W. Burt, E. Casas, H. H. Cheng, L. Clarke, C. Couldrey, B. P. Dalrymple, C. G. Elsik, S. Foissac, E. Giuffra, M. A. Groenen, B. J. Hayes, L. S. Huang, H. Khatib, J. W. Kijas, H. Kim, J. K. Lunney, F. M. McCarthy, J. C. McEwan, S. Moore, B. Nanduri, C. Notredame, Y. Palti, G. S. Plastow, J. M. Reecy, G. A. Rohrer, E. Sarropoulou, C. J. Schmidt, J. Silverstein, R. L. Tellam, M. Tixier-Boichard, G. Tosser-Klopp, C. K. Tuggle, J. Vilkki, S. N. White, S. H. Zhao, and H. Zhou. 2015. Coordinated international action to accelerate genome-to-phenome with FAANG, the Functional Annotation of Animal Genomes project. Genome Biol. 16:57.

Fortes, M. R. S., K. Kemper, S. Sasazaki, A. Reverter, J. E. Pryce, W. Barendse, R. Bunch, R. McCulloch, B. Harrison, S. Bolormaa, Y. D. Zhang, R. J. Hawken, M. E. Goddard, and S. A. Lehnert. 2013. Evidence for pleiotropism and recent selection in the PLAG1 region in Australian Beef cattle. Anim. Genet. 44:636–647. doi:10.1111/age.12075

Fortes, M. R. S., L. R. Porto-Neto, K. L. DeAtley, A. Reverter, M. G. Thomas, S. S. Moore, S. A. Lehnert, and W. M. Snelling. 2014. Genetic markers in transcription factors of differentially expressed genes associated with post-partum anoestrus predict pregnancy outcome in an independent population of beef cattle. In: 10th World Congr. Genet. Appl. Livest. Prod., Vancouver, Canada.

Fortes, M. R. S., A. Reverter, S. H. Nagaraj, Y. Zhang, N. N. Jonsson, W. Barris, S. Lehnert, G. B. Boe-Hansen, and R. J. Hawken. 2011. A single nucleotide polymorphism-derived regulatory gene network underlying puberty in 2 tropical breeds of beef cattle. J. Anim. Sci. 89:1669–1683. doi:10.2527/jas.2010-3681

Fortes, M. R. S., A. Reverter, Y. D. Zhang, E. Collis, S. H. Nagaraj, N. N. Jonsson, K. C. Prayaga, W. Barris, and R. J. Hawken. 2010. Association weight matrix for the genetic dissection of puberty in beef cattle. Proc. Natl. Acad. Sci. USA 107:13642–13647. doi:10.1073/pnas.1002044107

Hill, W. G. 2010. Understanding and using quantitative genetic variation. Philos. Trans. R. Soc., B 365:73–85. doi:10.1098/rstb.2009.0203

Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4:44–57.

Hudson, N. J., A. Reverter, Y. H. Wang, P. L. Greenwood, and B. P. Dalrymple. 2009. Inferring the transcriptional landscape of bovine skeletal muscle by integrating co-expression networks. PLoS One 4:e7249. doi:10.1371/journal.pone.0007249

Jauliac, S., C. Lopez-Rodriguez, L. M. Shaw, L. F. Brown, A. Rao, and A. Toker. 2002. The role of NFAT transcription factors in integrin-mediated carcinoma invasion. Nat. Cell Biol. 4:540–544. doi:10.1038/ncb816

Johnston, D. J. 2014. Genetic improvement of reproduction in beef cattle. 10th World Congr. Genet. Appl. Livest. Prod., Vancouver, Canada.

Johnston, D. J., S. A. Barwick, N. J. Corbet, G. Fordyce, R. G. Holroyd, P. J. Williams, and H. M. Burrow. 2009. Genetics of heifer puberty in two tropical beef genotypes in northern Australia and associations with heifer- and steer-production traits. Anim. Prod. Sci. 49:399–412. doi:10.1071/EA08276

Karim, L., H. Takeda, L. Lin, T. Druet, J. A. C. Arias, D. Baurain, N. Cambisano, S. R. Davis, F. Farnir, B. Grisart, B. L. Harris, M. D. Keehan, M. D. Littlejohn, R. J. Spelman, M. Georges, and W. Coppieters. 2011. Variants modulating the expression of a chromosome domain encompassing PLAG1 influence bovine stature. Nat. Genet. 43:405–413. doi:10.1038/ng.814

Klesert, T. R., D. H. Cho, J. I. Clark, J. Maylie, J. Adelman, L. Snider, E. C. Yuen, P. Soriano, and S. J. Tapscott. 2000. Mice deficient in Six5 develop cataracts: Implications for myotonic dystrophy. Nat. Genet. 25:105–109. doi:10.1038/75490

Laster, D. B., G. M. Smith, and K. E. Gregory. 1976. Characterization of biological types of cattle IV. Postweaning growth and puberty of heifers. J. Anim. Sci. 43:63–70.

Littlejohn, M., T. Grala, K. Sanders, C. Walker, G. Waghorn, K. Macdonald, W. Coppieters, M. Georges, R. Spelman, E. Hillerton, S. Davis, and R. Snell. 2012. Genetic variation in *PLAG1* associates with early life body weight and peripubertal weight and growth in *Bos taurus*. Anim. Genet. 43:591–594. doi:10.1111/j.1365-2052.2011.02293.x

Lomniczi, A., J. M. Castellano, H. Wright, B. Selcuk, K. Sonmez, and S. R. Ojeda. 2015a. Gene networks, epigenetics and the control of female puberty. In: J.-P. Bourguignon, J.-C. Carel, and Y. Christen, editors, Brain crosstalk in puberty and adolescence. p. 97–119.

Lomniczi, A., H. Wright, J. M. Castellano, V. Matagne, C. A. Toro, S. Ramaswamy, T. M. Plant, and S. R. Ojeda. 2015b. Epigenetic regulation of puberty via Zinc finger protein-mediated transcriptional repression. Nat. Commun. 6:10195. doi:10.1038/ncomms10195

Lomniczi, A., H. Wright, and S. R. Ojeda. 2015c. Epigenetic regulation of female puberty. Front. Neuroendocrinol. 36:90–107. doi:10.1016/j.yfrne.2014.08.003

Lopez-Rodriguez, C., C. L. Antos, J. M. Shelton, J. A. Richardson, F. M. Lin, T. I. Novobrantseva, R. T. Bronson, P. Igarashi, A. Rao, and E. N. Olson. 2004. Loss of NFAT5 results in renal atrophy and lack of tonicity-responsive gene expression. Proc. Natl. Acad. Sci. USA 101:2392–2397. doi:10.1073/pnas.0308703100

Lopez, R., M. G. Thomas, D. M. Hallford, D. H. Keisler, G. A. Silver, B. S. Obeidat, M. D. Garcia, and C. R. Krehbiel. 2006. Metabolic hormone profiles and evaluation of associations of metabolic hormones with body fat and reproductive characteristics of Angus, Brangus, and Brahman heifers. Prof. Anim. Sci. 22:273–282.

MacGregor, R. G., and N. H. Casey. 1999. Evaluation of calving interval and calving date as measures of reproductive performance in a beef herd. Livest. Prod. Sci. 57:181–191. doi:10.1016/S0301-6226(98)00158-4

McGettigan, P. A., J. A. Browne, S. D. Carrington, M. A. Crowe, T. Fair, N. Forde, B. J. Loftus, A. Lohan, P. Lonergan, K. Pluta, S. Mamo, A. Murphy, J. Roche, S. W. Walsh, C. J. Creevey, B. Earley, S. Keady, D. A. Kenny, D. Matthews, M. McCabe, D. Morris, A. O'Loughlin, S. Waters, M. G. Diskin, and A. C. O. Evans. 2016. Fertility and genomics: Comparison of gene expression in contrasting reproductive tissues of female cattle. Reprod. Fertil. Dev. 28:11–24. doi:10.1071/RD15354

Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer, and B. Wold. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5:621–628. doi:10.1038/nmeth.1226

Nishimura, S., T. Watanabe, K. Mizoshita, K. Tatsuda, T. Fujita, N. Watanabe, Y. Sugimoto, and A. Takasuga. 2012. Genome-wide association study identified three major QTL for carcass weight including the PLAG1-CHCHD7 QTN for stature in Japanese Black cattle. BMC Genet. 13:40. doi:10.1186/1471-2156-13-40

Ojeda, S. R., C. Dubay, A. Lomniczi, G. Kaidar, V. Matagne, U. S. Sandau, and G. A. Dissen. 2010. Gene networks and the neuroendocrine regulation of puberty. Mol. Cell. Endocrinol. 324:3–11. doi:10.1016/j.mce.2009.12.003

Ojeda, S. R., A. Lomniczi, C. Mastronardi, S. Heger, C. Roth, A. S. Parent, V. Matagne, and A. E. Mungenast. 2006. Minireview: The neuroendocrine regulation of puberty: Is the time ripe for a systems biology approach? Endocrinology 147:1166–1174. doi:10.1210/en.2005-1136

Pérez-Enciso, M., J. C. Rincón, and A. Legarra. 2015. Sequence- vs. chip-assisted genomic selection: Accurate biological information is advised. Genet. Sel. Evol. 47:43. doi:10.1186/s12711-015-0117-5

Perry, J. R. B., F. Day, C. E. Elks, P. Sulem, D. J. Thompson, T. Ferreira, C. Y. He, D. I. Chasman, T. Esko, G. Thorleifsson, E. Albrecht, W. Q. Ang, T. Corre, D. L. Cousminer, B. Feenstra, N. Franceschini, A. Ganna, A. D. Johnson, S. Kjellqvist, K. L. Lunetta, G. McMahon, I. M. Nolte, L. Paternoster, E. Porcu, A. V. Smith, L. Stolk, A. Teumer, N. Tsernikova, E. Tikkanen, S. Ulivi, E. K. Wagner, N. Amin, L. J. Bierut, E. M. Byrne, J. J. Hottenga, D. L. Koller, M. Mangino, T. H. Pers, L. M. Yerges-Armstrong, J. H. Zhao, I. L. Andrulis, H. Anton-Culver, F. Atsma, S. Bandinelli, M. W. Beckmann, J. Benitez, C. Blomqvist, S. E. Bojesen, M. K. Bolla, B. Bonanni, H. Brauch, H. Brenner, J. E. Buring, J. Chang-Claude, S. Chanock, J. H. Chen, G. Chenevix-Trench, J. M. Collee, F. J. Couch, D. Couper, A. D. Coviello, A. Cox, K. Czene, A. P. D'adamo, G. D. Smith, I. De Vivo, E. W. Demerath, J. Dennis, P. Devilee, A. K. Dieffenbach, A. M. Dunning, G. Eiriksdottir, J. G. Eriksson, P. A. Fasching, L. Ferrucci, D. Flesch-Janys, H. Flyger, T. Foroud, L. Franke, M. E. Garcia, M. Garcia-Closas, F. Geller, E. E. J. de Geus, G. G. Giles, D. F. Gudbjartsson, V. Gudnason, P. Guenel, S. Q. Guo, P. Hall, U. Hamann, R. Haring, C. A. Hartman, A. Heath, A. Hofman, M. J. Hooning, J. L. Hopper, F. B. Hu, D. J. Hunter, D. Karasik, D. P. Kiel, J. A. Knight, V. M. Kosma, Z. Kutalik, S. Lai, D. Lambrechts, A. Lindblom, R. Magi, P. K. Magnusson, A. Mannermaa, N. G. Martin, G. Masson, P. F. McArdle, W. L. McArdle, M. Melbye, K. Michailidou, E. Mihailov, L. Milani, R. L. Milne, H. Nevanlinna, P. Neven, E. A. Nohr, A. J. Oldehinkel, B. A. Oostra, A. Palotie, M. Peacock, N. L. Pedersen, P. Peterlongo, J. Peto, P. D. P. Pharoah, D. S. Postma, A. Pouta, K. Pylkas, P. Radice, S. Ring, F. Rivadeneira, A. Robino, L. M. Rose, A. Rudolph, V. Salomaa, S. Sanna, D. Schlessinger, M. K. Schmidt, M. C. Southey, U. Sovio, M. J. Stampfer, D. Stockl, A. M. Storniolo, N. J. Timpson, J. Tyrer, J. A. Visser, P. Vollenweider, H. Volzke, G. Waeber, M. Waldenberger, H. Wallaschofski, Q. Wang, G. Willemsen, R. Winqvist, B. H. R. Wolffenbuttel, M. J. Wright, Australian Ovarian Cancer Study, The GENICA Network, kConFab, The LifeLines Cohort Study, The InterAct Consortium, Early Growth Genetics (EGG) Consortium, D. I. Boomsma, M. J. Econs, K. T. Khaw, R. J. F. Loos, M. I. McCarthy, G. W. Montgomery, J. P. Rice, E. A. Streeten, U. Thorsteinsdottir, C. M. van Duijn, B. Z. Alizadeh, S. Bergmann, E. Boerwinkle, H. A. Boyd, L. Crisponi, P. Gasparini, C. Gieger, T. B. Harris, E. Ingelsson, M. R. Jarvelin, P. Kraft, D. Lawlor, A. Metspalu, C. E. Pennell, P. M. Ridker, H. Snieder, T. I. A. Sorensen, T. D. Spector, D. P. Strachan, A. G. Uitterlinden, N. J. Wareham, E. Widen, M. Zygmunt, A. Murray, D. F. Easton, K. Stefansson, J. M. Murabito, and K. K. Ong. 2014. Parent-of-origin-specific allelic associations among 106 genomic loci for age at menarche. Nature 514:92. doi:10.1038/nature13545

Reverter, A., and E. K. Chan. 2008. Combining partial correlation and an information theory approach to the reversed engineering of gene co-expression networks. Bioinformatics 24:2491–2497. doi:10.1093/bioinformatics/btn482

Reverter, A., N. J. Hudson, S. H. Nagaraj, M. Pérez-Enciso, and B. P. Dalrymple. 2010. Regulatory impact factors: Unraveling the transcriptional regulation of complex traits from expression data. Bioinformatics 26:896–904. doi:10.1093/bioinformatics/btq051

Reverter, A., Y. H. Wang, K. A. Byrne, S. H. Tan, G. S. Harper, and S. A. Lehnert. 2004. Joint analysis of multiple cDNA microarray studies via multivariate mixed models applied to genetic improvement of beef cattle. J. Anim. Sci. 82:3430–3439.

Roth, C. L., C. Mastronardi, A. Lomniczi, H. Wright, R. Cabrera, A. E. Mungenast, S. Heger, H. Jung, C. Dubay, and S. R. Ojeda. 2007. Expression of a tumor-related gene network increases in the mammalian hypothalamus at the time of female puberty. Endocrinology 148:5147–5161. doi:10.1210/en.2007-0634

Saatchi, M., R. D. Schnabel, J. F. Taylor, and D. J. Garrick. 2014. Large-effect pleiotropic or closely linked QTL segregate within and across ten US cattle breeds. BMC Genomics 15:442. doi:10.1186/1471-2164-15-442

Sartori, R., and C. M. Barros. 2011. Reproductive cycles in *Bos indicus* cattle. Anim. Reprod. Sci. 124:244–250. doi:10.1016/j.anireprosci.2011.02.006

Shannon, P., A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker. 2003. Cytoscape: A software environment for integrated models of biomolecular interaction networks. Genome Res. 13:2498–2504. doi:10.1101/gr.1239303

Shirley, K. L., M. G. Thomas, D. H. Keisler, D. M. Hallford, D. M. Montrose, G. A. Silver, and M. D. Garcia. 2006. A Chihuahuan desert Brangus breeding program: Feed efficiency, metabolic hormones, and puberty in heifers sired by bulls with differing expected progeny differences for growth and scrotal circumference. Prof. Anim. Sci. 22:48–58.

Snelling, W. M., R. A. Cushman, J. W. Keele, C. Maltecca, M. G. Thomas, M. R. S. Fortes, and A. Reverter. 2013. Breeding and genetics symposium: Networks and pathways to guide genomic selection. J. Anim. Sci. 91:537–552. doi:10.2527/jas.2012-5784

Tomikawa, J., Y. Uenoyama, M. Ozawa, T. Fukanuma, K. Takase, T. Goto, H. Abe, N. Ieda, S. Minabe, C. Deura, N. Inoue, M. Sanbo, K. Tomita, M. Hirabayashi, S. Tanaka, T. Imamura, H. Okamura, K. Maeda, and H. Tsukamura. 2012. Epigenetic regulation of Kiss1 gene expression mediating estrogen-positive feedback action in the mouse brain. Proc. Natl. Acad. Sci. USA 109:E1294–E1301. doi:10.1073/pnas.1114245109

Trama, J., Q. J. Lu, R. G. Hawley, and S. N. Ho. 2000. The NFAT-related protein NFATL1 (TonEBP/NFAT5) is induced upon T cell activation in a calcineurin-dependent manner. J. Immunol. 165:4884–4894. doi:10.4049/jimmunol.165.9.4884

Utsunomiya, Y. T., A. S. D. Carmo, R. Carvalheiro, H. H. R. Neves, M. C. Matos, L. B. Zavarez, A. M. P. O'Brien, J. Solkner, J. C. McEwan, J. B. Cole, C. P. V. Tassell, F. S. Schenkel, M. V. D. Silva, L. R. Porto Neto, T. S. Sonstegard, and J. F. Garcia. 2013. Genome-wide association study for birth weight in Nellore cattle points to previously described orthologous genes affecting human and bovine height. BMC Genet. 14:52. doi:10.1186/1471-2156-14-52

Wickramasinghe, S., G. Rincon, A. Islas-Trejo, and J. F. Medrano. 2012. Transcriptional profiling of bovine milk using RNA sequencing. BMC Genomics 13:45. doi:10.1186/1471-2164-13-45

Xie, H., H. M. Hoffmann, J. D. Meadows, S. L. Mayo, C. Trang, S. S. Leming, C. Maruggi, S. W. Davis, R. Larder, and P. L. Mellon. 2015. Homeodomain proteins SIX3 and SIX6 regulate gonadotrope-specific genes during pituitary development. Mol. Endocrinol. 29:842–855. doi:10.1210/me.2014-1279