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Single nucleotide polymorphisms in the imprinted bovine *insulin-like growth factor 2 receptor* gene (*IGF2R*) are associated with body size traits in Irish Holstein-Friesian cattle

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Summary

The regulation of the bioavailability of insulin-like growth factors (IGFs) is critical for normal mammalian growth and development. The imprinted *insulin-like growth factor 2 receptor* gene (*IGF2R*) encodes a transmembrane protein receptor that acts to sequester and degrade excess circulating insulin-like growth factor 2 (IGF-II) – a potent foetal mitogen – and is considered an important inhibitor of growth. Consequently, *IGF2R* may serve as a candidate gene underlying important growth- and body-related quantitative traits in domestic mammalian livestock. In this study, we have quantified genotype–phenotype associations between three previously validated intronic bovine *IGF2R* single nucleotide polymorphisms (SNPs) (*IGF2R*:g.64614T>C, *IGF2R*:g.65037T>C and *IGF2R*:g.86262C>T) and a range of performance traits in 848 progeny-tested Irish Holstein-Friesian artificial insemination sires. Notably, all three polymorphisms analysed were associated ($P \leq 0.05$) with at least one of a number of performance traits related to animal body size: angularity, body depth, chest width, rump width, and animal stature. In addition, the C-to-T transition at the *IGF2R*:g.65037T>C polymorphism was positively associated with cow carcass weight and angularity. Correction for multiple testing resulted in the retention of two genotype–phenotype associations (animal stature and rump width). None of the SNPs analysed were associated with any of the milk traits examined. Analysis of pairwise r^2 measures of linkage disequilibrium between all three assayed SNPs ranged between 0.41 and 0.79, suggesting that some of the observed SNP associations with performance may be independent. To our knowledge, this is one of the first studies demonstrating associations between *IGF2R* polymorphisms and growth- and body-related traits in cattle. These results also support the increasing body of evidence that imprinted genes harbour polymorphisms that contribute to heritable variation in phenotypic traits in domestic livestock species.

Keywords *Bos*, genetic imprinting, *IGF2R*, performance trait, single nucleotide polymorphism.

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Genetic imprinting, a form of epigenetic regulation, results in the preferential monoallelic expression of autosomal genes in a parent-of-origin manner of at least 100 mammalian genes, most of which play pivotal roles in directing pre- and post-natal mammalian growth and development (Feil 2009). While most studies of imprinted genes have focussed on mouse and human disease models, there is accumulating evidence suggesting that imprinted loci underlie performance in domesticated animals and hence

may serve as molecular targets for artificial selection and animal breeding strategies (Ruvinsky 1999; de Koning *et al.* 2002; Magee *et al.* 2010).

The *insulin-like growth factor 2* (*somatomedin A*) (*IGF2*) and *insulin-like growth factor 2 receptor* (*IGF2R*) genes represent two well-studied imprinted loci, because of their purported roles within the somatotropic axis, which regulates mammalian growth and development, and their relationship to biomedical disorders, particularly cancer (Foulstone *et al.* 2005). The paternally expressed *IGF2* gene encodes a potent foetal mitogen (IGF-II) that stimulates cellular proliferation via interactions with the transmembrane insulin-like growth factor 1 receptor (IGF-IR) protein (Chao & D'Amore 2008). *IGF2R* encodes a single transmembrane receptor (IGF-IIR) that reduces the mitogenic effects of IGF-II by sequestering and internalizing excess IGF-II for lysosomal degradation (Kornfeld 1992). *IGF2R* is thus regarded as an important inhibitor of mammalian growth (Ghosh *et al.* 2003; Brown *et al.* 2009). *IGF2R* exhibits madumnal expression in a range of mammalian species, including mice, sheep, pigs and cattle, although the imprinting status of the human *IGF2R* gene remains equivocal (Barlow *et al.* 1991; Xu *et al.* 1993; Killian *et al.* 2000, 2001; Monk *et al.* 2006; Kim *et al.* 2007).

Previous genetic studies have revealed that DNA sequence polymorphisms within the *IGF2* gene underlie important quantitative trait loci for production traits in domestic livestock species (Van Laere *et al.* 2003; Bagnicka *et al.* 2010). However, there is little published work on associations between the *IGF2R* gene and heritable phenotypic variation in domestic livestock populations. On the basis of the biological role of *IGF2R* in regulating mammalian growth and development, we adopted a candidate gene approach to assess genotype–phenotype associations between three previously validated intronic single nucleotide polymorphisms (SNPs) in the bovine *IGF2R* gene and a range of performance traits measured in 848 progeny-tested Irish Holstein-Friesian artificial insemination bulls.

In cattle, the *IGF2R* gene spans a ~101-kb region on *Bos taurus* chromosome 9 (BTA9) and consists of 48 translated exons (http://www.ensembl.org). At the time of laboratory analysis, 11 putative *B. taurus IGF2R* SNPs (all intronic) were deposited in the Ensembl database (Build 3.1 of the *B. taurus* genome assembly, Ensembl release 46, August 2007; http://aug2007.archive.ensembl.org/Bos_taurus). For SNP validation purposes, high-fidelity polymerase chain reaction (PCR) amplicons spanning six of these putative *IGF2R* SNPs, distributed across 90 592 base pairs (bp) of the *IGF2R* gene, were generated and sequenced in a panel of 17 European *B. taurus* animals (six Limousin, five Simmental and six Aberdeen Angus samples) (Magee *et al.* 2010). Only two of the six Ensembl-listed SNPs, rs41623543 (recoded here as *IGF2R*:g.64614T>C) and rs41623544 (recoded here as *IGF2R*:g.65037T>C), both of which are located in intron 20 of the *IGF2R* gene, were shown to be polymorphic in this panel of animals. These SNPs were re-formatted for medium throughput genotyping; the remaining four SNPs were monomorphic and not considered further (Table S1). In addition, two *de novo* polymorphic *IGF2R* SNPs (*IGF2R*:g.86262C>T and *IGF2R*:g.86531G>A, both located in intron 36 of the *IGF2R* gene), which were detected as part of the *IGF2R* SNP validation process and reported by Magee *et al.* (2010), were selected for further genotyping analysis in the current study. All four selected SNPs had minor allele frequencies between 0.196 and 0.210 in a population of 138 European *B. taurus* (Limousin) animals previously analysed by us (Magee *et al.* 2010).

Genotyping was performed commercially by Sequenom Inc. (San Diego, CA, USA) via their proprietary MassARRAY iPLEX platform (http://www.sequenom.com/iplex) using 914 Irish Holstein-Friesian artificial insemination bulls together with 25 additional independently extracted technical duplicate samples for quality control purposes. Prior to association analyses, quality control on all genotypic data was undertaken using the criteria outlined by Waters *et al.* (2011). The iterative algorithm used for data filtering removed a total of 66 sires and one SNP (*IGF2R*:g.86531G>A) from the data set, with the final edited data set consisting of genotypes for 848 sires based on three SNPs (*IGF2R*:g.64614T>C, *IGF2R*:g.65037T>C and *IGF2R*:g.86262C>T). All association analyses, summary statistics and estimates of linkage disequilibrium (LD) were performed and generated using this edited data set. The control duplicate DNA samples displayed a ≥99% SNP genotype concordance rate, and where discordance occurred, the genotype of the sample in question was set to missing. The genomic co-ordinates of the three analysed SNPs are presented in Table 1 and are based on the open reading frame gene model for the currently annotated bovine *IGF2R* gene in the ENSEMBL database (gene ID ENSBTAG00000002402, Build 4.0 of *B. taurus* genome assembly, Ensembl release 60, November 2010).

A range of phenotypic traits, grouped into seven broad categories, were analysed for genotype association: (i) milk production traits (milk yield, milk fat yield, milk protein yield, milk fat and protein percentage); (ii) udder/animal health (somatic cell count, SCC); (iii) carcass traits (culled cow carcass weight, progeny carcass weight, subcutaneous progeny carcass fat level and progeny carcass conformation score); (iv) growth- and size-related traits in live animals (animal stature, chest width, body depth, rump angle and rump width); (v) subjectively assessed subcutaneous fat level on live animals (angularity and body condition score); (vi) calving traits [direct calving difficulty attributed to the size of the calf, maternal calving difficulty (a function of maternal pelvic width) and perinatal mortality]; and (vii) fertility and survival (calving interval, gestation length and cow survival). A detailed description of each trait is provided in Table S2.

Table 1 Summary statistics for the *IGF2R* SNPs in the 848 Holstein-Friesian sires analysed in this study

SNP ID	Location of SNP within the bovine *IGF2R* gene	SNP position on BTA9	Genotype	Genotype frequencies	Minor allele frequency	Deviations from HWE (*P*-value)
IGF2R:g.64614T>C	Intron 20	100 112 956	TT	0.014	0.121 (T)	0.892
			CT	0.214		
			CC	0.772		
IGF2R:g.65037T>C	Intron 20	100 113 379	CC	0.062	0.253 (C)	0.757
			CT	0.382		
			TT	0.556		
IGF2R:g.86262C>T	Intron 36	100 134 604	CC	0.022	0.144 (C)	0.699
			CT	0.244		
			TT	0.734		

Genotype and allele frequencies, and the significance of deviations from HWE based on *P*-values obtained from chi-square test results are shown for all three *IGF2R* SNPs analysed. All SNP nucleotide positions were obtained from the Build 4.0 of the *Bos taurus* genome sequence in the ENSEMBL database (http://www.ensembl.org, release 60) or the UCSC genome browser (http://genome.ucsc.edu). The open reading frame gene model positions for each SNP are given based on the currently annotated bovine *IGF2R* gene in ENSEMBL (gene ID ENSBTAG00000002402).
HWE, Hardy–Weinberg equilibrium; SNP, single nucleotide polymorphism.

Sire predicted transmitting ability (PTA) was the dependent variable for all traits, with the exception of the milk production traits (including SCC), which were daughter yield deviations (DYDs) expressed on a PTA scale. DYDs were expressed on a PTA scale as opposed to the scale of estimated breeding values (i.e. twice the PTAs) used in some other studies. Predicted transmitting abilities, together with associated reliabilities, for a range of performance traits were available, as evaluated by the Irish Cattle Breeding Federation (http://www.icbf.com) in the January 2009 domestic genetic evaluations.

Models used in genetic evaluations in Ireland, as well as variance components, have been summarized in detail by Berry *et al.* (2007). Daughter yield deviations for 305-day milk, fat and protein yield as well as geometric mean somatic cell score (SCS) ($\log_e$ SCC) are estimated in Ireland using a repeatability animal model across the first five lactations. Predicted transmitting ability for calving interval and survival are estimated using a multi-trait animal model, including data from the first three lactations. Predicted transmitting abilities for milk yield are used to adjust PTAs for survival differences related to milk yield, and hence this survival trait reflects functional survival. PTAs for cull 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 are included in the evaluation of cull 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 are 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. Estimated breeding values are standardized to the mean and standard deviation of the base population.

All PTAs were de-regressed using the procedure outlined by Berry and colleagues (Berry *et al.* 2009). Only sires with a reliability score, minus parental contribution, of >60% were retained for inclusion in the association analysis. A total of 742 sires fulfilled this criterion for inclusion in the analysis of milk, fat and protein yield and milk fat and protein concentration. The numbers of sires included for calving interval and cow survival were 501 and 477, respectively. The numbers of sires for direct calving difficulty, maternal calving difficulty and perinatal mortality were 575, 506 and 201, respectively. A total of 446 sires had a reliability of >60% for the carcass traits, and between 484 and 551 sires had a reliability of >60% for the linear size traits.

The association between each SNP and performance was quantified using weighted mixed linear models in ASREML (Gilmour *et al.* 2006), with individual included as a random effect. Average expected relationships among individuals were accounted for through the numerator relationship matrix. Year of birth (divided into 5-yearly intervals) and percent Holstein of the individual sire were included as fixed effects in the model. Year of birth was grouped for two reasons: (i) to account for non-linearity in the effect, and (ii) because a small number of bulls had no known date of birth, and fitting as a class effect facilitated their inclusion in the model. The impact of including year of birth (or Holstein proportion) was small, because the effect was not highly significant and was not significant for all traits.

In all instances, the dependent variable was de-regressed PTA or DYD, weighted by their respective reliability minus

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the parental contribution. Genotype was included in the analysis as a continuous variable coded as the number of copies of a given allele. Bonferroni adjustment (Bonferroni 1936) for multiple testing of genotype–phenotype associations was undertaken assuming a total of 21 effective independent tests (i.e. three SNPs multiplied by seven groups of traits). The HAPLOVIEW package (Barrett *et al.* 2005) was used to measure r^2 values of LD between pairwise combinations of segregating SNPs (Hill & Robertson 1968).

Summary statistics, including genotype and allele frequencies, together with chi-square tests for deviations from Hardy–Weinberg equilibrium (HWE) for each of the assayed SNPs, are presented in Table 1. Minor allele frequencies ranged between 0.12 (*IGF2R*:g.64614T>C) and 0.25 (*IGF2R*:g.65037T>C). Heterozygosities for each SNP ranged between 0.214 (*IGF2R*:g.64614T>C) and 0.382 (*IGF2R*:g.65037T>C), with an average heterozygosity of 0.280 across all three loci. None of the SNPs deviated significantly from HWE. Only significant associations ($P \leq 0.05$) between the assayed SNPs and the performance traits investigated are presented in Table 2; non-significant associations with genotypes are not presented. In addition, underlined values in Table 2 indicate those associations that remained significant after adjustment for multiple testing (adjusted *P*-values ≤0.05).

In the current study, significant associations were observed between all three *IGF2R* SNPs and linear traits related to body size only (Table 2). No associations were observed with milk, animal health or calving traits. A C-to-T allele substitution at the *IGF2R*:g.86262C>T and *IGF2R*:g.65037T>C SNPs and a T-to-C transition at the *IGF2R*:g.64614T>C SNP were associated ($P \leq 0.05$) with taller, deeper animals with lower pins, while C-to-T allele substitutions at the *IGF2R*:g.86262C>T and *IGF2R*:g.64614T>C loci were also associated ($P \leq 0.01$) with greater chest depth. Furthermore, an association ($P \leq 0.05$) with cow carcass weight was observed at the *IGF2R*:g.65037T>C SNP, while the *IGF2R*:g.64614T>C SNP tended ($P \leq 0.10$) to be associated with this trait. Finally, only the C-to-T transition at the *IGF2R*:g.65037T>C SNP was associated with more angular cows; however, this SNP was not associated with body condition score. This was despite the fact that cow angularity and body condition score are genetically similar and yet represent opposite subjective assessments of the subcutaneous fat deposits on a live animal (Berry *et al.* 2004). Despite the similar allele substitution effects observed at all three loci and the relatively small genomic interval containing all three SNPs (~21.6 kb), inspection of the r^2 values of LD for all SNP pairwise combinations (*IGF2R*:g.64614T>C–*IGF2R*:g.65037T>C: 0.41; *IGF2R*:g.65037T>C–*IGF2R*:g-86262C>T: 0.52; *IGF2R*:g.64614T>C–*IGF2R*:g.86262C>T: 0.79) indicates that some of the phenotypic associations detected at these loci may be independent. Notably, only two genotype–phenotype associations remained significant (adjusted *P*-value ≤0.05) after Bonferroni adjustment for multiple testing: *IGF2R*:g.86262C>T and rump width, and *IGF2R*:g.65037T>C and animal stature.

Collectively, these data suggest that variation in the imprinted bovine *IGF2R* gene influences important animal body size/growth traits in cattle. This assertion is supported by the molecular role of the IGF-IIR protein in negatively regulating IGF-II-mediated growth and development by preventing the bioaccumulation of excess IGF-II mitogen. Simultaneous excess circulating IGF-II and reduced IGF-IIR levels have been shown to induce tumourigenesis in mice (Hassan & Howell 2000), while studies in humans have demonstrated that loss-of-function mutations in the human *IGF2R* gene result in the development of cancer, suggesting that *IGF2R* functions as a tumour suppressor gene (Foulstone *et al.* 2005). This is supported by previous studies which have shown that mice deficient in IGF-IIR display increased circulating IGF-II levels, resulting in internal organ enlargement and ultimately resulting in pre- and early post-natal lethality (Lau *et al.* 1994; Ludwig *et al.* 1996).

Only two of the observed genotype–phenotype associations in this candidate gene study remained significant after Bonferroni adjustment for multiple testing. However, preselection of loci for genetic association studies incorporating pre-existing biological information (a so-called 'candidate gene' approach) can reduce the incidences of both Type I

Table 2 Significant allele substitution effects between the three *IGF2R* SNPs and body size traits and milk performance traits

SNP	Allele substitution	Cow carcass weight (kg)	Body depth[1]	Stature[1]	Rump width[1]	Chest width[1]	Angularity[1]
IGF2R:g.64614T>C	T → C	1.55† (0.93)	0.32* (0.13)	0.35* (0.14)	0.40** (0.14)	0.45** (0.15)	–0.07 (0.15)
IGF2R:g.65037T>C	C → T	1.55* (0.72)	0.29** (0.10)	0.42*** (0.10)	0.28* (0.11)	0.07 (0.12)	0.23* (0.11)
IGF2R:g.86262C>T	C → T	1.41 (0.86)	0.32** (0.16)	0.36** (0.12)	0.41** (0.13)	0.38** (0.13)	–0.01 (0.13)

Values show the units of effective change per base change in the given allele substitution with standard error within brackets. *P* values are represented as follows: $^{\dagger}P \leq 0.10$, $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$. Underlined values indicate associations that remained significant after Bonferroni adjustment for multiple testing (adjusted *P*-values ≤0.05).

SNP, single nucleotide polymorphism.

[1]Measured in standard deviation units (SDU).

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error (i.e. the generation of false-positive associations with traits) and Type II error (i.e. the generation of false-negative associations because of stringent statistical correction for multiple testing) that are commonly encountered during genome-wide association studies (Hu *et al.* 2009; Jorgensen *et al.* 2009; Li *et al.* 2011). Accordingly, in the current study, based on the molecular role of *IGF2R* in regulating mammalian growth and development, associations with growth and animal size-related phenotypes were considered more likely than associations with non-growth-related traits (e.g. milk and fertility traits).

All SNPs analysed in this study were intronic, and therefore it is unlikely that they directly modulate *IGF2R* expression levels or alter the efficiencies of IGF-II/IGF-IIR interactions leading to heritable differences in animal growth. However, mutations occurring in non-amino protein coding DNA sequences (e.g. 5′ UTRs, introns and 3′ UTRs) can directly affect phenotypic variation in domestic livestock. For example, padumnal (paternally expressed) inheritance of the 'A' allele of the maternally imprinted porcine *IGF2* intron 3 g.3072G>A mutation is believed to prevent the binding of an IGF2 repressor protein, resulting in increased *IGF2* expression that in turn stimulates increase muscle growth (Van Laere *et al.* 2003; Goodall & Schmutz 2007; Stinckens *et al.* 2007, 2010). Similarly, it is possible that the SNPs analysed in this study affect *IGF2R* expression levels by preventing or enhancing the binding of repressor/activator molecules to *IGF2R* DNA sequences, which in turn affects the levels of IGF-IIR protein that are available for sequestering and degrading circulating IGF-II. In contrast, the SNPs analysed in the current study may be in strong LD with an unidentified regulatory SNP (or group of SNPs) located proximal to or within the bovine *IGF2R* gene, which themselves affect *IGF2R* expression levels or the ability/efficiency of IGF-IIR to bind to and regulate IGF-II. Indeed, a putative regulatory SNP in the human *IGF2R* ortholog, which is hypothesized to alter the binding affinity of lysosomal enzymes to IGF-IIR during IGF-II degradation, has been shown to be associated with human foetal growth (Kaku *et al.* 2007).

The observed association between the *IGF2R*:g.65037T>C SNP and angularity suggests that the *IGF2R* gene may somehow be involved in adipocyte maturation, although this was the only SNP to be associated with this trait. To our knowledge, there is little scientific evidence supporting a role for *IGF2R* in fat metabolism. For example, a recent study performed by Duffield *et al.* (2008) did not reveal any significantly altered *IGF2R* expression in adipose tissue in normal foetal sheep and sheep receiving restricted placental supplementation (which is known to result in increased post-natal visceral fat mass) during late gestation. Again, this may point towards an association between the *IGF2R*:g.65037T>C SNP and a putative regulatory SNP influencing fat deposition that is located proximal to the *IGF2R* locus on BTA9.

The molecular role of IGF-IIR in regulating the bioavailability of mitogenic IGF-II has often been cited as evidence for the conflict theory for the evolution of genetic imprinting in mammals. This theory proposes that paternally expressed imprinted genes act to recruit maternal biological resources during development, thereby promoting growth, whereas maternally expressed imprinted genes act to restrict demand on maternal biological resources by inhibiting growth (Moore & Haig 1991). The data presented in the current study lend some credence to this theory, whereby SNPs in the bovine *IGF2R* gene [a maternally expressed gene in tissues from normally developed animals (Killian *et al.* 2000, 2001; Suteevun-Phermthai *et al.* 2009)] are significantly associated with animal growth and body traits. These findings, however, lead to the apparent paradoxical observation that SNPs within a maternally expressed (i.e. paternally imprinted) gene are associated with sire-derived genetic merit scores for phenotypic traits. However, a possible explanation is that the genetic merit scores used here are based on the phenotypic data of the assayed sires' female progeny across many generations; consequently, a sire-derived madumnal (maternally expressed) allele could be associated with performance traits because of its transmission via female intermediaries.

It is important to note that recent studies involving the genetic dissection of quantitative traits incorporate parent-of-origin effects in their models; however, many of these investigations use data obtained from multi-generational structured livestock resource populations/pedigrees (de Koning *et al.* 2000, 2002; Uemoto *et al.* 2009; Boysen *et al.* 2010). The inclusion of parent-of-origin effects in the statistical analysis used in the present study was not possible, as the DNA samples used were derived solely from progeny-tested artificial insemination (AI) sires. Therefore, it is important to note that the analyses presented here may have reduced sensitivity to phenotypic effects for SNPs associated with imprinted genes.

To our knowledge, this study represents one of the first investigations describing genotype–phenotype associations between performance traits in domestic cattle and SNPs in the bovine *IGF2R* gene. Although the current study focussed on dairy animals, the association of SNPs in the bovine *IGF2R* gene with animal growth traits may be of value in beef production, where the selection of accelerated post-natal growth relative to birth weight for improved animal profitability is desired (MacNeil 2003; Bennett 2008; Bennett *et al.* 2008). Consequently, *IGF2R* may serve as a candidate gene for growth-related traits in beef populations. Furthermore, the results presented here add to an accumulating body of research showing that imprinted genes contribute to complex performance traits in domesticated species (Ruvinsky 1999; de Koning *et al.* 2000). These findings, together with the documented biological roles of imprinted genes in animal growth and development, suggest that imprinted genes represent an important reservoir

of molecular markers for future genetic improvement of livestock populations.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Ensemble database-listed SNPs analysed for the *IGF2R* gene.

Table S2 Details of the phenotypes analysed in this study.

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