Imprinted Gene Expression and Phenotype of Bovine Concepti with Bos taurus and Bos indicus Genetics

By

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A thesis submitted to the University of Adelaide in fulfilment of the requirement of the degree of doctor of philosophy (PhD) in Science

THE UNIVERSITY of ADELAIDE

The University of Adelaide

School of Animal and Veterinary Sciences

Discipline of Animal Science

July 2013

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Declaration

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* Xiang R, Ghanipoor-Samami M, Johns WH, Eindorf T, Rutley DL, et al. (2013) Maternal and Paternal Genomes Differentially Affect Myofibre Characteristics and Muscle Weights of Bovine Fetuses at Midgestation. PLoS ONE 8(1): e53402. doi:10.1371/journal.pone.0053402

Mani Ghanipoor Samami

July 2013

Dedications

I dedicate this work to my parents and my wife, Banafsheh Ashoordehi Noveir, for their great support with love during my life and the period of this study.

Acknowledgements

During my PhD, I have learnt a lot from many people. It is with great pleasure that I acknowledge the assistance provided by people who supported and helped me.

I would sincerely like to thank my principal supervisor, Prof. Stefan Hiendleder for his excellent supervision, guidance, trust and criticism over the course of my years of study. He encouraged me to always strive to improve the quality of my project. I would also like to thank Prof. Stefan Hiendleder for reviewing and commenting on the thesis chapters and components.

I am very grateful for the help from my co-supervisor, Dr. Karen Kind, who was always willing to understand, discuss, listen and encourage. Our discussions on analyses and writing were very beneficial. I would also like to thank Dr. Karen Kind for reviewing and commenting on the thesis chapters and components.

To the members of our laboratory group: Ali Javadmanesh, Ruidong Xiang, Dr Dana Thomsen and Ying Liu, thank you for your support and help with the laboratory work, and for your friendship. Dr. Dana Thomsen kindly read and edited the thesis.

I am very grateful for the J.S. Davies Bequest project funding. My sincere thanks also extend to Iran's Ministry of Science, Research and Technology for providing the PhD scholarship, without which this work would not have been possible. I thank the University of Adelaide for providing the opportunity for this project and support through university staff in particular the Graduate Centre.

My deepest gratitude goes to my mum and dad for being generous supporters over the past years. It was your love, support and strength that got me through the toughest time. Thank you for always being proud of my achievements.

I also thank my mother-in-law who supported my wife and I during our stay in Australia, and took care of our baby, allowing me to focus on my study. Most importantly, special thanks are given to my dear wife Banafsheh, who has constantly motivated me and

encouraged me in every way. You sacrificed sleep time to take care of our newborn girl and it was your support and hard work following my scholarship expiration that allowed me to continue and finish my study. Thank for all your support even during the most emotionally challenging time. It is your understanding and your love that enabled me to complete this work.

Abstract

Epigenetic parent-of-origin effects contribute significantly to phenotypic variation in animals. Imprinted genes, which show differential allelic expression in a parent of-origin dependent manner, are critical regulators of prenatal development. Altered epigenetic modifications of imprinted genes in response to maternal environmental stimuli are believed to impact on prenatal development with long-term consequences for postnatal phenotype, a process which is known as fetal programming. Most studies in the area of fetal programming have focused on the epigenetic link between intrauterine environment and fetal phenotype, and genetic programming of variation in pre- and postnatal growth traits remains largely unexplored.

We hypothesised that heterosis, i.e., the superiority of F1 hybrids compared to their parents, in growth traits is programmed prenatally through changes in expression patterns of imprinted genes. The purpose of this thesis was (i) to perform a comparative *in silico* analysis of promoter-specific transcripts and splice variants of the imprinted IGF2 and IGF2R genes as well as their imprinted regulatory non-coding RNAs, H19 and AIRN, (ii) to analyse expression patterns of all identified transcripts in bovine pre- and postnatal tissues, and (iii) to investigate fetal genetic effects on gene expression and their association with heterotic phenotype.

To this end, a bovine model was employed using two genetically and phenotypically distinct subspecies of domesticated cattle, *Bos taurus* and *Bos indicus*, and their reciprocal crosses. Real time quantitative PCR was used to quantify expression of *IGF2* promoter-specific transcripts, *IGF2R*, *H19* and *AIRN*, in liver, brain, heart, cotyledon, skeletal muscle, kidney, lung and testis at three developmental stages, Day-48 embryo, Day-153 fetus and 12-month juvenile. Heterotic effects on transcript abundances and expression patterns of imprinted genes and their correlations with fetal body weight and weights of fetal tissues were estimated with general linear models.

All studied imprinted genes were subject to developmental control of gene expression with the transcript abundance being downregulated in postnatal tissues. We identified *IGF2* promoter-specific transcripts and the bovine orthologue of human P0 promoter, and found a developmental shift in tissue specificity of P0 from fetal skeletal muscle to postnatal liver. Fetal body weight and absolute weights of fetal tissues, except brain, were subject to significant parent-of-origin effects, and were higher in fetal groups with *B. taurus* maternal genetics compared to *B. indicus* maternal genetics. Fetal placental weight, lung weight and relative muscle mass showed significant heterosis. Heterosis in fetal placental weight was associated with a polar overdominance imprinting pattern of *IGF2R* and *AIRN* in cotyledon with highest expression in *B. indicus* (sire) × *B. taurus* (dam) group, whereas the transcript abundance in the other reciprocal was close to purebred groups. In hybrid genetic groups, expression of *IGF2R* and *AIRN* in cotyledon was positively correlated with weight of fetal placenta and negatively correlated with placental efficiency, defined as fetal to placental weight ratio. *H19* expression in skeletal muscle was significantly affected by the interaction between parental genomes. With respect to significant negative association between *H19* expression and muscle mass, the negative heterotic effect on *H19* expression may explain the positive heterosis in relative muscle mass. Fetal genetics consistently influenced *H19* expression, which in *B. indicus* was between 1.5 to 2.2-fold higher than in *B. taurus* in all examined tissues, except brain. A negative relationship was observed between transcript abundance of *H19* and weights of fetal tissues, except brain. These results suggest that *H19* could be a molecular driver for differential subspecies-specific fetal phenotypes. By *in silico* search, we found *B. indicus*-specific nucleotide polymorphisms at CpG sites of the consensus sequence for the first CTCF binding site within the imprinting control region (ICR) located upstream of *H19* promoter. This led us to speculate that higher expression of *H19* in *B. indicus* tissues could be attributable to partial or complete relaxation of imprinting resulting from epigenetic changes in the ICR.

In conclusion, our results provide insight into the interplay between genetics and epigenetics and its consequences for genetic programming of phenotypic variation and heterosis. Significant interaction between parental genomes on expression of *H19*, a miRNA precursor and master regulator of an imprinted gene network, and negative relationship between *H19* expression and fetal muscle mass suggested imprinted genes and miRNA interference as mechanisms for differential effects of maternal and paternal genomes on fetal muscle.

Chapter 1

Introduction and literature review

1.1 Introduction

Prenatal development consists of a series of orchestrated processes which leads to differentiation of multiple specialised tissues from a single-cell zygote. Gene expression patterns in the specialised cells during the process of differentiation are controlled by fetal genetics and epigenetics (Reik *et al.*, 2001; Surani, 2001). Gene transcription at the genetic level is regulated by tissue-specific availability of transcription factors which bind specific regulatory DNA sequences and are thereby implicated in regulation of gene expression (Reik *et al.*, 2001; Reik, 2007). Epigenetics is defined as stable transmission of cell information determined by gene expression patterns across cell division which occurs independent of DNA sequence (Nafee *et al.*, 2008). The heritable epigenetic marks, including DNA methylation and histone modification, which create the epigenome, partition chromatin into active and inactive domains, and serve to store information as an epigenetic memory to regulate gene expression (Li, 2002). Genomic imprinting is the best example of epigenetic regulation of gene expression, which infringes Mendelian rules of inheritance, and is described as an imbalance in expression of alleles depending on parent-of-origin (Reik and Walter, 1998; Tilghman, 1999).

It is well known that epigenetic parent-of-origin effects significantly contribute to phenotypic variation of postnatal growth traits in animals (Cockett *et al.*, 1996; Van Laere *et al.*, 2003; Kim *et al.*, 2004; Neugebauer *et al.*, 2010a; Neugebauer *et al.*, 2010b; Imumorin *et al.*, 2011). Therefore, epigenetics imposes a source of non-additive genetic variation on the phenotype, which is unidentified in the classic genetic models for genetic evaluation of animals leading to reduced accuracy of estimated genetic parameters. It has been demonstrated that the use of inappropriate quantitative genetic models for deriving breeding values and population (co)variances between relatives may result in biased estimation of genetic parameters for traits controlled by imprinted genes (Engellandt and Tier, 2002; Santure and Spencer, 2006). On the other hand, epigenetic modifications are strong candidates for the interaction between genetics and environment, which compromise genetic

evaluation and improvement programs of farm animals. Any changes in environmental conditions, including maternal factors and fetal nutrition, result in developmental adaptations through transient alterations in epigenetic status of the epigenome, which have a permanent impact on postnatal growth and development, a process termed “fetal programming” (Barker and Clark, 1997). Although the concept of fetal programming has the most implications in the context of human disease, there is growing evidence that animal postnatal performance has its origin in prenatal development and is affected by maternal environmental conditions (Young *et al.*, 1998; Bell, 2006; Foxcroft *et al.*, 2006; Wu *et al.*, 2006; Gardner *et al.*, 2007; Funston *et al.*, 2010; Hill *et al.*, 2010). While studies in the area of fetal programming have focused on the epigenetic link between intrauterine environment and fetal phenotype, genetic effects on epigenetic regulation of gene expression and its association with phenotypic variation in prenatal development and consequences for postnatal growth traits and heterosis remain largely unexplored.

The insulin like growth factor 2 (*IGF2*) and insulin like growth factor 2 receptor (*IGF2R*) genes are the first identified imprinted genes, and have been studied extensively in many species. *IGF2* and *IGF2R* are expressed from the paternal and maternal allele, respectively, and are vital regulators of prenatal development. The imprinted expression of *IGF2* and *IGF2R* is regulated by the long non-coding RNA genes, *H19* and *AIRN*, respectively. Several lines of evidence demonstrated that epigenetic status and expression of the imprinted genes changes in response to prenatal environmental conditions and these effects persist into postnatal life, suggesting that these genes are implicated in developmental programming of postnatal performance (Young *et al.*, 2001; Micke *et al.*, 2011a; Micke *et al.*, 2011b). However, expression pattern of these genes and their contribution to phenotypic variation and heterosis in hybrids remains unexplored.

The focus of this research was to exploit an experimental model for the systematic analysis of fetal genetic and heterotic effects on tissue-specific transcript abundance of the genes controlled by genomic imprinting, and their link to prenatal phenotypic variation and

heterosis in the bovine. Additionally, this model facilitates analysis of the phenotypic patterns associated with the parent-of-origin (epi)genetic effects and their link to imprinted gene expression. Development of this model was achieved using reciprocal crosses of two phenotypically and genetically distinct subspecies of domesticated cattle, commonly known as *Bos taurus* (Angus) and *Bos indicus* (Brahman), in an attempt to attain maximum genetic diversity between parents. This model could be utilised to investigate the interplay between genetics and epigenetics to diversify prenatal phenotypes.

1.2 Epigenetics

Epigenetics describes the modifications and mechanisms beyond DNA sequence that cause heritable changes in gene expression patterns, and persist for one or more generation. (Bernstein *et al.*, 2007). DNA methylation and histone posttranscriptional modifications are key components of the epigenetic machinery, which interplay with each other, and with chromatin remodelling complexes and non coding RNAs, to regulate vital biological processes, including differentiation (Delcuve *et al.*, 2009).

DNA methylation occurs on the 5'-cytosine of CpG dinucleotides across the genome. Methylation of CpG sites in the promoters is usually associated with reduced gene expression (Kass *et al.*, 1997; Siegfried and Cedar, 1997; Bird, 2002). There are unmethylated CG-rich regions (CpG islands) in the genome that co-localise with the promoter of most constitutively expressed genes (Bird, 1986; Larsen *et al.*, 1992; Antequera, 2003; Zhu *et al.*, 2008). Although, the majority of CpG islands remain unmethylated, a small proportion acquire methylation during development, some of which play an essential role in X chromosome inactivation and genomic imprinting (Edwards and Ferguson-Smith, 2007; Reik, 2007).

Genomic DNA methylation patterns are dynamic during development. There are two major cycles of epigenetic reprogramming, in which genome wide demethylation and de novo methylation occurs: during germ cell development and after fertilisation (Monk *et al.*, 1987; Sanford *et al.*, 1987; Howlett and Reik, 1991; Kafri, 1992; Rougier *et al.*, 1998; Surani,

1998). A number of DNA methyltransferases (Dnmt) which transfer methyl group from *s*-adenosyl methionine onto C5 position of cytosine residues in the CpG dinucleotides have been identified in mammals (Bestor, 2000).

Histones are subject to posttranslational modifications, including acetylation (Sterner and Berger, 2000) and methylation of lysines (K) and arginines (R) (Zhang and Reinberg, 2001). Histone acetylation is always associated with transcriptional activation (Sterner and Berger, 2000), whereas methylation of lysines may result in gene activation or repression (Bannister and Kouzarides, 2005). It is believed that histone modifications are associated with changes in the structure, position and composition of nucleosomes. This process which is termed chromatin remodelling influences accessibility of DNA to transcriptional machinery (Peterson and Laniel, 2004).

Genome wide patterns of DNA methylation are the consequence of the interplay between DNA methyltransferases and chromatin remodelling machinery, including histone modifications (Dennis *et al.*, 2001; Meehan *et al.*, 2001; Geiman and Robertson, 2002; Klose and Bird, 2006). It is evident that histone methylation, which is thought to be affected by chromatin remodelling complexes, and other histone modifications such as acetylation and phosphorylation, is a prerequisite for proper DNA methylation patterns (Geiman and Robertson, 2002).

1.3 Genomic imprinting

Genomic imprinting is the best example of epigenetic regulation of gene expression which results in an imbalance in expression levels of alleles depending on their parent-of-origin, and thus, violates Mendelian rules of inheritance (Sha, 2008). So far, a group of 70 genes in human, 130 genes in mouse, 15 genes in ovine, 19 genes in bovine and 20 genes in porcine have been identified as imprinted (http://igc.otago.ac.nz/, www.geneimprint.com, www. mgu.har.mrc.ac.uk). *In silico* studies have predicted that 2.5% of mouse genes and 1% of human genes are subject to genomic imprinting (Luedi *et al.*, 2005; Luedi *et al.*, 2007). A

recent study has uncovered more than 1300 candidate imprinted loci with parent-of-origin-specific allelic expression in mouse (*Mus musculus castaneus/Mus musculus domesticus*) brain (Gregg *et al.*, 2010).

In mammals, most imprinted genes are in close proximity on the chromosome and organised into clusters (Reik and Walter, 2001; Verona *et al.*, 2003). Expression of imprinted genes within a cluster is co-ordinately controlled by regulatory sequences referred to as imprinting control regions (ICRs), which are differentially methylated regions (DMRs) or domains (DMDs) (Sutcliffe *et al.*, 1994; Wutz *et al.*, 1997; Thorvaldsen *et al.*, 1998; Arima *et al.*, 2001; Kanduri *et al.*, 2002; Reinhart *et al.*, 2002; Yatsuki *et al.*, 2002; Williamson *et al.*, 2006; Lin *et al.*, 2007; Shin *et al.*, 2008). These regions, which act in *cis*, are sites of differential methylation in a parent-of origin-dependent manner (Holmes and Soloway, 2006). There are two types of DMRs: primary DMRs, which acquire methylation imprints in the germ cells during gametogenesis and maintain it during development, and secondary DMRs, which undergo erasure and establishment of new methylation imprints during embryogenesis (Kobayashi *et al.*, 2006). The DMRs harbour binding sites for transcription regulatory proteins, such as methyl-CpG binding domains (MBDs), which target methylated DNA (Sha, 2008).

All ICRs in known imprinted clusters act in *cis* to repress expression of genes when unmethylated through different mechanisms (Koerner and Barlow, 2010). In the first mechanism, which has been observed in the *Igf2/H19* imprinted cluster, the unmethylated ICR acts as an insulator that binds the zinc finger transcription factor CTCF to block *Igf2* promoter access to its enhancer located downstream of H19 gene (Hark *et al.*, 2000). In the second model, which is represented in two imprinted clusters, *Igf2r* and *Kcnq1*, the unmethylated ICR contains an active promoter for the long non-coding RNAs, *Airn* and *Kcnq1ot1*, respectively, expression of which is associated with *cis*-silencing of the flanking genes (Sleutels *et al.*, 2002; Sleutels *et al.*, 2003; Mancini-DiNardo *et al.*, 2006).

1.4 The insulin like growth factor family: General overview

The insulin-like growth factor (IGF) family is part of a complex cell signalling system composed of ligands (IGF1 and IGF2, insulin), cell surface receptors (IGF1R and IGF2R, insulin receptor) and a group of six binding proteins (IGFBP1-6) in addition to IGFBP degrading proteases (Roith, 2003).

IGF1 and IGF2 are single-chain peptides of 70 and 67 amino acids, respectively, which are organised into four domains designated as B, C, A and D (in order from N- to C-terminus). Precursor forms of IGF1 and IGF2 contain the C-terminal E domain as well as N-terminal signal peptide, which are cleaved off before secretion. IGF1 and IGF2 are involved in a number of vital biological processes, including mitogenesis, anti-apoptosis, cell growth and differentiation, cell migration and metabolism. Their functions are mediated through binding to the insulin-like growth factor type 1 receptor (IGF1R).

Mechanisms of action and signal transduction pathways for IGF1R and the insulin receptor (IR) show significant similarities (Cheatham and Kahn, 1995; Leroith *et al.*, 1995). These heterotetrameric glycoproteins belong to the tyrosine kinase subfamily of transmembrane receptors (Leroith *et al.*, 1995). Two insulin receptor variant forms (IR-A and IR-B) resulting from alternative splicing of *IR* transcript have been identified (Sesti, 2000; Denley *et al.*, 2003; Denley *et al.*, 2005). As a result of the high level of similarity in structure, IGF1 αβ hemireceptor and insulin αβ hemireceptor are joined together to make up hybrid receptors in the tissues, but their functional roles in cell signalling are still unclear (Federici *et al.*, 1997a; Federici *et al.*, 1997b).

IGF2R serves as a scavenger receptor which binds IGF2 at the cell surface and internalises it for lysosomal degradation (Scott and Firth, 2004). On the other hand, IGF2R acts as a dual purpose receptor which binds proteins containing mannose-6-phosphate (M6P) recognition sites, including lysosomal enzymes in the *trans*-Golgi network, and transports them to lysosome (Kornfeld, 1992).

So far, a group of six IGF-binding proteins (IGFBP1-6) have been described. The IGFBPs have different affinity for IGF1 and IGF2 (Firth and Baxter, 2002) and carry most (99%) of the IGF1 in the bloodstream. The majority of serum IGF1 and IGF2 (70-80%) during human postnatal life are bound to a 150 kDa ternary complex consisting of IGFBP3 and a glycoprotein, acid labile subunit (ALS) (Frystyk, 2004). Ninety percent of IGFBP3 and 55% of IGFBP5 circulate in heterotrimeric complexes containing ALS in human adults. All IGFBPs also circulate in the free form or in binary complexes with IGFs. Free and IGF-bound IGFBPs are able to pass across the endothelium and diffuse into tissues, while ternary complexes are believed to remain in blood vessels (Baxter *et al.*, 2002; Firth and Baxter, 2002). IGFBPs prolong the half-life of plasma IGF ligands and regulate their bioavailability to the receptors. IGFBPs bind IGFs with higher affinity than IGF receptors and so can compete with IGF receptors in binding IGFs and prevent receptor access to IGF1 and IGF2 (Baxter, 2000; Firth and Baxter, 2002). This constitutes a mechanism to control IGF release in tissues and normal cell growth (Rajah *et al.*, 1999). IGFs are released from IGFBPs and delivered to their receptors following degradation of IGFBPs by specific proteases or binding of IGFBPs to the extracellular matrix (ECM) (Hwa *et al.*, 1999).

1.5 Insulin-like growth factor 2

1.5.1 Regulation of expression

1.5.1.1 Transcriptional regulation: Genomic imprinting

The Igf2 gene in mouse (*Mus musculus domesticus*) shows imprinted expression and is predominantly expressed from paternal allele, whereas expression from the maternal allele is repressed (DeChiara *et al.*, 1991). Mouse *Igf2* is part of a conserved imprinted cluster which consists of five imprinted genes. The gene is flanked 5’ by *Ins2* and 3’ by *H19*. The H19 gene, which encodes a long non-coding RNA, is located 90 kb downstram of *Igf2* and is maternally expressed (Bartolomei *et al.*, 1991). *IGF2* and *H19* imprinted expression is conserved in human (Rachmilewitz *et al.*, 1992; Giannoukakis *et al.*, 1993; Ohlsson *et al.*,

1993), rat (Overall *et al.*, 1997), porcine (Li *et al.*, 2008), ovine (*Ovis aries*) (Feil *et al.*, 1998; McLaren and Montgomery, 1999; Young *et al.*, 2003) and bovine (*B. taurus/B. gaurus*) (Dindot *et al.*, 2004a; Dindot *et al.*, 2004b; Zhang *et al.*, 2004). Observations on nascent RNA levels at the initial transcription sites in mouse (*Mus musculuc domesticus*) embryonic liver cells revealed numerous transcription patterns for *Igf2* and *H19*, including monoallelic and biallelic expression. This suggests that imprinted monoallelic expression of *Igf2* and *H19* is likely to be controlled at both transcriptional and post-transcriptional levels (Jouvenot *et al.*, 1999).

1.5.1.1.1 Differentially methylated regions

In mouse (*Mus musculus domesticus/Mus spretus*), there are three DMRs within the imprinted domain encompassing *Igf2*, two of which (DMR1 and DMR2) lie within the Igf2 gene with extensive paternal methylation (Feil *et al.*, 1994; Moore *et al.*, 1997). DMR1 is a CpG-rich element with tandem repeat sequences that lies 3 kb upstream of mouse (*Mus musculus domesticus*) *Igf2* P1 promoter and reveals mosaic patterns of methylation in sperm-derived DNA. DMR1 is believed to have a role in silencing the maternal allele, since deletion of this region led to biallelic expression of *Igf2* in mesodermally derived tissues, except muscle, without changing *H19* imprinting status or expression (Constancia *et al.*, 2000). Differential methylation of DMR1 on the transcriptionally active paternally-derived allele gives rise to the proposal that DMR1 contains repressor binding sites interacting with trans-acting repressors when unmethylated. A possible role for the protein GCF2 as a *trans*-acting repressor interacting with DMR1 has been elucidated (Eden *et al.*, 2001). However, the role of DMR1 in primary imprinting control of *Igf2* remains controversial by the fact that targeted deletion of mouse (*Mus musculus domesticus*/*Mus musculus castaneus*) *H19* causes loss of imprinting of *Igf2* (Leighton *et al.*, 1995a). DMR2, which is predominantly methylated in sperm-derived DNA, is situated close to the downstream part (last exon) of *Igf2* and appears to be involved in tissue-specific repression of the maternal allele. The methylation intensity of

DMR2 in fetal tissues changes depending on the expression level of *Igf2* in the tissues (Feil *et al.*, 1994). DMR2 does not appear to have a primary role in *Igf2* imprinting, as disruption of maternal DMR2 does not influence imprinting of *Igf2*. The third DMR (DMR0), located upstream of DMR1, is differentially methylated on the maternal allele in the mouse (*Mus musculus domesticus/Mus spretus*) placenta, whereas it is heavily methylated in both alleles in fetal tissues. Differential parental methylation of DMR0 in placenta is linked to the expression of *H19* in *cis*, whereas *H19* knockout does not have any effect on the placental DMR1 and DMR2 methylation. This can be interpreted as a tissue-specific mechanism involved in the regulation of imprinting (Moore *et al.*, 1997). The differentially methylated regions homologous to the mouse DMR0 and DMR2 with the same differential methylation pattern were identified in human (Reik *et al.*, 1995; Sullivan *et al.*, 1999; Cui *et al.*, 2002). Moreover, a region homologous to mouse DMR1 containing CpG island but without tandem repeats was identified in human. This region that spans exons 2 and 3 shows complete demethylation (Moore *et al.*, 1997). The presence of DMR2 and also DMR1 with the differential methylation on the paternal allele, has been elucidated in porcine (Han *et al.*, 2008a). However, *IGF2* imprinting in adult porcine liver, muscle and kidney was not dependent on methylation status of the DMR1 and DMR2 (Braunschweig *et al.*, 2011). In addition, an intragenic DMR within exon 10 of bovine *IGF2*, homologous to the mouse DMR2, has been found with significant difference in methylation between oocyte (16%) and sperm (99%) (Gebert *et al.*, 2006).

1.5.1.1.2 Imprinting control region

A paternally methylated DMR is located upstream of *H19* in mouse (*Mus musculus domesticus*/*Mus musculus castaneus*). This conserved DMR which acts as a *cis*-acting imprinting control region (ICR) is one of the major elements determining *IGF2* and *H19* imprinting as its deletion abolishes both *Igf2* and *H19* imprinted expression (Bartolomei *et al.*, 1993; Thorvaldsen *et al.*, 1998). The ICR harbours binding sites for the CTCF, a zinc-finger

transcription factor, which acts as an insulator (Kurukuti *et al.*, 2006). The conserved DMR upstream of the H19 gene has been identified in ovine (*Ovis aries*) (Young *et al.*, 2003), porcine (*Sus scrofa*) (Braunschweig *et al.*, 2011), bovine (*B. taurus*/*B. indicus*) (Curchoe *et al.*, 2009; Hansmann *et al.*, 2011; Robbins *et al.*, 2012) and human (Jinno *et al.*, 1996; Vu *et al.*, 2000).

Several models have been suggested to explain the mechanisms underlying imprinting of the Igf2/H19 gene domain. In the boundary model (Katharine, 2003), imprinting of *Igf2* in endoderm-derived tissues is believed to be controlled by interplay between tissue-specific enhancers, which are located downstream of *H19* (Yoo-Warren *et al.*, 1988), and ICR. The unmethylated ICR on the maternal allele functions as a boundary which blocks the interaction between downstream enhancers and the *Igf2* promoter by binding to CTCF. Methylation of CTCF binding sites on the paternal allele prevents CTCF binding and allows transcription of *Igf2* induced by the enhancers (Bell and Felsenfeld, 2000; Hark *et al.*, 2000; Kanduri *et al.*, 2000; Szabu *et al.*, 2000; Pant *et al.*, 2003). Paternal and maternal transmission of the enhancer deletion leads to loss of expression of *Igf2* and *H19*, respectively, in tissues of endodermal origin (Leighton *et al.*, 1995b).

A chromatin model has been suggested to explain mechanisms underlying imprinted expression of *Igf2* and *H19*. In this model, allelic expression of the *Igf2* and *H19* is regulated by the interaction between promoters and the enhancers which takes place possibly by forming loop-like structures along chromatin and is determined by the methylation status of *H19* DMR (ICR). The paternally derived methylated DMR suppresses expression of *H19* by spreading inactive chromatin state on its promoter allowing *Igf2* promoter to access its enhancer. The maternally derived unmethylated DMR prevents interaction between the *Igf2* promoter and the enhancers probably by forming a complex with insulator proteins leaving enhancers accessible to the *H19* promoter (Sasaki *et al.*, 2000).

Preferential interaction of the unmethylated *H19* DMR with *Igf2* DMRs, which are influenced by the epigenetic modifications of DMRs involving binding of protein factors,

partitions *Igf2* and *H19* into active and silent chromatin loops (Murrell *et al.*, 2004; Kurukuti *et al.*, 2006; Qiu *et al.*, 2008). In the enhancer tracking model, enhancers move over the length of chromosomes to recognise the promoters of relevant genes. In this model, insulator sequences located between enhancer and promoters stop the enhancer tracking along the chromosome (Engel *et al.*, 2008). There is evidence that cohesion, a protein involved in the cohesion of chromatids during cell division, co-localises with the CTCF at the ICR and plays an important role in facilitating and stabilizing CTCF-mediated chromatin looping. Gene depletion of cohesion in human abolished the chromatin conformation and was associated with the alteration in *IGF2* expression (Nativio *et al.*, 2009).

1.5.1.2 Posttranscriptional regulation

Although the complex patterns of *IGF2* transcription are thought to be involved in the regulation of expression at the transcription level, it is speculated that alternative 5' untranslated regions (5'UTRs) may be involved in the posttranscriptional regulation of gene expression, which influence mRNA translation (van der Velden and Thomas, 1999). A family of three *IGF2* mRNA binding proteins (IMP1, IMP2, IMP3) have been found in human that bind to the 5'UTR of the P3 derived mRNA (Nielsen *et al.*, 1995). The *IGF2* mRNA binding proteins have been found in both human and mouse and are thought to play an important role in temporal and spatial regulation of gene expression during late mammalian development.

The P4 derived transcripts are constitutively translated and maintain IGF2 generation at a basal level in all tissues, whereas translation of P3 derived transcripts is regulated in a tissue- and developmental stage-specific manner (Nielsen *et al.*, 1999). It has turned out that translation of human P2 derived transcripts is carried out in an eukaryotic translation initiation factor 4E (eIF4E) independent fashion which involves an internal ribosome entry site (IRES) allowing for translation initiation in the middle of the messenger RNA. Since cap-dependent translation may be compromised during cell division, the P2 derived transcripts may maintain IGF2 generation in the growing tissues of the embryo (Pedersen *et al.*, 2002). These data

show that the measurement of mRNA level *per se* does not necessarily reflect the total IGF2 protein expression in tissues. Further studies are required to show mechanisms controlling promoter-specific translation of *IGF2* in different tissues and developmental stages.

1.5.2 Tissue, developmental stage, and promoter-specific expression and imprinting

1.5.2.1 Tissue and developmental stage-specific expression of *IGF2* global transcripts

Developmental stage and tissue-specific imprinted expression of *IGF2* in a number of species is shown in Table 1.1. The mouse *Igf2* transcript is first seen at the two-cell stage of the embryo (Mizuki Ohno, 2001) and is frequently expressed shortly after implantation in the primitive endoderm, extraembryonic mesoderm, the anterior-proximal and lateral mesoderm cells and then in the mesoderm-derived tissues, including heart and somites (Lee *et al.*, 1990). In bovine, *IGF2* mRNA is present in mature oocytes and in the embryo throughout preimplantation and implantation period (Watson *et al.*, 1992; Wang *et al.*, 2009). In bovine fetuses, *IGF2* expression increases substantially by Day-180 of gestation. In bovine Day-150 fetuses, expression is higher in heart, liver and muscle than in brain (Kawase *et al.*, 2000).

Several lines of evidence have demonstrated tissue-specific and developmental regulation of *IGF2* with highest expression level in prenatal tissues of rat (Adams *et al.*, 1983; Brown *et al.*, 1986; Soares *et al.*, 1986; Gray *et al.*, 1987), bovine (Boulle *et al.*, 1993), ovine (Stylianopoulou *et al.*, 1988a; O'Mahoney *et al.*, 1991; Delhanty and Han, 1993) and human (Schofield and Tate, 1987). In a study to analyse the quantitative expression level of *IGF2* in bovine fetal tissues using northern blot and dot blot, it has been demonstrated that although liver *IGF2* expression level does not significantly change, muscle *IGF2* transcripts declined steadily after Day-150 of gestation. Also, the quantity of *IGF2* transcripts in bovine liver and muscle was remarkably reduced in postnatal, compared to prenatal developmental stage, with the postnatal decrease in expression being more severe in muscle compared to liver (Boulle *et*

al., 1993). In human, the level of *IGF2* transcripts during the first and second trimester of pregnancy was higher in muscle, compared with placenta, and was the lowest in fetal brain (Goshen *et al.*, 1993). Dot blot analysis of total RNA in ovine fetal tissues has revealed reduction of *IGF2* from Day-80 to Day-135 of gestation in liver, heart, spleen, skeletal muscle, kidney, and lung, whereas expression in placenta remained constant (O'Mahoney *et al.*, 1991). Expression of *IGF2* mRNA in ovine liver and kidney showed an increase from Day-50 to Day-100, then a decrease in late gestation (Day-120 to 145), while the mRNA level in muscle and choroid plexus declined as prenatal age advanced (Delhanty and Han, 1993). The relative abundance of rat *IGF2* transcripts was highest in fetal muscle, lung and liver and lowest in brain, heart and kidney (Brown *et al.*, 1986).

In human placenta, *IGF2* is transcribed in chorionic mesoderm and first trimester villous cytotrophoblast (Han, 1996), whereas in rhesus monkey placenta, *IGF2* is expressed in syncytiotrophoblast but not in chorionic mesoderm (Coulter and Han, 1996b; Coulter and Han, 1996a). In rodents, *Igf2* is detectable in the fetal mesodermal part of placenta and the trophoblast of labyrinth (Han and Carter, 2000). In ovine, *IGF2* mRNA is found in the fetal mesoderm close to trophoblast and also in the stroma of maternal caruncles (Reynolds *et al.*, 1997).

Igf2 is downregulated after birth in rodents (DeChiara *et al.*, 1991). *IGF2* in human continues to be expressed after birth with liver as the main source of IGF2 (de Pagter-Holthuizen *et al.*, 1987).

1.5.2.2 Tissue and developmental stage-specific expression of *IGF2* promoter-specific transcripts

Expression of *IGF2* promoter-specific transcripts at different developmental stages and in different tissues of the studied species is summarized in Table 1.2. In most species, including human, mouse, rat, porcine, ovine and bovine, *IGF2* is transcribed from distinct promoters which initiate transcription from different 5’ non-coding exons. This complex

promoter-specific transcription, which involves alternative splicing and polyadenylation, is believed to play an important role in regulation of transcription of *IGF2* in a tissue and developmental stage-specific manner. In human (Holthuizen *et al.*, 1990), bovine (Curchoe *et al.*, 2005; Goodall and Schmutz, 2007), porcine (Amarger *et al.*, 2002) and ovine (Ohlsen *et al.*, 1994), transcription from four promoters (P1-4) gives rise to a family of mRNA transcripts with different untranslated exons. In mouse, four distinct promoters (P0, P1, P2 and P3) initiate transcription from alternative untranslated exons, all of which splice onto the common protein-coding exons (Rotwein and Hall, 1990; Moore *et al.*, 1997). Recently, a novel mesoderm-specific promoter (*Igf2* Pm) has been identified in mouse whose transcription is initiated from a novel untranslated exon which is located downstream of exon U2 (Giang Tran *et al.*, 2012). A novel *IGF2* promoter which appears to be equivalent to the mouse placental-specific P0 promoter (Moore *et al.*, 1997) has been found to be active specifically in skeletal muscle of the human fetus (Monk *et al.*, 2006b).

In human, P2, P3 and P4 promoters are actively transcribed and drive transcripts in tissues during prenatal development, whereas P1 promoter is only active in human adult liver (Pedersen *et al.*, 2002). Expression patterns of *IGF2* alternative transcripts in bovine display considerable changes depending on the tissue and developmental stage. Three splice variants derived from P1 promoters were solely seen in the pre-term and postnatal liver, which is in agreement with the observation of a 4.4 kb adult liver-specific transcript reported previously (Boulle *et al.*, 1993), and the transcripts derived from P2, P3 and P4 promoters were observed in the liver and other tissues from newborn calves to 19 month old steers (Goodall and Schmutz, 2007). Contrary to these observations, in a later study, it was demonstrated that P1 derived transcripts are expressed in all bovine pre- and postnatal tissues, including liver, heart, lung, brain and kidney (Curchoe *et al.*, 2005). These contrary results can be explained by the utilisation of different primer pairs for amplification of different exonic regions of P1 derived transcripts. Curchoe *et al.* (2005) used primers inside exon 3, whereas Goodall and Schmutz (2007) utilised the forward primer in exon 1 and the reverse primer in exon 8. It appears that

primers spanning exons 1 and 8 specifically amplify P1 derived transcripts, which display evolutionarily conserved expression in postnatal liver. On the other hand, the primers located in exon 3 are supposedly able to amplify any putative transcripts containing exon 3.

1.5.2.3 Tissue, developmental stage, and promoter-specific imprinting

Developmental stage and tissue-specific imprinting of *IGF2* global and promoter-specific transcripts are shown in Tables 1.1 and 1.2. It is evident that monoallelic expression of the Igf2 gene in mouse (*Mus musculuc domesticus/Mus musculus molossinus*) is established at the blastocyst stage, while both alleles are transcriptionally active in the morula (Mizuki Ohno, 2001). In mouse (*Mus musculuc domesticus*), the Igf2 gene displays biallelic expression in choroid plexus and leptomeninges of brain (DeChiara *et al.*, 1991). Monoallelic expression of ovine (*Ovis aries*) *IGF2* occurs only after the blastocyst stage, before which both paternal and maternal copies can be detected (Thurston *et al.*, 2008). Conserved imprinted expression of ovine (*Ovis aries*) *IGF2* and *H19* has been documented at Day-25 of gestation (Young *et al.*, 2003). The IGF2 gene is imprinted in ovine (*Ovis aries*) fetal kidney, spleen and liver, and also in the kidney of juveniles but is biallelically expressed in fetal brain and adult liver (McLaren and Montgomery, 1999). In domestic pig, monoallelic paternal expression of *IGF2* was demonstrated using pyrosequencing in Day-30 embryos (Bischoff *et al.*, 2009).

Promoter-specific imprinting of *IGF2* has been studied in several species. In mouse (*Mus musculus/Mus spretus*), *Igf2* P1, P2 and P3 drive monoallelic paternal expression in all tissues, except the central nervous system (CNS), where biallelic expression from all promoters is observed (Hu *et al.*, 1995). In human, P2, P3 and P4 promoters direct monoallelic expression in fetal tissues. Biallelic expression of four promoters of *IGF2* in brain (leptomeninges and choroid plexus) has been reported in human (Ohlsson *et al.*, 1994; Zhan *et al.*, 1998). Moreover, the P1 promoter in human directs biallelic expression in postnatal liver (Vu and Hoffman, 1994; Ekstrom *et al.*, 1995). Monoallelic expression from P1 and

biallelic expression from other promoters was observed in tissues (heart, liver, brain, lung, kidney and muscle) of 1 week old porcine (Li *et al.*, 2008). Analysis of imprinted expression of *IGF2* during ovine (*Ovis aries*) development has demonstrated that expression of imprinted fetal transcripts in liver switches to biallelic expression after birth, which mimics the prenatal and postnatal imprinted expression pattern of *IGF2* in human and bovine (McLaren and Montgomery, 1999). *IGF2* monoallelic gene expression was seen in all examined fetal tissues of bovine (*B. taurus*), including liver, kidney, heart, lung and placenta, with the exception of brain, which showed biallelic expression, while more leaky expression from maternal allele or biallelic expression was observed in adult tissues (Curchoe *et al.*, 2005). Loss of imprinting (LOI) of P1 derived transcripts was observed in bovine (*B. taurus*) fetal liver at term and postnatal liver (Goodall and Schmutz, 2007). A study of promoter-specific loss of imprinting in heterozygous calves using the single nucleotide polymorphism (SNP) in exon 3 showed biallelic expression of the P1 derived transcript in liver and monoallelic expression in placenta, lung and heart (Curchoe *et al.*, 2005). The difference in imprinting status of transcripts containing exon 3 between liver and other tissues may result from tissue-specific differences in transcripts containing exon 3 and, as a result, their imprinting status. The majority of transcripts containing exon 3 in postnatal liver likely belong to *IGF2* P1 promoter, whereas in other tissues and developmental stages, they may derive from other overlapping genes and/or *IGF2* promoters.

Biallelic expression of the P3 derived transcript was observed in brain of fetus, heart of calf, and adult heart and liver, whereas loss of imprinting of P4 derived transcript occurred in brain of fetus, kidney of calf and adult liver and lung, suggesting that in bovine (*B. taurus*), P3 and P4 promoters direct loss of imprinting (Curchoe *et al.*, 2005). Evaluation of allelic expression of *IGF2* in a *Bos taurus* and *Bos gaurus* intercross, revealed paternal expression of *IGF2* in both placental and fetal tissues, including liver, lung and brain at Day-72 of gestation (Dindot *et al.*, 2004b). Preferential expression of *IGF2* from the paternal allele was also observed at Day-40 of gestation in nuclear transfer derived fetuses generated from an

interspecies cross between *Bos taurus* and *Bos gaurus* (Dindot *et al.*, 2004a). Further quantitative studies are needed to confirm promoter-specific loss of imprinting at different developmental stages.

1.5.3 Role of *IGF2* in development

1.5.3.1 Prenatal development

Evidence for the importance of *Igf2* in prenatal development has been provided by mouse gene knockout models. The crucial role of *Igf2* in mouse (*Mus musculus domesticus*) embryonic development has first been described by targeted disrupted of the gene on the paternal chromosome, which led to a significant reduction in birth weight (Dechiara *et al.*, 1990). Mouse (*Mus musculuc domesticus*) offspring that inherited a paternally disrupted *Igf2* allele were stunted in growth after birth, whereas transmission of a deleted maternal allele had no significant effects on progeny (DeChiara *et al.*, 1991). Studies in human demonstrated significant association of polymorphisms in the IGF2 gene with birth weight (Sayer *et al.*, 2002; Nagaya *et al.*, 2009; Adkins *et al.*, 2010).

The role of Igf2 in mesoderm differentiation has been documented in mouse (Morali *et al.*, 2000). Extensive studies have indicated the important role of IGF2 in muscle development during embryogenesis. Using a fusing skeletal muscle cell line in mouse, it was demonstrated that a switch takes place from low expression levels during myoblast proliferation to remarkably high expression levels over differentiation into myotubes. Disruption of *Igf2* mRNA by RNA interference, has blocked differentiation of multinucleated myofibers from myocytes and generation of muscle structural proteins (Wilson *et al.*, 2003). Suppression of *Igf2* expression results in downregulation of the genes targeted by MyoD via inhibition of the recruitment of transcriptional coactivators to the specific promoter elements of the muscle-specific genes, including myogenin. This suggests IGF2-induced signalling influences differentiation by regulating transcription factors involved in myogenesis (Wilson and Rotwein, 2006). *Igf2* is co-expressed with the fast myosin in the majority of myotubes and is

essential for proper specification of development of fast skeletal muscle fibers (Merrick *et al.*, 2007).

Studies on how changes in *IGF2* expression affect bovine prenatal development are limited. Bovine embryos produced by *in vitro* procedures may suffer from developmental abnormalities such as large offspring syndrome, which may be caused by dysregulation of the genes critical for prenatal development as a consequence of aberrant epigenetic alterations, including DNA methylation and histone modification (Niemann and Wrenzycki, 2000; Niemann *et al.*, 2002; Wrenzycki *et al.*, 2004). Expression level of *IGF2* transcripts increased dramatically at Day-63 of gestation in bovine fetuses generated by *in vitro* fertilisation (IVF) compared with their *in vivo* counterparts (Blondin *et al.*, 2000). Aberrant expression of *IGF2* was also observed in tissues of calves derived from somatic cell nuclear transfer (SCNT) that died shortly after birth (Yang *et al.*, 2005). It is known that *IGF2* imprinting is conserved in *B. gaurus/B. taurus* hybrids (Dindot *et al.*, 2004b). A differentially methylated region corresponding to mouse DMR2 was identified in the last exon of bovine *IGF2*, which is methylated in sperm but not in oocytes (Gebert *et al.*, 2004; Gebert *et al.*, 2006). Methylation pattern of this DMR was shown to be reprogrammed in a sex-specific manner in *in vivo* produced and SCNT-derived embryos (Gebert *et al.*, 2009). Insufficient methylation reprogramming of the DMR could be associated with developmental abnormalities in embryos derived by SCNT (Rideout *et al.*, 2001).

Several lines of evidence show a critical role for Igf2 in structural and functional development of placenta. One of the *Igf2* transcripts derived from the P0 promoter is specifically expressed in the mouse labyrinthine trophoblast, which acts as an exchange barrier in placenta (Moore *et al.*, 1997). Placental-specific P0 derived *Igf2* transcript is responsible for regulation of placental growth, and mice lacking this transcript exhibit decreased placental size and, as a consequence, retarded fetal growth in late gestation. Increased placental efficiency, which most likely results from elevated transport of glucose and amino acids as a consequence of an upregulated transporter genes, including *Slc2a3* and

Slc38a4, was also observed in P0-deficient mice (Constancia *et al.*, 2005). Such an increased expression of placental transporter genes is not observed in knockout fetuses that lack fetal *Igf2* and therefore have lower demand for nutrients. The P0 transcript was proposed to determine transfer capacity of the mouse placenta for nutrient exchange by regulating the development of the exchange barrier and therefore placental permeability (Constancia *et al.*, 2002; Constancia *et al.*, 2005). At embryonic Day-19, a reduction in diffusing capacity by 40%, in placental weight by 66%, and in birth weight by 76% has been reported in knockout mice for P0 promoter. Furthermore, there is a decrease in passive diffusion capacity of placentas lacking the P0 promoter, due to a reduced surface area and thickened exchange barrier (Sibley *et al.*, 2004).

1.5.3.2 Postnatal growth

Imprinted quantitative trait loci (iQTL) effects linked to *IGF2*, on postnatal growth related phenotypes, including body composition (de Koning *et al.*, 2000), heart weight and skeletal muscle growth (Jeon *et al.*, 1999), muscle mass and fat deposition (Nezer *et al.*, 1999; Nezer *et al.*, 2003), have been detected in porcine intercross experiments. A mutation in a CpG island located in intron 3 of *IGF2* explains the QTL effect of *IGF2* as a quantitative trait nucleotide (QTN). Individuals carrying the mutant copy derived from the sire, display a 3-fold higher expression level of *IGF2* in skeletal muscle after birth, and consequently have a significantly increased muscle mass (Van Laere *et al.*, 2003). Further experiments in porcine have revealed an association of *IGF2* polymorphisms with postnatal growth traits (Kolarikova *et al.*, 2003; Hou *et al.*, 2010).

In bovine, QTL effects associated with meat traits have been mapped to *IGF2* (MacNeil and Grosz, 2002; Casas *et al.*, 2003). Furthermore, single nucleotide polymorphisms within the IGF2 gene have been associated with bovine carcass and meat traits (Zhao *et al.*, 2002; Goodall and Schmutz, 2003; Han *et al.*, 2008b; Sherman *et al.*, 2008; Magee *et al.*, 2010; Zwierzchowski *et al.*, 2010), and grip strength in adult man (Sayer *et al.*, 2002).

Table 1.1 Tissue and developmental stage-specific imprinting and expression of *IGF2* global transcripts, consisting of all promoter-specific transcripts and splice variants, in different species

Species	Developmental stage	Tissue	Method	Expression	Imprinting status	Reference
Human	Embryo	Trophoblast	Insitu hybridisation	High steady state in trophoblast after implantation	ND	(Ohlsson *et al.*, 1989b)
Human	Embryo (first trimester)	Placenta	Insitu hybridisation	Proliferative cytotrophoblasts, villous cytotrophoblasts (low)	ND	(Ohlsson *et al.*, 1989a)
Human	Embryo	Placenta	Insitu hybridisation, Northern blot	Expressed	Biallelic (till 10 weeks), monoallelic (after 10 weeks)	(Jinno *et al.*, 1995)
Human	Embryo (first trimester)	Embryo, placenta	Nuclease protection assay	Expressed	Paternal expression	(Ohlsson *et al.*, 1993)
Human	Embryo	Trophoblast, kidney, liver, lung	Insitu hybridisation	- Kidney: High in metanephric blastema (6 weeks) - Cytotrophoblast: Increased between 18-69 days - Liver: Constant within the period - Lung: Low in airway epithelia before 12 weeks	ND	(Brice *et al.*, 1989)
Human	Fetus (first and second trimester)	Kidney	Insitu hybridisation	Reduced from 6 till 15 weeks	ND	(Brice *et al.*, 1989)
Human	Fetus (8-18 weeks)	Brain, liver, lung, muscle, placenta	Northern blot	Expressed	ND	(Monk *et al.*, 2006b)
Human	Fetus (8-12 weeks)	Liver, lung, kidney, brain, heart	Nuclease protection assay	Expressed	ND	(Schofield and Tate, 1987)
Human	Fetus (term)	Umbilical cord blood cells	PCR-RFLP	Expressed	Polymorphic imprinting	(Giannoukakis *et al.*, 1996)
Human	Postnatal (10 years old)	Liver, lung, kidney, brain, heart	Nuclease protection assay	- Postnatal decline - Developmental shift in promoter usage in adult liver	ND	(Schofield and Tate, 1987)
Human	Postnatal	Brain, heart, kidney, placenta, liver	PCR based assay	Expressed	Monoallelic (except liver)	(Wu *et al.*, 1997)
Ovine (*Ovis aries*)	Embryo (Day-14-35)	Placenta	In situ hybridisation & OD quantification	Increased by Day-35	ND	(Reynolds *et al.*, 1997)
Ovine (*Ovis aries*)	Fetus(Day-50 to term)	Lung, heart, liver, kidney, muscle, brain	Northern blot	- Decreased with fetal age	ND	(Delhanty and Han, 1993)
Ovine (*Ovis aries*)	Fetus (80, 105, 135 days)	Muscle, kidney, lung, heart, liver, placenta	Northern/dot blot	- Expressed - Adult liver-specific transcript - Decreased from Day-80 to 135 of gestation in tissues except placenta	ND	(O'Mahoney *et al.*, 1991)
Ovine (*Ovis aries*)	Fetus (Day-35-55)	Placenta	In situ hybridisation & OD quantification	Decreased after Day-35 in fetal mesoderm of placenta	ND	(Reynolds *et al.*, 1997)

Table 1.1 continued

Species	Developmental stage	Tissue	Method	Expression	Imprinting status	Reference
Ovine (*Ovis aries*)	Fetus (Day-121, 130, 150)	Liver	RT-PCR	Expressed	Monoallelic	(McLaren and Montgomery, 1999)
Ovine (*Ovis aries*)	Postnatal (Newborn, Day-7, Adult)	Muscle, kidney, lung, heart, liver, placenta	Northern/dot blot	- Adult liver-specific transcript - Postnatal decrease	ND	(O'Mahoney *et al.*, 1991)
Ovine (*Ovis aries*)	Postnatal (3 days, 4 days, 8 weeks, adult)	Lung, heart, liver, kidney, muscle, brain	Northern blot	- Postnatal decrease - Specific transcript in adult liver	ND	(Delhanty and Han, 1993)
Ovine (*Ovis aries*)	Postnatal (6-months old)	Liver	RT-PCR	Expressed	Biallelic	(McLaren and Montgomery, 1999)
Bovine	Embryo (2 cell, 16 cell, blastocyst)	-	RT-PCR	Expressed	ND	(Watson *et al.*, 1992)
Bovine	Embryo (blastocyst)	-	RT-PCR	Low	ND	(Kawase *et al.*, 2000)
Bovine (*Bos taurus*)	Fetus (113-280 days)	Liver, muscle	Dot blot	- Constant in liver - Increased at Day-150 then decreased	ND	(Boulle *et al.*, 1993)
Bovine	Fetus (30-180days)	Brain, liver, muscle, heart	RT-PCR	High	ND	(Kawase *et al.*, 2000)
Bovine (*Bos gaurus/Bos taurus*)	Fetus (Day-72)	Chorion, allantois, liver, lung, brain	RT-PCR	Expressed	Paternal expression	(Dindot *et al.*, 2004b)
Bovine (*Bos taurus*)	Postnatal (40-100 days, adult)	Liver, muscle	Dot blot	Downregulation	ND	(Boulle *et al.*, 1993)
Mouse (*Mus musculus/Mus spretus*)	Fetus (E14-17)	Brain	RFLP, Northern blot	Influence of parental species background on allelic expression	Biallelic	(Hemberger *et al.*, 1998)
Mouse	Fetus	Lung	RT-PCR and In situ hybridisation	Expressed	ND	(Maitre *et al.*, 1995)
Mouse	Postnatal (1, 4, 8 weeks)	Different tissues	Microarray, qPCR	Downregulation with age	ND	(Lui *et al.*, 2008)
Mouse	Postnatal (early, adult)	Lung	RT-PCR and In situ hybridisation	Not expressed	ND	(Maitre *et al.*, 1995)
Rat (*Rattus norvegicus*)	Fetus (Day-10-16)	Different tissues	In situ hybridisation	- Mesoderm (Muscles and chondrocytes) - Endoderm (liver and bronchial epithelium) - Ectoderm: (choroid plexus)	ND	(Stylianopoulou *et al.*, 1988a)
Rat	Fetus (Day-16-22)	Lung	Northern blot	Decreased in late gestation	ND	(Moats-Staats *et al.*, 1995)

Table 1.1 continued

Species	Developmental stage	Tissue	Method	Expression	Imprinting status	Reference
Rat	Fetus (Day-17-20)	Lung, liver	Northern blot and RNAse protection assay	- Higher in liver than lung (Day-20) - Decreased in late gestation (lung)	ND	(Batchelor *et al.*, 1995)
Rat (*Rattus norvegicus*)	Fetus (Day-8.5-20.5)	Heart, kidney, lung, liver, muscle	RT-PCR & southern blot	Expressed	Paternal expression except in leptomeninges and choroid plexus	(Overall *et al.*, 1997)
Rat (*Rattus norvegicus*)	Fetus (Day-16, 21)	Brain, liver, lung, kidney, muscle	Northern blot & densitometry	Higher in fetal muscle, lung, liver, and lower in brain, kidney, heart	ND	(Brown *et al.*, 1986)
Rat	Fetus (Day-18, 20)	Liver, muscle, heart, brain, lung	Northern blot	Multiple transcripts in fetal tissues	ND	(Soares *et al.*, 1986)
Rat	Postnatal (Day-2-13, 6 weeks)	Liver, muscle, heart, brain, lung	Northern blot	- Multiple transcripts in neonatal tissues - Repression in adult tissues except brain	ND	(Soares *et al.*, 1986)
Rat (*Rattus norvegicus*)	Postnatal (Day-2, 11, 22, 75)	Brain, liver, lung, kidney, muscle	Northern blot & densitometry	- Higher in neonatal muscle, lung, liver, and lower in brain, kidney, heart - Lower postnatal expression in lung, kidney, brain stem - Continuous high level through Day-22 after birth in muscle, liver, heart	ND	(Brown *et al.*, 1986)
Rat (*Rattus norvegicus*)	Postnatal (adult)	Brain, spinal cord	In situ hybridisation	Choroid plexus/leptomeninges	ND	(Stylianopoulou *et al.*, 1988b)
Rat	Postnatal (60 days)	Lung	Northern blot	Suppressed	ND	(Moats-Staats *et al.*, 1995)
Rat	Postnatal (Day-2, adult)	Brain, heart, liver, lung muscle	Northern blot	Different transcript lengths	ND	(Soares *et al.*, 1985)
Rat	Postnatal (Day-9)	Lung	Northern blot and RNAse protection assay	Low	ND	(Batchelor *et al.*, 1995)
Rat (*Rattus norvegicus*)	Postnatal (newborn)	Heart, kidney, lung, liver, muscle	RT-PCR & southern blot	Expressed	Paternal expression except in leptomeninges and choroid plexus	(Overall *et al.*, 1997)
Rhesus	Fetus (Day-65 till term)	Placenta	In situ hybridisation	No change with gestational age	ND	(Coulter and Han, 1996b)

* ND: not determined

Table 1.2 Tissue and developmental stage-specific imprinting and expression of *IGF2* promoter-specific transcripts in different species

Species	Promoter	Developmental stage	Tissue	Method	Expression	Imprinting status	Reference
Human	P0	Fetus (8-18 weeks)	Placenta, heart, brain, lung, liver, muscle, kidney	Northern blot	Specific in muscle	Maternally imprinted	(Monk *et al.*, 2006b)
Human	P1	Fetus	Liver	Northern blot	Not expressed	ND	(Depagter holthuizen *et al.*, 1987)
Human	P2	Fetus	Liver, placenta	Semi-qPCR	Transcript containing exon 4b in liver and placenta	ND	(Mineo *et al.*, 2000)
Human	P1-4	Fetus (18 weeks, prematurely born)	Liver	Nuclease protection assay	- P1-3: Peaking after birth (2 months) - P4: Peaking in fetus	ND	(Li *et al.*, 1996)
Human	P1-4	Fetus (11-17 weeks)	Brain	PCR-RFLP	All promoters in brain	Biallelic	(Zhan *et al.*, 1998)
Human	P1-4	Fetus (19-22 weeks)	Liver, chondrocyte	PCR-RFLP	All promoters in brain	Biallelic (P1), Monoallelic (P2-4)	(Vu and Hoffman, 1994)
Human	P1-4	Fetus (first trimester)	Different tissues	RNase protection & In situ hybridisation	- P1 in choroid plexus/leptomeninges and liver - P3: the most active promoter	ND	(Ohlsson *et al.*, 1994)
Human	P1-4	Fetus (7 weeks)	Liver	Nuclease protection assay	All promoters	Monoallelic (P2, P3, P4), Biallelic (P1)	(Ekstrom *et al.*, 1995)
Human	P1, P3	Fetus (6-12 weeks)	Brain	RT-PCR	P3 (predominant)	Biallelic (P3)	(Pham *et al.*, 1998)
Human	P2-4	Fetus	Placenta	Nuclease protection assay	- P3: Higher compared to P4 - All promoters expressed	Imprinted (placenta)	(Ekström *et al.*, 1995)
Human	P0	Postnatal (20-46 years)	Placenta, heart, brain, lung, liver, muscle, kidney	Northern blot	All tissues except brain	ND	(Monk *et al.*, 2006b)
Human	P1	Postnatal (20-46 years)	Placenta, heart, brain, lung, liver, muscle, kidney	Northern blot	Specific in liver	ND	(Monk *et al.*, 2006b)
Human	P1	Postnatal (adult)	Liver	Northern blot	Postnatal specific	ND	(Depagter holthuizen *et al.*, 1987)
Human	P1-4	Postnatal (2 months-3 years, 21-92 years)	Liver	Nuclease protection assay	- P1, P2, P4: Postnatal decrease after 2 months, constant level after 18 months - P3: Postnatal rapid decline after 2 months, no expression at 18 months	ND	(Li *et al.*, 1996)
Human	P1-4	Postnatal (adult)	Brain	PCR-RFLP	All promoters in brain	Biallelic	(Zhan *et al.*, 1998)

Table 1.2 continued

Species	Promoter	Developmental stage	Tissue	Method	Expression	Imprinting status	Reference
Human	P1-4	Postnatal (adult)	Liver, chondrocyte	PCR-RFLP	All promoters in brain	Biallelic (P1), Monoallelic (P2-4)	(Vu and Hoffman, 1994)
Human	P1-4	Postnatal (16 weeks, 9-18 months)	Different tissues	RNase protection & In situ hybridisation	- P2, P3, P4 in early postnatal: liver, choroid plexus and paraganglia - P1 in choroid plexus/leptomeninges and liver	Biallelic (choroid plexus/ leptomeninges) and monoallelic (other tissues) in early postnatal	(Ohlsson *et al.*, 1994)
Human	P1-4	Postnatal (neonatal, 9-18 months, 3 years, 21-62 years)	Liver	Nuclease protection assay	All promoters	Monoallelic (P2, P3, P4 in infant), Biallelic (P1 in postnatal)	(Ekstrom *et al.*, 1995)
Human	P1, P3	Postnatal (adult)	Brain	RT-PCR	- P3 (predominant) - P1 (less abundant)	Biallelic (P3), monoallelic (P1)	(Pham *et al.*, 1998)
Human	P2-4	Postnatal (9 months old)	Liver	Nuclease protection assay	- P3: Higher compared to P4 - All promoters expressed	ND	(Ekström *et al.*, 1995)
Mouse (*Mus musculus domesticus /Mus spretus*)	P0	Fetus (E10-E18)	Placenta	Northern blot, RT-PCR	Specific in labyrinthine trophoblast	Maternally imprinted	(Moore *et al.*, 1997)
Bovine (*Bos taurus*)	P1	Fetus	Liver, heart, lung, brain, kidney, placenta	RT-PCR	Expressed	ND	(Curchoe *et al.*, 2005)
Bovine (*Bos taurus*)	P2	Fetus	Liver, heart, lung, brain, kidney, placenta	RT-PCR	- Truncated form in most tissues - Full length transcript in liver, lung, kidney	Maternally imprinted in fetus	(Curchoe *et al.*, 2005)
Bovine (*Bos taurus*)	P2 (exon 4)	Fetus (preterm)	Liver	RT-PCR	Expressed	ND	(Goodall and Schmutz, 2007)
Bovine (*Bos taurus*)	P2 (exon 4-5)	Fetus (preterm)	Liver	RT-PCR	Expressed	ND	(Goodall and Schmutz, 2007)
Bovine (*Bos taurus*)	P3	Fetus	Liver, heart, lung, brain, kidney, placenta	RT-PCR	Truncated form in brain and placenta	ND	(Curchoe *et al.*, 2005)
Bovine (*Bos taurus*)	P4	Fetus	Liver, heart, lung, brain, kidney, placenta	RT-PCR	- Truncated form in brain	ND	(Curchoe *et al.*, 2005)
Bovine (*Bos taurus*)	P1-4	Fetus (113-280 days)	Liver	Northern blot	- P1: Specific in preterm	ND	(Boulle *et al.*, 1993)

Table 1.2 continued

Species	Promoter	Developmental stage	Tissue	Method	Expression	Imprinting status	Reference
Bovine (*Bos taurus*)	P1	Postnatal (newborn, adult)	Liver, heart, lung, brain, kidney	RT-PCR	All tissues except newborn heart	ND	(Curchoe *et al.*, 2005)
Bovine (*Bos taurus*)	P1	Postnatal (5 weeks, 19 months)	Brain, lung, heart, kidney, liver, muscle	RT-PCR	Specific in liver	Biallelic	(Goodall and Schmutz, 2007)
Bovine (*Bos taurus*)	P2	Postnatal (newborn, adult)	Liver, heart, lung, brain, kidney, placenta	RT-PCR	- Truncated form in most tissues - Full length transcript in newborn liver, lung, kidney	ND	(Curchoe *et al.*, 2005)
Bovine (*Bos taurus*)	P2 (exon 4)	Postnatal (5 weeks, 19 months)	Brain, lung, heart, kidney, liver, muscle	RT-PCR	- 19 months: Brain, kidney, liver, muscle - 5 weeks: Heart, kidney, liver, muscle	ND	(Goodall and Schmutz, 2007)
Bovine (*Bos taurus*)	P2 (exon 4-5)	Postnatal (5 weeks, 19 months)	Brain, lung, heart, kidney, liver, muscle	RT-PCR	5 weeks: Expressed	ND	(Goodall and Schmutz, 2007)
Bovine (*Bos taurus*)	P3	Postnatal (newborn, adult)	Liver, heart, lung, brain, kidney	RT-PCR	Adult liver and brain (weak)	ND	(Curchoe *et al.*, 2005)
Bovine (*Bos taurus*)	P3	Postnatal (5 weeks, 19 months)	Brain, lung, heart, kidney, liver, muscle	RT-PCR	5 weeks: Expressed	ND	(Goodall and Schmutz, 2007)
Bovine (*Bos taurus*)	P4	Postnatal (newborn, adult)	Liver, heart, lung, brain, kidney, placenta	RT-PCR	- Truncated form in adult brain and liver - Adult kidney (weak)	ND	(Curchoe *et al.*, 2005)
Bovine (*Bos taurus*)	P4	Postnatal (5 weeks, 19 months)	Brain, lung, heart, kidney, liver, muscle	RT-PCR	5 weeks: Expressed	ND	(Goodall and Schmutz, 2007)
Bovine (*Bos taurus*)	P1-4	Postnatal (40-100 days, adult)	Liver	Northern blot	- P1-4: Newborn to 19-months - P1: Specific in postnatal liver	ND	(Boulle *et al.*, 1993)
Porcine	P1	Fetus	Liver, muscle, kidney, ham, heart, brain, placenta, lung	RT-PCR	- Exons 1, 3: Brain and placenta - Exons 1, 2, 3: Ham and liver (strong), brain	ND	(Amarger *et al.*, 2002)
Porcine	P2	Fetus	Liver, muscle, kidney, ham, heart, brain, lung	RT-PCR	Exon 4 and exons 4-4b: Expressed	ND	(Amarger *et al.*, 2002)
Porcine	P3, P4	Fetus	Liver, muscle, kidney, ham, heart, brain, placenta, lung	RT-PCR	Expressed	ND	(Amarger *et al.*, 2002)
Porcine	P1	Postnatal (adult)	Liver, muscle, kidney, ham, heart, brain, lung	RT-PCR	- Exon 1: Liver (strong) - Exons 1, 3: Liver (strong), muscle and kidney - Exons 1, 2, 3: Liver	ND	(Amarger *et al.*, 2002)

Table 1.2 continued

Species	Promoter	Developmental stage	Tissue	Method	Expression	Imprinting status	Reference
Porcine	P2	Postnatal (adult)	Liver, muscle, kidney, ham, heart, brain, lung	RT-PCR	Exon 4 and exons 4-4b: Expressed	ND	(Amarger *et al.*, 2002)
Porcine	P3, P4	Postnatal (adult)	Liver, muscle, kidney, ham, heart, brain, lung	RT-PCR	Expressed	ND	(Amarger *et al.*, 2002)
Porcine (*Sus scrofa*)	P1-4	Postnatal (1 week)	Different tissues	SSCP	P2-P4 (Predominant)	Biallelic (P1), monoallelic (P2-4)	(Li *et al.*, 2008)
Ovine (*Ovis aries*)	P1-4	Fetus (Day-75)	Liver	RT-PCR	- P1: Not expressed - P2 (exon 4-5), P3 and P4: Expressed	ND	(Ohlsen *et al.*, 1994)
Ovine (*Ovis aries*)	P1-4	Postnatal (adult)	Liver	RT-PCR	- P1, P2 (exon 4-5), P3 and P4: xpressed	ND	(Ohlsen *et al.*, 1994)

* ND: not determined

Taken together, these observations suggest that the *IGF2* significantly influences postnatal productive traits in domestic animals, however, it remains to be understood whether *IGF2* directly affects postnatal growth, or postnatal manifestation of growth traits results from the effect of *IGF2* on prenatal development and fetal programming.

1.6 *H19* expression and role in development

The H19 gene is located downstream to the *IGF2* gene being part of an imprinted cluster and organised into 5 exons with a total length of 2373 bp in bovine. The sequence of *H19* shows high conservation between bovine, ovine and porcine (Zhang *et al.*, 2004). The gene encodes a long RNA of 2048 bp length (in bovine), which is exported to the cytoplasm and shows common features of RNA polymerase 2 derived mRNAs undergoing polyadenylation and splicing, however, the transcript lacks a conserved open reading frame (ORF) (Brannan *et al.*, 1990; Hurst and Smith, 1999).

The gene is imprinted on the paternal chromosome and expressed from the maternal allele in human (Rachmilewitz *et al.*, 1992; Zhang and Tycko, 1992; Kalscheuer *et al.*, 1993),

mouse (*M. m. domesticus*/*M. m. castaneus*/*M. m. musculus*/*M. spretus*) (Bartolomei *et al.*, 1991), bovine (*B. taurus/B. indicus*) (Zhang *et al.*, 2004; Robbins *et al.*, 2012), porcine (Li *et al.*, 2008) and ovine (*Ovis aries*) (Young *et al.*, 2003). A considerable variation in *H19* allelic expression was observed in human placenta ranging from 1-25%, which was higher in the first trimester compared to term placenta (Buckberry *et al.*, 2012). Results of an earlier study revealed that *H19* loss of imprinting in human placenta is cell-type-specific and occurs in the extravillous cytotrophoblasts without affecting *IGF2* imprinting status (Adam *et al.*, 1996). The imprinted expression of *IGF2/H19* domain is regulated by a differentially methylated ICR which lies upstream of *H19* promoter (Tremblay *et al.*, 1995; Tremblay *et al.*, 1997; Thorvaldsen *et al.*, 1998).

High expression of *H19* in fetal tissues derived from mesoderm and endoderm followed by postnatal downregulation has been documented in mouse (Pachnis *et al.*, 1984; Poirier *et al.*, 1991), which is closely linked to the expression pattern of the adjacent *Igf2* (Lee *et al.*, 1990). Similar tissue-specific and developmental expression patterns have been reported in ovine (Lee *et al.*, 2002) and bovine (Khatib and Schutzkus, 2006). Results from human studies have postulated that the expression pattern of *H19* changes depending on the tissue and prenatal developmental stage (Goshen *et al.*, 1993; Lustig *et al.*, 1994).

The conserved structure and sequence of *H19* across species suggests a functional role for non coding RNA (Juan *et al.*, 2000). It has been demonstrated that H19 serves as a miRNA precursor (miR-675) (Cai and Cullen, 2007) which is also conserved in marsupials (Smits *et al.*, 2008), suggesting its possible involvement in posttranscriptional regulation of gene expression. The H19 was shown to be associated with polysomes in numerous cell types in human and mouse, suggesting a *trans*-functional role for H19 in regulation of *IGF2* translation (Li *et al.*, 1998). This strongly argues against the hypothesis that *H19* transcription serves to silence expression of *IGF2* in *cis* (Webber *et al.*, 1998). Another clue to the involvement of H19 in posttranscriptional regulation of *IGF2* comes from the observation that H19 has binding sites for the oncofetal *IGF2* mRNA binding proteins (IMPs) (Runge *et al.*,

2000). This is further supported by the evidence that H19 functions as a trans regulator of the newly described imprinted gene network (IGN) in mouse skeletal muscle and is involved in downregulation of the growth regulatory imprinted genes in the IGN (Gabory *et al.*, 2009). Processing of the miR-675 from H19 was shown to be developmentally regulated and exclusive expression of miR-675 in placenta coincides with ceased placental growth. Furthermore, in the mouse lacking *H19*, placental growth persists and the miRNA targets, including *Igf1r*, show upregulation (Keniry *et al.*, 2012). Further evidence of a growth inhibitory role of H19 in placenta has come from the observation that H19 suppresses trophoblast proliferation via miR-675 mediated targeting of the Nodal Modulator 1 (*NOMO1*) and its downregulation (Gao *et al.*, 2012).

An antisense to *H19* transcript (*91H*) with the potential length of 120 kb has been identified in mouse and human. This transcript, which is unstable and nuclear localised, is predominantly expressed from the maternal allele and is implicated in regulation of *IGF2* expression, since its downregulation results in reduced expression of *IGF2* (Berteaux *et al.*, 2008). Upregulation of the mouse *Igf2* by 91H occurs in *trans* through activation of a novel mesodermal-specific promoter (*Igf2* Pm) which is located downstream of *Igf2* exon U2. Enhanced expression of *Igf2* by *91H* is countered by overexpression of *H19* (Giang Tran *et al.*, 2012).

Evidence for the importance of *H19* in embryonic development came from the demonstration that *H19* knockout mice (*Mus musculus domesticus*/*Mus musculus castaneus*) displayed over expression of *Igf2* due to activation of the maternal allele, and a 27% rise in birth weight, when the disrupted *H19* allele belongs to the mother. However, it was not clear whether loss of *H19* function or gain of *Igf2* maternal allele expression contributes to somatic overgrowth (Leighton *et al.*, 1995a; Ripoche *et al.*, 1997). Although it is known that *H19* is a negative regulator of growth in human and mouse, there is no evidence for the effect of variation in *H19* expression on growth of tissues during prenatal development and its programming potential for postnatal performance in animals. A splice variant of H19 lacking

exon 4 has been described in human with the spatial expression being subject to genetic background and its parent-of-origin (Lin *et al.*, 1999). Significant association of maternal genetics genotyped by the SNPs in H19 gene with offspring birth weight was shown in human. Also, a significant association of SNPs with birth weight was found depending on whether the allele is paternally or maternally transmitted (Adkins *et al.*, 2010).

1.7 Insulin-like growth factor 2 receptor

1.7.1 Mechanisms underlying genomic imprinting

Igf2r is part of an imprinted cluster that comprises several imprinted genes, including *Slc22a2* and *Slc22a3*, extending over 400 kb (Sleutels *et al.*, 2002). In mouse (*Mus musculus domesticus*), there are two differentially methylated regions (DMRs) in this domain; DMR1, which lies within the promoter of *Igf2r* sense transcript, contains a secondary imprint with its paternal hypermethylation pattern being established after implantation, and DMR2, located in intron 2, which is within a CpG island and acts as an imprinting control region (ICR), and contains the primary imprint with a maternally-derived hypermethylation pattern established during oogenesis (Stoger *et al.*, 1993). The DMR2 encompasses the promoter for a long non-coding RNA gene which is transcribed in an antisense direction to *Igf2r*, designated as antisense *Igf2r* non-coding RNA (*Airn*) (Sleutels *et al.*, 2002). The CpG island within intron 2 of *Igf2r* is essential for *Airn* transcript initiation and elongation and retains an unmethylated state of the *Airn* promoter on the paternal allele (Koerner *et al.*, 2012).

As in the mouse, two CpG islands have been found in human *IGF2R*. Unlike the mouse, the region 1 located in the promoter of the sense *IGF2R* is not methylated on both parental alleles, while the region 2, which is located within intron 2, is differentially hypermethylated on the maternal allele and can explain biallelic expression of *IGF2R* in human (Riesewijk *et al.*, 1996). Conservation of the ICR within intron 2 of *IGF2R* has been shown in bovine with high sequence homology to mice and ovine (Long and Cai, 2007). Expression of *AIRN* transcript has been postulated in all bovine fetal tissues (Suteevun-Phermthai *et al.*, 2009).

Igf2r and *Airn* show reciprocal imprinted expression patterns in mouse (*Mus musculus/Mus spretus*) fetal, placental and adult tissues with maternal expression of *Igf2r* and paternal expression of *Airn* (Barlow *et al.*, 1991). Two other genes in the imprinted cluster, *Slc22a2* and *Slc22a3,* display monoallelic maternal expression only in extraembryonic tissues and are biallelically expressed in embryonic and fetal tissues (Regha *et al.*, 2006). In mouse (*Mus musculus domesticus*), *Igf2r* is expressed from both alleles before implantation from the two-cell stage onwards (Harvey and Kaye, 1991), but imprinted expression of *Igf2r* arises during implantation (Lerchner and Barlow, 1997). It is now evident that expression of *Airn* is required for silencing of *Igf2r*, *Slc22a2* and *Slc22a3* paternally derived alleles (Sleutels *et al.*, 2002). In the central nervous system of the neonatal mouse (*Mus musculus domesticus*/*Mus musculus musculus*) and of the mouse fetus, *Igf2r* is biallelically expressed notwithstanding the imprinted expression of *Airn* and presence of the differentially methylated ICR. There is evidence that *Igf2r* relaxation of imprinting in the CNS occurs in a tissue-specific manner and is associated with neuron-specific histone modifications in the DMR1 and absence of the *Airn*, whereas reciprocal imprinted expression of *Igf2r* and *Airn* takes place in glial cells and fibroblasts (Yamasaki *et al.*, 2005).

Studies on expression of *Igf2r* in mouse ES cells revealed initially low expression from both paternal and maternal alleles. During differentiation of ES cells, the maternally-derived allele exhibits a 10-fold increase in expression, while expression of the paternally derived allele remains at the same initial low level, suggesting an expression bias as the cause of imprinted expression of *Igf2r* (Latos *et al.*, 2009).

The DMR2 on the paternal allele is marked by H3K4 methylation and H3 acetylation, but on the maternal allele is marked by H3K9 methylation (Fournier *et al.*, 2002). It has been proposed that histone modifications specific to the promoters of *IGF2R* and *AIRN* are more critical than differentially methylated regions and AIRN non-coding RNA to determine imprinted expression of *IGF2R* in human and mouse (*Mus musculus domesticus*/*Mus spretus*) (Vu *et al.*, 2004).

The Airn in mouse shows the characteristics of messenger RNAs that is, it is capped and polyadenylated, is transcribed by RNA polymerase II and undergoes alternative splicing resulting in four splice variants all of which originate from a promoter located within intron 2 of *Igf2r*. The majority of the Airn escape from splicing leading to a mature 108 kb noncoding RNA which is not able to enter the cytoplasm. These observations raise the possibility that the act of transcription, not the RNA itself, may play a role in silencing of the maternal alleles in *cis* (Seidl *et al.*, 2006). Recent evidence confirms that *Igf2r* silencing results from transcriptional overlapping with *Airn* which interferes with recruitment of RNA polymerase II to *Igf2r* promoter and does not require splice variants or full length nuclear localised RNA product (Latos *et al.*, 2012).

1.7.2 Expression and imprinting status in prenatal development

IGF2R expression is first detected in the bovine blastocyst but is expressed at very low levels. However, a number of studies showed the presence of *IGF2R* transcript in all stages of bovine preimplantation embryos from mature oocyte (Watson *et al.*, 1992; Wang *et al.*, 2009). IGF2R protein was detected in the cytoplasmic membrane of immature and matured oocytes, and two-cell stage embryos. In the bovine blastocyst stage, IGF2R protein is distributed in the trophectoderm whereas is less abundant in the inner cell mass (Wang *et al.*, 2009). *IGF2R* mRNA has been detected in the mesodermal tissues of ovine placenta at Day-40 of gestation, and its abundance was reduced towards late gestation (Day-140) (Lacroix *et al.*, 1995). *IGF2R* expression occurs in the microvillous and plasma membrane of human trophoblast (Fang *et al.*, 1997).

Analysis of *IGF2R* expression in bovine fetal tissues by RT-PCR showed that this gene is highly expressed between the Day-30 and Day-180 of gestation with less expression obtained in brain than in liver, heart and muscle at Day-150 of gestation (Kawase *et al.*, 2000). In bovine fetuses, *IGF2R* mRNA expression was shown to be higher in liver, lung, kidney and placenta compared with brain and heart (Suteevun-Phermthai *et al.*, 2009).

However, immunoblotting revealed that IGF2R protein is highly expressed in tissues of bovine fetuses at midgestation with the highest expression in heart (10-28 weeks) (Pfuender *et al.*, 1995). In human, *IGF2R* expression is present during preimplantation stages of development, including the oocyte (Lighten *et al.*, 1997). Study of tissue-specific expression of *IGF2R* in rat revealed highest expression of transcript and protein in embryonic heart (Senior *et al.*, 1990). In contrast, quantitative analysis of *IGF2R* expression in bovine Day-75 of gestation showed higher expression in liver, lung, kidney and cotyledon compared with heart and brain (Suteevun-Phermthai *et al.*, 2009). The IGF2R concentration in rat fetal tissues was highest in heart, placenta, lung, muscle, kidney, liver and brain, respectively (Sklar *et al.*, 1989). This corresponds to expression level of mRNA in fetal rat tissues which was highest in heart, muscle, lung, kidney, liver, brain, respectively (Sklar *et al.*, 1992).

Imprinting status of *IGF2R* in different tissues and developmental stages of the studied species has been summarised in Table 1.3. In mouse (*Mus musculus/Mus spretus*), *Igf2r* is paternally imprinted in peripheral tissues but shows biallelic expression in the CNS (Hu *et al.*, 1998). Imprinting status of the human *IGF2R* remains ambiguous. An early investigation of imprinting status of human *IGF2R* using a polymorphic dinucleotide repeat and a tetranucleotide insertion/deletion within the 3'UTR has revealed biallelic expression of the gene in the fetal tissues of lung, kidney, brain, liver, heart and muscle (Kalscheuer *et al.*, 1993). Because the reverse transcriptase PCR (RT-PCR) was employed in this study, the quantitative differences in expression between paternal and maternal alleles could not be determined. Also, a number of other investigations have demonstrated that human *IGF2R* is not imprinted (Ogawa *et al.*, 1993; Treacy *et al.*, 1996; Killian *et al.*, 2001a). However, further studies in human showed that *IGF2R* imprinting is a polymorphic trait, and *IGF2R* is imprinted in some individuals (Xu *et al.*, 1993; Xu *et al.*, 1997; Oudejans *et al.*, 2001; Monk *et al.*, 2006a). Polymorphic imprinting of *IGF2R* suggests that epigenetic regulation of this gene is modified as a consequence of genetic background and/or environmental conditions.

Table 1.3 Imprinting status of *IGF2R* in tissues of different developmental stages across species

Species	Developmental stage	Tissue	Marker	Method	Imprinting status	Reference
Human	Fetus (11-24 weeks)	Lung, kidney, brain, liver, heart, muscle	Dinucleotide repeat, tetranucleotide In/Del in 3'UTR	PCR and gel electrophoresis	Biallelic	(Kalscheuer *et al.*, 1993)
Human	Fetus (12-17 weeks)	Muscle, kidney	Tetranucleotide In/Del in 3'UTR	PCR and gel electrophoresis	Biallelic	(Ogawa *et al.*, 1993)
Human	Fetus (1st and 2nd trimester, term placenta)	Placenta, lung, heart, kidney	CA repeat polymorphism	PCR and gel electrophoresis	Polymorphic imprinting	(Xu *et al.*, 1993)
Human	Fetus (7-20 weeks, term placenta)	Kidney, placenta	CA repeat polymorphism	PCR and gel electrophoresis	Polymorphic imprinting in fetus, loss of imprinting in term placenta	(Xu *et al.*, 1997)
Human	Fetus (6-8 weeks)	Placenta	ACAA In/Del in 3'UTR	PCR and gel electrophoresis	Polymorphic imprinting	(Oudejans *et al.*, 2001)
Human	Fetus (first trimester)	Kidney	SNPs in exons 6, 9, 12, 16, 34, 40, ACAA In/Del in 3'UTR	Sequencing, PCR and gel electrophoresis	Biallelic	(Killian *et al.*, 2001a)
Human	Fetus (6-12 weeks, 37-42 weeks)	Placenta	SNPs	Pyrosequencing	Biallelic	(Buckberry *et al.*, 2012)
Human	Postnatal (juvenile)	Kidney	Tetranucleotide In/Del in 3'UTR	PCR and gel electrophoresis	Biallelic	(Ogawa *et al.*, 1993)
Human	Postnatal (24-years old)	Blood lymphocyte	Chromosomal translocation (one gene copy)	Semi-qPCR, gene dosage and protein measurement	Lower expression (50 %) compared with control	(Treacy *et al.*, 1996)
Human	Postnatal	White blood cell	CA repeat polymorphism	PCR and gel electrophoresis	Polymorphic imprinting	(Xu *et al.*, 1993)
Porcine (*Sus scrofa*)	Fetus (8 weeks)	Brain, liver, muscle, kidney	SNPs in exon 37 and 3'UTR	PCR-RFLP, sequencing	Biallelic	(Braunschweig, 2012)
Porcine	Fetus	Liver	SNP in exon 48	PCR-sequencing	Monoallelic	(Killian *et al.*, 2001a)
Porcine (*Sus scrofa*)	Postnatal (1 day, 8-10 months)	Brain, liver, muscle, kidney	SNPs in exon 37 and 3'UTR	PCR-RFLP, sequencing	Biallelic	(Braunschweig, 2012)
Porcine	Postnatal (piglet, hog)	-	SNP in exon 48	PCR-RFLP-SSCP	Maternal expression	(Li *et al.*, 2010)
Ovine (*Ovis aries*)	Embryo (Day-21)	-	Parthenogenesis	Northern blot	Higher expression in parthenogenetic samples	(Young *et al.*, 2003)
Ovine (*Ovis aries*)	Embryo (Day-21)	Blastocyst, chorioallantois	Parthenogenesis	Semi-qPCR	Higher expression in parthenogenetic fetus	(Thurston *et al.*, 2008)
Ovine (*Ovis aries*)	Fetus	Liver	SNP in exon 19	PCR-sequencing	Monoallelic	(Killian *et al.*, 2001a)
Bovine (*Bos taurus*)	Embryo (Day-25, 45)	Trophectoderm	G/A SNP in 3'UTR	Single stranded conformational polymorphism	Monoallelic	(Suteevun-Phermthai *et al.*, 2009)

Table 1.3 continued

Species	Developmental stage	Tissue	Marker	Method	Imprinting status	Reference
Bovine (*Bos taurus*)	Fetus (Day-75, 250-280)	Brain, cotyledon, heart, liver, lung, kidney	G/A SNP in 3'UTR	Single stranded conformational polymorphism	Monoallelic in fetal tissues, biallelic in near term brain	(Suteevun-Phermthai *et al.*, 2009)
Bovine	Fetus	Liver	SNP in exon 48	PCR-sequencing	Monoallelic	(Killian *et al.*, 2001a)
Mouse (*Mus musculus domesticus*)	Pre- (E4.5) and post- (E 6.5) implantation embryo	-	*Igf2r*-LacZ expression	Localisation of β-galactosidase activity on cryosections	Biallelic before implantation and monoallelic after implantation	(Lerchner and Barlow, 1997)
Mouse (*Mus musculus/Mus spretus*)	Fetus (Day-15)	-	Deletion on maternal chromosome	Northern blot	Maternal expression	(Barlow *et al.*, 1991)
Mouse (*Mus musculus domesticus*)	Fetus (E13.5)	Heart, choroid plexus, tongue, small intestine	*Igf2r*-LacZ expression	Localisation of β-galactosidase activity on cryosections	Monoallelic	(Lerchner and Barlow, 1997)
Mouse (*Mus musculus domesticus*/*Mus musculus musculus*)	Fetus (E10, E15)	Cerebral cortices, cortical neurons, glial cells, embryonic fibroblasts	RFLP	PCR-RFLP and gel electrophoresis	Biallelic in neurons, maternal expression in glial cells	(Yamasaki *et al.*, 2005)
Mouse (*Mus musculus/Mus spretus*)	Fetus (E15)	Liver, kidney, heart, lung, muscle, CNS	RFLP	PCR-RFLP and gel electrophoresis	Biallelic in CNS and maternal expression in peripheral tissues	(Hu *et al.*, 1998)
Mouse (*Mus musculus/Mus spretus*)	Postnatal (newborn, 1-month old)	Brain, heart, lung, liver, kidney	RFLP	PCR-RFLP and gel electrophoresis	Maternal expression in tissues, biallelic in brain	(Hu *et al.*, 1999)
Mouse (*Mus musculus domesticus*/*Mus musculus musculus*)	Postnatal (Day-1, 42)	Cerebral cortices, cortical neurons, glial cells, embryonic fibroblasts	RFLP	PCR-RFLP and gel electrophoresis	Biallelic in neurons, maternal expression in glial cells	(Yamasaki *et al.*, 2005)
Mouse (*Mus musculus/Mus spretus*)	Postnatal (Day-1, 2, 3, week-2, month-2)	Liver, kidney, heart, lung, muscle, CNS regions	RFLP	PCR-RFLP and gel electrophoresis	Biallelic in CNS and maternal expression in peripheral tissues	(Hu *et al.*, 1998)

The discrepancy in imprinting status of *IGF2R* in human studies may be due to different experimental methods or different markers used for imprinting analysis. For example, it was shown that the results of imprinting analysis of the *IGF2R* employing the CA repeat polymorphism at the 3' untranslated region of *IGF2R* (Goto *et al.*, 1992) are not reliable since one of the primers to amplify the DNA fragment flanking this SNP was located on the ACAA In/Del. Also, it has been speculated that stochastic amplifications in PCR cycles beyond 35 cycles due to low amounts of the template may render the imprinting results difficult to interpret (Killian *et al.*, 2001b).

Recently, a pyrosequencing approach to quantify allelic expression of *IGF2R* revealed equal transcript abundance of paternal and maternal alleles in the first trimester and term placenta (Buckberry *et al.*, 2012), which is consistent with previously reported biallelic expression of *IGF2R* in human term placenta (Daelemans *et al.*, 2010). Biallelic expression of *IGF2R* observed in porcine fetal and adult tissues (Braunschweig, 2012) is in contrast to the previously reported monoallelic *IGF2R* expression in fetal liver of porcine (Killian *et al.*, 2001a) and tissues of piglets (Li *et al.*, 2010). Since the fetal and postnatal age is not clear in the latter (Killian *et al.*, 2001a), the conflicting results obtained in these studies may be the outcome of different developmental stages at which allelic expression of *IGF2R* was measured.

Imprinted maternal expression of *IGF2R* was found to be conserved in the ovine (*Ovis aries*) fetus (Killian *et al.*, 2001a; Young *et al.*, 2003). In this species, *IGF2R* is biallelically expressed in the blastocyst, while monoallelic imprinted expression is observed in concepti from Day-21 (Thurston *et al.*, 2008). Monoallelic expression of *IGF2R* was shown in fetal liver of ovine (*Ovis aries*), bovine and porcine (Killian *et al.*, 2001a). Apart from biallelic expression of *IGF2R* in the brain of newborn calves, predominant monoallelic expression of *IGF2R* in all tissues (heart, brain, liver, spleen, lung, cotyledon, intercotyledon) of fetuses at different developmental stages (Days-25, 45 and 75 of gestation) and neonatal calves was observed, though the parental origin of the expressed allele was not determined due to lack of parental tissues (Suteevun-Phermthai *et al.*, 2009).

1.7.3 Role in prenatal development

IGF2R is a multifunctional protein that is known to be implicated in multiple cellular processes in addition to regulation of IGF2 bioavailability. Some of the known cellular pathways involving IGF2R include T-cell induced apoptosis through granzyme B uptake (Motyka *et al.*, 2000), endocytosis and trafficking lysosomal enzymes (Kornfeld, 1992; Hille-Rehfeld, 1995; Le Borgne and Hoflack, 1998; Dahms and Hancock, 2002), endothelial-cell

migration and angiogenesis through binding proliferin (Groskopf *et al.*, 1997), growth inhibition and apoptosis induced through binding retinoid acid (Kang *et al.*, 1999), proteolytic activation of latent form of transforming growth factor-β1 (TGF-β1), which is a growth inhibitor (Dennis and Rifkin, 1991; Ghahary *et al.*, 1999; Godár *et al.*, 1999; Leksa *et al.*, 2005), control of cell invasion and migration through binding urokinase-type plasminogen activator receptor (uPAR) as well as targeting uPAR to lysosomes for degradation (Nykjær *et al.*, 1998; Godár *et al.*, 1999; Leksa *et al.*, 2002; Schiller *et al.*, 2009), regulation of plasminogen activation (Godár *et al.*, 1999; Leksa *et al.*, 2002), T-cell activation via binding dipeptidyl peptidase IV (CD26) on the cell surface (Ikushima *et al.*, 2000) and inducing transendothelial cell migration by binding soluble CD26 released from T-cells (Ikushima *et al.*, 2002). Although the mitogenic function of IGF2 is believed to be exerted through IGF1R (Nakae *et al.*, 2001) and insulin receptor isoform A (Frasca *et al.*, 1999), a number of researchers have raised the controversial suggestions that IGF2R is involved in cellular IGF2 signalling (Murayama *et al.*, 1990; Kornfeld, 1992; Minniti *et al.*, 1992; Nishimoto, 1993; Ikezu *et al.*, 1995; Groskopf *et al.*, 1997; Zhang *et al.*, 1997; Ikushima *et al.*, 2000; Tsuruta *et al.*, 2000; McKinnon *et al.*, 2001; Zygmunt *et al.*, 2005; Chu *et al.*, 2008; Sferruzzi-Perri *et al.*, 2008; Maeng *et al.*, 2009; Harris *et al.*, 2011). The functions of IGF2R are more complicated considering different cell types that have been exploited in these studies. In fact, specific roles of the IGF2R may be more complex and vary with tissue and developmental stage. Therefore, it is necessary to investigate tissue-specific association of *IGF2R* transcript abundance with organ growth at specific stages of development.

A significant association of polymorphisms within *IGF2R* with birth weight was observed in human (Kaku *et al.*, 2007; Adkins *et al.*, 2010), whereas inconsistent results were obtained in other studies (Kukuvitis *et al.*, 2004; Petry *et al.*, 2005). The critical role of *Igf2r* gene in prenatal development has been documented in knockout mouse experiments and by the observation of aberrant expression of the gene in some growth-related disorders. Mice with a disrupted *Igf2r* gene suffer from overgrowth syndrome and die shortly after birth (Lau

et al., 1994; Wang *et al.*, 1994; Ludwig *et al.*, 1996), whereas mosaic mice with tissue-specific disruption (liver, skeletal and cardiac muscle) in *Igf2r* possess no obvious abnormality (Wylie *et al.*, 2003). Death before birth accompanied by organomegaly and congenital heart defects, hyperdactyly and kinky tail have been shown in the mice lacking functional *Igf2r*, suggesting a key role for Igf2r in differentiation and organogenesis during prenatal development, a function that needs to be further investigated (Lau *et al.*, 1994; Wang *et al.*, 1994). Heart failure as a result of cardiac hyperplasia is the major cause of death in the *Igf2r* null mice, suggesting a critical role for Igf2r in heart.

A soluble type of IGF2R (sIGF2R) comprising the extracellular domain of cellular IGF2R has been reported in the serum and amniotic fluid of rat (Kiess *et al.*, 1987), ovine (Gallaher *et al.*, 1994), bovine (Li *et al.*, 1991), monkey (Gelato *et al.*, 1988) and human (Causin *et al.*, 1988). This isoform which is a truncated form resulting from proteolytic cleavage of transmembrane and intracellular domains of the cell membrane IGF2R has been shown to block IGF2-induced DNA synthesis (Scott and Weiss, 2000). Soluble IGF2R (sIGF2R) appears to take part in regulating bioavailability of IGF2, as it accounts for 50% of total IGF2 binding in fetal ovine blood but is dramatically downregulated in adults (Gelato *et al.*, 1989; Gallaher *et al.*, 1994). The ratio of cord blood IGF2 to soluble IGF2R was shown to be positively related to placental and birth weight (Ong *et al.*, 2000). Transgenic mice which express a mutated *Igf2r* complementary DNA encoding a soluble receptor showed decreased size of organs (Zaina *et al.*, 1998). It has been suggested that reduction of organ size as a result of increased levels of sIGF2R occurs through both IGF2-dependent and IGF2-independent pathways (Zaina and Squire, 1998).

1.7.4 Expression and role in postnatal growth

IGF2R expression is downregulated in rat postnatal tissues (Kiess *et al.*, 1987; Sklar *et al.*, 1989; Senior *et al.*, 1990; Sklar *et al.*, 1992). Developmental downregulation of soluble IGF2R protein was reported postnatally in human, bovine, ovine and monkey serum (Gelato

et al., 1988; Gelato *et al.*, 1989; Li *et al.*, 1991; Xu *et al.*, 1998; Costello *et al.*, 1999). *Igf2r* was shown to be expressed throughout mouse growth plate and is postnatally upregulated with age in the growth plate, perichondrium and bone, whereas *Igf2* expression is downregulated with age (Parker *et al.*, 2007; Andrade *et al.*, 2010).

Studies of the association between polymorphisms in *IGF2R* and phenotypic traits are rare and the results are inconsistent. In an attempt to examine single nucleotide polymorphisms in *IGF2R* affecting beef performance traits in the bovine, none of the studied SNPs were significantly associated with meat production traits (Magee *et al.*, 2010). The reliability of these results may be questioned since the imprinting effects were not included in the model used for association analysis. In a subsequent study, the SNPs located in the intronic regions of *IGF2R* were shown to be associated with body size traits and carcass weight and angularity (Berkowicz *et al.*, 2012). A polymorphism resulting in a missense mutation has been reported in human which was associated with height and weight gain in children (Petry *et al.*, 2005).

1.8 Imprinted genes and genetic programming of postnatal performance: Implications for heterosis

It has been documented that postnatal growth and health is largely determined by prenatal development, which is in turn subject to fetal genetics, epigenetics and maternal factors, a process termed “fetal programming” (Barker and Clark, 1997). What was initially known as fetal programming is now believed to occur throughout development and includes oocyte programming (Gilchrist *et al.*, 2004; Krisher, 2004; Sirard *et al.*, 2006; Doblado and Moley, 2007; Jungheim *et al.*, 2010; Parry and Singson, 2011; Tian and Diaz, 2013), embryo (fetal) programming (Barker, 2004; Simmons, 2006) and early postnatal programming (Neu *et al.*, 2007; Nilsson *et al.*, 2008; Baum, 2010; Wright, 2010). In addition, there is increasing evidence of intergenerational programming and paternal influence on intrauterine development (Godfrey *et al.*, 2001; Drake and Walker, 2004; Veena *et al.*, 2004; Drake *et al.*,

2005; Temple *et al.*, 2006; Whitaker *et al.*, 2010). Although the concept of fetal programming has long been developed in human epidemiological research there is growing evidence that animal postnatal performance has its origin in prenatal development and is affected by maternal environmental conditions (Young *et al.*, 1998; Bell, 2006; Foxcroft *et al.*, 2006; Wu *et al.*, 2006; Gardner *et al.*, 2007; Funston *et al.*, 2010; Hill *et al.*, 2010). Altered epigenetic modifications of imprinted genes, including those in IGF system, in response to maternal environmental stimuli are believed to impact on prenatal development with long-term consequences for postnatal phenotype (Dean *et al.*, 1998; Brameld *et al.*, 2000; Khosla *et al.*, 2001; Young *et al.*, 2001; Wu *et al.*, 2004; Dong *et al.*, 2005; Kwong *et al.*, 2006; Igwebuike, 2010; Micke *et al.*, 2011a; Micke *et al.*, 2011b).

Most studies in the area of fetal programming have focused on the epigenetic link between intrauterine environment and fetal phenotype, and the role of imprinted genes in genetic programming of variation in pre- and postnatal growth traits remains largely unexplored. There is evidence that genetics impacts on expression of imprinted genes (Hemberger *et al.*, 1998; Van Laere *et al.*, 2003). Further evidence for genetic effects on imprinted genes comes from association of genetic polymorphisms within imprinted gene loci and phenotypes (Zhao *et al.*, 2002; Han *et al.*, 2008b; Sherman *et al.*, 2008; Zwierzchowski *et al.*, 2010; Berkowicz *et al.*, 2012) and also imprinted QTL effects mapped to *IGF2* on growth-related phenotypes in porcine (Jeon *et al.*, 1999; Nezer *et al.*, 1999; Van Laere *et al.*, 2003).

Changes in (epi)genetic mechanisms implicated in regulation of gene expression as caused by intra-species hybridisation are likely to be significant contributors to postnatal phenotype, including heterosis. Heterosis is defined as superiority of hybrids in a trait compared to the average of their parental purebreds (Veitia and Vaiman, 2011). Mendelian genetic models have been used to describe non-additive gene expression and heterosis (Davenport, 1908; Shull, 1908; Bruce, 1910; Keeble, 1910; Jones, 1917; Powers, 1944; Crow,

1948; Hochholdinger and Hoecker, 2007) and models with non-Mendelian (epi)genetic effects, including parent-of-origin imprinting effects, have not yet been viewed.

A model of reciprocal crosses between two genetically distant groups has been used in most studies to examine parent-of-origin (iQTL) effects on phenotypes. In such models, the parent-of-origin effects on phenotypes are analysed based on the difference between two reciprocal groups, which is interpreted as the effects of parent-of-origin dependent gene expression associated with genomic imprinting (Wolf *et al.*, 2008b). Significant heterosis for birth weight (Brown *et al.*, 1993) and significant differences between reciprocal hybrids for growth and carcass characteristics of calves (Rohrer *et al.*, 1994; Amen *et al.*, 2007a; Amen *et al.*, 2007b) have been detected in a bovine model using *Bos taurus* × *Bos indicus* crosses. Accumulating evidence suggests that the difference between reciprocals could be attributed to non-Mendelian epigenetic inheritance of imprinted genes (Jiang *et al.*, 2007; Loschiavo *et al.*, 2007; Cheverud *et al.*, 2008; Hager *et al.*, 2008; Wolf *et al.*, 2008a). However, the role of imprinted genes in driving heterotic phenotypes has not been investigated.

Reciprocal intra-species hybridisation models enable the study of associations of parent-of-origin imprinting patterns and heterotic phenotypes at the molecular and phenotypic level. This can provide insights into the molecular basis of natural phenotypic variation and is relevant not only in studies aimed at understanding molecular mechanisms involved in heterosis, but is also likely to shed light on the (epi)genetic architecture and prenatal programming of complex traits associated with growth and disease in human.

Bovine provide a unique animal model for studying parent-of-origin epigenetic effects on complex traits in human because of generally outbred genetics, similar reproductive characteristics, including singleton pregnancies, similar gestation length and lifetime reproductive performance. Bovine are one of the most important agricultural species providing foods for nearly 6.6 billion humans globally (Elsik *et al.*, 2009). The bovine has become an important model organism for assisted reproductive technologies, including *in vitro* fertilization (IVF) (Ménézo and Hérubel, 2002). There are two genetically and

phenotypically distinct subspecies of bovine (Lin *et al.*, 2010), which provide an excellent source of genetic diversity for the study of genomic imprinting, the interplay of genetics and epigenetics, and its effect on pre- and postnatal growth.

1.9 Hypothesis and research objectives

The *IGF2* and *IGF2R* belong to the insulin like growth factor system and are the best known imprinted genes in ruminants. Although it is understood that imprinted genes are implicated in environmental programming of postnatal growth little is known about their role in (epi)genetic programming of phenotypes, and effect of genetics on expression of imprinted genes and its consequence for non-additive heterotic gene expression and phenotypic variation of growth-related traits in prenatal development has not been examined.

In this thesis, we hypothesised that

i) Expression of imprinted genes in tissues of *B. taurus/B. indicus* prenatal development is subject to fetal genetics.

ii) Heterosis, i.e., the superiority of F1 hybrids compared to their parents, in *B. taurus/B. indicus* growth traits is (epi)genetically programmed in prenatal development through changes in expression patterns of imprinted genes.

The objectives of this work were:

i. to perform a comparative *in silico* analysis of promoter-specific transcripts and splice variants of the imprinted IGF2 and AIRN genes.

ii. to evaluate tissue-specific changes in transcript abundance of imprinted genes, *IGF2* global and promoter-specific transcripts, *IGF2R*, and their regulatory long non-coding RNA imprinted genes, *H19* and *AIRN* across pre- and postnatal developmental stages.

iii. to analyse the effect of fetal genetics and heterosis on fetal weight and weight of fetal tissues and also on tissue-specific expression of the imprinted genes.

iv. to investigate association of transcript abundance of imprinted genes with heterotic phenotypes.

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Chapter 2

Comparative *in silico* analysis of *IGF2* promoter-specific transcript variants and *AIRN* transcripts in bovine

2.1 Introduction

The bovine insulin-like growth factor 2 gene (*IGF2*) spans 18606-bp on the distal end of chromosome 29 (Schmutz *et al.*, 1996; Goodall and Schmutz, 2003). IGF2 is a major regulator of prenatal development (Dechiara *et al.*, 1990; Vrana *et al.*, 1998; Sayer *et al.*, 2002; Nagaya *et al.*, 2009; Adkins *et al.*, 2010) and a QTL affecting postnatal performance (Jeon *et al.*, 1999; Nezer *et al.*, 1999; de Koning *et al.*, 2000; Nezer *et al.*, 2003; Van Laere *et al.*, 2003). The gene is subject to a complex transcription regulation through genomic imprinting, promoter-specific transcription, differential splicing and alternative polyadenylation (Ikejiri *et al.*, 1991; Hu *et al.*, 1996; Li *et al.*, 1996; Overall *et al.*, 1997; Yun *et al.*, 1998; Constancia *et al.*, 2005; Curchoe *et al.*, 2005; Thurston *et al.*, 2008; Buckberry *et al.*, 2012).

The IGF2 gene is part of an evolutionarily conserved domain that is linked to INS and H19 genes on its upstream and downstream sides, respectively. These three genes cover a region of approximately 150 kb on human chromosome 11 and mouse chromosome 7 (Zemel *et al.*, 1992; Onyango *et al.*, 2000). In mouse, the gene comprises upstream exons U1 and U2, previously known as pseudo-exons, followed by six exons (Rotwein and Hall, 1990). The IGF2 gene is composed of 10 exons in human (Mineo *et al.*, 2000), bovine (Goodall and Schmutz, 2007) and porcine (Amarger *et al.*, 2002). In all species, the IGF2 protein is translated from the last three exons.

In all species studied, including human, mouse, rat, porcine, ovine and bovine, *IGF2* is transcribed from distinct promoters which initiate transcription from different 5' non-coding exons. This complex promoter-specific transcription, which involves alternative splicing and polyadenylation, is believed to play an important role in regulation of transcription of *IGF2* in a tissue and developmental stage-specific manner (Davies, 1994; Vu and Hoffman, 1994; Ekstrom *et al.*, 1995; Li *et al.*, 1996; Curchoe *et al.*, 2005; Li *et al.*, 2008a; Giang Tran *et al.*, 2012). In human (Holthuizen *et al.*, 1990), bovine (Curchoe *et al.*, 2005; Goodall and Schmutz, 2007), porcine (Amarger *et al.*, 2002) and ovine (Ohlsen *et al.*, 1994), transcription

from four promoters (P1-4) gives rise to a family of mRNA transcripts with different untranslated exons. A developmental switch in *IGF2* promoter usage occurs in bovine (Boulle *et al.*, 1993), ovine (O'Mahoney *et al.*, 1991; Ohlsen *et al.*, 1994), porcine (Amarger *et al.*, 2002) and human (Depagterholthuizen *et al.*, 1987; Pedersen *et al.*, 2002; Monk *et al.*, 2006) in which transcription from the P2, P3 and P4 promoters falls sharply after birth and the P1 promoter becomes active in liver at the time of birth. Furthermore, a fifth promoter (P0) has been characterised upstream of human *IGF2* exon 2 (Monk *et al.*, 2006). In mouse, transcription of *IGF2* is initiated by three promoters (P1-3) in fetal tissues as well as a promoter (P0) that is only active in placenta (Rotwein and Hall, 1990; Moore *et al.*, 1997).

Bovine *IGF2R* is located on the distal end of chromosome 9 at position 9q26. It is a large gene with 48 exons spanning 102612 bp. The transcript length is 9075 bp with 7500 bp encoding the mature IGF2R protein. The *IGF2R/Igf2r* shows imprinted expression and is expressed from maternal allele in the studied animal species (Barlow *et al.*, 1991; Killian *et al.*, 2001; Young *et al.*, 2003; Bebbere *et al.*, 2013). In mouse (*Mus musculus domesticus*), imprinted expression of *Igf2r* is regulated by the reciprocally imprinted paternally expressed long non-coding RNA, Airn. The promoter of *Airn* is located in intron 2 of Igf2r gene and is transcribed in an antisense direction to *Igf2r* (Sleutels *et al.*, 2002). Although, *AIRN* was shown to be expressed in bovine prenatal development (Suteevun-Phermthai *et al.*, 2009; Farmer *et al.*, 2013), its gene and transcript structure and regulatory effect on *IGF2R* imprinted expression remains unclear.

Several sense and antisense transcripts have been characterised in the region encompassing *INS/IGF2* in mouse, human and porcine (Moore *et al.*, 1997; Braunschweig *et al.*, 2004; Monk *et al.*, 2006). Many of these transcripts, which overlap exonic regions of *IGF2* transcripts, have not been identified in bovine and therefore complicate analysis of expression of *IGF2* promoter-specific transcripts. In order to be able to identify the putative previously unknown transcripts in bovine and to design appropriate primers for specific amplification of *IGF2* promoter-specific transcripts, we performed a comprehensive

comparative analysis of sequence and exon/intron structures of the known transcripts surrounding the INS/IGF2 gene domain in bovine, human, porcine and mouse using previous findings from literature and sequence information available in public databases.

2.2 Materials and methods

Sequences and exon/intron structures of the studied genes and transcripts, including *IGF2* promoter-specific transcripts, *INSIGF2* hybrid transcripts, *IGF2* antisense transcripts for human (*Homo sapiens*), bovine (*Bos taurus*), mouse (*Mus musculus*) and porcine (*Sus scrofa*) were retrieved from literature and the public databases, National Center for Biotechnology Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov/genbank/) and Ensembl project database (http://www.ensembl.org). Sequence similarity search between bovine and other species was carried out using Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/ (Altschul *et al.*, 1990). The dbEST of NCBI GenBank (Boguski *et al.*, 1993) was used to explore the Expressed Sequence Tags (ESTs) corresponding to the putative *IGF2R* antisense transcript (*AIRN*) and *IGF2* antisense transcript (*IGF2AS*) in bovine. The corresponding ESTs were identified using sequence similarity search by BLAST against bovine EST database (http://blast.ncbi.nlm.nih.gov/).

CTCF-binding sites within the ICR upstream of H19 gene, were predicted with CTCFBSDB (Bao *et al.*, 2008). Comparison of the sequences of seven CTCF binding sites between *Bos taurus* and *Bos indicus* was performed using BLAST with *Bos indicus* genome, in NCBI (http://www.ncbi.nlm.nih.gov/genomes/geblast.cgi?taxid=9915).

2.3 Results

Comparative exon/intron structure of *INS/IGF2* for human, bovine, porcine and mouse is shown in Figure 2.1. The IGF2 gene shows structural homology in the studied species. The gene shows more similarity between human, bovine and porcine as compared with mouse and is composed of 10 exons (exons 1 to 9 in human and porcine with exon 4b corresponding to exon 5 in bovine). In human, bovine and porcine, the first upstream seven exons are not translated and are part of the 5'UTR in the transcripts derived from different promoters. In human, bovine and porcine, four promoters (P1, P2, P3 and P4) drive transcription from the leading exons 1, 4, 5 (exon 6 in bovine) and 6 (exon 7 in bovine), respectively. A novel promoter (P0) has been found in human that drives transcription from a region upstream to exon 2 in fetal skeletal muscle. Despite the complexity in transcription, all promoter-specific transcripts possess the protein coding exons 7, 8 and 9 (8, 9 and 10 in bovine) and lead to the same protein. A conserved microRNA (*MIR483*) is in intron 7, 8 and 4 of human, bovine and mouse, respectively. The respective microRNA has not been reported in pig.

In mouse, promoters P1, P2 and P3 drive transcription from untranslated exons 1, 2 and 3, respectively, which are extended to the protein coding exons 4, 5 and 6. Furthermore, a transcript consisting of the upstream exons U1 and U2 and the last three protein coding exons is derived from promoter P0 located upstream of exon U1 (Figure 2.1).

Sequence information of *IGF2* transcripts in the studied species was extracted from public databases. For human, the transcript containing exon 1 derived from P1 promoter was not found in NCBI and Ensembl databases. Two transcripts initiating from upstream region to exon 2 and containing exon 3, as well as two transcripts initiating from the leader exon 3, were found in human with different polyadenylation sites (Figure 2.2). Three transcripts with different transcription initiation and polyadenylation sites are derived from the P2 promoter in human and contain exons 4 and 4b. The P2 derived splice variant with the leading exon 4 (but not exon 4b) is not available in the databases. Five transcripts derived from the P3 promoter in human were found with different transcription initiation and polyadenylation sites.

Transcripts derived from the P4 promoter in human show different polyadenylation and the same transcription initiation sites. A processed non translated transcript with a leader exon located upstream to exon 6 was found in human (Figure 2.2).

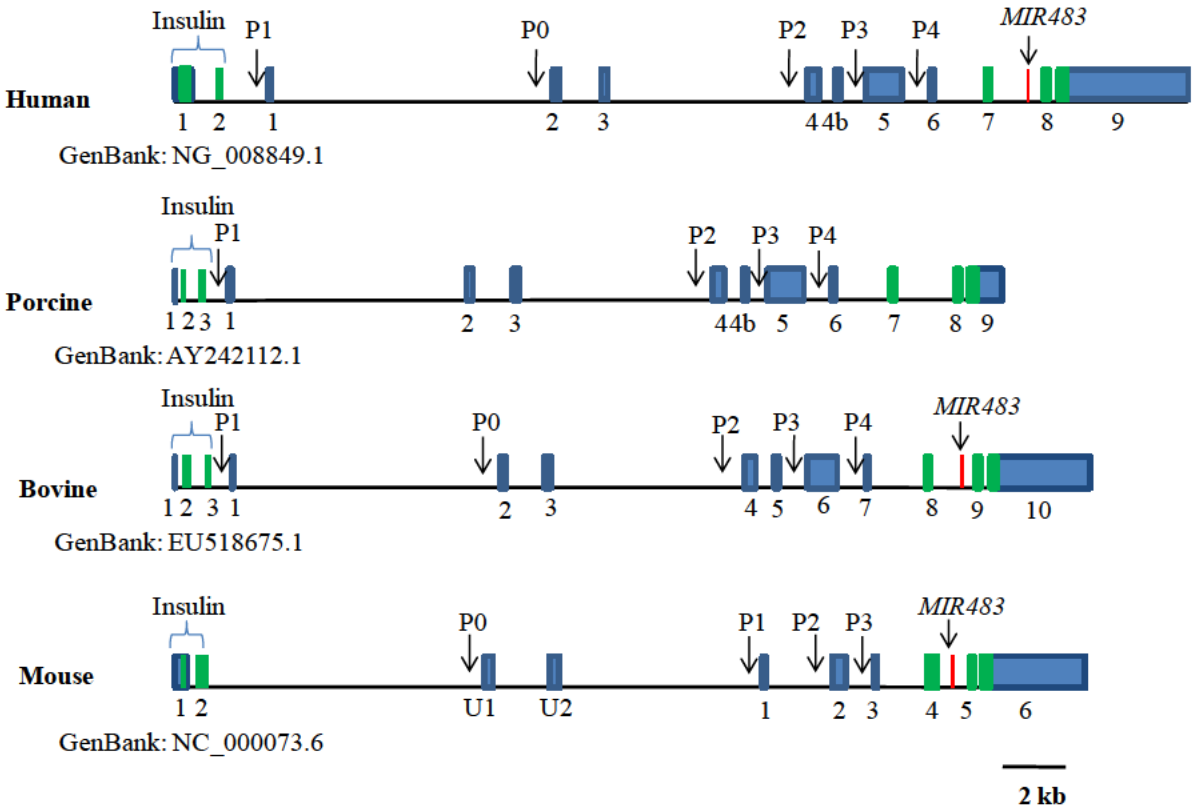

Figure 2.1 Comparative exon-intron organisation of insulin (INS) and insulin-like growth factor 2 (IGF2) genes in human, porcine, bovine and mouse.
Green boxes represent protein-coding exons/exonic regions and blue boxes represent non-coding exons/exonic regions. U1 and U2 stand for untranslated exons 1 and 2, which are present in the placental-specific transcript derived from P0 promoter in mouse. Locations of P0, P1, P2, P3 andP4 promoters and the encoded microRNA (*MIR483*) are shown. Sequence information was obtained from National Center for Biotechnology Information (NCBI) database (www ncbi.nlm.nih.gov/).

Three major transcript variants of *IGF2* are available in NCBI, which are translated into two isoforms of prepro-IGF2 protein. The transcript variants 1 (derived from P3 promoter) and 2 (derived from P0 promoter) correspond to the isoform 1 with 180 amino acids. The use of an alternate translation start codon (AUG) in the transcript variant 3 (derived from P2 promoter) leads to translation of exon 4b and a longer (236 amino acids) isoform (2) with distinct N-terminus compared with isoform 1 (Figure 2.2).

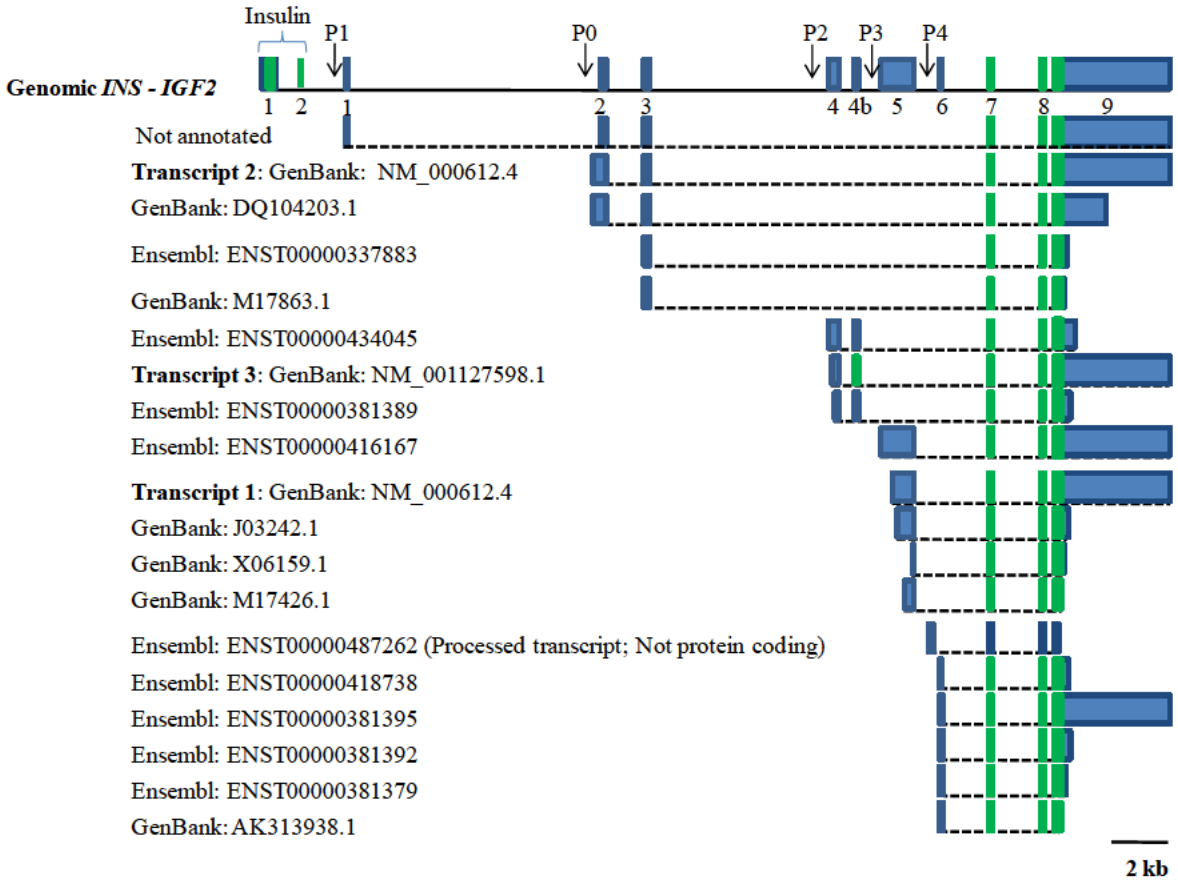

Figure 2.2 Schematic scale diagram of insulin-like growth factor 2 (*IGF2*) transcripts originating from distinct functional promoters in human.
Structure of insulin (INS) and IGF2 genes with location of *IGF2* promoters (P0, P1, P2, P3, P4) is shown. Different transcription start sites along with alternative polyadenylations lead to transcription units with varying lengths. Three main transcripts (transcript 1, 2 and 3) are available in NCBI. Green boxes represent protein-coding exons/exonic regions and blue boxes represent non-coding exons/exonic regions. Sequence information was obtained from National Center for Biotechnology Information (NCBI) and Ensembl databases (www.ncbi nlm nih.gov/, www.ensembl.org/).

For mouse, three major transcripts derived from P1 (variant 2), P2 (variant 1) and P3 (variant 3) promoters are present in NCBI (Figure 2.3). Transcript variant 1 corresponds to the IGF2 preproprotein isoform 1 (191 amino acids), whereas translation from a downstream in-frame start codon of the transcript variants 2 and 3 results in a 180-amino acid preproprotein (isoform 2) with a shortened N terminus. We did not find transcripts representing the known P0 transcript in mouse (Moore *et al.*, 1997). However, a transcript containing exons U1, 4, 5 and coding part of exon 6 is present in Ensembl. There are two transcripts present in Ensembl whose transcription start sites are not within the known exonic regions of the mouse *IGF2* ending in the protein coding exons 4 and 5. An alternative splice variant of P3 transcript in

which exon 6 splits into three exons was found in Ensembl. Two processed non protein coding transcripts were also found in Ensembl (Figure 2.3).

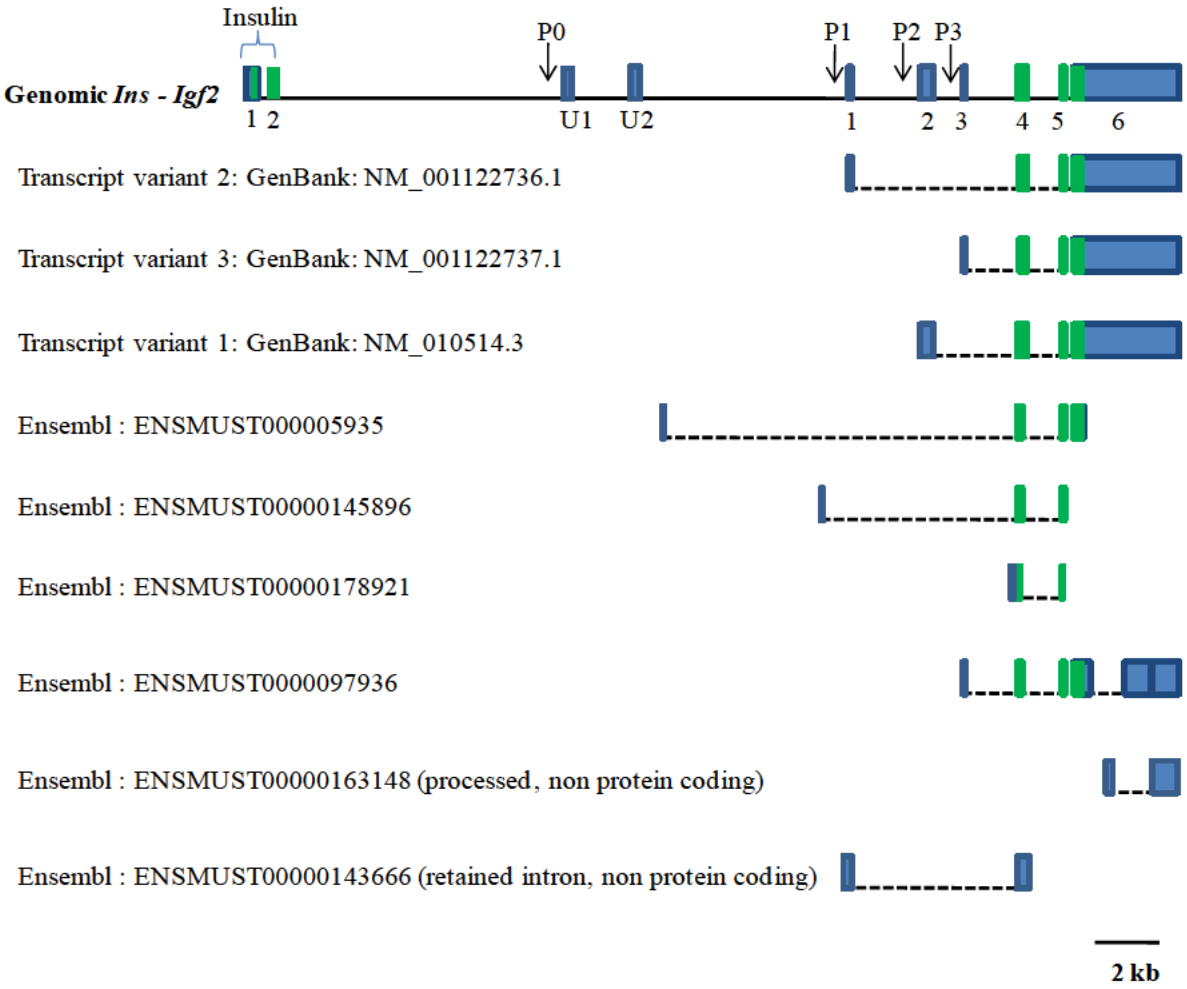

Figure 2.3 Schematic scale diagram of insulin-like growth factor 2 (*Igf2*) transcripts originating from distinct functional promoters in mouse.
Structure of insulin (Ins) and Igf2 genes in mouse, with location of promoters (P0, P1, P2, P3) is shown. Three main transcripts (transcript variant 1, 2 and 3) and two processed non-protein coding transcripts are available in NCBI and Ensembl, respectively. U1 and U2 stand for untranslated exons 1 and 2, which are present in the placental-specific transcript derived from P0 promoter in mouse. Green boxes represent protein-coding exons/exonic regions and blue boxes represent non-coding exons/exonic regions. Sequence information was obtained from National Center for Biotechnology Information (NCBI) and Ensembl databases (www ncbi.nlm.nih.gov/, www.ensembl.org/).

In porcine, four promoters (P1, P2, P3 and P4) initiate transcription from the leading exons 1 4, 5 and 6, respectively (Figure 2.4). Three splice variants arising from the P1 promoter are transcribed from exon 1 and differentially spliced into exons 2, 3 and protein coding exons. The only transcript available in NCBI is the splice variant containing

untranslated leader exons 1 and 3. Two splice variants of P2 promoter as well as P3 and P4 transcripts have been identified in porcine (Figure 2.4).

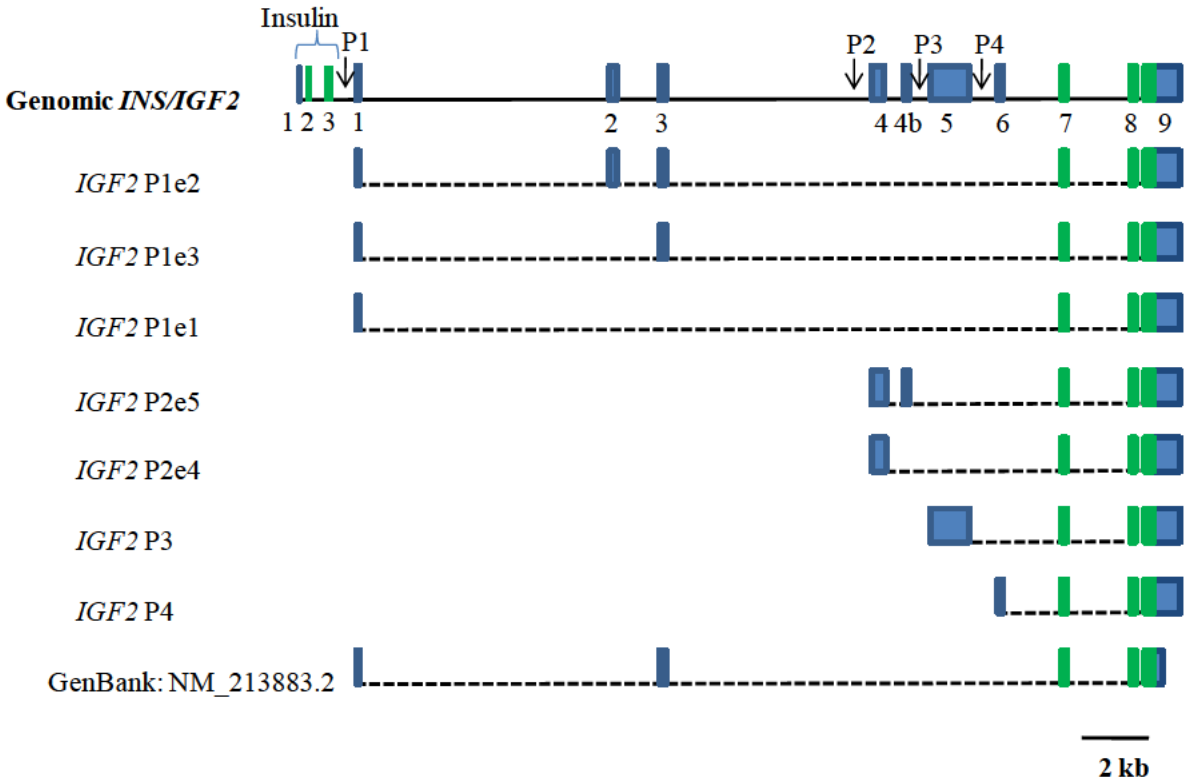

Figure 2.4 Schematic scale diagram of insulin-like growth factor 2 (*IGF2*) transcripts originating from distinct functional promoters in pig.
Structure of insulin (INS) and IGF2 genes in porcine, with location of promoters (P1, P2, P3, P4) is shown. Green boxes represent protein-coding exons/exonic regions and blue boxes represent non-coding exons/exonic regions. Sequence information and exon/intron structures for *IGF2* promoters were obtained from National Center for Biotechnology Information (NCBI) database (www.ncbi nlm nih.gov/) and (Amarger *et al.*, 2002).

In human, two types of hybrid transcripts (long and short) originating from insulin exon 1 and splicing into *IGF2* exons 2 and 3 have been characterised in pancreas. Long transcripts further splice onto *IGF2* exons 7, 8 and 9 with differential polyadenylation, whereas the short transcript ends in an exonic region downstream to *IGF2* exon 3. The hybrid transcripts have an open reading frame comprising insulin exon 1 and *IGF2* exons 2 and 3 (Figure 2.5).

In human, mouse and porcine, transcripts have been identified which are transcribed in an opposite (antisense) direction to *IGF2*. In human, two variants of *IGF2* antisense transcripts (*IGF2AS*) have been identified that originate from *IGF2* intron 4 and splice onto parts of intron 3, exon 3 and intron 2. The *IGF2AS* in human has an open reading frame and

encodes a putative protein. The *IGF2AS* in porcine has similar structure to its orthologue in human. The *Igf2as* in mouse is transcribed from intron 1 and extends into exon 1, introns U1 and U2, and exons U1 and U2. The first and last exons of *Igf2as* transcript in mouse are longer than those in human and pig. The *IGF2AS/Igf2as* in mouse and porcine does not contain an open reading frame (Figure 2.6).

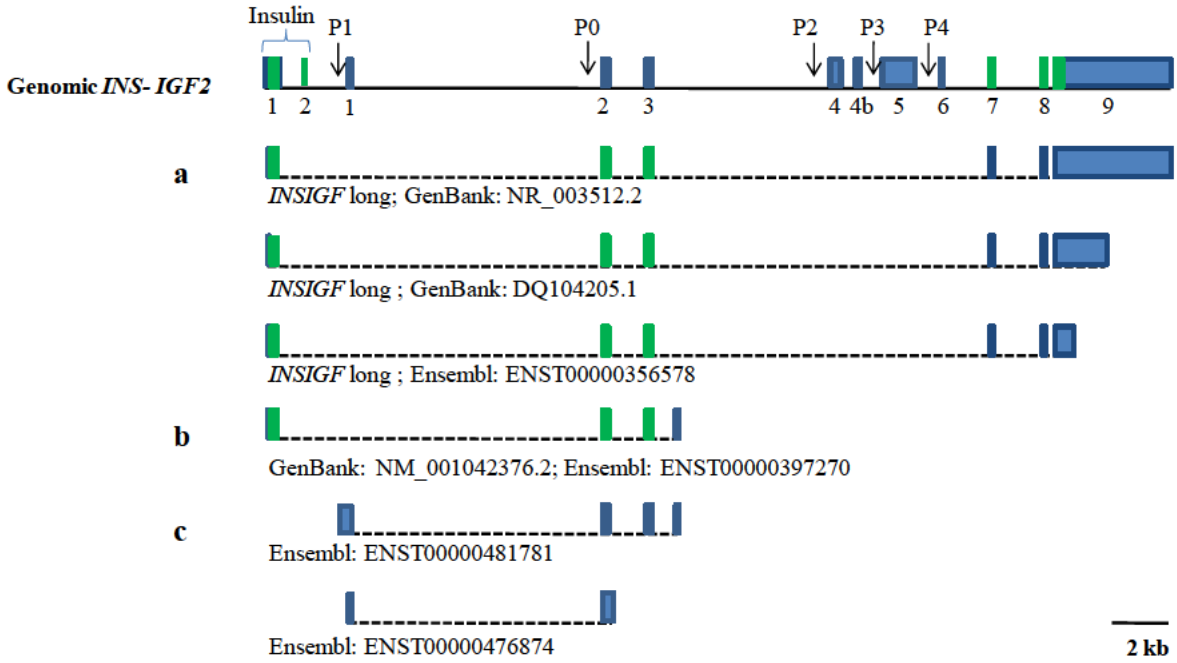

Figure 2.5 Schematic scale diagram of insulin (*INS*) and insulin-like growth factor 2 (*IGF2*) hybrid transcripts in human.
Structure of IGF2 and INS genes with the locations of distinct promoters (P0, P1, P2, P3, P4) is shown. *INS-IGF2* hybrid long **(a)** and short **(b)** transcripts as well as two untranslated transcripts **(c)** were identified. Green boxes represent protein-coding exons/exonic regions and blue boxes represent non-coding exons/exonic regions. Sequence information was obtained from National Center for Biotechnology Information (NCBI) and Ensembl databases (www.ncbi.nlm.nih.gov/, www.ensembl.org/).

We searched the bovine expressed sequence tag (EST) database (dbEST, NCBI) for evidence of *IGF2* antisense transcripts and found a number of ESTs in intron 3 of *IGF2*. Most of these ESTs were close to exon 3, whereas four ESTs encompassed exon 4 and the intronic region upstream of exon 4 (Figure 2.7).

The bovine *IGF2* transcripts derived from promoters P1 (three splice variants), P2 (two splice variants), P3 and P4 initiating transcription from exons 1, 4, 6 and 7, respectively, are shown in Figure 2.8. The transcript with the leader exons 2 and 3 splicing onto the coding exons 8, 9 and 10 is not available in NCBI. Also, the P4 transcript with a shorter 3'UTR is

available in Ensembl. The sequences for other promoter-specific transcripts are present in NCBI as partial sequences for *IGF2* mRNAs (Figure 2.8).

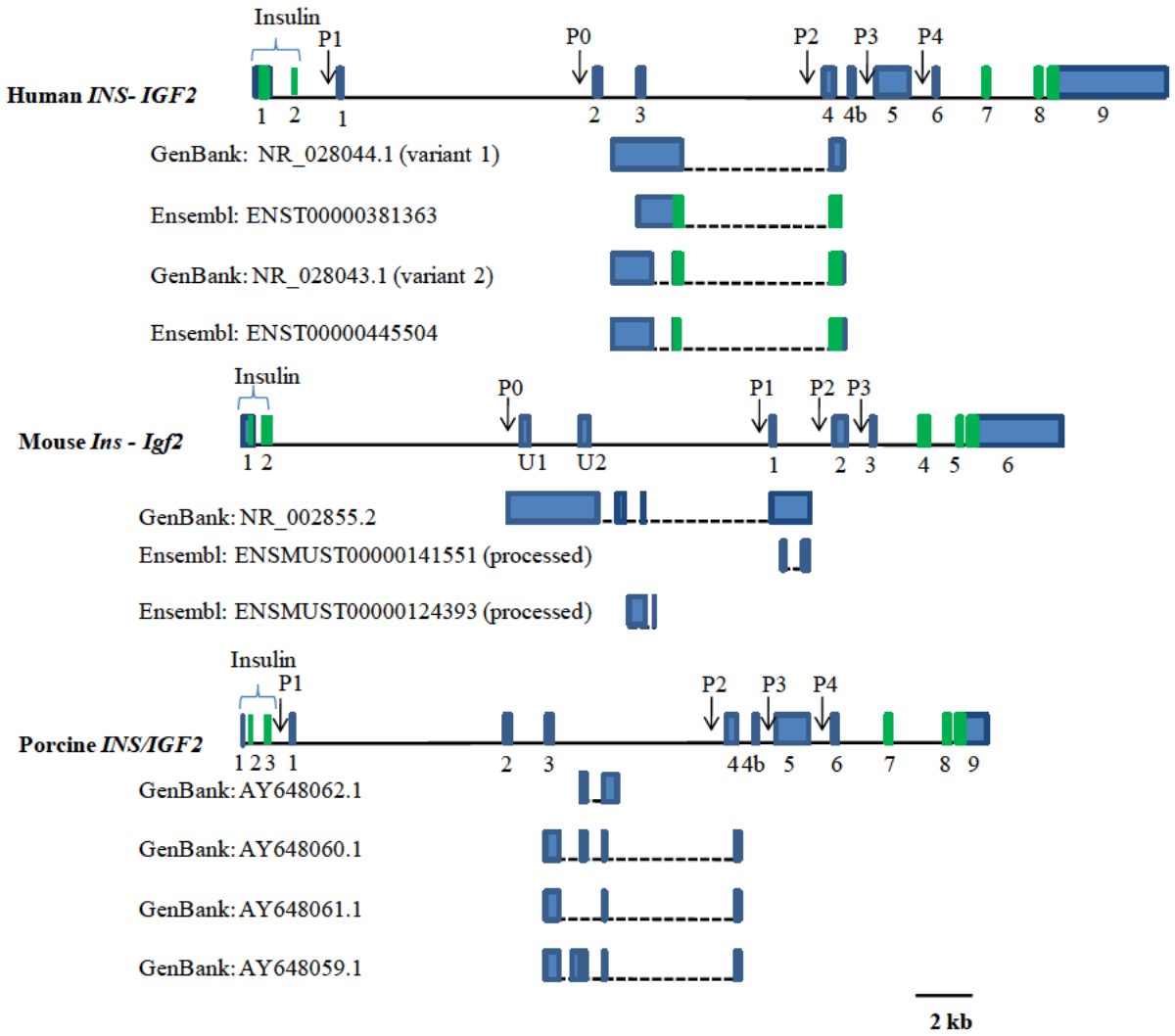

Figure 2.6 Schematic scale diagram of insulin-like growth factor 2 (*IGF2*) antisense transcripts (*IGF2AS*) in human, mouse and pig.
Structure of insulin (INS) and IGF2 genes with the locations of distinct promoters (P0, P1, P2, P3, P4) is shown. U1 and U2 stand for untranslated exons 1 and 2, which are present in the placental-specific transcript derived from P0 promoter in mouse. Green boxes represent protein-coding exons/exonic regions and blue boxes represent non-coding exons/exonic regions. Sequence information was obtained from National Center for Biotechnology Information (NCBI) and Ensembl databases (www.ncbi nlm nih.gov/, www.ensembl.org/).

In mouse, four splice variants of Airn originating from a promoter in intron 2 of *Igf2r*, differentially splice onto the intergenic region upstream of the Igf2r and Mas1 gene (Seidl *et al.*, 2006) (Figure 2.9). Therefore we searched the bovine EST database (dbEST, NCBI) for evidence of *AIRN* and found several ESTs in *IGF2R* intron 2 which were close to the CpG island harbouring the putative promoter for *AIRN* (Figure 2.10).

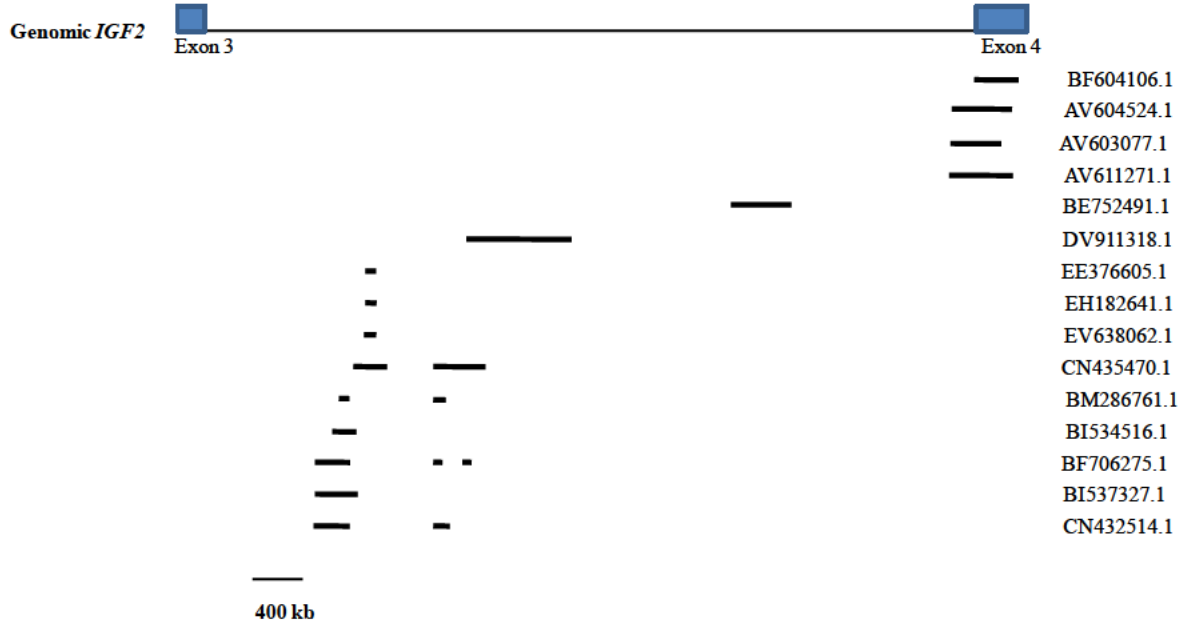

Figure 2.7 Schematic view of the bovine expressed sequence tags (ESTs) corresponding to the insulin-like growth factor 2 (*IGF2*) intron 3.
These ESTs indicate existence of the putative *IGF2AS* transcript. The locations of ESTs (thick black lines) compared to the *IGF2* intron 3 were shown. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) database (http://blast.ncbi nlm nih.gov/).

We looked for evidence of genetic polymorphisms between *Bos taurus* and *Bos indicus* within the imprinting control region (ICR) and found seven CTCF binding sites within a region of 6 kb upstream of H19 gene. Analysis of sequence similarity of this region against the *Bos indicus* genome revealed a GCG to AAA polymorphism in the consensus sequence of the first CTCF binding site (the furthest upstream of *H19*) (Figure 2.11)

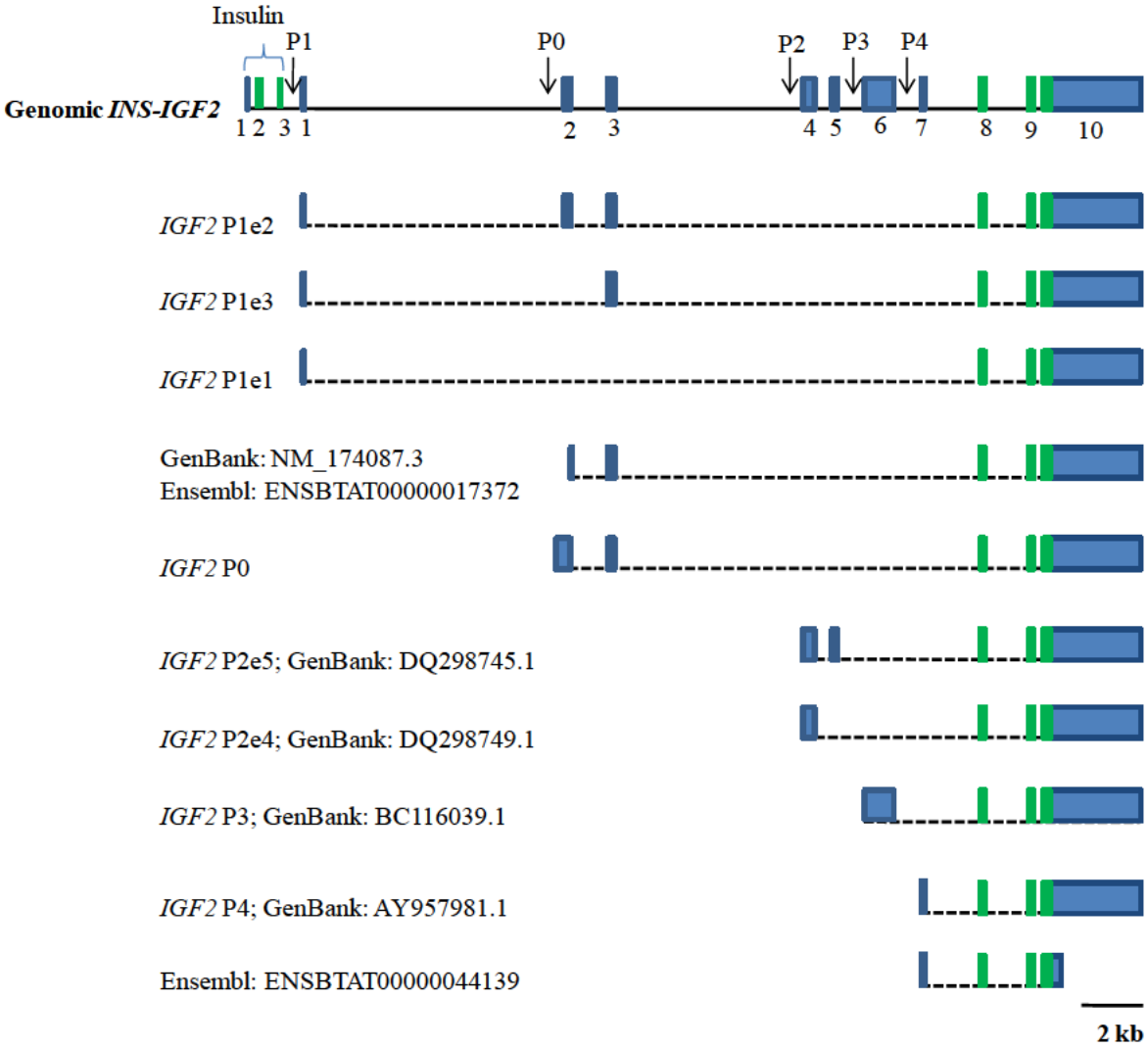

Figure 2.8 Schematic scale diagram of insulin-like growth factor 2 (*IGF2*) transcripts originating from distinct functional promoters in bovine.

Structures of insulin (INS) and IGF2 genes with location of promoters (P0, P1, P2, P3, P4) is shown. Green boxes represent protein-coding exons/exonic regions and blue boxes represent non-coding exons/exonic regions. Sequence information and exon/intron structures for *IGF2* promoters were obtained from National Center for Biotechnology Information (NCBI) and Ensembl databases (www ncbi.nlm.nih.gov/, www.ensembl.org/) and (Curchoe *et al.*, 2005; Goodall and Schmutz, 2007).

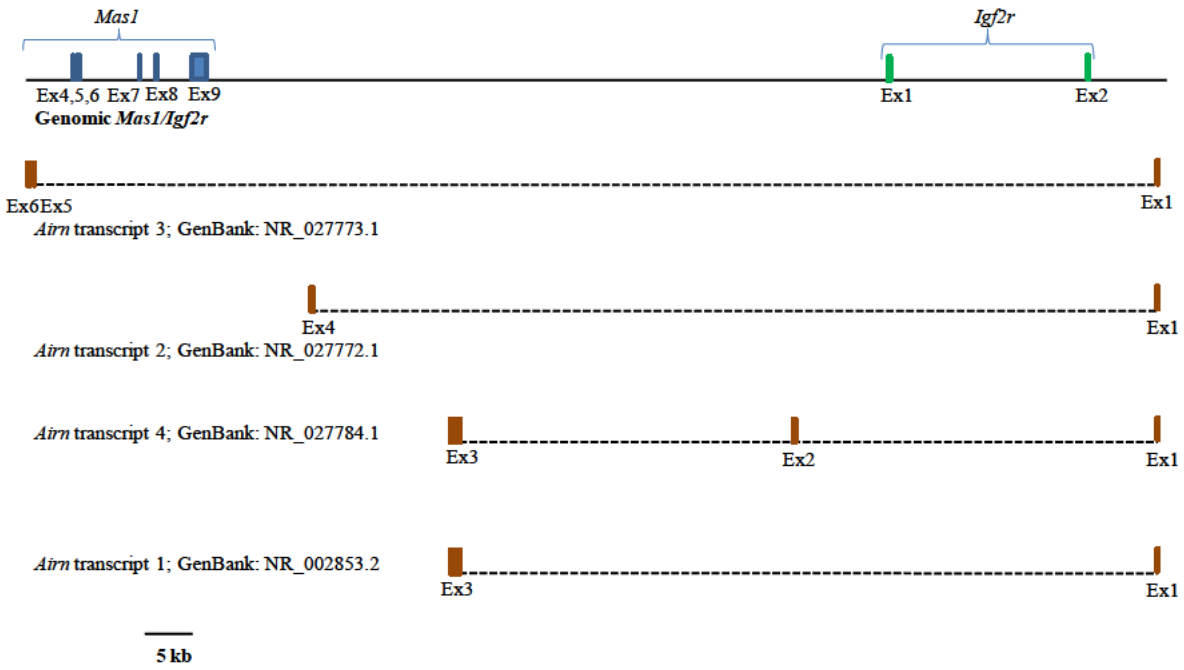

Figure 2.9 Four antisense *Igf2r* (insulin-like growth factor 2 receptor) RNA noncoding (Airn) splice variants. The splice variants contain distinct exonic regions (exons 1-6 in brown), but all originate from the promoter located within *Igf2r* intron 2. The mouse *Igf2r* exons 1-2 (green) and Mas1 oncogene (*Mas1*) exons 4-9 (blue) are shown. Sequence information was obtained from National Center for Biotechnology Information (NCBI) database (www ncbi.nlm.nih.gov/, www.ensembl.org/).

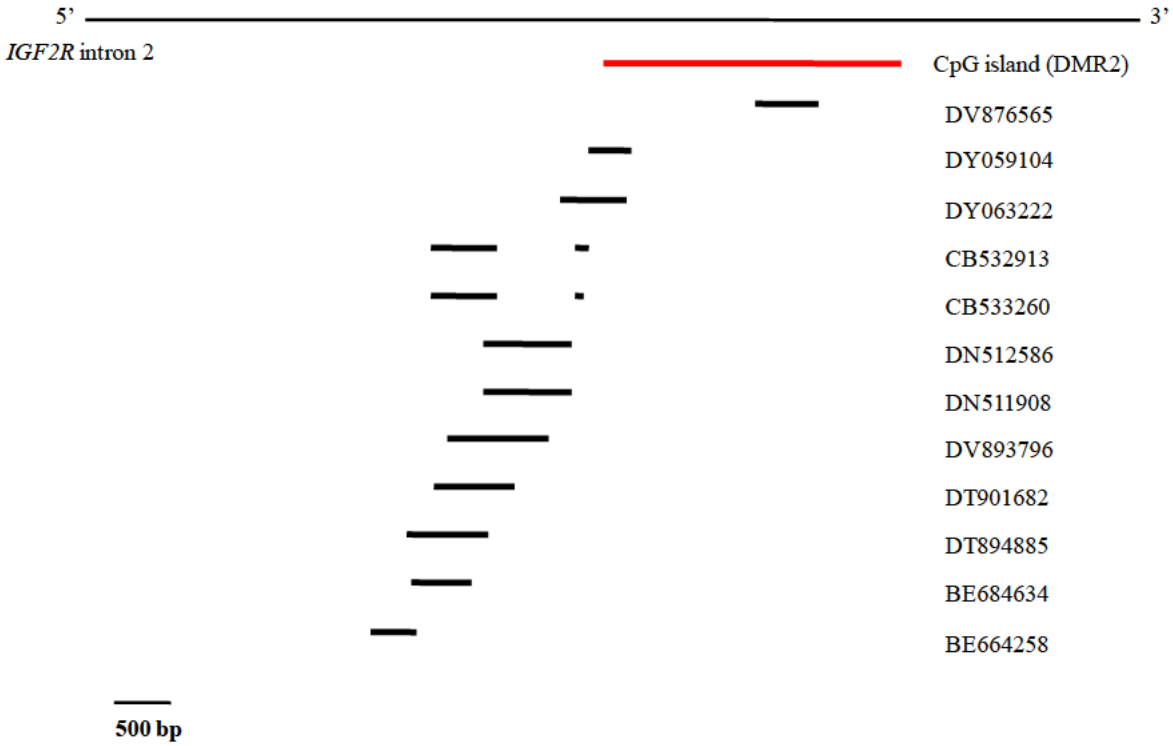

Figure 2.10 Schematic view of the expressed sequence tags (ESTs) corresponding to the insulin-like growth factor 2 (*IGF2R*) intron 2 in bovine.
These ESTs indicate existence of the putative *AIRN* transcript. The locations of ESTs (thick black lines) and CpG islands (thick red line) compared to the *IGF2R* intron 2 are shown. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) database (http://blast.ncbi nlm nih.gov/).

CTCF-BS1:	GCGGCCGCGAGGCGGCAGTGCAGGCTCATGCATCA	*Bos taurus*
CTCF-BS1:	GCGGCCAAAAGGCGGCAGTGCAGGCTCATGCATCA	*Bos indicus*
CTCF-BS2:	GCGGCCGCGGGGCGGCAGTGTGGGCTCACACATCA	*Bos taurus*
CTCF-BS2:	GCGGCCGCGGGGCGGCAGTGTGGGCTCACACATCA	*Bos indicus*
CTCF-BS3:	GCGGCCGCGAGGCGGCAGTGCAGGCTCATGCATCA	*Bos taurus*
CTCF-BS3:	GCGGCCGCGAGGCGGCAGTGCAGGCTCACACATCA	*Bos indicus*
CTCF-BS4:	GCGGCCGCGGGGCGGCGGTGTGGGCTCACACATCA	*Bos taurus*
CTCF-BS4:	GCGGCCGCGGGGCGGCGGTGTGGGCTCACACATCA	*Bos indicus*
CTCF-BS5:	GCGGCCGCGAGGCGGCAGTGCAGGCTCACACATCA	*Bos taurus*
CTCF-BS5:	GCGGCCGCGAGGCGGCAGTGCAGGCTCACRCATCA	*Bos indicus*
CTCF-BS6:	GCGGCTGCGAGGTGGCAGTGCAGGCTCACACATCA	*Bos taurus*
CTCF-BS6:	GCGGCTGCGAGGTGGCAGTGCAGGCTCACACATCA	*Bos indicus*
CTCF-BS7:	GCGGCCGCGAGGCGGCGGTGCAGGCGCCAACATCA	*Bos taurus*
CTCF-BS7:	GCGGCCGCGAGGCGGCGGTGCAGGCGCCAACATCA	*Bos indicus*

Figure 2.11 Comparison of the sequences of seven CTCF binding sites (CTCF-BS1-7) between *Bos taurus* and *Bos indicus*.
The boxed sequences depict the consensus sequences which are conserved across species. The single nucleotide polymorphisms are marked in red. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) with *Bos indicus* genome, in National Center for Biotechnology Information (NCBI) database (http://www ncbi.nlm.nih.gov/genomes/geblast.cgi?taxid=9915).

2.4 Discussion

Despite the complex patterns of transcription, all *IGF2* transcripts are translated into the same protein (Li *et al.*, 1997; Curchoe *et al.*, 2005). However, truncated forms of *IGF2* mRNA missing the major portion of exon 10 have been found in bovine tissues where full-length transcripts are not present (Curchoe *et al.*, 2005). Exon 10 translates the major part of E domain of the IGF2 precursor protein which is cleaved off to form the mature IGF2 protein (Curchoe *et al.*, 2005). The truncated forms of mouse *Igf2* transcripts missing exon 6 are present in Ensembl (Figure 2.3). It is speculated that truncation of *IGF2* transcripts is a mechanism for control of IGF2 protein levels in tissues via translational regulation of IGF2 protein and E-domain. This is supported by data in rat in which the level of E-domain peptide changes during development (Hylka and Straus, 1990).

Variant forms of the IGF2 protein have been identified in human blood (Hampton *et al.*, 1989; Straczek *et al.*, 1990; Vandenbrande *et al.*, 1990) and placenta (De Ceuninck *et al.*, 1995). Furthermore, higher molecular weight forms of the IGF2 (unprocessed proIGF2s) have been found in human plasma (Zumstein *et al.*, 1985; Gowan *et al.*, 1987) and cerebrospinal fluid (Haselbacher and Humbel, 1982). Presence of variant forms of IGF2 in human tissues implies that regulatory mechanisms which are active at the translation level may be linked to the transcriptional regulation as the result of differential splicing, which influence IGF2 bioavailability and binding affinity to its receptors and binding proteins. For example, a variant isoform of IGF2 has been shown to have a 2 to 3-fold lower affinity for IGF1R compared to the IGF1 and IGF2 (Hampton *et al.*, 1989). Although, the biological role of *IGF2* transcript variants is poorly understood, their tissue and developmental stage-specific regulation of transcription and association of these variants with the protein levels needs to be investigated. There are no reports of bovine proIGF2 protein variants, however, based on the information in human and rodents, they are also likely to be present in bovine. The identification of putative IGF2 protein variants in bovine tissues and their association with *IGF2* transcript variants can be of particular interest.

In mouse, three distinct promoters, P1, P2 and P3, which are equivalent to human P2, P3 and P4 promoters, respectively, regulate *IGF2* expression in fetal tissues (Rotwein and Hall, 1990). Furthermore, a unique promoter (P0) drives a transcript containing exons U1 and U2 specifically in mouse placenta which is necessary for placental growth (Moore *et al.*, 1997).

A novel *IGF2* transcript has been found in human that appears to be equivalent to the mouse placental-specific transcript derived from P0 transcript (Monk *et al.*, 2006). The human exons 2 and 3 exhibit sequence similarity of 81% and 53% to the mouse exons U1 and U2 respectively (Rotwein and Hall, 1990). The promoter for the human P0 transcript is located within intron 1 which initiates transcription from a unique sequence 5' to exon 2 extending 256 bp towards upstream of intron 1 (Figure 2.1). The transcript is subsequently spliced onto exon 3 as well as the last three coding exons of the IGF2 gene (Monk *et al.*, 2006) (Figure 2.2). These results show an evolutionary shift in tissue specificity of P0 promoter from placental-specific activity in rodents to fetal skeletal muscle-specific activity in other mammals.

P1 transcripts containing exon 1 in human and bovine are not present in the databases. We found transcripts in human and mouse whose leader exon is exon 3 and U2, respectively, raising the possibility of a new as yet unknown promoter upstream of exons 3 in human and U2 in mouse. The sequence information on promoter-specific transcripts in human, bovine, mouse and porcine is not complete in NCBI. For example, human P4 and P1 sequence, mouse P0 sequence and porcine P2, P3 and P4 are not available in NCBI. In bovine, there is a transcript beginning from exon 2 splicing onto exon 3. This transcript appears to be P0 transcript with an alternative transcription start site or P1 transcript whose exon 1 is missing in the database.

Hybrid transcripts (*INSIGF*) which originating from the upstream insulin exonic regions and splice to the downstream *IGF2* exons have been identified in human pancreas (Monk *et al.*, 2006). Two long and short isoforms of *INSIGF2* mRNA result from alternative splicing of the hybrid transcripts. The *INSIGF* short transcript is composed of *INS* exon1, *IGF2* exons 2,

3 as well as a specific exonic region downstream of exon 3 (Figure 2.5). This region, which is also present in the human *IGF2* antisense transcript, is highly conserved in bovine (data not shown), suggesting that hybrid *INSIGF* transcripts may exist in bovine. There is no evidence regarding the presence of the hybrid transcripts in mouse and other species. The *INSIGF2* transcript contains an open reading frame (ORF) which extends to *IGF2* exon 3. Translation of this ORF results in a novel protein that includes the insulin leader sequence and B-chain peptide as well as an additional peptide at the C-terminal end (Monk *et al.*, 2006).

An *IGF2* antisense transcript has been detected in human (Okutsu *et al.*, 2000), mouse (Rivkin *et al.*, 1993) and porcine (Braunschweig *et al.*, 2004). Human *IGF2AS* lies within the IGF2 gene and shares some exonic regions (exons 2, 3, 4), exhibiting about 50% homology with the mouse *Igf2as* (Okutsu *et al.*, 2000). In mouse, three *Igf2as* transcripts encompassing DMR0, which overlap P0- and P1- derived transcripts, have been detected (Moore *et al.*, 1997). Notwithstanding, there is no evidence of a link between *Igf2as* and *Igf2* expression. Human *IGF2AS* is imprinted and exclusively expressed from the paternal allele. There is no information on the possible regulatory effects of *IGF2AS* on *IGF2* expression and imprinting. *IGF2* and *IGF2AS* are overexpressed in human Wilms' tumors at levels over 10 and 100 times higher than that in normal fetal tissues, indicating their expression is co-regulated (Okutsu *et al.*, 2000). Human *IGF2AS* has an ORF encoding a putative peptide, while the corresponding mouse *Igf2* does not have such an ORF. Although no relevant proteins for *IGF2AS* have been identified this gene can affect tumorogenesis by its protein products or acting as a non-coding RNA (Okutsu *et al.*, 2000). Analysis of the expression pattern of *IGF2* and *IGF2AS* in human fetal tissues has revealed that *IGF2AS* is ubiquitously expressed in most tissues at levels similar to P2-derived transcripts, so it is possible that both transcripts are under control of the same regulatory elements (Vu *et al.*, 2003). This can be explained by the locations of *IGF2* and *IGF2AS* promoters which are adjacent to each other and may share the same regulatory mechanisms. Dissociation of imprinting of *IGF2* and *IGF2AS* was inferred from the study of loss of imprinting (LOI) of *IGF2AS* in Wilms' tumors (Vu *et al.*,

2003). No *IGF2* antisense transcripts were reported in bovine, but our current knowledge on *IGF2AS* in human, mouse and porcine can postulate a putative antisense transcript with the potential for a regulatory role in pre and postnatal development. Investigation of the bovine EST database revealed evidence for the existence of the putative *IGF2AS*. These ESTs matched the sequences in intron 3 of bovine *IGF2* and show splicing patterns. Interestingly, the corresponding region in porcine harbours exons 2 and 3 of *IGF2AS*.

Results from an early study of cross-hybridisation of bovine mRNA using human-derived probes covering different *IGF2* exons revealed seven transcripts of different size from P3 (5.2, 3.4, 2.8, 2.1 kb), P4 (4, 1.1 kb) and a liver-specific transcript of 4.4 kb (Boulle *et al.*, 1993). According to the known sequence lengths of bovine *IGF2* exons/introns, sizes of the transcripts obtained in this study differ from the expected sizes of different transcripts (4 kb for P1 promoter; 4.4 kb for P3 promoter; 3.6 kb for P4 transcript). This raises the possibility of alternative functional polyadenylation signals located downstream to the distal polyadenylation signal of 3'UTR. An alternative explanation is that the measurement of transcript length based on size markers might not be accurate.

Some *IGF2* exons overlap exonic regions of the putative sense and antisense transcripts, including *INSIGF2* and *IGF2AS*. Therefore, primers for amplification of *IGF2* promoter-specific transcripts must be designed considering the overlapping exons. Postnatal liver-specific expression of P1 transcript in bovine was demonstrated by PCR-amplification of the transcript containing exon 1 (Goodall and Schmutz, 2007). Curchoe *et al.* (2005) used exon 3, which is shared between P0 and P1 transcripts, to analyse expression of P1 transcript and reported non-specific expression of transcripts containing exon 3 in bovine fetal and adult tissues, including liver, heart, lung, brain and kidney, although the developmental stage of fetuses was not clear in this work (Curchoe *et al.*, 2005). The ubiquitous expression of P1 transcript in adult tissues using primers within exon 3 (Curchoe *et al.*, 2005) likely results from amplification of *IGF2AS* which entirely overlaps *IGF2* exon 3 (Figure 2.6).

We investigated the bovine expressed sequence tag (EST) database for the putative *AIRN* transcript and found 12 ESTs corresponding to intron 2 of *IGF2R*, two of which show splicing patterns (Figure 2.11). These data support the existence of *AIRN* in bovine which originate from the CpG island within intron 2. Therefore, primers located within *IGF2R* intron 2 in a region close to the CpG island likely amplify all putative variants of *AIRN*. The putative *AIRN* transcript was previously amplified in bovine fetal tissues by placing primers in *IGF2R* intron 2 (Suteevun-Phermthai *et al.*, 2009) and intron 1/exon 2 (Farmer *et al.*, 2013).

Single nucleotide polymorphisms were identified in the consensus sequence of the first CTCF binding site, which is conserved across species (Young *et al.*, 2003). These SNPs, which disrupted two CpG dinucleotides, are likely to affect regulation of imprinting and expression of *IGF2* and *H19*. Several studies demonstrated that mutations in the CTCF binding sites and ICR influence epigenetic state and imprinted expression of *H19* and *IGF2* with consequent effects on growth (Szabó *et al.*, 2002; Engel *et al.*, 2004; Pant *et al.*, 2004; Han *et al.*, 2008; Li *et al.*, 2008b; Lee *et al.*, 2010; Matsuzaki *et al.*, 2010; Quenneville *et al.*, 2011; Singh *et al.*, 2012)

In conclusion, the bovine *IGF2* expression pattern may be much more complicated than previously thought. A number of sense and antisense transcripts found in human, mouse and porcine IGF2 gene region have not been identified in bovine. These antisense transcripts share exonic regions with the IGF2 gene. The discrepancies in results of expression analyses of *IGF2* promoter-specific transcripts in bovine likely result from amplification of different *IGF2* exons which overlap exonic regions of other sense and/or antisense transcripts. Further studies are required to elucidate complete sequence and exon/intron structure of different *IGF2* transcripts and their tissue-specific and developmental expression in bovine. The high sequence homology between human and bovine and the intron-exon structure demonstrate that the human IGF2 gene is closer to bovine than mouse. Thus, the bovine could provide a suitable model for studying the role of this gene in human prenatal development.

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Chapter 3

Tissue and developmental stage-specific expression of the imprinted IGF2, IGF2R, H19 and AIRN genes in bovine

3.1 Introduction

The imprinted IGF2 and IGF2R genes play a key role in regulating prenatal growth and development (Dechiara *et al.*, 1990; Lau *et al.*, 1994; Wang *et al.*, 1994; Ludwig *et al.*, 1996). Furthermore, several lines of evidence showed that they are associated with postnatal growth (Jeon *et al.*, 1999; Petry *et al.*, 2005; Berkowicz *et al.*, 2012). This requires that expression of these genes is controlled in a developmental stage-specific manner. The imprinted expression of *IGF2* and *IGF2R* is regulated by the reciprocally imprinted H19 and AIRN long non-coding RNA genes (Steenman *et al.*, 1994; Ripoche *et al.*, 1997; Sleutels *et al.*, 2002).

IGF2 is a potent growth promoter and essential for prenatal development. The *IGF2* gene is subject to genomic imprinting and paternally expressed in mouse (*Mus musculuc domesticus*), human, ovine (*Ovis aries*) and bovine (*B. taurus/B. gaurus*) (DeChiara *et al.*, 1991; Giannoukakis *et al.*, 1993; McLaren and Montgomery, 1999; Dindot *et al.*, 2004). Postnatally, *IGF2* has a significant role in muscle growth and fat deposition in porcine (Jeon *et al.*, 1999; Nezer *et al.*, 1999; Van Laere *et al.*, 2003) and bovine (Zhao *et al.*, 2002; Goodall and Schmutz, 2003; Sherman *et al.*, 2008; Zwierzchowski *et al.*, 2010). In human, bovine, ovine and porcine, four promoters (P1-4) are involved in regulation of *IGF2* transcription in a manner depending on tissue and developmental stage (Ohlsen *et al.*, 1994; Amarger *et al.*, 2002; Curchoe *et al.*, 2005; Goodall and Schmutz, 2007).

The IGF2R is a cell surface receptor possessing multiple functions. It acts as a scavenger receptor which binds and internalises IGF2 for lysosomal degradation, thus, regulating its bioavailability to cells (Braulke, 1999). The IGF2R gene is imprinted and expressed from the maternal allele in *Mus musculus/Mus spretus* (Barlow *et al.*, 1991), ovine (*Ovis aries*) (Young *et al.*, 2003), bovine and porcine (Killian *et al.*, 2001), whereas it is likely polymorphically imprinted in human (Xu *et al.*, 1993; Xu *et al.*, 1997; Buckberry *et al.*, 2012). Associations of polymorphisms in *IGF2R* with postnatal growth-related traits in bovine also suggest a role for *IGF2R* in postnatal growth (Berkowicz *et al.*, 2012). Imprinted expression of the *IGF2R* is controlled by the reciprocally imprinted long non-coding RNA gene, *AIRN*. While expression

of *IGF2R* was shown to be under developmental regulation, evidence of tissue-specific developmental expression of *AIRN* is lacking.

The H19 gene is located downstream of *IGF2* being part of an imprinted cluster. The gene encodes a long RNA which shows common features of RNA polymerase II derived mRNAs undergoing polyadenylation and splicing but lacks a conserved open reading frame (Brannan *et al.*, 1990). The gene is imprinted on the paternal chromosome and expressed from the maternal allele in human (Rachmilewitz *et al.*, 1992; Zhang and Tycko, 1992; Buckberry *et al.*, 2012), mouse (Bartolomei *et al.*, 1991), bovine (Zhang *et al.*, 2004), porcine (Li *et al.*, 2008) and ovine (*Ovis aries*) (Young *et al.*, 2003). The conserved structure and sequence of *H19* across species suggests a functional role for the non coding RNA (Juan *et al.*, 2000). *H19* is highly expressed during embryogenesis in mouse mesoderm and endoderm-derived tissues and its expression is fully suppressed in postnatal tissues, except skeletal muscle and heart (Poirier *et al.*, 1991).

Several lines of evidence in different species showed that expression patterns of the imprinted genes belonging to the IGF system are developmentally regulated (Brown *et al.*, 1986; Soares *et al.*, 1986; Gray *et al.*, 1987; Schofield and Tate, 1987; Sklar *et al.*, 1989; Ballesteros *et al.*, 1990; Senior *et al.*, 1990; O'Mahoney *et al.*, 1991 ; Sklar *et al.*, 1992; Boulle *et al.*, 1993; Delhanty and Han, 1993a; Moats-Staats *et al.*, 1995; Pfuender *et al.*, 1995). However, quantitative tissue-specific expression of *IGF2*, *IGF2R* and their regulatory transcripts across key developmental time points has not been systematically examined in any species.

The aim of the present study was (i) to investigate the distribution of transcript abundance of IGF2, IGF2R, H19 and AIRN genes in different tissues of bovine and (ii) to compare developmental changes in expression of these genes between Day-48 embryos, Day-153 fetuses and 12-month old juvenile calves.

3.2 Materials and methods

3.2.1 Animals and tissue

All animal experiments and procedures described in this study were approved by the University of Adelaide Animal Ethics Committee (No. S-094-2005 and S-094-2005A). Dams and sires of the Angus and Brahman breeds were used to generate purebred and crossbred offspring at three developmental stages, Day-48 embryo, Day-153 fetus and 12-month-old juvenile. Angus and Brahman females, which had not given birth previously and were approximately 16–20 months of age received standard commercial estrous cycle synchronization (e.g. http://www.absglobal.com/aus/resources/beef-resources/synchronisation-programs---cue-mate-1/). Cidirol - Heat Detection & Timed Insemination (HTI) and Cidirol - Timed Insemination (TI) was used. Briefly this consisted of an initial injection of 1 ml of 1 mg/ml estradiol benzoate (Cidirol, Genetics Australia Co-operative Ltd., Bacchus Marsh, Australia) and insertion of a progesterone-releasing vaginal insert (Eazi-Breed CIDR, DEC International, Hamilton, New Zealand). The vaginal inserts were removed after 7–9 days and heifers injected with 2 ml of a prostaglandin analogue (0.26 mg of cloprostenol sodium/ml (Estrumate), Schering-Plough Animal Health, Baulkam Hills, Australia). Estrus detection devices (Kamar, Agrigene, Wangaratta, Australia) were placed on all animals. In HTI, animals that showed estrus two days later were inseminated, while animals not in estrus received an additional 0.5 ml injection of estradiol benzoate and were inseminated 24 h later. In TI, animals received 0.7 ml estradiol benzoate the day after removal of vaginal inserts and were inseminated 24 h later. Synchronization/insemination was repeated in HTI and TI with estradiol benzoate injection of all animals after removal of vaginal inserts, followed by a final round of insemination and natural breeding in HTI animals without further synchronization measures. Angus and Brahman paternal genetics were used in HTI and Ti. Animals were pregnancy tested by ultrasound scanning (Anand-Ivell *et al.*, 2011).

Embryos, fetuses and offspring were generated using at least two sires of each breed. Dams were pregnancy tested by ultrasound scanning, and embryos and fetuses recovered in

an abattoir. Embryos and fetuses were removed from the uterus, eviscerated, vacuum packed and stored frozen at -20°C until further processing.

Tissues were collected from 60 Day-48 embryos, 100 Day-153 fetuses (term ~ 283 days), with both sexes represented, and 23 calves recovered by cesarian section (Day-278 of gestation) and 12-14-month old heifers and steers. At Day-48 of gestation, all organs have not yet formed and embryos still have a mesonephros, but some organs (heart, brain, liver) are clearly visible and could be sampled. The amount of tissue available was another criterion to opt for Day-48 rather than earlier stages that often require pooling of individual samples or amplification procedures that can impact on the quality of data obtained. Dissected tissues were immediately placed into RNA-later® (Qiagen, Chadstone Centre, VIC, Australia) and stored at -80°C after equilibration for 24 hours at 2–4°C. Heart, brain, and liver samples were collected from Day-48 embryos. Heart, brain, liver, lung, kidney, skeletal muscle (*M. semitendinosus*) and testis were collected from Day-153 fetuses and the same tissues, with the exception of testis, were collected at 12 months of age. Cotyledon (*placenta fetalis*) was collected at Day-48, Day-153 and following delivery by caesarean section at Day-278 term. All tissue samples were provided by Prof. Stefan Hiendleder.

3.2.2 RNA isolation and reverse transcription

Tissue specimens (40-50 mg of each tissue) were homogenised by PRECELLYS®24 lyser / homogeniser (Bertin Technologies, Saint Quentin en Yvelines Cedex, France). Homogenisation of samples was carried out in 1 ml of TRIzol reagent (Ambion, Life Technologies™, Inc., Carlsbad, CA, USA) in tubes containing 1.4 mm ceramic beads (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) (for embryonic liver, fetal lung, liver, kidney and brain, and juvenile brain) or 2.8 mm ceramic beads (for fetal heart and cotyledon, juvenile muscle, kidney, heart, liver and lung, and term cotyledon). Homogenisation runs were performed at the speed of 6500 rpm in one (embryonic liver, fetal brain, kidney and lung, and juvenile liver, lung and brain) or two (fetal liver, heart and cotyledon, term cotyledon, and

juvenile muscle, kidney and heart) cycles, with a cycle duration of 7 seconds (fetal brain), 10 seconds (fetal heart and cotyledon), 12 seconds (fetal kidney and lung, embryonic liver, near-term cotyledon, juvenile muscle, kidney, heart and brain), or 15 seconds (fetal liver and juvenile liver and lung). In cases where tissue samples underwent two cycles of homogenisation, the waiting time between two cycles was 10 seconds (fetal heart and cotyledon) or 12 seconds (fetal liver, term cotyledon, and juvenile muscle, kidney and heart). Total RNA was extracted from fetal tissues using TRI Reagent® (Ambion, Life Technologies™, Inc., Carlsbad, CA, USA). After removing insoluble materials from the homogenate by centrifugation at 12000 g for 10 minutes at 2 to 8 °C and incubation of the supernatant on ice for 5 minutes, 0.2 ml of chloroform was added, followed by incubation on ice for 3 minutes and centrifugation for 20 minutes at 12000 g (4°C). The aqueous phase was separated and mixed with 0.6ml of isopropyl alcohol and incubated for overnight at -20° C, followed by centrifugation at 13000 g (4°C). After removing the supernatant, the pellet was washed by adding 1.2 ml of 75% ethanol and re-pelleted by centrifugation at 12000 g for 5 minutes (4°C). After removing the supernatant, the RNA pellet was dissolved in 50-100 µl of nuclease-free water (GIBCO UltraPure™ Distilled Water, Invitrogen™, Inc., Auckland, NZ). Due to small sample size, AllPrep™ DNA/RNA Micro (Qiagen GmbH, Inc., Hilden, Germany) was used for extraction of RNA from embryonic heart and brain tissues according to the manufacturer's instructions (Appendix 1). 8-10 mg of embryonic heart and brain was used for DNA/RNA extraction. RNA quality and integrity was assessed by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Inc., Wilmington, DE, USA) and Agilent RNA 6000 Nano Kit with Bioanalyzer 2100 (Agilent Technology, Inc., Santa Clara, CA, USA) and RIN (RNA Integrity Number) values averaged 7.16 and 8.21 for skeletal muscle in juvenile and fetus, 7.43 and 8.85 for lung in juvenile and fetus, 4.16 and 7.03 for kidney in juvenile and fetus, 7.66, 9.00 and 8.45 for heart in juvenile, embryo and fetus, 7.54, 8.93 and 8.05 for liver in juvenile, embryo and fetus, 6.74 and 8.38 for brain in juvenile and fetus, 5.49, 6.35 and 7.16 for cotyledon in calf, embryo and fetus, and 5.85 for testis in fetus, respectively. The

integrity of RNA molecules is a major concern in gene expression studies, which can affect the results. RIN values are based on an algorithm which is a user-independent, automated and reliable procedure for standardisation of RNA quality control (Schroeder *et al.*, 2006). RNA samples were provided by Ali Javadmanesh.

Prior to RT-PCR, the extracted RNAs were treated by DNase (RQ1-DNase, Promega, Inc., Madison, WI, USA). Reactions were performed in a total volume of 10 µl containing RNase-Free DNase 10X reaction buffer (1 µl), RNase-Free DNase (1 U/µg RNA), RNA solution and nuclease-free water incubated at 37°C for 30 minutes. 1 µl of DNase stop solution was added to the mixture, followed by incubation at 65 °C for 10 minutes.

Complementary DNA (cDNA) was synthesised from 500 ng of total RNA using SuperScript™ III First-Strand Synthesis System (Invitrogen, Life Technologies™, Inc., Carlsbad, CA, USA) and random hexamer oligonucleotides according to the manufacturer's instructions. Briefly, 10 µl of a mixture containing total RNA, 1 µl of 50 ng/µl random hexamers, 1 µl of 10 mM dNTP mix and nuclease-free water was incubated at 65 °C for 5 minutes and placed on ice for 1 minute. The mixture was mixed with 10 µl of a cDNA synthesis mix containing 2 µl of 10X RT buffer, 4 µl of 25 mM MgCl2, 2 µl of 0.1 M DTT, 1 µl of 40 U/µl RNaseOUT^{TM} and 1 µl of 200 U/µl SuperScript^{TM} III RT, and incubated at 25 °C for 10 minutes, followed by 50 °C for 50 minutes. The reaction was terminated at 85 °C for 5 minutes and then chilled on ice. The mixture was incubated at 37 °C for 20 minutes after adding 1 µl of RNase H. Equal quantities of 60 embryonic cDNA samples, 100 fetal cDNA samples and 23 juvenile cDNA samples were mixed to create a pooled representive cDNA sample for each tissue and developmental stage. The pooled cDNA samples were then used as templates to run quantitative real time RT-PCR reactions in three technical replicates.

3.2.3 Quantitative real time RT-PCR

Relative abundance of the following transcripts was analysed: *IGF2* global and promoter-specific transcripts, including P0, P1 (two transcripts, P1e2 and P1e3), P2 (two splice variants, P2e4 and P2e5), P3 and P4; *IGF2R*, *H19* and *AIRN*. Details of primers used are summarised in Appendix 2. Schematic location of primers for amplification of *IGF2* promoter-specific transcripts is presented in Figure S1 (Appendix 8). We amplified two splice variants of P2 transcripts. The splice variant containing leader exon 4 was amplified by forward primer located at the junction of exons 4 and 8 and reverse primer within exon 8. The other splice variant with leader exons 4 and 5 was amplified by forward and reverse primers placed in exons 5 and 8, respectively. Forward and reverse primers for amplification of P3 transcript were placed in exons 6 and 8, and for amplification of P4 transcript were located in exons 7 and 8, respectively. *IGF2* global transcripts were amplified by placing primers in exons 8 and 9, which are found in all known *IGF2* transcripts. Although all RNA samples were DNase I treated we designed most primers spanning an intron to avoid potential amplification of genomic DNA.

Quantitative real time PCR reactions were performed using Fast Start Universal SYBR Green Master (Roche Diagnostics GmbH, Mannheim, Germany) in an Eppendorf Mastercycler® pro S thermal cycler (Eppendorf, Inc., Hamburg, Germany). The pooled sample from each tissue and developmental stage was used as a cDNA template in the reactions in triplicate and the mean of the three CTs used to calculate the mean amount of target transcript and standard error for three technical replicates. Reactions were performed in a total volume of 12 µl containing 6 µl of SYBR master mix (2×), 4 µl of cDNA (40-time diluted from stock cDNA, equivalent to 12.5 ng of starting RNA), 0.8 µl of primers (5 pmol/µl) and 1.2 µl of double distilled nuclease-free water (ddH_2O). A non-template control was included in all experiments to check for DNA contamination of reagents used for amplification. Thermocycling reactions were carried out with a 10-minute initial denaturation/activation step at 95°C, followed by 40 cycles of 95°C for 20 seconds

(denaturation), 57-62°C for 30 seconds (annealing, depending on the primer pair) and 72°C for 20 seconds (extension). Product specificity and integrity was confirmed via sequencing on a 3730*xl* DNA Analyzer (Applied Biosystems, Inc., Foster City, CA, USA), plots of the melting curve derived by Mastercycler® ep Realplex software (Eppendorf, Inc., Hamburg, Germany), and electrophoresis on a 2% agarose gel (Agarose low EEO, AppliChem GmbH, Darmstadt, Germany) stained with GelRed™ Nucleic Acid Stain (Biotium, Inc., Hayward, CA, USA) and visualised under UV with Gel Doc™ 1000 Single Wavelength Mini-Transilluminator, using Quantity One image analysing software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

An equal proportion of cDNA from all tissues and developmental stages was pooled to generate a cDNA template for standard curve analysis. The standard curve included a 3-fold serial dilution of initial pooled cDNA template over eight data points. Three replicates were used for each dilution of the cDNA template. The CT (threshold cycle) values of the standards were used to derive a standard curve which shows the CT values as a linear function of natural logarithm of the specified arbitrary amounts of cDNA. The relative abundance of each target transcript was calculated by the relative standard curve method with determination of PCR amplification efficiency using the following equation:

$$\text{Relative transcript abundance (arbitrary units)} = \exp\left(\frac{\text{CT} - \text{intercept}}{\text{slope}}\right)$$

where, "exp" indicates the exponential function, CT is threshold cycle for each sample, intercept is the point at which the standard curve intersects with the Y-axis and slope is the increase in standard curve. PCR amplification efficiencies (E) were calculated by the following equation:

$$\text{E} = 10^{\left(\frac{-1}{\text{slope}}\right)} - 1$$

CT values and transformed quantities (relative transcript abundances, in terms of standard curve), as well as standard curve parameters, including amplification efficiency and coefficient of determination (R-squared), were automatically calculated by Mastercycler® ep

Realplex software (Eppendorf, Inc., Hamburg, Germany). Amplification efficiency and coefficient of determination for the experiments are shown in Appendix 6. Since pooled cDNA was used in the quantitative real time RT-PCR reactions, there was no need to normalise the expression data using housekeeping gene expression. In addition, we deemed it not appropriate to perform statistical significance tests on technical replicates to compare the average transcript abundances between tissues and developmental stages. Rather, we presented means and their respective standard errors.

3.3 Results

3.3.1 *IGF2*, global transcript

Transcript abundance of global *IGF2* was lowest in brain at all developmental stages (Figure 3.1). In Day-48 bovine embryos, highest abundance of *IGF2* transcript was in liver, with expression levels 330-fold higher than in brain. Expression of *IGF2* in embryonic heart and cotyledon was 77- and 30-fold higher than in brain, respectively. In the fetus, highest *IGF2* mRNA transcript abundance was observed in fetal lung, muscle and liver, followed by kidney. Despite a 4-fold decrease in global *IGF2* transcript abundance in postnatal liver, when compared to mid gestation, liver displayed the highest expression of *IGF2* compared with other postnatal tissues, i.e., approximately 13-fold higher than *IGF2* expression in postnatal skeletal muscle. The expression level of *IGF2* fell markedly after birth in all other tissues, with the highest fold decrease between pre and postnatal developmental periods being observed in lung (356-fold), kidney (122-fold) and muscle (60-fold). In general, expression levels of global *IGF2* transcript are higher at the fetal stage in all examined tissues. In cotyledon, a slightly higher transcript abundance was observed in embryos compared with fetuses, and *IGF2* expression was down-regulated 2-fold at term.

3.3.2 *IGF2R*

IGF2R expression was detected in all tissues studied at all three developmental stages (Figure 3.1). Expression of *IGF2R* in embryonic liver was (more than 2-fold) higher than in other embryonic tissues, whereas in the fetus, expression of *IGF2R* in heart, lung and kidney was comparable to liver. Fetal skeletal muscle, cotyledon and brain showed the lowest mRNA abundance of *IGF2R* compared to all other fetal tissues.

Relative expression level in brain as compared to other tissues for *IGF2R* (around 10 fold less than liver) was substantially higher than that for *IGF2* (around 300-fold less than liver). Postnatal expression of *IGF2R* was highest in liver, followed by skeletal muscle. The transcript abundance of *IGF2R* was highest in the fetus and decreased dramatically after birth.

The highest fold change from fetal to postnatal developmental stage occurs in lung (67-fold decrease), heart (35-fold decrease) and kidney (25-fold decrease).

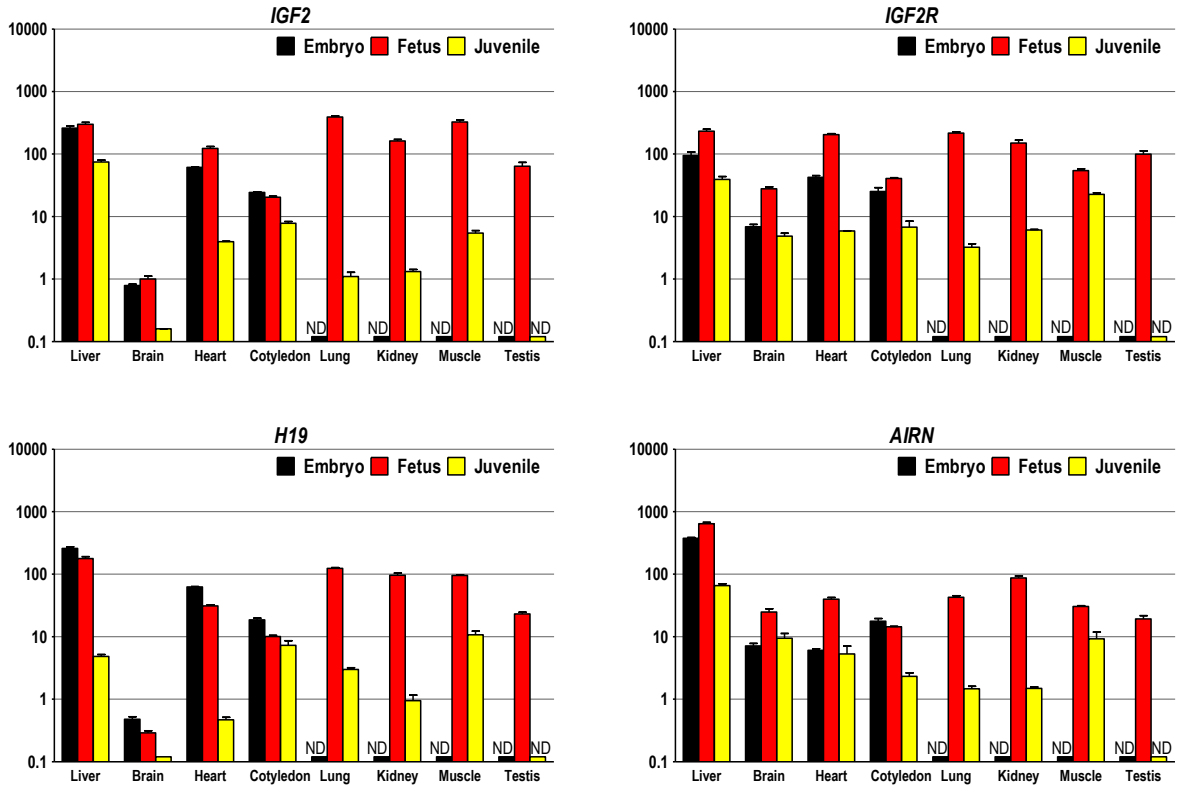

Figure 3.1 Means for transcript abundances of *IGF2*, *IGF2R*, *H19* and *AIRN* in tissues of three developmental stages, including embryo, fetus and juvenile.
Standard errors of means for each tissue were calculated on three technical replicates of pooled cDNA made from 60 embryonic cDNA samples, 100 fetal cDNA samples and 23 juvenile cDNA samples. Cotyledon was taken from Day-48 embryo, Day-153 fetus and Day-278 calves. Transcript abundance was calculated by the standard curve method and expressed in arbitrary logarithmic units (ND: not determined).

3.3.3 *H19*

The abundance of *H19* transcript was highest in liver and lowest in brain, with more than 500-fold difference during prenatal development (Figure 3.1). In the current study, skeletal muscle displayed the highest expression of *H19* at the postnatal developmental stage compared with other tissues.

In all examined tissues, expression of *H19* declined with advancing developmental age. The relative expression of *H19* in cotyledon from C-section delivered calves was 2.5-fold lower compared to cotyledons sampled at Day-48 gestation. Transcript abundance of *H19*

decreases in all tissues after birth with highest and lowest postnatal fold change occurring in kidney (100-fold decrease) and skeletal muscle (9-fold decrease), respectively.

3.3.4 *AIRN*

We showed expression of *AIRN* in all tissues of each developmental stage (Figure 3.1). Liver displayed the highest mRNA abundance of *AIRN* at all developmental stages, which in embryos was at least 21-fold higher than other tissues. The transcript abundance of *AIRN* in liver of fetuses and juveniles was more than seven times higher than in other tissues at these developmental stages.

Expression of *AIRN* was highest in tissues obtained at midgestation compared with the other developmental stages. Expression of *AIRN* increased from Day-48 to Day-153 in liver, brain and heart by 2, 3 and 7-fold, respectively. The level of *AIRN* mRNA in cotyledon was relatively constant across embryonic and fetal stages but decreased by 6-fold in late gestation. *AIRN* expression declined postnatally, with the postnatal fold decrease being highest in kidney (58-fold) and lowest in brain (3-fold).

3.3.5 *IGF2* P0

By performing a sequence similarity search, we identified a region upstream of bovine *IGF2* exon 2 that corresponded to the human P0 promoter with the upstream sequence highly conserved in bovine (data not shown). Therefore, we hypothesised the existence of a putative orthologous promoter in bovine and were able to amplify the corresponding transcript by specifically designed primers. We demonstrated P0-specific expression in skeletal muscle and testis during fetal development (Figure 3.2), with 30-fold lower expression in testis compared to muscle. We also demonstrated for the first time, a developmental shift in tissue specificity of *IGF2* P0 activity. During the postnatal period, the promoter is turned off in bovine skeletal muscle and becomes active specifically in liver. The transcript is expressed in postnatal liver at levels resembling those observed in fetal muscle.

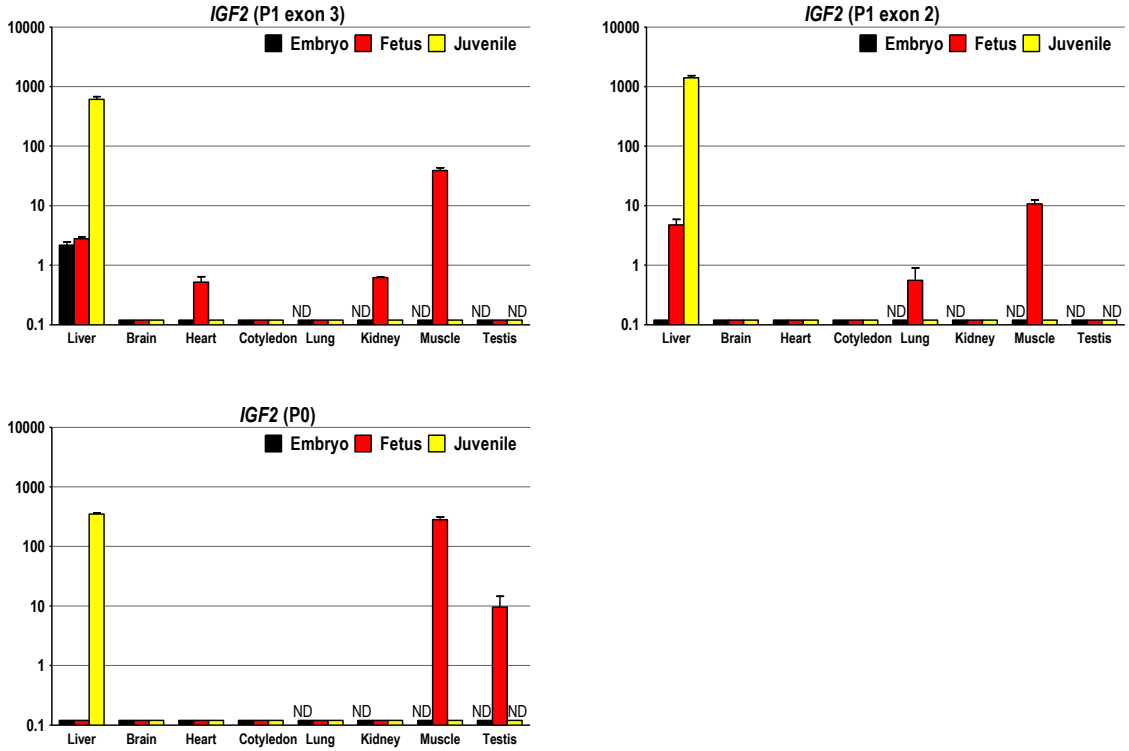

Figure 3.2 Means for transcript abundances of transcript abundances of *IGF2* promoters, P0 and P1, and splice variants, P1 exon 2 and P2 exon 3, in tissues of three developmental stages, including embryo, fetus and juvenile.
Standard errors of means for each tissue were calculated on three technical replicates of pooled cDNA made from 60 embryonic cDNA samples, 100 fetal cDNA samples and 23 juvenile cDNA samples. Cotyledon was taken from Day-48 embryo, Day-153 fetus and Day-278 calves. Transcript abundance was calculated by the standard curve method and expressed in arbitrary logarithmic units (ND: not determined).

3.3.6 *IGF2* P1

We used two pairs of primers located in exons 3 and 8 (*P1e3*) and also exon 2 (*P1e2*) of the bovine IGF2 gene as specific amplification of P1 transcript failed using primers located in exon 1. As exons 2 and 3 are found in P0 and P1 transcripts, the *P1e3* and *P1e2* amplicons could potentially belong to both P0 and P1 transcripts depending on tissue and developmental stage. In the fetus, expression level for *IGF2* transcripts containing exon 3 (*P1e3*) and exon 2 (*P1e2*) was highest in skeletal muscle (Figure 3.2). Both *P1e3* and *P1e2* are expressed in fetal liver, while only *P1e3* transcript is present in embryonic liver. Expression of P1 transcripts amplified by *P1e3* and *P1e2* primers is substantially elevated in postnatal liver, which was more than 200 times the transcript abundance in fetal liver.

3.3.7 *IGF2* P2

Two splice variants are derived from P2 promoters which comprise exon 4 (*IGF2* P2e4) and exons 4 and 5 (*IGF2* P2e5) as well as protein coding exons of 8, 9 and 10. The transcript that includes exons 4 and 5 was amplified by placing forward and reverse primers within exons 5 and 8, respectively. The forward primer for specific amplification of the splice variant that includes only exon 4 was located at the junction of exons 4 and 8 with the reverse primer placed within exon 8.

The *IGF2* P2e5 is expressed in liver, cotyledon and heart at Day-48 of gestation but was not detected in embryonic brain (Figure 3.3). The transcript was also expressed in all fetal tissues studied, with lowest expression in brain. Highest expression of the *IGF2* P2e5 transcript was in liver, with expression in embryos, fetuses and juveniles being at least 13, 3 and 8-fold higher than in other tissues, respectively. Decreased activity of P2 promoter was observed after birth in all studied tissues with the highest fold decrease in lung (177-fold), muscle (34-fold) and liver (11-fold). No expression was detected in juvenile brain, heart and kidney.

Transcript abundance of *IGF2* P2e4 in liver of embryos, fetuses and juveniles was at least 75, 10 and 5-fold higher than other tissues, respectively. The transcript is completely absent in brain, is repressed in postnatal heart, lung and kidney, and is dramatically downregulated in postnatal liver (70-fold decrease) and muscle (32-fold decrease).

3.3.8 *IGF2* P3

Expression of *IGF2* P3 transcripts was present in all embryonic tissues studied, with highest expression in liver and heart and lowest expression in brain (Figure 3.3). In the fetus, P3 transcript was also expressed in all studied tissues, with the highest activity in lung and lowest in brain. Expression of P3 transcripts in cotyledon displayed a progressive decline with gestational age, with transcript abundance in embryos being 3 and 76-fold higher than in fetuses and at term, respectively. Postnatally, P3 transcripts were detected only in muscle,

heart and liver, with abundance declining sharply by 225, 140 and 90-fold, respectively. Expression of P3 transcript in liver, heart and cotyledon was highest in the embryo compared with other developmental stages.

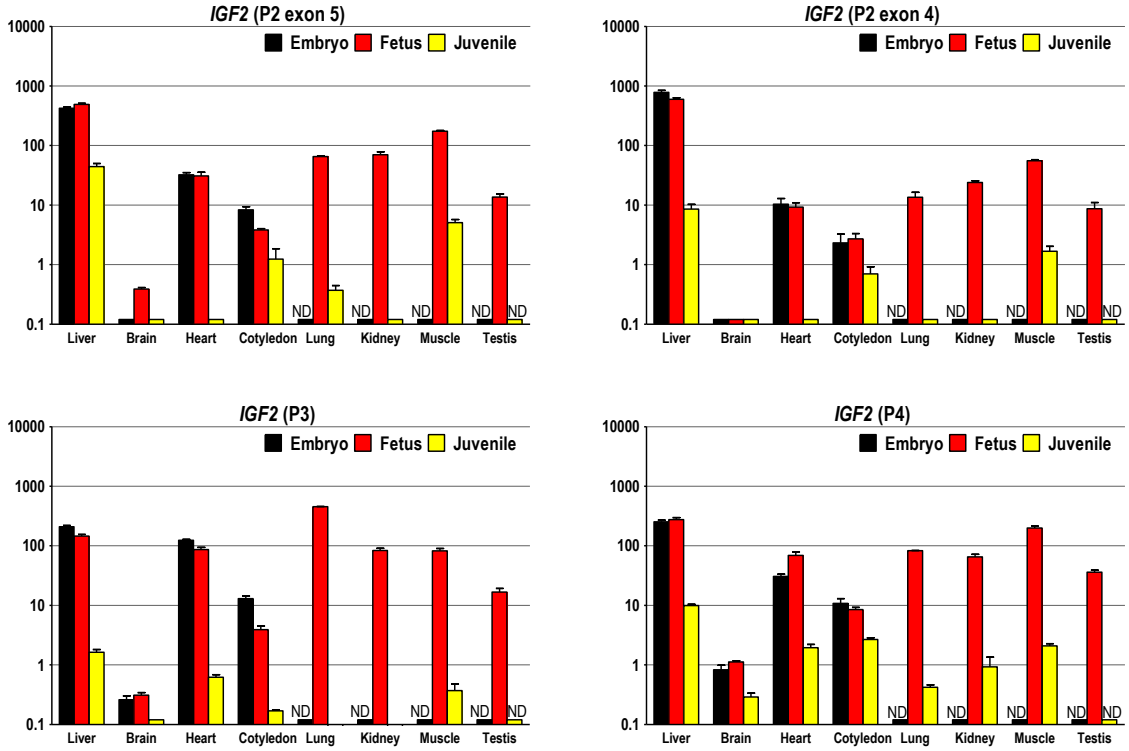

Figure 3.3 Means for transcript abundances of *IGF2* promoters, P2, P3 and P4, and splice variants, P2 exon 4 and P2 exon 5, in tissues of three developmental stages, including embryo, fetus and juvenile.
Standard errors of means for each tissue were calculated on three technical replicates of pooled cDNA made from 60 embryonic cDNA samples, 100 fetal cDNA samples and 23 juvenile cDNA samples. Cotyledon was taken from Day-48 embryo, Day-153 fetus and Day-278 calves. Transcript abundance was calculated by the standard curve method and expressed in arbitrary logarithmic units (ND: not determined).

3.3.9 *IGF2* P4

The transcript derived from P4 promoter was expressed in all studied tissues of all three developmental stages (Figure 3.3). In the fetus, expression of P4 transcript was highest in liver and skeletal muscle, and lowest in brain. In embryonic tissues, P4 transcript abundance in liver was 8, 23 and 300-fold higher than heart, cotyledon and brain, respectively, and in juvenile, expression level in liver was at least 4-fold higher than in other tissues, and lowest expression was observed in brain. Expression from P4 was highest in fetal tissues compared with embryonic tissues, except cotyledon, and decreased dramatically in postnatal tissues. The

fold decrease was highest in lung (195-fold), muscle (95-fold) and kidney (70-fold). The developmental expression pattern of P4 in cotyledon was similar to that of global *IGF2* transcripts.

3.3.10 Expression ratio of *IGF2/H19*, *IGF2R/AIRN* and *IGF2/IGF2R*

Since imprinted expression of *IGF2* and *IGF2R* is known to be controlled by *H19* and *AIRN*, we examined relative transcript abundance of *IGF2* to *H19* and *IGF2R* to *AIRN* and showed that these parameters change in a manner depending on the tissue and developmental stage (Figure 3.4).

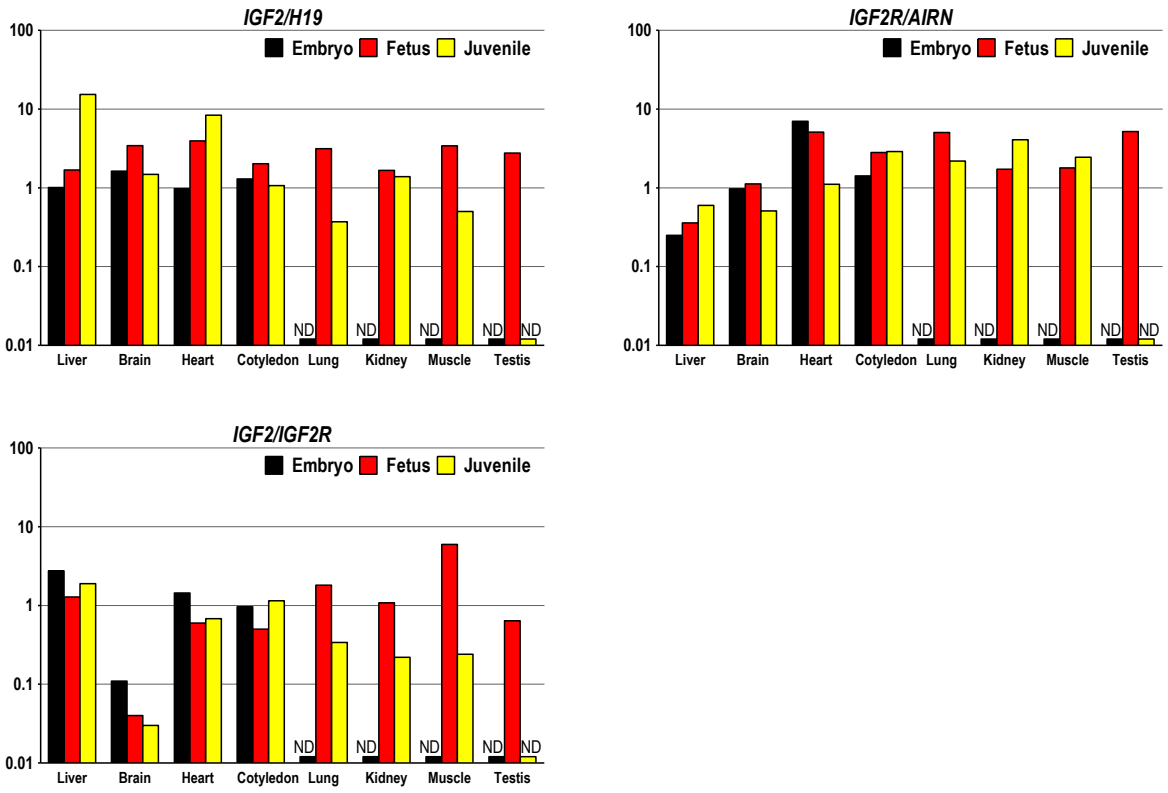

Figure 3.4 Means for ratio of *IGF2/H19*, *IGF2R/AIRN* and *IGF2/IGF2R* transcript abundance in tissues of three developmental stages, including embryo, fetus and juvenile.
Cotyledon was taken from Day-48 embryo, Day-153 fetus and Day-278 calves. Transcript abundance was calculated by the standard curve method and expressed in arbitrary logarithmic units (ND: not determined).

Relative expression of *IGF2* to *H19* was higher in tissues of Day-153 compared with Day-48 of gestation. The *IGF2/H19* ratio was highest in postnatal liver and heart but declined

in brain, lung and skeletal muscle of juveniles. Liver and brain displayed the lowest ratio of *IGF2R/AIRN* at all developmental stages. The relative transcript abundance of *IGF2R* to *AIRN* was highest in embryonic heart. The ratio of *IGF2/IGF2R* was lowest in brain at all developmental stages and was higher in embryos compared with fetuses. The *IGF2/IGF2R* ratio was highest in fetal skeletal muscle and substantially declined in postnatal lung, kidney and skeletal muscle, whereas it did not change markedly in liver and heart of the juvenile compared with the fetus.

3.4 Discussion

In the present study, the use of pooled cDNA revealed drastic differences in transcript abundances between tissues and developmental stages. The magnitude of differences in transcript abundances between genetic groups and sexes, analysed by individual cDNAs, was much smaller (see chapter 4) compared with the differences in expression levels between tissues and developmental stages, analysed by pooled cDNA.

The five studied *IGF2* promoters showed differential expression patterns depending on tissue and developmental stage. Global *IGF2* expression in bovine fetal tissues was consistent with relative abundance of *IGF2* in rat fetal tissues (Brown *et al.*, 1986). Overall, expression of total *IGF2* transcripts in juvenile liver and other tissues decreased compared with those in prenatal developmental stages. A postnatal decrease in *IGF2* expression level has been previously reported in tissues of bovine (Boulle *et al.*, 1993), ovine and human (Schofield and Tate, 1987; O'Mahoney *et al.*, 1991; Delhanty and Han, 1993b), and rat (Brown *et al.*, 1986; Soares *et al.*, 1986; Gray *et al.*, 1987).

In cotyledon, abundance of *IGF2* transcript was relatively constant across embryonic and fetal developmental stages, followed by a slight decrease at term. *IGF2* expression level in placenta was higher at term compared with midgestation in human (Gray *et al.*, 1987), whereas did not change after midgestation in ovine (O'Mahoney *et al.*, 1991; Reynolds *et al.*, 1997) and rhesus monkey (Coulter and Han, 1996).

Specific expression of *IGF2* P0 in bovine fetal skeletal muscle is in agreement with results obtained in human (Monk *et al.*, 2006), whereas specific expression of P0 in fetal testis has not been previously reported in bovine or any other species. Whether expression of P0 in testis is developmentally controlled or whether it plays a role in testis development and function remains to be elucidated. In contrast to specific expression of P0 in bovine postnatal liver, ubiquitous expression of P0 in adult human (20-46 years old) tissues has been observed (Monk *et al.*, 2006). The discrepancy between the postnatal expression pattern of *IGF2* P0 in

human and bovine may relate to the different developmental ages, with bovine offspring studied as juveniles.

Since transcripts derived from P0 are expressed in fetal muscle, and exons 2 and 3 are present in both P0 and P1 derived transcripts, the *P1e3* and *P1e2* amplicons detected in skeletal muscle could be derived from both P0 and P1 promoters. The amplicons containing exons 2 and 3 in fetal liver most likely belong to P1 splice variants, as P0 is not active in prenatal liver. These results demonstrate that the P1 promoter is active throughout the prenatal stage in liver and is upregulated postnatally, which is in agreement with the observations in porcine (Amarger *et al.*, 2002). A postnatal developmental switch in *IGF2* promoter usage has been reported in human (Depagterholthuizen *et al.*, 1987; Monk *et al.*, 2006), porcine (Amarger *et al.*, 2002), ovine (O'Mahoney *et al.*, 1991; Ohlsen *et al.*, 1994) and bovine (Boulle *et al.*, 1993), when P2-4 transcription drops sharply and P1 transcription becomes predominant in liver. Our results provide further evidence for a developmental shift in promoter usage in the *IGF2* gene in bovine.

The two splice variants derived from the *IGF2* P2 promoter showed similar patterns of expression, with the exception that relative transcript abundance in liver compared with other tissues was higher for P2e4 compared with P2e5. Furthermore, they exhibited different expression patterns in embryonic and fetal cotyledon. Both P2 derived splice variants were previously detected in tissues of bovine and porcine fetuses (Amarger *et al.*, 2002; Curchoe *et al.*, 2005). The transcript that includes exons 4 and 5 has also been identified in ovine fetal and adult liver (Ohlsen *et al.*, 1994) and in human liver and placenta (Ikejiri *et al.*, 1991; Mineo *et al.*, 2000). Expression of P2 derived splice variants in postnatal tissues has been analysed in bovine (Curchoe *et al.*, 2005; Goodall and Schmutz, 2007), porcine (Amarger *et al.*, 2002), human (Li *et al.*, 1996) and ovine (Ohlsen *et al.*, 1994).

The high expression level of total *IGF2* transcripts in fetal lung was consistent with highest transcript abundance of *IGF2* P3 in fetal lung, suggesting that P3 is the most active promoter in fetal lung. Expression of *IGF2* P3 transcripts was reported in a range of tissues

from bovine, porcine and human fetal and postnatal tissues (Li *et al.*, 1996; Amarger *et al.*, 2002; Curchoe *et al.*, 2005). Results of tissue-specific expression of *IGF2* P3 in postnatal tissues were not consistent in different studies (Curchoe *et al.*, 2005; Goodall and Schmutz, 2007). Amplification of low abundant transcripts in postnatal tissues is highly variable depending on the experimental conditions and may lead to inconsistent results in different studies. Transcript abundance of *IGF2* P3 was highest in embryonic tissues, except brain, compared with other studied developmental stages, whereas expression of the *IGF2* P2 and P4 promoters was highest in fetal tissues. P3 was shown to be the most active promoter in tissues during human prenatal development (Holthuizen *et al.*, 1993; Ohlsson *et al.*, 1994).

In agreement with our results, a postnatal decrease in *IGF2* P4 transcript was reported in human liver (Li *et al.*, 1996). The transcript was shown to be expressed in bovine and porcine fetal tissues (Amarger *et al.*, 2002; Curchoe *et al.*, 2005) but was not found in juvenile bovine lung and heart (Goodall and Schmutz, 2007).

Consistent with the results presented here, a high level of *IGF2R* expression in fetal heart has been shown in rat (Sklar *et al.*, 1989; Ballesteros *et al.*, 1990; Senior *et al.*, 1990; Sklar *et al.*, 1992), human (Funk *et al.*, 1992) and bovine fetuses (Pfuender *et al.*, 1995), whereas in another study, expression of *IGF2R* in fetal heart was lower than other tissues (Suteevun-Phermthai *et al.*, 2009). We demonstrated that *IGF2R* expression changes across pre and postnatal developmental stages. Developmental regulation of *IGF2R* has been shown in rat with highest expression in fetal tissues (Sklar *et al.*, 1989; Ballesteros *et al.*, 1990; Senior *et al.*, 1990; Sklar *et al.*, 1992; Moats-Staats *et al.*, 1995).

In the bovine fetus, liver exhibited a higher level of *H19* expression compared with other tissues. Studies on tissue-specific expression of *H19* during bovine development are limited. In bovine fetuses, the highest expression of *H19* was detected in amnion, chorion, and allantois, and the lowest expression was in brain (Khatib and Schutzkus, 2006). In the current study, transcript abundance of *H19* was highest in skeletal muscle of juveniles compared with other postnatal tissues, which agrees with previous data from bovine (Khatib and Schutzkus,

2006) and mouse (Gabory *et al.*, 2009). These observations suggest that *H19* may be important in postnatal growth of skeletal muscle. *H19* expression in cotyledon decreased from Day-48 to Day-153 of gestation and did not change significantly until term. *H19* is expressed at a constant level throughout prenatal development in human placenta (Goshen *et al.*, 1993). Expression of the H19 gene is dramatically downregulated in the investigated postnatal tissues and is fully repressed in brain, which is consistent with the observation in human adult brain (Pham *et al.*, 1998). A postnatal decrease in *H19* expression has previously been reported in bovine (Khatib and Schutzkus, 2006) ovine (Lee *et al.*, 2002) and mouse (Pachnis *et al.*, 1984; Poirier *et al.*, 1991) tissues. The higher expression level of *H19* in embryonic tissues, compared with fetal tissues, implies that *H19* may be more important in regulation of earlier stages of prenatal development.

We demonstrated for the first time that expression of *AIRN* is developmentally controlled and is highest in fetal tissues. Transcripts of *AIRN* were previously detected in brain, cotyledon, kidney, liver and lung of bovine Day-75 fetuses (Suteevun-Phermthai *et al.*, 2009), and fetal liver at Day-35-55 and 70 of gestation (Farmer *et al.*, 2013). Higher relative transcript abundance of *IGF2R* and *AIRN* in brain, compared with the relative expression level of *IGF2* and *H19* in brain, suggests that the *IGF2R/AIRN* system may play an important role in brain development and/or function.

We demonstrated that relative expression of *IGF2* to *H19*, *IGF2R* to *AIRN* and *IGF2* to *IGF2R* is controlled in a tissue and developmental stage-specific manner. Highest relative transcript abundance of *IGF2* to *H19* in postnatal liver is attributed to the upregulation of the *IGF2* P1 promoter transcript, which is biallelically transcribed with dissociated expression from *H19* (Vu and Hoffman, 1994; Ekstrom *et al.*, 1995; McLaren and Montgomery, 1999; Goodall and Schmutz, 2007). The highest expression ratio of *IGF2R/AIRN* in embryonic heart suggests that specific co-regulation of *IGF2R* and *AIRN* expression in heart may be important at the embryonic stage. The importance of IGF2R in heart development was shown in *IGF2R*-deficient mice which showed abnormal heart growth and perinatal death, due to heart failure

secondary to cardiac hyperplasia (Lau *et al.*, 1994; Wang *et al.*, 1994; Ludwig *et al.*, 1996). Relative expression of *IGF2* to *IGF2R*, which is interpreted as an indicator of free IGF2 available to the tissues, was highest in fetal skeletal muscle, suggesting that IGF2 is important in prenatal muscle development. This is supported by several lines of evidence that show importance of IGF2 in skeletal muscle differentiation and development (Florini *et al.*, 1996; Stewart and Rotwein, 1996; Coolican *et al.*, 1997; Morali *et al.*, 2000; Prelle *et al.*, 2000; Wilson *et al.*, 2003; Wilson and Rotwein, 2006; Alzhanov *et al.*, 2010).

In conclusion, our findings provide further evidence that developmentally important imprinted genes are subject to significant spatial and temporal regulation of gene expression. We used quantitative real time PCR to examine expression patterns of the imprinted genes across bovine development. Most studies in bovine and other species have exploited northern blot or semi-quantitative RT-PCR techniques for studying expression patterns of imprinted genes which may produce conflicting results depending on experimental conditions. The results of the present study enhance our understanding of how expression patterns of functionally linked imprinted genes and their regulatory non-coding RNAs change during pre- and postnatal development. Further studies are required to elucidate the imprinting status of the studied genes at different developmental stages and association of alterations in imprinting with changes in overall expression of the genes and phenotype during pre and postnatal development.

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Chapter 4

Genetic effects on transcript abundances of the imprinted IGF2, IGF2R, H19 and AIRN genes in fetal tissues and their associations with growth-related phenotypes

4.1 Introduction

Fetal growth is a complex process governed by the interplay of genetic, epigenetic and environmental factors. Any changes in environment, including maternal factors and fetal nutrition, result in developmental adaptations which have a permanent impact on postnatal growth and development, a process termed "fetal programming" (Barker and Clark, 1997). Although the concept of fetal programming has the most implications in the context of human disease there is growing evidence that animal postnatal performance has its origin in prenatal development and is affected by maternal environmental conditions (Young *et al.*, 1998; Bell, 2006; Foxcroft *et al.*, 2006; Wu *et al.*, 2006; Gardner *et al.*, 2007; Funston *et al.*, 2010; Hill *et al.*, 2010). Changes in genetic and epigenetic mechanisms and effects as caused by intra-species hybridisation are likely to be significant contributors to postnatal phenotype, including heterosis.

Heterosis is defined as superiority of hybrids in a trait compared to the average of their parental purebreds (Veitia and Vaiman, 2011). A number of agricultural systems, including the beef industry, benefit from heterosis. There are two subspecies of domesticated cattle, *Bos taurus* and *Bos indicus* (Hiendleder *et al.*, 2008; Elsik *et al.*, 2009), which have been widely used in crossbreeding programs to improve performance via heterosis (Franke, 1980; Koger, 1980; Huffman *et al.*, 1990; Johnson *et al.*, 1990; Brown *et al.*, 1993).

Classical genetic models, including dominance (Davenport, 1908; Bruce, 1910; Keeble, 1910; Jones, 1917), overdominance (Shull, 1908; Crow, 1948) and more recently epistasis (Powers, 1944; Hochholdinger and Hoecker, 2007), have been proposed to explain heterosis, but molecular mechanisms underlying this phenomenon remain enigmatic and the role of the genes conferring non-Mendelian (epi)genetic effects is largely unexplored.

Several lines of evidence suggested epigenetic parent-of-origin effects as part of molecular mechanisms that explain phenotypic variation of complex traits and heterosis (Cubas *et al.*, 1999; Manning *et al.*, 2006; Shindo *et al.*, 2006; Ni *et al.*, 2009; He *et al.*, 2010; Groszmann *et al.*, 2011). Alteration in gene expression as a result of changes in methylation

of differentially methylated regions may play a role in heterosis (Shen *et al.*, 2012). Imprinted genes, whose expression is regulated by epigenetic mechanisms in a parent-of-origin dependent manner (Reik and Walter, 2001), significantly contribute to phenotypic variation of complex traits (de Koning *et al.*, 2000; Mantey *et al.*, 2005; Wolf *et al.*, 2008b) and are likely to be candidate genes underlying heterosis and differences between reciprocal hybrids. However, the significance of parent-of-origin dependent gene expression associated with genomic imprinting in heterosis has not been studied. Genomic imprinting can mimic the effects that arise from hybrid vigor (Chakraborty, 1989). Loss of imprinting of imprinted genes under genetic influence has been shown in mouse interspecific hybrids of *Mus musculus domesticus*/*Mus musculus castaneus* and *Peromyscus polionotus*/*Peromyscus maniculatus* (Jiang *et al.*, 1998; Vrana *et al.*, 1998). Parent-dependent loss of gene silencing has also been documented in Arabidopsis interspecies hybrids (Josefsson *et al.*, 2006).

It is evident that the imprinted IGF2, IGF2R and their regulatory noncoding genes have an indispensable function in prenatal development and are subject to epigenetic regulation in response to changes in prenatal environment, suggesting involvement in fetal programming (Dean *et al.*, 1998; Sinclair *et al.*, 2000; Khosla *et al.*, 2001; Young *et al.*, 2001; Holt, 2002; Igwebuike, 2010; Micke *et al.*, 2011b; Micke *et al.*, 2011a). However, genetic effects on tissue-specific expression patterns of imprinted genes and their contribution to prenatal phenotypic variation and programming of postnatal performance and heterosis are largely unexplored.

We hypothesised a major role for *IGF2/H19* and *IGF2R/AIRN* in prenatal programming of heterosis. The objective of this study was to explore non-additive expression of the imprinted genes in tissues of hybrid fetuses and its link to heterosis in phenotype. Therefore, we sought to examine genetic and heterotic effects on tissue-specific expression patterns of these imprinted genes at Day-153 of gestation in a bovine model of two genetically and phenotypically distinct subspecies, *Bos taurus* (Angus, A), *Bos indicus* (Brahman, B) and their reciprocal cross hybrids (BA and AB, sire indicated first).

4.2 Materials and methods

4.2.1 Animal model, fetuses and tissues

All animal experiments and procedures described in this study were approved by the University of Adelaide Animal Ethics Committee (No. S-094-2005 and S-094-2005A). Angus (A) and Brahman (B) breeds were used to study differential expression of imprinted genes at midgestation. These two breeds represent two subspecies of domesticated cattle, *Bos taurus* (Bt) and *Bos indicus* (Bi) (Elsik *et al.*, 2009), which have also been classified as *Bos primigenius taurus* and *Bos p. indicus* based on ancestral wild subspecies (Hiendleder *et al.*, 2008). Forty-five Angus and 28 Brahman females which had not given birth previously and were approximately 16–20 months of age received standard commercial estrous cycle synchronization (e.g. http://www.absglobal.com/aus/resources/beef-resources/synchronization-programs---cue-mate-1/). Cidirol - Heat Detection & Timed Insemination (HTI) was used. Briefly this consisted of an initial injection of 1 ml of 1 mg/ml estradiol benzoate (Cidirol, Genetics Australia Co-operative Ltd., Bacchus Marsh, Australia) and insertion of a progesterone-releasing vaginal insert (Eazi-Breed CIDR, DEC International, Hamilton, New Zealand). The vaginal inserts were removed after 7–9 days and heifers injected with 2 ml of a prostaglandin analogue (0.26 mg of cloprostenol sodium/ml (Estrumate), Schering-Plough Animal Health, Baulkam Hills, Australia). Estrus detection devices (Kamar, Agrigene, Wangaratta, Australia) were placed on all animals. Animals that showed estrus two days later were inseminated, while animals not in estrus received an additional 0.5 ml injection of estradiol benzoate and were inseminated 24 h later. Synchronization/insemination was repeated with estradiol benzoate injection of all animals after removal of vaginal inserts, followed by a final round of insemination and natural breeding without further synchronization measures. Angus (n = 3) and Brahman (n = 2) paternal genetics of average breeding value for birth weight were used to generate fetuses. Animals were pregnancy tested by ultrasound scanning (Anand-Ivell *et al.*, 2011; Xiang *et al.*, 2013).

Fetuses were sired by three Angus and two Brahman bulls. Dams were pregnancy tested by ultrasound scanning, and fetuses were recovered at Day-153 ± 1 in a commercial abattoir. Fetuses were removed from the uterus, eviscerated, vacuum packed and stored frozen at -20°C until further processing. A total of 73 fetuses, including 23 Bt×Bt (11 males and 12 females), 22 Bi×Bt (5 males and 17 females), 13 Bt×Bi (7 males and 6 females) and 15 Bi×Bi (4 males and 11 females) genetics (sire breed indicated first), were used in this study. Therefore, 45 and 28 fetuses were representative of Bt and Bi maternal genetics, respectively, and 36 and 37 fetuses were representative of Bt and Bi paternal genetics, respectively (Appendix 7).

Fetal phenotype, including fetal weight and weights of individual fetal tissues (liver, heart, brain, kidney, lung), was recorded immediately after collection and fetal placenta weight was recorded as weight of the fetal membranes. *Musculus supraspinatus*, *M. longissimus dorsi*, *M. quadriceps femoris* (consisting of *M. rectus femoris, M. vastus medialis*, *M. vastus intermedius* and *M. vastus lateralis*) and *M. semimembranosus* were dissected from both sides of the fetus. *M. longissimus dorsi* was defined from the 7th rib to the natural caudal end of the muscle, at the apophysis of the lumbosacral. Dissected muscles from both sides of the fetus were weighed, and absolute muscle weight was recorded as the mean weight for each muscle. Combined muscle weights were calculated as the sum of mean weight of each dissected muscle. Relative muscle weights, reflecting fetal muscle proportions, were calculated as muscle weight divided by the fetal body weight. Samples of fetal tissues, including brain, cotyledon (*placenta fetalis*), heart, kidney, liver, lung and skeletal muscle (*M. semitendinosus*), were collected in RNA-later® (Qiagen, Chadstone Centre, VIC, Australia) immediately after recovery of fetuses in the abattoir and stored at -80°C after equilibration in RNA-later® for 24 hours at 2–4°C. All fetal samples and phenotypes were provided by Professor Stefan Hiendleder.

4.2.2 RNA extraction and cDNA synthesis

Tissue specimens (40-50 mg of each tissue) were homogenised by PRECELLYS®24 lyser / homogeniser (Bertin Technologies, Saint Quentin en Yvelines Cedex, France). Homogenisation of samples was carried out in 1 ml of TRIzol reagent (Ambion, Life Technologies™, Inc., Carlsbad, CA, USA) in tubes containing 1.4 mm ceramic beads (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) (for lung, liver, kidney and brain) or 2.8 mm ceramic beads (for heart and cotyledon). Homogenisation runs were performed at the speed of 6500 rpm in one (brain, kidney and lung) or two (liver, heart and cotyledon) cycles with cycle duration of 7 seconds (brain), 10 seconds (heart and cotyledon), 12 seconds (kidney and lung) and 15 seconds (liver). In cases where tissue samples underwent two cycles of homogenisation, the waiting time between two cycles was 10 seconds (heart and cotyledon) or 12 seconds (liver). Total RNA was extracted from fetal tissues using TRI Reagent® (Ambion, Life Technologies™, Inc., Carlsbad, CA, USA). After removing insoluble materials from the homogenate by centrifugation at 12000 g for 10 minutes at 2 to 8 °C and incubation of the supernatant on ice for 5 minutes, 0.2 ml of chloroform was added, followed by incubation on ice for 3 minutes and centrifugation for 20 minutes at 12000 g (4°C). The aqueous phase was separated and mixed with 0.6ml of isopropyl alcohol and incubated for overnight at -20° C, followed by centrifugation at 13000 g (4°C). After removing the supernatant, the pellet was washed by adding 1.2 ml of 75% ethanol and re-pelleted by centrifugation at 12000 g for 5 minutes (4°C). After removing the supernatant, the RNA pellet was dissolved in 50-100 µl of nuclease-free water (GIBCO UltraPure™ Distilled Water, Invitrogen™, Inc., Auckland, NZ). RNA quality and integrity was assessed by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Inc., Wilmington, DE, USA) and Agilent RNA 6000 Nano Kit with Bioanalyzer 2100 (Agilent Technology, Inc., Santa Clara, CA, USA) and RIN (RNA Integrity Number) values averaged 8.21 for skeletal muscle, 8.85 for lung, 7.03 for kidney, 8.45 for heart, 8.05 for liver, 8.38 for brain and 7.16 for cotyledon. RNA samples were provided by Ali Javadmanesh.

Prior to RT-PCR, the extracted RNAs were treated by DNase (RQ1-DNase, Promega, Inc., Madison, WI, USA). Reactions were performed in a total volume of 10 μl, containing RNase-Free DNase 10X reaction buffer (1 μl), RNase-Free DNase (1 U/μg RNA), RNA solution and nuclease-free water incubated at 37°C for 30 minutes. 1 μl of DNase stop solution was added to the mixture, followed by incubation at 65 °C for 10 minutes.

Five hundred ng of total RNA was reverse transcribed to cDNA using SuperScript™ III First-Strand Synthesis System (Invitrogen, Life Technologies™, Inc., Carlsbad, CA, USA) and random hexamer oligonucleotides according to the manufacturer's manual. Briefly, 10 μl of a mixture containing total RNA, 1 μl of 50 ng/μl random hexamers, 1 μl of 10 mM dNTP mix and nuclease-free water was incubated at 65 °C for 5 minutes and placed on ice for 1 minute. The mixture was mixed with 10 μl of a cDNA synthesis mix containing 2 μl of 10X RT buffer, 4 μl of 25 mM MgCl2, 2 μl of 0.1 M DTT, 1 μl of 40 U/μl RNaseOUT^{TM} and 1 μl of 200 U/μl SuperScript^{TM} III RT and incubated at 25 °C for 10 minutes, followed by 50 °C for 50 minutes. The reaction was terminated at 85 °C for 5 minutes and then chilled on ice. The mixture was incubated at 37 °C for 20 minutes after adding 1 μl of RNase H. For each tissue, equal quantities of all individual cDNAs were then combined to generate a pooled representative cDNA sample.

4.2.3 Quantitative real time RT-PCR

Expression of *IGF2R*, *AIRN*, *IGF2*, *H19* as well as *IGF2* promoter-specific transcripts (P0, P1, P2, P3, P4) and two splice variants derived from *IGF2* P2 promoter (P2e4 and P2e5) were analysed in fetal tissues by quantitative real time PCR using gene-specific primers. Sequences, locations and annealing temperatures of the primers are listed in Appendix 2. Schematic exon/intron structure of the bovine IGF2 gene and locations of the primers designed for amplification of the *IGF2* promoter-specific transcripts and splice variants are shown in Figure S1 (Appendix 8). We amplified two splice variants of P2 transcripts. The

splice variant containing leader exon 4 was amplified by forward primer located at the junction of exons 4 and 8 and reverse primer within exon 8. The other splice variant with leader exons 4 and 5 was amplified by forward and reverse primers placed in exons 5 and 8, respectively. Forward and reverse primers for amplification of P3 transcript were placed in exons 6 and 8, and for amplification of P4 transcript were located in exons 7 and 8, respectively. *IGF2* global transcripts were amplified by placing primers in exons 8 and 9 that are found in all known *IGF2* transcripts. We designed most primers spanning an intron to avoid amplification of genomic DNA.

Quantitative real time PCR reactions were performed using Fast Start Universal SYBR Green Master (Roche Diagnostics GmbH, Mannheim, Germany) in an Eppendorf Mastercycler® pro S thermal cycler (Eppendorf, Inc., Hamburg, Germany) using 4µl of cDNA (40-time diluted from stock cDNA, equivalent to 12.5 ng of starting RNA), 0.8 µl of primers (5 pmol/µl) and 1.2 µl of double distilled nuclease-free water (ddH_2O) in a final volume of 12 µl. A non-template control was included in all experiments to check for DNA contamination of reagents used for amplification. Thermocycling reactions were carried out with a 10-minute initial denaturation/activation step at 95°C, followed by 40 cycles of 95°C for 20 seconds (denaturation), 57-62°C for 30 seconds (annealing, depending on the primer pair) and 72°C for 20 seconds (extension). Product specificity and integrity was confirmed via sequencing on a 3730*xl* DNA Analyzer (Applied Biosystems, Inc., Foster City, CA, USA), plots of melting curve derived by Mastercycler® ep Realplex software (Eppendorf, Inc., Hamburg, Germany), and electrophoresis on a 2% agarose gel (Agarose low EEO, AppliChem GmbH, Darmstadt, Germany) stained with GelRed™ Nucleic Acid Stain (Biotium, Inc., Hayward, CA, USA) and visualized under UV with Gel Doc™ 1000 Single Wavelength Mini-Transilluminator using Quantity One image analysing software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each qPCR experiment was performed in duplicate and the mean of both CTs used to calculate the amount of target transcript.

An equal proportion of cDNA from all fetuses in each tissue was pooled to generate a cDNA template for standard curve analysis. The standard curve included a 2-fold serial dilution of initial pooled cDNA template over eight data points. Three replicates were used for each dilution of the cDNA template. The CT (threshold cycle) values of the standards were used to derive a standard curve which shows the CT values as a linear function of natural logarithm of the specified arbitrary amounts of cDNA. The relative abundance of each target transcript was calculated by the relative standard curve method with determination of PCR amplification efficiency using the following equation:

$$\mathrm{TB_{Sample}\ (arbitrary\ units)} = \exp\left(\frac{\mathrm{CT_{Sample}} - \mathrm{intercept}}{\mathrm{slope}}\right)$$

where, TB_{Sample} is relative transcript abundance of the gene of interest in each sample, "exp" indicates the exponential function, CT_{Sample} is threshold cycle for each sample, intercept is the point at which the standard curve intersects with the Y-axis, and slope is the increase in standard curve. PCR amplification efficiencies (E) were calculated by the following equation:

$$\mathrm{E} = 10^{\left(\frac{-1}{\mathrm{slope}}\right)} - 1$$

CT values and transformed quantities (relative transcript abundances, in terms of standard curve), as well as standard curve parameters, including amplification efficiency and coefficient of determination (R-squared), were automatically calculated by Mastercycler® ep Realplex software (Eppendorf, Inc., Hamburg, Germany). Amplification efficiency and coefficient of determination for transcripts of target and housekeeping genes are shown in Appendix 4 and 5.

We determined expression levels of seven putative housekeeping genes, including actin beta (*ACTB)*, ribosomal protein S9 (*RPS9*), ubiquitin B (*UBB*), H3 histone family 3A *(H3F3A)*, TATA box binding protein *(TBP)*, vacuolar protein sorting 4 homolog A (*VPS4A*) and cyclin G associated kinase (*GAK*) (Appendix 3), in each fetal tissue. In cotyledon, expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was determined instead of *H3F3A*. The geNorm program version 3.5 (Vandesompele *et al.*, 2002) was used to

identify *RPS9* and *VPS4A* in brain and cotyledon, and *GAK* and *VPS4A* in heart, kidney, liver, lung and skeletal muscle as the most stable genes for normalisation of the target genes. Expression levels of the genes in each tissue were normalised to the geometric mean of the expression levels of the selected housekeeping genes (TB_{HK}), referred to as "normalisation factor" (Vandesompele *et al.*, 2002):

$$\text{Normalised } TB_{Sample} = \frac{TB_{Sample}}{\text{Geometric mean}(TB_{HK1}, TB_{HK2})}$$

The qPCR reactions and data analysis for the housekeeping genes in fetal liver, brain, heart, muscle and cotyledon, and also data analysis for housekeeping genes in lung and kidney, were performed by Ali Javadmanesh.

4.2.4 Statistical analysis

The normalised expression data were checked for normality. Logarithmic transformation was performed on the data which were not normally distributed. When transformation was needed, the results for least square means and standard errors of means were presented after back-transformation. Gene expression data and fetal phenotype, including fetal weight and tissue weights, were analysed by Univariate Analysis of Variance (ANOVA) using the general linear model procedure of SPSS 19 (SPSS Inc, an IBM Company). Data were fitted into the following linear model to analyse the effects of fetal genetics and sex:

$y_{ijk} = G_i + S_j + e_{ijk}$

where y_{ijk} is the normalised relative gene expression level or fetal phenotype, G_i is fetal genetic effect (i = AA, BA, AB, BB), S_j is fetal sex effect (j = male, female) and e_{ijk} is the residual effect.

The following model was used to analyse heterotic effects on gene expression and fetal phenotype:

$y_{ijkl} = H_i + G_j(H_i) + S_k + e_{ijkl}$

where y_{ijkl} is the normalised relative gene expression level or fetal phenotype, H_i is heterotic effect (i = purebred, crossbred), $G_j(H_i)$ is fetal genetic effect nested within heterotic

effect (j = AA, BB nested within purebred, j = BA, AB nested within crossbred), S_k is fetal sex (k = male, female) and e_{ijkl} is the residual effect.

Coordinate expression of genes in each tissue was analysed using Pearson's correlation coefficients with the two-tailed tests of significance. Associations between fetal phenotypic traits and relative gene expression levels were analysed using simple linear regression models. Dependent and/or independent variables were subjected to logarithmic transformation in order to meet assumptions of regression analysis, including linearity, normality and homoscedasticity.

The following linear models were used to estimate relative contribution of each promoter-specific transcript (P0, P2, P3 and P4) to the total *IGF2* expression in each tissue:

IGF2 (skeletal muscle) = *IGF2* P0 + *IGF2* P2e4 + *IGF2* P2e5 + *IGF2* P3 + *IGF2* P4

IGF2 (liver) = *IGF2* P2e4 + *IGF2* P2e5 + *IGF2* P3 + *IGF2* P4

IGF2 (cotyledon, heart, lung, kidney) = *IGF2* P2e5 + *IGF2* P3 + *IGF2* P4

Where *IGF2* is the relative expression (normalised to the housekeepers) of global *IGF2* and *IGF2* P2e4 (transcript with untranslated leader exon 4) *IGF2* P2e5 (transcript with untranslated leader exons 4 and 5) are relative expression of the splice variants derived from P2 promoter. *IGF2* P0, *IGF2* P3 and *IGF2* P4 are relative expression of the transcripts derived from P0, P3 and P4 promoters, respectively. The contribution of each promoter-specific transcript to total *IGF2* variation was calculated from the type III sums of squares (SSIII) of relative transcript abundance for the promoter divided by total SSIII for all covariates included in the model.

4.3 Results

We analysed the effects of fetal genetics and estimated heterosis effects on body weight as well as absolute and relative organ weights in Day-153 fetuses of Angus (AA), Brahman (BB), and reciprocal cross hybrids (BA and AB, sire given first) (Figure 4.1). Moreover, we examined heterotic and fetal genetic effects on tissue-specific transcript abundances of the *IGF2* promoters, *IGF2R*, *H19*, *AIRN* and expression ratios of *IGF2* promoters to *H19*, *IGF2R* to *AIRN* and *IGF2* to *IGF2R*, and their correlations with weights of the relevant tissues in Day-153 fetuses.

4.3.1 Genetic effects on fetal body weight, relative and absolute weights of fetal tissues

Fetal weight and absolute weights of all the examined fetal tissues, except brain, were significantly affected by fetal genetics, (P<0.001) with higher fetal weight observed in AA compared with BB fetuses. Significant differences were observed between reciprocal hybrids in fetal body and organ weights, which were higher in the BA compared with AB fetuses. Absolute lung and fetal placental weight displayed significant heterosis (P<0.05). Fetal weight and absolute weights of fetal tissues, except placenta, were significantly higher in male fetuses compared with females (Figure 4.1.a).

Relative weights of liver, skeletal muscle, heart and brain were significantly influenced by fetal genetics. Relative liver and brain weight was higher in BB compared with AA fetuses, whereas relative muscle and heart weight was higher in AA compared with BB fetal groups. A significant fetal genetic effect (P<0.001) was observed on placental efficiency (defined as the ratio of fetal weight to fetal placental weight), which was lowest in BA fetuses.

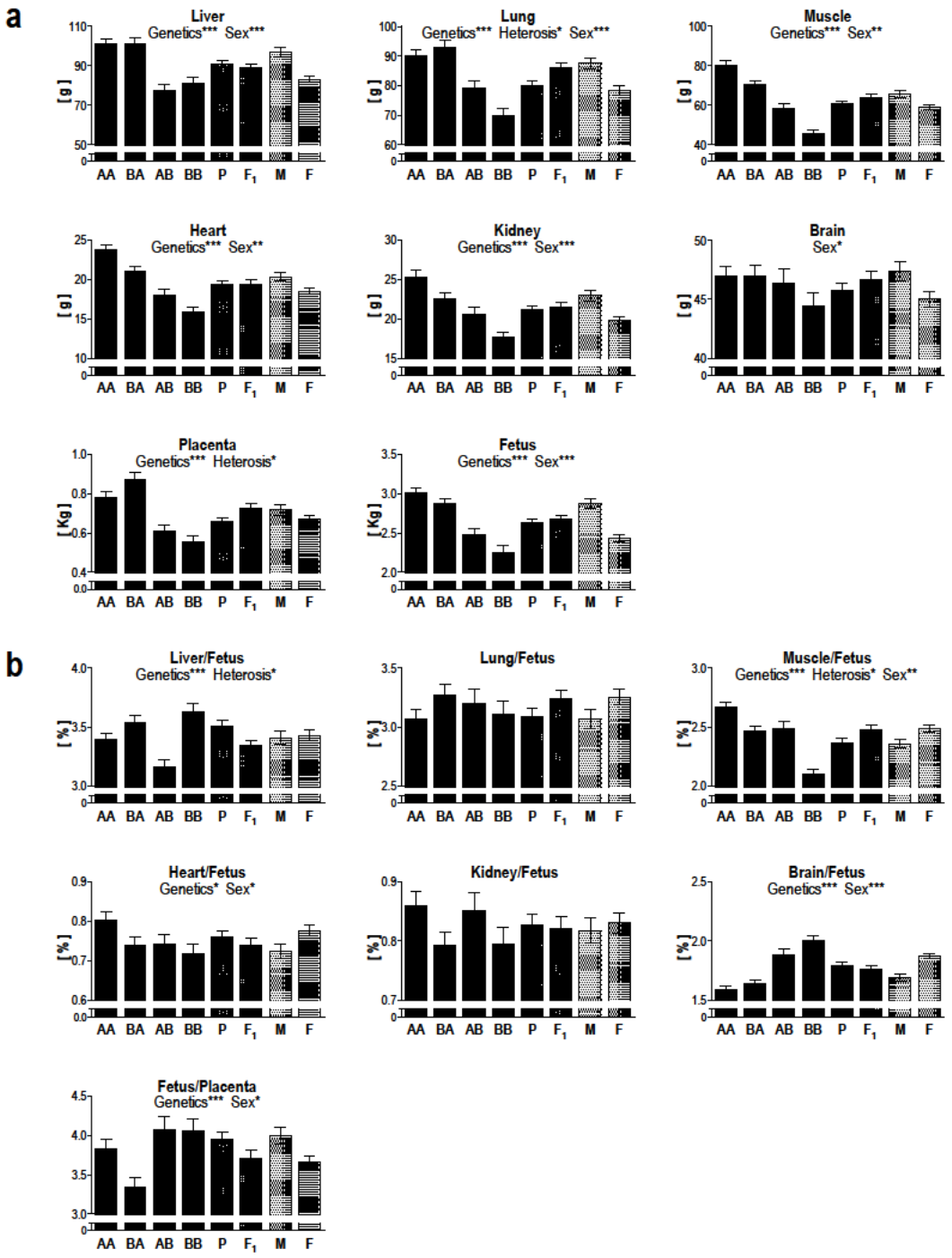

Figure 4.1 Effect of heterosis, fetal genetics and sex on fetal weight and absolute and relative weights of fetal tissues.

(a) Least square means and standard errors of means for fetal weight and weights of fetal organs, including liver, lung, skeletal muscle, heart, kidney, brain and placenta, in Day-153 fetuses with Angus (AA), Brahman (BB) and reciprocal crossbred genetics (BA and AB, sire given first), as well as purebred (P, consisting of AA and BB) and crossbred (F_1, consisting of BA and AB) genetics. **(b)** Least square means and standard errors of means for the ratio of fetus to placenta weight and relative weights of fetal organs, including liver, lung, skeletal muscle, heart, kidney, brain and placenta, to fetus weight in Day-153 fetuses with Angus (AA), Brahman (BB) and reciprocal crossbred genetics (BA and AB, sire given first), as well as purebred (P, consisting of AA and BB) and crossbred (F_1, consisting of BA and AB) genetics. The means were also shown for male (M) and female (F) fetal groups. Skeletal muscle weight was calculated as the combined weights of *M. supraspinatus*, *M. longissimus dorsi*, *M. quadraceps femoris*, *M. semimembranosus* (each measured as the average weight of left and right muscles). Effects of genetics, heterosis and sex were shown where significant (*F*-test). Statistical tests were considered significant at the levels of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

Relative skeletal muscle, heart and brain weight was higher in female fetuses compared with males, whereas placental efficiency was significantly (P<0.05) higher in male fetuses compared with females. Relative weights of liver and skeletal muscle were significantly (P<0.05) affected by negative and positive heterosis, respectively (Figure 4.1.b).

4.3.2 Genetic effects on expression of *IGF2*, *H19* and *IGF2/H19* in fetal tissues and associations with fetal tissue weights

Fetal genetics, sex and heterosis did not impact on transcript abundance of *IGF2* global transcript in the examined fetal tissues, with the exception of lung. Expression of *IGF2* in lung was significantly (P<0.001) affected by fetal genetics and negative heterosis and was lower in BA compared with the other genetic groups (Figure 4.2).

Transcript abundance of *H19* in the examined tissues, with the exception of brain, was significantly (P<0.001) influenced by fetal genetics and was higher in BB compared with AA fetuses. Expression of *H19* in liver and lung showed a significant difference between reciprocals and was higher in AB compared with BA fetuses, whereas in the other tissues, *H19* expression was not different between reciprocals. A negative heterotic effect (P<0.05) was observed on transcript abundance of *H19* in fetal lung and skeletal muscle (Figure 4.2). *H19* expression was negatively correlated with fetal tissue weight in all examined tissues (P<0.01), except cotyledon and brain (Figure 4.3). Independent regression analyses in purebred (AA and BB) and hybrid (BA and AB) fetal groups showed that *H19* transcript abundance was significantly correlated with lung, muscle mass, heart, kidney and fetal placental weight in purebreds only. *H19* expression was negatively correlated with absolute and relative liver weight in hybrids (P<0.01) and also with absolute liver weight in purebred fetuses (P<0.05) (Figure 4.3).

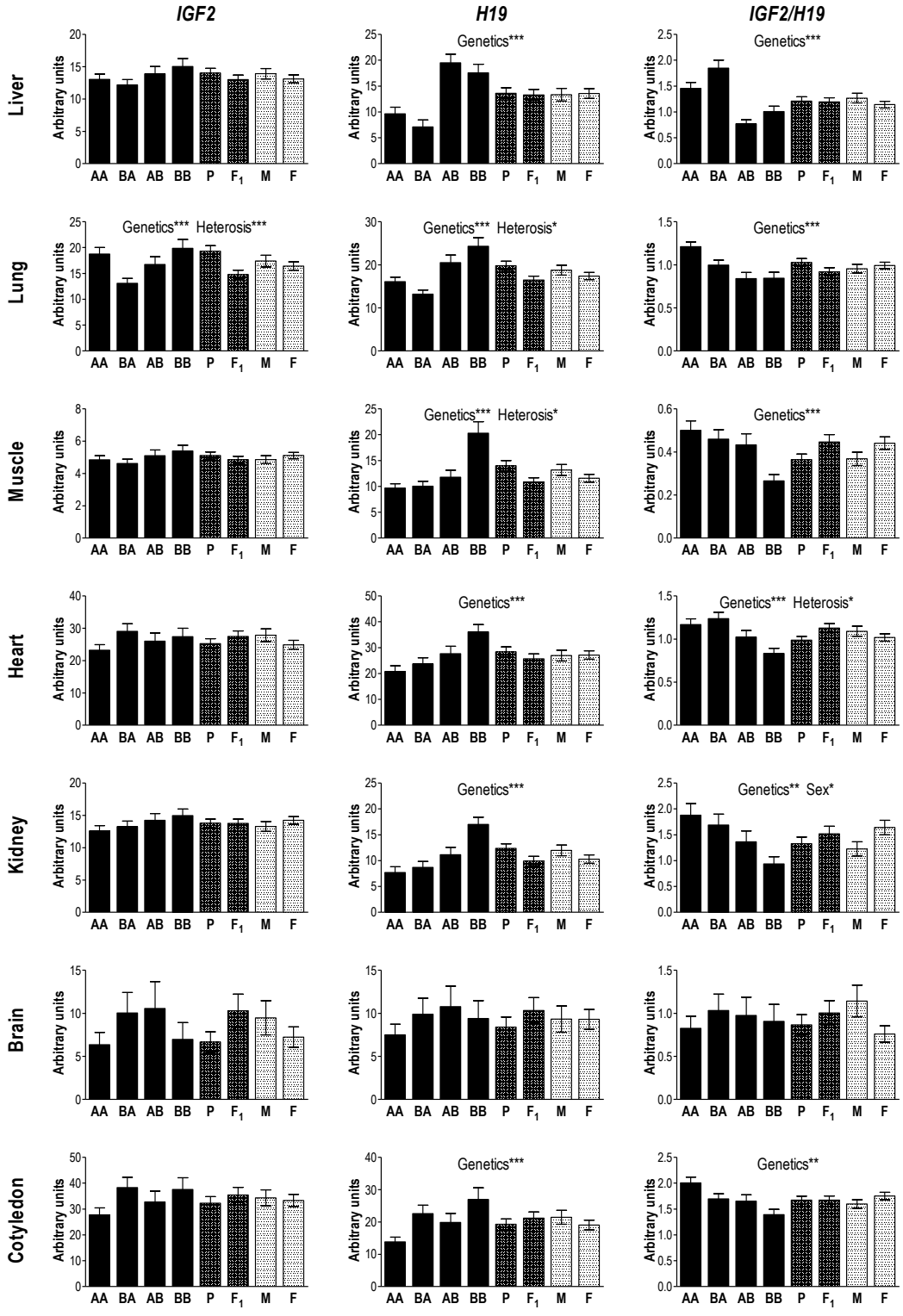

Figure 4.2 Effect of heterosis, fetal genetics and sex on transcript abundance of *IGF2* and *H19*, and the ratio of *IGF2* to *H19* transcript abundance in fetal tissues.

Least square means and standard errors of means for relative transcript abundance of (calculated by standard curve method, normalised to the housekeeper genes and expressed in arbitrary units) *IGF2* and *H19*, and the ratio of *IGF2* to *H19* transcript abundance in liver, lung, skeletal muscle (*M. semitendinosus*), heart, kidney, brain and cotyledon of Day-153 fetuses with Angus (AA), Brahman (BB) and reciprocal crossbred genetics (BA and AB, sire given first), as well as purebred (P, consisting of AA and BB) and crossbred (F_1, consisting of BA and AB) genetics. The means were also shown for male (M) and female (F) fetal groups. Effects of genetics, heterosis, and sex were shown where significant (*F*-test). Statistical tests were considered significant at the levels of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

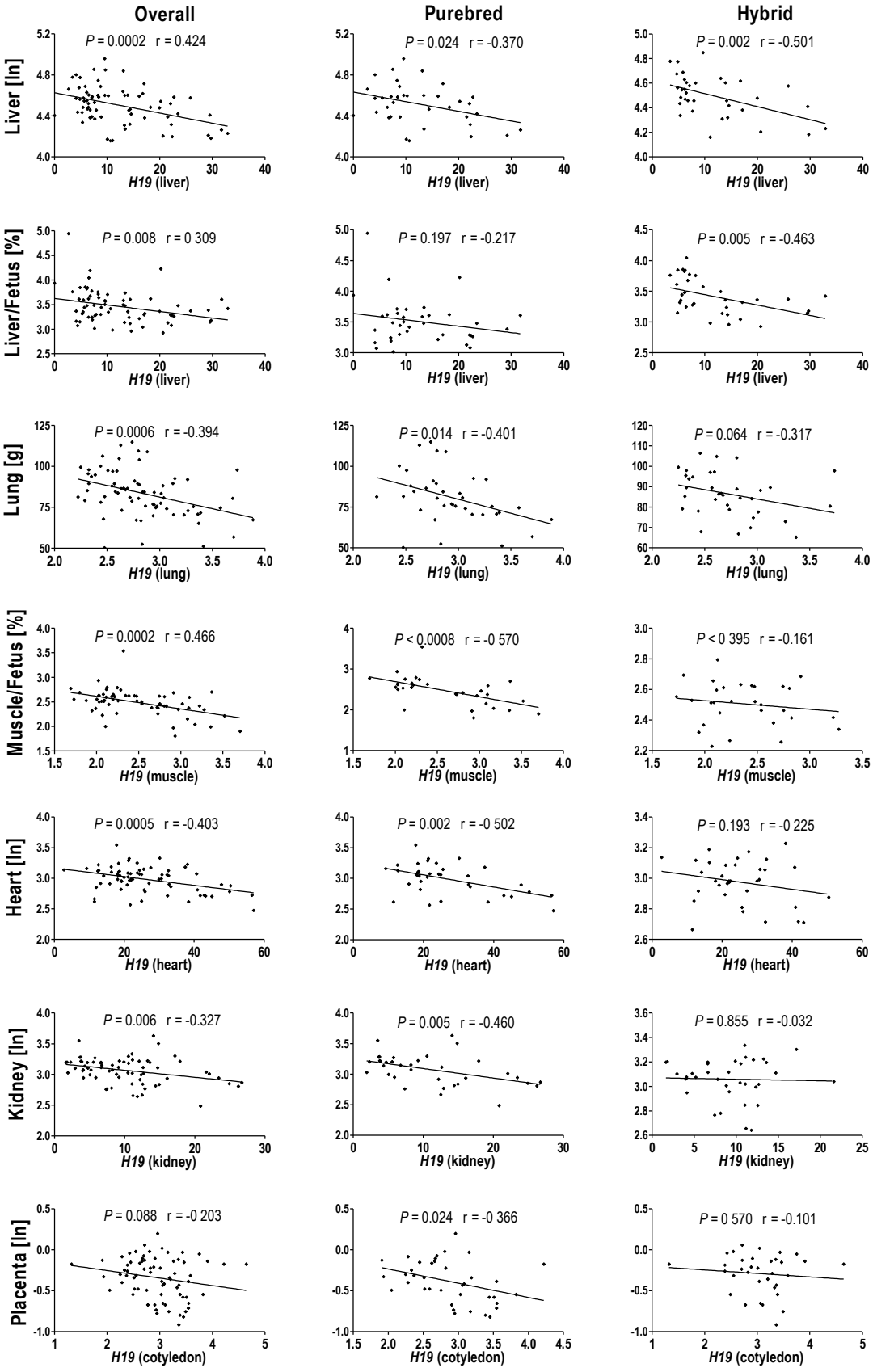

Figure 4.3 Regression of absolute weight of fetal liver, lung, heart, kidney and placenta, and relative weight of liver and skeletal muscle on *H19* transcript abundance in overall (including fetuses with purebred and hybrid genetics), purebred and hybrid genetic groups.

P-values and correlation coefficients are shown above each graph. Logarithmic transformations (ln) were performed for dependent and/or independent variable where necessary to meet assumptions of regression analyses.

The relative expression level of *IGF2* to *H19* was affected by fetal genetics ($P<0.01$) in all studied tissues, except brain, and was higher in AA compared with BB fetuses. The *IGF2/H19* expression ratio in liver was significantly higher in fetuses from the BA compared with AB group. The *IGF2/H19* expression ratio was significantly influenced by positive heterosis ($P<0.05$) in heart and by fetal sex ($P<0.05$) in kidney (Figure 4.2). The *IGF2/H19* ratio in heart was positively correlated with heart weight ($P<0.01$) in all fetuses combined, and in the purebred genetic groups (Figure 4.10). *IGF2/H19* was significantly positively correlated with weights of fetal tissues, except cotyledon (data not shown).

4.3.3. Genetic effects on expression of *IGF2* promoter-specific transcripts, their ratio to *H19* expression, and associations with fetal tissue weights

We analysed tissue-specific expression of the *IGF2* promoter-specific transcripts, including P0, P1 (two transcripts P1e2 and P1e3 which may derive from putative P0 and/or P1 promoters), P2 (two splice variants P2e4 and P2e5), P3 and P4. We also analysed relative expression of *IGF2* promoters to *H19*. Analysis of regression of global *IGF2* transcript abundance on expression levels of promoter-specific transcripts revealed that P4 is the most abundant transcript in most tissues accounting for 66 % (in skeletal muscle), 84 % (in heart), 92 % (in cotyledon), 82 % (in lung), 88 % (in kidney), and 42 % (in liver) of global *IGF2* transcripts. P2 is the most abundant transcript in liver which accounts for 53 % of global *IGF2* transcripts and the second most abundant transcript in heart (14 %), lung (17 %), and kidney (10 %) (Figure S2, Appendix 8). Transcript abundances of P2 (P2e5) and P4 in lung and P3 in liver were significantly affected by fetal genetics ($P<0.01$) being higher in AB compared with BA reciprocal fetuses. P4 transcript abundance in cotyledon and P2e4 transcript abundance in liver were significantly affected by fetal genetics ($P<0.01$) and were higher in fetuses with paternal Brahman genetics (BA and BB) compared to those with paternal Angus genetics (Figures 4.4 and 4.6). Significant negative heterosis affected P4 transcript abundance in liver ($P<0.05$), lung ($P<0.01$) and kidney ($P<0.05$). P3 transcript

abundance in kidney was higher (P<0.05) in male fetuses compared with females (Figure 4.4).

Fetal genetics affected the expression ratios of *IGF2* promoter-specific transcripts to *H19* in all examined tissues (except for *IGF2* P3*/H19* in liver), with the expression ratios being higher in AA compared with BB fetuses (with the exception of *IGF2* P2e4*/H19* in liver, which did not differ significantly between purebred fetal groups). A substantial difference between BA and AB reciprocals was observed for *IGF2* P2*/H19* in heart and cotyledon, *IGF2* P3*/H19* in muscle and heart, *IGF2* P4*/H19* in liver and heart (Figure 4.5) and *IGF2* P2e4*/H19* in liver (Figure 4.6). The *IGF2* P2*/H19* in kidney (Figure 4.5), and *IGF2* P0*/H19* (Figure 4.6) and *IGF2* P1e3*/H19* (Figure 4.6) in muscle were significantly higher in females compared with males (P<0.05). Furthermore, positive heterotic effects were significant on *IGF2* P2e4*/H19* in muscle (P<0.01), *IGF2* P3*/H19* in kidney (P<0.05), *IGF2* P4*/H19* in muscle (P<0.05) and also the expression ratio of skeletal muscle-specific transcripts (P0, P1e2 and P1e3) to *H19* (P<0.01) (Figures 4.5 and 4.6). The ratio of expression of *IGF2* skeletal muscle-specific promoter to *H19* was positively correlated (P<0.001) with relative muscle mass. Analysis within genetic subgroups revealed that the correlation was significant (P<0.01) only for purebred fetuses (Figure 4.10).

4.3.4. Genetic effects on expression of *IGF2R*, *AIRN*, *IGF2R/AIRN* and *IGF2/IGF2R*, and associations with fetal tissue weights

Expression of *IGF2R* in lung was significantly influenced by fetal genetics and negative heterosis (P<0.001) with the lowest transcript abundance observed in BA group. Furthermore, relative transcript abundance of *IGF2R* to *AIRN* in lung was subject to significant fetal genetic and negative heterotic effects (P<0.01). *IGF2R* and *AIRN* in cotyledon showed similar patterns of expression with transcript abundances being higher (P<0.001) in BA compared with the other three genetic groups (Figure 4.7). *AIRN* expression in cotyledon was positively related to placental weight and was negatively related to placental efficiency (P<0.01).

Regression analysis within the genetic subgroups showed that these correlations were only significant in hybrid fetuses (P<0.05). *IGF2R* expression in cotyledon displayed a positive correlation (P<0.05) with fetal placental weight only in the hybrid fetal group (Figure 4.9).

Relative expression of *IGF2* to *IGF2R* in cotyledon was affected by fetal genetics (P<0.05) and was lower in BA fetuses compared with the other fetal groups (Figure 4.8). The *IGF2/IGF2R* expression ratio in cotyledon was negatively correlated with fetal placental weight (P<0.05) and positively correlated with placental efficiency (P<0.01) in overall (including fetuses with purebred and hybrid genetics), and hybrid genetic groups (Figure 4.9).

Expression of *AIRN* in liver was significantly impacted by fetal genetics (P<0.05) and was higher in fetal groups with maternal Brahman genetics (AB and BB) compared to those with maternal Angus genetics. *AIRN* transcript abundance in brain was higher in BA compared with the other three genetic groups (P<0.001) and was subject to heterosis (P<0.05) (Figure 4.7). Expression of *AIRN* was positively correlated with relative brain weight in hybrid fetuses (P<0.05) (Figure 4.10). Fetal genetics and negative heterosis had significant effects on *IGF2R/AIRN* expression ratio in brain (P<0.001), which was lower in BA compared with the other fetal groups (Figure 4.7). Significant positive heterosis was also observed (P<0.05) for *IGF2/IGF2R* expression ratio in brain (Figure 4.8).

The relative expression of *IGF2R* to *AIRN* in heart was influenced by fetal genetics (P<0.01) and sex (P<0.05) and was higher in fetuses with maternal Brahman genetics compared to those with maternal Angus genetics (Figure 4.7). Regression analysis showed a negative correlation of *IGF2R/AIRN* expression in heart, with heart weight (P<0.05) in overall, and also purebred genetic groups (Figure 4.10).

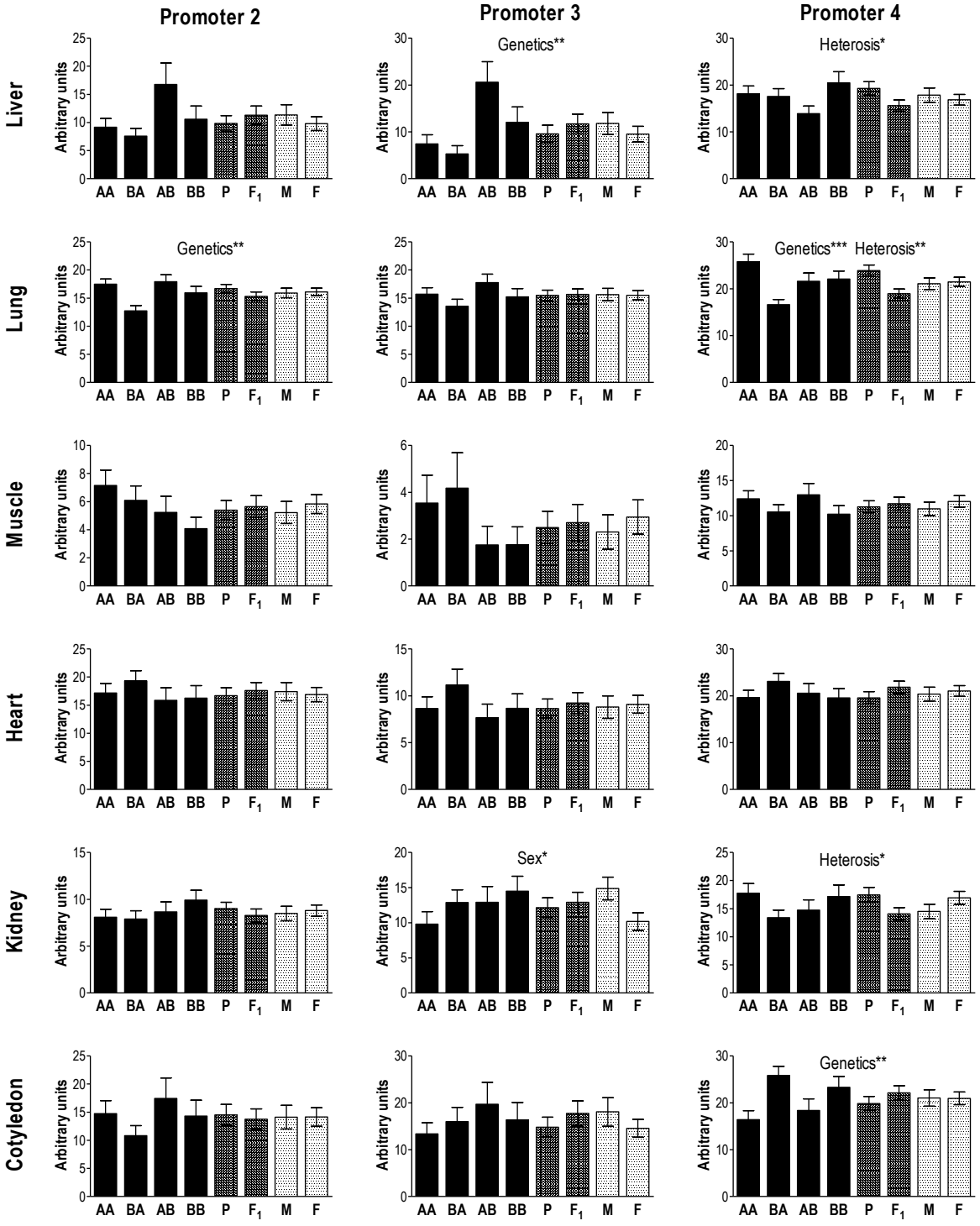

Figure 4.4 Effect of heterosis, fetal genetics and sex on transcript abundance of *IGF2* promoter-specific transcripts, P2, P3 and P4 in fetal tissues.

Least square means and standard errors of means for relative transcript abundance (calculated by standard curve method, normalised to the housekeeper genes and expressed in arbitrary units) of *IGF2* transcripts derived from promoters *P2*, *P3* and *P4* in liver, lung, skeletal muscle (*M. semitendinosus*), heart, kidney and cotyledon of Day-153 fetuses with Angus (AA), Brahman (BB) and reciprocal crossbred genetics (BA and AB, sire given first), as well as purebred (P, consisting of AA and BB) and crossbred (F_1, consisting of BA and AB) genetics. The means were also shown for male (M) and female (F) fetal groups. Effects of genetics, heterosis, and sex were shown where significant (*F*-test). Statistical tests were considered significant at the levels of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***). The *IGF2* P2 transcript (*IGF2* P2e5) is the splice variant consisting of non protein coding exons 4 and 5 plus protein coding exons 8, 9 and 10.

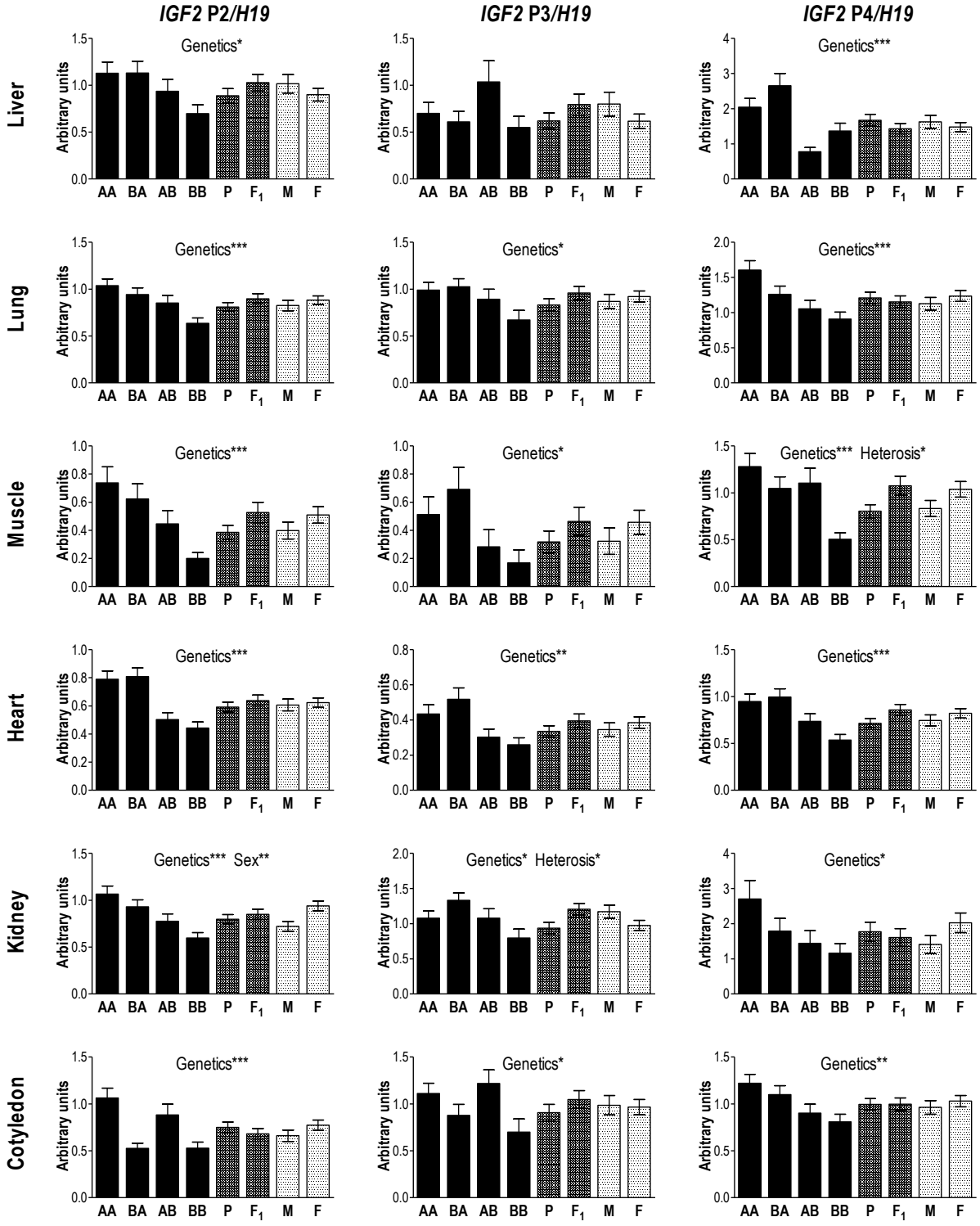

Figure 4.5 Effect of heterosis, fetal genetics and sex on the ratio of transcript abundance of *IGF2* promoter-specific transcripts, P2, P3 and P4, to *H19* in fetal tissues.

Least square means and standard errors of means for the ratio of *IGF2* P2 (P2e5), P3 and P4 to *H19* transcript abundance (calculated by standard curve method, normalised to the housekeeper genes and expressed in arbitrary units) in liver, lung, skeletal muscle (*M. semitendinosus*), heart, kidney and cotyledon of Day-153 fetuses with Angus (AA), Brahman (BB) and reciprocal crossbred genetics (BA and AB, sire given first), as well as purebred (P, consisting of AA and BB) and crossbred (F_1, consisting of BA and AB) genetics. The means were also shown for male (M) and female (F) fetal groups. Effects of genetics, heterosis, and sex were shown where significant (*F*-test). Statistical tests were considered significant at the levels of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***). The *IGF2* P2 transcript (*IGF2* P2e5) is the splice variant consisting of non protein coding exons 4 and 5 plus protein coding exons 8, 9 and 10.

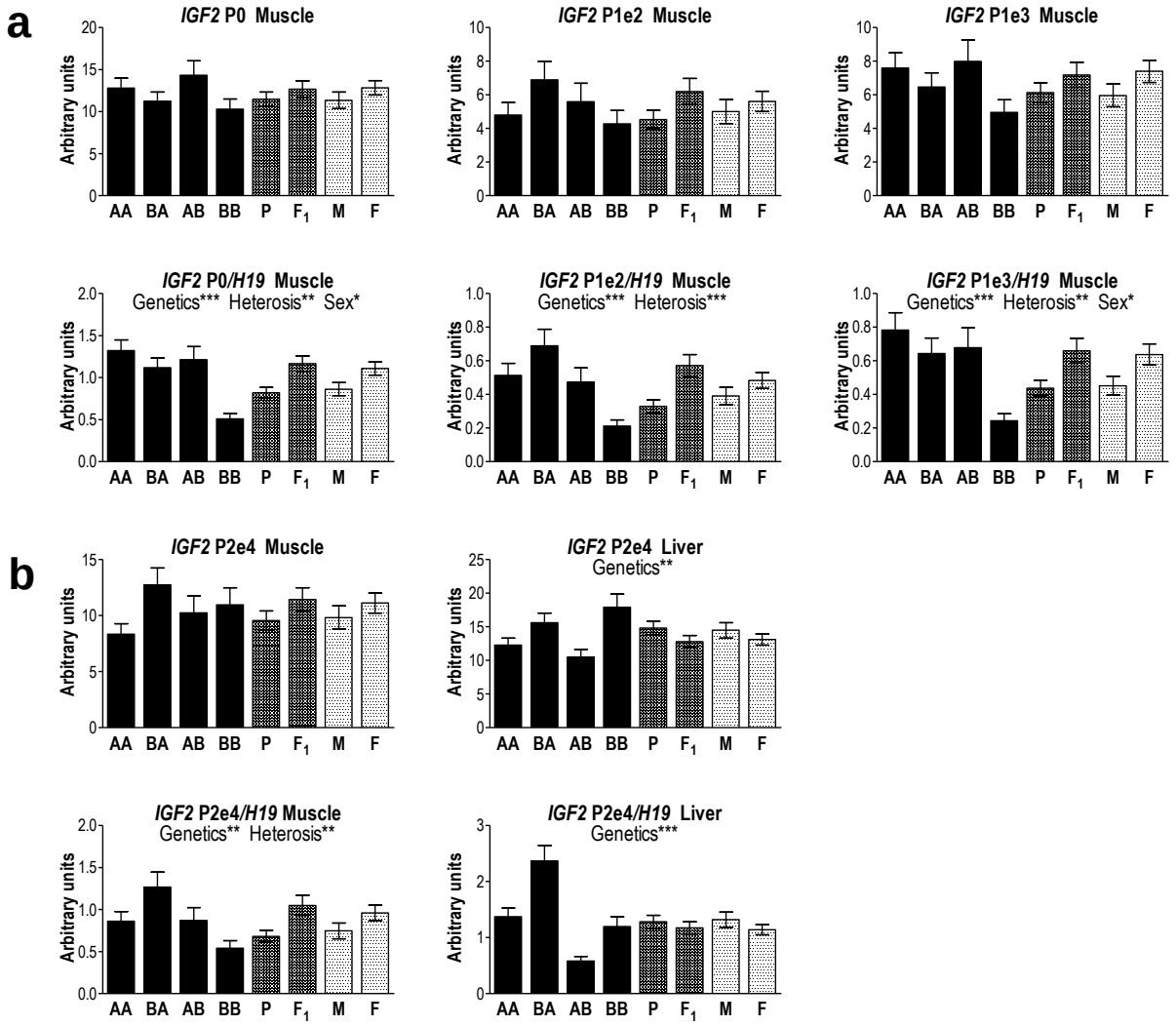

Figure 4.6 Effect of heterosis, fetal genetics and sex on transcript abundance of *IGF2* promoter-specific transcripts, P0, P1e2, P1e3, and P2e4, and their ratio to *H19* transcript abundance in fetal muscle and liver.
Least square means and standard errors of means for relative transcript abundance (calculated by standard curve method, normalised to the housekeeper genes and expressed in arbitrary units) of *IGF2* promoter-specific transcripts, including **(a)** P0, P1e2, P1e3 in skeletal muscle (*M. semitendinosus*), and **(b)** *P2e4* in skeletal muscle (*M. semitendinosus*) and liver of Day-153 fetuses with Angus (AA), Brahman (BB) and reciprocal crossbred genetics (BA and AB, sire given first), as well as purebred (P, consisting of AA and BB) and crossbred (F_1, consisting of BA and AB) genetics. The ratios of transcript abundances of *IGF2* promoters to *H19* were also shown. The means were also shown for male (M) and female (F) fetal groups. Effects of genetics, heterosis, and sex were shown where significant (*F*-test). Statistical tests were considered significant at the levels of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***). Expression of the splice variant (*IGF2* P2e4) comprising exon 4 plus exons 8, 9 and 10 is confined to skeletal muscle and liver. Relative expression levels of all transcripts to *H19* were shown.

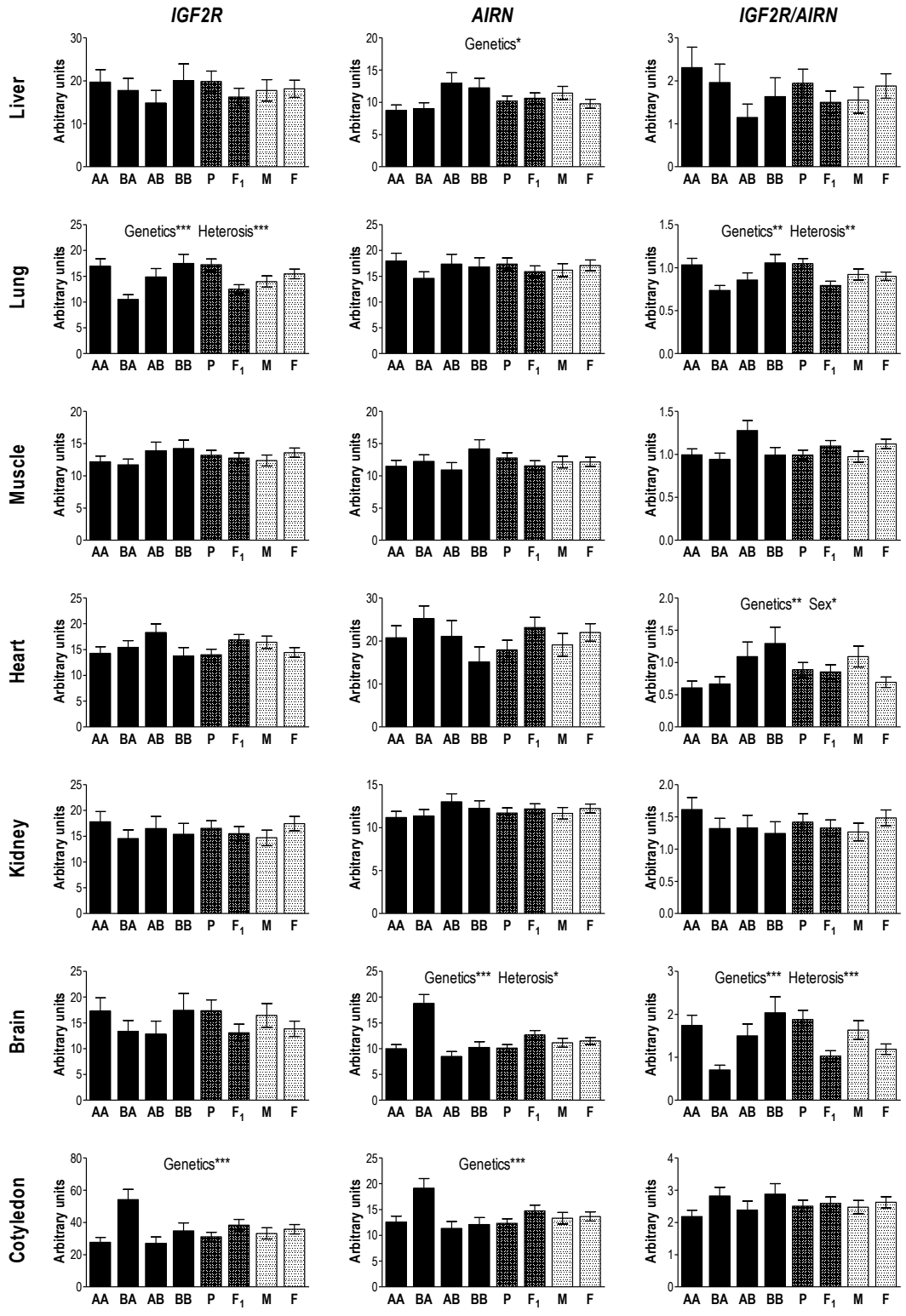

Figure 4.7 Effect of heterosis, fetal genetics and sex on transcript abundance of *IGF2R* and *AIRN*, and the ratio of *IGF2R* to *AIRN* transcript abundance in fetal tissues.

Least square means and standard errors of means for relative transcript abundance (calculated by standard curve method, normalised to the housekeeper genes and expressed in arbitrary units) of *IGF2R* and *AIRN*, and the ratio of *IGF2R* to *AIRN* transcript abundance in liver, lung, skeletal muscle (*M. semitendinosus*), heart, kidney, brain and cotyledon of Day-153 fetuses with Angus (AA), Brahman (BB) and reciprocal crossbred genetics (BA and AB, sire given first), as well as purebred (P, consisting of AA and BB) and crossbred (F_1, consisting of BA and AB) genetics. The means were also shown for male (M) and female (F) fetal groups. Effects of genetics, heterosis, and sex were shown where significant (*F*-test). Statistical tests were considered significant at the levels of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

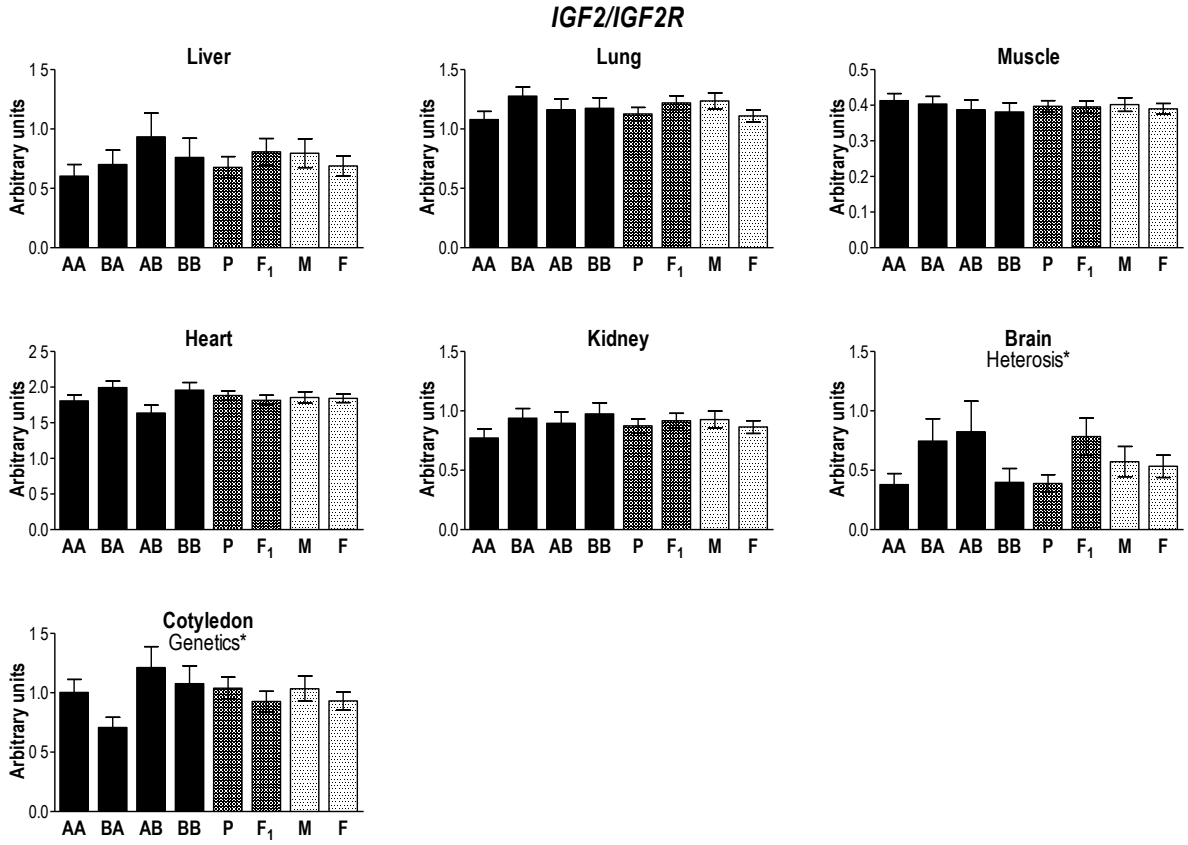

Figure 4.8 Effect of heterosis, fetal genetics and sex on the ratio of *IGF2* to *IGF2R* transcript abundance in fetal tissues.

Least square means and standard errors of means for the ratio of *IGF2* to *IGF2R* transcript abundance (calculated by standard curve method, normalised to the housekeeper genes and expressed in arbitrary units) in liver, lung, skeletal muscle (*M. semitendinosus*), heart, kidney, brain and cotyledon of Day-153 fetuses with Angus (AA), Brahman (BB) and reciprocal crossbred genetics (BA and AB, sire given first), as well as purebred (P, consisting of AA and BB) and crossbred (F_1, consisting of BA and AB) genetics. The means were also shown for male (M) and female (F) fetal groups. Effects of genetics, heterosis, and sex were shown where significant (*F*-test). Statistical tests were considered significant at the levels of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

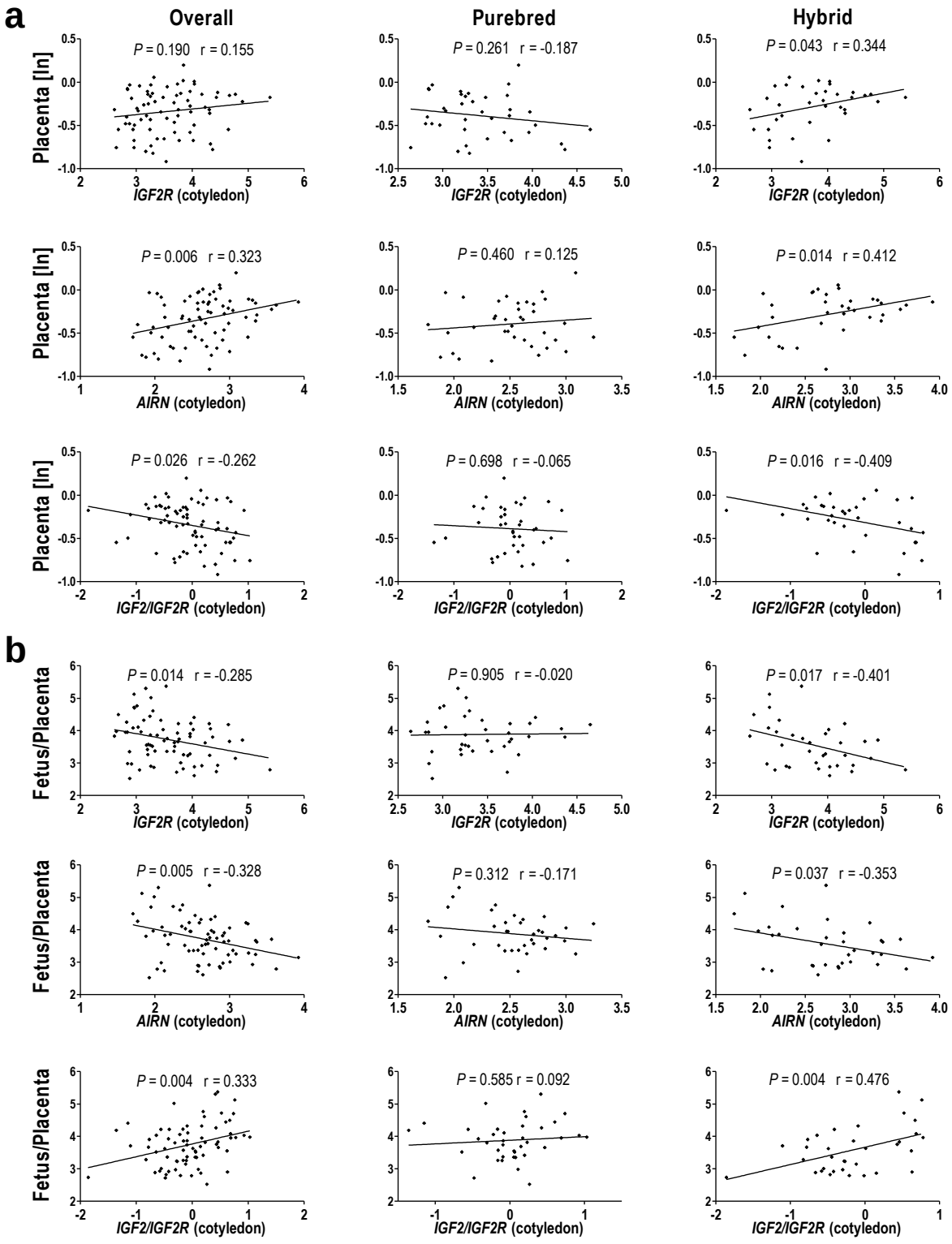

Figure 4.9 Regression of **(a)** fetal placental weight and **(b)** placental efficiency on transcript abundance of *IGF2R*, *AIRN* and the ratio of *IGF2/IGF2R* expression in cotyledon.

Placental efficiency was defined as the ratio of fetal body weight to fetal placental weight. The regressions were analysed in overall (including fetuses with purebred and hybrid genetics), purebred and hybrid genetic groups. *P*-values and correlation coefficients are shown above each graph. Logarithmic transformations (ln) were performed for dependent and/or independent variable where necessary to meet assumptions of regression analyses.

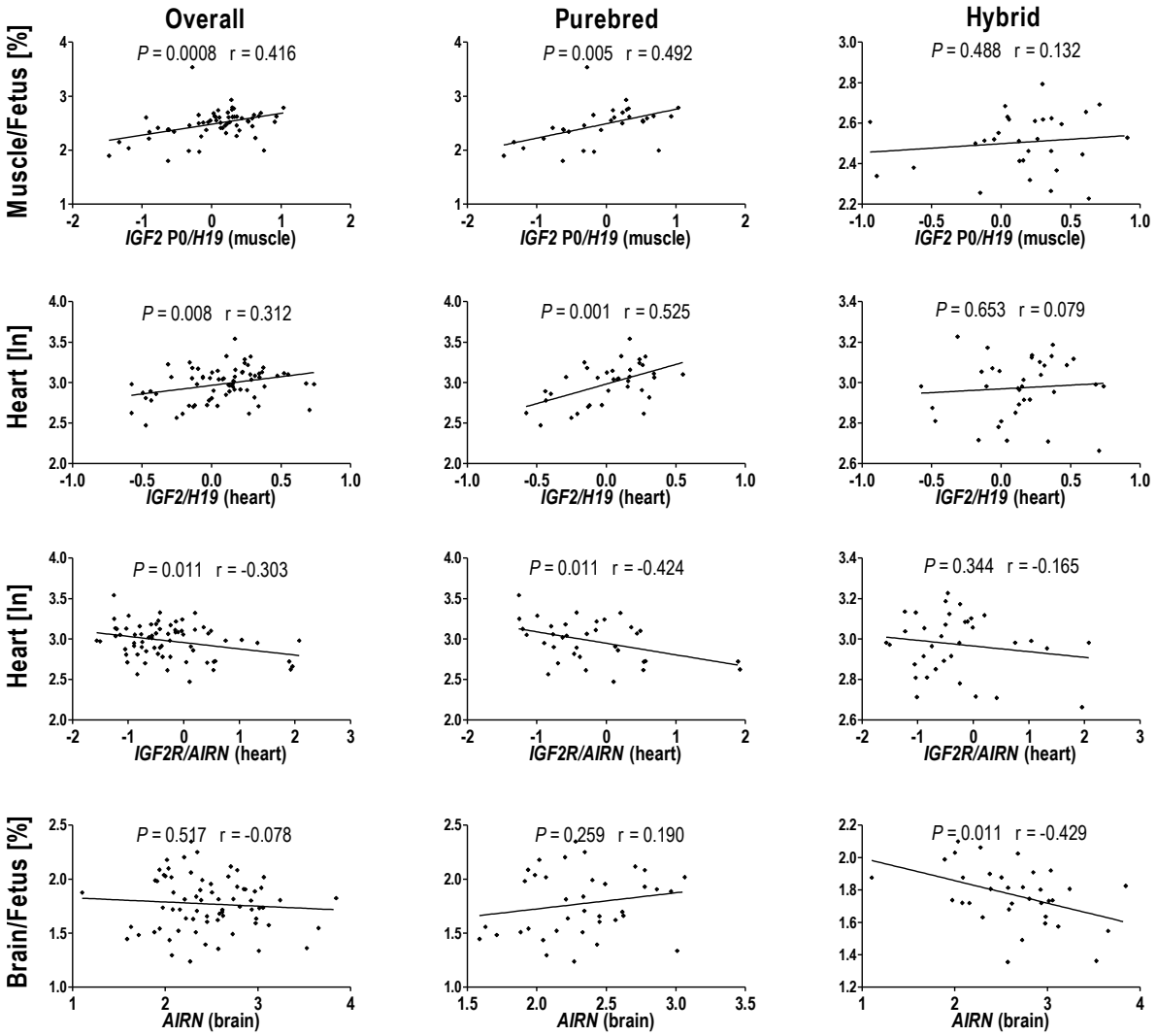

Figure 4.10 Regression of absolute/relative weight of fetal tissues on transcript abundance/expression ratio of imprinted genes significantly affected by genetics, in overall (including fetuses with purebred and hybrid genetics), purebred and hybrid genetic groups.

P-values and correlation coefficients are shown above each graph. Logarithmic transformations (ln) were performed for dependent and/or independent variable where necessary to meet assumptions of regression analyses.

4.4 Discussion

Fetal body weight, absolute weights of fetal tissues, with the exception of brain, and relative brain weight showed phenotypic patterns with parent-of-origin (maternal) genetic effects. Genomic imprinting is a plausible mechanism that may explain phenotypic manifestation of the difference between two reciprocals (Wolf *et al.*, 2008b). However, parent-of-origin phenotypic patterns caused by maternal effects may be confounded by genomic imprinting (Hager *et al.*, 2008). A genome-wide analysis of the imprinted QTLs revealed a complex phenotypic pattern of genomic imprinting, including dominance imprinting (Wolf *et al.*, 2008a). A phenotype-based approach has been exploited in this study to describe the phenotypic patterns associated with genomic imprinting according to the difference between two reciprocals. Although the underlying molecular mechanisms that lead to a specific expression pattern, e.g. miRNA interference, are disregarded, this approach can also be applied to quantitative data on transcript abundances to characterise imprinting patterns of gene expression and their link to manifestation of parent-of-origin dependent phenotypes and heterosis.

Analysis of phenotypic data at Day-153 of gestation revealed positive heterosis for fetal lung and placental weight and relative muscle mass, and negative heterosis in relative liver weight. Heterosis in fetal placenta, with the highest placental weight in BA fetuses, is in agreement with heterosis reported on birth weight, which was higher in the BA hybrid (Brown *et al.*, 1993). This is consistent with the significant role of the placenta in programming pre and postnatal growth (Godfrey, 2002; Jansson and Powell, 2007; Fowden *et al.*, 2008).

Expression of *H19* in cotyledon was significantly affected by fetal genetics with differential *B. taurus* and *B. Indicus*-specific expression. Spatial expression of a splice variant of *H19* in human placenta was shown to be associated with genetic polymorphism and was affected by fetal genetics (Lin *et al.*, 1999). A negative relationship was found between fetal placental weight and *H19* expression in only purebreds. A negative role of *H19* in placental

growth has been documented in mice with targeted disruption of the *H19* gene, which showed increased fetal and placental weight (Leighton *et al.*, 1995; Ripoche *et al.*, 1997; Esquiliano *et al.*, 2009; Angiolini *et al.*, 2011; Keniry *et al.*, 2012). Transcript abundance of *H19* in cotyledon was not significantly different between the two reciprocals and was not subject to heterotic effects. Lack of significant correlation between *H19* expression and placental weight in hybrids implies that *H19* could not explain the heterotic phenotypic pattern of fetal placenta.

The expression level of *IGF2R* in cotyledon was higher in the BA group, which could account for the higher placental weight observed in this fetal group. This is supported by the observation that transcript abundance of *IGF2R* in hybrid genetics was positively correlated with fetal placental weight, raising the possibility that IGF2R may be implicated in cellular pathways that lead to increased placental weight. The traditional role of IGF2R in suppressing placental growth has been documented in mouse gene knockout experiments (Wylie *et al.*, 2003). However, several lines of evidence have suggested that IGF2R is involved in signalling pathways mediating IGF2 function in stimulation of trophoblast cell migration, mitogenesis and survival of trophoblast (McKinnon *et al.*, 2001; El-Shewy *et al.*, 2006; El-Shewy *et al.*, 2007; Harris *et al.*, 2011).

Expression of *AIRN* was higher in the BA group and was positively correlated with fetal placental weight in hybrids. The role of *AIRN* in placental growth remains to be explored. *Airn* in mouse (*Mus musculus domesticus*) placenta is engaged in regulation of imprinted expression of *Igf2r* and transporter genes within an imprinted domain (Sleutels *et al.*, 2002; Nagano *et al.*, 2008).

Placental efficiency, which is indicative of placental capacity to provide nutrients to the growing fetus and is defined as the ratio of fetal weight to placental weight (Wilson and Ford, 2001), fell sharply in the BA group. Our data showed that expression of *IGF2R* and *AIRN*, which is affected by fetal genetics, is negatively correlated with placental efficiency in the hybrid fetuses.

Relative transcript abundance of *IGF2/IGF2R*, which may be interpreted as an indicator of the relative level of free IGF2 protein available to the tissue, was lowest in the BA group. The *IGF2/IGF2R* ratio was positively correlated with placental efficiency and negatively correlated with placental weight. The negative correlation of *IGF2/IGF2R* with placental weight is not consistent with the generally accepted function of IGF2R in growth suppression by reducing cellular bioavailability of IGF2 raising the possibility that IGF2R in placenta may also be involved in mediating growth stimulatory pathways. This speculation is in agreement with previous findings on the potential signalling role of IGF2R in placenta (Harris *et al.*, 2011). However, a positive correlation between the molar ratio of cord blood IGF2 to soluble IGF2R, and placental and birth weight, has been observed in human (Ong *et al.*, 2000).

The positive correlation of *IGF2/IGF2R* with efficiency of fetal placenta could be indicative of increased placental efficiency as a consequence of elevated bioavailability of IGF2. Gene ablation studies in mouse demonstrated that Igf2 is involved in regulation of expression of glucose and amino acid transporter genes (Matthews *et al.*, 1999; Constancia *et al.*, 2005). Disruption of *Igf2* and *Igf1r* similarly altered expression patterns of amino acid transporters in mouse placenta (Matthews *et al.*, 1999). However, null mutation of mouse *Igf2* resulted in decreased placental weight, while null mutation of *Igf1r* did not display such an effect, suggesting that IGF2 function in placental growth is mediated by receptors other than the Igf1r (Baker *et al.*, 1993). In guinea pig, Leu^{27}-IGF2, an IGF2 analogue which can bind IGF2R but not IGF1R influenced placental efficiency and fetal weight, suggesting that the role of IGF2 in enhancing placental functional development and nutrient transfer is also mediated in part by IGF2R (Sferruzzi-Perri *et al.*, 2008).

According to imprinting patterns described previously (Wolf *et al.*, 2008a), absolute and relative muscle mass showed partial imprinting (with difference between reciprocals) and dominance non-imprinting (with no difference between reciprocals) phenotypic patterns, respectively. The significant negative correlation of *H19* transcript abundance with muscle mass phenotypes in purebreds suggests that *H19* could be a major determinant of skeletal

muscle phenotype. The importance of *H19* in prenatal growth has been shown by the associations of the genetic polymorphisms within the human *H19* gene with birth weight (Petry *et al.*, 2005; Petry *et al.*, 2011).

Positive heterosis on relative muscle mass could be attributed to a negative heterotic effect on *H19* transcript abundance in skeletal muscle, although the correlation between *H19* expression and muscle mass was not significant in the hybrid group. The relative expression of the skeletal muscle-specific promoter, *IGF2* P0 to *H19* exhibited positive heterosis, which was consistent with the heterotic phenotypic pattern of relative muscle mass with respect to positive relationship between *IGF2* P0*/H19* ratio and relative muscle mass. Relative expression of *IGF2* P4 and *IGF2* P2e4 to *H19* was also subject to positive heterotic effects. This suggests that fetal genetics differentially impacts on relative expression of *IGF2* promoter-specific transcripts to *H19*.

Absolute liver weight showed a phenotypic pattern of maternal expression, as described previously (Wolf *et al.*, 2008a). The negative correlation of *H19* expression with absolute liver weight in both purebred and hybrid fetal groups suggests that *H19* could be a driver of the parent-of-origin effect on absolute liver weight. Relative liver weight was higher in fetuses with BB genetics compared with AA genetics, which could not be explained by the molecular data produced in this study. The significant negative correlation of *H19* expression with relative liver weight in only hybrids suggests that the negative heterosis on relative liver weight, which was lowest in the AB group, could be driven by *H19* with highest transcript abundance in the AB group. Furthermore, the negative heterosis on relative liver weight was consistent with the negative heterotic effects on *IGF2* P4 expression in liver, which was lowest in the AB group, but this was not supported by the regression data. Although global *IGF2* expression in liver was highly stable and was not affected by fetal genetics, sex or heterosis, the abundance of *IGF2* P3 and P2e4 transcripts was influenced by fetal genetics, suggesting that genetics differentially drives expression of *IGF2* promoters. Expression of *AIRN* in liver was affected by fetal genetics and was higher in fetuses with maternal Brahman

genetics, which is against the accepted imprinting status of a maternally imprinted gene. Further studies are required to elucidate imprinting status of *AIRN* in liver.

Considering the negative relationship between *H19* expression and lung weight, positive heterosis in lung weight could be explained by the negative heterotic effect on *H19* expression. However, no significant correlation was observed between *H19* expression and lung weight in hybrids and the phenotypic difference between reciprocals may not be caused by *H19*. Despite a genetic and negative heterotic effect on *IGF2* and *IGF2R* expression in fetal lung, which showed a significant difference between reciprocals, correlation of lung weight with *IGF2* and *IGF2R* expression was not significant. This could be explained by the indication that relative expression of *IGF2* to *IGF2R* in lung did not significantly change across genetic groups.

Heart weight showed a phenotypic pattern of maternal expression associated with partial imprinting, as described previously (Wolf *et al.*, 2008a). *H19* expression in heart did not exhibit a significant difference between reciprocals and was negatively related to heart weight in purebred fetuses. Heterotic effect on expression ratio of *IGF2* to *H19* is not related to the phenotypic pattern of heart in hybrids, and *IGF2/H19* had a positive relationship with heart weight only in purebreds. This could indicate that phenotypic manifestation of the altered expression ratio of *IGF2/H19* in favour of heterosis in heart does not occur at mid-gestation developmental stage. Relative expression of *IGF2R* to *AIRN* was under the influence of fetal genetics and was negatively correlated with heart weight in purebreds. These observations suggest that both *IGF2/H19* and *IGF2R/AIRN* imprinted gene systems could be implicated in differential manifestation of heart weight in Angus and Brahman. A critical role of Igf2r in heart development has been postulated in mice (*Mus musculus domesticus*) carrying a disrupted maternal allele of *Igf2r* which show phenotypes of heart enlargement and heart failure (Lau *et al.*, 1994). The molecular systems studied here do not explain the phenotypic appearance of heart in hybrids.

According to the imprinting patterns classified previously (Wolf *et al.*, 2008a), kidney weight showed a phenotypic pattern of maternal expression associated with partial imprinting. Differential expression of *H19* in Angus and Brahman and negative correlation of *H19* expression with kidney weight in purebreds could explain breed (genetic) effect on kidney weight.

Although absolute brain weight did not change significantly across genetic groups relative brain weight was significantly under fetal and parent-of-origin genetic effects. Significance of parent-of-origin effects on brain development has been previously postulated (Keverne *et al.*, 1996; Gregg *et al.*, 2010). In brain, *AIRN* displayed a polar overdominance imprinting pattern of expression with elevated transcript abundance in the BA group and consequently a heterotic effect. Despite a negative correlation between *AIRN* transcript abundance and relative brain weight in hybrids, a negative heterosis on *IGF2R/AIRN* in fetal brain, and a positive heterosis on *IGF2/IGF2R* in fetal brain, heterotic effects at molecular level in brain are not phenotypically manifested at Day-153 of gestation raising the possibility that such effects on imprinted gene expression may manifest in brain phenotype at subsequent stages of prenatal development or after birth. Our molecular data, including *H19* expression, are not consistent with the phenotypic pattern of relative brain weight in purebreds. Further studies are required to elucidate imprinting status of imprinted genes and their role in programming brain development.

In the present study, we showed that transcript abundance of *H19* was consistently influenced by fetal genetics in all examined tissues, except brain. These observations along with the demonstration of negative correlations between *H19* expression and weights of fetal tissues, except brain, in the purebred genetic group suggest that *H19* is associated with the differential prenatal phenotypes in *B. indicus* and *B. taurus* and could be involved in developmental programming. *H19* is a master regulator involved in downregulation of the genes in the newly described imprinted gene network (IGN), including *Igf2* and *Igf2r* in mouse skeletal muscle (Gabory *et al.*, 2009). Regression analysis revealed that correlation of

H19 expression with tissue weight was highest in skeletal muscle compared with other tissues, suggesting that a similar regulatory role for *H19* exists in bovine skeletal muscle.

To our knowledge, this is the first study to examine genetic effects on tissue-specific expression of imprinted genes and their relationship with growth-related phenotypes in prenatal development. We demonstrated that fetal genetics affects fetal weight, weights of fetal tissues and tissue-specific expression of imprinted genes. Furthermore, we showed that transcript abundances of imprinted genes are correlated with weight of the corresponding tissues in a manner depending on fetal tissue and genetic background. This is the first demonstration that imprinted genes are subject to non-additive heterotic expression and could be implicated in genetic programming of heterosis.

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Chapter 5

Maternal and paternal genomes differentially affect myofibre characteristics and muscle weights of bovine fetuses at midgestation

Maternal and paternal genomes differentially affect myofibre characteristics and muscle weights of bovine fetuses at midgestation

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Citation: Xiang R, Ghanipoor-Samami M, Johns WH, Eindorf T, Rutley DL, et al. (2013) Maternal and Paternal Genomes Differentially Affect Myofibre Characteristics and Muscle Weights of Bovine Fetuses at Midgestation. PLoS ONE 8(1): e53402. doi:10.1371/journal.pone.0053402

Editor: Alejandro Lucia, Universidad Europea de Madrid, Spain

Received May 16, 2012; Accepted November 30, 2012; Published January 14, 2013

Funding: This work was funded by the JS Davies Bequest with support from the Queensland Government through the Department of Agriculture, Fisheries and Forestry's Reinvestment Fund. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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PLoS ONE – 2013, 8(1), e53402

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Abstract

Postnatal myofibre characteristics and muscle mass are largely determined during fetal development and may be significantly affected by epigenetic parent-of-origin effects. However, data on such effects in prenatal muscle development that could help understand unexplained variation in postnatal muscle traits are lacking. In a bovine model we studied effects of distinct maternal and paternal genomes, fetal sex, and non-genetic maternal effects on fetal myofibre characteristics and muscle mass. Data from 73 fetuses (Day-153, 54% term) of four genetic groups with purebred and reciprocal cross Angus and Brahman genetics were analysed using general linear models. Parental genomes explained the greatest proportion of variation in myofibre size of *Musculus semitendinosus* (80-96%) and in absolute and relative weights of *M. supraspinatus*, *M. longissimus dorsi*, *M. quadriceps femoris* and *M. semimembranosus* (82-89% and 56-93%, respectively). Paternal genome in interaction with maternal genome ($P<0.05$) explained most genetic variation in cross sectional area (CSA) of fast myotubes (68%), while maternal genome alone explained most genetic variation in CSA of fast myofibres (93%, $P<0.01$). Furthermore, maternal genome independently (*M. semimembranosus*, 88%, $P<0.0001$) or in combination (*M. supraspinatus*, 82%; *M. longissimus dorsi*, 93%; *M. quadriceps femoris*, 86%) with nested maternal weight effect (5-6%, $P<0.05$), was the predominant source of variation for absolute muscle weights. Effects of paternal genome on muscle mass decreased from thoracic to pelvic limb and accounted for all (*M. supraspinatus*, 97%, $P<0.0001$) or most (*M. longissimus dorsi*, 69%, $P<0.0001$; *M. quadriceps femoris*, 54%, $P<0.001$) genetic variation in relative weights. An interaction between maternal and paternal genomes ($P<0.01$) and effects of maternal weight ($P<0.05$) on expression of *H19*, a master regulator of an imprinted gene network, and negative correlations between *H19* expression and fetal muscle mass ($P<0.001$), suggested imprinted genes and miRNA interference as mechanisms for differential effects of maternal and paternal genomes on fetal muscle.

5.1 Introduction

Skeletal muscle accounts for up to half of mammalian body mass (Du *et al.*, 2010b) and has important functions in metabolic homeostasis (Daniel *et al.*, 1977; Wolfe, 2006). It is a major source of endocrine factors, including insulin-like growth factors -I (IGF1) and -II (IGF2), key components of the insulin-like growth factor (IGF) system and growth hormone – IGF axis, which are major regulators of pre- and postnatal muscle development and growth (Adams, 2002; Chang, 2007; Pedersen and Febbraio, 2008; Sawitzky *et al.*, 2012). Skeletal muscle is composed of two major fibre types, type I (slow oxidative) fibres and type II (fast) fibres (Daniel *et al.*, 1977). Myofibres originate from mesenchymal stem cells which differentiate into myoblasts during embryonic development (Relaix, 2006). Myoblasts fuse to form myotubes which develop into myofibres at the fetal stage (Picard *et al.*, 2002). In ruminants, myofibres differentiate during late fetal development into type I, type IIA (fast oxidative-glycolytic) and type IIX (fast glycolytic) myofibres (Scott *et al.*, 2001; Greenwood *et al.*, 2009). Thus, myofibre number is established during fetal development and postnatal skeletal muscle mass is largely determined prenatally (Picard *et al.*, 2002; Du *et al.*, 2010a) by the interplay of a complex network of genetic and epigenetic factors (Brand-Saberi, 2005; Baar, 2010; Ge and Chen, 2011; Bentzinger *et al.*, 2012).

Studies on postnatal muscle tissue of human, porcine and bovine revealed that genetics explained up to 45% of variation in slow myofibre percentage (Simoneau and Bouchard, 1995), up to 58% of variation in myofibre number (Larzul *et al.*, 1997) and 74% of variation in myofibre size (Rehfeldt *et al.*, 1999), respectively. Similarly, using proxies such as lean body mass and lean tissue percentage, studies in human (Seeman *et al.*, 1996; Arden and Spector, 1997) and porcine (Larzul *et al.*, 1997) demonstrated that genetics accounted for approximately 50-80% of variation in postnatal muscle mass. Apart from genetic factors that follow Mendelian rules of inheritance, prenatal muscle development and postnatal muscle phenotype may be affected by genetic and epigenetic factors with Non-Mendelian modes of inheritance. This includes effects of mitochondrial genome (Mannen *et al.*, 1998), X- and Y-

chromosomes (Engellandt and Tier, 2002; Amen *et al.*, 2007a), non-random X-inactivation (Amen *et al.*, 2007b), microRNA (miRNA) interference (Clop *et al.*, 2006) and genomic imprinting (Engellandt and Tier, 2002; Boysen *et al.*, 2010; Neugebauer *et al.*, 2010a; Neugebauer *et al.*, 2010b). Genomic imprinting, i.e., parent-of-origin dependent allele-specific gene expression (Reik and Walter, 2001), has been described for genes with pivotal roles in myogenesis, including *IGF2* and its receptor *IGF2R* (Nezer *et al.*, 1999; Young *et al.*, 2001). In porcine, mapping and gene expression studies demonstrated that *IGF2* alleles explained up to 30% of variation in postnatal muscle mass (Van Laere *et al.*, 2003). The ovine callipyge (CLPG) mutation has provided an example of complex genetic and epigenetic effects on postnatal muscle phenotype. The CLPG mutation causes postnatal muscle hypertrophy only in heterozygous offspring and only when inherited through the paternal germline (Cockett *et al.*, 1996). This polar overdominance changes imprinted gene expression, presumably by miRNA interference (Caiment *et al.*, 2010), and affects absolute and relative weights of specific muscles and muscle groups of the torso (e.g. *M. longissimus lumborum*) and pelvic limb (e.g. *M. semimembranosus, M. quadriceps femoris*), but not of the thoracic limb (e.g. *M. supraspinatus*) (Koohmaraie *et al.*, 1995; Jackson *et al.*, 1997). The increased muscle mass of CLPG sheep is due to fast myofibre hypertrophy and results in higher glycolytic metabolism of affected muscles (Carpenter *et al.*, 1996; Jason *et al.*, 2008). A similar paternal polar overdominance effect on postnatal myofibre characteristics, muscle mass and growth has been described in porcine (Kim *et al.*, 2004). Furthermore, the ovine Carwell locus, which exerts paternal effects on weight of *M. longissimus dorsi* and a shift from type IIA to type IIX myofibres, was mapped to the same chromosome region as the CLPG mutation (Nicoll *et al.*, 1998; Cockett *et al.*, 2005; Greenwood *et al.*, 2006). More recently, statistical modelling revealed significant parent-of-origin effects attributed to genomic imprinting on postnatal absolute and relative weights of specific muscles in porcine (Neugebauer *et al.*, 2010a) and bovine (Neugebauer *et al.*, 2010b).

Nutritional effects on prenatal myogenesis are well documented (Dwyer *et al.*, 1994; Greenwood *et al.*, 1999; Zhu *et al.*, 2004; Du *et al.*, 2010a), but data on parental genetic and epigenetic effects are lacking. To our knowledge, only one previous study investigated genetic effects on mammalian prenatal muscle. This report described significant individual sire effects on bovine fetal biceps weight in the last trimester of gestation (Anthony *et al.*, 1986). However, the study was designed to test only for effects of different sires and did not address differential effects of maternal and paternal genomes. In the present study, we generated the largest fetal resource to date for the study of (epi)genetic effects on mammalian prenatal muscle development. This collection of defined bovine fetuses consists of both purebreds and reciprocal hybrids with Angus and Brahman genetics. The taurine (Angus) and indicine (Brahman) breeds are subspecies of the domestic cattle, currently named *Bos taurus taurus* and *Bos taurus indicus*, respectively (Sequencing *et al.*, 2009). Both subspecies originated from the wild aurochs (*Bos primigenius*) and are commonly referred to as *Bos taurus* and *Bos indicus* (*Linnaeus, 1758*; *Bojanus, 1827*; loc. cit. http://www.itis.gov) (Hiendleder *et al.*, 2008). This unique intra-species model with well defined divergent parental genomes allowed us to dissect maternal and paternal genome effects on fetal myofibre characteristics and absolute and relative muscle weights at midgestation (Day-153, 54% term). We show, for the first time, significant differential effects of parental genomes, independently or in combination with non-genetic maternal effects, on specific fetal muscles. Furthermore, we correlated expression of the imprinted non-coding RNA H19, which harbors miRNAs and is involved in regulation of *IGF2* and *IGF1R*, with fetal muscle mass, demonstrating that imprinted genes and miRNA interference provide plausible mechanisms for observed differential effects of parental genomes on fetal muscle phenotype.

5.2 Materials and Methods

5.2.1 Cattle and fetuses

All animal experiments and procedures described in this study were approved by The University of Adelaide Animal Ethics Committee (No. S-094-2005 and S-094-2005A). We used animals and semen of the Angus and Brahman breeds to study differential parental genome effects on fetal muscle phenotype at midgestation. The two breeds are subspecies of domestic cattle, commonly referred to as *Bos taurus* and *Bos indicus*, respectively (Hiendleder *et al.*, 2008; Sequencing *et al.*, 2009). Nulliparous Angus and Brahman dams which were approximately 16–20 months of age were purchased from farms in South Australia and Queensland and transferred to, and maintained at, Struan Agricultural Centre, South Australia. Animals were on pasture supplemented by silage. After an adjustment period of 3-4 weeks the animals received standard commercial estrous cycle synchronization as described previously (Anand-Ivell *et al.*, 2011). All fetuses were sired by two Brahman and three Angus bulls. Dams were pregnancy tested by ultrasound scanning and fetuses recovered in an abattoir at Day-153±1 of gestation. Fetuses were removed from the uterus, eviscerated, vacuum packed and stored frozen at –20ºC until further processing. Final maternal weight (FMW) was recorded and average maternal daily weight gain (MDG) was calculated as FMW minus weight at conception divided by gestation length (Figure S6, Appendix 8). We analysed 73 fetuses in total, including 23 Bt × Bt, 22 Bi × Bt, 13 Bt × Bi and 15 Bi × Bi (paternal genetics listed first) with both sexes represented in each genetic group. The distribution of Bt and Bi maternal and paternal genomes, and of females and males, are shown in Appendix 7**.**

5.2.2 Muscle dissection and weights

Fetuses were thawed and the head removed by disarticulation between the *Os occipitale* and first cervical vertebra atlas. *Musculus supraspinatus*, *M. longissimus dorsi*, *M. semimembranosus* and *M. quadriceps femoris* (consisting of *M. rectus femoris, M. vastus*

medialis, M. vastus intermedius and *M. vastus lateralis*) were dissected from both sides of the fetus. *M. longissimus dorsi* was defined from the 7th rib to the natural caudal end of the muscle, at the apophysis of the lumbosacral. The dissection protocol was based on Budras and Habel (Budras and Habel, 2003) and muscle nomenclature according to Tucker (Tucker, 1952). *M. semimembranosus* was obtained from 61 fetuses due to damage to some specimens from sampling adjacent *M. semitendinosus* for immunohistochemistry, described below. Dissected muscles from both sides of the fetus were weighed and absolute muscle weight was recorded as the mean weight for each muscle. Combined muscle weights were calculated as the sum of mean weight of each dissected muscle. Relative muscle weights, reflecting fetal muscle proportions, were calculated as muscle weight divided by the weight of the decapitated eviscerated fetus (see Figure S4, Appendix 8).

5.2.3 Muscle immunohistochemistry

At the time of fetus collection, a section of *M. semitendinosus* was cut from the centre of the muscle and mounted using gum tragacanth (Sigma Chemical Company, St. Louis, MO; prepared 5% wt/vol in distilled, deionized H_2O) onto a cork block, with muscle fibres running perpendicular to the cork block. Samples were frozen by immersion in iso-pentane cooled to approximately −160°C in liquid nitrogen, before storage at −80°C. Muscle tissue preparation and immunohistochemical staining followed the protocol by Greenwood et al. (Greenwood *et al.*, 2009). Briefly, 10-µm-thick, serial cross-sections were cut from each frozen sample using a cryostat microtome (ThermoShandon AS 620 Cryostat SME, Thermotrace Ltd., Noble Park, Victoria, Australia). After air-drying, cross-sections were stained against type I (slow) (clone WBMHC, Novocastra, Newcastle upon Tyne, UK; diluted 1:100 in PBS) and type II (fast) (clone MY-32, Sigma; diluted 1:400 in PBS) myosin heavy chain isoforms. Staining using these antibodies was previously shown to discern these myofibre types in ruminant fetal muscle (Greenwood *et al.*, 1999). They were revalidated in bovine fetal muscle using myofibrillar ATPase staining for the present experiment. The stained sections were

dehydrated and cleared using graded ethanols and xylenes to produce slides using a xylene-based mounting medium.

5.2.4 Myofibre classification and morphometry

Microscopic image analysis was used to classify and measure myofibres on stained slides. A Zeiss AxioPlan2 microscope fitted with Plan-Neofluar objectives (Carl Zeiss Pty. Ltd., Goettingen, Germany) and a Fujix colour digital camera (FUJIFILM Australia Pty. Ltd.) were used to produce images. Images were generated using a 40 × objective, and were captured using Analysis FIVE software (Soft Imaging System Corp. 12596 W. Bayaud Ave. Suite 300 Lakewood CO 80228, USA) and analysed using Image Pro Plus 6.0 software (Media Cybernetics, Inc. 4340 East-West Hwy, Suite 400 Bethesda, MD 20814-4411 USA). Fibre type was identified based on staining characteristics (Picard *et al.*, 1998). Myotubes were defined as cells that appeared hollow in cross-section, the remainder were considered myofibres (Picard *et al.*, 1994; Picard *et al.*, 2002). Myofibres and myotubes were classified as type I (slow) myofibre, type I (slow) myotube, type II (fast) myofibre and type II (fast) myotube (Figure S3, Appendix 8).

Morphological measurements were conducted by manually tracing anti-laminin-stained (rabbit anti-laminin, affinity isolated antibody: Sigma; diluted 1:500 in PBS) margins of cells using the draw/merge object function of Image Pro Plus 6.0. For each fetus, the serial slow or fast stained myosin heavy chain slide with highest contrast was chosen to measure myofibre characteristics. Three fields (40 × objective) of each chosen slide were analysed. For each field, cross-sectional area (CSA) and number of type I (slow) myotubes and myofibres, type II (fast) myotubes and myofibres were measured. Furthermore, number and CSA were measured irrespective of cell type. All counted cells in the field comprised total cell number, and CSA of counted cells in the field was total cell CSA. For each myofibre characteristic an average was calculated of the three fields measured. For each fetus the average number of cells measured was 369, ranging from 152 to 705 cells. The average standard deviation between

replicated fields for myofibre number was 1.3 for slow myotubes, 0.9 for slow myofibres, 5.1 for fast myotubes and 16.9 for fast myofibres. The average standard deviation between replicated fields for CSA was 43.3μm^2 for slow myotubes, 38.3μm^2 for slow myofibres, 19.7μm^2 for fast myotubes and 10.7μm^2 for fast myofibres.

5.2.5 Expression of *H19* in skeletal muscle

Samples from *M. semitendinosus* were collected into RNA later (Qiagen, Chadstone Centre, VIC, Australia) immediately after recovery of fetuses in the abattoir and stored at −80°C after equilibration for 24 hours at 2-4°C. Total RNA was extracted from *M. semitendinosus* of all fetuses by TRI Reagent® Solution (Ambion, Life Technologies™ Inc., Carlsbad, CA, USA) according to the manufacturer's instructions and RQ1-DNase treated (Promega, Madison, WI, USA). Reverse transcription was carried out using SuperScript™ III First-Strand synthesis system for RT-PCR (Invitrogen, Life Technologies™ Inc., Carlsbad, CA, USA) on 500 ng of total RNA with random hexamer oligonucleotides according to the manufacturer's instructions. Amplification of *H19* from cDNA was performed using a forward primer located at the junction of exons 3 and 4, and a reverse primer located within exon 5 (Appendix 2). Total length of this amplicon was 171 bp. Real time quantitative PCR (qPCR) reactions were performed using Fast Start Universal SYBR Green Master (Roche Diagnostics GmbH, Mannheim, Germany) in an Eppendorf Mastercycler® pro S thermal cycler (Eppendorf Inc., Hamburg, Germany) on 4μl of 40-fold diluted cDNA in a final volume of 12 μl with 6 μl of SYBR master mix (2×) at an annealing temperature of 60 ºC. Product specificity and integrity were confirmed using plots of melting curve and electrophoresis on a 2% agarose gel stained with GelRed™ Nucleic Acid Stain (Biotium Inc., Hayward, CA, USA). All qPCR experiments were performed in duplicate and the mean of both Cts used to calculate the amount of target transcript. We used the standard curve method with determination of PCR amplification efficiency. A two-fold serial dilution over eight data points was produced on a mixture of pooled cDNAs from all fetuses with equal proportions.

Three replicates were used for each dilution of the cDNA template. Non-template control was included in all experiments. We determined relative expression levels of seven putative housekeeping genes including actin beta (*ACTB)*, ribosomal protein S9 (*RPS9*), ubiquitin B (*UBB*), H3 histone family 3A *(H3F3A)*, TATA box binding protein *(TBP)*, vacuolar protein sorting 4 homolog A (*VPS4A*) and cyclin G associated kinase (*GAK*) and used geNorm program version 3.5 (Vandesompele *et al.*, 2002) to identify *GAK* and *VPS4A* (see Appendix 3) as the most stable genes for normalisation of the target gene. Expression levels of *H19* were normalised to the geometric mean of the expression levels of the selected housekeeping genes. As the normalised expression data were not normally distributed, we performed statistical analysis after logarithmic transformation of the data. The results for least square means and standard errors of means were presented after back-transformation.

5.2.6 Statistical estimation of effects and means

All data were analysed by Univariate Analysis of Variance (ANOVA) using the general linear model (GLM) procedure of SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Initially, data were fitted to the following full linear model:

$$y_{ijk} = M_i + P_j + S_k + gain(M_i) + weight(M_i) + F \times F + F \times F \times F + C \times C(M_i) + C^2(M_i) + F \times C(M_i) + F \times C \times C(M_i) + F \times C^2(M_i) + e_{ijk}$$

where y_{ijk} were myofibre characteristics, muscle weights and transcript abundance, M_i was maternal genome effect (j = Angus, Brahman), P_j was paternal genome effect (i = Angus, Brahman), S_k was fetal sex effect (k = male, female), *gain* was post-conception daily weight gain and *weight* was final maternal weight. M_i, P_j and S_k were fitted as fixed factors (F) and *gain* and *weight* were fitted as covariates (C). The covariates fitted in the model were nested within maternal genome (M_i) in order to adjust for effects of *gain* and *weight* within each of the two dam breeds. Interactions between factors and covariates were tested as follows: $F \times F$ was 2-way interaction between factors, $M_i \times P_j$, $M_i \times S_k$ and $P_j \times S_k$, $F \times F \times F$ was the 3-way

interaction between factors, $M_i \times P_j \times S_k$; $C \times C(M_i)$ was the 2-way interaction of covariates nested within maternal genome, *gain*×weight(M_i); $C^2(M_i)$ was the quadratic term of covariates nested within maternal genetics, $gain^2(M_i)$ and $weight^2(M_i)$; $F \times C(M_i)$ was the 2-way interaction between factors and covariates nested within maternal genetics, $P_j \times gain(M_i)$ and $S_k \times gain(M_i)$, $P_j \times weight(M_i)$ and $S_k \times weight(M_i)$; $F \times C \times C(M_i)$ was the 3-way interaction between factors and the two covariates nested within maternal genetics, $P_j \times gain \times weight(M_i)$ and $S_k \times weight \times gain(M_i)$; $F \times C^2$ was the interaction between factors and quadratic terms of covariates nested within maternal genetics, $P_j \times gain^2(M_i)$, $S_k \times gain^2(M_i)$, $P_j \times weight^2(M_i)$ and $S_k \times weight^2(M_i)$.

Backward stepwise elimination was used to reduce the model for each measured parameter based on type III sums of squares (SSIII) at significance level (*P*) of 0.05. Type III sums of squares are independent of the order that effects are fitted in the model (Shaw and Mitchell-Olds, 1993). Specifically, elimination started with the least significant (largest *P*-value) interaction or effect. Insignificant variables were removed stepwise according to marginality rules (Nelder, 1994) i.e. independent variables cannot be eliminated until after the interaction is eliminated due to insignificance, and lower order interactions cannot be eliminated until after the corresponding higher order interaction is eliminated. Main effects were also considered to be marginalized by corresponding nested effects of covariates. Elimination continued until only significant effects and interactions remained, or had to be retained to maintain the marginality requirements. Main effects of M_i, P_j and S_k were retained in the final model, irrespective of the significance levels. This approach retained factors of the experimental design and produced models with relatively large coefficients of determination (R^2). R^2 values, model significance levels and significance levels of factors and nested covariates in the final model for each measured parameter are shown in Table 5.1. Means for effects of factors and interactions (with *P*-values from *t*-tests of the contrast, Figures 5.3, 5.4, 5.6, 5.7) and regression slopes for nested effects of covariates (Figure 5.5, 5.7 and Figure S5, Appendix 8) were plotted according to marginal means and estimated parameters obtained

from the final model. *P*-values of maternal and/or paternal genome effects on fast myotube CSA, absolute weights of *M. supraspinatus, M. longissimus dorsi and M. quadriceps femoris*, and *H19* transcript abundance were not determined. The significant effects of final maternal weight nested within maternal genetics and/or significant interaction effects of maternal and paternal genome, would have biased *P*-values for corresponding main effects estimated with type III sums of squares (Table 5.1., Figure 5.3, 5.4, 5.7).

Only one nested quadratic effect was significant when tested; $weight^2(M_i)$ explained a significant (P = 0.007) amount of variation in absolute *M.quadriceps femoris* weight. However, examination of plotted curves with individual data points revealed that this effect was dependent upon two heavy dams with high leverage. Therefore, this quadratic effect was removed from the model and the linear effect retained. The graph for the initial quadratic effect is presented in Figure S5, Appendix 8.

The contribution of maternal genome (M_i), paternal genome (P_j), fetal sex (S_k) and significant interaction and nested effects (P<0.05) to explained variation in myofibre characteristics, muscle weights and *H19* transcript abundance, was calculated from type I sums of squares (SSI). Type I sums of squares are dependent on the order in which effects are fitted in the model and sum to the total model SS (Shaw and Mitchell-Olds, 1993; Nelder, 1994) (Figures 5.1, 5.2).

Final maternal weight (FMW) may contain both genetic and non-genetic effects as a function of breed and permanent environmental effect from origin of dam. Dams were sourced from different properties and had, therefore, been subject to different environments prior to recruitment for the experiment. By using SSI and fitting the maternal genome effect before *weight* in the model, we apportioned all the maternal genetic effect to maternal breed (M_i) and left only environmental effects attributable to *weight*. Specifically, variables and/or interactions were fitted into the final SSI model in the following order:

1) $M_i, P_j, S_k, F \times F$ and $C(M_i)$ (M_i before P_j)
2) $P_j, M_i, S_k, F \times F$ and $C(M_i)$ (P_j before M_i)

The SSI values of P_j and M_i were averaged from both models, assuming equal importance of maternal and paternal genomes. SSI values of other variables and interactions were identical for models 1 and 2. The SSI contribution of an interaction was apportioned equally to each component of the interaction. The contributions of maternal genetics (M_i), paternal genetics (P_j), fetal sex (S_k) and final maternal weight (*weight*) to myofibre characteristics, muscle weights and transcript abundance were calculated from the SSI of M_i, P_j, S_k and *weight* as a percentage of total SSI, respectively (Figure 5.1). The contribution of *weight* was defined as the non-genetic maternal effect, since the estimation of SSI values of *weight* were independent of maternal genome. The relative proportions of maternal and paternal genomes to total genetic variation in myofibre characteristics, muscle weights and transcript abundance were calculated by totalling respective contributions (Figure 5.2).

The regressions and Pearson correlation coefficients (r) for absolute and relative combined muscle weights and *H19* transcript abundance were estimated in SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

5.3 Results

5.3.1 Proportion of variation explained by parental genomes, fetal sex and non-genetic effects

Myofibre characteristics determined in *M. semitendinosus* samples included number and cross-sectional area (CSA) of type I (slow) and type II (fast) myotubes and myofibres and total cell number and total cell CSA (Figure S3, Appendix 8). Wet weights were determined for *M. supraspinatus*, *M. longissimus dorsi*, *M. quadriceps femoris* and *M. semimembranosus*. Since the four fetal groups with specific combinations of *Bos taurus taurus* (Bt) and *Bos taurus indicus* (Bi) genomes showed significant differences in carcass weights (Figure S4, Appendix 8), relative muscle weights were analyzed in addition to absolute muscle weights to identify effects of parental genomes on muscle mass independent of fetal size.

Significant final statistical models for studied muscle parameters with adjusted R^2 values and significance levels of retained variables are presented in Table 5.1. Parental genomes, fetal sex, and effects of maternal weight, caused by non-genetic variation and nested within maternal genomes (see methods), each contributed differentially to muscle parameters (Figure 5.1). Parental genome was the most important source of variation for all studied traits with significant final statistical models. Maternal and paternal genomes together explained most of the variation in myofibre size (80-96%), absolute muscle weights (82-89%) and relative muscle weights (56-93%). Fetal sex contributed less to variation in myofibre characteristics (4-20%) and absolute (2-13%) and relative muscle weights (7-44%). Non-genetic maternal effects of final maternal weight accounted for some variation in absolute weights of *M. supraspinatus*, *M. longissimus dorsi* and *M. quadriceps femoris* (5-6%). Combined absolute and relative muscle weight showed parental genome contributions of 94% and 72%, respectively (Figure 5.1).

The relative contributions of maternal and paternal genomes to total explained (epi)genetic variation in myofibre size and muscle weights are shown in Figure 5.2 Maternal

genome explained most of the (epi)genetic variation in fast myofibre CSA (93%) whereas the

Table 5.1 Summary of the final general models (type III sums of squares) for myofibre characteristics, muscle weight parameters and H19 gene expression with adjusted R2 values and significance levels (*P*-values) of models and variables.
Only *P*-values for factors, interactions and nested effects retained in the final model are shown.

Myofibre characteristics	R^2	***P*-values**					
		Model	**Maternal genome**	**Paternal genome**	**Fetal sex**	**Maternal×Paternal genome** [b]	**Final maternal weight (Maternal genome)** [c]
Fast myotube CSA[a]	0.152	0.0043	ND	ND	0.4337	0.0129	
Fast myofibre CSA[a]	0.111	0.0117	0.0031	0.7345	0 1390		
Total cell CSA[a]	0.101	0.0160	0.0076	0.4280	0 1434		
Absolute muscle weights							
M. supraspinatus	0.689	8.7E-17	ND	2.3E-07	7.0E-04		0.0112
M. longissimus dorsi	0.649	1.2E-15	ND	6.9E-08	0 2828		0.0420
M. quadriceps femoris	0.666	1.0E-14	ND	2.1E-05	0.0457		0.0256
M. semimenbranosus	0.595	7.2E-12	5.1E-12	0.04974	0.0026		
Combined muscles	0.667	2.9E-14	5.0E-13	3.3E-05	0.0095		
Relative muscle weights							
M. supraspinatus	0.210	3.3E-04	0.5294	2.7E-05	0 2327		
M. longissimus dorsi	0.441	4.8E-09	0.0014	9.8E-08	1.6E-04		
M. quadriceps femoris	0.332	1.6E-06	0.0048	1.2E-04	1.4E-04		
M. semimenbranosus	0.136	0.0115	0.0176	0.4209	0.0637		
Combined muscles	0.517	2.1E-09	2.3E-04	2.2E-06	5 9E-06		
***H19* expression**	0.350	4.0E-06	ND	ND	0 1288	0.0051	0.0296

[a]Total cell CSA: Average cross-sectional area of muscle cells irrespective of cell type.
[b]Maternal × paternal genome: Effect of maternal and paternal genome interaction.
[c]Final maternal weight (maternal genome): Effect of final maternal weight nested in maternal genome. ND: Not determined because of significant interaction and/or nested effect of final maternal weight.

paternal genome accounted for most of the variation in fast myotube CSA (68%). Maternal genome again explained most of the variation in total cell CSA (82%). Maternal genome also explained most of the genetic variation (59-88%) in all absolute muscle weights. Paternal genome, in contrast, explained most of the genetic variation (54-97%) in relative weights of *M. supraspinatus*, *M. longissimus dorsi* and *M. quadriceps femoris*. However, maternal genome accounted for 82% of genetic variation in relative weight of *M. semimembranosus.* Combined absolute muscle weight was predominantly affected by maternal genome (73%) while combined relative muscle weight showed a stronger effect of paternal genome (63%).

Overall, the data clearly showed a distinct pattern of effects of maternal and paternal genomes with an increase of maternal genome contributions (or conversely, a decrease of paternal genome contributions) to variation in absolute and relative weights of muscles from the thoracic limb (*M. supraspinatus*) to muscles from the torso (*M. longissimus dorsi*) and pelvic limb (*M. quadriceps femoris* and *M. semimembranosus*) (Figure 5.2).

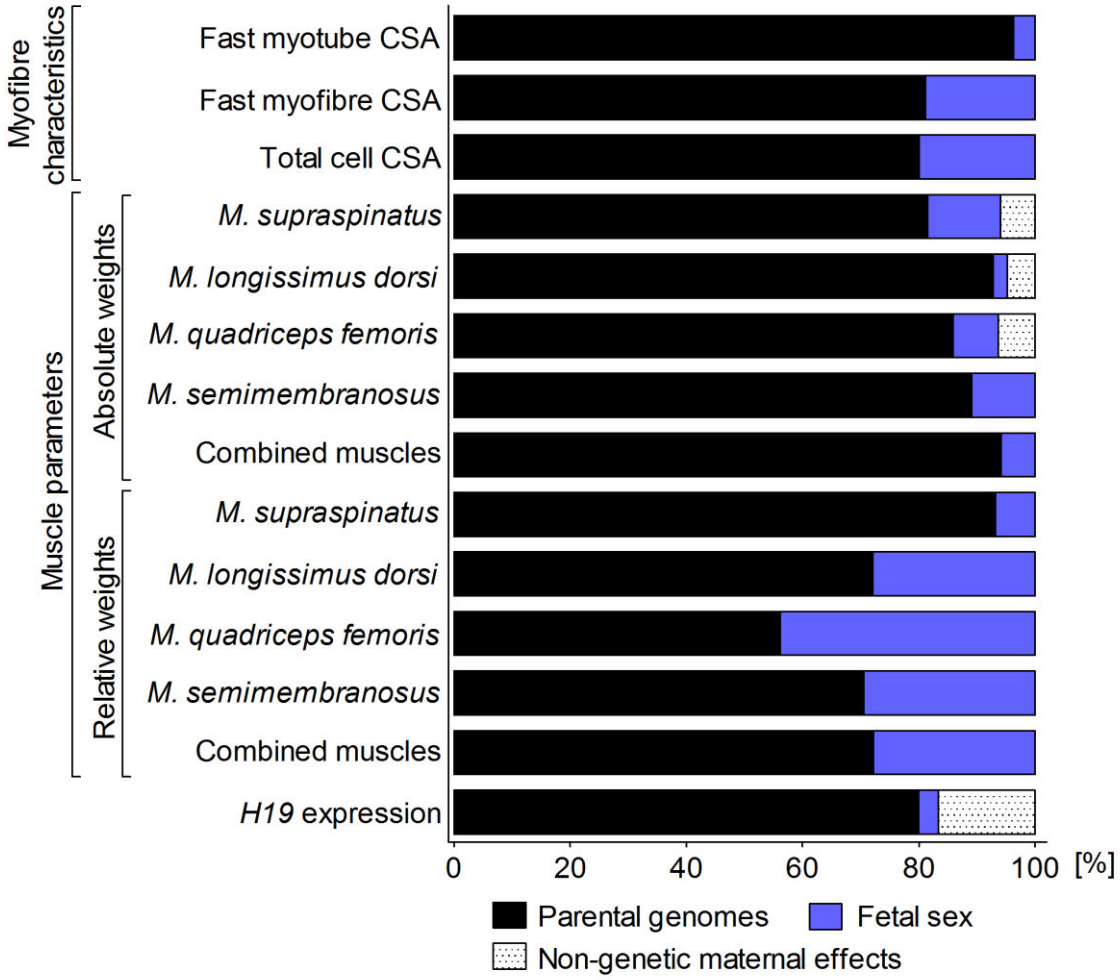

Figure 5.1 Relative contributions of parental genomes, fetal sex and non-genetic maternal effects to explained variation in fetal myofibre characteristics, absolute and relative muscle weights, and *H19* transcript abundance. Myofibre characteristics were determined in *M. semitendinosus.* Maternal and paternal genome, fetal sex and other significant effects were retained in the final general linear models as presented in Table 5.1. Non-genetic maternal effect: Final maternal weight at mid-gestation. CSA: Cross-sectional area. Total cell: All myofibres measured regardless of cell type. Combined muscle weights: Sum of *M. supraspinatus, M. longissimus dorsi, M. semimembranosus* and *M. quadriceps femoris* weight. Relative muscle weight: Absolute muscle weight divided by decapitated and eviscerated fetal carcass weight.

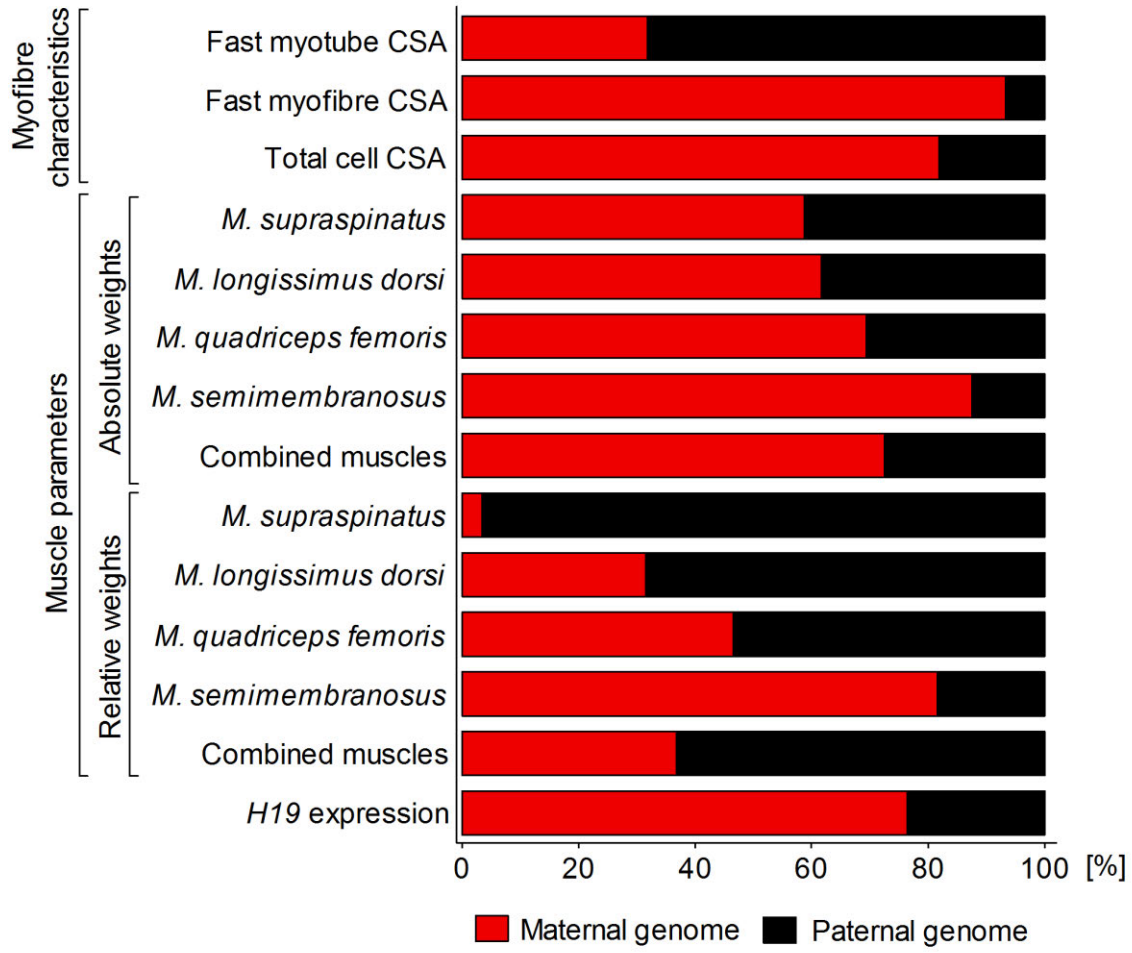

Figure 5.2 Relative contributions of maternal and paternal genome to genetic variation in fetal myofibre characteristics, absolute and relative muscle weights, and *H19* transcript abundance.
bre characteristics were determined in *M. semitendinosus.* CSA: Cross-sectional area. Total cell: All myofibres measured regardless of cell type. Combined muscle weights: Sum of *M. supraspinatus*, *M. longissimus dorsi*, *M. semimembranosus* and *M. quadriceps femoris* weight. Relative muscle weight: Absolute muscle weight divided by decapitated and eviscerated fetal carcass weight.

5.3.2 Specific effects of Bt and Bi genomes, fetal sex and maternal weight

Least square means for specific effects of *Bos taurus taurus* (Bt, Angus) and *B. taurus indicus* (Bi, Brahman) maternal and paternal genomes, fetal sex and non-genetic maternal effects of final maternal weight, as detailed in statistical models for myofibre characteristics and muscle weights (Table 5.1.), are presented in Figure 5.3-5.6 Fast myotube CSA was affected by a significant interaction between maternal and paternal genomes ($P<0.05$). Fetuses with Bt × Bt genomes had larger CSA ($P<0.05 – 0.01$) than fetuses of other genetic combinations (Figure 5.3 A). Maternal genome significantly affected fast myofibre CSA and total cell CSA (both $P<0.01$) with Bt genomes causing larger CSA than Bi genomes (Figure 5.3 B,C).

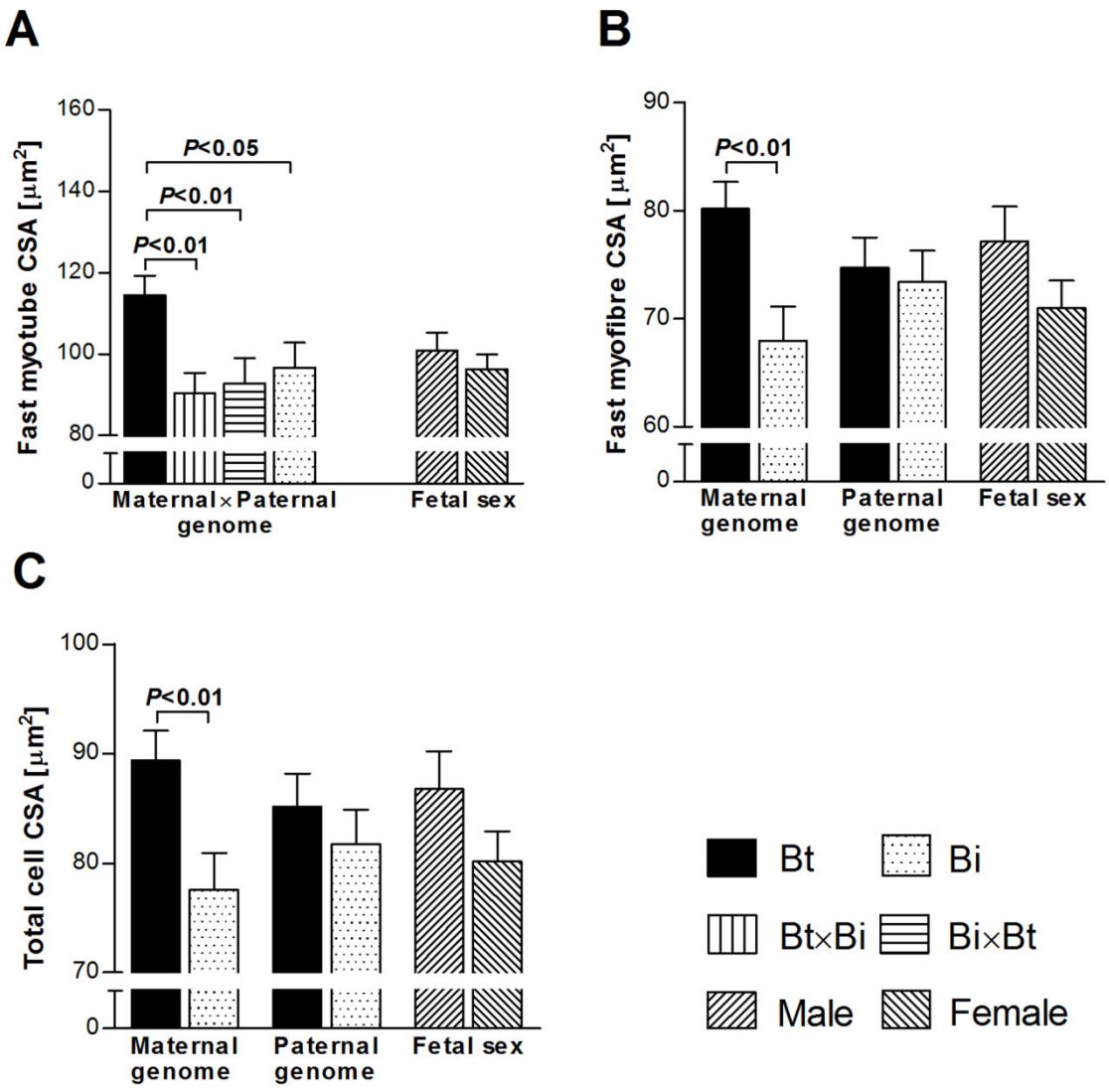

Figure 5.3 Specific effects of maternal genomes, paternal genomes and fetal sex on fetal myofibre characteristics of M. semitendinosus at midgestation.
Least square means with standard errors of means are shown and *P*-values for significant differences (*t*-test) between means for fast myotube CSA (**A**), fast myofibre CSA (**B**) and total cell CSA (**C**) are indicated. CSA: Cross-sectional area. Total cell: All myofibres measured regardless of cell type. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

Maternal genome significantly affected absolute weights of all muscles (Figure 5.4 A-D), but *M. supraspinatus, M. longissimus dorsi* and *M. quadriceps femoris* also showed significant non-genetic effects of final maternal weight nested within maternal genome (all $P<0.05$, see below). Maternal genome effects, independent of maternal weight, were detected for *M. semimembranosus* ($P<0.0001$). Paternal genome, in contrast, independently and strongly affected absolute weights of *M. supraspinatus, M. longissimus dorsi* and *M.*

quadriceps femoris (all $P<0.0001$), but not *M. semimembranosus*, a muscle strongly affected by maternal genome (see above).

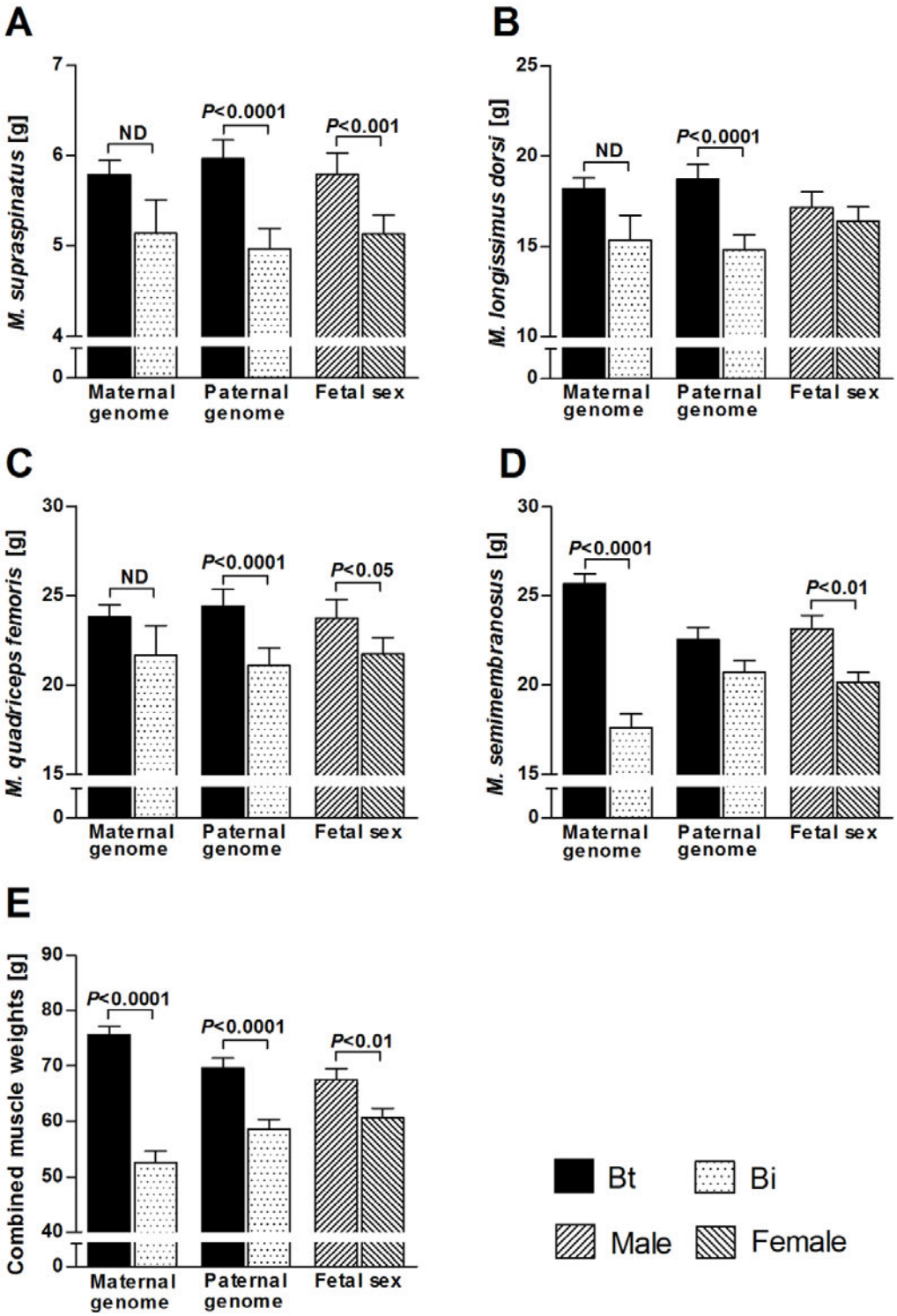

Figure 5.4 Specific effects of maternal genomes, paternal genomes and fetal sex on fetal absolute muscle weights at midgestation.

Least square means with standard errors of means are shown and *P*-values for significant differences (*t*-test) between means for *M. supraspinatus* (**A**), *M. longissimus dorsi* (**B**), *M. quadriceps femoris* (**C**), *M. semimembranosus* (**D**) and combined muscle weight (sum of weights of dissected muscles) (**E**) are indicated. ND: Not determined because of significant nested effect of final maternal weight (see Figure 5.5). Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

Combined muscle weights showed significant effects of maternal and paternal genome that were stronger for the maternal genome. Irrespective of maternal or paternal origin Bt genome always increased, and Bi genome always decreased, absolute muscle weights. Fetal sex significantly affected absolute weights of *M. supraspinatus* ($P<0.001$), *M. quadriceps femoris* ($P<0.05$) and *M. semimembranosus* ($P<0.01$) with heavier muscles in males than in females (Figure 5.4 A,C,D). Non-genetic effects of final maternal weight, nested within maternal genome, on absolute weights of *M. supraspinatus*, *M. longissimus dorsi* and *M. quadriceps femoris* ($P<0.05$) indicated positive linear relationships for Bi and Bt, but with a higher intercept and less slope in Bt (Figure 5.5 A-C). Only one of the quadratic maternal weight effects tested yielded a significant result (*M. quadriceps femoris*, $P<0.01$). Examination of plotted curves with individual data points revealed that this was dependent upon two heavy dams with high leverage (see methods and Figure S5, Appendix 8). Therefore, we fitted linear effects throughout. Nested effects of post conception maternal daily weight gain were not significant for any of the investigated muscle parameters.

Maternal genome had moderate effects on relative weights of *M. longissimus dorsi* ($P<0.01$), *M. quadriceps femoris* ($P<0.01$) and *M. semimembranosus* ($P<0.05$), but not *M. supraspinatus*. Paternal genome showed strong effects on *M. supraspinatus* ($P<0.0001$), *M. longissimus dorsi* ($P<0.0001$) and *M. quadriceps femoris* ($P<0.001$), but not *M. semimembranosus*. Combined relative muscle weight showed stronger effects of the paternal genome. Again, as for absolute muscle weights, Bt genome increased relative muscle weights irrespective of parental origin (Figure 5.6 A-D). Strong fetal sex effects were present for relative weights of *M. longissimus dorsi* ($P<0.001$) and *M. quadriceps femoris* ($P<0.001$), with greater weights in females than in males (Figure 5.6 B,C).

5.3.3 Expression of the H19 lincRNA

Expression of the H19 large intergenic non-coding RNA (lincRNA) was measured by real-time quantitative PCR in *M. semitendinosus* samples. Transcript abundance was

significantly affected by an interaction between maternal and paternal genomes ($P<0.01$) (Table 5.1.). Fetuses with Bi × Bi genome showed higher levels of *H19* transcript ($P<0.01$) than fetuses of other genetic combinations (Figure 5.7 A). Transcript abundance was also affected by final maternal weight ($P<0.05$) nested within maternal genome (Figure 5.7 B). Subsequent regression analyses revealed significant negative relationships ($P<0.001$) between *H19* transcript abundance and combined absolute and relative muscle weight (Figure 5.8 A,B).

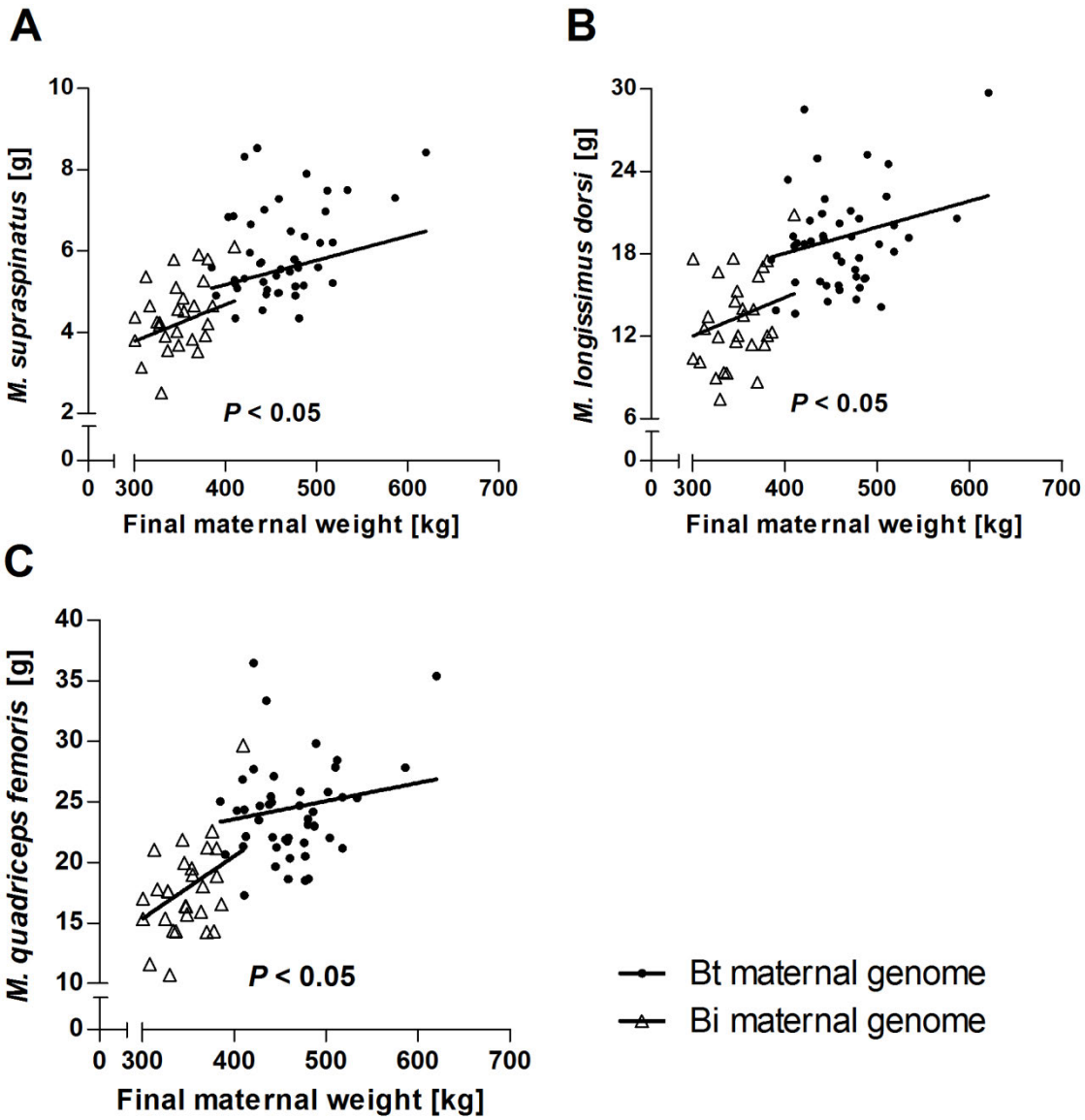

Figure 5.5 Effects of final maternal weight nested within maternal genomes on fetal absolute muscle weights at midgestation.

P-values (ANOVA) of significant linear regressions within Bt and Bi maternal genetics on absolute weights of *M. supraspinatus* (**A**), *M. longissimus dorsi* (**B**) and *M. quadriceps femoris* (**C**) are indicated. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

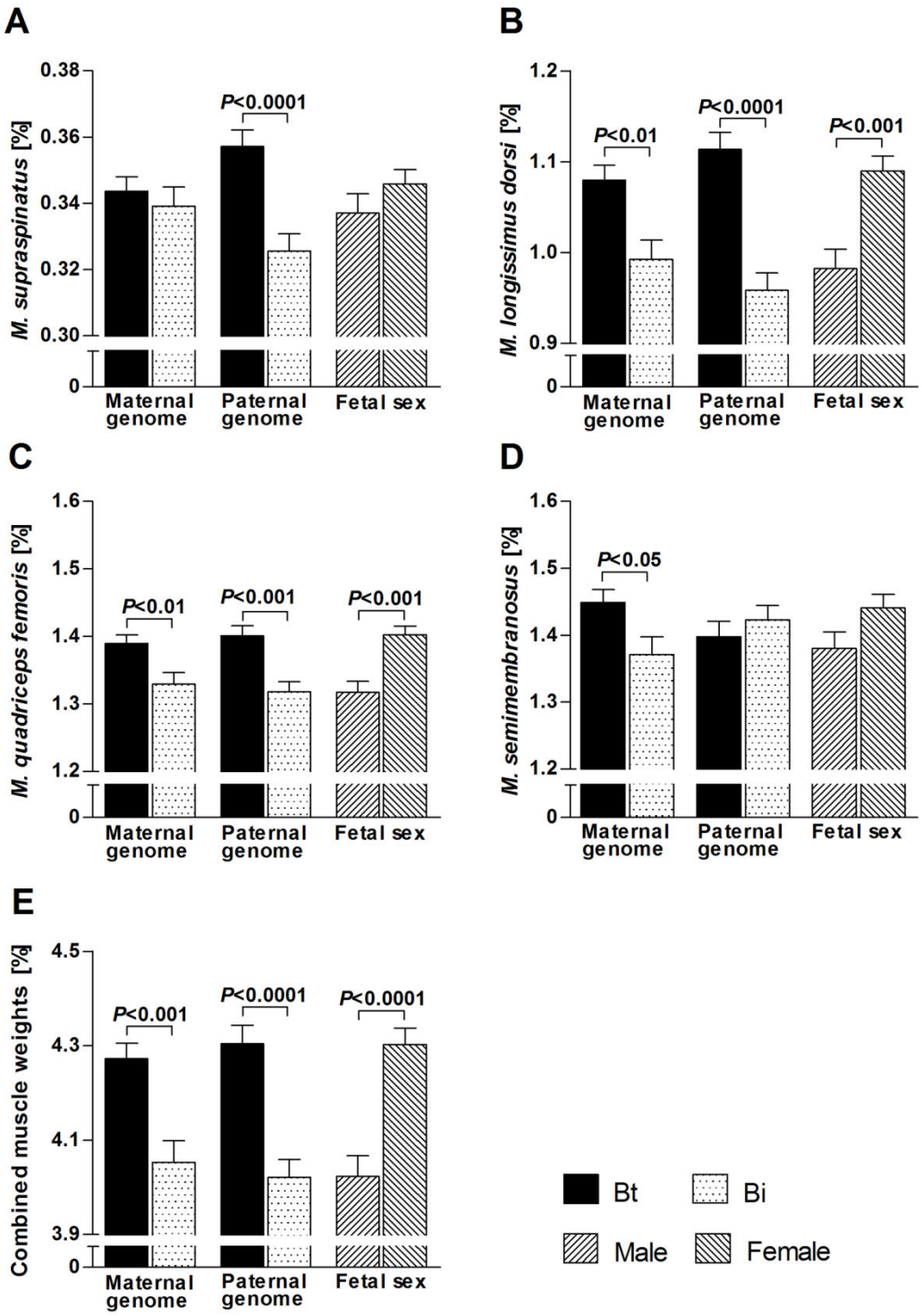

Figure 5.6 Specific effects of maternal genomes, paternal genomes and fetal sex on fetal relative muscle weights at midgestation.

Relative muscle weights were calculated as absolute muscle weight divided by fetal carcass weight. Least square means with standard errors of means and *P*-values for significant differences (*t*-test) between means for *M. supraspinatus* (**A**), *M. longissimus dorsi* (**B**), *M. quadriceps femoris* (**C**) and *M. semimembranosus* (**D**) are indicated. Combined relative muscle weight is the sum of relative weights of dissected muscles. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

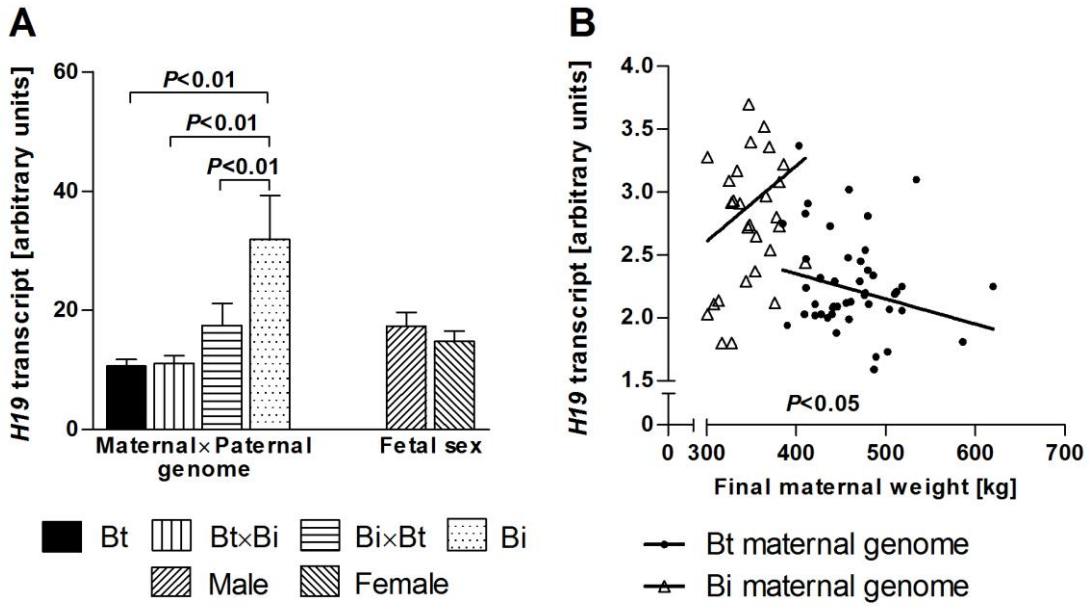

Figure 5.7 Effects of interaction of maternal and paternal genomes, fetal sex and final maternal weight nested within maternal genetics on *H19* transcript abundance in fetal M. semitendinosus at midgestation.
Least square means with standard error of means and *P*-values for significant differences (*t*-test) between means **(A)** and significant regressions of final maternal weight nested within Bt and Bi maternal genomes **(B)** are shown. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

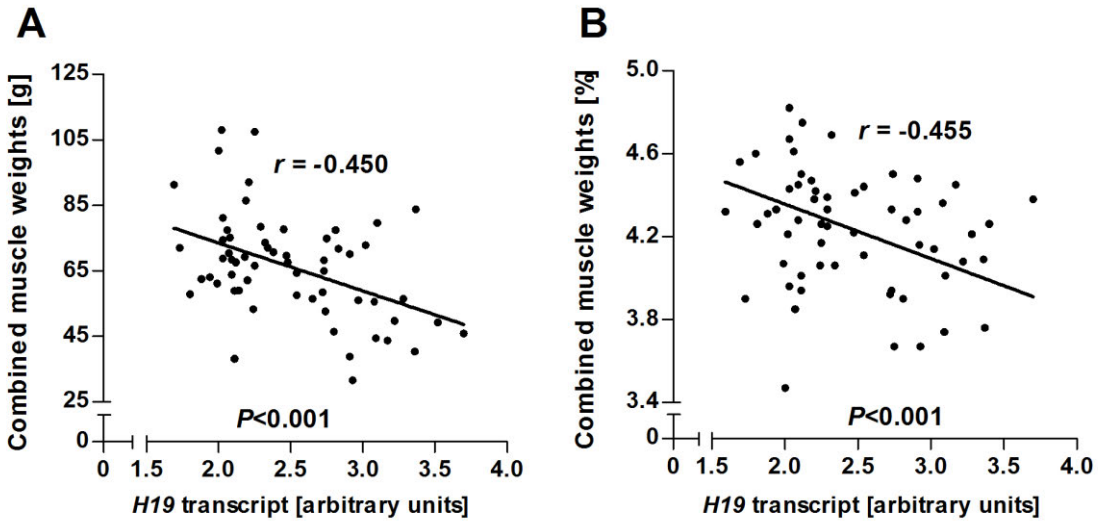

Figure 5.8 Regressions of fetal muscle mass at midgestation on *H19* transcript abundance. (A) Absolute muscle mass and (B) relative muscle mass.
Muscle mass is combined absolute and relative weights of *M. supraspinatus*, *M. longissimus dorsi*, *M. quadriceps femoris* and *M. semimembranosus.* *P*-values and Pearson correlation coefficients (*r*) are indicated.

5.4 Discussion

To our knowledge, this is the first study to examine effects of maternal and paternal genome on fetal myofibre characteristics and muscle mass. Our results showed that differential effects of parental genomes were the most important determinants of fetal muscle phenotype at midgestation. Fetal sex and non-genetic effects of final maternal weight had a significant but lesser impact on some investigated muscle parameters (Figure 5.1). Considering the fetal programming of skeletal muscle development (Picard *et al.*, 2002; Du *et al.*, 2010a), these findings are consistent with generally medium to high heritabilities reported for postnatal myofibre size and muscle mass in mammals, including bovine (Larzul *et al.*, 1997; Rehfeldt *et al.*, 1999; Engellandt and Tier, 2002; Smith *et al.*, 2007; Mansan Gordo *et al.*, 2012). Since myotubes are immature myofibres that decrease in size as myogenesis progresses (Martyn *et al.*, 2004), both the predominant contribution of the paternal genome to variation in fast myotube cross sectional area (CSA), and the predominant contribution of the maternal genome to variation in fast myofibre CSA (Figure 5.2), indicate specific roles of maternal and paternal genomes in myofibre differentiation and maturation.

The observed differences between *Bos taurus taurus* (Bt) and *Bos taurus indicus* (Bi) genomes likely result from allelic differences in genes with parent-of-origin effects controlling myofibre development. Evidence for subspecies differences in postnatal fibre type ratios and size, and in absolute postnatal muscle weights of Bt and Bi breeds has been reported previously (Whipple *et al.*, 1990; Ferrell, 1991; Strydom and Smith, 2010). Differential parental effects were masked in total cell CSA, which was predominantly affected by maternal genome (Figure 5.2). Muscle specific differences in fibre type composition and size (Totland and Kryvi, 1991) could explain some of the varying contributions of maternal and paternal genomes to different muscles. The present data suggest that maternal genes are important determinants of myofibre development and muscle mass.

Variation in the maternally inherited mitochondrial genome has been associated with effects on postnatal muscle mass (Mannen *et al.*, 1998), but specific effects of maternal genes

in myogenesis remain, to our knowledge, unexplored. The present results are in agreement with recent data obtained by statistical modelling and imprinted quantitative trait loci (QTL) analyses which suggested significant maternal parent-of-origin effects for postnatal muscle traits (Boysen *et al.*, 2010; Neugebauer *et al.*, 2010a; Neugebauer *et al.*, 2010b). In contrast, paternally expressed genes with effects on myogenesis have been identified previously and were studied in detail. This includes the imprinted Delta-like 1 homolog (*DLK1*), which has been implicated in the commitment and/or proliferation of fetal myoblasts (Jason *et al.*, 2008) and in increased postnatal myofibre diameter and muscle mass (Davis *et al.*, 2004; Jason *et al.*, 2008). Further examples of gene-specific genetic and epigenetic regulatory mechanisms that could explain effects of maternal and paternal genomes on fetal muscle phenotype observed in the present study are found in the IGF1-AKT/PKB pathway (Schiaffino and Mammucari, 2011). In the mouse embryo, paternally expressed IGF2 is required for fibre type specification (Merrick *et al.*, 2007). This imprinted gene has been identified as a QTL for postnatal muscle mass (Jin-Tae Jeon, 1999; Nezer *et al.*, 1999) and encodes a miRNA in intron 2 that targets transcripts of the non-imprinted *IGF1* gene (Wang, 2008). Several other genes in this pathway, including *PTEN*, a gatekeeper for the accretion of muscle mass (Sawitzky *et al.*, 2012), are also targeted by miRNAs (Crist and Buckingham, 2009; Ge and Chen, 2011). The significance of allelic differences in miRNA target sequences for regulation of muscle mass by epistatic miRNA interference has been demonstrated with myostatin alleles in the ovine model (Clop *et al.*, 2006). Genome sequences of *Bos taurus taurus* and *Bos taurus indicus* revealed genomic variation (Sequencing *et al.*, 2009; Canavez *et al.*, 2012) that provides a basis for maternal and paternal (epi)genetic effects on myogenesis described in the present study.

The imprinted long intergenic non-coding (linc) RNA H19 is maternally expressed at high levels in embryonic and fetal tissues, including skeletal muscle (Lee *et al.*, 2002; Gabory *et al.*, 2006). The H19 gene is located immediately downstream of *IGF2* and involved in regulation of *IGF2* expression. More recently, *H19* has been identified as the master regulator

of an imprinted gene network with important roles in growth and development (Gabory *et al.*, 2010). The *H19* transcript was further shown to harbor a miRNA that suppresses *IGF1R* expression and prenatal growth (Cai and Cullen, 2007; Keniry *et al.*, 2012). Gene expression data generated in the present study demonstrated significant differences in *H19* transcript abundance of *M. semitendinosus* from fetuses with different parental combinations of Bt and Bi genomes (Figure 5.7). In human, *H19* expression is also affected by genetic background (Lin *et al.*, 1999). Furthermore, *H19* expression was significantly negatively correlated with absolute and relative fetal muscle mass (Figure 5.8). This is consistent with the previously reported role of *H19* as a negative regulator of prenatal growth and development (Keniry *et al.*, 2012). Thus, imprinted gene expression and miRNA interference are plausible mechanisms for differential effects of maternal and paternal genomes observed in the present study.

Our data indicated predominant contributions of the maternal genome to variation in absolute fetal muscle weights and predominant contributions of the paternal genome to variation in relative fetal muscle weights (Figure 5.2). With respect to maternal genome, these results are in agreement with data available from an analysis of parent-of-origin effects on postnatal bovine muscle, where absolute muscle weights were predominantly affected by imprinted maternal genetic factors (Neugebauer *et al.*, 2010b). The genetic conflict hypothesis of genomic imprinting states that paternally expressed genes promote, and maternally expressed genes limit, fetal growth (Moore and Haig, 1991). Accordingly, maternal genes are expected to control fetal size to avoid detrimental effects for the mother that are associated with higher nutrient transfer to the fetus and increased birthweight (Moore and Haig, 1991). In the present study, fetuses with different maternal and paternal combinations of Bt and Bi genomes showed significant differences in carcass weight (Figure S4, Appendix 8) that are consistent with a phenotypic pattern of genomic imprinting for maternally expressed genes (see Figure 1 in (Wolf *et al.*, 2008)) affecting fetal size. Correlations between absolute muscle weights and fetal carcass weight ranged from r = 0.88 (*M. longissimus dorsi*, $P<0.0001$) to

r = 0.95 (*M. quadriceps femoris*, $P<0.0001$). Effects of the maternal genome on absolute muscle weights are, therefore, likely to be primarily correlated effects of maternal (epi)genetics on fetal size, presumably via imprinted genes (Moore and Haig, 1991; Wolf *et al.*, 2008) and/or epistatic interaction of miRNAs and their target sites (see above). However, mitochondrial DNA (Mannen *et al.*, 1998; Hiendleder *et al.*, 2004b), or X-chromosome effects (Amen *et al.*, 2007a; Amen *et al.*, 2007b) could also contribute to Bt and Bi maternal (epi)genetic effects on muscle phenotype (Figure 5.3, 5.4).

Predominance of parental genomic contributions to muscle weights varied from maternal for absolute weights to paternal for relative weights. An exception was *M. semimembranosus*, which showed only a weak maternal ($P<0.05$) and no paternal genome effect (Figure 5.2, 5.4, 5.6). Considering the genetic conflict hypothesis (Moore and Haig, 1991), it appears that the full extent of paternal genome effects on muscle mass and shape should manifest postnatally, without causing detrimental effects to mother or fetus at parturition. Such effects could nevertheless be expected to be programmed prenatally (Picard *et al.*, 2002; Du *et al.*, 2010a) and to be independent of absolute fetal muscle weights. This interpretation is consistent with the imprinting status of major regulators of fetal muscle development and growth in bovine e.g. paternally expressed growth promoting *IGF2* and maternally expressed growth inhibiting *IGF2R* (Dindot *et al.*, 2004; Hiendleder *et al.*, 2004a). Imprinted gene effects with paternal mode of expression responsible for increased muscle mass in ovine (*DLK1*) and porcine (*IGF2*) manifest postnatally (Jin-Tae Jeon, 1999; Nezer *et al.*, 1999; Davis *et al.*, 2004; Cockett *et al.*, 2005).

Analyses of the proportion of parental contributions to muscle traits revealed that contributions of the maternal genome to absolute and relative fetal muscle mass increased (or conversely, contributions of the paternal genome decreased) from thoracic limb to torso and pelvic limb. This novel spatial effect of the maternal genome mirrored paternal effects on muscle mass observed in sheep with the polar overdominant callipyge mutation (Koohmaraie *et al.*, 1995; Cockett *et al.*, 1996; Jackson *et al.*, 1997). Consistent with our findings, a recent

study in porcine identified a quantitative trait locus (QTL) with maternal polar overdominance that affected postnatal pelvic limb muscle mass (Boysen *et al.*, 2010). Moreover, statistical modelling of parent-of-origin effects on postnatal muscle mass in porcine and bovine also showed a preponderance of maternal effects attributed to genomic imprinting (Neugebauer *et al.*, 2010a; Neugebauer *et al.*, 2010b). The significant switch in gene expression, including imprinted transcripts from the *DLK1-DIO3* region, in ovine *M. longissimus dorsi* from fetus to neonate (Byrne *et al.*, 2010), could indicate developmental stage specific roles of maternal and paternal genomes in myogenesis. Interestingly, the imprinting status of genes can change from monoallelic to non-imprinted biallelic expression during development (Davies, 1994; McLaren and Montgomery, 1999; Goodall and Schmutz, 2007). Statistical analyses of experimental data for postnatal growth and development in mouse identified multiple imprinted QTL with complex temporal patterns of parent-of-origin effects (Wolf *et al.*, 2008). It is tempting to speculate that such effects could also be spatial.

Significant effects of sex on postnatal muscle mass of mammals, including bovine, have been reported (Seideman and Crouse, 1986; Fortin *et al.*, 1987; Uttaro *et al.*, 1993; Larzul *et al.*, 1997), but the present study is the first to examine sex effects in prenatal myogenesis. In agreement with fetal programming of postnatal muscle mass discussed above (see maternal and paternal genomes), sex explained greater proportions of variation in relative fetal muscle weights than in absolute muscle weights (Figure 5.1). Male fetuses had higher absolute muscle weights but lower relative muscle weights than females (Figure 5.4, 5.6). The latter findings are in agreement with results for postnatal muscle weights in porcine (Fortin *et al.*, 1987) and ovine (Santos *et al.*, 2007). In the present study, fetal sex had no effect on relative weight of *M. supraspinatus*, a shoulder muscle, but significantly affected the relative weights of *M. longissimus dorsi* (loin) and *M. quadriceps femoris* (pelvic limb) (Figure 5.6). This is again similar to results obtained for postnatal muscle mass in ovine (Santos *et al.*, 2007), where sex had no effect on shoulder muscle percentage but significantly affected loin muscle percentage, with greater muscle percentage in females than in males. An explanation for these

results could be that fetal shoulder muscle mass is under strong selection because of its relevance for birthing difficulties and thus survival. The loin and pelvic limb region of females may require a higher relative muscle weight to maintain sex-specific postnatal proportions and reproductive functions, which may be programmed during fetal development.

Our analyses identified significant contributions of final maternal weight (FMW) to variation in absolute fetal muscle weights and *H19* expression at midgestation (Figure 5.1). These non-genetic maternal effects were estimated as nested effects within maternal genetics using type I sums of squares in the final linear models, allowing the removal of maternal genetic contributions from effects of FMW (see methods). Non-genetic maternal components can be explained by differences in environmental factors acting on dams before they were recruited for the experiment. These environmental effects could not be erased during several weeks of adjustment under a controlled environment prior to the start of the experiment. To our knowledge, pre-conception non-genetic maternal contributions to variation in fetal muscle mass have not been reported previously. The estimated regression coefficients suggested that the same mechanisms affect fetal muscle mass in dams with Bt and Bi genomes (Figure 5.5, 5.7).

In conclusion, we have shown for the first time, that fetal muscle development is differentially affected by maternal and paternal genome, independently, or in combination with non-genetic maternal effects. Our statistical analyses of effects of parental genomes, and molecular data for the imprinted maternally expressed lincRNA H19, suggested that imprinted gene networks (Gabory *et al.*, 2010) and epistatic miRNA interference (Clop *et al.*, 2006) could be major drivers of the observed parental effects on fetal muscle traits. Our conclusions are supported by results from statistical modelling of postnatal muscle traits (Engellandt and Tier, 2002; Neugebauer *et al.*, 2010a; Neugebauer *et al.*, 2010b) which identified parent-of-origin effects attributed to imprinted genes as a major source of variation. Detailed molecular profiles are now required to elucidate genetic, epigenetic and non-genetic components and interactions that control variation in prenatal muscle traits. Our data further

suggest that specific combinations of (epi)genetic and non-genetic factors can be used to optimise fetal, and therefore, postnatal muscle development and phenotype. Non-Mendelian (epi)genetic and non-genetic maternal effects can help understand unexplained variation in postnatal muscle traits. These traits may be highly variable within populations, even when genetics and environment are well controlled (Reverter *et al.*, 2003; Greenwood *et al.*, 2007).

Acknowledgments

RX is a recipient of China scholarship and Adelaide University China Fee Scholarship provided by China Scholarship Council and The University of Adelaide. MG-S is recipient of a PhD scholarship from the Iranian Ministry of Science, Research & Technology. SH is a JS Davies Fellow. CTR is a NHMRC Senior Research Fellow (APP1020749). We would like to thank Mick Deland, John Cooper and Struan Research Centre farm staff (SARDI) for animal management, Sarah Truran and David Lines for assistance in fetus collection and Lin Lin for help with figures.

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Chapter 6

General discussion

6.1 Developmental changes in transcript abundance of imprinted genes in bovine tissues

In the present study, we have characterised tissue-specific quantitative changes in expression of the imprinted genes *IGF2*, *IGF2R*, and their regulatory lincRNA genes, *H19* and *AIRN*, in key stages of bovine prenatal development and postnatal life. We also analysed tissue and developmental stage-specific expression patterns of alternative *IGF2* promoters. To our knowledge, this is the first comprehensive study to examine developmental changes in transcript abundance of these imprinted genes using a quantitative method, real time qPCR. We confirmed that the studied genes are highly active during bovine prenatal development and are downregulated after birth. The genes studied here belong to the *IGF* system, which is essential in prenatal development (Heyner *et al.*, 1989; Rappolee *et al.*, 1992; Adamson, 1993; Baker *et al.*, 1993; Heyner *et al.*, 1993; Schultz *et al.*, 1993; Oksbjerg *et al.*, 2004; Randhawa and Cohen, 2005). The *IGF2* global transcripts displayed a similar tissue-specific pattern of expression as *IGF2R*, with the exception of brain and cotyledon. The highest level of *IGF2* transcription in cotyledon was observed in the embryo and persisted to the fetal stage, and expression of *IGF2R* in cotyledon peaked in midgestation. The relative expression level of *IGF2/IGF2R* in the studied fetal tissues, which can be interpreted as an indicator of the relative level of free IGF2 protein available to the tissues, was subject to developmental change and was lower in tissues from midgestation fetuses compared with the embryonic stage. This could indicate that more free IGF2 is required by fetal tissues at the earlier stage of prenatal development compared with midgestation. Differential relative expression of *IGF2* and *IGF2R* at embryonic and fetal stages could be linked to their functions at different stages of prenatal development. Accumulating evidence suggests that *IGF2* plays a key role in placental development and function particularly at earlier stages of gestation (Kanai-Azuma *et al.*, 1993; Kniss *et al.*, 1994; Irving and Lala, 1995; Hamilton *et al.*, 1998; McKinnon *et al.*, 2001; Hills *et al.*, 2004). It has been shown that *IGF2* affects placental growth via interaction

with *IGF2R*, which activates signal transduction pathways (McKinnon *et al.*, 2001; Sferruzzi-Perri *et al.*, 2008; Harris *et al.*, 2011).

Relative *IGF2R* transcript abundance in brain, compared with other tissues, was higher than that for *IGF2*, which is compatible with a widespread distribution of Igf2r transcript/protein observed in rat brain (Hawkes and Kar, 2004). Several lines of evidence demonstrated that IGF2 can promote growth, proliferation and differentiation of a variety of neuronal cell types (Lenoir and Honegger, 1983; Lim *et al.*, 1985; Recio-Pinto *et al.*, 1986; Knusel *et al.*, 1990; Liu and Lauder, 1992; Neff *et al.*, 1993; Konishi *et al.*, 1994; Sondell *et al.*, 1997; Pu *et al.*, 1999; Silva *et al.*, 2000). Our results revealed the lowest ratio of *IGF2/IGF2R* transcript abundance in brain compared with other tissues. The significance of the relatively high level of *IGF2R* transcripts and specific functions of IGF2R in brain remains unclear. Several studies suggest different functions for IGF2R in brain. For example, IGF2R is involved in internalisation and degradation of LIF (leukemia inhibitory factor) (Blanchard *et al.*, 1999), activation of TGF-β precursor form (Dennis and Rifkin, 1991; Ghahary *et al.*, 1999; Villevalois-Cam *et al.*, 2003) and mediating the growth-inhibitory effect of retinoic acid (Kang *et al.*, 1997; Kang *et al.*, 1998; Kang *et al.*, 1999) all of which are known to modulate the functions of the nervous system (Murphy *et al.*, 1997; Zetterström *et al.*, 1999; Böttner *et al.*, 2000; Malik *et al.*, 2000; Turnley and Bartlett, 2000; Krieglstein *et al.*, 2002; Thompson Haskell *et al.*, 2002; Bauer *et al.*, 2003; Maden and Hind, 2003; McCaffery *et al.*, 2003; Luo *et al.*, 2004; Haskell and LaMantia, 2005; Lane and Bailey, 2005; Clagett-Dame *et al.*, 2006; McCaffery *et al.*, 2006; Aoto *et al.*, 2008). However, the importance of the *Igf2/Igf2r* system in brain development has been challenged by the observations that mutant mice carrying disrupted *Igf2* or *Igf2r*, and also transgenic mice overexpressing *Igf2* show normal brain size and morphology (Rogler *et al.*, 1994; Wolf *et al.*, 1994; van Buul-Offers *et al.*, 1995; D'Ercole *et al.*, 2002). One interpretation for such a result is that activity of these genes may be compensated by the action of other gene systems. In addition, ontogenetic changes may occur in growth of specific cell types in brain.

The ratio of *IGF2/IGF2R* decreased considerably in postnatal lung, kidney and muscle, which coincides with reduced growth rate. Nevertheless, the ratio of *IGF2/IGF2R* did not change significantly in heart, brain and liver at the postnatal compared with fetal stage. The significance of *IGF2/IGF2R* transcript ratio remaining at high levels in some tissues during postnatal development is unclear.

In this thesis, we investigated developmental and tissue-specific changes in *IGF2* expression from alternative promoters. *IGF2* is a complex gene which is expressed from distinct promoters overlapping a number of sense and antisense transcription units (Moore *et al.*, 1997). The bovine orthologues for some human and mouse *IGF2* promoters and overlapping transcripts are unknown. Expression analysis of *IGF2* promoter-specific transcripts, without prior knowledge of their exon/intron structure, can yield misleading results. Therefore, we performed *in silico* comparative sequence analysis of *IGF2* promoter-specific transcripts in bovine, human, mouse and porcine to identify evolutionarily conserved regions which may belong to putative, as yet unknown, transcripts in bovine. A sequence similarity search showed that the promoter and first exon of the human P0 transcript is conserved in bovine. In agreement with the observation in human (Monk *et al.*, 2006b), we found that bovine *IGF2* P0 promoter is specifically active in skeletal muscle during prenatal development. In contrast, the equivalent P0 promoter in mouse is only active in placenta (Moore *et al.*, 1997). Our finding gives further evidence for an evolutionary shift in tissue-specific activity of P0 promoter from placenta in mouse to skeletal muscle in human and bovine prenatal development and indicates that the bovine *IGF2* transcription unit is evolutionarily closer to its human orthologue compared with mouse. Thus, the bovine provides a suitable model for studying the role of *IGF2* in prenatal development.

The significance of the P0 promoter in skeletal muscle development remains enigmatic. Since we could not obtain skeletal muscle tissue from the embryo, further research is required to elucidate dynamic changes in P0 expression across prenatal development and role of P0 expression in skeletal muscle differentiation and growth. Interestingly, *IGF2* P0 becomes

inactive in postnatal skeletal muscle and is specifically expressed in postnatal liver. Our results provide evidence for developmental shift in tissue-specific expression of an *IGF2* promoter. Developmental changes in tissue-specific expression of a gene were previously reported (Yoon *et al.,* 1987; Schmidt-Ullrich *et al.,* 1996).

Transcription of *IGF2* from distinct promoters is thought to be engaged in regulation of gene expression at multiple layers, including transcription and post-transcription. Different isoforms of IGF2 protein are derived from different promoters in human and mouse (see chapter 3). However, the functional significance of these isoforms remains unexplored. It is evident that *IGF2* expression is also controlled at the translational level so that *IGF2* promoter-specific transcripts display different translational efficiency (Nielsen *et al.,* 1990; De Moor *et al.,* 1994; Moor *et al.,* 1994; Teerink *et al.,* 1994; Nielsen and Christiansen, 1995; Pedersen *et al.,* 2002; Polesskaya *et al.,* 2007; Dai *et al.,* 2011). It is interesting to note that translational efficiency of *IGF2* promoter-specific transcripts is developmentally controlled (Newell *et al.,* 1994; Teerink *et al.,* 1994; Nielsen *et al.,* 1995; Nielsen *et al.,* 1999). Differential regulation of mRNA stability by site-specific endonucleolytic cleavage provides another layer of tissue- and developmental stage-specific posttranscriptional regulation of *IGF2* promoter-specific expression (Meinsma *et al.,* 1991; Meinsma *et al.,* 1992; Nielsen and Christiansen, 1992; Christiansen *et al.,* 1994; Scheper *et al.,* 1995; Scheper *et al.,* 1996a; Scheper *et al.,* 1996b; Van Dijk *et al.,* 1998; van Dijk *et al.,* 2000; van Dijk *et al.,* 2001). Transcriptional regulation of *IGF2* promoters occurs at the genetic level via differential interaction with tissue and developmental stage-specific transcription factors (Vandijk *et al.,* 1991; van Dijk *et al.,* 1992; Holthuizen *et al.,* 1993; Rietveld *et al.,* 1997; Rodenburg *et al.,* 1997; Rietveld *et al.,* 1999) and enhancer elements (Yoo-Warren *et al.,* 1988; Leighton *et al.,* 1995; Ishihara *et al.,* 2000; Kaffer *et al.,* 2001; Eun *et al.,* 2013) and at the epigenetic level via genomic imprinting.

Changes in imprinting status resulting from epigenetic modifications are plausible mechanisms involved in tissue and developmental stage-specific expression of imprinted

genes (Latham, 1995; Jaenisch and Bird, 2003). Changes in imprinting of *H19* in human placenta during development has been associated with DNA methylation levels in the imprinting control region upstream of *H19* (Buckberry *et al.*, 2012). Furthermore, in human placenta, higher rates of loss of imprinting have been observed during the first trimester of pregnancy compared with term (Jinno *et al.*, 1995; Yu *et al.*, 2009; Pozharny *et al.*, 2010). It is interesting to note that *IGF2* imprinting in liver of human, bovine (*B. taurus*) and ovine (*Ovis aries*) is developmentally regulated and shifts from monoallelic paternal expression during prenatal development to non-imprinted biallelic expression after birth (Vu and Hoffman, 1994; Ekstrom *et al.*, 1995; McLaren and Montgomery, 1999; Goodall and Schmutz, 2007). Further evidence for developmental regulation of imprinting came from developmental stage-specific relaxation of imprinting of *Kvlqt1* in mouse interspecific model of *Mus musculus domesticus*/*Mus musculus castaneus* (Jiang *et al.*, 1998). Further investigation of the quantitative differences between expression of parental alleles are required to determine the extent to which tissue and developmental stage-specific changes in gene expression are caused by alterations in imprinting status.

6.2 Genetic effects on transcript abundance of imprinted genes: Implications for mammalian heterosis

In this thesis, we analysed fetal genetic and heterotic effects on expression of imprinted genes which are known to be involved in prenatal development and their associations with fetal body and tissue weight and heterosis in a bovine intraspecies model at midgestation. Significant genetic effects and heterosis was observed on fetal placental weight, which was highest in *Bos indicus* (Brahman) × *Bos taurus* (Angus) group (sire listed first). Evidence for parent-of-origin and fetal genetic influence on placental growth came from the interspecific crosses in the mouse (*Peromyscus polionotus*/*Peromyscus maniculatus* and *Mus musculus*/*Mus spretus*/*Mus macedonicus*) (Rogers and Dawson, 1970; Zechner *et al.*, 1996; Zechner *et al.*, 1997; Zechner *et al.*, 2002) and equids (*Equus asinus*/*Equus caballus*) (Allen,

1975; Allen *et al.*, 1993), which showed altered placental growth in hybrids and a striking difference between reciprocals in placental weight and size.

Accumulating evidence demonstrates that placental adaptive responses to intrauterine environment, including changes in placental size and capacity for nutrient transport, occur through epigenetic changes in regulatory regions of imprinted domains and subsequently altered expression of imprinted genes which influences prenatal development with long term consequences for postnatal growth, suggesting a key role for placenta in fetal programming (Godfrey, 2002; Fowden *et al.*, 2006a; Fowden *et al.*, 2006b; Myatt, 2006; Jansson and Powell, 2007; Fowden *et al.*, 2008; Coan *et al.*, 2011; Fowden *et al.*, 2011; Vaughan *et al.*, 2011; Burton and Fowden, 2012).

Considering a positive correlation between placental and birth weight (Thomson *et al.*, 1969; Echternkamp, 1993; Jiang *et al.*, 2009; Mericq *et al.*, 2009), the heterosis observed in placenta at midgestation is likely to program birth and early postnatal weight. This is consistent with the heterosis reported for birth weight that was higher in the BA hybrid (Brown *et al.*, 1993). The heterotic effect observed in placental weight was not yet manifested in fetal weight, which could be explained by reduced placental efficiency in the BA group. It is evident that adaptive changes in placental efficiency take place in response to alterations in placental size to regulate fetal nutrient acquisition (Frank *et al.*, 2002; Angiolini *et al.*, 2006; Coan *et al.*, 2008a; Coan *et al.*, 2008b; Fowden *et al.*, 2009). *H19*-deficient mice with placental and fetal overgrowth displayed decreased expression of transporter genes and placental efficiency, whereas reduced placental growth in mice by downregulation of the *Igf2* P0 promoter resulted in increased transport capacity and placental efficiency (Angiolini *et al.*, 2011).

The finding that highest placental weight is countered by lowest placental efficiency in the BA group could be evolutionarily explained by the parent-offspring conflict theory (Trivers, 1974; Haig, 1992) where changes in placental efficiency take place probably by the

effects of maternally expressed genes like *IGF2R* (Moore and Haig, 1991) to protect the mother from detrimental effects of fetal overgrowth.

Our results suggested that *IGF2R* and *AIRN* could be involved in phenotypic variation of placental weight in hybrids, and heterosis in placental phenotype is likely to be caused by an expression pattern of polar overdominance imprinting (Wolf *et al.*, 2008). Results from this study provide evidence for genetic effects on transcript abundances of imprinted genes which impact on placental weight and efficiency with possible consequences for fetal programming. Placental overgrowth observed in mouse interspecific crosses (*Peromyscus polionotus*/*Peromyscus maniculatus* and *Mus musculus/Mus spretus*) was shown to be associated with changes in transcript abundances of imprinted genes, including *Igf2*, and relaxation of imprinting (Vrana *et al.*, 1998; Vrana *et al.*, 2000; Zechner *et al.*, 2002). The molecular (epi)genetic mechanisms underlying the specific expression pattern of *IGF2R/AIRN* in cotyledon remain to be understood. One possibility for the phenotypic polar overdominance expression pattern of *IGF2R* and *AIRN* is relaxation of imprinting in the *B. indicus* (sire) × *B. taurus* (dam) reciprocal. This speculation is supported by the observation in mouse that *IGF2R* imprinting is disrupted and *IGF2R* is biallelically expressed in placenta of one of the reciprocals in the interspecific *Peromyscus polionotus*/*Peromyscus maniculatus* hybrids (Vrana *et al.*, 1998). Further evidence in support of our hypothesis comes from genetic background-dependent relaxation of imprinting of *Kvlqt1* in the mouse interspecific model of *Mus musculus domesticus*/*Mus musculus castaneus* (Jiang *et al.*, 1998). Biallelic expression of *IGF2R* (Buckberry *et al.*, 2012), polymorphic imprinting of *IGF2R* (Xu *et al.*, 1993; Monk *et al.*, 2006a) and loss of imprinting for a panel of imprinted genes (Monk *et al.*, 2006a; Diplas *et al.*, 2009; Pozharny *et al.*, 2010) have been shown in human placenta.

Relative muscle weight displayed significant heterosis. The phenotypic pattern for relative muscle mass differed from that for placental weight. Relative muscle mass was not different between reciprocal fetuses and showed a phenotypic expression pattern of dominance (Wolf *et al.*, 2008), whereas the reciprocal fetuses varied considerably in placental

weight. Therefore, the molecular mechanisms causing heterosis in skeletal muscle may be different from those in placenta. Notwithstanding the negative heterotic effect on *H19* expression, skeletal muscle weight was not significantly correlated with *H19* expression in hybrids. Therefore, it is unclear whether our studied molecular systems can explain heterosis in muscle mass.

Our results suggest that imprinted genes in the IGF system could be implicated in prenatal programming of heterosis in brain. Our molecular data suggest a change in brain gene expression in favour of a heterotic pattern which could manifest in phenotype at later stages of development. The observation of overexpression of *AIRN* in one of the reciprocals and correlation of *AIRN* expression with relative brain weight in hybrids implies that *AIRN* could have a role in programming hybrid brain phenotypes. It has been postulated that genetic programs predominantly direct early stages of brain development (Rubenstein and Rakic, 1999). To our knowledge, heterosis in bovine pre and postnatal brain weight has not been explored. Several studies in mouse have investigated heterosis in brain weight. Heterosis in mouse body and brain weight has been shown at Days-42 and 100 after birth (Henderson, 1973; Seyfried and Daniel, 1977; Bulman-Fleming *et al.*, 1991), while no heterosis (overdominance effect) was seen at Day-70 of mouse postnatal life (Hahn and Haber, 1978).

To our knowledge, this is the first study indicating that imprinted genes could be involved in prenatal programming of heterosis. Our results suggest that heterosis is programmed at the (epi)genetic level via changes in transcript abundance of imprinted genes in hybrids. In contrast to the heterosis previously reported for birth weight in a cross between *B. taurus* and *B. indicus* (Brown *et al.*, 1993), no heterosis was observed for fetal body weight at midgestation. As midgestation is the beginning of the exponential phase of bovine fetal growth (Evans and Sack, 1973; Eley *et al.*, 1978; Prior and Laster, 1979), it is reasonable to deduce that heterotic effects programmed at the molecular level in early stages of development, are manifested in phenotype in the exponential phase of prenatal growth or after birth. The study of heterosis at different developmental stages in the rat revealed that heterotic

responses become more pronounced during the rapid growth phase of postnatal development and with increasing age (Naso *et al.*, 1975).

6.3 Imprinted genes as molecular drivers of parent-of-origin effects on fetal growth-related phenotypes

Fetal body weight and absolute weights of fetal tissues, except brain, were higher in fetuses with maternal *B. taurus* genetics compared with those with *B. indicus* maternal genetics. Although absolute brain weight did not change significantly across genetic groups relative brain weight was higher in fetuses with maternal *B. indicus* genetics compared with those with maternal *B. taurus* genetics. A maternal effect on adult brain weight has previously been reported in the mouse (*Mus musculus*) (Henderson, 1973; Hahn and Haber, 1978; Wahlsten, 1983). While absolute weights of fetal tissues, except brain, were correlated with *H19* expression, relative brain weight did not show significant relationship with *H19* expression. This suggests that (epi)genetic systems which drive the differential parent-of-origin effect on relative brain weight, are different from those affecting fetal weight and weights of other studied fetal tissues.

Maternal effects on fetal phenotypes may be caused by parent-of-origin effects of imprinted genes (Amen *et al.*, 2007a) or epistatic interaction of miRNAs and their target sites (Clop *et al.*, 2006). Moreover, mitochondrial DNA variations (Schutz *et al.*, 1994; Mannen *et al.*, 1998; Mannen *et al.*, 2003; Hiendleder *et al.*, 2004) or X chromosome effects (Amen *et al.*, 2007a; Amen *et al.*, 2007b) may contribute to Bt × Bi maternal (epi)genetic effects on phenotypes. It has been shown that at the phenotypic level, parent-of-origin effects resulting from maternal effects can be confounded by genomic imprinting (Hager *et al.*, 2008).

The parent-of-origin effects on fetal phenotypic traits studied here are consistent with a phenotypic pattern of genomic imprinting for maternally expressed genes (Wolf *et al.*, 2008). Therefore, we hypothesised a role for imprinted genes in causing parent-of-origin effects on

phenotypes and studied expression of the genes which are known to be paternally (*H19* and *IGF2R*) and maternally (*IGF2* and *AIRN*) imprinted in bovine prenatal development.

We did not find a phenotypic pattern of genomic imprinting associated with transcript abundances for paternally expressed genes (Wolf *et al.*, 2008). This may be explained by lack of fetal genetic effects on transcript abundance of the paternally expressed IGF2 gene in most fetal tissues. Also, in most fetal tissues, *IGF2* expression was not correlated with fetal phenotypes. In agreement with the parental genetic conflict hypothesis (Moore and Haig, 1991), the paternally expressed *IGF2*, which is a growth enhancer, did not contribute to prenatal phenotypic variations, which is to be expected to protect the mother from detrimental effects caused by fetal overgrowth, and *IGF2* effects on phenotype are likely to be manifested postnatally. A genome wide scan of the mouse genome for imprinted QTL revealed that effects of most iQTLs are manifested in the traits expressed at later stages of postnatal life (Wolf *et al.*, 2008). Several studies in porcine have revealed that *IGF2* is a candidate QTL with a major effect on postnatal muscle mass and other growth related phenotypes (Jeon *et al.*, 1999; Nezer *et al.*, 1999).

Surprisingly, the results of the current study indicated that differences in fetal body and tissue growth between *B. taurus* and *B. indicus* could be attributed to differential expression of the maternally expressed gene, *H19*. In addition, the parent-of-origin phenotypic patterns were consistent with the expression pattern of the maternally expressed H19 gene for all the studied tissues, except brain. With respect to the negative relationship of *H19* expression with fetal body weight and weights of fetal tissues, including skeletal muscle, liver, lung, heart, kidney and placenta, our results suggest that maternal effects on fetal phenotypes are likely to be mediated by *H19*. *H19* might exert its effect through fine-tuning of an imprinted gene network (Gabory *et al.*, 2009) and regulation of growth-suppressing miRNA (Keniry *et al.*, 2012). Expression of *H19* in skeletal muscle was significantly influenced by the interaction between paternal and maternal genomes. Interestingly, final maternal weight of *B. taurus* and *B. indicus*, which represents maternal environmental conditions, was differentially correlated

with *H19* expression in fetal skeletal muscle. *H19* expression in fetal skeletal muscle was positively correlated with *B. indicus* final maternal weight and negatively correlated with *B. taurus* final maternal weight. The evolutionary explanation for these observations may be that *B. indicus* has evolved to survive in poor environmental conditions with restricted food supply and therefore signals to enhance activity of fetal growth suppressing genes to protect the mother from detrimental effects of fetal overgrowth and consequently increased fetal nutrient demand. These observations suggest an important role of *H19* in developmental programming of fetal growth.

6.4 *B. taurus* / *B. indicus* polymorphisms in the imprinting control region (ICR) of *H19*: Plausible mechanisms for differential expression of *H19*

Analysis of *H19* expression revealed that *H19* transcript abundance in *B. indicus* was between 1.5 to 2.2-fold higher than in *B. taurus* in all examined tissues, except brain. We sought to find plausible molecular mechanisms underlying genetic effects on differential *B. taurus / B. indicus H19* expression. The observations that (epi)genetic mutations in the *IGF2/H19* ICR have been implicated in aberrant imprinting and developmental abnormalities (Hark *et al.*, 2000; Pant *et al.*, 2003; Schoenherr *et al.*, 2003; Pant *et al.*, 2004; Sparago *et al.*, 2004; Szabó *et al.*, 2004; Li *et al.*, 2005) led us to hypothesise that genetic polymorphisms in the regulatory sequences of *H19* imprinted domain could account for the varying transcript abundances between subspecies. We performed a *B. taurus / B. indicus* sequence similarity search in the *H19* ICR and found major polymorphisms within the conserved consensus sequence of the first CTCF binding site (the most upstream to *H19* promoter) where two CpG sites are disrupted in *B. indicus*. This CTCF binding site resides within an evolutionarily conserved region and reveals sequence homology with human CTCF binding site 6 (Hansmann *et al.*, 2011). In human, only CTCF binding site 6 is differentially methylated on the paternal allele and its methylation status is linked to *H19* expression (Takai *et al.*, 2001).

This suggests an important functional role for the bovine CTCF binding site 1. Different theories could be postulated to explain how polymorphisms in the CTCF binding site are linked to the altered *H19* expression. One hypothesis is that polymorphisms in the CpG sites may cause reduced methylation of the CTCF binding site on the paternal chromosome without changing their CTCF binding properties that result in biallelic expression of *H19* in *B. indicus* but does not change allelic expression of *IGF2*. In support of this hypothesis, there are two lines of experimental evidence. In mouse (*Mus musculus castaneus*), the paternal ICR was substituted by two copies of chicken beta globin insulator (ChβGI)2 which had two CTCF binding sites and was of similar size to ICR with sufficient number of CpG dinucleotides (Szabó *et al.*, 2002; Lee *et al.*, 2010). The (ChβGI)2 on the paternal chromosome was prevented from *de novo* methylation in the male germ cells and acted as an insulator through binding the CTCF. This led to reduced *IGF2* expression, overactivation of paternal *H19* allele and reduced fetal growth by 44-61% of normal fetal size. This model does not explain the stable expression of *IGF2* in all genetic groups, which was observed in our results. Hypomethylation of the *H19* differentially methylated region has been shown to be correlated with biallelic *H19* expression in cloned bovine (*B. taurus/B. indicus*), but was not associated with *IGF2* allelic expression (Zhang *et al.*, 2004; Curchoe *et al.*, 2009; Suzuki *et al.*, 2011). An alternative theory is that polymorphisms in the consensus sequences change binding ability of the CTCF-binding site resulting in epigenetic modification in the imprinted domain and subsequently altered *H19* expression. This is supported by several lines of evidence that suggest CTCF is the major epigenetic organiser of the maternal *H19* imprinted domain (Han *et al.*, 2008; Li *et al.*, 2008; Singh *et al.*, 2010a; Singh *et al.*, 2010b; Singh *et al.*, 2011). It is also possible that mutation in one of the CTCF binding sites interacts with other CTCF binding sites and change their methylation status (Pant *et al.*, 2004). Another possibility is that the polymorphism may occur in the binding site for another regulatory protein that overlaps CTCF binding motifs and subsequently changes epigenetic status of chromatin in the ICR and leads to activation of the paternal *H19*. It has been shown that

mutations in the Zfp57-Trim28-Setdb1 repressor complex binding site, which overlaps CTCF binding motifs, without affecting CTCF binding sites result in CTCF binding and induced insulation on the paternal ICR due to reduced ICR methylation (Engel *et al.*, 2004; Matsuzaki *et al.*, 2010; Quenneville *et al.*, 2011; Singh *et al.*, 2012). Further studies are needed to elucidate *B. taurus*- and *B. indicus*-specific differential methylation of parental alleles in the *H19* ICR and promoter and its association with *H19* overall expression and imprinting.

Together, these findings led us to speculate that higher expression of *H19* in *B. indicus* tissues could be attributable to partial or complete relaxation of imprinting resulting from epigenetic changes in the ICR. In this scenario, changes in DNA methylation patterns are the consequence of gene regulation rather than being its cause. Recently, it has been suggested that sequence polymorphisms in regulatory regions of genes alter their methylation status and consequently lead to changes in binding characteristics (Schübeler, 2012). The interplay between genetics and epigenetics has been shown to affect spatial expression pattern of *H19* (Lin *et al.*, 1999).

6.5 General conclusions

Our results demonstrate that fetal genetics significantly influences tissue-specific transcript abundance of imprinted genes and induces specific expression patterns that cause heterotic effects on gene expression. This suggests that imprinted genes could be implicated in prenatal programming of postnatal heterosis in phenotypes. Our findings suggest that the imprinting pattern of polar overdominance, as described previously (Wolf *et al.*, 2008), could explain the phenotypic patterns associated with heterosis. We identified a key role of *H19* as a driver of differential parent-of-origin phenotypic patterns in prenatal development, suggesting that imprinted gene networks and epistatic miRNA interference could be the molecular basis for observed parent-of-origin effects on fetal growth related traits. We posited that the sequence variation in the functional regulatory intergenic CTCF binding site may be responsible for the differential subspecies-specific expression pattern of *H19* by causing

altered epigenetic status in the imprinted domain. Taken together, our findings significantly enhance understanding of the interplay between genetics and epigenetics and its contribution to variations in prenatal growth-related phenotypes with possible consequences for postnatal performance and heterosis. These results have significance in animal breeding and can help realise the unexplained sources of variations in muscle traits attributed to non-Mendelian (epi)genetic effects and interactions, which lead to improved efficiency of selection programs.

6.6 Future works

– Tissue-specific DNA methylation studies of regulatory elements of *IGF2/H19* (i.e. CTCF binding sites located within *H19* ICR) and *IGF2R/AIRN* (i.e. DMR1 and DMR2) in the genetic groups and developmental stages. Altered DNA methylation patterns of DMR2 in bovine embryos produced by somatic cell nuclear transfer (SCNT) were correlated with altered *IGF2R* expression (Long and Cai, 2007). In another study, DNA methylation of DMR2 and allelic expression of *IGF2R* did not alter in bovine fetuses derived from *in vitro* fertilisation (IVF) (Bebbere *et al.*, 2013). In addition, the intergenic ICR containing putative CTCF binding sites with differential DNA methylation patterns have been characterised upstream of bovine (*B. taurus*/*B. indicus*) *H19* (Curchoe *et al.*, 2009; Hansmann *et al.*, 2011; Robbins *et al.*, 2012).

– Identification of subspecies-specific genetic polymorphisms in regulatory sequences of *IGF2/H19* and *IGF2R/AIRN*.

– Study of the link between genetic polymorphisms and DNA methylation differences in regulatory sequences of *IGF2/H19* and *IGF2R/AIRN*.

– Detailed studies of *IGF2/H19* and *IGF2R/AIRN* tissue-specific imprinting in the genetic groups and developmental stages.

– Study of correlation between differences in tissue-specific imprinting status and overall expression of *IGF2/H19* and *IGF2R/AIRN*.

– Study of correlation between differences in tissue-specific DNA methylation and overall expression of *IGF2/H19* and *IGF2R/AIRN*.

– Study of correlation between differences in tissue-specific DNA methylation and imprinting status of *IGF2/H19* and *IGF2R/AIRN*.

This will link changes in epigenetric modification and mechanisms with differences in gene expression and phenotype.

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Appendices

Appendix 1 Protocol of RNA extraction using AllPrep™ DNA/RNA Micro (Qiagen GmbH, Inc., Hilden, Germany).

1. Add 350 µl Buffer RLT Plus to the tissue (maximum 10 mg).
2. Perform homogenisation using ceramic beads, as optimised.
3. Centrifuge the lysate for 3 min at full speed. Carefully remove the supernatant by pipetting, and transfer it to an AllPrep DNA spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 30 s at ≥8000 x *g* (≥10,000 rpm).
4. Place the AllPrep DNA spin column in a new 2 ml collection tube (supplied), and store at room temperature (15–25ºC) or at 4ºC for later Genomic DNA purification.
5. Add 1 volume (usually 350 µl) of 70% ethanol to the flow-through from step 4, and mix well by pipetting.
6. Transfer the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flowthrough.
7. Add 700 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
8. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
9. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.
10. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.
11. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the centre of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

Appendix 2 Details of primers used for amplification of transcripts of target genes.

Target transcript	Primer sequence	Annealing temperature (°C)	Fragment length (bp)	Exon/intron	Accession No.
IGF2R (F)	GATGGTAATGAGCAGGCTTACC	60	123	*IGF2R* (Exon 47)	NM_174352.2
IGF2R (R)	ATCTCCTCCATCAGCCACTC	60	123	*IGF2R* (Exon 48)	NM_174352.2
AIRN (F)	AATCTCTTGCGGAGTGTTCAT	57	136	*IGF2R* (intron 2)	DQ835615.1
AIRN (R)	CTCTGTTGTATCGTGTCTTTCG	57	136	*IGF2R* (intron 2)	DQ835615.1
IGF2 (F)	CTTCGCCTCGTGCTGCTATG	60	134	*IGF2* (Exon 8)	NM_174087.3
IGF2 (R)	GTCGGTTTATGCGGCTGGAT	60	134	*IGF2* (Exon 9)	NM_174087.3
H19 (F)	TCAAGATGACAAGAGATGGTGCTA	60	171	*H19* (Exons 3_4)	NR_003958.2
H19 (R)	GGTGTGGGTCGTCCGTTC	60	171	*H19* (Exon 5)	NR_003958.2
IGF2 P0 (F)	CACGCTCTAAAAATGCCCTTCA	60	103	*IGF2* (intron 1)	EU518675 1
IGF2 P0 (R)	TGCTCTGGCTGTGGTGCTCA	60	103	*IGF2* (Exon 2)	EU518675 1
IGF2 P1e2 (F)	CCTCAGCCTCATCCCCTCCTTTGC	60	217	*IGF2* (Exon 2)	EU518675 1
IGF2 P1e2 (R)	CTGTGCTCTATTTGCTGTGTTGTCT	60	217	*IGF2* (Exon 2)	EU518675 1
IGF2 P1e3 (F)	GGTCAGCCCTTTGCCCAG	62	179	*IGF2* (Exon 3)	NM_174087.3
IGF2 P1e3 (R)	CACCAGCACCGACTTTCCT	62	179	*IGF2* (Exon 8)	NM_174087.3
IGF2 P2e4 (F)	TCCAGCCTCGCGACATCA	61	66	*IGF2* (Exons 4-8)	DQ298749.1
IGF2 P2e4 (R)	CAAGAAGGCAAGAAGCACCA	61	66	*IGF2* (Exon 8)	DQ298749.1
IGF2 P2e5 (F)	TACGCAAGTCCAACGCATAGA	60	160	*IGF2* (Exon 5)	DQ298745.1
IGF2 P2e5 (R)	CAAGAAGGCAAGAAGCACCA	60	160	*IGF2* (Exon 8)	DQ298745.1
IGF2 P3 (F)	AGACAGCCCGTCCTCCCTA	60	246	*IGF2* (Exon 6)	BC116039 1
IGF2 P3 (R)	CACCAGCACCGACTTTCCT	60	246	*IGF2* (Exon 8)	BC116039 1
IGF2 P4 (F)	CAGCGAGCCTCCTGTCCA	60	64	*IGF2* (Exon 7)	AY957981.1
IGF2 P4 (R)	CACCAGCACCGACTTTCCT	60	64	*IGF2* (Exon 8)	AY957981.1

Appendix 3 Details of primers used for amplification of transcripts of housekeeping genes.

Target gene	Primer sequence	Annealing temperature (°C)	Fragment length (bp)	Accession No.
VPS4A (F)	GAAGACAGAAGGCTACTCGGGTG	60	106	NM_001046615.1
VPS4A (R)	ACAGACCTTTTTGAAGTGTGTTGCT	60	106	NM_001046615.1
GAK (F)	CACGACCATCTCACACTACCCA	60	128	NM_001046084.2
GAK (R)	AGTTTGAGTACAAGTCCACAATTTCC	60	128	NM_001046084.2
TBP (F)	GCAACAGTTCAGTAGTTATGAGCCAG	60	164	NM_001075742.1
TBP (R)	GAATAGGGTAGATGTTCTCAAAGGCT	60	164	NM_001075742.1
H3F3A (F)	ACTGCTACAAAAGCCGCTC	60	231	XM_003586223.1
H3F3A (R)	ACTTGCCTCCTGCAAAGCAC	60	231	XM_003586223.1
UBB (F)	AGATCCAGGATAAGGAAGGCAT	62	198	NM_174133.2
UBB (R)	GCTCCACCTCCAGGGTGAT	62	198	NM_174133.2
ACTB (F)	CTCTTCCAGCCTTCCTTCCT	62	245	NM_173979.3
ACTB (R)	CCAATCCACACGGAGTACTTG	62	245	NM_173979.3
RPS9 (F)	TAGGCGCAGACGGGCAAACA	60	136	NM_001101152.2
RPS9 (R)	CCCATACTCGCCGATCAGCTTCA	60	136	NM_001101152.2
GAPDH (F)	GGGTCATCATCTCTGCACCT	62	173	NM_001034034.2
GAPDH (R)	CATAAGTCCCTCCACGATGC	62	173	NM_001034034.2

Appendix 4 Amplification efficiency and coefficient of determination for quantitative real time PCR runs for target transcripts in the studied fetal (Day-153) tissues.

Target transcript	Tissue	Amplification efficiency	Coefficient of determination (R^2)
IGF2R	Muscle	0.88	0.992
IGF2R	Heart	0.97	0.993
IGF2R	Liver	0.99	0.993
IGF2R	Brain	0.99	0.990
IGF2R	Cotyledon	0.92	0.992
IGF2R	Lung	0.92	0.996
IGF2R	Kidney	0.95	0.993
IGF2	Muscle	0.96	0.999
IGF2	Heart	0.96	0.995
IGF2	Liver	0.96	0.997
IGF2	Brain	0.90	0.991
IGF2	Cotyledon	0.97	0.996
IGF2	Lung	0.98	0.993
IGF2	Kidney	0.88	0.99
H19	Muscle	0.95	0.997
H19	Heart	0.96	0.994
H19	Liver	0.97	0.996
H19	Brain	0.96	0.991
H19	Cotyledon	0.95	0.996
H19	Lung	0.99	0.993
H19	Kidney	1.00	0.994
AIRN	Muscle	0.88	0.992
AIRN	Heart	0.87	0.993
AIRN	Liver	0.96	0.995
AIRN	Brain	1.00	0.990
AIRN	Cotyledon	0.96	0.993
AIRN	Lung	0.98	0.992
AIRN	Kidney	0.96	0.992
IGF2 P0	Muscle	0.80	0.988
IGF2 P1e2	Muscle	0.94	0.987
IGF2 P1e3	Muscle	0.88	0.992
IGF2 P2e5	Muscle	1.00	0.994
IGF2 P2e5	Heart	0.95	0.99
IGF2 P2e5	Liver	0.98	0.993
IGF2 P2e5	Cotyledon	0.98	0.993
IGF2 P2e5	Lung	0.95	0.995
IGF2 P2e5	Kidney	0.94	0.989
IGF2 P2e4	Muscle	0.90	0.990
IGF2 P2e4	Liver	1.00	0.996
IGF2 P3	Muscle	1.09	0.990
IGF2 P3	Heart	1.06	0.992
IGF2 P3	Liver	1.03	0.995
IGF2 P3	Cotyledon	1.00	0.997
IGF2 P3	Lung	1.03	0.994
IGF2 P3	Kidney	1.08	0.996
IGF2 P4	Muscle	0.97	0.993
IGF2 P4	Heart	0.96	0.995
IGF2 P4	Liver	0.98	0.994
IGF2 P4	Cotyledon	0.91	0.993
IGF2 P4	Lung	0.96	0.991
IGF2 P4	Kidney	0.94	0.991

Appendix 5 Amplification efficiency and coefficient of determination for quantitative real time PCR runs for housekeeping genes in the studied fetal (Day-153) tissues.

Target transcript	Tissue	Amplification efficiency	Coefficient of determination (R^2)
ACTB	Muscle	0.83	0.995
RPS9	Muscle	0.87	0.998
UBB	Muscle	0.95	0.983
H3F3A	Muscle	0.82	0.997
TBP	Muscle	1.00	0.981
VPS4A	Muscle	0.88	0.993
GAK	Muscle	0.72	0.989
ACTB	Heart	0.88	0.997
RPS9	Heart	0.93	0.998
UBB	Heart	0.89	0.995
H3F3A	Heart	0.89	0.995
TBP	Heart	1.10	0.989
VPS4A	Heart	0.91	0.99
GAK	Heart	0.78	0.993
ACTB	Liver	0.89	0.997
RPS9	Liver	0.90	0.998
UBB	Liver	0.87	0.994
H3F3A	Liver	0.94	0.995
TBP	Liver	0.92	0.99
VPS4A	Liver	0.93	0.995
GAK	Liver	0.72	0.991
ACTB	Brain	0.89	0.998
RPS9	Brain	0.94	0.998
UBB	Brain	0.95	0.989
H3F3A	Brain	0.92	0.984
TBP	Brain	1.04	0.981
VPS4A	Brain	0.91	0.991
GAK	Brain	0.78	0.985
ACTB	Cotyledon	0.85	0.994
RPS9	Cotyledon	0.97	0.996
UBB	Cotyledon	0.93	0.994
GAPDH	Cotyledon	0.98	0.998
TBP	Cotyledon	0.95	0.988
VPS4A	Cotyledon	0.94	0.996
GAK	Cotyledon	0.78	0.990
ACTB	Lung	0.85	0.993
RPS9	Lung	0.88	0.996
UBB	Lung	0.85	0.992
H3F3A	Lung	0.90	0.995
TBP	Lung	0.88	0.990
VPS4A	Lung	0.94	0.995
GAK	Lung	0.77	0.992
ACTB	Kidney	0.90	0.994
RPS9	Kidney	0.91	0.994
UBB	Kidney	0.85	0.992
H3F3A	Kidney	0.86	0.995
TBP	Kidney	0.97	0.986
VPS4A	Kidney	0.90	0.993
GAK	Kidney	0.76	0.992

Appendix 6 Amplification efficiency and coefficient of determination for quantitative real time PCR runs for analysis of expression of target transcripts in the tissues of three developmental stages.

Target transcript	**Amplification efficiency**	**Coefficient of determination (R^2)**
IGF2R	0.89	0.990
IGF2	0.96	0.998
H19	1.00	0.997
AIRN	0.94	0.991
IGF2 P0	0.78	0.986
IGF2 P1e2	0.96	0.988
IGF2 P1e3	0.94	0.989
IGF2 P2e5	0.98	0.992
IGF2 P2e4	1.11	0.986
IGF2 P3	1.16	0.987
IGF2 P4	0.88	0.992

Appendix 7 Summary of distribution of maternal and paternal genomes and sex of fetuses.

		Number of fetuses
Maternal genome	Angus	45
	Brahman	28
Paternal genome	Angus	36
	Brahman	37
Fetal gender	Male	27
	Female	46

Appendix 8 Supplementary figures

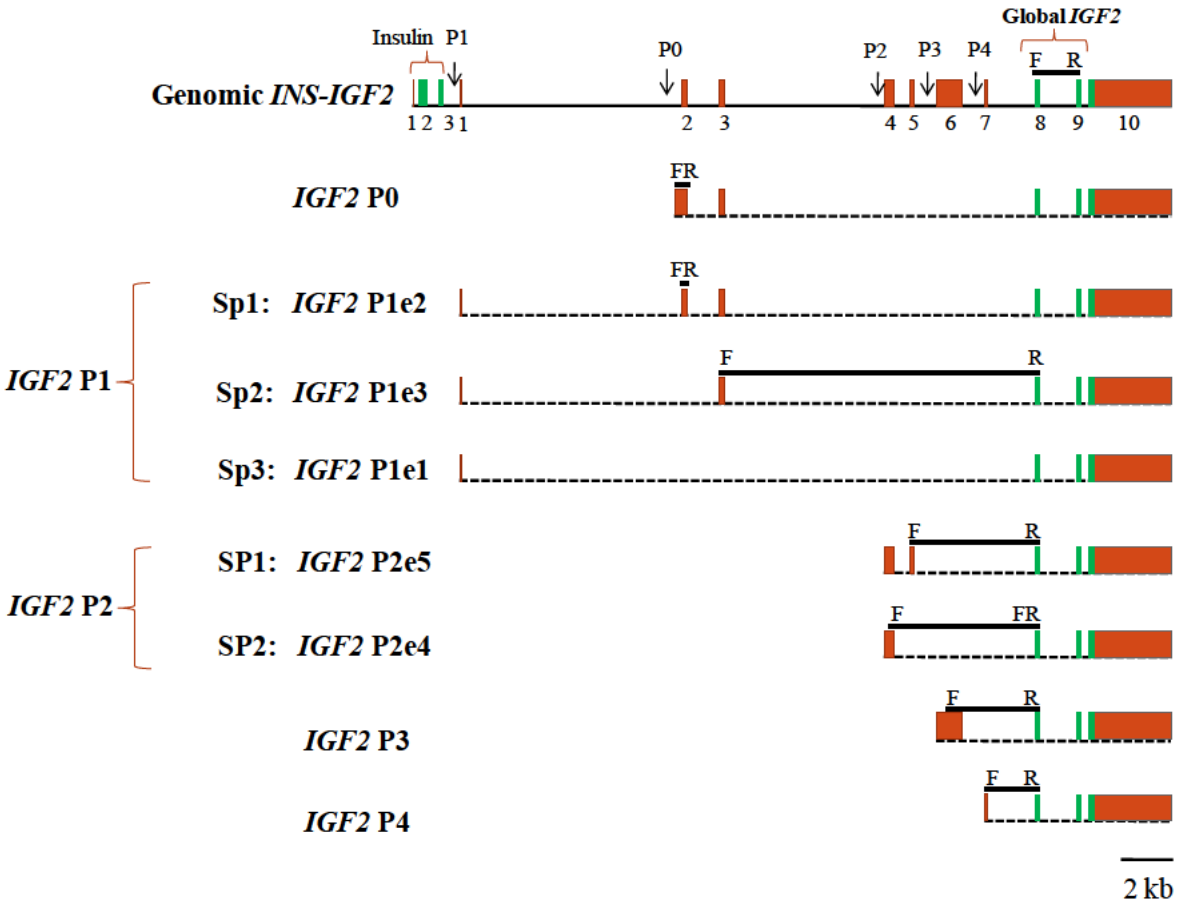

Figure S1 Transcript structure and location of forward (F) and reverse (R) primers for amplification of *IGF2* promoter-specific transcripts and splice variants (SP).

Exon/intron structure of bovine insulin/insulin-like growth factor 2 (*INS/IGF2*) with locations of five promoters (P0, P1, P2, P3 and P4) are shown. We could not specifically amplify *IGF2P1e1* by placing a primer within exon 1 as it is a short GC-rich sequence. Primers for amplification of *IGF2P1e2* and *IGF2P1e3* can amplify P0 transcript. As *IGF2P1* is mostly active in postnatal liver, the majority of transcripts amplified by *IGF2P1e2* and *IGF2P1e3* in skeletal muscle are expected to be derived from the P0 promoter. Red and green boxes depict untranslated and protein coding exons, respectively.

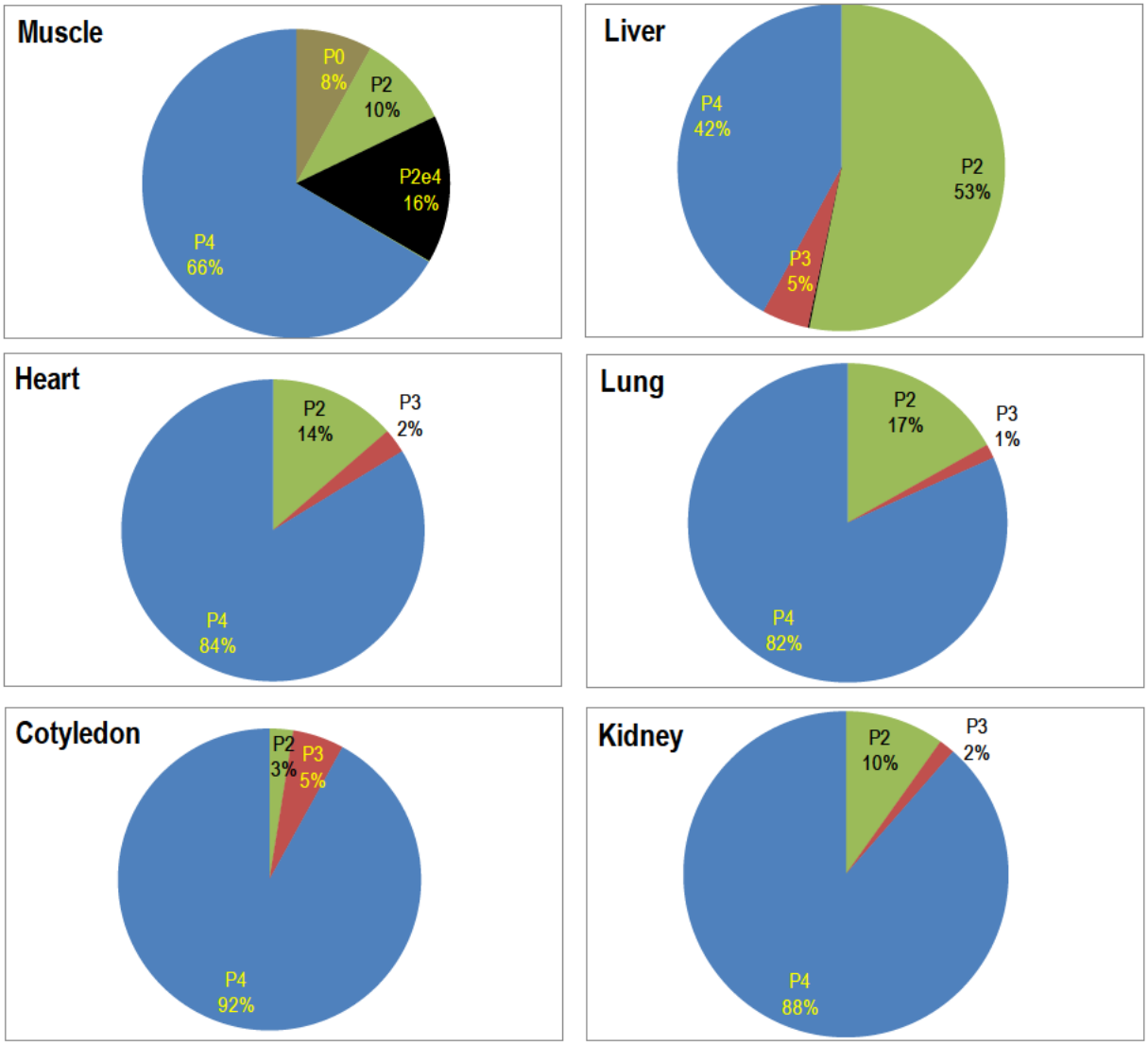

Figure S2 Contribution of *IGF2* promoter-specific transcripts to the variation in global *IGF2* transcript abundance in the studied fetal (Day-153) tissues.
P2e4 is the splice variant derived from P2 promoter containing untranslated exon 4, whereas the other splice variant (P2) contains untranslated exons 4 and 5.

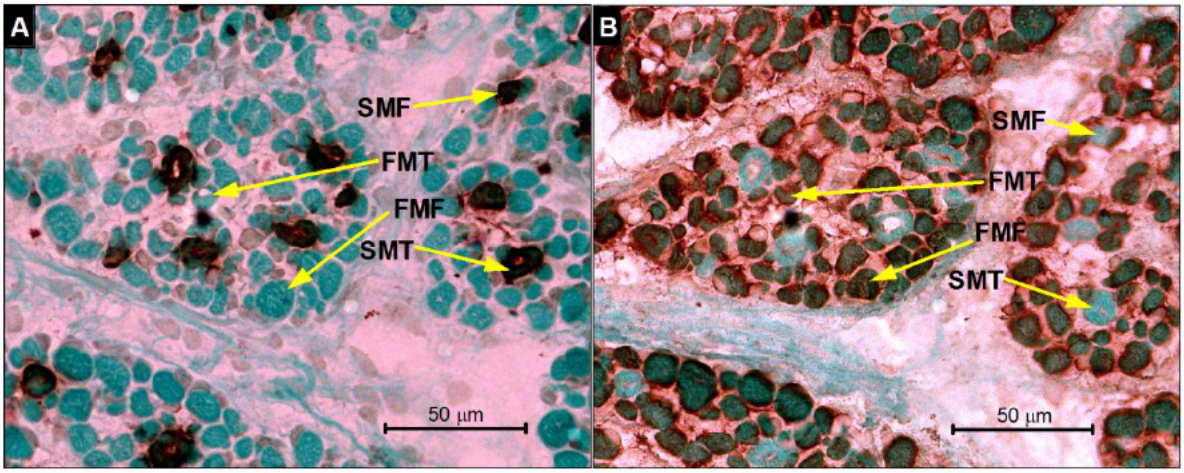

Figure S3 Example of immunohistochemical staining for fetal slow and fast myofibres in M. semitendinosus at midgestation. (A) and (B) show serial stained sections of muscle tissue from one fetus against slow and fast myosin heavy chain isoforms, respectively.
Arrows indicate slow myotubes (SMT), slow myofibres (SMF), fast myotubes (FMT) and fast myofibres (FMF).

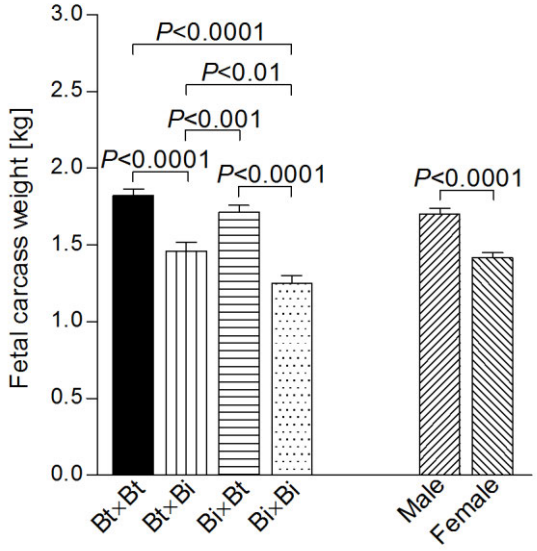

Figure S4 Fetal carcass weights for the four different combinations of maternal and paternal genomes and fetal sex at midgeststion.

Least square means with standard errors of means and *P*-values for significant differences (*t*-test) between means are indicated. Data were analyzed with a general linear model in SPSS 17.00 that included the factors fetal genetic group *i*, *i* = Bt × Bt, Bt × Bi, Bi × Bt, Bi × Bi (paternal genetics given first) and fetal sex *j*, *j* = male, female. The interaction between fetal genetic group and fetal sex was included in the model but removed as it was not significant (*P*>0.05).

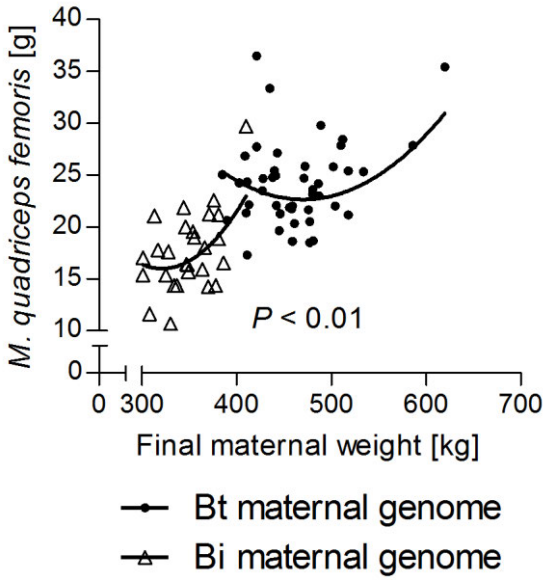

Figure S5 Quadratic effects of final maternal weight nested within maternal genomes on absolute weight of fetal M. quadriceps femoris at midgestation.

The *P*-value (ANOVA) of this nested effect is indicated. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.

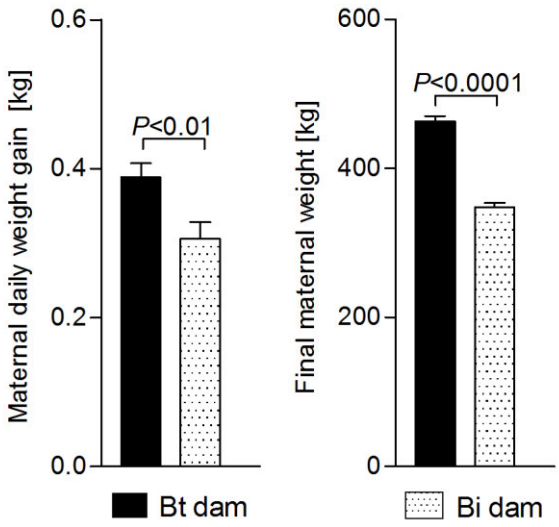

Figure S6 Daily weight gain and final weight for *Bos taurus taurus* and *Bos taurus indicus* dams.
(A) Post-conception maternal daily gain: Final maternal weight – weight at conception divided by days of gestation. (B) Final maternal weight: Weight before slaughter on Day-153 of gestation. *P*-values for significantly different means (*t*-test) are indicated. Bt: *Bos taurus taurus*, Angus. Bi: *Bos taurus indicus*, Brahman.