



Single nucleotide polymorphisms in the insulin-like growth factor 1 (IGF-1) gene are associated with performance in Holstein-Friesian dairy cattle

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Insulin-like growth factor 1 (IGF-1) has been shown to be associated with fertility, growth, and development in cattle. The aim of this study was to (1) identify novel single nucleotide polymorphisms (SNPs) in the bovine *IGF-1* gene and alongside previously identified SNPs (2) determine their association with traits of economic importance in Holstein-Friesian dairy cattle. Nine novel SNPs were identified across a panel of 22 beef and dairy cattle by sequence analysis of the 5' promoter, intronic, and 3' regulatory regions, encompassing ~5 kb of *IGF-1*. Genotyping and associations with daughter performance for milk production, fertility, survival, and measures of body size were undertaken on 848 Holstein-Friesian AI sires. Using multiple regression analysis nominal associations ($P < 0.05$) were identified between six SNPs (four novel and two previously identified) and milk composition, survival, body condition score, and body size. The C allele of *AF017143* a previously published SNP (*C-512T*) in the promoter region of *IGF-1* predicted to introduce binding sites for transcription factors HSF1 and ZNF217 was associated ($P < 0.05$) with increased cow carcass weight (i.e., an indicator of mature cow size). Novel SNPs were identified in the 3' region of *IGF-1* were associated ($P < 0.05$) with functional survival and chest width. The remaining four SNPs, all located within introns of *IGF-1* were associated ($P < 0.05$) with milk protein yield, milk fat yield, milk fat concentration, somatic cell score, carcass conformation, and carcass fat. Results of this study further demonstrate the multifaceted influences of *IGF-1* on milk production and growth related traits in cattle.

Keywords: single nucleotide polymorphism, performance, Holstein-Friesian dairy cattle

INTRODUCTION

Insulin-like growth factor 1 (IGF-1) plays an important physiological role in growth regulation, development, metabolism, and lactation in cattle (Daughaday and Rotwein, 1989; Hossner et al., 1997; Breier, 1999; Lucy, 2008). It regulates differentiation including the maintenance of differentiated function in numerous tissues and in specific cell types (Werner et al., 1994). IGF-1 also stimulates the anabolic and mitogenic activity of growth hormone in various tissues (Laron, 2001). The primary source of circulatory IGF-1 is the liver however it is also produced locally in a tissue specific manner (Miller et al., 1981; Schwander et al., 1983; Thissen et al., 1994; Etherton, 2004).

Systemic IGF-1 has been associated with a variety of fertility and reproductive performance measurements in cattle including age at first calving, post-partum resumption of ovarian cyclicity, conception rate to first service, twin ovulation rate, and preimplantation embryo development (Echternkamp et al., 2004; Velazquez et al., 2005; Yilmaz et al., 2006; Patton et al., 2007; Wathes et al., 2007; Velazquez, 2008). Serum IGF-1 has been shown to be highly heritable, with repeatability estimates to the order of 0.48 during the post-weaning period in Angus

cattle (Davis and Simmen, 1997). This suggests a strong additive genetic control of IGF-1, a gene which is regulated at both a transcriptional and translational level (Wang et al., 2003). IGF-1 has been shown to contribute to genetic variation in traits such as carcass fatness, live and carcass weight, average daily gain, as well as body size, food conversion efficiency, milk production, and fat deposition (Davis et al., 1995; Davis and Simmen, 2000; Johnston et al., 2001).

Quantitative trait loci (QTL) overlapping the *IGF-1* region on BTA5 have been reported (Allan et al., 2009; Kim et al., 2009; Sahana et al., 2010). However despite the physiological importance of IGF-1 there are few published studies analyzing the relationship between *IGF-1* variants and performance. Some studies have analyzed a single nucleotide polymorphism (SNP) in the promoter region (*C-512T*) of *IGF-1*, *AF017143*, and a SNP located between exon 3 and 4, *rs29012855*. *AF017143* has been shown to be positively associated with growth traits in Angus cattle (Ge et al., 2001). More recently, Islam et al. (2009), genotyped two purebred cattle populations including 204 Angus and 186 Charolais steers and a hybrid population of 455 cattle for *AF017143* and *rs29012855*. Both SNPs associated with back fat thickness, average back fat, and lean meat

yield in the Angus population although only *rs29012855* associated with slaughter and carcass weight in the Angus steers and rib eye area in the Charolais cattle (Islam et al., 2009).

The objective of the present study was to identify novel SNPs in *IGF-1* and together with *AF017143* and *rs29012855* determine their association with a number of performance traits in Holstein-Friesian cattle.

MATERIALS AND METHODS

IGF-1 SNP DISCOVERY

DNA extraction

Blood samples were obtained from 22 cattle of differing breeds including four Belgian Blue – Holstein-Friesian crossbreds, four Aberdeen Angus – Holstein-Friesian crossbreds as well as four, four and six purebred Charolais, Simmental and Holstein-Friesian cattle respectively. DNA was extracted from blood using a proteinase K/salting out/ethanol precipitation extraction method (adapted from Montgomery and Sise, 1990). DNA was also extracted from thawed–frozen semen from Holstein-Friesian AI bulls ($n = 914$) using a Maxwell[®] 16 instrument and Maxwell[®] 16 Tissue DNA Purification Kits from Promega (Southampton, UK). Prior to DNA extraction washed semen was incubated overnight in lysis buffer (Heyen et al., 1997). Following extraction, the quality and quantity of DNA derived from both blood and semen were assessed using a Nanodrop[®] spectrophotometer (Thermo Scientific, USA) and agarose gel electrophoresis.

Re-sequencing and SNP discovery

Putative SNPs in *IGF-1* (including 5 kb upstream and downstream of the gene to encompass promoter and regulatory regions) were identified using the ENSEMBL database¹ and the Btau_4.0 (October, 2007) assembly (accession no. ENSBTAG00000011082). Bovine specific PCR primers were designed using the Primer3 program (Rozen and Skaletsky, 2000) and the NCBI primer BLAST tool² to amplify approximately 1000 bp fragments of the gene flanking the SNPs identified in ENSEMBL (Table 1). Cycling conditions were as

follows: initial denaturation at 94°C for 4 min followed by 30 cycles 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, and a final extension step of 72°C for 5 min. Each PCR reaction was carried out in 50 μ l final volume and contained 20 ng genomic DNA, 1 μ M primers, 2 mM MgCl₂, 1 U Platinum[®] Taq polymerase, and 1 \times PCR buffer (Invitrogen Life Sciences, Dublin, Ireland). PCR products were purified and sequenced commercially by Eurofins MWG Operon (Martinsried, Germany). Sequence data were manually checked for quality including background noise, peak intensity, and accuracy via the chromatograms using Chromas Lite v2.01³ and analyzed using the BLAST tool from NCBI to confirm their identity and position on bovine chromosome 5⁴.

Sequence alignments and identification of SNPs including comparison with the reference Ensembl sequence and published literature were performed using Clustal W (Larkin et al., 2007) and Chromas Lite v2.01 programs⁵. This allowed validation of published SNPs and the identification of putative novel SNPs within and flanking *IGF-1*.

ASSOCIATION STUDY

Genotyping

All SNPs identified during the re-sequencing process ($n = 10$) alongside additional published SNPs ($n = 6$; *AF017143*, *rs43434843*, *rs43434842*, *rs43434841*, *rs43434839*, and *rs43434840*) were genotyped across 914 Holstein-Friesian sires. The SNP genotyping was carried out commercially using the Sequenom MassArray[®] iPLEX Gold assay (Sequenom, San Diego, CA, USA). As a quality control measure, 25 animals, originating from 25 different samples of extracted genomic DNA were genotyped twice for all SNPs. Mean concordance across all *IGF-1* SNPs and all duplicates was 99.8%. Where discordance existed, the SNP genotype for the sample in question was discarded.

Data editing and phenotypes

An iterative algorithm was used to simultaneously discard both SNPs and individuals with poor genotype call rates (Waters et al., 2011). This resulted in genotypes of 66 individuals being discarded with all *IGF-1* SNPs remaining in the study. A measure of linkage disequilibrium (r^2 ; Hill and Robertson, 1968) was calculated between each pair-wise combination of the segregating SNPs (Emigh, 1980).

Daughter yield deviations (DYD; expressed on the scale of predicted transmitting ability; PTA) and PTAs, as well as associated data reliabilities, for a range of performance traits evaluated by the Irish Cattle Breeding Federation in the January 2009 domestic genetic evaluations were available for inclusion in the analysis. Models used in genetic evaluations in Ireland, as well as variance components, are summarized in detail by Berry et al. (2007). DYD for 305 day milk, fat, and protein yield as well as geometric mean somatic cell score (SCS; log_e somatic cell count)

¹<http://www.ensembl.org> accessed April 2008.

²<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>

Table 1 | Nucleotide sequence and PCR product sizes for the *IGF-1* PCR primers.

Primer set name	Primer	Sequences	Tm (°C)	Product size (bp)
IGF1_PR_1	For	5'-ACCCAGGCTCATAGCATACC-3'	57.5	1020
	Rev	5'-GCAAAGCACCTGCAACTGTA-3'	58.5	
IGF1_PR_2-3	For	5'-CAGTGACCATTCTGTGGCTAA-3'	58.2	1140
	Rev	5'-GCAGCTTCCAATGAAAAGGA-3'	58.8	
IGF1_PR_4	For	5'-GCTAAGACTCCCCAATGCAG-3'	58.3	1020
	Rev	5'-GGCAACATGTGGCTAAAACA-3'	58.0	
IGF1_PR_5	For	5'-TTAGCCCCAAATTTTCAGCA-3'	59.1	964
	Rev	5'-GTCCTTTTGCAGCCCTAACA-3'	58.8	
IGF1_PR_6	For	5'-ACCTCACAGGGGTGGTATGA-3'	58.7	1020
	Rev	5'-CGGAGGACTAGAAGCCACTC-3'	57.5	

For, forward primer; Rev, reverse primer.

³http://www.technelysium.com.au/chromas_lite.htm, manual inspection of sequence data, accessed 14th October 2008.

⁴<http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid = 9913>, confirmation of sequence data with bovine database, accessed 14th October 2008.

⁵http://www.technelysium.com.au/chromas_lite.htm, manual inspection of sequence data, accessed 14th October 2008.

are estimated in Ireland using a repeatability animal model across the first five lactations. PTA for calving interval and survival are estimated using a multi-trait animal model, including data from the first three lactations. PTA for milk yield are used to adjust survival for differences in genetic merit of milk yield; hence, this survival trait is functional survival. PTA for cow carcass weight, progeny carcass weight, progeny carcass fat score, and progeny carcass conformation score, measured at slaughter, are estimated in a multi-trait animal model that includes weaning weight, live-weight of the animal between 300 and 600 days of age, feed intake, and skeletal and muscular linear traits. Cows slaughtered between 875 and 4000 days of age were included in the evaluation of cow carcass weight while male progeny slaughtered between 300 and 1200 days of age and female progeny slaughtered between 300 and 875 days of age were included in the evaluation of the remaining three carcass traits. Genetic evaluations for linear type traits are undertaken as part of a joint evaluation in the UK and Ireland. The estimated breeding values (EBVs) were standardized to the mean and standard deviation of the base population. PTAs were de-regressed using the procedure outlined by Berry et al. (2009):

$$\tilde{\mathbf{y}} = \mathbf{R}(\mathbf{R}^{-1} + \mathbf{A}^{-1})\hat{\mathbf{a}},$$

Where $\tilde{\mathbf{y}}$ is the de-regressed PTA, $\hat{\mathbf{a}}$ is a vector of PTAs from the genetic evaluations, \mathbf{R} is a diagonal matrix where each element is $1/(\text{Reliability of the respective animal} - 1)$, and \mathbf{A} is the numerator relationship matrix.

Parental contribution to the reliability of each DYD or PTA was removed using the approach of Harris and Johnson (1998):

$$\tilde{R} = \frac{R_{\text{TRAD}} - R_{\text{PA}}}{R_{\text{TRAD}} - R_{\text{PA}} + 1 - 2R_{\text{PA}}},$$

Where \tilde{R} is the reliability less the parental contribution, R_{TRAD} is the reliability from the traditional genetic evaluation (i.e., includes information from all relatives), R_{PA} is parental average reliability.

Only sires with a reliability (less parental contribution) of >60% for the trait under investigation were retained for inclusion in the association analysis. A total of 742 sires fulfilled these criteria for inclusion in the analysis of milk, fat, and protein yield as well as milk fat and protein concentration; the number of sires included in the association analysis with SCS, calving interval, and survival

was 703, 501, and, 477, respectively. The number of sires with a reliability of >60% for the carcass traits was 446 and the number of sires with a reliability of >60% for the linear type traits varied from 484 to 551.

Statistical analysis

The association between each SNP and performance was quantified using weighted mixed models in ASREML (Gilmour et al., 2009) with genotyped individual included as a random effect and average expected relationships amongst individuals accounted for through the numerator relationship matrix. Year of birth (divided into five yearly intervals) and percent Holstein of the individual sire were included as fixed effects in the model. In all instances the dependent variable was DYD for milk, yield, fat yield, protein yield, and SCS and de-regressed PTA for the remaining traits, weighted by their respective reliability less the parental contribution.

Genotype was included in the analysis as a continuous variable coded as the number of copies of a given allele. Bonferroni correction of the significance levels was undertaken to account for multiple testing across all SNPs by all traits. Additionally, a multiple regression model was progressively built for each trait where at least one SNP was associated ($P < 0.05$) with the trait in the univariate analyses.

TRANSCRIPTION FACTOR AND microRNA BINDING SITE ANALYSIS

Bioinformatic analysis was performed on SNPs in the promoter regions of the *IGF-1* gene to examine the effects of allele substitution on predicted transcription factor binding sites using MatInspector software package (Quandt et al., 1995) and microRNA binding sites using MicroInspector software (Rusinov et al., 2005).

RESULTS

RE-SEQUENCING AND SNP DISCOVERY

Ten SNPs were identified via sequencing in the 22 cattle, including nine which were putatively novel and one SNP which was previously published, *rs29012855*. The frequency of these 10 SNPs in the 22 cattle SNP discovery panel is shown in **Table 2**. In addition to these detected SNPs, one SNP, *AF017143* previously discovered 512 bp upstream from the *IGF-1* start codon (Ge et al., 1997, 2001) and the five published SNPs originally identified in Ensembl were also included in the genotyping process.

Table 2 | Minor allele frequency of the 10 SNPs identified in the 22 cattle SNP discovery panel.

N	Breed	IGF-1 SNPs*									
		<i>IGF1i1</i>	<i>IGF1i2</i>	<i>IGF1i3</i>	<i>rs29012855</i>	<i>IGF1i5</i>	<i>IGF1i6</i>	<i>IGF1i7</i>	<i>IGF1i8</i>	<i>IGF1i9</i>	<i>IGF1i10</i>
		T → A	T → C	A → G	A → G	T → C	A → G	G → A	G → T	G → A	C → T
4	Simmental	0.13	0.25	0.13	0.50	0.00	0.13	0.13	0.00	0.00	0.00
4	Angus × HF	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	BB × HF	0.00	0.38	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00
6	HF	0.25	0.08	0.00	0.08	0.08	0.00	0.00	0.08	0.08	0.08
4	Charolais	0.38	0.25	0.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00

HF, Holstein-Friesian; BB, Belgian blue. *Only frequencies of the minor alleles (indicated by the arrow) are presented. *AF017143* was not screened for in the 22 cattle panel, it was identified from the literature.

The seven previously published SNPs were all located within introns of the gene, with the exception of *AF017143* and *rs43434840*. Of the nine putative novel SNPs, three (*IGF1r8*, *IGF1r9*, and *IGF1r10*) were identified <2 kb downstream of *IGF-1*, while six SNPs (*IGF1i1*, *IGF1i2*, *IGF1i3*, *IGF1i5*, *IGF1i6*, *IGF1i7*) were all located within introns of *IGF-1*. To the knowledge of the authors, the nine putative novel SNPs discovered in the current study have not been described previously in the literature or in dbSNP and have been submitted to dbSNP⁶.

ASSOCIATION STUDY

Summary statistics

Of the total 16 SNPs examined, five previously identified SNPs (*rs43434843*, *rs43434842*, *rs43434841*, *rs43434839*, and *rs43434840*) were monomorphic in this population of Holstein-Friesian sires and were therefore excluded from the association analyses. Details of the 11 segregating SNPs including location on BTA5 relative to *IGF-1* is illustrated in **Figure 1**, while the nomenclature, flanking sequences including summary statistics of allele frequencies are described in **Table 3**. The minor allele frequency (MAF) for the 11 segregating SNPs ranged from 0.03 (*IGF1i7* and *IGF1r9*) to 0.43 (*AF017143*). Eight of the 11 SNPs (*IGF1i3*, *rs29012855*, *IGF1i5*–*r10*) had MAF between 0.03 and 0.05 with minor alleles present in the heterozygous genotype only for *IGF1r8* and *IGF1r10* and heterozygote frequencies between 5 and 10%. The remaining three SNPs (*AF017143*, *IGF1i1*, and *IGF1i2*) had MAF between 0.32 and 0.43 and heterozygote frequencies of 46–47%. None of the SNPs deviated from Hardy–Weinberg equilibrium.

Table 4 summarizes the extent of linkage disequilibrium among the 11 segregating SNPs in the Holstein-Friesian sample population. Strong LD existed between *IGF1r8*, *IGF1r9*, and *IGF1r10*, all of

which were within 390 base pairs. *IGF1i5*, *IGF1r8*, and *IGF1r9* were also in complete LD and as expected *IGF1i5* was in strong LD with *IGF1r10*. With the exception of *IGF1i6* and *IGF1i7*, the LD between all remaining SNPs was weak. Since *IGF1i5* and *IGF1r9* were in complete LD with *IGF1r8* and the latter SNP had the greatest call rate, *IGF1i5* and *IGF1r9* were omitted from further analysis.

Association analysis

The significant allelic substitution effects of the 11 SNPs on milk yield, milk composition, survival, carcass, and body size traits, when included individually in the model, are listed in **Table 5**. However following adjustment for multiple testing no significant association remained. Significant associations, when estimated using a multiple regression model, are presented in **Table 6** and only these are discussed herein and the significance levels are nominal values and not adjusted for multiple testing. Due to the relatively low LD and low MAF for some of the SNPs haplotype analysis did not provide any further insight into the associations and are therefore not presented.

Milk production, somatic cell count, calving interval, and survival

No significant association between any SNPs and either milk yield, milk protein concentration, or calving interval was evident. The G allele of *rs29012855* was associated ($P < 0.05$) with reduced milk fat percentage. Similarly, the G allele of *IGF1i6* and the G allele of *IGF1i3* were associated independently ($P < 0.05$) with reduced fat yields. Also, *IGF1i3* was the only SNP associated with protein yield with the G allele associated with decreased yield. Only one SNP, the G allele of *IGF1i3* was associated ($P < 0.05$) with decreased somatic cell count. The T alleles in both *IGF1r8* and *IGF1r10* SNPs associated ($P < 0.05$) with improved survival. However, when either *IGF1r8* or *IGF1r10* were included in the model, the association between survival and *IGF1r8* or *IGF1r10* was no longer significant (**Table 6**), attributable to the strong LD between both SNPs.

Carcass and body size related traits

In the multiple regression analysis the G allele of *rs29012855* was associated ($P < 0.05$) with decreased carcass weight while the G allele of *AF017143* was associated ($P < 0.05$) with increased cow carcass weight. *IGF1i3* was associated with carcass conformation; the G allele associated with improved conformation and reduced rump angle although no homozygous GG animals were present in this sample population. *IGF1i2* (T allele) was associated ($P < 0.05$) with decreased carcass fat.

No SNP was significantly associated ($P > 0.05$) with stature, angularity, or rump width (data not shown). However, *IGF1i2* (T allele) and *IGF1r10* (T allele) were all negatively associated ($P < 0.05$) with chest width. *IGF1i6* (G allele) was positively associated ($P < 0.05$) with body condition score.

DISCUSSION

There is a dearth of information available on polymorphisms in bovine *IGF-1* and their effects on economically important traits in cattle. The present study describes the identification of previously unreported SNPs in the intronic and in the 3' non-coding regions of *IGF-1* which associated with milk composition, somatic cell count, survival, cow carcass weight, carcass fat, body condition score, and body size. Following

⁶<http://www.ncbi.nlm.nih.gov/projects/SNP>, registered SNP data with dbSNP 11th September 2009.

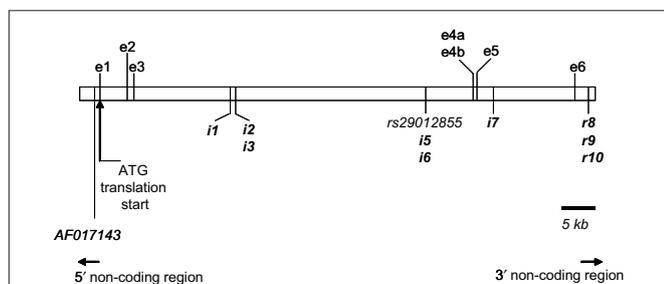


FIGURE 1 | Annotation of *IGF-1* (white box) on BTA5 as per build 4.0 of the *Bos taurus* genome sequence. The physical location of each of the 11 segregating SNPs used in this study is indicated in relation to contig BTA5. The putative nine novel SNPs are highlighted in bold (with the *IGF1* prefix omitted for clarity). The two previously reported SNPs are listed as per their dbSNP accession number (<http://www.ncbi.nlm.nih.gov/projects/SNP>) or GenBank reference (<http://www.ncbi.nlm.nih.gov/genbank/>). Three of the reported alternatively spliced *IGF-1* transcripts [ENSBTAT00000014713 (*IGF-1*); ENSBTAT00000057516 (Q27962_BOVIN) and ENSBTAT00000043595 (Q7YS34_BOVIN)] are shown here. Exon 4a and 4b share overlapping sequences. The *IGF-1* transcript is transcribed by exons 1, 3, 4b, and 6; Q27962_BOVIN is transcribed from exons 4a and 5 and Q7YS34_BOVIN is transcribed from exons 2, 3, 4a, and 6.

Table 3 | Location, nomenclature, genotype frequency, minor allele frequency (MAF), and significance of deviation from Hardy–Weinberg equilibrium (HWE) for the 11 segregating SNPs in the 848 Holstein-Friesian sires.

SNP	Location relative to <i>IGF-1</i>	Nucleotide position on BTA5 ^a	SNP alleles with flanking nucleotide sequence	Status	Reference	Genotype	Genotype frequency (%)	MAF (%)	HWE
<i>AF017143</i>	5'	71198324	GCCCATCCTC[C/T]ACGAATATTC	Reported	<i>AF017143</i> ^b	C/C C/T T/T	19.6 47.5 32.9	43	0.37
<i>IGF1i1</i>	Intron	71176219	TCCTCTAGAT[T/A]GGCACATATT	Novel	<i>ss161151663</i> ^c	A/A A/T T/T	12.4 45.9 41.7	35	0.99
<i>IGF1i2</i>	Intron	71175753	CTGTCAAGCC[C/T]GTAGTRGTTG	Novel	<i>ss161151664</i> ^c	C/C C/T T/T	11.0 42.9 46.1	32	0.61
<i>IGF1i3</i>	Intron	71175747	AGCCYGTAGT[A/G]GTTGTGTGTA	Novel	<i>ss161151665</i> ^c	A/A A/G G/G	91.4 8.2 0.4	4	0.45
<i>rs29012855</i>	Intron	71150007	GGAAGAAAGG[A/G]GGAGCATACC	Reported	<i>CH296968</i> ^b <i>rs29012855</i> ^{a,c}	A/A A/G G/G	89.5 10.0 0.5	5	0.87
<i>IGF1i5</i>	Intron	71149996	GGAGCATACC[T/C]GCCCAGCTAG	Novel	<i>ss161151666</i> ^c	C/C C/T T/T	0.1 7.9 92.0	4	1.00
<i>IGF1i6</i>	Intron	71149881	TTCAGTGCAA[A/G]GCGTGCAAGT	Novel	<i>ss161151667</i> ^c	A/A A/G G/G	92.9 6.7 0.4	4	0.21
<i>IGF1i7</i>	Intron	71138849	GGGCGGGGGC[G/A]GGGGGGAGTC	Novel	<i>ss161151668</i> ^c	A/A A/G G/G	0.4 5.3 94.3	3	0.07
<i>IGF1r8</i>	3'	71124854	AAAACACTTT[G/T]CAAATAACT	Novel	<i>ss161151669</i> ^c	G/G G/T T/T	92.0 7.9 0.1	4	1.00
<i>IGF1r9</i>	3'	71124671	ACTAATAATC[A/G]AATAATTGT	Novel	<i>ss161151670</i> ^c	A/A A/G G/G	0.0 6.9 93.1	3	0.75
<i>IGF1r10</i>	3'	71124464	CACAGAGCCA[C/T]CTGGGAAACC	Novel	<i>ss161151671</i> ^c	C/C C/T T/T	91.7 8.3 0.0	4	0.51

^aEnsembl; ^bGenBank accession number; ^cdbSNP. Sequences are written in the 5' to 3' direction of *IGF-1* on the reverse strand of the BTA5 as per build 4.0 of the *Bos taurus* genome sequence.

Table 4 | Linkage disequilibrium as measured by r^2 between the 11 segregating SNPs in this study.

SNP	<i>AF017143</i>	<i>IGF1i1</i>	<i>IGF1i2</i>	<i>IGF1i3</i>	<i>rs29012855</i>	<i>IGF1i5</i>	<i>IGF1i6</i>	<i>IGF1i7</i>	<i>IGF1r8</i>	<i>IGF1r9</i>
<i>IGF1i1</i>	0.423									
<i>IGF1i2</i>	0.251	0.261								
<i>IGF1i3</i>	0.062	0.026	0.098							
<i>rs29012855</i>	0.040	0.094	0.021	0.003						
<i>IGF1i5</i>	0.012	0.023	0.020	0.002	0.001					
<i>IGF1i6</i>	0.052	0.021	0.003	0.001	0.002	0.002				
<i>IGF1i7</i>	0.041	0.017	0.003	0.001	0.002	0.001	0.939			
<i>IGF1r8</i>	0.012	0.023	0.021	0.002	0.001	1.000	0.002	0.001		
<i>IGF1r9</i>	0.010	0.020	0.017	0.002	0.001	1.000	0.001	0.001	1.000	
<i>IGF1r10</i>	0.013	0.024	0.020	0.002	0.002	0.953	0.002	0.001	0.953	0.981

Table 5 | Allelic substitution effect (standard error in parenthesis) between the 11 segregating SNPs and milk production, somatic cell score (SCS), survival, carcass, and body size traits when included individually in the statistical model.

SNP	Allele substitution ³	MFY (kg)	MPY (kg)	MFC ¹ (%*100)	SCS (units*100)	Sur ¹ (%*100)	CCW (kg)	CW (kg)	CC	CF	CW ²	BCS ²	RA ²
<i>AF017143</i>	T → C						1.29* (0.61)						
<i>IGF1i1</i>	A → T												
<i>IGF1i2</i>	C → T									-5.14** (1.77)	-0.23* (0.10)		
<i>IGF1i3</i>	A → G	-1.96* (0.78)	-1.31* (0.61)		-2.90* (1.30)					12.33** (4.60)		0.35 [†] (0.21)	-0.50* (0.25)
<i>rs29012855</i>	A → G				-3.07* (1.50)		2.71* (1.30)	2.48* (1.01)					
<i>IGF1i6</i>	A → G	-1.91* (0.86)	-0.81 (0.67)				-3.5* (1.50)			7.45 [†] (4.24)	0.57* (0.26)	0.42* (0.21)	
<i>IGF1i7</i>	A → G	1.77 [†] (0.95)					3.8* (1.65)			-9.97* (4.63)			
<i>IGF1r8²</i>	G → T					78.56* (34.50)					-0.55** (0.21)		
<i>IGF1r10</i>	C → T					76.46* (35.90)				-7.8 [†] (4.62)	-0.6** (0.23)		

Significance from difference from 0. [†] $P < 0.10$, * $P < 0.05$, ** $P < 0.01$; Presented significant associations do not persist after adjusting for multiple testing therefore P -values are nominal. Column headings are abbreviated phenotypes; MFY, milk fat yield; MPY, milk protein yield; MFC, milk fat concentration; SCS, somatic cell score; Sur, survival; CCW, cull cow weight; CW, carcass weight; CC, carcass conformation [scale 100 (poor) to 1500 (good)]; carcass fat [scale 100 (low) to 1500 (high)]; CW, chest width; BCS, body condition score; RA, rump angle. ¹A value of 1, prior to multiplication by 100, equates to 1% unit; ²*IGF1i5* and *IGF1r9* associations not presented because they are identical to *IGF1r8*; ³Allele substitutions are reported from the reverse strand of *IGF-1*.

adjustment for multiple testing no SNP remained significantly associated with performance. However, identification of patterns/clusters of associations between adjacent SNPs, as well as results from multiple regression analysis, can provide useful information into regions of the genes that may be associated with performance.

Although all 11 SNPs were in Hardy–Weinberg equilibrium, eight were segregating with low MAFs of $\leq 5\%$ and two SNPs were present in the heterozygote form only. Other studies from our group have shown almost identical MAF for 10 of these SNPs (excluding *AF017143*) in a separate population of 610 commercial Holstein-Friesian dairy cows (Lynch et al., 2010). This provides supporting evidence of their segregation at low MAF within Holstein-Friesian cattle. Low frequency variants have been speculated to contribute to the missing heritability described from large scale genome wide association studies of complex traits in many species including humans (Maher, 2008; Manolio et al., 2009). Although substantially larger sample sizes are required to establish with confidence the effects of low frequency or rare variants on complex traits, the SNPs identified herein may represent such variants. This study therefore highlights the benefits of re-sequencing in addition to SNP chip based genome wide association analyses to facilitate detection of uncommon or rare variants affecting complex phenotypes.

The C allele of *AF017143* has previously been documented to be positively associated with higher *IGF-1* gene and protein expression levels in Polish Holstein-Friesian cattle (Maj et al., 2008). Ge et al. (2001) also reported a positive association between the C allele and post-weaning weight gain, ultrasound back fat thickness, and average carcass back fat. However, Islam et al. (2009) reported a negative association with meat yield in Angus beef cattle. In our study we

failed to observe any association between the *AF017143* SNP in Holstein-Friesian sires and carcass fat scores in their progeny. The inconsistency between studies may be a result of breed differences with Angus beef cattle maturing earlier and having greater carcass fat at slaughter compared with Holstein-Friesian cattle. It may also be due to different LD patterns within breeds which may account for the absence of an association of this SNP in the Charolais cattle analyzed by Islam et al. (2009). However, in the present study, the C allele of *AF017143* was associated with heavier cow carcasses but not associated with progeny carcass weight, suggesting that *AF017143* is associated with mature cow size rather than size at a younger age. Siadkowska et al. (2006) reported *AF017143* positively associated with live body weight at slaughter and cold carcass weight in a population of 131 fifteen-month-old Holstein-Friesian bulls. These observations are consistent with the role of the somatotrophic axis through the action of IGF-1. It plays a key role in the regulation of the metabolism and physiology of mammalian growth and is the main regulator of post-natal somatic growth, stimulating anabolic processes such as cell division, skeletal growth, and protein synthesis (Curi et al., 2005).

Potential mechanisms for an association between *AF017143* and carcass weight may lie in its location in the promoter region of *IGF-1* and the possible influence on transcriptional factor binding sites (TFBS). Analysis of TFBS *in silico* predicted that the C allele of *AF017143* introduced two new TFBS that are abrogated by the T allele (data not shown). One of which is for Heat Shock Factor 1 (HSF1), a known transcriptional repressor (Xie et al., 2002) which may act to repress *IGF-1* expression. Similarly the C allele introduces a predicted TFBS for Zinc finger protein 217 from the

Table 6 | Associations ($P < 0.05$) between *IGF-1* SNPs and milk composition, survival, body condition score, and carcass traits when estimated using multiple regression models¹.

SNP	Allele substitution ⁴	Fat yield (kg)	Protein yield (kg)	Fat concentration ² (%*100)	SCS (units*100)	Survival ² (%*100)	Cow carcass weight (kg)	Carcass weight (kg)	Carcass conformation [scale 100 (poor) to 1500 (good)]	Carcass fat [scale 100 (thin) to 1500 (fat)]	Chest width ³	Body condition score ³	Rump angle ²
AF017143	T → C					1.29 (0.61)							
IGF12	C → T									-5.14 (1.77)	-0.24 (0.11)		
IGF13	A → G	-2.17 (0.78)	-1.31 (0.61)		-2.90 (1.30)				12.33 (4.60)				-0.50 (0.25)
rs29012855	A → G			-3.07 (1.54)				2.48 (1.01)					
IGF1i6	A → G	-2.16 (0.86)										0.42 (0.21)	
IGF1r10	C → T					78.56 (34.48)					-0.51 (0.23)		

¹Multiple regression analysis only presented for trait where associations existed between more than 1 SNP in the univariate analyses; Presented significant associations do not persist after adjusting for multiple testing therefore P -values are nominal. ²A value of 1, prior to multiplication by 100, equates to 1% unit. ³Expressed in genetic standard deviation units. ⁴Allele substitutions are reported from the reverse strand of *IGF-1*.

zinc finger family of transcription factors. Although less is known about ZNF217, research in other species suggests that it too acts as a transcriptional repressor (Banck et al., 2009).

It is well established that gene transcription is extensively and co-ordinately regulated. While introns are known to carry regulatory sequences, they may not have a direct involvement in the regulation of transcription of highly expressed genes. Systematic differences in motif distributions do suggest that introns play a role in the rate of transcription (Zhang et al., 2008). Of the seven SNPs located within introns of the *IGF-1* gene, six including five novel (*IGF1i2*, *IGF1i3*, *IGF1i5*, *IGF1i6*, *IGF1i7*) and one previously reported (*rs29012855*) were associated with at least two performance traits, emphasizing the integral role of *IGF-1* in milk production, growth, and development in cattle.

In the current study, *IGF1i2* associated with carcass fat and chest width, while other studies from this group (Lynch et al., 2010) reported associations between *IGF1i2* and body condition score at calving in a cohort of 241 dairy cows. Body condition score in the current study was associated with *IGF1i6* (despite its low MAF). Both *IGF1i2* and *IGF1i6* are located between exons 3 and 4 of the *IGF-1* gene and it is possible a causative polymorphism affecting body condition score is in LD with this region of *IGF-1* exhibiting different LD patterns between the cattle populations. Indeed, previous studies have described a relationship between systemic IGF-1 and carcass fat; Davis and Simmen (2000) reported that Angus bulls with lower plasma IGF-1 concentrations had higher marbling scores and back fat thickness. Similarly, circulating IGF-1 was found to be negatively correlated with carcass fat percentage, fat accretion rate, and fat thickness in Simmental crossbred bulls (Anderson et al., 1988). Interestingly, the stage of differentiation of precursor cells into mature fat cells is accompanied by enhanced expression of *IGF-1* in transgenic mice indicating a role for IGF-1 in fat cell developmental processes (Rajkumar et al., 1999).

Systemic IGF-1 has been shown to be positively correlated with milk yield and milk fat concentrations (Moyes, 2004; Rose et al., 2005). However there are no reports in the literature of associations between *IGF-1* SNPs and milk production traits. Furthermore none of the SNPs analyzed in this study displayed an association with milk yield. However the G alleles of two novel SNPs, *IGF1i3*, and *IGF1i6*, were associated with decreased milk fat and protein (*IGF1i3* only) yield. Consistent with this, IGF-1 is known to play an important role in mammary gland growth and function by regulating several cellular processes (Akers, 2006) including the stimulation of protein synthesis in the epithelial cells of the mammary gland (Burgos and Cant, 2010). Additionally, significant associations (*IGF1i3*) were observed with somatic cell count (SCC). Liebe and Schams (1998) reported that the concentration profile of IGF-1 in milk corresponded well with SCC and concluded that this was possibly an important measurement in monitoring the state of udder health. Ruffer (2003) also found that IGF-1 concentrations increased significantly in milk collected from healthy udder quarters that were adjacent to quarters showing SCC of more than 100,000/ml and mastitis.

To the knowledge of the authors, associations between *rs29012855* and performance have only been previously reported by Islam (2009) in 455 hybrid, 206 Angus, and 186 Charolais beef cattle. Islam (2009) reported positive associations between the G allele and lean meat yield, slaughter, and carcass weight in the Angus steers. Islam (2009) also reported negative associations between the G allele and back fat thick-

ness in the Angus steers and rib eye area in the Charolais cattle. This is in agreement with our findings where the G allele associated with heavier carcasses but also decreased milk fat percentage. This is the first reporting of *rs29012855* being associated with milk production variables. A MAF of 1.9, 5.1, and 3.5% was observed for *rs29012855* in the Angus, Charolais, and hybrid populations respectively, providing further evidence that *rs29012855* is segregating at low MAF in cattle (Islam, 2009). The low MAF of *rs29012855* and other SNPs in this study suggests a potential antagonistic relationship with other variants under positive selection. The minor alleles of *IGF1i3*, *rs29012855*, *IGF1i6*, and *IGF1i7* were all significantly negatively associated with milk production variables and their low frequency in Holstein-Friesian cattle may be due to the intense selection for milk production over the last four decades in dairy cattle (Diskin et al., 2006).

The importance of mutations located in the 3'UTR was evident in a study by Clop et al. (2006), where a G to an A transition in the 3'UTR of the myostatin gene introduced illegitimate miRNA target sites resulting in muscular hypertrophy in Texel sheep. None of the three 3'UTR novel SNPs in this study (*IGF1r8*, *IGF1r9*, and *IGF1r10*) occur in any region of the *IGF-1* where miRNA are predicted to bind. However these three mutations are in high LD, within 390 bp, and significantly associated with functional survival. Although none of the SNPs in this study associated with reproductive performance measured as calving interval, reproductive performance, particularly in grass based production systems, is a major determinant of cow survival (Diskin et al., 2006). Therefore this may indicate an indirect effect on calving interval perhaps through fitness *per se*.

Other studies from our group using this population of sires have identified SNPs in other candidate genes including other members of the somatotropic axis, i.e., Growth Hormone (Mullen et al., 2010) and Growth Hormone Receptor (Waters et al., 2011). The

possible effects of these and *IGF-1* variants on epistasis especially in established pathways such as the somatotropic axis is unknown. Although untested, the multiple associations within traits and pleiotropic effects observed with *IGF-1* and the other genes analyzed in this population suggests independent effects. This may contribute to the multifactorial nature of complex phenotypes. Whether the effects observed herein are due to these *IGF-1* SNPs directly, or with LD with causative variants, affecting systemic IGF-1 levels remains to be ascertained. Future work could include targeted enrichment encompassing entire genes and regulatory regions or whole genome sequencing approaches in an attempt to identify causative polymorphisms. If such studies were augmented with functional genomic analyses and validated in independent populations, greater insight into the genetics underpinning performance would be gained.

CONCLUSION

The effects of IGF-1 on mammalian post-natal growth and developmental processes including metabolism and nutrient partitioning are well established. To what degree polymorphisms in *IGF-1* contribute to these effects is unknown. However this study indicates, polymorphisms including low frequency variants located within *IGF-1* or nearby regions of the genome are affecting performance in cattle.

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