CONTRIBUTION OF INSULIN-LIKE GROWTH FACTOR SYSTEM AND GROWTH HORMONE-INSULIN-LIKE GROWTH FACTOR 1 AXIS GENES TO HETEROSIS IN A BOVINE FETUS MODEL

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Abstract

Heterosis, the superiority of hybrids over the average of purebred parents, has been used for centuries to obtain higher yields in animal production. Molecular mechanisms of heterosis remain poorly understood and traditional genetic models based on dominance and overdominance largely fail to explain heterosis. This thesis is based on a bovine (*Bos taurus* × *Bos indicus*) heterosis model with high levels of heterosis in birthweight and postnatal growth and development associated with increased plasma insulin-like growth factor 1 (IGF1) concentrations. We hypothesised that heterosis is programmed prenatally and orchestrated by the IGF system and the growth hormone (GH)-IGF1 axis, which are fundamental for pre- and postnatal growth and development. We further hypothesised that epigenetic mechanisms involved in control of IGFs, i.e. as miRNA interference and retrotransposon insertion, contribute to heterosis.

The aims of this project were to study the contribution of IGF system and GH-IGF1 axis transcripts, and epigenetic regulatory elements, on fetal growth and development of purebred Angus and Brahman cattle and their reciprocal crosses. Quantitative real time-PCR was used to quantify transcript abundance of *IGF1* overall transcript, *IGF1* class 1 and class 2, *IGF1R*, insulin receptor *(IR*) overall transcript, *IR-A, IR-B, GH, GHR* overall transcript, *GHR-1A, GHR-1B, GHR-1C*, insulin-like growth factor binding protein 1 (*IGFBP1*), *IGFBP2*, *IGFBP3*, *IGFBP4*, *IGFBP5*, *IGFBP6*, *IGFBP7* and *IGFBP8*, in brain, cotyledon, heart, kidney, liver, lung, skeletal muscle and testis of Day-48 embryos, Day-153 fetuses and 12-month old juveniles. A miRNA abundance profile of fetal liver was obtained using miRNA arrays. Genetic, fetal sex and heterosis effects on transcript abundances were estimated using general linear models. Lack of data on developmental-stage and tissue-specific expression required an initial comparative gene expression study across key developmental stages and tissues.

IGF system and GH-IGF1 axis transcripts showed tissue-specific expression patterns that differed across developmental stages. There was no detectable *GH* mRNA in tissues studied. The abundance of most transcripts in juvenile tissue was lower than in fetal tissue, except in liver, which showed increased *IGF1*, *GHR* and *IGFBP4* expression and no change for *IR*, *IGFBP1*, *IGFBP3* and *IGFBP6* transcript.

Our data showed negative or no molecular heterosis for liver *IGFBP* transcripts. Reduced expression of these IGF-modulators suggested an increase in available IGF1 in the fetus. We found molecular heterosis in liver *GHR*, as the major GHR downstream pathways involve IGF1, we concluded that heterosis in liver *IGF1* class 2 transcripts was a result of increased liver *GHR*-1A mRNA.

Several miRNAs, predicted to target 3' UTRs of IGF system genes and *GHR*, were differentially expressed in different genotypes and may have a regulatory role in transcription of *IGF* system and *GHR* genes in bovine fetal liver. However, more experiments with an increased sample size for miRNA profiling are required to assess this further. Among studied tissues, fetal liver appears to be the most important tissue to study the molecular mechanisms of heterosis.

In conclusion, it was demonstrated that mRNA transcripts and miRNAs in the developmentally important *IGF* system, and *GHR* transcripts, contribute to molecular heterosis in the bovine model. We propose that liver *GHR-1A - IGF1 class 2* transcripts are important factors in molecular heterosis which may contribute to the reported heterosis in birth weight. Furthermore, miRNAs that regulate IGF system/GHR transcripts may contribute to bovine molecular and phenotypic heterosis postnatally.

Declaration

I hereby certify that this thesis is my original research work and has not been submitted for a degree at any university. This thesis, to best of my knowledge, contains no material previously published or written by another person, except where referencing is done to acknowledge publication of other authors.

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Abbreviations

ACTB	Actin beta
ANOVA	Analysis of variance
Bi	*Bos indicus*
bp	Base pair
Bt	*Bos taurus*
cDNA	Complementary deoxyribonucleic acid
Cq	Quantification cycle
D	Day
GAK	Cyclin-G associated kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GHR	Growth hormone receptor
H3F3A	H3 histone family 3A
IGF1	Insulin–like growth factor 1
IGF2	Insulin–like growth factor 2
IGF1R	Type 1 insulin–like growth factor receptor
IGF2R	Type 2 insulin–like growth factor receptor
IGFBP	Insulin-like growth factor binding protein
IGFBP-rP	Insulin-like growth factor binding protein-related protein
IR	Insulin receptor
Kb	Kilo base pair
kDa	Kilo dalton
L1	LINE-1
LINE-1	Long Interspersed Element–1
mRNA	Messenger ribonucleic acid
miRNA	Micro RNA
qPCR	Quantitative real-time polymerase chain reaction

QTL	Quantitative trait loci
QTN	Quantitative trait nucleotide
PCR	Polymerase chain reaction
PL	Placental lactogene
PRL	Prolactin
PRLR	Prolactin receptor
RPS9	Ribosomal protein S9
SEM	Standard error of the mean
SD	Standard deviation
TAE	Tris-acetic acid EDTA buffer
TBE	Tris-boric acid EDTA buffer
TBP	TATA box binding protein
UBB	Ubiquitin B
VPS4A	Vacuolar protein sorting 4 homolog A

Protein symbols are presented in normal script and gene/transcript symbols are presented by italic script.

Chapter 1

Literature review

1.1 Heterosis

Heterosis or hybrid vigour is the increase in performance of crossbred animals or plants over their purebred parents. From an evolutionary point of view, heterosis is defined as higher fitness of heterozygote individuals compared to homozygotes in a population (Chen, 2010). This phenomenon was described in 1876 by Charles Darwin. He observed that progeny of cross-pollinated maize (*Zea mays*) were 25% taller than progeny of inbred maize (Hochholdinger and Hoecke, 2007). Heterosis has an important role in improving the productivity of plants and animals. It has been used for over a century to obtain benefits in animal production industries (Simm, 1998; Sheridan, 1981; Dickerson, 1973). In agriculture, a large proportion of crops, including maize, rice and sorghum, are grown mainly as hybrids (Chen, 2010).

In the field of animal breeding and genetics, heterosis was defined by Lush (1945) as "the superiority of the out-bred animals over the average of their parents in individual merit". Robinson et al. (1956) defined heterosis more precisely as the higher average performance of reciprocal crosses than the average performance of parents. Heterosis can be negative when the desired quantitative trait is lower in hybrid individuals than in parents or homozygotes, and positive when the desired trait is higher in hybrids than in parents or homozygotes (Comings and MacMurray, 2000). Heterosis is organism, population, age and/or environment dependent (Budak et al, 2002; Gregory et al. 1992). This could explain the conflicting results of studies involving experiments designed to investigate the mechanism of heterosis. The

units used for measurement of heterosis are often defined as a percentage (Kress and Nelsen, 1998; Brown et al., 1997 and 1993) and can be calculated as:

Heterosis % = [(crossbred average-purebred average)/purebred average] × 100

Generally, the degree of heterosis is proportional to the genetic differences of parental strains. In other words, the level of heterosis increases as genetic distance between parents increases (Chen, 2010).

1.1.1 Heterosis effects in cattle

Crossbreeding is a widely accepted production tool in the cattle industry and may increase the level of production by up to 30% (Cundiff et al., 1992). Crossbreeding systems benefit from heterosis and differences among breeds to enhance performance of economically important traits and traits involved in adaptability to different climatic and nutritive environments (Chase et al., 2004; Koch, 1985).

Traits which are most noticeably affected by heterosis are often described with low or moderate heritability, such as survivability and fertility related traits (Table 1.1) (Drake and Phillips, 2006; Kress and Nelsen, 1998). The highest level of heterosis is seen in the F_1 generation and it breaks down rapidly in the F_2 and subsequent generations (Gregory et al., 1994; Sacco et al., 1991). Heterosis retention is the hybrid vigour which will be expressed in F2 and subsequent generations of crossbred animals (Bourdon, 2000; Gregory et al., 1999). Heterosis effects for some specific traits in beef cattle of *Bos taurus* are detailed in Table 1.2.

Table 1.1 Summary of heterosis effects for trait categories with different heritabilities in *Bos taurus* cattle (Kress and Nelsen, 1998).

Trait	Total heterosis
Carcass measurements Skeletal measurements Mature weight	Low (0 to 5%)
Growth rate Early weights Milk production	Medium (5 to 10%)
Maternal ability Reproduction Health Cow longevity Overall productivity	High (10 to 30%)

Table 1.2 Average levels of heterosis for specific beef cattle traits (Kress and Nelsen, 1998).

Trait	Heterosis (%)
Gestation length	0
Calving rate	6
Weaning rate	8
Female age at puberty	-3
Survival birth	1
Survival weaning	4
Calving difficulty	2
Birth weight	6
Weaning weight	11
Yearling weight	4
Cow mature weight	1
Cow mature height	1
Carcass weight	3
Dressing %	0
Loin eye area	2
Fat thickness	6
Kidney fat	5
Carcass yield grade	5
Feed conversion (TDN / gain)	-2
Tenderness	0
Palatability	0
Trimmed retail cuts	3
Cow milk production	9
Calf weaning wt. / cow exposed	18
Cow-calf TDN consumed	3
Calf weaning wt. / cow wt.	8
Cow longevity	38
Cow lifetime productivity	25

Bos taurus (or taurine) and *Bos indicus* (or indicine) are subspecies of domestic cow (Hiendleder et al., 2008). The breeds of cattle used in crossbreeding can be of the same subspecies, such as Angus and Hereford, or a cross between 2 different subspecies, such as crosses between *Bos taurus* and *Bos indicus* breeds. When subspecies are crossed, a greater amount of heterosis is expected due to the fact that their genetics differ at more loci than between two *Bos taurus* breeds or two *Bos indicus* breeds. Gregory and Cundiff (1980) stated that "although the performance of purebred Bos indicus cattle that have been available has generally been low for most economic traits, the performance of crosses of *Bos indicus* and *Bos taurus* cattle has been impressive for most traits that contribute to maternal performance".

The *Bos taurus* × *Bos indicus* cross benefits from traits seen in both species, such as heat and drought tolerance of the *Bos indicus* and reproductive performance and carcass qualities seen in some Bos taurus breeds (Prayaga, 2003). Specifically, there is a noticeable difference in the performance of reciprocal cross (*Bos indicus* bulls bred to *Bos taurus* cows, versus *Bos taurus* bulls to *Bos indicus* cows) calves for several size and growth traits (Amen et al., 2007) and carcass characteristics (Elzo et al., 2012) (Table 1.3).

Table 1.3 Average heterosis (%) in the economically important beef traits when crossing divergent breeds of cattle (Adapted from Cundiff et al., 1994 and Elzo et al., 2012).

Trait	***Bos taurus* × *Bos taurus***	***Bos indicus* × *Bos taurus***
Birth weight	2.4	11.1
Weaning weight	3.9	12.6
Post-weaning gain	2.6	16.2
Hot carcass weight	2.6	10.8

In addition, Peacock et al. (1978) showed that Brahman-Angus crossbred calves had 12% higher weaning weight in comparison to purebred individuals. Brown et al. (1993) reported that Brahman × Angus and Angus × Brahman crossbred cows and their calves were more tolerant to the negative effects from grazing tall fescue than purebred contemporaries. Crossbred calves had significantly heavier birth weights and body weight at D-205. The results of their study showed significantly higher heterosis effects can be achieved when the bull is Brahman rather than Angus in crossbred calves (Figure 1.1, A). Similarly it has been shown that heterosis for birth weight and weaning weight of Brahman-Angus calves is 12.4% and 13.1%, respectively (Riley et al., 2007), where Brahman was the sire of hybrid animals.

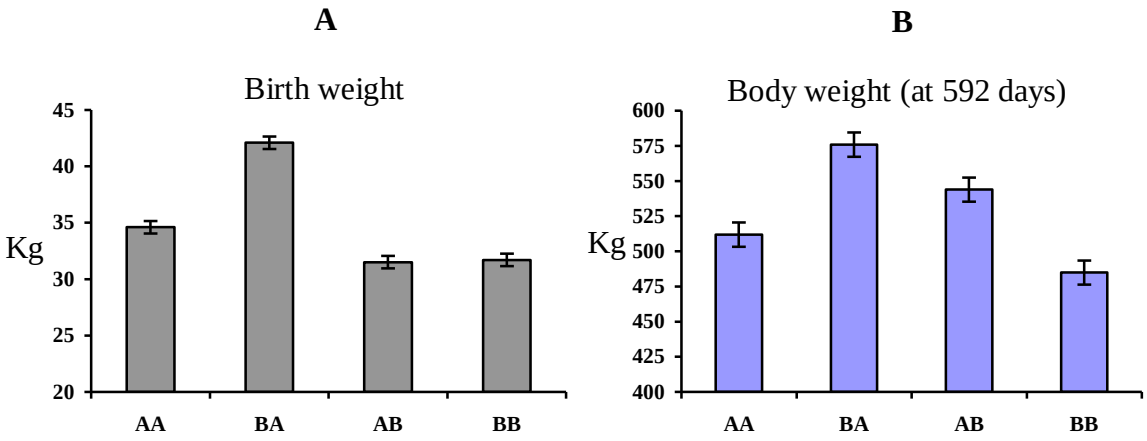

Figure 1.1 Heterosis effects in bovine inter-subspecies hybrids. A: Angus, B: Brahman, first letter indicates sire breed (A: data from Brown et al., 1993 and B: data from Riley et al., 2012).

It was shown that when the sire is Brahman rather than Angus, heterosis effects are significantly higher in comparison to other combinations (Riley et al., 2007). Hybrid calves of Angus and Brahman showed 12.3% heterosis in body weight at the average age of 592 days.

However, body weight of hybrids with a Brahman sire was significantly heavier (Figure 1.1, B) (Riley et al., 2012).

Crossbreeding between taurine and indicine cattle breeds is practiced to gain benefits from combining diverse genotypes in the hybrid offspring by achieving higher levels of heterosis. Significant reciprocal differences in pre- and post-weaning traits can be observed between *Bos taurus* × *Bos indicus* and *Bos indicus* × *Bos taurus* crosses (Peacock and Koger, 1980). Reciprocal crosses have the same "nuclear genomes"; this suggests an imbalance in expression of some genes could underlie the divergent growth phenotypes in reciprocal hybrids (Guo et al., 2004).

1.1.2 Theories to explain heterosis

Effective prediction of hybrid performance with traditional classical genetic models is not possible (Zhang et al., 1996). Despite the rediscovery of heterosis about a century ago and the suggestion of various genetic models to explain this phenomenon the mechanism(s) underlying heterosis have remained elusive to scientists (Sanghera et al., 2011; Chen, 2010; Hochholdinger and Hoecker, 2007; Budak et al., 2002).

Classic quantitative genetics has put forward several theories to explain the genetic basis of heterosis. The first hypothesis is dominance which means that heterosis results from different unfavourable alleles present in the inbred parental lines. Those alleles are complemented in the hybrid individuals by superior alleles from the other parent (Jones, 1917). Thus, harmful recessive mutations of one or the other parental line are masked by the dominant alleles and cannot reappear in the F_1-hybrid offspring resulting in a masking effect and consequent superiority in the performance of hybrids compared to their homozygous parents. The next

historical explanation for heterosis is overdominance which indicates that allelic interactions that occur in the hybrid and the heterozygotes perform better than either homozygous class (Birchler et al., 2003). The most recent possible explanation proposed is epistasis, which was defined by Dickerson (1952) as effects of alleles of a gene on the expression of alleles of other genes. Forms of epistasis which could cause the superior performance in F_1 hybrid offspring include the combinations of alleles of genes that have become fixed over time in different breeds (Dickerson, 1969; Lush, 1946). Dominance, overdominance and epistasis refer to non-additive genetic effects, although they have one common characteristic which is "allelic interaction". These terms were coined before the molecular concepts of genetics were discovered and are not connected with molecular principles. Therefore, they are not adequate for describing the molecular parameters that cause heterosis (Alexander et al., 2009; Birchler et al., 2003).

Recent studies in plants have determined the roles of small RNAs and epigenetic regulation in hybrid vigour (Ding et al., 2012; Chen, 2010). Groszmann et al. (2011) showed that reciprocal hybrids of *Arabidopsis thaliana* have a decreased level of 24-nt small RNA (sRNA) relative to the inbred parents. This change in sRNA level was correlated with gene expression and DNA methylation (Groszmann et al., 2011). Since none of the classical quantitative genetic theories can sufficiently explain heterosis we hypothesised that epigenetic mechanisms, such as RNA associated silencing, may explain observed phenotypic differences of hybrid individuals (Groszmann et al., 2013). More details of epigenetics and RNA associated silencing will be presented later in this chapter.

1.1.3 Possible candidate genes and pathways for heterosis

Candidate genes have known biological functions related to the development or physiology of an economically important trait in farm animals (Rothschild et al., 1997). These genes can be responsible for producing structural proteins, enzymes or a component of a regulatory or biochemical pathway affecting the expression of the trait and can be evaluated as putative QTLs (Yao et al., 1996). Growth in animals is controlled by a complex system, in which the somatotropic axis has a crucial role. The genes that play key roles in the somatotropic axis are mainly growth hormone (GH) or somatotropin that acts on the growth of bones and muscles which is mediated through insulin-like growth factor-1 (IGF1) or somatomedin C (Laron 2001; Sellier, 2000).

The bovine chromosome 5 contains QTLs that affect growth and carcass traits (Moody et al., 1996; Casas et al., 2000), reproduction (Kirkpatrick et al., 2000; Lien et al., 2000), birth weight (Gasparin et al., 2005) and milk production (Kalm et al., 1998). The location of some of these QTLs approaches the position of the *IGF1* and *IGFBP6* genes and, since IGF1 plays a fundamental role in regulation of growth and development, this gene is considered a strong candidate for the QTL effect (Machado et al., 2003).

In sheep and cattle, it has been shown that high birth weight observed in *in vitro* produced (IVP) embryos and nuclear transfer (NT) derived, known as large offspring syndrome (LOS), is associated with IGF components such as IGF2R and IGF1 (Wrenzycki and Niemann, 2003). Both serum IGF1 and IGF2 levels were strongly correlated to fetal body weight and growth rate during normal fetal development in cattle (Holland et al., 1997). Hiendleder et al. (2006) showed that plasma IGF1 level is correlated with bovine fetus weight in IVF produced embryos. It has also been shown that fetal and cord serum IGF1 concentrations are correlated

with birth weight in human (Fowden, 2003; Leger et al., 1996; Spencer et al., 1995). Elevated IGF2 concentration was significantly associated with higher birth weight in human (Hoyo et al., 2012). In contrast to positive association between IGF1 and IGF2 and birth weight, IGFBPs have been shown to have a negative association with birth weight (Asvold et al., 2011; Randhawa and Cohen, 2005; Boyne et al., 2003; Rajkumar et al., 1995). These studies indicate that IGF system components are important genes affecting prenatal growth and development and birth weight. Caldwell et al. (2011) demonstrated that IGF1 serum level is significantly ($P<0.05$) higher in hybrid calves of Brahman × Angus at D-84 of age (Figure 1.2).

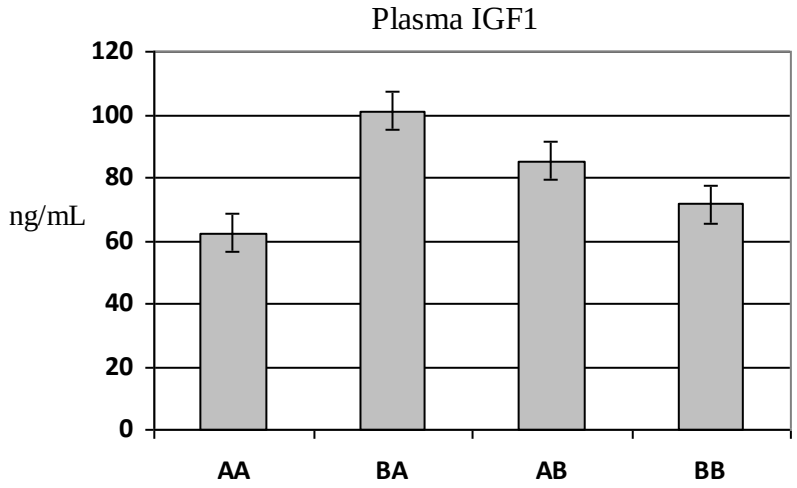

Figure 1.2 IGF1 plasma concentrations in D-84 calves of purebred and hybrid individuals. A is Angus, B is Brahman and the first and second letters in a genotype indicate the breed of sire and dam, respectively (with data from Caldwell et al, 2011).

Brown et al. (1993) showed that a larger amount of heterosis between hybrid calves belonged to offspring of Brahman bulls and Angus cows (Refer to Figure 1.1) which is similar to the

IGF1 level in the hybrid calves with the same breeds as published by Caldwell et al. (2011). Outcrossing of inbred mice showed a positive heterosis for serum concentrations of IGF1 in heterozygote animals (Adamo et al., 2006).

A QTL study in pigs showed a major effect on muscle growth that was mapped to the IGF2 gene (Van Laere et al., 2003). This QTL loci is actually a QTN (quantitative trait nucleotide) which is an SNP located in intron 3 of the IGF2 gene suggesting that the QTL effect was most likely due to a regulatory rather than a structural mutation (Van Laere et al., 2003).

QTL studies have shown growth hormone and its receptor to be important in cattle. Taylor et al. (1998) reported a QTL effect on subcutaneous fat mapped to a region of bovine chromosome 19 which is harbouring the GH gene. Also a number of QTL studies in cattle breeds have shown effects on milk yield and composition, as well as carcass traits, on bovine chromosome 20 close to the location of the growth hormone receptor (GHR) (Khatkar et al., 2004). It has been shown that SNPs located in the IGF system and GH-IGF1 axis in cattle can be considered as potential candidate genes associated with reproduction traits (Luna-Nevarez et al., 2011).

A number of studies in human, mouse and livestock have revealed that a majority of postnatal traits are affected by prenatal growth and development (Silveira et al., 2007). Based on the similar trend observed between heterosis of birth weight and plasma IGF1 level, and also the crucial role of somatotropin in growth and development, we hypothesised that the IGF system and GH-IGF1 axis could contribute to the altered prenatal development observed in bovine heterosis.

1.2 The IGF system and GH-IGF1 axis

The IGF system is composed of two ligands (IGF1, IGF2), two types of IGF receptors (IGF1R and IGF2R), the insulin receptor (IR) and IGF binding proteins (IGFBPs) (Moore et al., 2007; Denley et al., 2005) (Figure 1.3). The GH-IGF1 axis consists of GH and GHR in addition to IGF1 and its related IGF system components.

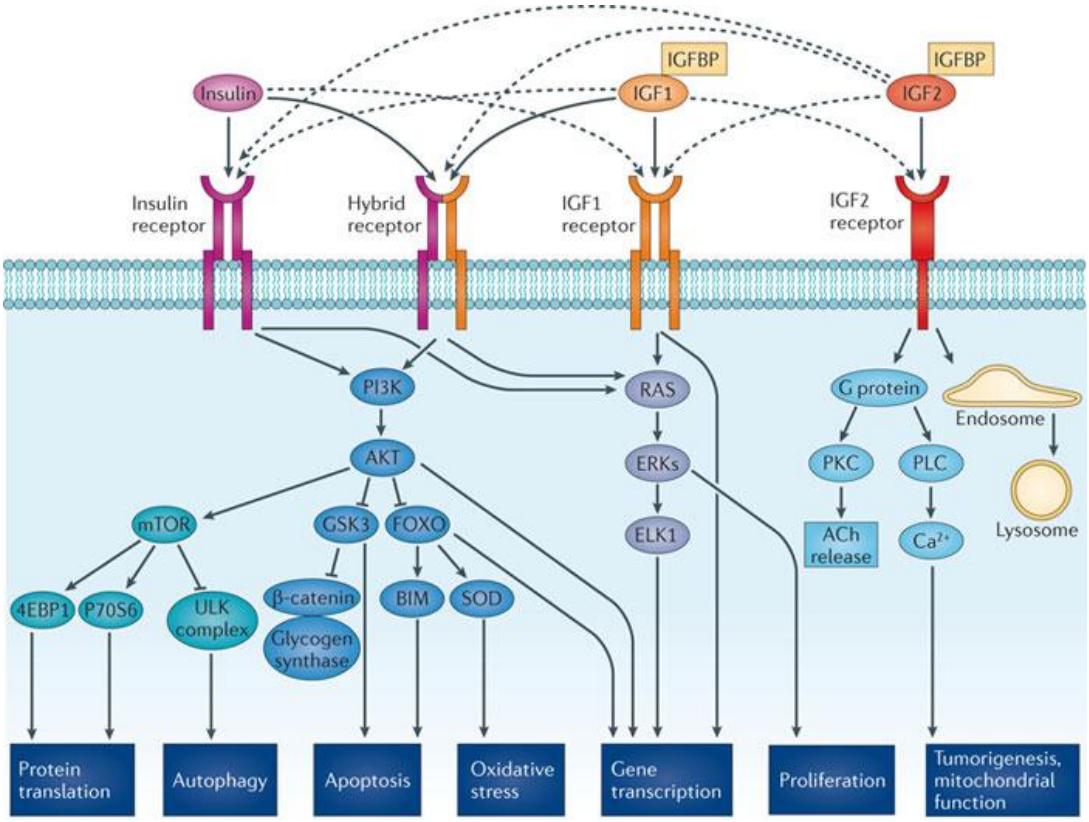

Figure 1.3 Schematic illustration of pathways involving the insulin-like growth factor system (Fernandez and Torres-Alemán, 2012).

IGF ligands were shown to be important in maintaining normal tissue growth and development pre and postnatally, cell proliferation and differentiation, and IGFBPs can act as potentiators of cell proliferation (Firth and Baxter, 2002; Jones and Clemmons, 1995).

However, altered function of the IGF system is also associated with a wide variety of cancers, such as breast, prostate, ovary and lung cancers (Reviewed by Maki, 2010; Firth and Baxter, 2002).

1.2.1 Insulin-like growth factor type 1 and type 2

Bovine *IGF1* and *IGF2* genes are located on chromosomes 5 (Miller et al., 1992) and 29, respectively (Goodall and Schmutz, 2003). They consist of 4 and 10 exons with lengths of 71.80 kb and 18.62 kb, respectively (NCBI GenBank accession numbers: NC_007303 and NC_007330, respectively). *IGF1* transcription is initiated from both exon 1 and exon 2, generating IGF1 RNA containing either exon 1 (*IGF1 class 1* mRNA) or exon 2 (*IGF1 class 2* mRNA) as the leader exon (Zhang et al., 2011). Thus, the two classes of *IGF1* mRNA encode prepro-IGF1 proteins differing only in length of the signal peptide. *IGF1 class 1* mRNA is expressed at relatively high levels in all studied tissues, whereas *IGF1 class 2* mRNA is tissue-specific and predominantly expressed in liver (Wang et al., 2003).

IGF1 is classified as an anabolic and mitogenic hormone; it stimulates protein and glycogen synthesis, increases DNA synthesis, stimulates cell cycle progression and inhibits apoptosis (Jones and Clemmons, 1995). Mice carrying null mutations of the *IGF1* gene are born small and grow poorly postnatally (Baker et al., 1993).

Most circulating IGF1 is produced in the liver, postnatally. Conditional liver knockout of the *IGF1* gene in mice results in significantly suppressed serum levels of IGF1, but without any corresponding decrease in postnatal growth (Sjogren et al., 1999). This suggests that paracrine/autocrine IGF1 effects might be more important than circulating IGF1 for longitudinal bone growth (Sjogren et al., 1999). Insulin-like growth factor 2 is a potent

growth promoter and essential for prenatal development. The *IGF2* gene is maternally imprinted and paternally expressed in human and cattle (Dindot, 2004; Giannoukakis, 1993). Targeted disruption of the paternal *IGF2* allele in mouse caused a significant reduction in birth weight (Dechiara, 1990). The expression pattern of the *IGF2* gene in cattle has not yet been fully studied in detail, but significant differences in expression patterns have been seen among species (Amarger et al., 2002). In human and bovine, *IGF2* has four promoters (P1-4) that drive transcription from a total of 10 exons, with untranslated leader exons 1 (P1), 4 and 5 (P2), 6 (P3) and 7 (P4). Alternative splicing of P2 transcripts yields two splice variants with leader exon 4 or leader exons 4 and 5. Exons 8, 9 and 10 are protein coding and present in all transcripts (Goodall and Schmutz, 2007; Curchoe et al., 2005; Amarger et al., 2002; Ohlsen et al., 1994).

1.2.2 Insulin-like growth hormone receptors

Bovine *IGF1R* and *IGF2R* are located on chromosome 21 and 9 and contain 20 and 48 exons, respectively. The full length of bovine *IGF1R* and *IGF2R* genes are 59.56 and 101.13 kb (NC_007319 and NC_007307). The structure of the IGF1R and the insulin receptor (IR) is similar, but their physiological roles are distinct. The biological actions of IGF1 are mediated by the IGF1R which belongs to tyrosine kinase receptor family. In normal physiological conditions, IGF1R stimulates linear body growth, promotes neuronal survival, postnatal mammary gland development and lactation and is also associated with bone formation and kidney function (Jones and Clemmons, 1995). The cellular action of both IGF1 and IGF2 is mediated through tyrosine kinase receptors, IGF1R and IR (Denley et al., 2006). Signal transduction for IGF1 and IGF2 occurs predominantly through IGFIR (Cohick and Clemmons, 1993). Expression of IGF1R is stimulated by hormones such as GH (LeRoith et al., 1995). The IGF1R binds IGF1 with high affinity while IGF2 is bound to IGF1R with only

20% of the affinity of IGF1 (Denley et al., 2004). Virtually every tissue and cell type expresses *IGF1R* mRNA, although it is a low abundance transcript (LeRoith et al., 1995). *IGF1R* knockout in mice results in more severe growth failure than deletion of IGF1, and the IGF1R null mice die of respiratory failure within minutes after birth (Baker et al., 1993), showing the vital importance of IGF1R in prenatal development.

The IGF2R, also known as the cation-independent mannose-6-phosphate receptor, is imprinted and expressed from the maternal allele in cattle (Bebbere et al., 2013; Killian, 2001). The IGF2R gene does not initiate signal transduction, but controls extracellular IGF2 concentrations by mediating the endocytosis of IGF2 and its subsequent degradation in lysosomes (Hawkes and Kar, 2003). Soluble IGF2R appears to take part in regulating bioavailability of IGF2 as it accounts for 50% of total IGF2 binding in the fetal sheep blood, but is dramatically downregulated in adults (Gallaher et al., 1994). It has been shown that IGF2R is an important factor affecting birth weight in mouse. Bi-allelic expression of IGF2R causes a 25% reduction in weight and let the embryonic development to persist to adulthood in mice (Wutz et al., 2001).

1.2.3 Insulin receptor

The *insulin receptor* (*IR*) gene is located on bovine chromosome 7, contains 22 exons (NCBI accession number NC_007305) and spans 120.45 kbp. Human and cattle *IR* exist in two isoforms determined by alternative splicing of exon 11 located at the carboxy-terminus of the receptor α-subunit. *Isoform A* (*IR-A*) lacks exon 11, whereas *isoform B* (*IR-B*) contains a 12 amino acid extension encoded by this small exon (Moller et al., 1989). The relative expression of these two isoforms varies in a tissue-specific manner. The isoform A displays higher affinity for IGF2 than IGF1 and can be considered as a second physiological receptor

for IGF2, while IR-B is very specific to insulin (Pandini et al., 2002). The IR-A isoform is expressed in fetal tissues and promotes cell growth in response to IGF1 and IGF2 (Frasca et al., 1999). Each of the IR isoforms is equally able to hybridise with the IGF1R, but isoform A has 1.7 fold more affinity for insulin than isoform B (Mosthaf et al., 1990) (Figure 1.4). Furthermore, hybrid receptors that result from the dimerization of the IGF1R and IR hybrid receptors have different affinities for IGF1, IGF2 and insulin. IGF1R/IR-A hybrids bind IGF1, IGF2, and insulin, while IGF1R/IR-B hybrids bind IGF1 with high affinity, IGF2 with low affinity and does not bind insulin (Pandini et al., 2002). Also, there is a heterodimer receptor which consists of both IR-A and IR-B (HIR-AB) (Pollak, 2012). IR is structurally and functionally related to IGF1R. The structural similarities allow formation of hybrid receptors in which an IGF1R αβ-chain is connected to an IR-A or -B αβ-chain. Hybrid receptors, particularly those incorporating IR-A, bind IGF1 and IGF2 with high affinity, which can result in both proliferative and anti-apoptotic responses (Pandini et al., 2002). Despite the similar structure and actions of IR and IGF1R, mice lacking IR have a 10-20% retardation of growth and are viable (Louvi et al., 1997), but mice lacking IGF1R have 50-60% growth retardation and die shortly after birth (Liu et al., 1993) which may indicate a compensating effect of IGF1R in the absence of IR during fetal development. Mice lacking both IR and IGF1R showed 70% growth retardation (Louvi et al., 1997).

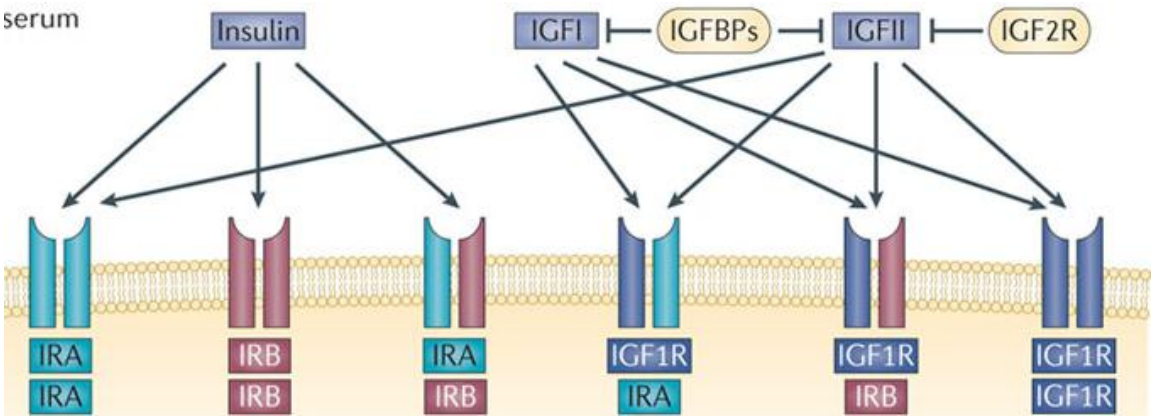

Figure 1.4 Overview of different combinations of insulin receptor and type 1 insulin-like growth factor receptor and their relationship with ligands (Pollak, 2012).

1.2.4 Insulin-like growth hormone binding proteins

Genes encoding IGFBPs are located on different chromosomes of cattle (Table 1.4). Locally produced IGFs and IGFBPs regulate tissue growth and differentiation. The IGFBPs are thought to modulate the action of IGFs in several ways, including (I) an inhibitory model in which IGFBPs sequester IGFs from their receptors, (II) an enhancing model in which IGFBPs transport IGFs to their site of action, or (III) by an IGF-receptor-independent model that may involve direct interaction of IGFBPs with IGFBP receptors (Allan et al., 2001; Cohen et al., 1993; Angervo et al., 1991).

Table 1.4 Chromosomal locations of bovine insulin-like growth factor binding proteins and affinity to IGF1 and IGF2.

Gene symbol	Chromosome number	Gene bank accession number	Length of the gene (bp)	IGF1/IGF2 affinity
IGFBP1	4	NW_003103902.1	4419	IGF1 = IGF2
IGFBP2	2	NW_001494682.3	28487	IGF1 < IGF2
IGFBP3	4	NT_181996.1	7984	IGF1 = IGF2
IGFBP4	19	NM_174557.3	11590	IGF1 = IGF2
IGFBP5	2	NM_001105327.1	3334	IGF1 < IGF2
IGFBP6	5	NM_001040495.1	3846	IGF1< IGF2
IGFBP7	6	NW_001495197.4	79393	Insulin>IGF1&IGF2
IGFBP8	9	NT_182009.1	3236	?

Around 99 % of circulating IGFs are bound to IGFBPs (Hossner et al., 1997). The modulation of IGF levels by IGFBPs is further regulated by IGFBP proteases which cleave high affinity IGFBPs into fragments with lower affinity for IGFs, thereby increasing free IGF bioavailability. This process leads to reduced inhibition of cell growth by IGFBPs (Conover et al., 1995). Each of IGFBPs can undergo proteolysis, which results in decreased affinity for IGFs. While some IGFBP proteases can use multiple IGFBPs as substrates, there are apparently proteases that are specific for individual IGFBPs (Schneider et al., 2002).

There are a limited number of IGFBP deletion models published that investigate the role during development and, in the mouse model, it seems that IGFBP deletion does not have severe effects (Firth and Baxter, 2002). IGFBP2 knockout mice showed no overall growth retardation, but they had smaller spleen and larger liver size than normal mice (Wood et al., 2000). Interestingly, IGFBP3 and IGFBP5 double knockout mice showed lower plasma IGF1 concentration, but heavier body weight, with greater relative organ weight, including kidneys and spleen, compared with normal adult mice (Murali et al., 2012). Over expression of

IGFBP1, IGFBP2 and IGFBP3 led to reduction in brain weight and abnormal brain development in mice (Silha and Murphy, 2002).

IGFBP1 was the first characterised member of the IGFBP family and was originally isolated from human amniotic fluid and temporarily termed “amniotic fluid-binding protein“. The affinity of IGFBP1 is 5 -10 times higher to IGF1 than to IGF2 in human and rat (Kostecka and Blahovec, 2002). Also, IGFBP1 concentration in adult human serum is 100 times less than IGFBP3. It has been shown that IGFBP1 is expressed predominantly from liver in mid-gestation rat fetuses (E14) (Ooi et al., 1990), and mouse fetuses (Cerro et al., 1993). IGFBP3 is growth hormone-dependent and has similar affinity to both IGF1 and 2. This binding protein is the predominant IGFBP in the serum of postnatal animals (Forbes et al., 2012). Baxter et al., (1989) showed that IGFBP3 can bind and carry 75% of IGF1 and 2 in serum of human.

IGFBP4 is the smallest binding protein and is unique in that it has been consistently shown to inhibit IGF actions (Wetterau et al., 1999). It binds IGF1 and IGF2 with similar affinities and is expressed by a large range of cell types and tissues (Zhou et al., 2003). IGFBP6 has a 20-100 fold higher affinity to IGF2 than IGF1 and can be considered a specific inhibitor of IGF2 (Bach et al., 1991).

In addition to the IGFBPs, IGFBP-related proteins (IGFBP-rP) have also been described, leading to the proposal of an IGFBP superfamily (Hwa and Rosenfeld, 1999). There are a number of recognised members in the IGFBP-related protein family including IGFBP-rP1 (mac25 or IGFBP7), IGFBP-rP2 (connective tissue growth factor (CTGF) or IGFBP8), IGFBP-rP3 (NovH or IGFBP9) and IGFBP-rP4 (Cyr61 or IGFBP10) (Kim et al., 1997) which are considered as low affinity IGFBPs, and IGFBP related proteins (Figure 1.5). IGFBP7 is a

high-affinity insulin binding protein which blocks insulin binding to the insulin receptor and insulin action (Yamanaka et al., 1997). IGFBP8 is the least studied IGFBP and has been demonstrated to specifically bind IGFs with low affinity. It is considered to be a member of the IGFBP superfamily and key cytokine in the fibrogenesis of tissues and organs (Hwa et al., 1999). The amino acid sequence of IGFBP8 shares an overall 28–38% identity to IGFBPs and it contains conserved sequences in the amino terminus (Vorwerk et al., 2000). There are a number of studies demonstrating the importance of low affinity IGFBPs during development. IGFBP8-deficient mice showed perinatal death due to respiratory failure and generalised chondrodysplasia (Ivkovic et al, 2003). Also, IGFBP10 knockout mice showed lethality in the embryonic period or shortly after birth (24 hour) (Mo et al., 2002).

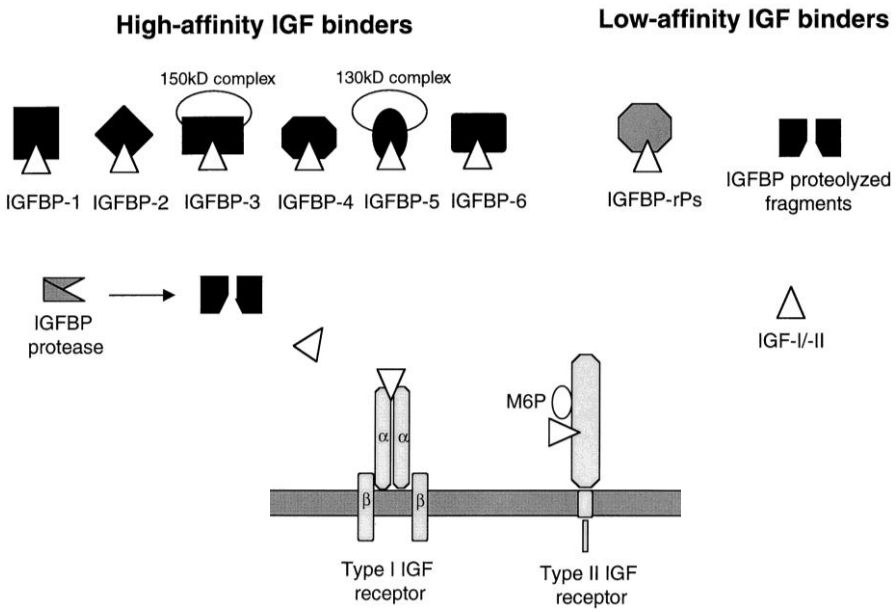

Figure 1.5 Insulin-like growth factor binding (IGFBP) family including high affinity IGFBPs and potential low affinity IGFBPs or IGFBP related proteins (rPs) (modified from Hwa and Rosenfeld, 1999).

1.2.5 Growth hormone and its receptor

The *GH* gene is located on bovine chromosome 19 and consists of 5 exons with total length of 1.6 kbp (NCBI accession number NW_001493688). *GH* gene expression is mainly from the pituitary gland and pituitary GH is a key stimulator of IGF1 production and also affects IGF2 production in liver (von Horn et al., 2002).

GH expression is not confined to the pituitary gland, and is also present in many extra-pituitary tissues, postnatally, in which it may act as an autocrine or paracrine growth factor (Harvey, 2010). Among those tissues, placental GH seems to be important for growth and development of the fetus. Since placenta GH cannot be detected in the fetal circulation and only presents in maternal blood and amniotic fluid, the direct role of placental GH in prenatal development is still controversial (Skottner, 2012; Edmonson et al., 1995; Garcia-Aragon et al., 1992). Spencer et al. (1995) measured umbilical blood concentrations of IGF1 and GH in normal and growth retarded human newborns, finding that IGF1, but not GH, is significantly lower in growth retarded individuals.

Fetal pituitary produces GH from the second trimester in some species including human, cow and sheep (Waters and Kaye, 2002). However, GH deficient mice, due to mutations in the genes encoding GH-releasing hormone receptor, showed normal birth weight (Efstratiadis, 1998). In sheep, a transcript identical to pituitary GH mRNA is expressed in the placenta after D-27 of the first trimester of pregnancy (Lacroix et al., 1999). In humans, the pituitary *GH* gene (*GH1*) is not expressed in the placenta, but a placental *GH* (*GHV* or *GH2*) is transcribed and translated into several placental GH proteins (Harvey, 2010). In humans, both *GH1* and *GH2* are located next to each other on chromosome 17 with 98% homology (Vnencak-Jones et al., 1988) as well as in sheep (chromosome 11) (Vacca et al., 2013; Lacroix et al., 2002)

and goats (Wallis et al., 1998). *GH1* secretion is pulsatile, while *GH2* has lower expression and is not pulsatile (Harvey, 2010). Furthermore, placenta GH can be detected from week 6 in maternal circulation, but not in the fetus, suggesting that it has no direct role in fetal development in human (Wu et al., 2003). Interestingly, there is not any known duplicated *GH* gene (or *GH2*) in mice and cattle (Skotner, 2012). GH actions are mediated through growth hormone receptor (GHR). Binding of GH to GHR initiates the transcription of many genes including IGF1 (Jiang et al., 2007).

It has been shown that *GHR* transcripts are present in fetal tissues in rat (Edmonson et al., 1995), cattle (Lucy et al., 1998) and human (Hill et al., 1988), which maybe an indicator of a possible prenatal role for GH. The GHR mediates the biological actions of GH on target cells by transducing the stimulating signal across the cell membrane and also inducing the transcription of many genes, including *IGF1* (Rotwein et al., 1991) (Figure 1.6). Upon binding of GH to GHR, signal transduction initiates with phosphorylation of cytosolic Janus kinase 2 (JAK2) which activates STAT pathways. STAT5b is directly involved in regulation of IGF1 transcription (Hwa et al., 2011).

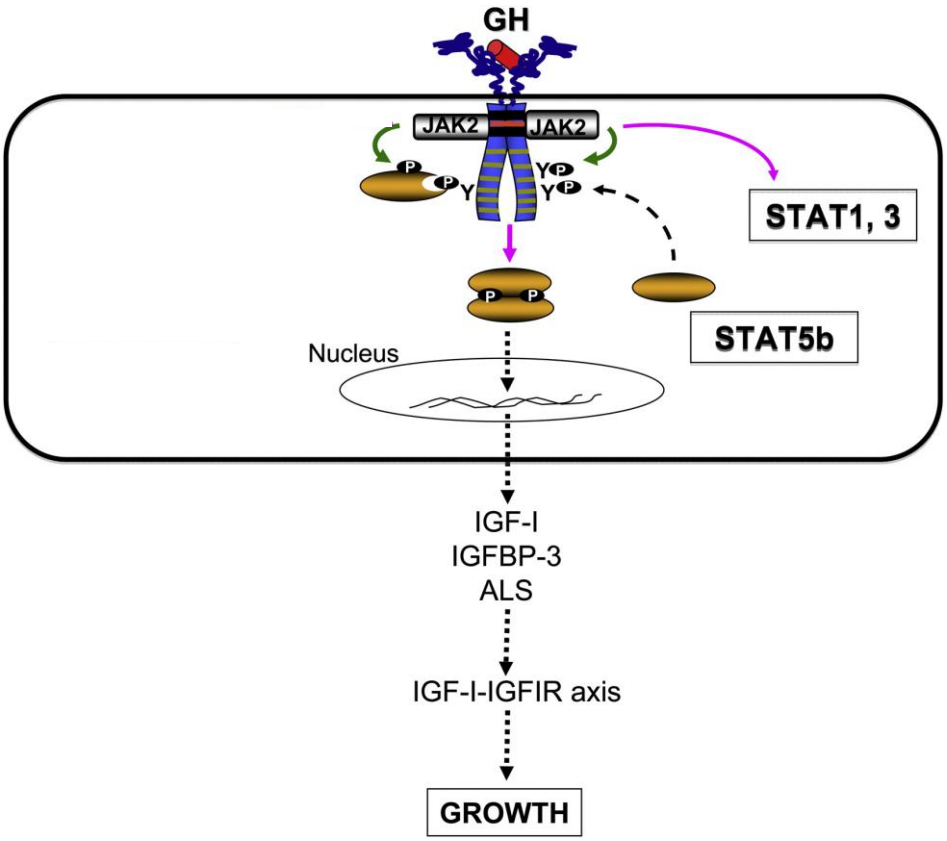

Figure 1.6 Schematic representation of the IGF-GH axis. Most of anabolic actions of GH are mediated by IGF1, which is produced in many different tissues, with most circulating IGF1 being derived from the liver. IGF1 acts through the IGF1 receptor by autocrine, paracrine and classical endocrine mechanisms (modified from Hwa et al., 2011).

GHR and GH are closely related to a family of hormone-receptors include prolactin (PRL), prolactin receptor (PRLR) and placental lactogen (PL) (Kelly et al., 1991). There is a high degree of similarity between GH and PRL and their receptors (GHR and PRLR) amino acid sequences, gene structures and functions (Goffin and Kelly, 2001). It has been shown that GHR can be a receptor for PRL and PL hormones, and interestingly, GHR and PRLR can form a functional heterodimer receptor (Goffin and Kelly, 2001). Upon binding either of GH, PRL or PL to GHR, signal transduction initiates with phosphorylation of cytosolic Janus kinase 2 (JAK2) which activates STAT pathways (Figure 1.7) (Hwa et al., 2011).

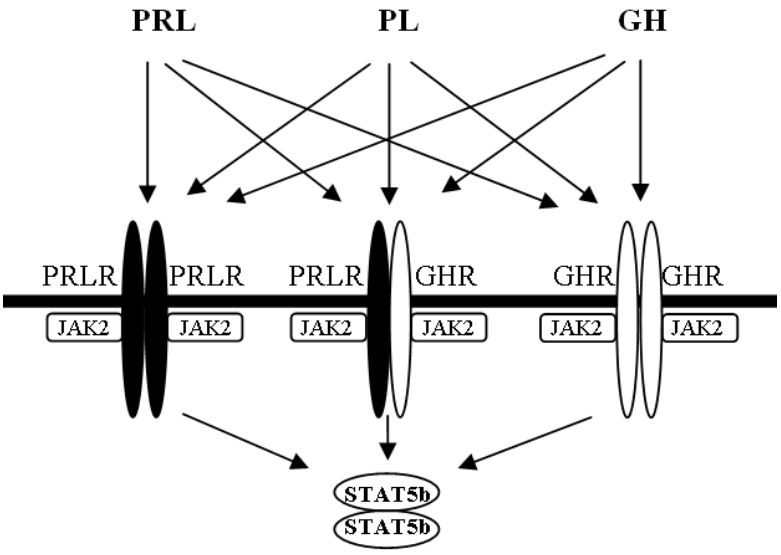

Figure 1.7 The actions of growth hormone (GH), prolactin (PRL) and placental lactogen (PL) are mediated by prolactin receptor (PRLR) and growth hormone receptor (GHR) homodimers or heterodimers of PRLR and GHR that stimulate formation of STAT5 homodimers.

The *GHR* gene is located on bovine chromosome 20 and contains 9 coding exons (exons 2 to 10) spanning 173.71 kbp (NCBI accession number NC_007318). It has a long 5' non-coding region that includes nine untranslated exons, 1A–1I resulting in 9 variants of *GHR* mRNA (Jiang and Lucy 2001). Among them, only variants 1A, 1B and 1C are well characterised, with the existence of exons 1D–1I being based on rapid amplification of cDNA end analyses. Variants 1D-1I altogether account for only 10 % of overall *GHR* transcripts (Jiang and Lucy 2001). *GHR* transcripts have been detected in a variety of bovine adult tissues including liver, muscle, kidney, lung, mammary gland, adipose tissue, and fetal tissues of placenta, liver, lung, kidney and skeletal muscle, with the highest level of expression detected in liver (Jiang and Lucy 2001). Expression of *GHR-1A* mRNA is specific to the liver and *GHR-1A* comprises the majority of liver *GHR* mRNA (Lucy et al., 1998). The *GHR-1A* mRNA is different from *GHR-1B* and *1C* mRNA because of its liver specificity and regulation by GH

(Butler et al., 2003). Knockout mice models showed no significant body size or weight effects among normal and GHR knockout newborns; however, GHR deficient mice (Laron mice) were significantly smaller 3 weeks after birth (Zhou et al., 1997). Laron mice had 90% less circulating IGF1, and interestingly these mice live significantly longer (up to 50%) than normal mice (Coschigano et al., 2000).

IGF system and *GH-IGF1* axis genes are described to be important for normal growth and development in several species. These genes and their transcripts have been studied in a range of developmental stages in human, mouse and cattle. To our knowledge, there is not a comprehensive study covering mRNA expression of the *IGF* system and *GH-IGF1* axis genes in bovine. To capture an image of developmental changes of the *IGF* system and *GH-IGF1* axis transcripts, a comparative gene expression study across key developmental stages and different tissues is required. Such a study, would lead to a more effective understanding of the contribution of the *IGF* system and *GH-IGF1* axis genes to the bovine heterosis model.

1.3 Epigenetics

In the 1940s, epigenetics was defined by Waddington as the study of alteration in the expression of genes during development in the absence of any change affecting the gene itself (Redei et al., 2006; Waddington, 1953). Epigenetic mechanisms regulate all biological processes from conception to death, including genome reprogramming during early embryogenesis and gametogenesis, cell lineage differentiation and maintenance of a committed lineage (Delcuve et al., 2009). Today, it is well established that epigenetic modification of the genome ensures proper gene expression during development (Ruvinsky, 1999). The epigenetic marks of germ cells which are of DNA methylation and histone modifications, changes dynamically during development (Sasaki and Matsui, 2008). In

mammals there are two periods in which methylation patterns and chromatin configuration are reprogrammed across genome; in germ cells and in preimplantation embryos (Reik et al., 2001). During germ cell development, epigenetic reprogramming of DNA methylation resets parent-of-origin specific marking of imprinted genes and restores totipotency to gametes. Immediately after fertilization, the paternal genome undergoes a genome-wide DNA demethylation while the maternal genome seems to be unaffected (Dean et al., 2003; Li, 2002).

Epigenetic mechanisms including genomic imprinting; X-chromosome inactivation and developmental stage and tissue specific gene silencing/activation depend on epigenetic modification including DNA methylation and histone modification. Epigenetic mechanisms are critical for normal development (Bernstein et al., 2007).

Another major regulatory mechanism that controls gene expression (Wang et al., 2007) is microRNA interference which is considered as a epigenetic mechanism, although many microRNA-mRNA interactions can be considered as epistasis.

1.3.1 DNA methylation and histone modifications

In mammals, DNA methylation occurs at the 5'-cytosine residues of CpG (cytosine phosphate diester guanine) dinucleotides, especially in CpG islands in the promoter regions of genes, and can result in gene silencing. CpG dinucleotides are typically clustered in GC rich regions termed CpG islands (Walsh and Bestor, 1999). The complementary strand of 3'GpC 5' is methylated and with the CpG together, exhibit a three dimensional structure prominent in the major groove of the double stranded DNA. In most mammals, 60-90% of all CpG sequences in the genome are methylated which equates to approximately 3×10^7 methylated cytosines).

The methylation profile of DNA is maintained after each replication by methyltransferases, thus DNA methylation becomes a heritable feature. Methylation of DNA has an effect on expression of individual genes, but can also contribute to regulatory mechanisms that involve a large segment of DNA that may contain a cluster of genes (Bestor, 2000).

The histone proteins that constitute the main body of nucleosomes are modified by several chemical changes such as acetylation, methylation, phosporylation, and ubiquitination. These epigenetic modifications correlate with chromatin structure and the expression or repression of genes. Distinct histone modifications can influence each other and may also interact with DNA methylation (Bernstein et al., 2007). Similar to the genetic code which consists of nucleotides, there is a histone code that has a fundamental regulatory effect on tissue specific gene expression during development (Jenuwein and Allis, 2001; Rice and Allis, 2001). This code can be read by regulatory proteins to affect transcription, chromosome condensation and replication during mitosis. The histone code can explain a possible link between chemical modification of histones and epigenetic regulation of gene expression (Godde and Ura, 2008).

1.3.2 RNA associated silencing

Biological dissection of RNA silencing processes in many eukaryotes indicates that multiple pathways operate to control the expression of target genes, often in a development or tissue-specific manner (McFarlane and Wilhelm, 2010, Raybak, 2009; Stefani and Slack, 2008). Recent work has demonstrated that small RNAs are involved in suppressing the translation of coding genes (Hsu et al., 2006). Small RNAs are non-protein-coding RNAs, including small nucleolar RNAs (snoRNAs), microRNAs (miRNAs), short interfering RNAs (siRNAs) and small double-stranded RNAs (Kawaji and Hayashizaki, 2008; Mattick and Makunin, 2005). Messenger RNA degradation, translational repression or heterochromatin formation are three

mechanisms which define RNA-mediated silencing (Figure 1.8). It involves a small double-stranded RNA (dsRNA), such as siRNA or miRNA. Short interfering RNAs have been found to be the trigger of an evolutionary conserved mechanism known as RNA interference (RNAi) (Wilson and Doudna, 2013; Morris, 2008).

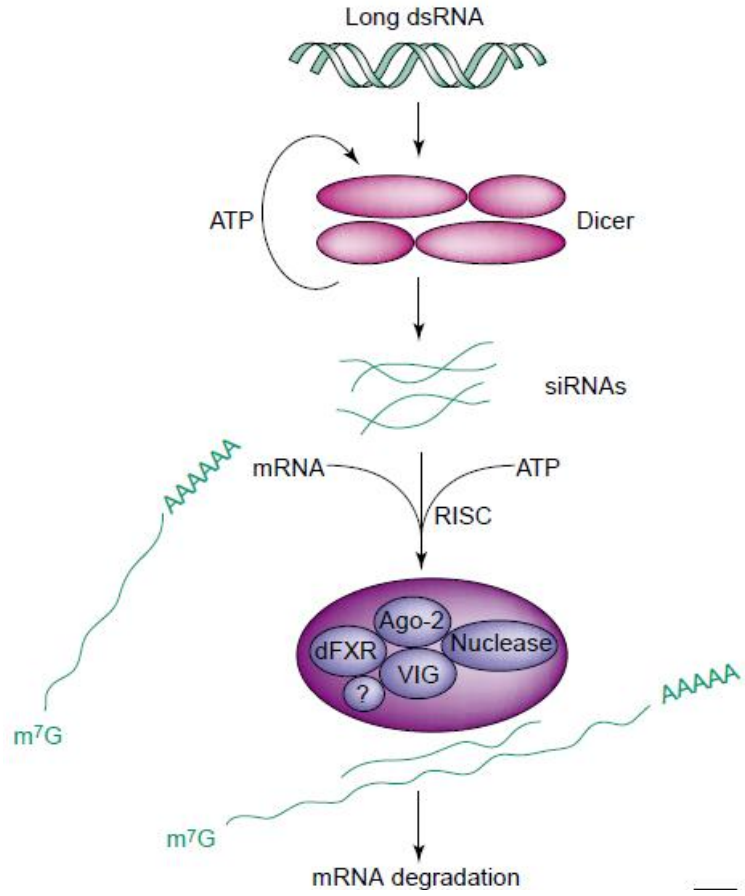

Figure 1.8 mRNA degradation pathway of RNAi. Dicer dimers cleave dsRNAs to form small-interfering RNAs (siRNAs) and siRNAs are incorporated in the RNA-induced silencing complex (RISC). Unwinding of siRNAs activates RISC therefore active RISC is guided to degrade the specific target mRNAs (Denli and Hannon, 2003).

Short RNAs are increasingly recognised to play multiple roles in affecting gene expression at many levels including RNA stability, translation, and quite possibly

transcription and splicing (Ghildiyal and Zamore, 2009; Morris, 2008; Keene, 2007). These small RNAs are predicted to constitute almost 1% of the entire human genome and are assumed to be responsible for regulating up to one third of all mRNAs (Wang et al., 2007; Aravin and Tuschl, 2005). Coding sequences for small regulatory RNA molecules have been located throughout the genome and include intergenic, exonic and intronic regions (Kim and Kim, 2007).

1.3.2.1 MicroRNAs

MicroRNAs are 21-23 nucleotide regulatory RNAs, first discovered in 1993 in *Caenhorhabditis elegans* (Liu et al., 2009). MiRNAs are initially transcribed as pri-miRNAs, can be several kilobases in length and contain a characteristic hairpin loop structure (Lee et al., 2004; 2002). Pre-miRNAs transcripts are cleaved by the RNase III endonuclease Drosha which works in combination with its co-factor DGCR8 to produce a ~70 nucleotide stem-loop intermediate precursor miRNA (Kim, 2004). Following Drosha cleavage, the pre-miRNA stem-loop structure is exported from the nucleus to the cytoplasm by Exportin-5 through a Ran- GTP mechanism (Yi et al., 2003) where it is then cleaved by Dicer to produce the mature form of miRNA (Lee et al., 2002). There appear to be two main mechanisms by which miRNAs impose translational regulation on their specific mRNA target(s): repression and cleavage/degradation (Wang et al., 2007) (Figure 1.9).

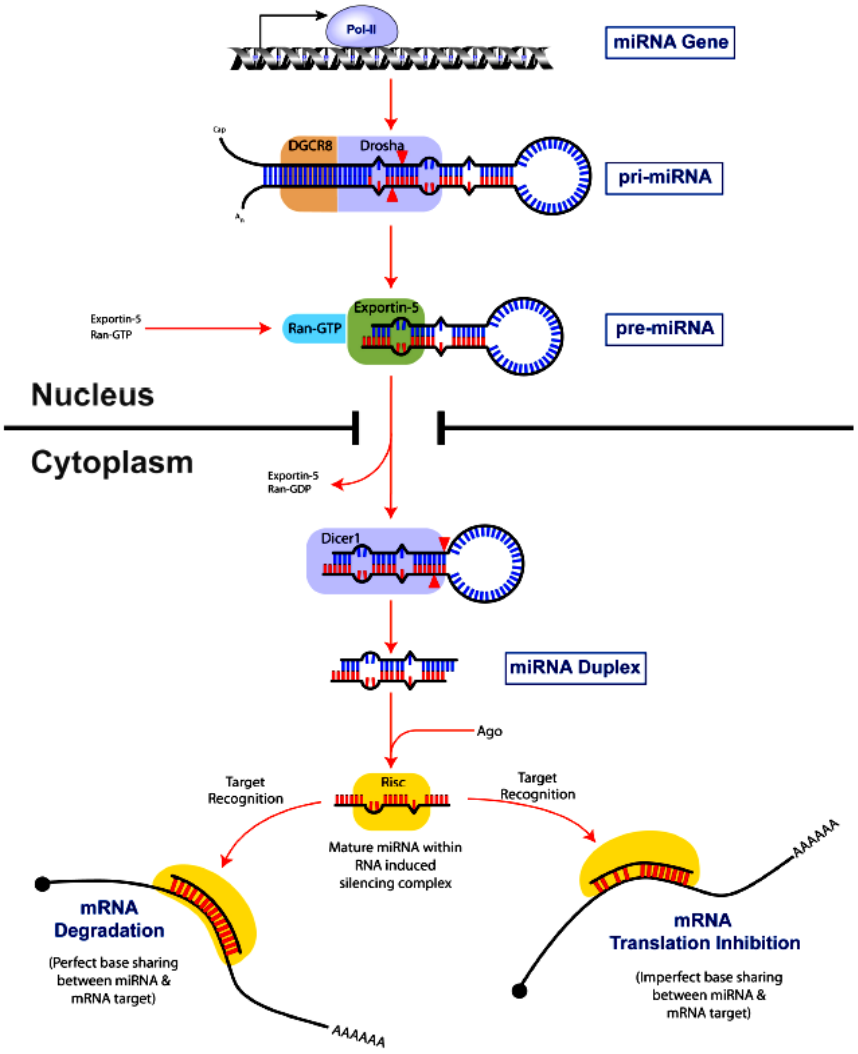

Figure 1.9 Graphical representation of microRNA synthesis, target recognition and inhibition (Fiedler, 2008).

The majority of miRNAs target sites are within the 3'UTR of mRNA molecules (Didiano and Hobert, 2008; Brennecke et al., 2005). By targeting the mRNA of protein-coding genes, miRNAs play a critical role in a variety of biological processes like development, cell growth, proliferation, lineage determination and metabolism (Cai et al., 2009; Morris, 2008; Filipowicz et al., 2008; Alvarez-Garcia et al., 2005). MiRNA studies in model invertebrates showed that small RNA molecules are involved in cell proliferation, control of developmental

timing, left – right patterning, fat metabolism and apoptosis (Bartel, 2004). Experimental data and computational prediction suggested that each miRNA may potentially target multiple (ten to hundreds) mRNAs which indicates that over 30% of all human genes may be regulated by miRNAs (Rybak et al., 2009; Cheng et al., 2009). In mammals, miRNAs have been shown to regulate a large number of pathways including B-cell differentiation (Chen et al., 2004), adipocyte differentiation (Esau et al., 2004) and insulin secretion (Poy et al., 2004). Knockout of the miRNA-producing enzyme Dicer1 in mice leads to lethality early in development, where embryos died before D-8 of gestation and did not develop any cell lineage. This is a clear indicator of the crucial roles of miRNAs in early development (Bernstein et al., 2003). A miRNA profiling study in bovine D-30 embryo showed that some miRNAs are differentially expressed among different tissues including thymus, small intestine and mesenteric lymph node (Coutinho et al., 2007). Another study on D-17 bovine embryo showed that some miRNA are differentially expressed between IVF and cloned embryos (Castro et al., 2010).

MiRNAs can target multiple mRNA molecules because of multiple recognition sequences inside of 3'UTR for target genes (Griffiths-Jones et al., 2006). This multiple recognition is due to the small area of pairing to the 5' region of the miRNA centered on nucleotides 2–7, which is called the miRNA "seed" (Figure 1.10) (Bartel, 2009; Brennecke et al., 2003). The seed region is the most conserved region of mammalian miRNA (Lim et al., 2003) and is considered to be very important to target recognition (Brennecke et al., 2003). Complete matching of seed nucleotides is an essential condition to form RISC complex and silencing procedure. It is crucial to uncover the functions of miRNAs by identifying their targets sites.

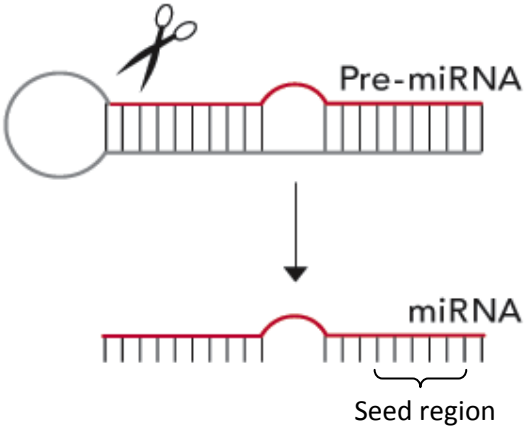

Figure 1.10 Position of seed region in a mature miRNA (Brennecke et al., 2003).

Single nucleotide polymorphisms (SNPs) can potentially create or eliminate the target recognition site of a miRNA in the target mRNA. A SNP inside a pre-miRNA sequence (stem and loop), or in the seed region, can change the secondary structure and consequently impact the maturation process of a miRNA. On the other hand, a SNP can either create a new target site for a miRNA or eliminate an existing target site. Recent findings have linked miRNA SNPs to human diseases and phenotypic variations in farm animals. Hiard et al. (2010) introduced "The Patrocles database" (http://www.patrocles.org/), a database which consists of SNPs that are predicted to perturb miRNA gene regulation. A G to A mutation in 3'UTR of ovine myostatin gene generated a new target site for 2 miRNAs in Texel sheep (Clop et al., 2006). It has been shown that two miRNAs (MiR-1 and miR-206), which are strongly expressed in muscle tissue, inhibit translation of myostatin gene and are responsible for muscle hypertrophy found in Texel sheep (Bignell, 2010).

1.3.2.2 MiRNAs and regulation of IGF system genes

It has been shown that miRNAs regulate IGF system genes and that let-7b is a major regulator of *GHR* expression in chicken (Lin et al., 2012). Elia et al. (2009) described a critical role of miR-1 in mediating the effects of the IGF1 pathway in human heart and skeletal muscle and demonstrated a feedback loop between miR-1 expression and the IGF1 signal transduction cascade. Ge et al. (2011) demonstrated that IGF2 is a critical regulator of skeletal myogenesis in mouse and is a direct target of miR-125b. There is a target site for miR-125 in the 3'UTR of mouse *IGF2* and biogenesis of this myogenic miRNA is negatively correlated with production of IGF2. It has been shown that miR-223 has a functional target in the *IGF1R* 3'UTR and can suppress proliferation of HeLa cells (Jia et al., 2011). Interestingly, Jia et al. (2011) showed knockdown of *IGF1R* mimicked miR-223 inhibition and decreased cell viability. Let-7 and Lin28 miRNAs has been shown to regulate glucose metabolism pathways in the mouse model. Overexpression of these two miRNAs can repress multiple components of the insulin pathway including IGF1R and IR (Zhu et al., 2011).

1.3.2.3 Prediction of miRNA target sites

Since experimental identification of miRNA targets is difficult and time consuming, computer software (e.g. TargetScan, http://targetscan.org) to predict target sites was developed. Seed region base pairing has been shown to be a reliable strategy to predict targets based on perfect complementary pairing (Lewis et al., 2005; 2003). Several web-based algorithms are available for computational prediction of miRNA targets, which include TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org) and PicTar (http://www.pictar.org). Also, experimentally validated microRNA target sites are available at TarBase, miRecords and miRTarBase (Kozomara and Griffiths-Jones, 2011).

MiRBase is the primary repository and database resource for miRNA data. The database has three main functions including miRNA registry, sequence of miRNA loci and their target sites (Griffiths-Jones et al., 2007). MiRBase has been developing since 2002 and started with 218 miRNA sequence in version 1.0. Currently, based on the latest version of MiRBase (version 19.0, Aug 2012), more than 25,141 mature miRNAs in 193 species, ranging from viruses to humans, have been identified (www.mirbase.org, 2013) of which 766 are classified as bovine, specifically *Bos taurus*. The majority of these miRNAs have been identified based only on sequence similarity to known vertebrate miRNA orthologs and have never been confirmed experimentally. However, there is an increasing number of studies describing prediction, cloning and experimental validation of novel miRNAs in cattle (Muroya et al., 2013; Huang et al., 2011; Glazov et al., 2009).

The profiling of miRNA expression patterns, functional analysis and validation experiments continues to be an evolving and active area of research because there are tissue specific miRNA types which need to be tested in different developmental stages (Dunn et al., 2009; Strozzi et al., 2009).

1.4 Hypothesis and objectives

Despite the rediscovery of heterosis about a century ago, and the suggestion of various genetic models to explain this phenomenon, the mechanism(s) underlying heterosis have remained elusive to scientists. IGF1 is a suitable candidate to investigate the mechanism of heterosis in a bovine model as it is a member of the IGF system and the GH-IGF1 axis which are responsible for pre- and postnatal growth and development. This axis has been studied in a number of species, but there are still significant gaps in knowledge about developmental- and tissue-specific expression patterns of IGF system and GH-IGF1 axis genes in mammals in

general and bovine in particular. In addition, the roles of IGF system and GH-IGF1 axis transcripts and their epigenetic regulators including microRNAs in heterosis have not yet been described.

The hypotheses to be tested are:

1. IGF system and GH-IGF1 axis genes contribute to heterosis in a bovine fetal model.

2. MicroRNAs are involved in heterosis by regulation of transcription in the IGF system and GH-IGF1 axis.

The objectives of this study were:

1. Evaluation of tissue specific changes in transcript abundance of IGF system and GH-IGF1 axis genes, including *IGF1, IGF1 class1, IGF1 class2, IGF1R, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP6, IGFBP7, IGFBP8, IR, IR-A, IR-B, GHR, GHR-1A, GHR-1B* and *GHR-1C* transcripts across key embryonic, fetal and postnatal developmental stages in a bovine model.

2. Determine effects of heterosis, fetal genetics and sex on expression profile of selected fetal liver miRNAs which target *IGF* system and *GH-IGF1* axis transcripts. Determine if expression levels of IGF system and GH-IGF1 axis gene transcripts contribute to heterosis by investigating the association between IGF system and GH-IGF1 axis transcripts and fetal phenotypes including bovine fetus and fetal organs.

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Chapter 2

Materials and methods

2.1 Animals and sampling

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). The two breeds used in this research are subspecies of domestic cow, commonly referred to as *Bos taurus* (or taurine) and *Bos indicus* (or indicine) (Hiendleder et al., 2008). Nulliparous heifers between 16 and 20 months of age and semen/sires of the Angus and Brahman breeds were used to generate both purebred and reciprocal hybrid D-48 embryos, D-153±1 fetuses, D-277/278 caesarean section calves and 12-14 month old juveniles. Heifers were subjected to standard commercial estrous cycle synchronisation protocols (http://www.absglobal.com/Websites/absglobal/images/ABS%20Global%20Home/Beef/Beef%20Information/2012beefcowprotocol_1.pdf) as described previously (Anand-Ivell et al., 2011). Briefly, Cidirol - Heat Detection and Timed Insemination (HTI) and Cidirol - Timed Insemination (TI) were used. 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 were 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. Synchronisation/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 synchronisation measures. Pregnancy testing was confirmed by ultrasound scanning, and embryos and fetuses were recovered in a commercial abattoir.

Tissues which represent all three germ layers and trophectoderm (Yu et al., 2010), were collected from 60 embryos (D-48, 29 male embryos), 73 fetuses (D-153±1, 39 male fetuses) and 23 juveniles aged 12-14 months (heifers n=12, steers n=11). 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 D-48 embryos. Heart, brain, liver, lung, kidney, skeletal muscle (*M. semitendinosus*) and testis samples were collected from D-153±1 fetuses. Heart, brain, liver, lung, kidney and skeletal muscle were collected from 12-month-old juveniles. Cotyledon (*Placenta fetalis*) was collected at D-48, 153±1 and following delivery by caesarean section at D-277/278 of term. All fetal phenotype data, including organ weights, and all tissue samples were generously provided by Prof. Stefan Hiendleder. Combined skeletal muscle weight data including *M. supraspinatus, M. longissimus dorsi, M. quadriceps femoris and M. semimembranosus* (each of them measured as the average weight of both left and right muscles) was kindly provided by Ruidong Xiang.

2.2 Nucleic acid extraction

Genomic DNA was extracted from fetal liver, maternal placenta and sire semen. Liver DNA extraction was performed using AquaPure Genomic DNA isolation Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Placental DNA was extracted by phenol/chloroform (Sigma-Aldrich, St Louis, MO, USA) procedures as described by Sambrook et al. (1989). Semen DNA was extracted with the salting-out method described by Heyen et al. (1997). Quality and quantity of DNA were assessed by agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), respectively. Extracted DNA was treated with RNase A (Qiagen GmbH, Hilden, Germany) and diluted to concentrations of 50 or 100 ng/µl as working solution.

Total RNA was extracted from tissues using TRI Reagent® (Ambion, Life Technologies™, Inc., Carlsbad, CA, USA), according to manufacturer's instructions. Homogenisation of fetal and juvenile tissue samples was carried out with ceramic beads (MoBio Laboratories, Carlsbad, CA, USA) and the PRECELLYS®24 homogeniser/grinder (Bertin Technologies, Saint Quentinen Yvelines Cedex, France). Embryonic tissues were homogenised using ceramic beads and the PowerLyzer™ 24 homogeniser (MoBio Laboratories, Carlsbad, CA, USA). Due to the small size of embryonic heart and brain samples, AllPrep™ DNA/RNA Micro Kits (Qiagen GmbH, Inc., Hilden, Germany) were used for extraction of RNA from these tissues, according to the manufacturer's instructions. All RNA samples were treated with DNase (RQ1-DNase, Promega, Madison, WI, USA).

RNA quality (integrity) was assessed using the Agilent RNA 6000 Nano Kit with a Bioanalyzer 2100 (Agilent Technology Inc., Santa Clara, CA, USA).

Average RIN (RNA Integrity Number) values of RNA samples were calculated and are shown in Table 2.1.

Table 2.1 Average RNA integrity number (RIN) values of extracted RNA from different tissues. Means and standard deviations of means are shown.

	Muscle	**Lung**	**Kidney**	**Heart**	**Liver**	**Brain**	**Cotyledon**	**Testes**
Embryo	-	-	-	9.00±0.55 (n=28)	8.93±0.44 (n=35)	8.60±0.40 (n=38)	6.35±1.44 (n=59)	-
Fetus	8.21±0.41 (n=97)	8.85±1.37 (n=49)	7.41±1.69 (n=48)	8.45±0.40 (n=45)	8.05±0.45 (n=100)	8.38±0.45 (n=56)	7.16±1.26 (n=51)	5.85±1.3 (n=24)
Juvenile	7.16±0.77 (n=11)	7.43±0.76 (n=12)	4.16±1.72 (n=10)	7.67±0.17 (n=9)	7.54±0.67 (n=9)	6.74±0.48 (n=10)	5.49±1.82 (n=8)	-

Quantity and purity of extracted RNAs were assessed using NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

2.3 Complementary DNA synthesis

Complementary DNA (cDNA) was synthesised from 500 or 2000 ng 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. For each tissue, at each developmental stage, equal quantities of all individual cDNAs were then combined to generate a pooled representative cDNA sample as template cDNA for standard curve points.

2.4 Electrophoresis of DNA fragments

Electrophoresis of extracted DNA was done on a 0.7% agarose gel with TAE 1X running buffer for 45 min with voltage of 100 volt. Electrophoresis of PCR amplicons was done on 1.0-2.0 % agarose gel with TBE 0.5X running buffer for approximately 30 min at 80 volt.

All agarose gels were stained by Biotium GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) and visualised by Gel Doc 1000 UV transilluminator (Bio-Rad Laboratories, Hercules, CA, USA).

2.5 Primer design

All primers for standard PCR (Table 2.2) and quantitative real-time PCR (qPCR) (Table 2.3) were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, SUA). Primer sequences were analysed with BLAST software (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) against *Bos primigenius taurus* and *Bos primigenius indicus* sub-species (Hiendleder et al., 2008) genomes to ensure specificity and to avoid possible SNPs in primer sequences.

Table 2.2 Primer sequences used for PCR amplification and sequencing of and IGF system and GH-IGF1 axis genes.

Primer name with target gene symbol	Primer sequence	Annealing temperature (°C)	Amplicon length (bp)	Target region	Accession No. of target sequence
IGF1-E1 (F)	CTCCCAGTGCCGAAACAATG	56	595	Exon 1	NW_003103925.1
IGF1-E1 (R)	TAATGTCTGCTCCTCTTGTCACTAAC			Exon 1	
IGF1-E2 (F)	CCACAGGCAGTCATTCAGTTCTTC	56	263	Exon 2	NW_003103925.1
IGF1-E2 (R)	AGATGACCCTCCTTCTGCTTTTTC			Exon 2	
IGF1-E3 (F)	GTATGAATTACTCTTCGGATGCTG	55	384	Exon 3	NW_003103925.1
IGF1-E3 (R)	GCAGTGAACACAGCCTATTATCC			Exon 3	
IGF1-E4 (F)	AACAGCAATCTACCAACTCCAG	54	412	Exon 4	NW_003103925.1
IGF1-E4 (R)	GTTGTCTATTAAACTGACTGGTGAGA			Exon 4	
GHR-Retro (F2)	CAT TCA GTT ACT TTC AGG TCT TGG C	57	2200 in Angus 700 in Brahman	Growth Hormone Receptor retrotransposon insertion	NW_001493950.3
GHR-Retro (R3)	AAA AGA TTA GAG CAC ACA ACG CAT T				
Let7/miR98 1st (F)	CGA CAG ACA CTC AGG ACA CAA GGC	61	211	1st Target site for Let-7/miR98 miRNA in IGF1 3'UTR	NW_001494007.3
Let7/miR98 1st (R)	GCA GGT ATG TTC AGG GCA ATG TG				
Let7/miR98 2st (F)	ACA GGA GCG ACG CCA AAT GAG	59	486	2st Target site for Let-7/miR98 miRNA in IGF1 3'UTR	NW_001494007.3
Let7/miR98 2st (R)	TTG CTT ATC AGT AGT TTC AGT CCC AC				
Let7/miR98 3st (F)	ACA ACC ACA GGT GAT GGA TGC T	57	409	3st Target site for Let-7/miR98 miRNA in IGF1 3'UTR	NW_001494007.3
Let7/miR98 3st (R)	TTT TCG GTA ATG TAA AGA ATC CAG AG				
Let7/miR98 loci (F)	ATA TTT GCC TCA CAC TAC ATA TCA CC	57	1375	Let-7/miR98 miRNA loci	NW_001508802.3
Let7/miR98 loci (R)	GCT TAT CCT CCA TCC AGG TCA TAT				
miR 483 locus (F)	CAG GAA GTG GCA CCG CAG T	56	500	IGF2-Intron3 IGF2-Intron3	NW_001494547.4
miR 483 locus (R)	CAG AAA GGA CAG AAG GGA CAG TG				
M13 (F)	GTA AAA CGA CGG CCAG	55	N/A	M13 universal primer	N/A
M13 (R)	CAG GAA ACA GCT ATG AC		N/A		

Table 2.3 Primer sequences used for quantitative real-time PCR amplification of IGF system and GH- IGF1 axis transcripts and housekeeper genes

Primer name with target gene symbol	Primer sequence	Annealing temperature (°C)	Amplicon length (bp)	Target region	Accession No. of target sequence
IGF1 (F)	GAT GCT CTC CAG TTC GTG TGC	58	140	Exon 2	NW_003103925.1
IGF1 (R)	TCC AGC CTC CTC AGA TCA CAG			Exon 3	
IGF1Class1 (F)	TTC AGA AGC AAT GGG AAA AAT CAG	58	115	Exon 1	NW_003103925.1
IGF1Class1 (R)	ATA GAA GAG ATG CGA GGA GGA TGT G			Exon 2	
IGF1Class2 (F)	TCA TAA TAC CCA CCC TGA CCT GC	58	105	Exons 1 & 2	NW_003103925.1
IGF1Class2 (R)	ATA GAA GAG ATG CGA GGA GGA TGT G			Exon 2	
IGF-1R (F)	GAT CCC GTG TTC TTC TAC GTT C	58	100	Exon 13	XM_606794.3
IGF-1R (R)	AAG CCT CCC ACT ATC AAC AGA A			Exon 14	
IR (F)	GGA GCC CAA GGA ACC CAA CG	62	105	Exon 13	NC_007305.4
IR (R)	AGA GCA TAA TGT CGG CGG GAG A			Exon 14	
IR-A (F)	TCC TCA AGG AGC TGG AGG AGT	59	89	Exon 10	AJ488553
IR-A (R)	TTT CCT CGA AGG CCT GGG GAT			Exons 10 and 12	
IR-B (F)	TCC TCA AGG AGC TGG AGG AGT	59	110	Exon 10	AJ320235
IR-B (R)	TAG CGT CCT CGG CAA CAG G			Exon 11	
IGFBP-1 (F)	ACC AGC CCA GAG AAT GTG TC	59	119	Exon 2	NW_003103902.1
IGFBP-1 (R)	CTG ATG GCA TTC CAG AGG AT			Exon 2	
IGFBP-2 (F)	CAC ATC CCC AAC TGT GAC AA	58	114	Exon 3	NW_001494682.3
IGFBP-2 (R)	GAT CAG CTT CCC GGT GTT AG			Exon 4	
IGFBP-3 (F)	CTA CGA GTC TCA GAG CAC AG	58	103	Exon 2	NT_181996.1
IGFBP-3 (R)	GTG GTT CAG CGT GTC TTC C			Exon 3	
IGFBP-4 (F)	ATG TGC CTG ATG GAG AAA GG	57	106	Exon 4	NM_174557.3
IGFBP-4 (R)	GCC ATC CTG TGA CTT CCT GT			5' UTR	
IGFBP-5 (F)	CAA GCC AAG ATC GAA AGA GAC T	60	85	Exon 1	NM_001105327.1
IGFBP-5 (R)	AAG ATC TTG GGC GAG TAG GTC T			Exon 2	
IGFBP-6 (F)	GGA GAG AAT CCC AAG GAG AGT A	60	100	Exon 2	NM_001040495.1
IGFBP-6 (R)	GAG TGG TAG AGG TCC CCG AGT			Exon 2	
IGFBP-7 (F)	CTG CGA GGT CAT CGG AAT CCC CAC	62	110	Exon 2	NW_001495197.4
IGFBP-7 (R)	CCA GGT TGT CTC GGT CAC CAG GCA			Exon 3	
IGFBP-8 (F)	GCT GAC CTG GAG GAG AAC ATT A	58	112	Exon 4	NT_182009.1
IGFBP-8 (R)	CTC GGT ATG TCT TCA TGC TGG			Exon 5	
GH (F)	TGG CTG CTG ACA CCT TCA AAG AGT T	57	174	Exon 2	NW_001493688.4
GH (R)	CCA AGC CAC GAC TGG ATG AGG AG			Exon 4	
GHR (F)	AGT AGG GGG TCC ACA CAG AGG TAT	60	150	Exon 4	NW_001493950.3
GHR (R)	CTA ATC ACA GTT TAC AGA GCC CAG G			Exon 3	
GHR-1A (F)	AGC GAC ATT ACA CCA GCA GGA A	60	173	Exon-10	DQ062716.1
GHR-1A (R)	GGC CAG GGC AAT GTA CTT TT			Exon-10	
GHR-1B (F)	GAG GCT CGG CTC GCA GGT CC	60	110	Exon-1B	NM_176608
GHR-1B (R)	AAA GCT GGT GTG GCT TCA CT			Exon-1B	
GHR-1C (F)	AAC TGC TCG AGG CAA GAG AG	60	120	Exon-1C	AF036292
GHR-1C (R)	AAA GCT GGT GTG GCT TCA CT			Exon-1C	
VPS4A (F)	GAA GAC AGA AGG CTA CTC GGG TG	60	106		NM_001046615.1
VPS4A (R)	ACA GAC CTT TTT GAA GTG TGT TGC T				
GAK (F)	CAC GAC CAT CTC ACA CTA CCC A	60	128		NM_001046084.2
GAK (R)	AGT TTG AGT ACA AGT CCA CAA TTT CC				
TBP (F)	GCA ACA GTT CAG TAG TTA TGA GCC AG	60	164		NM_001075742.1
TBP (R)	GAA TAG GGT AGA TGT TCT CAA AGG CT				
H3F3A (F)	ACT GCT ACA AAA GCC GCT C	60	231		XM_003586223.1
H3F3A (R)	ACT TGC CTC CTG CAA AGC AC				
UBB (F)	AGA TCC AGG ATA AGG AAG GCA T	62	198		NM_174133.2
UBB (R)	GCT CCA CCT CCA GGG TGA T				
ACTB (F)	CTC TTC CAG CCT TCC TTC CT	62	245		NM_173979.3
ACTB (R)	CCA ATC CAC ACG GAG TAC TTG				
RPS9 (F)	TAG GCG CAG ACG GGC AAA CA	60	136		NM_001101152.2
RPS9 (R)	CCC ATA CTC GCC GAT CAG CTT CA				
GAPDH (F)	GGG TCA TCA TCT CTG CAC CT	60	264		NW_003103940.1
GAPDH (R)	CAT AAG TCC CTC CAC GAT GC				

2.6 Standard PCR and quantitative real-time PCR

Standard PCR amplification of exonic and regulatory regions of IGF system and GH-IGF1 axis genes was carried out using AmpliTaq Gold (Roche Molecular Systems, Inc., Branchburg, NJ) according to the manufacturer's instructions in an Eppendorf Mastercycler® pro S thermal cycler (Eppendorf Inc., Hamburg, Germany). The reaction volume was 20 µl and the ingredients consisted of 50-100 ng of genomic DNA, 2 µl of 10X PCR buffer, 2-4 µl of 25 mM $MgCl_2$, 0.4 µl of 10 mM dNTPs, 3 µl of primer mix (5 pMol/µl), 0.75 U of *Taq* polymerase and UltraPure™ DNase/RNase-Free Distilled Water (Gibco Laboratories) added as required up to the total reaction volume. All reactions were performed in duplicate.

Quantitative real-time PCR reactions were performed using Fast Start Universal SYBR Green Master (Roche Diagnostics GmbH, Mannheim, Germany) in an Eppendorf Mastercycler® ep realplex Real-time PCR System (Eppendorf Inc., Hamburg, Germany) following MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments) (Bustin et al., 2009). Reactions were conducted in duplicate or triplicate in a total volume of 12 µl, containing 6 µl of SYBR master mix (2X), 5.2 µl of cDNA (20-fold diluted from stock cDNA, equivalent to 6* or 24 ng of starting RNA) and 0.8 µl of mixed forward and reversed primers (5 pmol/µl). A non-template control was included in all experiments to confirm the absence of genomic DNA contamination of reagents used for amplification. Thermal cycling was carried out with a 5-minute initial denaturation/activation step at 95°C, followed by 40 cycles of 95°C for 20 seconds (denaturation), 55-62°C for 30 seconds (annealing, depending on the primer pair), and 72°C for 20 seconds (extension). Product specificity was confirmed

* Quantity of template cDNA used in qRT-PCR reactions was equal to 6 ng of starting RNA except in chapters 3 and 5 which was 24 ng.

by sequencing, plots of the melting curve derived by Mastercycler® ep Realplex software (Eppendorf Inc., Hamburg, Germany) and agarose gel electrophoresis of PCR products.

An equal proportion of cDNA from all samples 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 8 data points. Three replicates were used for each dilution of the cDNA template. The Cq (threshold cycle) values of the standards were used to derive a standard curve which shows the Cq 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 using the following equation:

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

where, exp indicates the exponential function, Cq is threshold cycle for each sample, intercept is the point at which the standard curve intersects with the Y-axis, and slope is the the slope of the standard curve plot.

PCR amplification efficiencies (E) were calculated by the following equation:

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

Cq 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 were higher than 0.90 and 0.99, respectively.

Eight housekeeping genes from different pathways were selected (Hruz et al., 2011; Lisowski et al., 2008) and all housekeeper transcripts were determined for all analysed individuals and in all studied tissues. House keeper genes were 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), cyclin-G associated kinase (GAK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Reactions were done in duplicate or triplicate. To choose the best housekeeper genes, average expression stability values (M) and pair wise variation (V) of all housekeeping genes were calculated using geNorm version 3.5 software for each tissue (Vandesompele et al., 2002). In this approach, the M value is defined as the mean pairwise variation of a gene with all the other reference genes in a given set of samples so that a low M value is indicative of more stable expression. A limit of M = 1.5 was defined as an acceptable cut-off for selection of reference genes. A cut off of 0.15 was considered for pairwise variation where inclusion of an additional control gene was not required (Vandesompele et al., 2002). In cotyledon, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was assessed, instead of *H3F3A*, due to initial studies that indicated that *H3F3A* expression was not constant in cotyledon. The geometric mean of the housekeeper genes identified as most stable was calculated for normalisation of the gene expression data. *GAK* and *RSP9* were identified as the two genes with the most stable, and readily detectable, expression. Since expression levels of *GAK* transcript was low as a consequence high value of threshold cycles achieved in all tissues which did not meet the MIQE standards (Bustin et al., 2009), the *RPS9* was used as the next best housekeeper gene after *GAK*. Therefore, we decided to use *VPS4A* and *RPS9* as our two best housekeeper genes.

2.7 Cloning and sequencing

Cloning of PCR products was done using TOPO® TA Cloning® Kit for Subcloning, with TOP10 *E. coli* (Applied Biosystems Inc., Foster City, CA) according to manufacturer's instructions. Transformation was done with competent *E. coli* TOP10 cells provided by the manufacturer. The transformed cells were then plated onto Luria-Bertani agar (Sigma-Aldrich, St Louis, MO, USA) plates supplemented with kanamycin (Sigma-Aldrich, St Louis, MO, USA) and incubated overnight at 37°C. Recombinant and native (negative control) plasmids were extracted by PureLink™ Quick Plasmid Miniprep Kit provided by the manufacturer. The size of inserts was determined by PCR with flanking vector M13 forward primer and reverse primers provided by manufacturer followed by electrophoresis on a 1% agarose gel.

All PCR products were sequenced using the BigDye® Terminator Sequencing Cycle Kit (Applied Biosystems Inc., Foster City, CA) on an ABI PRISM Model 3700 Genetic Analyser (Applied Biosystems Inc., Foster City, CA) and chromatograms were analysed using Chromas lite version 2.1.1 software (Technelysium Pty Ltd, QLD, Australia). Sequenced fragments were subjected to sequence alignment with the computer program BioEdit 7.0.0 (Hall, 1999) and "Blast 2 sequences" (Zhang et al., 2000).

2.8 Microarray assessment of microRNAs

GeneChip® miRNA 3.0 (Affymetrix, Santa Clara, CA, USA) was used to profile the bovine microRNAs in fetal liver samples. This new chip contains 19724 miRNA probes including mature and pre-miRNA belonging to 153 organisms including bovine. Probe design of this chip is based on miRBase version 17 (http://www.mirbase.org). Cell data were normalised

and transformed to text file by Affymetrix® Expression Console™ Software 1.3 (Affymetrix, Inc.). Data was analysed by BRB-Array tools 4.3 software package (Simon et al, 2007).

2.9 Data analysis

Gene expression data and fetal phenotype, including fetal weight and organ weights, were analysed by Univariate Analysis of Variance (ANOVA) using the general linear model procedure of JMP statistical package version 4.0 (SAS Institute Inc.). As the normalised gene expression data were not normally distributed, statistical analysis was performed after logarithmic transformation of the data. The results for least square means and standard errors of means are presented after back-transformation. Data were fitted into the following linear model to analyse the effects of fetal genetics, sex and interactions:

$$y_{ijk} = G_i + S_j + (G*S)_{ij} + e_{ijk}$$

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), $(G*S)_{ij}$ is fetal genetic by sex interaction and e_{ijk} is the residual effect.

The following model was used to estimate heterosis effects on gene expression and fetal phenotype:

$$y_{ijkl} = H_i + G_j(H_i) + S_k + (H*S)_{ik} + e_{ijkl}$$

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

[†] A is Angus, B is Brahman and the first and second letters in a genotype indicating the breed of sire and dam, respectively.

AA, BB nested within purebred, j = BA, AB nested within crossbred), S_k is fetal sex (k = male, female), $(H*S)_{ik}$ is heterosis effect-by-sex interaction and e_{ijkl} is the residual effect.

Values are presented as least square means and their associated standard errors. To illustrate where group differences existed, individual pairwise comparison of least-squares means was performed using Student's t-tests.

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.

Pearson correlation coefficients among different gene expression measurements were determined using JMP version 4.0 (SAS Inc.).

Graphs were plotted using GraphPad Prism 6.0 (GraphPad Software Inc, San Diego, CA, USA). For presentation, least square means and their associated standard errors for IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP6, INSR-A and INSR-B in liver tissue; IGF1 class1 and IGF1 class2 in skeletal muscle tissue; and GHR-1B and GHR-1C in heart and brain tissues have been divided by 100.

2.10 References

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Chapter 3

Expression of genes from the insulin-like growth factor system and growth hormone – insulin-like growth factor 1 axis in embryonic, fetal and postnatal bovine tissues

3.1 Introduction

The insulin-like growth factor (IGF) system and growth hormone – insulin-like growth factor 1 (GH-IGF1) axis play key roles in regulating fetal and postnatal growth and development. This complex consist of three ligands, GH, IGF1 and IGF2, four receptors, growth hormone receptor (GHR), IGF type 1 (IGF1R), IGF type 2 (IGF2R) and insulin (IR) receptors, six IGF binding proteins (IGFBP1-6) (Rosenfeld and Roberts, 1999) and also low affinity IGFBPs including IGFBP7 to IGFBP10 (Kim, 1997). The tissue and developmental stage specific expression of IGF system and GH-IGF1 axis in cattle is poorly understood.

The IGF1 peptide has endocrine and paracrine actions to promote growth and development in a range of pre and postnatal tissues (Nakae et al., 2001). *IGF1* is expressed in preimplantation embryo in cattle (Lonergan et al., 2000), human (Lighten et al., 1997) and mouse (Rao et al., 1990). Mice with an *IGF1* null allele have lower birth weight than their wild-type littermates and most of them die early after birth. Surviving mice grow poorly during postnatal development (LeRoith et al., 2001a). In several species, including rat (Fu et al., 2009), human (Jansen et al., 1992) and sheep (Dickson et al., 1991), *IGF1* transcription is initiated from both exon 1 and 2, generating *IGF1* mRNA containing either exon 1 (*IGF1 class 1* mRNA) or exon 2 (*IGF1 class 2* mRNA) as the leader exon. Thus, the two classes of *IGF1* mRNA

encode prepro-IGF1 proteins differing in the length of the signal peptide; however mature IGF protein produced by either of them is identical (LeRoith and Roberts, 1991).

The type 1 insulin-like growth factor receptor (IGF1R) belongs to the tyrosine kinase receptor family has been found to be expressed in all normal adult and fetal human tissues and cell types (LeRoith et al., 2001b). The IGF1R receptor binds IGF1 with high affinity and can also binds to IGF2 and insulin, but with a six- and 100-fold lower affinity than IGF1, respectively (LeRoith, 2003). The structure of IGF1R and IR is similar and they can form a hybrid receptor which has higher affinity for IGF1 than IGF2 (Arnaldez and Helman, 2012).

The IGF1R gene is expressed ubiquitously among tissues and its main role is maintaining tissue growth and development (Leventhal and Feldman, 1997). It is known that mutations in *IGF1R* can lead to growth retardation in human postnatally (Hwa et al., 2013). In mouse models, *IGF1R* deletion results in more severe growth failure than *IGF1* deletion, and *IGF1R* null mice die of respiratory failure at birth (Liu et al., 1993), showing the important role of IGF1R for prenatal development.

Insulin and IR signalling pathways are very similar to IGF1 and IGF1R pathways (Belfiore et al., 2009). In human and cattle, alternative splicing of the IR transcript produces two splice variants or isoforms, IR-A and IR-B that either exclude or include exon 11 at the carboxyl terminus of the IR α-subunit (Moller et al., 1989). Each of the IR isoforms is equally able to form hybrids with IGF1R, but the IR-A isoform has 1.7 fold more affinity for insulin than the IR-B isoform (Mosthaf et al., 1990). The IR-A isoform displays higher affinity for IGF2 than IGF1 and can be considered as a second physiological receptor for IGF2 while IR-B is very specific to insulin (Pandini et al., 2002). There is also a heterodimer receptor which consists of both IR-A and IR-B (HIR-AB) (Pollak, 2012). The contribution of IR-A to embryo

development has been studied in mouse, where IGF2 acts as an IR ligand in the promotion of embryonic growth. Mice lacking IR achieved approximately 80-90% growth of normal mice (Louvi et al., 1997). Humans lacking functional IR due to a mutation showed more severe intrauterine growth retardation as compared to mouse (Jospe et al., 1996). This difference between growth retardation in human and mouse due to IR deficiency might be caused by differences in developmental timing. Rodents are born at a developmental age equal to an approximately 26 week old human fetus (Otis and Brent, 1954).

Insulin-like growth factor binding proteins (IGFBPs) are thought to modulate actions of IGFs since they have 2-50 fold higher affinity to IGF1 and 2 than IGF1 receptor (Allan et al., 2001; Hwa et al., 1999). A number of *IGFBP* deletion models have been used to further investigate the roles of IGFBPs during development. It appears that *IGFBP* deletion in mice does not have severe effects similar to those resulting from deletion of other IGF components (Firth and Baxter, 2002). *IGFBP2* knockout mice showed no overall growth retardation but they had smaller spleen and larger liver size than controls (Wood et al., 2000). Interestingly, *IGFBP3* and *IGFBP5* double knockout mice showed lower plasma IGF1 concentration, but heavier body weights with greater relative organ weights, including kidneys and spleen, compared with control adult mice (Murali et al., 2012). Overexpression of *IGFBP1*, *IGFBP2* and *IGFBP3* led to reduction in brain weight and abnormal brain development in mice (Silha and Murphy, 2002). IGFBP1-6 are expressed in all normal tissues at mid-gestation in mouse and rat (Cerro et al., 1993). Each IGFBP has a distinct expression pattern in rat and mouse fetal tissues and appears to be tightly regulated during development of specific tissues (Allan et al., 2001). In addition to the IGFBPs, IGFBP-related proteins have been described, leading to the proposal of an IGFBP superfamily (Kostecka and Belahovec, 2002; Hwa and Rosenfeld 1999; Kim, 1997). These lower affinity IGFBPs are also considered as IGFBP related proteins (Figure 1.4). There are a number of recognised members in the IGFBP-related protein family,

including IGFBP-rP1 (mac25 or IGFBP7), IGFBP-rP2 (connective tissue growth factor (CTGF) or IGFBP8), IGFBP-rP3 (NovH or IGFBP9) and IGFBP-rP4 (Cyr61 or IGFBP10) (Kim et al., 1997), all of which are considered low affinity IGFBPs. The IGFBP7 is a high-affinity insulin binding protein and it blocks insulin binding to the insulin receptor and insulin action (Yamanaka et al., 1997). IGFBP8 is considered to be a key cytokine in fibrogenesis of tissues and organs (Hwa et al., 1999). Mice deficient in IGFBP8 died in the perinatal period due to respiratory failure and showed generalised chondrodysplasia (Ivkovic et al, 2003). An IGFBP10 knocked out mouse model showed lethality in the embryonic period or shortly after birth (Mo et al., 2002).

Growth hormone (GH) gene expression is mainly from the pituitary gland, and pituitary GH is a key stimulator of IGF1 production, but also affects IGF2 production in liver (von Horn et al., 2002). Evidence for *GH* expression has been found in some extra-pituitary tissues, where it may act as an autocrine or paracrine growth factor (Harvey, 2010). Among those tissues, placental GH seems to be very important for growth and development of the fetus. Since in human placental GH cannot be detected in the fetal circulation, and only presents in maternal blood and amniotic fluid, a direct role of GH in prenatal development is still controversial (Skottner, 2012; Osafo et al., 2005; Edmonson et al., 1995; Garcia-Aragon et al., 1992). However, mice deficient in GH due to mutations in the genes encoding GH-releasing hormone receptor, showed normal birth weight (Efstratiadis, 1998). In sheep, a transcript identical to pituitary GH mRNA is expressed from the placenta after D-27 of the first trimester of pregnancy (Lacroix et al., 1999). In humans, the pituitary GH gene (*GH1*) is not expressed in the placenta, but a placental GH gene (*GHV* or *GH2*) is transcribed and translated into several placental GH proteins (Harvey, 2010). In humans both *GH1* and *GH2* genes are located next to each other on chromosome 17 with 98% homology (Vnencak-Jones et al., 1988); this is similar to sheep, where both GH genes are found on chromosome 11

(Vacca et al., 2013; Lacroix et al., 2002), and also goat (Wallis et al., 1998). Interestingly, there is so far no evidence for a duplicated *GH* gene (or *GH2*) in mice and cattle (Skottner, 2012).

GH exerts its biological effects by binding to specific cell surface growth hormone receptors (GHRs) (Kelly et al., 1991). Expression of *GHR* is regulated by age, nutritional intake, GH itself, steroid hormones and insulin. The GHR belongs to the cytokine/hematopoietin receptor family that includes the receptors for prolactin (PRL), erythropoietin, leptin, interferons, granulocyte colony stimulating factor and interleukins (Horseman and Yu-Lee, 1994; Cosman et al., 1990). It has a long 5' non-coding region that includes nine untranslated exons, 1A–1I resulting in up to nine variants of *GHR* mRNA (Jiang and Lucy, 2001). Only variants 1A, 1B and 1C are well characterised, the description of exons 1D–1I is based on rapid amplification of cDNA end analyses. Variants 1D-1I altogether account for only 10% of overall *GHR* transcripts (Jiang and Lucy, 2001). *GHR* transcripts have been detected in a variety of bovine adult tissues, including liver, muscle, kidney, lung, mammary gland, adipose tissue, and fetal tissues of placenta, liver, lung, kidney and skeletal muscle with the highest level of expression detected in liver (Jiang and Lucy, 2001). Knockout mouse models showed no significant body size or weight differences between normal and *GHR* knockout newborns. However, GHR deficient mice (Laron mice) were significantly smaller 3 weeks after birth (Zhou et al., 1997). Laron mice showed 90% less circulating IGF1 and significant reduction of IGFBP3. Interestingly, these mice live up to 50% longer than control mice (Coschigano et al., 2000). This research clearly indicated the role of GH/GHR in expression of IGF1 and the important role of the GH-IGF1 axis in postnatal growth and development.

Expression of IGF system and GH-IGF1 axis transcripts have been extensively studied in cattle, human and mouse tissues; however, tissue-specific expression of IGF system and GH-

IGF1 axis transcripts has not been conducted systematically across key developmental time points in bovine or other species.

The aims of this chapter were to investigate the expression of *IGF1, IGF1 class 1* and *class 2*, *IGF1R, IGFBP1* to 8, *IR, IR-A, IR-B, GH, GHR, GHR-1A, GHR-1B* and *GHR-1C* transcript in different tissues of cattle and to compare developmental changes in transcript abundances between D-48 embryos, D-153±1 fetuses, D-277/278 caesarean section calves and 12-14 month old juveniles.

3.2 Materials and methods

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).

Tissues were selected to represent the three germ layers ectoderm, mesoderm, endoderm, and extraembryonic tissue, originating from trophectoderm (Yu et al., 2010). Tissues were collected from 60 D-48 embryos, 73 D-153±1 fetuses, 6 D-277/278 caesarean section calves and 23 juveniles aged 12-14 months as described in Chapter 2, section 2.1. Heart, brain, and liver were collected at all developmental stages. Lung, kidney and skeletal muscle (*M. semimembranosus*) were collected from D-153 fetuses and from 12 month old juveniles. Testicular tissue was collected only from fetuses. Cotyledon was collected at D-48, D-153 and following caesarean section delivery at D-277/278. Total RNA was extracted and cDNA synthesised as described in Chapter 2, section 2.2.

For each tissue, at each developmental stage, equal quantities of all individual cDNAs were combined to generate pooled cDNA representing tissue and developmental stage specific cDNA samples (Table 3.1).

Table 3.1 Summary of pooled cDNA samples representative of every tissue at each developmental stage used to investigate transcript abundances.

Tissues / Developmental Stage	Liver	Brain	Heart	Cotyledon	Lung	Kidney	Skeletal muscle	Testis
Embryo Day 48 (n=60)	✓	✓	✓	✓				
Fetus Day 153 (n=73)	✓	✓	✓	✓	✓	✓	✓	✓
Caesarean section Day 277/278(n=6)				✓				
Juvenile 12-14 months (n=23)	✓	✓	✓		✓	✓	✓	

Relative abundances of the following transcripts were analysed: *IGF1* overall transcript and *class 1* and *class 2* transcripts; *IGF1R*, *IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP4*, *IGFBP5*, *IGFBP6*, *IGFBP7*, *IGFBP8; IR* overall transcript and variants *IR-A* and *IR-B*; *GH*, *GHR* overall transcript and variants *GHR-1A*, *GHR-1B* and *GHR-1C*. Primer details are shown in Table 2.3 in Chapter 2.

Quantitative real time PCR reactions were performed in triplicate using pooled cDNA representing every tissue across all developmental stages sampled (Table 3.1). The relative abundance of each target transcript was calculated using the standard curve method with determination of PCR amplification efficiency. In this experiment, since each analysed sample was a mixture of a large number of individuals (see Chapter 2, section 2), and all developmental stages were analysed in parallel, we assumed differences in individual RNA quality and quantity would not impact on results. Thus, we did not use housekeeper genes to normalise relative gene expression data. The cDNA template used to generate the standard

curve was a mixture containing equal proportions of all pooled cDNAs which included all tissues and all developmental stages.

Data was analysed with a general linear model in JMP version 4.0 (SAS Inc.). Values are reported as means ± standard deviation of the mean.

3.3 Results

3.3.1 *Insulin-like growth factor 1 (IGF1) overall transcript* expression

IGF1 mRNA expression was detected in all tissues examined at each developmental stage (Figure 3.1). In embryos, *IGF1* expression was highest in liver and lowest in brain. At D-153 of gestation, *IGF1* expression was the highest in skeletal muscle, kidney and cotyledon had the lowest expression levels. In juveniles, liver had the highest expression of *IGF1*, which was about 35-fold higher than muscle and kidney tissues. Lung and cotyledon showed the lowest *IGF1* expression. Hepatic *IGF1* expression level appeared to remain constant from D-48 to 153 of gestation, but increased 40-fold after birth. *IGF1* transcript abundance in cotyledon showed a progressive decrease as gestation advanced. The greatest change in *IGF1* expression occurred between mid-gestation and 12 months of age in lung, where a 60-fold decrease occurred. The lowest fold change, a 3-fold decrease, was observed in heart from mid-gestation to 12 months of age.

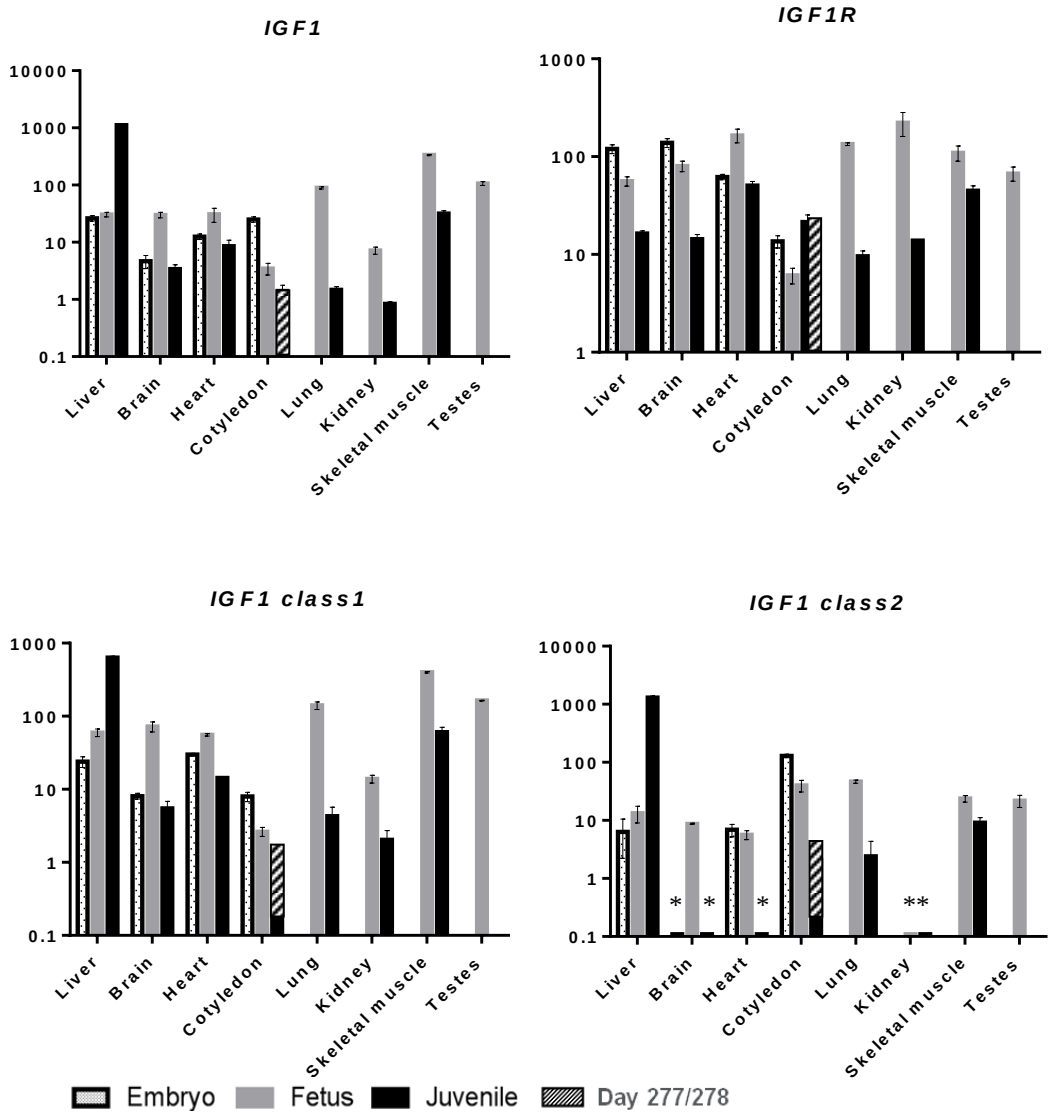

Figure 3.1 Means and standard deviations of means for 3 technical replicates of measured transcript abundances of insulin-like growth factor 1 (*IGF1*), type 1 insulin-like growth factor (*IGF1R*), *IGF1 class 1* and *IGF1 class 2* in bovine tissues of different developmental stages. Tissue and developmental stage specific transcript abundance was calculated by the standard curve method and expressed in arbitrary logarithmic units (*: measured but not expressed in this tissue/developmental stage).

3.3.*2 Insulin-like growth factor class 1* and *2* (*IGF1 class 1* and *2*) expressions

We showed that the *IGF1 class 1* transcript was expressed in all tissues and developmental stages studied, with similar trends as for *IGF1* overall transcript (Figure 3.1). The highest expression of *IGF1 class 1* transcript was observed in the liver of embryos and juveniles, where in fetuses, skeletal muscle showed the highest level of *IGF1 class 1* transcript followed by liver. At D-48 of gestation, the *IGF1 class 2* transcript was expressed in liver, heart and cotyledon, but was not detected in embryonic brain (Figure 3.1). In the fetus, *IGF1 class 2* transcript was expressed in most tissues, except kidney, with the highest expression in lung and cotyledon. Postnatally, this transcript showed tissue-specific expression restricted to liver and skeletal muscle.

3.3.3 *Type 1 insulin-like growth factor receptor* (*IGF1R*) expression

IGF1R was expressed in all studied tissues and at all developmental stages (Figure 3.1). At D-48 of gestation, *IGF1R* expression was highest in brain and liver and lowest in cotyledon. In the fetus, *IGF1R* was expressed in all studied tissues, with the lowest expression in cotyledon. In the juvenile, *IGF1R* was highly expressed in heart and muscle tissue and expressed at 4-5 times lower levels in lung and kidney. Similar to *IGF1*, *IGF1R* had higher expression prenatally in all studied tissues and the biggest decrease in postnatal tissues (16-fold) was observed in kidney.

3.3.6 *Insulin-like growth factor binding protein* (*IGFBP*) expression

Relative transcript abundances of high affinity (*IGFBP1* to *IGFBP*6) and low affinity IGFBPs (*IGFBP7* and *IGFBP8*) transcripts are shown in Figures 3.2 and 3.3, respectively.

3.3.6.1 High affinity *IGFBP* expression

We showed that liver was the main tissue producing *IGFBP1* transcript at all developmental stages. On average, liver had 500-1000 fold higher expression in comparison to other tissues. Very low expression of *IGFBP1* was observed in fetal skeletal muscle and kidney. In juveniles, *IGFBP1* was expressed at very low levels in kidney at ~ 500 fold lower levels than in liver. Brain, heart, lung and skeletal muscle did not reveal *IGFBP1* expression in juveniles as well as fetal lung and testes.

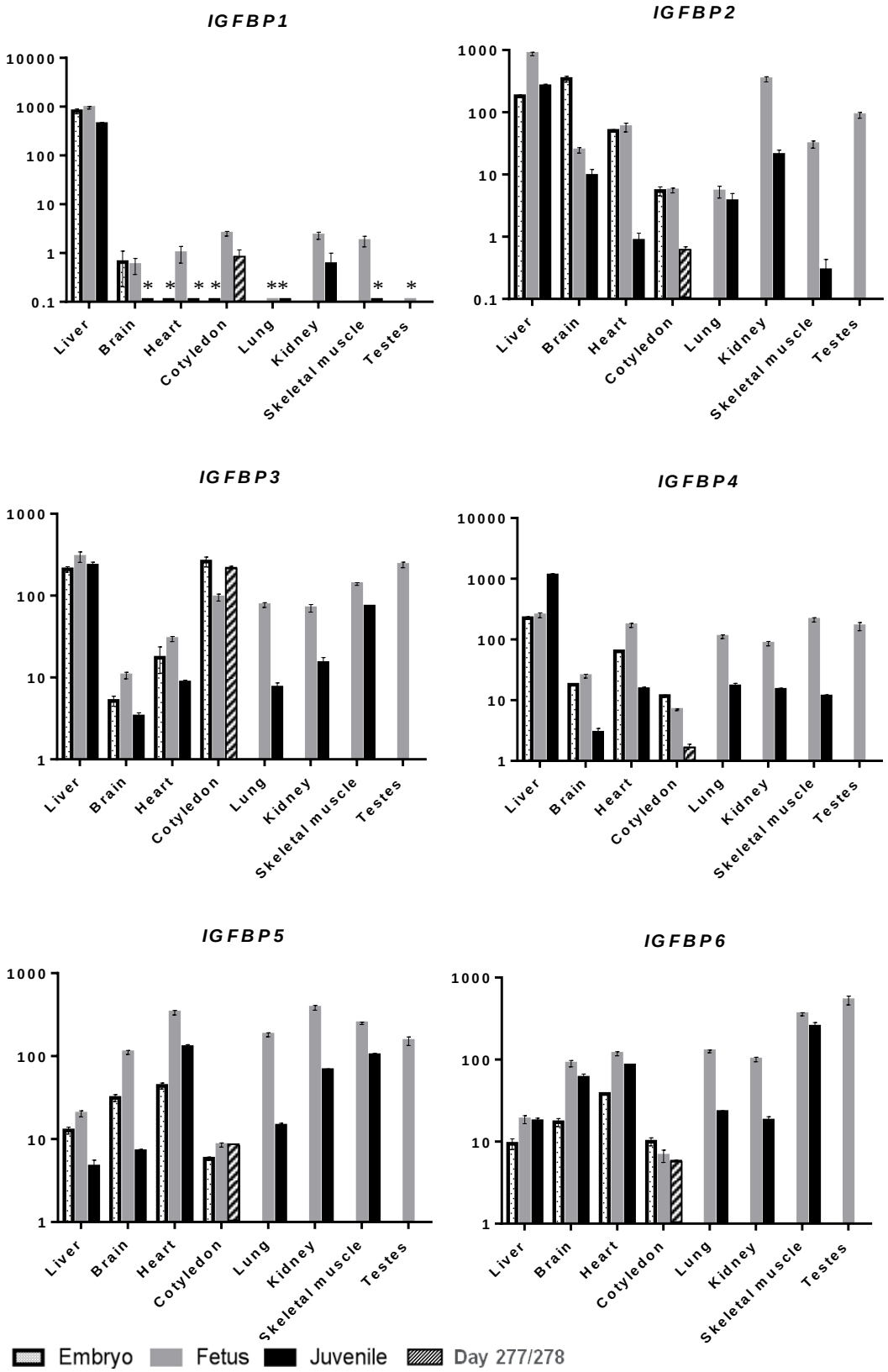

Figure 3.2 Means and standard deviation of means for 3 technical replicates of measured transcript abundances of insulin-like growth factor binding protein 1 (*IGFBP1*) to *IGFBP6* in bovine tissues of different developmental stages. Tissue and developmental stage specific transcript abundance was calculated by the standard curve method and expressed in arbitrary logarithmic units (*: measured but not expressed).

IGFBP2 was expressed ubiquitously in all embryonic and fetal tissues studied. Embryos showed high levels of *IGFBP2* expression in brain, followed by liver and heart. In the fetus, liver and kidney had the highest level of expression of *IGFBP2*. In the juvenile, *IGFBP2* was expressed at readily detectable levels in liver, brain, lung and kidney, with lower levels of expression in heart and skeletal muscle. In general, *IGFBP2* expression level was higher in fetal tissues and decreased after birth. However, liver *IGFBP2* expression remained high.

IGFBP3 was expressed in all studied tissues. Liver, cotyledon and testis had the highest and brain had the lowest levels of expression at all developmental stages. Prenatally, most tissues expressed a high level of *IGFBP3*, but liver and skeletal muscle showed the highest level of expression.

Liver had the highest and brain and cotyledon had the lowest levels of *IGFBP4* transcript abundance in all developmental stages. The level of this transcript decreased postnatally in all tissues but liver had the reverse pattern, with an increase observed postnatally.

Heart, kidney and muscle had the highest, and cotyledon the lowest, expression levels of *IGFBP5* at all developmental stages with a decrease in transcript abundance postnatally. Heart had the highest and liver the lowest level of *IGFBP6* transcript in embryos. Testicular tissue had the highest expression and cotyledon had the lowest transcript abundance in fetuses. Muscle had the highest and liver the lowest level of *IGFBP6* transcript in juveniles.

3.3.6.1 Low affinity *IGFBPs* expression

Skeletal muscle, followed by heart, showed the highest levels of *IGFBP7* expression in all developmental stages. Cotyledon always had the lowest level of expression of *IGFBP7*.

IGFBP8 transcript was expressed at relatively high levels in embryos, fetuses and juvenile tissues. Heart followed by skeletal muscle and testis had the highest level of *IGFBP8* expression.

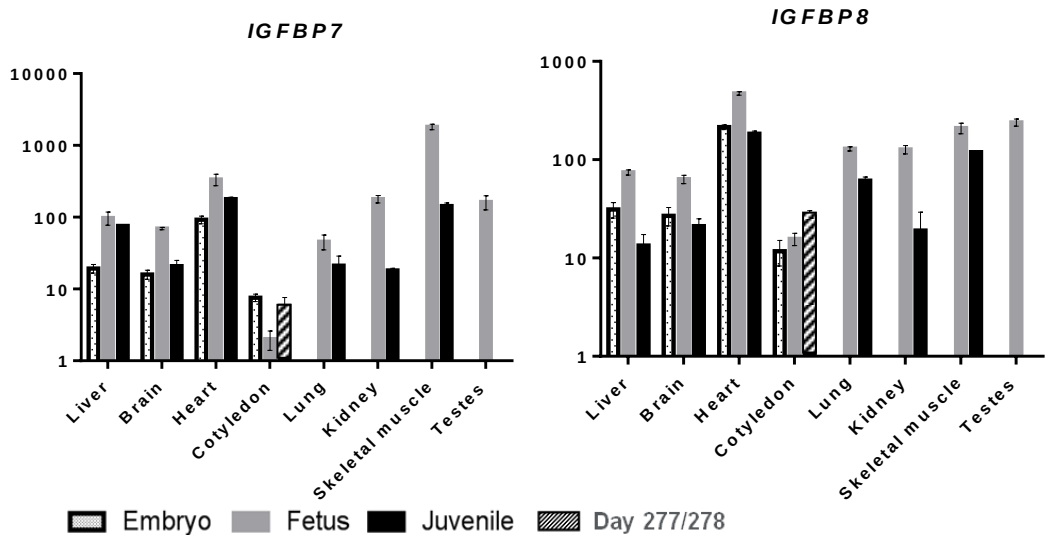

Figure 3.3 Means and standard deviation of means for 3 technical replicates of measured transcript abundances of insulin-like growth factor binding protein 7 (*IGFBP7*) and *IGFBP8* in bovine tissues of different developmental stages. Tissue and developmental stage specific transcript abundance was calculated by the standard curve method and expressed in arbitrary logarithmic units.

3.3.4 *Insulin receptor (IR)* expression

3.3.4.1 *IR overall transcript* expression

The insulin receptor was highly expressed in all tissues at all developmental stages studied (Figure 3.4). Liver, followed by skeletal muscle and heart, showed the highest level of expression at all developmental stages. Kidney showed the lowest *IR* expression in juveniles; 2-fold less than liver. Cotyledon had the lowest *IR* expression prenatally. Levels of expression in fetal and embryonic tissues were similar to those observed in juveniles, with a small decrease in some tissues, especially lung and kidney, postnatally.

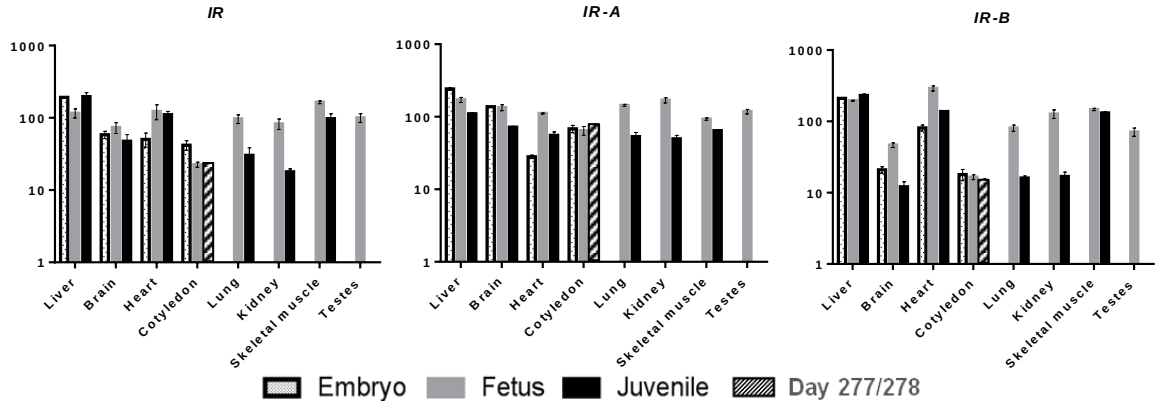

Figure 3.4 Means and standard deviation of means for 3 technical replicates of measured transcript abundances of insulin receptor (*IR*), *IR variant A* (*IR-A*) and *IR-B* in bovine tissues of different developmental stages. Tissue and developmental stage specific transcript abundance was calculated by the standard curve method and expressed in arbitrary logarithmic units.

3.3.4.2 *IR-A* and *B* expressions

The expression pattern of *IR-A* was very similar to *IR* overall transcript in all tissues, but slightly higher in embryonic cotyledon, compared to other developmental stages.

Developmental change did not bring about a dramatic change in expression level of *IR-A*. Also, *IR-A* was expressed at high levels among all tissues pre and postnatally. *IR-B* expression in some postnatal tissues such as brain and cotyledon was lower than in other tissues and it was almost 10-fold less than in liver or skeletal muscle and heart.

IR-B was expressed in all studied tissues. In contrast to *IR-A*, *IR-B* showed a decrease in some tissues postnatally, such as kidney, lung, heart and brain. Liver, skeletal muscle and cotyledon did not show notable change during development.

3.3.5 *Growth hormone* (*GH*) and *Growth hormone receptor* (*GHR*) expression

3.3.5.1 *GH* expression

Growth hormone mRNA was not detected in any of the studied tissues at any developmental stage.

3.3.5.2 *GHR overall transcript* expression

Cotyledon showed the lowest level of *GHR* transcript at all stages. Liver in embryos and juveniles had the highest levels of *GHR* mRNA, although adult skeletal muscle also showed relatively high levels of *GHR* transcript. In the fetal stage, skeletal muscle had the highest level of *GHR* expression, followed by liver.

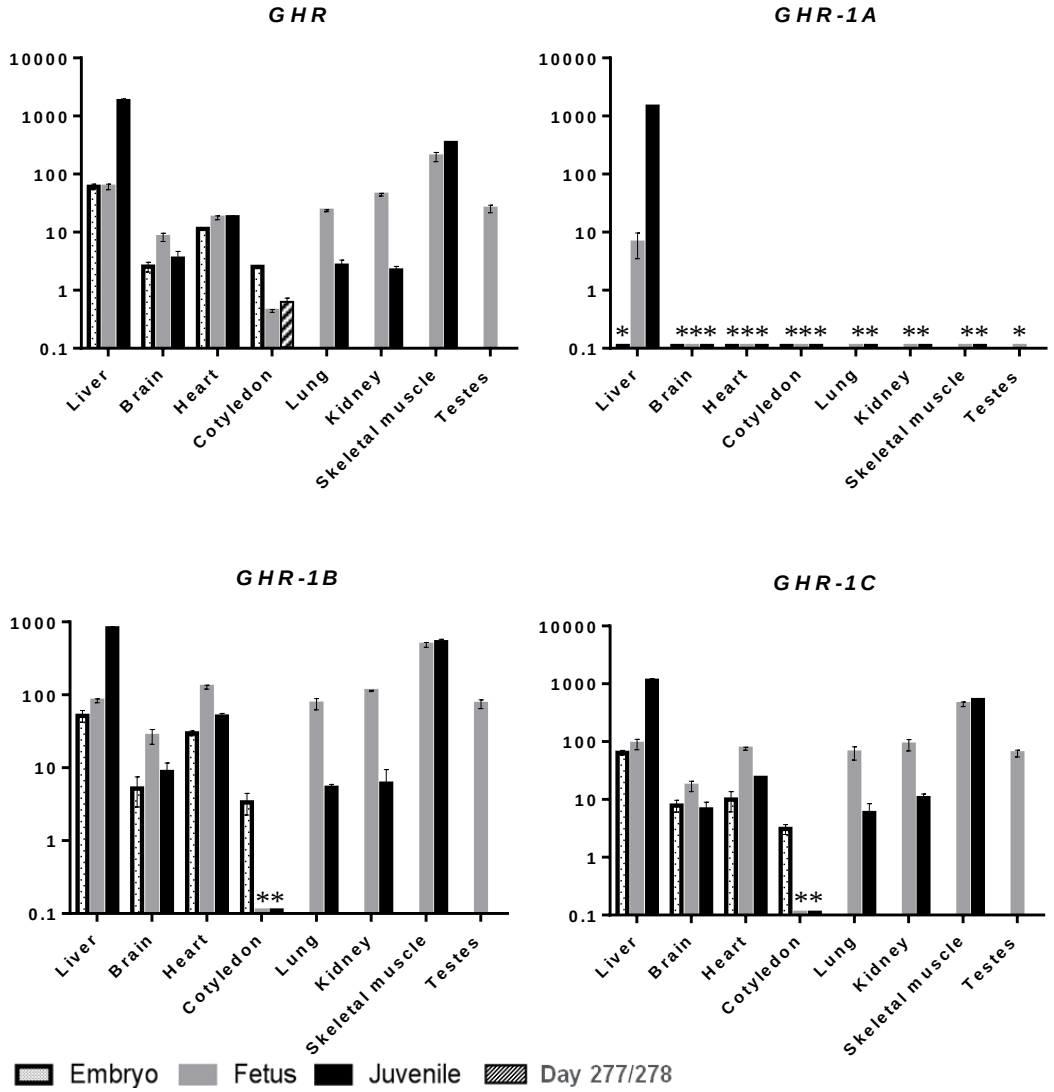

Figure 3.5 Means and standard deviation of means for 3 technical replicates of measured transcript abundances of growth hormone receptor (*GHR*), *GHR-1A, GHR-1B* and GHR-1C in bovine tissues of different developmental stages. Tissue and developmental stage specific transcript abundance was calculated by the standard curve method and expressed in arbitrary logarithmic units (*: measured but not expressed).

3.3.5.3 *GHR-1A, -1B* and *-1C* expression

GHR-1A showed a liver specific expression pattern in fetal and juvenile tissues. However, it could not be detected in embryonic liver.

GHR-1B and *-1C* transcripts showed very similar patterns to the overall transcript but there was no detectable transcript in fetal cotyledon and cotyledon near term (C-sectioned calves). Liver and skeletal muscle showed higher mRNA levels for *GHR-1B* and *1C* than other tissues.

3.4 Discussion

3.4.1 *IGF1*

We showed that *IGF1* transcript was expressed in all of the tissues examined at each developmental stage. This is in accordance with other reports in cattle (Wang et al., 2003; Zhang et al., 2011). Skeletal muscle showed the highest level of *IGF1* expression at D-153 of gestation, whereas kidney and cotyledon had the lowest expressions at the fetal stage. Similar results have been reported for the late gestation sheep and pig fetuses, with highest *IGF1* expression found in ovine and porcine skeletal muscle and fat, and lowest expression levels in kidney and placenta (Kind et al., 1995; Ramsay et al., 1994). In accordance with our results, kidney had the lowest level of *IGF1* mRNA in the human fetus at week 14-16 of gestation (Hill, 1990). The present data suggested that hepatic *IGF1* expression levels could remain constant from D-48 to 153 of gestation in the bovine fetus. Gore et al (1994) studied hepatic IGF1 expression in bovine fetuses at D-100 and 200 gestation, and in one month old calves, and also reported no differences in expression across these development stages. The latter study investigated bovine D-100 and D-200 fetuses and one month old calves. Furthermore, fetal serum IGF1 showed an increase of 5-fold from D-85 to D-240 in the normal developing bovine fetus (Holland et al., 1997).

In juveniles, liver had the highest expression of *IGF1*, which was almost 40-fold higher than in skeletal muscle and lung had the lowest *IGF1* expression. Similar findings have been

reported in other species. It was shown in steers, cows and bulls that adult liver had the highest level of *IGF1* transcript (Wang et al., 2003; Cordano et al., 2000; Gore et al., 1994). Similar results were obtained in human (Bornfeldt et al., 1989) mouse (Williams et al., 2011; Yakar et al., 1999; Baker et al., 1993), rat (Cohick and Clemmons, 1993), sheep (Pell et al., 1993; Dickson et al., 1991), chicken (McMurtry et al., 1997) and fish (Shamblott and Chen, 1993; Duguay et al., 1996). Liver is the main source of circulating IGF1, compared to other tissues of adult mammals (Sjogren et al., 1999; Yakar et al., 1999; Phillips et al., 1998), although we showed that *IGF1* expression in skeletal muscle was at relatively high levels in comparison to liver in fetuses and juveniles.

IGF1 transcript level decreased postnatally in all studied tissues except liver, supporting the suggested role for autocrine/paracrine actions of IGF1 in regulating growth and development of prenatal tissues (Clemmons, 2007).

In juveniles we showed that liver expressed the highest level of *IGF1 class 1* transcript. It was previously shown that liver had the highest *IGF1 class 1* mRNA levels in adult cattle (Wang et al., 2003; Zhang et al., 2011), sheep (Dickson et al., 1991), mouse (Ohtsuki et al., 2005) and pig (Xiao et al., 2009).

The *IGF1 class 2* transcript also showed a tissue specific expression, which was in accordance with data obtained by Shemer et al. (1992) in rats and Jensen et al. in human (1991). We demonstrated that *IGF1* mRNA variants are differentially expressed during development in bovine. Tissue specific expression of *IGF1 class 1* and *class 2* mRNA has also been demonstrated in other species including human (Nagaoka et al., 1991), mouse (Ohtsuki et al., 2005), rat (Adamo et al., 1989), fish (Shamblott and Chen, 1993) and rabbit (Yang et al., 1996). In general, *IGF1 class 1* transcript showed relatively higher abundances than *IGF1*

class 2 transcript in our experiment. A similar relationship between these two transcripts was shown in adult cattle (Wang et al., 2003), human (Nagaoka, 1991), pig (Xiao, 2009) and mouse (Ohtsuki et al., 2005). *IGF1 Class 1* transcript was expressed at 10 times higher levels than *IGF1 class 2* in adult human liver (Nagaoka et al., 1991) and mouse liver (Ohtsuki et al., 2005). It has been shown that both *IGF1 class 1* and *2* levels increased in liver after administration of GH in cattle. However, *IGF1 class 2* showed a significantly greater increase than *IGF1 class 1* (Wang et al., 2003). Similar result have been reported in sheep (Pell et al., 1993), showing that *IGF1 class 2* transcript is more responsive to GH than *IGF1 class 1*.

3.4.2 *IGF1R*

We found that *IGF1R* was expressed in all studied tissues at all developmental stages, but with higher expression in all studied prenatal tissues. A limited number of studies have investigated developmental stage specific expression of *IGF1R*. It has been demonstrated that *IGF1* receptor mRNA was present in pre-implantation bovine embryos (Wang et al., 2009) as well as 4-6 weeks old human embryos (Coppola, 2009), which shows the very early potential role of *IGF1R* in prenatal development. At the embryonic stage, *IGF1R* expression was the highest in brain and liver. In the fetus, *IGF1R* was expressed in all studied tissues, with cotyledon showing the lowest expression. Similar results were published for chicken, where kidney and brain had relatively high expression levels of *IGF1R*, while no transcript could be detected in heart and skeletal muscle tissues at the E16 stage (Holzenberger et al., 1996). *IGF1R* was shown to be very important in normal brain growth and development during prenatal period. In several studies it was shown that *IGF1R* knockout mice developed severe brain growth retardation (Okusky and Ye, 2012; Dupont and Holzenberger, 2003; Liu et al., 1993).

In the juvenile, *IGF1R* was highly expressed in heart and muscle tissues, and expressed at 4-5 fold lower levels in lung and kidney. In addition to its essential role in brain development, *IGF1R* is critical for muscle growth. Mice lacking *IGF1R* showed lower muscle mass than control mice and 50-60% growth retardation (Louvi et al., 1997).

3.4.3 *IGFBPs*

3.4.3.1 High affinity *IGFBPs*

The present data showed that liver was the major source of *IGFBP1* transcript at all developmental stages. However, there was very low expression of *IGFBP1* in fetal muscle and kidney. Similar findings are reported for rat which showed that *IGFBP1* is expressed predominantly from liver in fetuses at 21 days of gestation, followed by kidney (Ooi et al., 1990). Postnatally, Ooi et al. (1990) showed similar *IGFBP1* expression pattern in rats at the age of 65 days. In another study in adult rats, the highest level of *IGFBP1* mRNA was found in the liver, followed by kidney (Murphy et al., 1990). In contrast to our results, it has been reported that *IGFBP1* is exclusively expressed in human fetal liver during week 10-16 post conception (Han et al., 1996) and mouse liver from mid to late gestation (Cerro et al., 1993). The liver-specific expression pattern of *IGFBP1* was only observed in mouse and human. We might expect mouse and rat to have similar gene expression patterns. However, it was shown that *IGFBP1* expression is specific to mouse liver, but this pattern was not observed in rat. We need to consider that these data were obtained by *in situ* hybridisation which may not be as accurate as qPCR (Rosa et al., 2009; Marino et al., 2003).

Expression of *IGFBP2* was detected in all tissues studied. Embryos showed high levels of *IGFBP2* expression in brain, followed by liver and heart. The developmental expression patterns observed in liver and muscle in the present study were quite similar to those

described by Gerrard et al. (1999) in pig, where the fetal liver and kidney had the highest level of *IGFBP2* expression. In the human fetus, *IGFBP2* mRNA was present in moderate abundance in every tissue with the highest level observed in liver (Han, 1996). Fetal liver was also the predominant site of *IGFBP2* expression in the rat, although kidney, stomach, lung, and brain also expressed moderate levels. Levels of *IGFBP2* mRNA was higher in prenatal than in adult rat tissues (Orlowski et al., 1990). In the juvenile, *IGFBP2* was readily detectable in liver, brain, lung and kidney, with lower levels of expression in heart and skeletal muscle. Even in fish such as the common carp (*Cyprinus carpio*), *IGFBP2* was reported to be expressed at the highest level in liver amongst studied tissues, followed by testes and ovary, while kidney showed very low expression of *IGFBP2* transcript (Chen et al., 2009). In general, bovine *IGFBP2* expression levels were higher prenatally and decreased postnatally. However, liver *IGFBP2* expression remained high. This was in accordance with *IGFBP2* expression studies in prenatal tissues in sheep (Delhanty and Han, 1993), rat (Batchelor et al., 1995) and pig (Gerrard et al., 1999).

Liver, cotyledon and testis had the highest and brain had the lowest level of *IGFBP3* expression in all developmental stages. High levels of *IGFBP3* mRNA were found in adult rat liver while brain showed very low levels of *IGFBP3* mRNA (Albiston and Herington, 1992). This is similar to results of the present study. In human fetuses at weeks 10-16, *IGFBP3* was reported as highly expressed in muscle and heart but not liver, which contrasts with our results (Han et al., 1996).

Liver showed the highest, and brain and cotyledon the lowest, levels of *IGFBP4* transcript at all developmental stages. *IGFBP4* was expressed in multiple tissues of adult rats, with liver exhibiting the highest expression (Cohick and Clemmons, 1993). We showed that transcript levels decreased postnatally in all tissues with the exception of liver, which showed the

reverse pattern. Han et al. (1996) reported that *IGFBP4* was expressed moderately and equally from all tissues, including liver, heart, muscle, skin, spleen and stomach in human fetuses at weeks 10-16. In addition, Carr et al. (1995) showed that IGFBP4 protein levels in fetal sheep plasma increased linearly from D-40 of gestation until birth. The fact that liver *IGFBP4* expression dramatically increased (4-fold) postnatally compared to prenatal liver, and that it was expressed 10-fold higher than other adult tissues, may suggest that liver is a major source of circulating IGFBP4. In contrast to our results, Batchelor et al. (1995) showed that *IGFBP4* mRNA increased in postnatal lung tissue of rat in comparison to the fetal stage. However, comparing late prenatal and early postnatal differences of murine models to bovine might not be appropriate since newborn rodents are not as well developed as the new born calf (Otis and Brent, 1954). Therefore, developmental timing differences between rodents and cattle would impact gene expression comparisons, especially for transcripts involved in prenatal development.

The present data showed that heart, kidney and muscle had the highest levels of expression of *IGFBP5* at all developmental stages. *IGFBP5* has an important role in skeletal muscle growth and differentiation (Mukherjee et al., 2008). It has been shown that increased *IGFBP5* expression in mice caused a reduction of whole-body growth and retarded muscle development (Salih et al., 2004). These phenotypic changes are mainly caused by the blocking of IGFs by excess *IGFBP5* (Mukherjee et al., 2008). In the human fetus, skin and muscle tissues had the highest, and brain the lowest, *IGFBP5* expression (Han et al., 1996). Similar to our results, *IGFBP5* mRNA transcript has been detected in all examined tissues of adult rats and several tissues have higher levels of *IGFBP5* mRNA compared to liver tissue, with the highest expression in kidney (Shimasaki and Ling, 1991b). Similar results were reported in adult goats where kidney had the highest level of *IGFBP5* transcript followed by spleen, brain, lung and liver (Wang et al., 2012).

Bovine *IGFBP6* was expressed in all studied tissues, which is similar to data reported for adult rat (Shimasaki et al., 1991a). We showed that in contrast to other *IGFBPs*, liver did not have the highest level of *IGFBP6* transcript. *IGFBP6* also has the highest affinity for IGF2 in comparison to other IGFBPs. Transgenic mice with overexpression of *IGFBP6* showed significantly smaller body size and smaller size of cerebellum and lower male reproduction rate compared to controls (Bienvenu et al., 2004).

3.4.3.2 Low affinity *IGFBPs*

The IGFBP7 gene was expressed in all studied bovine tissues. Transcripts of this gene have been found in a variety of human tissues, including liver, uterus, testis, skeletal muscle, brain and kidney, but not in fat (Adachi et al., 2001; Degeorges et al., 2000). We showed that skeletal muscle showed the highest levels of *IGFBP7* expression, followed by heart, at all developmental stages. It has been shown that *IGFBP7,* as expressed by skeletal myoblasts, may have a role in stimulating myoblast proliferation in responses to IGFs (Haugk et al., 2000).

The *IGFBP8* transcript was expressed at relatively high levels in embryos, fetuses and juvenile tissues. Heart, followed by skeletal muscle and testicular tissue, had the highest level of *IGFBP8* transcript. This was similar to expression of *IGFBP7*. The *IGFBP8* gene is widely expressed in human tissues and has critical roles in embryonic development, impacting on cell proliferation (De Winter et al., 2008).

Fewer studies have been conducted regarding the developmental stage specific expression of low affinity IGFBPs in comparison to IGFBPs 1-6. We showed that *IGFBP* transcripts in most tissues decreased postnatally and this reduction was greater in kidney and lung than in other tissues. Postnatal reduction of *IGFBP* transcription may highlight the importance of

IGFBPs during prenatal growth and development in cattle. We found that, except for *IGFBP1*, all studied *IGFBPs* were expressed across all tissues and all developmental stages. IGFBP knockout in mouse models showed relatively modest phenotypic effects on growth and development (Silha and Murphy, 2002) in comparison to IGF knockout or knockout of their receptors (Louvi et al., 1997). These modest effects may be due to the compensating effect of other members of the large IGFBP family rather than IGFBPs being of lesser importance for growth and development.

3.4.4 IR

The insulin receptor was highly expressed in all bovine tissues and across all developmental stages studied. Liver, skeletal muscle and heart tissue had the highest levels of expression in all developmental stages. It was shown that the major tissues targeted by insulin in adult human are skeletal muscle, liver and fat (Pezzino et al., 1989).

The bovine *IR-A* expression patterns were very similar to the pattern of *IR* overall transcript. The *IR-A* was expressed at high levels among all tissues pre- and postnatally, while postnatal *IR-B* expression was almost 10-fold less in some tissues, such as brain and cotyledon, as compared with liver or skeletal muscle and heart. It was also shown that *IR-A* in adult rats was highly expressed in muscle and had the lowest expression level in kidney; liver showed the highest levels of *IR-B* expression (Serrano et al., 2005).

The *IR-A* (fetal IR in human) is a high affinity receptor for IGF2 in the human fetus and essential for prenatal growth and development (Frasca et al., 1999). In human, *IR-A* is the predominant prenatal transcript, suggesting *IR-A* might play a role in mediating IGF2 effects (Belfiore et al., 2009). However, our findings in the bovine model showed that both IR-A and IR-B were expressed at all developmental stages at relatively high levels and do not suggest

that *IR-A* is predominant over *IR-B* in prenatal life. We showed that levels of *IR-B* expression in fetal and embryonic tissues are similar to juvenile, with small decreases in some tissues, especially lung and kidney, postnatally.

3.4.5 *GH*

Pituitary gland is the primary source of GH in mammals but GH can be expressed from a duplicated *GH* gene, *GH2* or *GH-V,* which is active in the placenta of some mammals including human, goat and sheep (Gootwine, 2004). There is no known duplicated gene in cattle to produce placental GH, although small amounts of GH can be produced from other tissues including reproductive, respiratory and skeletal muscle tissues (Harvey, 2010).

Since we did not measure GH transcript from the pituitary gland as a primary source of GH, there cannot be any statement on the potential roles of GH transcription in bovine prenatal growth and development from this research. However, we did not find any extra-pituitary GH transcripts in our studied tissues.

3.4.6 GHR

The expression of *GHR* has been studied in a variety of mammals and transcript was detected in a range of embryonic and fetal tissues (Waters and Kaye, 2002). In accordance with the present results, it has been shown that in adult human, liver and skeletal muscle had the highest levels of *GHR* expression (Ballesteros et al., 2000). Liver, heart and skeletal muscle also showed highest levels of *GHR* expression in adult rabbit (Ymer and Herington, 1992). A study of bovine *GHR* expression showed that liver followed by skeletal muscle and kidney had the highest level of expression in adults and in 8-month old fetuses (Jiang et al., 1999). In our study, *GHR* overall transcript was expressed at low levels in fetal cotyledon and cotyledon

obtained by C-section close to term, but none of the measured *GHR* transcripts including *GHR-1A, GHR-1B* and *GHR-1C* were detectable. This might indicate that other possible transcript variants of *GHR* might be more expressed in cotyledon. Variants A, B and C are responsible for 90% of total *GHR* transcription (Jiang and Lucy, 2001).

GHR-1A showed a liver specific expression pattern in bovine fetal and juvenile tissues. However, this transcript could not be detected in embryonic liver. It has been shown that *GHR-1A* is exclusively expressed in liver of mammals, including human (Zhou et al., 1997), mouse (Menon et al., 1995) and cattle (Jiang and Lucy, 2001). In contrast to our result, Lucy et al. (1998) reported that *GHR-1A* was not expressed in 8-month old bovine fetal liver. It has been demonstrated that administration of recombinant bovine somatotropin increased expression of *GHR-1A,* but not of the other *GHR* transcripts in cattle liver (Kobayashi et al., 1999). This may suggest GH-dependent IGF1 action in liver is more correlated with liver *GHR-1A* mRNA and not with other liver transcripts of *GHR* (Kobayashi et al., 1999).

GHR-1B and *-1C* transcripts showed very similar patterns to overall transcript abundance but there were no detectable transcripts in fetal cotyledon or near term (C-section) cotyledon. Liver and skeletal muscle showed higher mRNA levels for *GHR-1B* and *1C* than other tissues. Jiang and Lucy (2001) reported that bovine fetal and adult liver expressed the highest levels of *GHR-1B* and *1C*. However, in contrast to our result, they showed that fetal kidney expressed the second highest level of *GHR-1B* and not skeletal muscle. We showed that bovine adult liver has the highest level of *GHR* mRNA expression which was ~5.5 fold higher than in skeletal muscle. Since *GHR-1B* and *1C* had almost 2-fold higher expression levels in liver than in skeletal muscle, it appears that *GHR-1A* contributes a significant proportion of total GHR transcript in adult liver.

GHR and GH are closely related to a family of hormones and receptors including prolactin (PRL), prolactin receptor (PRLR) and placental lactogen (PL). There is a high degree of similarity between GH and PRL and their receptors (GHR and PRLR) amino acid sequences, gene structures, and functions (Goffin and Kelly, 1997). It has been shown that GHR can be a receptor for PRL and PL hormones and GHR and PRLR can form a functional heterodimer receptor (Goffin and Kelly, 1997). Upon binding either of GH, PRL or PL to GHR, signal transduction initiates with phosphorylation of cytosolic Janus kinase 2 (JAK2) which activates STAT pathways. STAT5b is directly involved in regulation of IGF1 transcription (Hwa et al., 2011).

GHR is the mediator of GH actions and binding of GH to GHR initiates the transcription of many genes including IGF1 (Jiang et al., 2007). Knockout mouse models showed no significant body size or weight differences among normal and GHR knockout newborns. However, GHR deficient mice (Laron mice) were significantly smaller 3 weeks after birth (Zhou et al., 1997). Laron mice had 90% less circulating IGF1 and lived up to 50% longer than controls (Coschigano et al., 2000). It has been shown that *GHR* expression is highly correlated with *IGF1* expression in cattle liver (Kobayashi et al., 1999). Liver tissue can be considered a key organ of the GH-IGF1 axis postnatally. However, a GH-independent role of IGF1 appears to be the predominant mechanism responsible for growth and development in the prenatal period (Osafo et al., 2005; LeRoith et al., 2001a).

The present data showed that GHR transcript was expressed in a variety of bovine fetal and embryonic tissues. Since there is no published evidence of a direct role of GH in prenatal growth and development (Osafo et al., 2005), and since the ability of binding other ligands such as PRL and PL, which are specific to gestational age, there could be other mechanisms contributing to differential GHR transcription during bovine fetal development.

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Chapter 4

Heterosis in insulin-like growth factor family member transcript abundance

4.1 Introduction

Heterosis or hybrid vigour is the superiority of crossbred animals over the average of purebred parents (Dickerson, 1973). From an evolutionary point of view, heterosis is defined as higher fitness of heterozygote individuals, compared to homozygotes in a population (Chen, 2011).

Heterosis has an important role in improving the productivity of plants and animals and has been used for centuries to obtain benefits in animal production industries (Simm, 1998; Dickerson, 1973). Despite the long history of using heterosis in industry, the molecular mechanisms underlying this phenomenon remain unclear. There are several hypotheses proposed from quantitative genetics, including dominance, over dominance and epistasis, but none are able to fully explain the mechanism of heterosis (Alexander et al., 2009; Birchler et al., 2003).

As a basic principle of crossbreeding, heterosis has been used extensively in the beef industry (Dickerson, 1973). In general, heterosis increases as the genetic distance of the parents increases (Chen, 2010). Thus, when breeds from two subspecies are crossed (e.g. *Bos taurus* × *Bos indicus*), a higher level of heterosis is expected compared to a cross between two similar breeds. Cundiff et al. (1994) showed that performance in carcass and meat characteristics of *Bos indicus* and *Bos taurus* crossbred cattle is greater than purebreds. Similarly, Brown et al. (1993) demonstrated that Brahman-Angus hybrid calves had higher

birth weight compared to Angus or Brahman purebreds. Since heterosis in cattle can be detected as early as birth, the molecular mechanism driving heterosis may be initiated during embryonic and fetal development.

A number of studies have been performed to investigate possible physiological contributors to increased growth associated with heterosis in cattle. This includes assessment of the potential role of insulin-like growth factor 1 (IGF1) in heterosis (Caldwell et al., 2011). Plasma and serum concentrations of IGF1 have been linked to many economically important traits in cattle. Johnston et al. (2001) found positive correlations between the circulating concentration of IGF1 and traits such as birth weight and carcass weight in cattle. A significant linear correlation between plasma IGF1 and weight of D-80 bovine IVF and AI fetuses was demonstrated by Hiendleder et al. (2006). It has also been shown that circulating IGF1 is significantly higher at D-84 in Angus-Brahman crossbred calves compared to Angus or Brahman purebred individuals (Caldwell et al., 2011).

IGF1 is an anabolic and mitogenic hormone, that stimulates protein and glycogen synthesis, increases DNA synthesis, stimulates cell cycle progression and inhibits apoptosis (Jones and Clemmons, 1995). From a broader perspective, IGF1 belongs to the IGF family which plays an important role in regulating pre- and post-natal growth and development (Alexander et al., 2010). Translation and transcription of IGF system components has been extensively studied in mammals (Moore et al., 2007; Kind et al., 1995; Baker et al., 1993; Rotwein et al., 1987). The IGF family is composed of two ligands (IGF1, IGF2); two types of IGF receptors (IGF1R and IGF2R), the insulin receptor (IR) and six high affinity IGF binding proteins (IGFBP1-6) (Moore et al., 2007; Pandini et al., 2002) and at least two low affinity IGF binding proteins (IGFBP7-8) (Kim, 1998) (Refer to Figures 1.3 and 1.4).

A studies showed an association of IGF1 and heterosis in postnatal cattle (Caldwell et al., 2011), but tissue specific expression analyses of IGF family members, including different transcript variants, have not been conducted in prenatal stages in the context of heterotic phenotypes.

The aim of this chapter is to investigate potential heterosis effects in transcript abundance of IGF system components, including *IGF1*, *IGF1 class1*, *IGF1 class2*, *IGF1R*, *IGFBP1* to *6*, *IR*, *IR-A* and *IR-B* in heart, brain, skeletal muscle, liver and cotyledon tissue of both reciprocal hybrid and purebred fetuses of *Bos indicus* (Brahman) and *Bos taurus* (Angus).

4.2 Materials and methods

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). The two breeds used in this research are subspecies of domestic cow, commonly referred to as *Bos taurus* (or taurine) and *Bos indicus* (or indicine) (Hiendleder et al., 2008). Four different groups of D-153 fetuses were used in this study: purebred Angus (n=23) and Brahman (n=15) and the two reciprocal crosses (AB[†], n=13 and BA, n=22). Fetal tissues including heart, brain, skeletal muscle (*M. semimembranosus*), liver and cotyledon were collected as described in Chapter 2, section 2.1. RNA was extracted and cDNA synthesised as described in Chapter 2, section 2.2. The tissues were selected to represent all three germ layers, including ectoderm, mesoderm and endoderm. Cotyledon tissue represented the trophectoderm lineage (Yu et al., 2010).

[†] A is Angus, B is Brahman and the first and second letters in a genotype indicating the breed of sire and dam, respectively.

Relative abundance of 13 transcripts from the IGF family, including *IGF1*, *IGF1 class1*, *IGF1 class2*, *IGF1R*, *IGFBP1* to *6*, *IR*, *IR-A* and *IR-B*, were assessed by quantitative real-time PCR (qPCR). All reactions, including housekeeper genes, were conducted in duplicate or triplicate (Chapter 2, section 2.6).

To identify the most stable genes for purposes of normalising qPCR data for RNA/cDNA input, seven housekeeping genes were used. These were 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*) (Hruz et al. 2011; Lisowski et al., 2008). The qPCR reactions for housekeeper genes were performed in triplicate and all of desired genes were assessed in each of the tissue types, with the exception of cotyledon. In cotyledon, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was assessed, instead of *H3F3A*, due to initial studies that indicated that *H3F3A* expression was not stable in cotyledon. Computer software, geNorm version 3.5 was used to determine the most appropriate two housekeeper genes in each tissue, based on a method described by Vandesompele et al. (2002). *VPS4A* and *RSP9* were identified as the two genes with the most stable and readily detectable expression.

Gene expression data and fetal phenotype including fetal and organ weights were analysed by Univariate Analysis of Variance (ANOVA) using the general linear model procedure of JMP statistical package version 4.0 (SAS Institute Inc.).

Data were fitted into following linear models:

1- To analyse the effects of fetal genetics, fetal sex and interactions between these factors:

$$y_{ijk} = G_i + S_j + (G*S)_{ij} + e_{ijk}$$

where y_{ijk} is the normalised relative gene expression level, or fetal phenotype, G_i is fetal genetics effect (i = AA, BA, AB, BB)[†], S_j is fetal sex effect (j = male, female), $(G*S)_{ij}$ is fetal genetics by sex interaction and e_{ijk} is the residual effect.

2- To estimate heterosis effects on gene expression:

$$y_{ijkl} = H_i + G_j(H_i) + S_k + (H*S)_{ik} + e_{ijkl}$$

where y_{ijkl} is the normalised relative gene expression level or fetal phenotype, H_i is heterosis effect (i = purebred, crossbred), $G_j(H_i)$ is fetal genetics effect nested within heterosis effect (j = AA, BB nested within purebred, j = BA, AB nested within crossbred), S_k is fetal sex (k = male, female), $(H*S)_{ik}$ is heterosis effect-by-sex interaction and e_{ijkl} is the residual effect.

Associations between fetal phenotypic traits and relative gene expression levels were analysed using simple linear regression models using JMP 4.0 (SAS Institute Inc.).

Fetal phenotype traits included weights of fetus, liver, heart, brain, fetal placenta and combined muscles, including *M. supraspinatus, M. longissimus dorsi, M. quadriceps femoris and M. semimembranosus* (each measured as the average weight of both left and right muscles). Phenotype data were provided by Prof. Stefan Hiendleder and Ruidong Xiang (combined muscle weight). Values are presented as least square means and their associated standard errors. Group differences were tested by pairwise comparison of least-squares means using Student's t-tests.

Heterosis for phenotypic and molecular parameters was calculated as heterosis percentage:

Heterosis % = [(crossbred average-purebred average)/purebred average] ×100

[†] A is Angus, B is Brahman and the first and second letters in a genotype indicating the breed of sire and dam, respectively.

Differences were considered significant at $P<0.05$.

4.3 Results

4.3.1 Heterosis, genetic and sex effects on phenotype

There was no effect of heterosis on fetus weight or absolute fetal organ weights. Fetus weight and the weights of all fetal organs were significantly heavier in males than females, with the exception of fetal placenta (Figure 4.1). Fetal weight and all organ weights were affected by genetics, with the exception of fetal brain. Fetuses with BB and/or AB genotypes always had lower organ and fetus weights compared with individuals with AA or BA genotypes, except in brain. Also BA fetuses showed significantly higher weights for fetus and organs, compared with AB fetuses (except in brain).

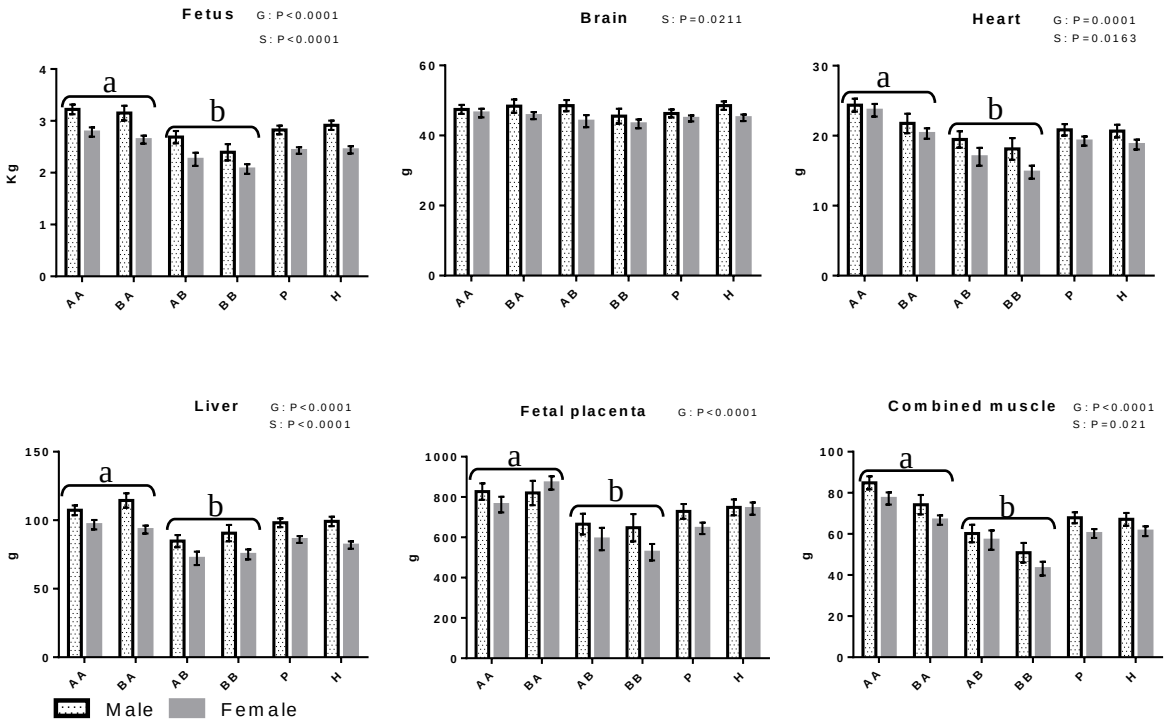


Figure 4.1 Least square means and associated standard errors of means for fetal and absolute organ weights including brain, heart, liver, fetal placenta and combined skeletal muscles in D-153 fetuses with four different genetics. Means for Angus (AA) (n=23), Brahman (BB) (n=15) and reciprocal crossbred genetics (BA, n=13, and AB, n=22, sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. Combined muscle weight was calculated as the combined weights of *M. supraspinatus*, *M. longissimus dorsi*, *M. quadraceps femoris*, *M. semimembranosus* (each of them measured as the average weight of both left and right muscles). Effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) in ANOVA are indicated when significant (P<0.05). Genetic groups with different superscripts differ significantly (t-test P <0.05).

Effects of heterosis, genetics and sex on relative organ weights are shown in Figure 4.2. Females had significantly higher relative organ weights, except for heart and liver. Relative brain weight was significantly higher in BA and BB fetuses. None of the investigated factors were significant for relative heart weight. Purebred fetuses had a significantly higher relative liver weight than hybrid fetuses and AB fetuses had significantly lower liver relative weight than all other genetic groups. Relative fetal placenta weight showed a significant effect of

genetics and BA individuals had significantly higher relative fetal placenta weights than all other genetic groups. Also BB fetuses showed a significantly lower relative (combined) skeletal muscle weight.

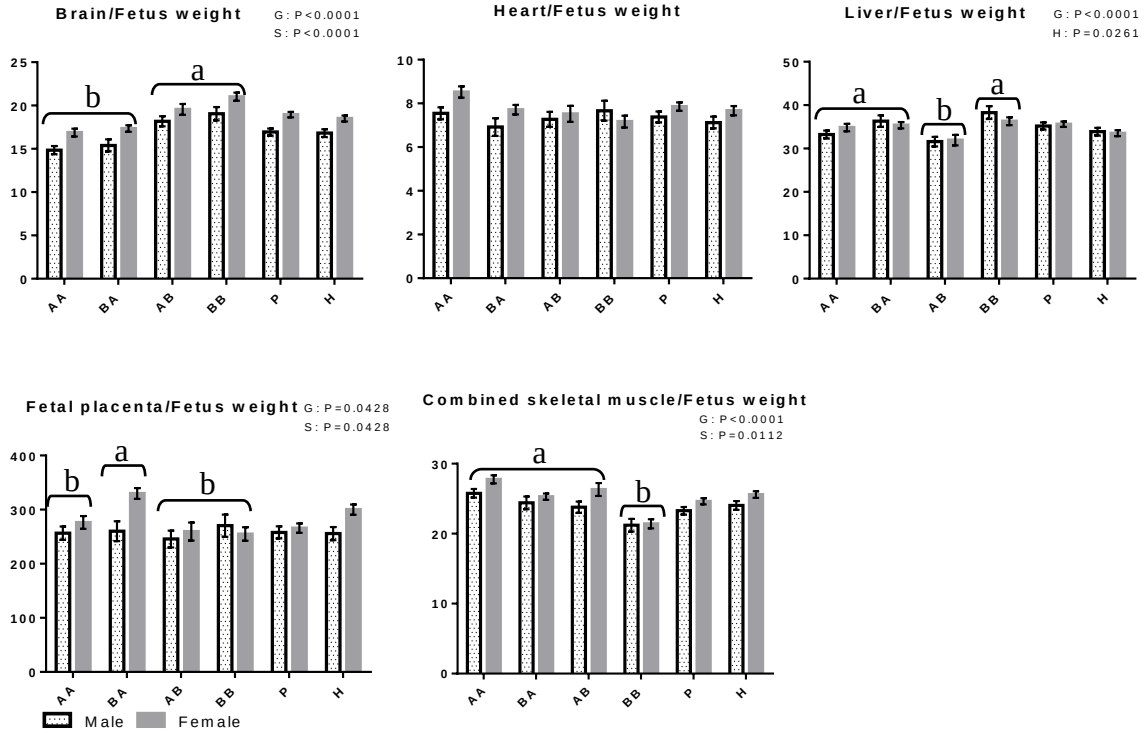


Figure 4.2 Least square means and associated standard errors of means for relative organ weights to fetus weight including brain, heart, liver, fetal placenta and combined skeletal muscles in in D-153 fetuses with four different genetics. Means for Angus (AA) (n=23), Brahman (BB) (n=15) and reciprocal crossbred genetics (BA, n=13 and AB, n=22, sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. Combined muscle weight was calculated as the combined weights of *M. supraspinatus*, *M. longissimus dorsi*, *M. quadraceps femoris*, *M. semimembranosus* (each of them measured as the average weight of both left and right muscles). Effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) in ANOVA are indicated when significant (P<0.05). Genetic groups with different superscripts differ significantly (t-test P <0.05).

4.3.2 Heterosis, genotype and sex effects on *IGF* family member transcript abundances

4.3.2.1 *IGF* and *IGF1R* expression

Expression levels of *IGF1*, *IGF1 class1* and *IGF1R* are shown in Figure 4.3. In fetal brain, transcript abundance of *IGF1* was significantly higher in purebred Angus individuals in comparison to hybrids. In fetal liver, *IGF1* expression was affected by heterosis and genetic effects. Hybrid AB and BA fetuses had significantly higher expression of *IGF1* in liver. Interestingly, male hybrids and female purebreds had higher levels of *IGF1* compared to the opposite sex. There was no effect of fetal genetics on *IGF1* expression in heart, skeletal muscle or cotyledon tissues.

Expression of *IGF1R* transcript was significantly higher in brain of purebred fetuses. *IGF1R* mRNA levels were higher in liver of AB fetuses. In liver, female hybrids and female AB fetuses also showed higher *IGF1R* expression. *IGF1R* transcript abundance was not significantly affected by hybrid, genetics or sex effects in other tissues.

IGF1 class1 transcript was only expressed in liver and skeletal muscle tissues at a quantifiable level and its expression in liver resembled that of *IGF1* general transcript. *IGF1 class1* mRNA was affected by heterosis, genotype and interaction effects. Hybrid fetuses expressed the highest, and BB genotype showed the lowest, level of *IGF1 class 1* transcript.

Expression level for *IGF1 class2* transcript was too low to be measured consistently in heart, brain and cotyledon. This transcript was affected significantly by heterosis and genetics in liver. The BB genotype showed significantly lower *IGF1 class 2* expression in liver. *IGF1 class 2* expression in skeletal muscle was not affected by heterosis or genetics.

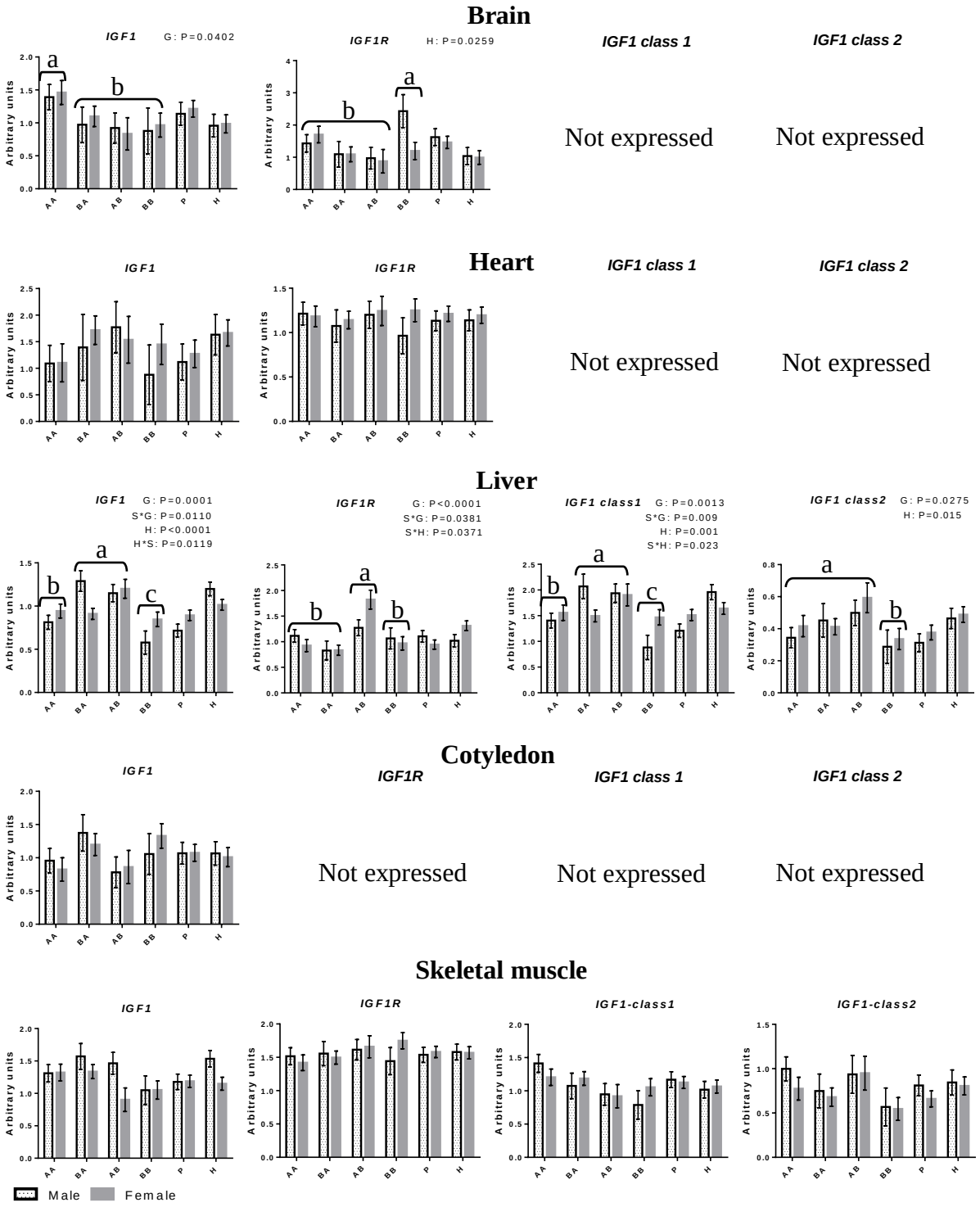


Figure 4.3 Least square means and associated standard errors of means for relative transcript abundance of *IGF1*, *IGF1R* and *IGF1 class1* and *2* in brain, heart, liver, fetal placenta and skeletal muscles (*M. semimembranosus*) in D-153 fetuses with four different genetics. Means for Angus (AA) (n=23), Brahman (BB) (n=15) and reciprocal crossbred genetics (BA, n=13 and AB, n=22 sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. Effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) in ANOVA are indicated when significant (P<0.05). Genetic groups with different superscripts differ significantly (t-test P <0.05).

4.3.2.2 *IGFBP1* to *6* expression

Expression of *IGFBP1*, *IGFBP2* and *IGFBP3* is shown in Figure 4.4. *IGFBP1* could only be measured in liver and showed significant sex and genetic effects. Expression of *IGFBP1* was highest in liver of AA fetuses. Expression of *IGFBP1* was higher in males compared to females. *IGFBP2* expression was not affected by heterosis, fetal genetics or sex effects in heart, liver and skeletal muscle, but was affected by genetics in cotyledon tissue, where fetuses with BA genotype showed a significantly higher level of expression. *IGFBP3* expression was not affected by heterosis, genetic or sex effects in any of the studied fetal tissues.

Transcript abundances for *IGFBP4*, *IGFBP5* and *IGFBP6* are presented in Figure 4.5. *IGFBP4* was not affected by heterosis, genetic or sex effects, except in liver where female hybrids showed a significantly higher level of *IGFBP4* transcript. *IGFBP5* expression in fetal tissues was not affected by heterosis, fetal genetic or sex effects. *IGFBP6* transcript was affected by fetal genetic and heterosis effects in liver tissue. AB fetuses showed significantly lower *IGFBP6* expression in liver. *IGFBP6* mRNA abundance was higher in AA fetuses in skeletal muscle compared to other genetic groups. Purebred fetuses also showed higher *IGFBP6* expression in both liver and skeletal muscle, but only differences in liver was significant.

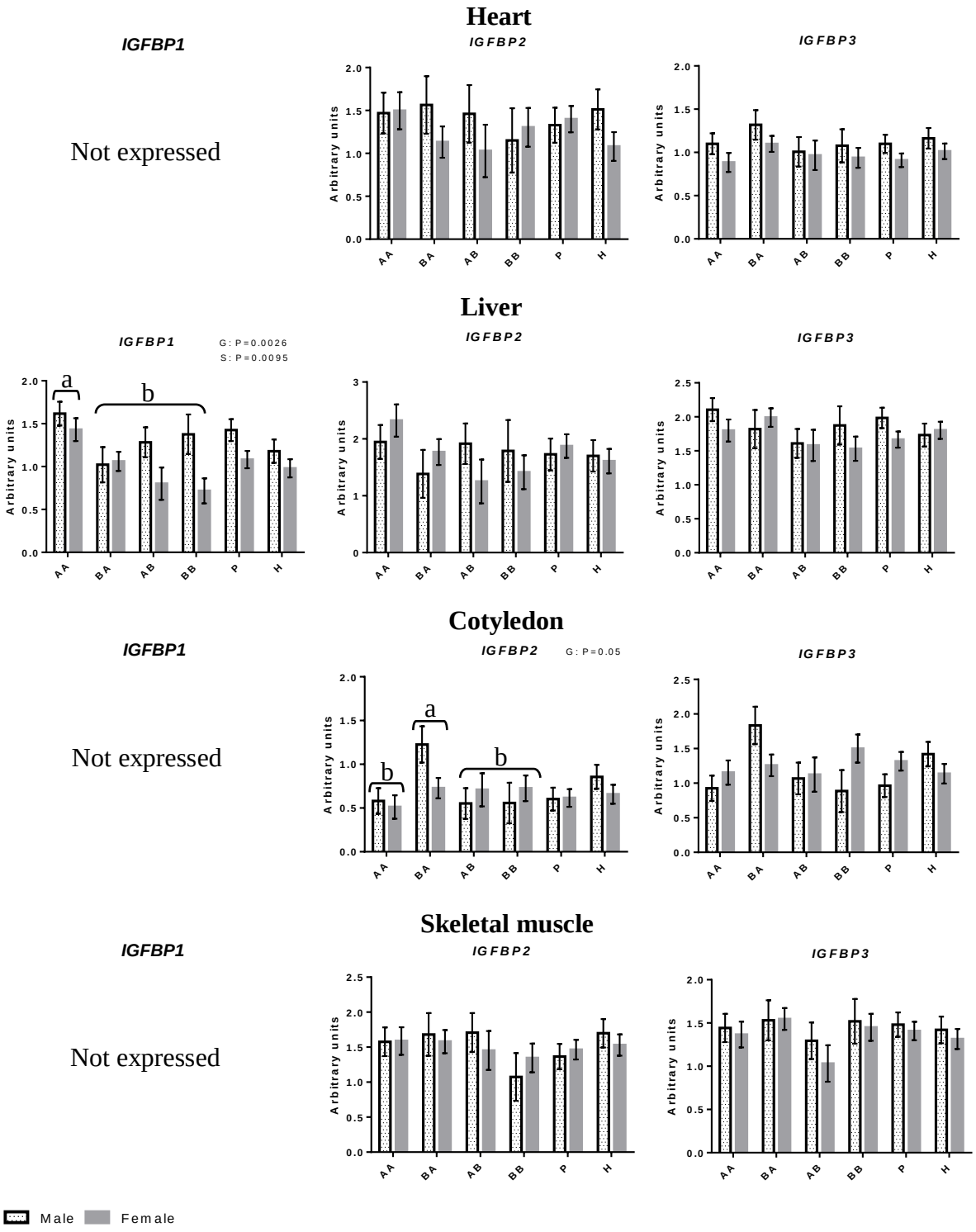


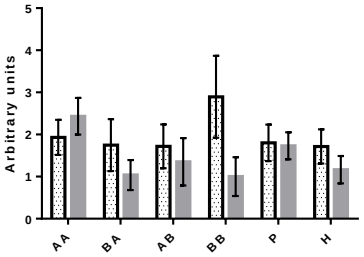
Figure 4.4 Least square means and associated standard errors of means for relative transcript abundance of *IGFBP1*, *IGFBP2* and *IGFBP3* in heart, liver, fetal placenta and skeletal muscles (*M. semimembranosus*) in D-153 fetuses with four different genetics. Means for Angus (AA) (n=23), Brahman (BB) (n=15) and reciprocal crossbred genetics (BA, n=13 and AB, n=22, sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. Effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) in ANOVA are indicated when significant (P<0.05). Genetic groups with different superscripts differ significantly (t-test P <0.05).

Brain

IGFBP4

Not expressed

IGFBP5

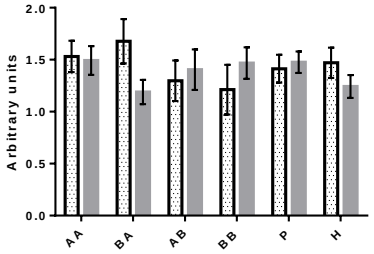


IGFBP6

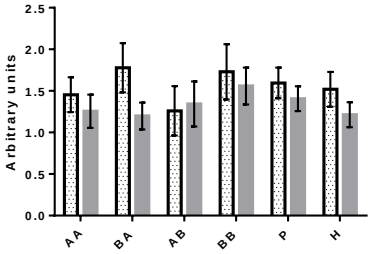
Not expressed

Heart

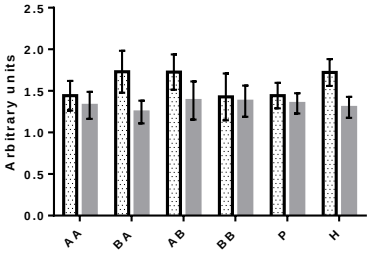
IGFBP4



IGFBP5

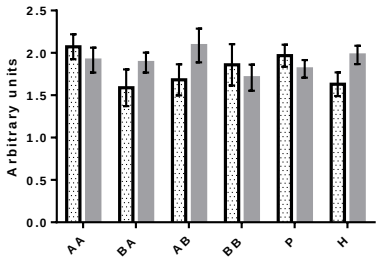


IGFBP6

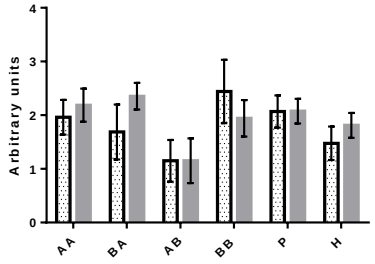


Liver

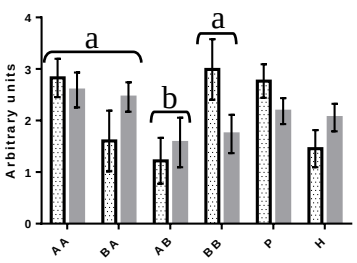
IGFBP4 S* H: P=0.0443



IGFBP5

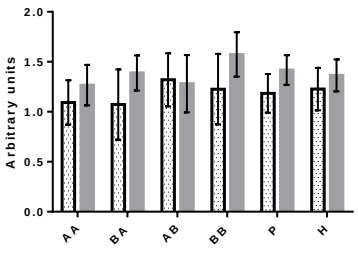


IGFBP6 G: P=0.0197
H: P=0.0196

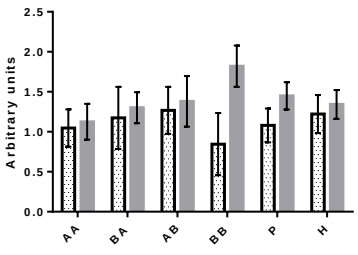


Cotyledon

IGFBP4



IGFBP5

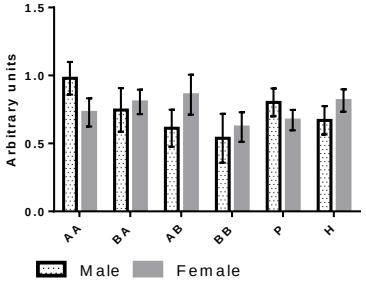


IGFBP6

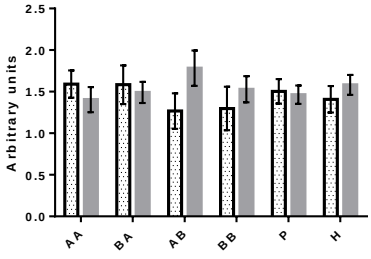
Not expressed

Skeletal muscle

IGFBP4



IGFBP5



IGFBP6 G: P=0.0018

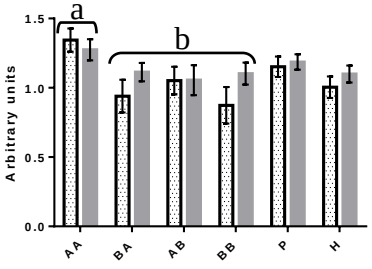


Figure 4.5 Least square means and associated standard errors of means for relative transcript abundance of *IGFBP4*, *IGFBP5* and *IGFBP6* in brain, heart, liver, fetal placenta and skeletal muscles (*M. semimembranosus*) in D-153 fetuses with four different genetics. Means for Angus (AA) (n=23), Brahman (BB) (n=15) and reciprocal crossbred genetics (BA, n=13 and AB, n=22, sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. Effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) in ANOVA are indicated when significant (P<0.05). Genetic groups with different superscripts differ significantly (t-test P <0.05).

4.3.2.3 *IR*, *IR-A* and *IR-B* expression

Fetal heterosis, genetic and sex effects on *IR*, *IR-A* and *IR-B* expression in different tissues are shown in Figure 4.6. In heart, expression of *IR* was significantly higher in AA fetuses. *IR-A* was not affected by sex, genotype and heterosis effects in any tissues. *IR-B* expression level was significantly lower in liver of AB fetuses in comparison to other groups.

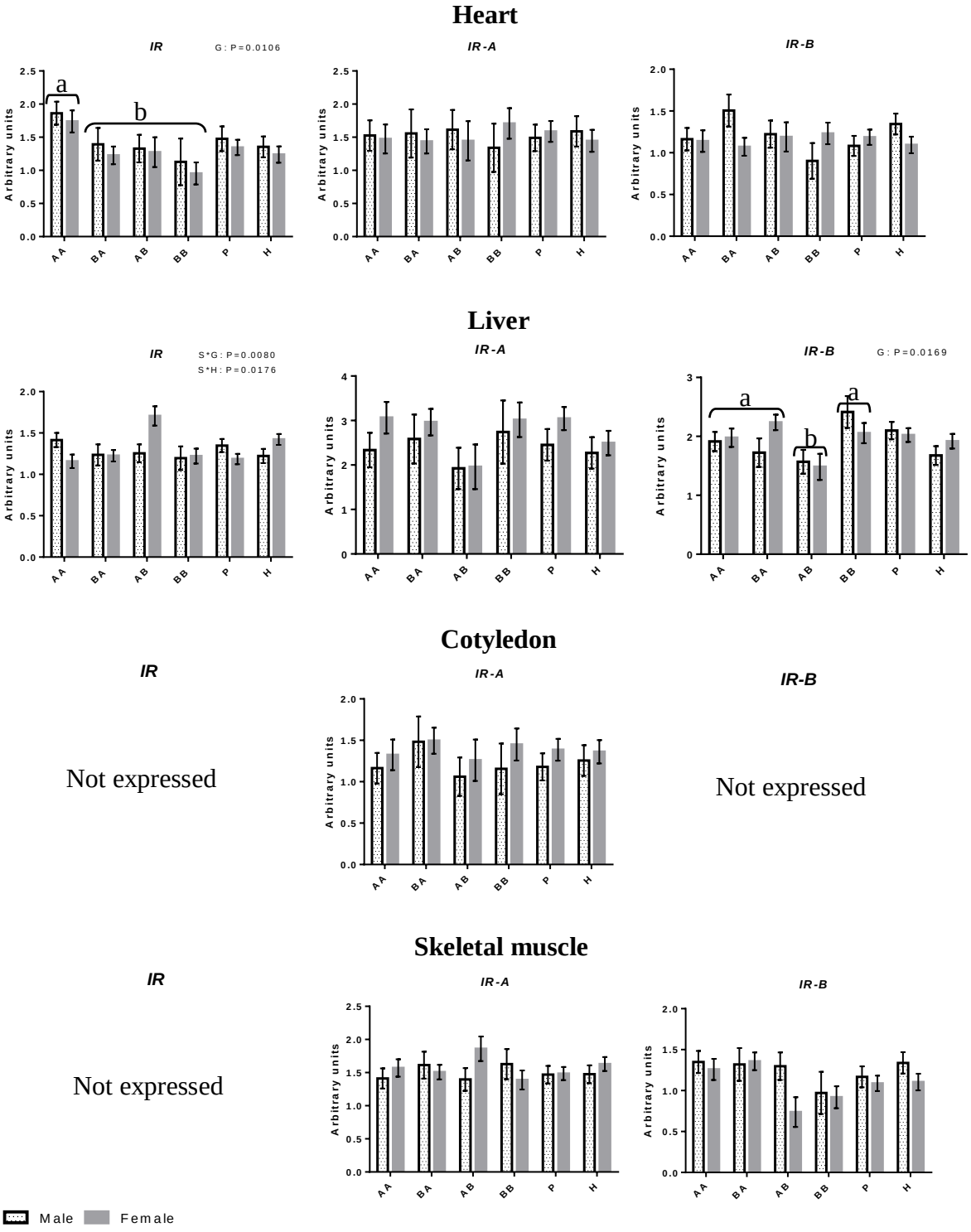


Figure 4.6 Least square means and associated standard errors of means for relative transcript abundance of *IR*, *IR-A*, and *IR-B* in heart, liver, fetal placenta and skeletal muscles (*M. semimembranosus*) in D-153 fetuses with four different genetics. Means for Angus (AA) (n=23), Brahman (BB) (n=15) and reciprocal crossbred genetics (BA, n=13 and AB, n=22, sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. Effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) in ANOVA are indicated when significant (P<0.05). Genetic groups with different superscripts differ significantly (t-test P <0.05).

4.3.3 Summary of phenotypic heterosis and molecular heterosis effects

An overview of phenotypic and molecular heterosis effects is presented in Tables 4.1 and 4.2. The highest positive heterosis effect among fetal organ weights was observed for fetal placenta which increased by 10%. This heterosis effect was approaching significance (*P*=0.08). Fetus weight and fetal organ weight did not show heterosis effects.

Table 4.1 Overview of heterosis effects in fetus and organ weights of bovine D-153 fetuses (n=73). Positive and negative deviations of the reciprocal F_1 mean from the parental mean as estimated in linear models are shown. Combined muscle weight was calculated as the combined weights of *M. supraspinatus*, *M. longissimus dorsi*, *M. quadraceps femoris*, *M. semimembranosus* (each of them measured as the average weight of both left and right muscles).

	Brain	**Heart**	**Liver**	**Fetal Placenta**	**Combined Muscle**	**Fetus weight**
Heterosis	1.4	- 4.5	- 2.1	10.5	- 2.8	0.0

In general, brain showed considerable negative molecular heterosis for all transcripts and fetal placenta showed positive molecular heterosis for all of the transcripts.

Expression of *IGF1* showed a negative 20.2 % heterosis in brain, although it was not significant. Molecular heterosis for *IGF1* was found to be positive in heart (40%) and liver (26%). Heart *IGF1* transcript showed the highest level of molecular heterosis at 40.7 %. In contrast, brain *IGF1R* showed the biggest negative molecular heterosis with 32.7 %. Both *IGF1 class 1* and *class 2* transcripts only showed significant levels of heterosis in liver.

Table 4.2 Overview of molecular heterosis effects in organs of bovine D-153 fetuses. Positive and negative deviations of the reciprocal F_1 mean from the parental mean as estimated in linear models are shown. The significance level considered as $P<0.05$ and indicated with *. *P*-values are from ANOVA (see methods).

organs / Transcripts	Brain	Heart	Liver	Fetal Placenta	Skeletal Muscle
IGF1	- 20.2	40.7*	26.2*	3.9	8.2
IGF1 class1	-	-	26.6*	-	-8.2
IGF1 class2	-	-	37.1*	-	22.9
IGF1R	-32.7*	- 2.5	9.9	-	0.6
IGFBP1	-	-	- 17.8	-	-
IGFBP2	-	-11.5	- 12.8	26.7	8.9
IGFBP3	-	12.2	0.0	12.3	- 1.4
IGFBP4	-	-10.2	- 3.3	1.5	4.1
IGFBP5	- 28.6	- 9.3	- 12.5	4.0	2.7
IGFBP6	-	4.3	- 21.1*	-	- 11.7
IR	-	-14.0	4.8	-	-
IR-A	-	- 3.2	- 10.1	4.7	5.4
IR-B	-	3.5	- 6.2	-	6.0

4.4 Discussion

Brain showed a negative heterosis for expression of *IGF1* and its receptor, and for *IGFBP5*. However, we did not observe any concurrent negative heterosis in brain weight. Relative brain weight was affected by genetics and sex. Both AB and BB genotypes showed higher relative brain weight than other genotypes. Males had significantly higher absolute brain weight, but they showed lower relative brain weight than females.

All liver *IGFBP* transcripts showed negative or no molecular heterosis effects. This could indicate low levels of IGF1 modulators and therefore higher free IGF1 availability, especially in hybrids, to bind to its receptors and initiate its wide range of actions.

The present study did not reveal any heterosis effects in D-153 fetus and organ weights. However, there were high levels of heterosis for *IGF1* transcripts. It was shown that a 10-12% heterosis can be achieved for birth weight in Brahman sired Angus-Brahman hybrids (Cundiff et al., 1994; Brown et al., 1993). Similar levels of heterosis can be observed in plasma IGF1 of calves (Caldwell et al., 2011). It was also shown that plasma IGF1 levels are correlated with fetus and birth weight (Hiendleder et al., 2006; Fowden, 2003).

Organ weights were significantly higher in male fetuses except for fetal placenta, but local *IGF1* transcripts did not show the same pattern. Among other IGF system transcripts only *IGFBP1* expression was affected by sex.

It appears that despite no detectable heterosis for weight at D-153 in bovine hybrid fetuses, the molecular and endocrinological mechanisms of hybrid superiority in growth rate have already developed at mid-gestation. Increased IGF1 expression will clearly impact overall prenatal growth and development until parturition.

IGF1 class2 transcript showed higher levels of heterosis in both liver and skeletal muscle than *IGF1 class1* transcript. *IGF1 class 2* transcript was reported to be more associated with heterosis in circulating IGF1 hormone in hybrid mice in comparison to *IGF1 class1* transcript (Adamo et al., 2006). It was demonstrated in cattle that both *IGF1 class1* and *2* transcript levels in liver increased after administration of GH. However, *class 2* transcript showed a significantly greater increase than *class 1* transcript (Wang et al., 2003). A similar result has

been reported in sheep, where Pell et al. (1993) showed that *IGF1 class 2* transcript is more responsive to GH than *class* 1 transcript.

The present results confirm that high levels of observed molecular heterosis in liver *IGF1* transcript are in accordance with circulating IGF1 levels observed in hybrid calves (Caldwell et al., 2011; Johnston et al., 2001) and increased birth weight and postnatal growth of hybrid calves (Riley et al., 2012). The lack of heterosis effects in weight of D-153 fetuses, and the previously reported heterosis in birth weight in cattle (Elzo et al., 2012; Johnston et al., 2001; Kress and Nelsen, 1998; Brown et al., 1993) suggest that mechanisms controlling heterosis in cattle, such as liver *IGF1* mRNA, are initiated during the prenatal stage. This prenatal mechanism may impact growth rate in later stages of gestation.

The present data showed a high level of molecular heterosis in liver *IGF1 class 2* transcript. This demonstrates an overall higher contribution of *class 2* transcript to heterosis than *IGF1 class 1* transcript in the bovine fetal model. The higher correlation of *IGF1 class 2* transcripts with GH administration (Wang et al., 2003; Pell et al., 1993) suggests an overall higher contribution of *class 2* transcript to heterosis than *IGF1 class1* transcript in the bovine model. It is interesting to speculate that mechanisms involving *IGF1 class 2* transcript continue to operate postnatally and contribute or cause the extensive heterosis effects observed in postnatal growth and development of hybrids.

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Chapter 5

Heterosis in growth hormone receptor transcript abundance

5.1 Introduction

Heterosis or hybrid vigour is the superiority of first generation hybrids over the average of the purebred parents (Dickerson, 1973). Heterosis has an important role in improving the productivity of plants and animals and has been used for over a century to obtain benefits in animal production industries (Simm, 1998; Dickerson, 1973). Despite the long history of using heterosis in industry, the molecular mechanisms underlying this phenomenon remain unclear. There are several hypotheses proposed from quantitative genetics, including dominance, over dominance and epistasis, but none fully explain the mechanism of heterosis (Alexander et al., 2009; Birchler et al., 2003).

As a basic tool of animal breeding, crossbreeding and resulting heterosis effects have been used extensively in the beef industry (Dickerson, 1969). In general, the amount of heterosis is higher when the genetic distance of the parents is increased (Chen, 2010). In cattle, when two subspecies are crossed (e.g. *Bos taurus*×*Bos indicus*), a higher level of heterosis is expected as compared to a cross between similar species. Cundiff et al. (1994) showed that performance in carcass and meat characteristics of crosses of *Bos indicus* and *Bos taurus* cattle is greater than in purebreds. Similarly, Brown et al. (1993) demonstrated that Brahman-Angus hybrid calves had higher birth weight (BW) compared to Angus or Brahman purebreds. Since heterosis in cattle can be detected as early as birth, the molecular mechanism driving heterosis may be initiated during fetal development.

Growth hormone and its receptor have been shown to be among important quantitative trait loci (QTLs) for reproduction (Luna-Nevarez et al., 2011), growth rate and carcass characteristics, as well as milk production in cattle. Taylor et al. (1998) reported a QTL effect on subcutaneous fat mapped to a region of bovine chromosome 19 which harbours the GH gene. Also, a number of QTL studies in cattle breeds have shown effects on milk yield and composition, as well as carcass traits, on bovine chromosome 20 close to the location of growth hormone receptor (GHR) (Khatkar et al., 2004).

Growth hormone gene expression is mainly from the pituitary gland, and pituitary GH is a key stimulator of IGF1 production (Laron 2001). Also it upregulates the transcription of IGF2 gene in liver (von Horn et al., 2002). However, GH expression is not confined to the pituitary gland, and is also present in many extra-pituitary tissues postnatally, in which it may act as an autocrine or paracrine growth factor (Harvey, 2010). Among those tissues, placental GH seems to be important for growth and development of the fetus. Since placenta GH cannot be detected in the fetal circulation and only presents in maternal blood and amniotic fluid, the direct role of placental GH in prenatal development is controversial (Skottner, 2012; Edmonson et al., 1995; Garcia-Aragon et al., 1992). Fetal pituitary gland produces GH from the second trimester in some species, including human, cow and sheep (Waters and Kaye, 2002). However, mice deficient in GH due to mutations in the genes encoding GH-releasing hormone receptor, showed normal birth weight (Efstratiadis, 1998).

GHR is the mediator of GH actions and binding of GH to GHR initiates transcription of many genes including the *IGF1* gene (Figure 5.1) (Jiang et al., 2007). It has been shown that *GHR* expression is highly correlated with *IGF1* expression in adult cattle liver (Kobayashi et al., 1999). Adult GHR knockout mice have a 90% reduction in circulating IGF1. However, their prenatal growth is not altered (Coschigano et al., 2000). This indicated different roles for the

IGF-system and GH-IGF1 axis in prenatal and postnatal growth and development. Interestingly, another study demonstrated that liver-specific *GHR* knockout mice also have a 90% reduction in circulating IGF1 (Fan et al., 2009). This study highlighted that liver GHR expression is more important for regulation of circulating IGF1 than total overall GHR expressed by a range of tissues. Furthermore, the major proportion of liver GHR consists of GHR-1A which is expressed only in liver in cattle, suggesting that these effects may be mediated through GHR-1A (Jiang et al., 1999).

The *GHR* has a long 5' non-coding region that includes nine untranslated exons, 1A–1I resulting in up to nine variants of *GHR* mRNA (Jiang and Lucy, 2001). Variants 1A, 1B and 1C are well studied and altogether account for 90% of overall *GHR* transcripts (Jiang and Lucy, 2001). *GHR* transcripts have been detected in a variety of adult bovine tissues e.g., liver, muscle, kidney, lung, mammary gland, adipose tissue, and fetal tissues of placenta, liver, lung, kidney and skeletal muscle, with the highest level of expression detected in liver of both adult and fetal stages (Jiang et al., 1999). Eight variants of *GHR* mRNA were detected in human, including V1-V8, where V1 is a liver specific variant (Pekhletsky et al., 1992).

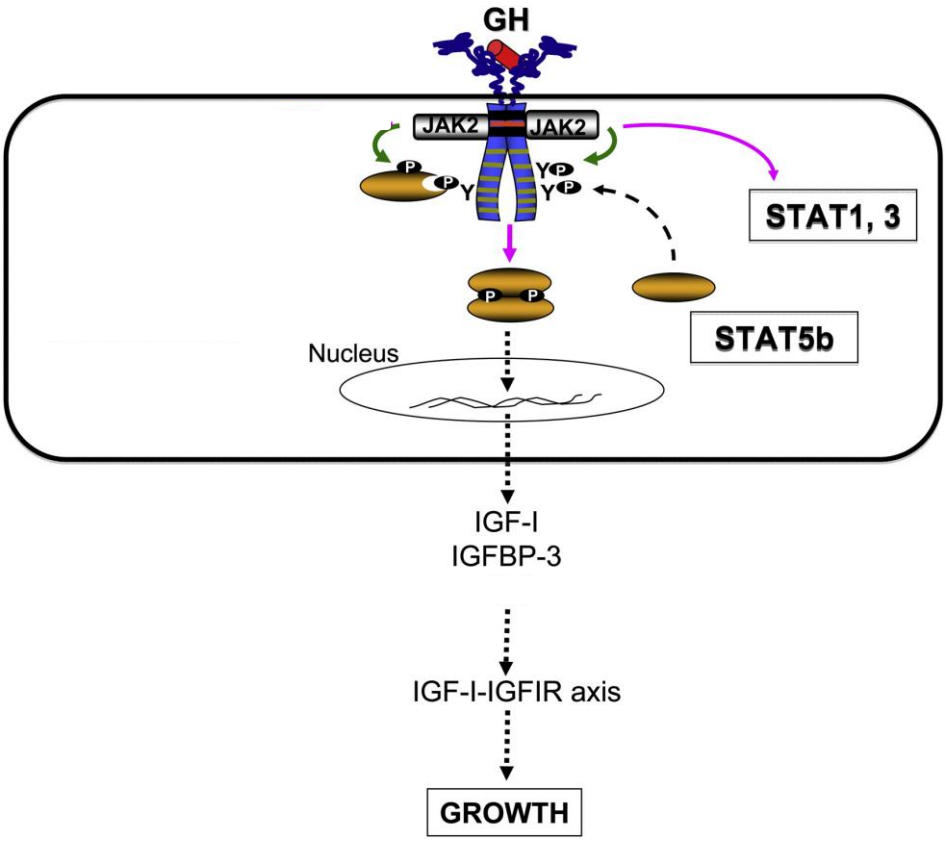

Figure 5.1 The growth hormone (GH)-GH receptor (GHR) signalling pathways which can lead to *IGF1* transcription. Upon binding of GH to GHR, signal transduction is initiated with phosphorylation of cytosolic Janus kinase 2 (JAK2) which activates STAT pathways. STAT5b is directly involved in regulation of *IGF1* transcription (modified from Hwa et al., 2011).

The bovine *GHR* gene is located on chromosome 20 and contains nine coding exons (exons 2 to 10) spanning 173.71 kb (Figure 5.2) (NCBI accession number NC_007318). It has been demonstrated that there is a retrotransposon element insertion in the liver-specific promoter of the bovine *GHR* gene (Lucy et al., 1998). This insertion was found to be specific to *Bos taurus* (Ohkubo et al., 2006; Lucy et al., 1998). A similar insertion was reported in mouse (Moffat et al., 1999) and goat (Maj and Zwierzchowski, 2005), but has not been reported in other mammals, including human and sheep. The inserted retrotransposon, with a length of

1.2 kb, is a long interspersed nuclear element (LINE-1 element, or L1) and belongs to the non-long terminal repeat (non-LTR) family of retrotransposons (Furano et al., 2004). Any L1 insertion in the genome can potentially impact on regulation of transcription, especially when a gene is disrupted by a L1 insertion (Britten, 1997).

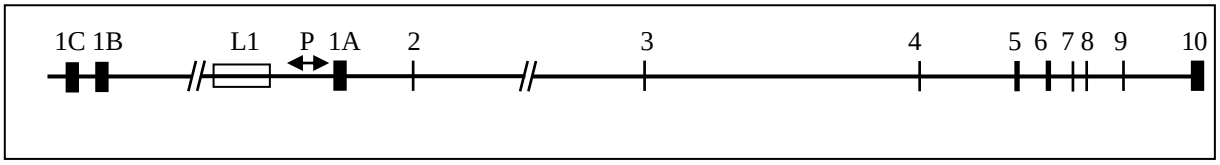

Figure 5.2 Structure of the bovine (*Bos taurus*) growth hormone receptor gene with retrotransposon (L1) insertion at 5' of the liver specific promoter (P) area. Exons are numbered from 2 to 10. The leader exons are 1A, 1B and 1C.

Altered gene expression caused by L1 insertion was shown in several species including fruit fly, human and mouse (Britten, 1997). It was demonstrated that L1 insertion in the 5' region of the *Agouti* gene in Normande cattle causes an overexpression of the *Agouti* gene which may be responsible for the brindle coat colour pattern of Normande cattle (Girardot et al., 2006). In human there are ~ 500,000 L1 elements, covering ~18% of the total genome length (Graham and Boissinot, 2006; Lander et al., 2001). L1 can be considered a potential mutagen and activation of L1 occurs frequently in cancer (Cruickshanks et al., 2013). Since there are ~6000 full-length L1 transposable elements in the human genome (Brouha et al., 2003), mechanisms for inactivation of L1 are required; DNA methylation is one of these regulatory mechanisms (Shi et al., 2007; Hata and Sakaki, 1997). L1 is down-regulated by cytosine methylation of its promoter in mammals (Yoder et al., 1997). Another example of a functional

retrotransposon insertion was reported in mouse by Morgan et al. (1999). They showed that the methylation status of an L1 insertion upstream of the *Agouti* gene can cause abnormal expression of Agouti protein and as a consequence those mice have yellow fur, as well as obesity and diabetes.

L1 element insertions and their potential effects on gene expression have been widely studied (Beck et al., 2011; Rodic et al., 2013). However, the potential role of L1 insertion in bovine GHR promoter in GHR transcription and potential contribution of GHR mRNA expression in bovine heterosis remain unknown.

The aims of this chapter were to investigate the presence of L1 retrotransposon insertion in the promoter area of the *GHR* gene and possible contribution to heterosis. Investigated parameters were abundance of *GHR* overall transcript, *GHR-1A*, *GHR-1B* and *GHR-1C* transcript in fetal tissues including heart, brain, skeletal muscle and liver of hybrid and purebred Brahman and Angus.

5.2 Materials and methods

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). The two breeds used in this research are subspecies of domestic cow, commonly referred to as *Bos taurus* (or taurine) and *Bos indicus* (or indicine) (Hiendleder et al., 2008). Four different groups of D-153 fetuses were used in this study; purebred Angus (n=23) and Brahman (n=15) and the two reciprocal crosses (AB[†], n=13 and BA, n=22). Fetal tissues including heart, brain, skeletal muscle (*M. semimembranosus*), liver and cotyledon were collected as described in

[†] A is Angus, B is Brahman and the first and second letters in a genotype indicating the breed of sire and dam, respectively.

Chapter 2, section 2.1. RNA was extracted and cDNA synthesised as described in Chapter 2, section 2.2. The tissues were selected to represent all four germ layers including ectoderm, mesoderm, endoderm and trophectoderm (Yu et al., 2010).

The presence of inserted L1 retrotransposon element was verified using standard PCR reactions followed by agarose gel electrophoresis. We used a specific high performance DNA polymerase, GoTaq® (Promega, Madison, WI, USA), to amplify multiple, large fragments in one reaction with similar efficiencies. TA sub-cloning, followed by standard sequencing, identified the inserted DNA sequence. Details of reactions and methods are described in Chapter 2, sections 2.6 and 2.7. "Blast 2 sequences" was used to detect sequence identity between our result and a reference sequence (Zhang et al., 2000).

Quantitative real-time PCR reactions were performed using SsoAdvanced SYBR® Green supermix (Bio-Rad Laboratories, Hercules, CA, USA) in a Bio-Rad CFX384 Touch™ Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) following MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments) (Bustin et al., 2009). Reactions were conducted in duplicate or triplicate in a total volume of 10 µl, containing 5 µl of SYBR master mix (2X), 4.2 µl of cDNA (10-fold diluted from stock cDNA, equivalent to 24 ng of starting RNA) and 0.8 µl of mixed forward and reverse primers (5 pmol/µl). A non-template control was included in all experiments to confirm the absence of genomic DNA contamination in reagents used for amplification. Automated PCR reaction setup was performed with a liquid handling robot (QIAgility, Qiagen, Hilden, Germany) in 384-well white plates (Bio-Rad Laboratories, Hercules, CA, USA). Product specificity was confirmed by sequencing, plots of the melting curve derived by Bio-Rad CFX384 Manager software version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA) and agarose gel

electrophoresis of PCR products. Details of primer sequence and cycling temperatures are explained in Chapter 2 sections 2.5 and 2.6.

All reactions, including housekeeper genes, were conducted in duplicate or triplicate. To identify the most stable genes for purpose of normalising, seven housekeeping genes were used 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) (Hruz et al., 2011; Lisowski et al., 2008). The qRT-PCR reactions for housekeeper genes were performed in triplicate and all desired genes were assessed in every tissue type, with the exception of cotyledon. In cotyledon, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was assessed, instead of *H3F3A*, due to initial studies that indicated that *H3F3A* expression was not constant in cotyledon. Computer software, geNorm version 3.5 was used to determine the most appropriate two housekeeper genes in each tissue, based on a method described by Vandesompele et al. (2002). VPS4A and RSP9 were identified as the two genes with the most stable, and readily detectable, expression.

Gene expression data and fetal phenotype including fetal and organ weights were analysed by Univariate Analysis of Variance (ANOVA) using the general linear model procedure of JMP statistical package version 4.0 (SAS Institute Inc.).

Data were fitted into following linear models:

1- To analyse the effects of fetal genetics, sex and interactions between these factors:

$$y_{ijk} = G_i + S_j + (G*S)_{ij} + e_{ijk}$$

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), $(G*S)_{ij}$ is fetal genetics by sex interaction and e_{ijk} is the residual effect.

2- To estimate heterosis effects on gene expression:

$$y_{ijkl} = H_i + G_j(H_i) + S_k + (H*S)_{ik} + e_{ijkl}$$

where y_{ijkl} is the normalised relative gene expression level or fetal phenotype, H_i is heterosis effect (i = purebred, crossbred), $G_j(H_i)$ is fetal genetic effect nested within heterosis effect (j = AA, BB nested within purebred, j = BA, AB nested within crossbred), S_k is fetal sex (k = male, female), $(H*S)_{ik}$ is heterosis effect by sex interaction and e_{ijkl} is the residual effect. Associations between fetal phenotypic traits and relative gene expression levels were analysed using simple linear regression models using JMP 4.0 (SAS Institute Inc.).

Fetal phenotype traits included weights of fetus, liver, heart, brain and combined muscle weight (*M. supraspinatus, M. longissimus dorsi, M. quadriceps femoris and M. semimembranosus*, each measured as the average weight of both left and right muscles). Values are presented as least square means and their associated standard errors. To illustrate where group differences existed, individual pairwise comparison of least-squares means was performed using Student's t-tests.

To investigate any relationship between tissue specific transcript abundances of *GHR*, *GHR-1A*, *-1B* and *-1C* with fetal and organ weights, associations were analysed by ANOVA using

[†] A is Angus, B is Brahman and the first and second letters in a genotype indicating the breed of sire and dam, respectively.

the general linear model procedure of JMP statistical package version 4.0 (SAS Institute Inc.). The same models as described above were used.

Heterosis for all investigated parameters was calculated as percent heterosis:

Heterosis % = [(crossbred average-purebred average)/purebred average] ×100

Significance level was as $P<0.05$.

5.3 Results

5.3.1 LINE-1 retrotransposon insertion in promoter of growth hormone receptor

5.3.1.1 Validation of LINE-1 insertion

Standard PCR followed by electrophoresis confirmed the presence of a retrotransposon insertion in the liver-specific promoter of the *GHR* gene in fetuses with at least one *Bos taurus* (Angus, A) allele (Figure 5.3). Homozygous AA fetuses produced a fragment with an approximate length of 1900 bp and homozygous BB fetuses produced a smaller fragment with an approximate length of 700 bp. The LINE-1 insertion was not found in BB individuals. All AB/BA fetuses were identified as heterozygous for this insertion and showed both fragments. Insertion length was calculated as ~1.2 kb.

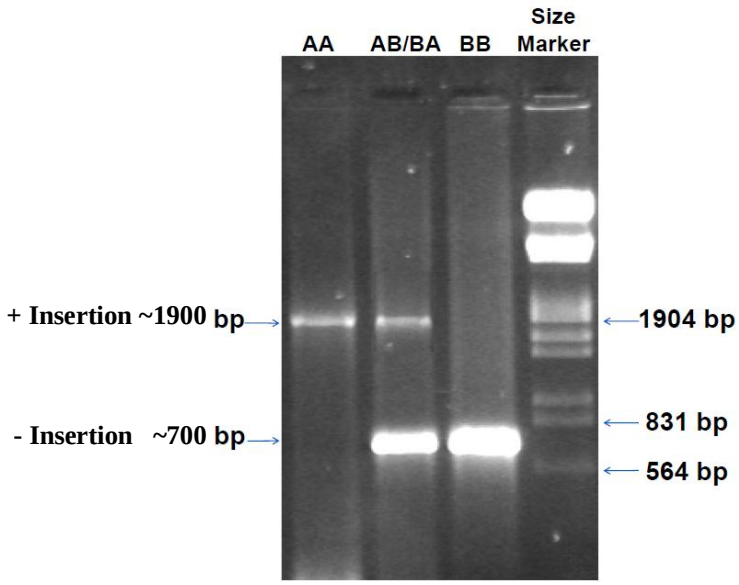

Figure 5.3 Typing of L1 insertion in the liver-specific *GHR* promoter using 1% agarose gel electrophoresis of amplicons. A is Angus and B is Brahman. Phage lambda DNA/*Hind* III was used as size marker.

5.3.1.2 Sequencing

Sequencing of the sub-cloned fragment from homozygous fetuses revealed that the inserted fragment is a partial L1 retrotransposon with a length of 1196 bp starting from nucleotide position -1667 and ending at -493 from the *GHR* transcription initiation site (Figure 5.4). The full length L1 retrotransposon was reported as approximately 6 kb in human (Lee et al., 2007) and 8 kb in bovine (Girardot et al., 2006).

```
   1  CTGGGAGACC CACCTCCCAG AATATTGGAA ATAAAAGCAA AAATAAACAA ATGGGACCTA ATTAACCTTA
  71  AAAGCTTCTG CACATCAAAG GAAACTATTA GCAAGGTGAA AAGACAGCCT TCAGAATGGG AGAAAATAAT
 141  AGCAAATGAA GCAACCGACA AACAACTAAT CTCAAAAATA TACAAGCAAC TCCTACAGCT CAACTCCAGA
 211  AAAATAAACG ACCCAATCAA AAAATGGGCC AAAGAACTAA ATAGACATTT CTCCAAAAAA GACATACAGA
 281  TGGCTAACAA ACACATGAAA AGATGCTCAA CATCACTCAT TATCAGAGAA ATGCAAATCA AAACCACTAT
 351  GAGGTACCAT TTCACACCAG TCAGATTGGC TGCGATCCAA AAGTACAAAT AATAAATGCT GGAGAGGGTG
 421  TGGAGAAAAG GGAACCCTCT TACACTGTTG GTGGGAATGC AAACTAGTAC AGCCACTATG GAGAACAGTG
 491  TGGAGATTCC TTAAAAAACT GGAAATAGAA CTGCCTTATG ATCCAGCAAC CCCACTGCTG GGCATACACA
 561  CTGAGGAAAC CAGAAGGGAA AGAGACACGT GTACCCCAAT GTTCATCGCA GCACTGTTTA TAATAGCCAA
 631  GACATGGAAG CAACCTAGAT GTCCATCAGC AGATGAATGG ATAAGAAAGC TGTGGTACAT ATACACAATG
 701  GAGTATTACT CAGCCATTAA AAAGAATACA TTTGAATCAG TTCTAATGAG GTGGATGAAA CTGGAGCCTA
 771  TTATACAGAG TGAAGTAAGC CAGAAGGAAA AACATAAATA CAGTATACTA ACGCATATAT ATGGGATTTA
 841  GAAAGATGGT AACAATAACC CGGTGTACGA GACAGCAAAA GAGACACTCA TGTATAGAAC AGTCTTATGG
 911  ACTCTGTGGG AGAGGGAGAG GGTGGGAAGA TTTGGGAGAA TGGCAATGAA ACATGTGAAA TATCATGTAG
 981  GAAACGAGTT GCCAGTCCAG GTTCGATGCA TGATGCTGGA TGCTTGGGGC TGGAGCACTG GGACGGCCCA
1051  GAGGGATGGT ATGGGGAGGG AGGAGGGAGG AGGGTTCGGG ATGGGGAACA CATGTATACC TGTGGCGGAT
1121  TCATTTTGAT ATTTGGCAAA ACTAATACAA TTATGTAAAG TTTAAAAATA AAATAAAATT GGAAGAAAAA
1191  AAAAAT
```

Figure 5.4 DNA sequence of partial LINE-1 element with a length of 1196 bp inserted in the promoter of growth hormone receptor (GHR) gene in *Bos taurus* (Angus) cattle.

Alignment of the partial L1 element showed 97% homology with the 3' end of a full length bovine L1 (NCBI accession number: DQ000238) (Figure 5.5). The size of the complete bovine L1 element is 8390 bp and the length of the partial L1 was 1196 bp and resulted in 14% coverage.

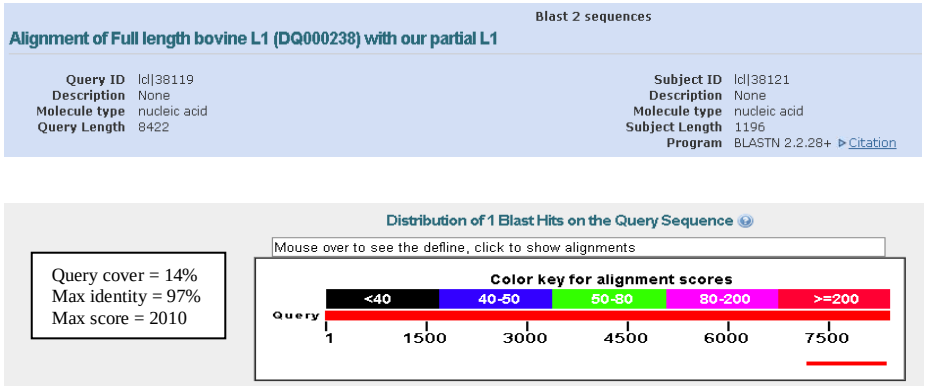

Figure 5.5 Alignment of a full length LINE-1 element with the partial LINE-1 inserted in the promoter of growth hormone receptor gene in *Bos taurus* (Angus) cattle.

We identified a GT microsatellite located at -90 bp upstream of transcript initiation between the L1 insertion and the liver specific GHR promoter, which has been previously described (Lucy et al., 1998). Interestingly, we found that this microsatellite had 18 repeats in purebred Angus individuals and 11 in Brahman purebreds (Figure 5.6).

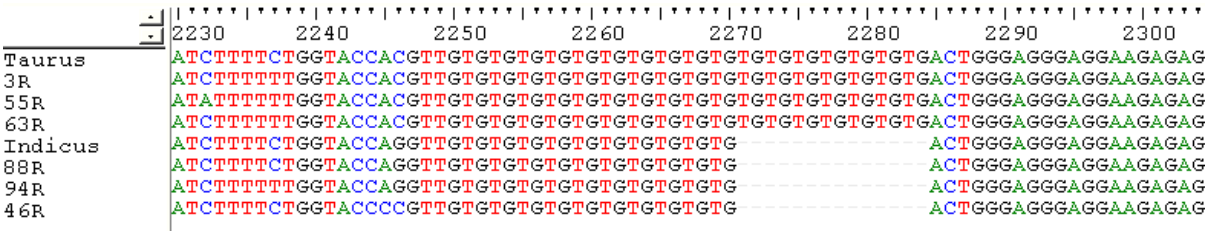

Figure 5.6 Microsatellite repeat variations between purebred *Bos taurus* (Angus) (Shown as Taurus) and *Bos indicus* (Brahman) (Shown as Indicus) individuals.

5.3.2 Heterosis, genetic and sex effects on *GHR* transcript abundances

5.3.2.1 *GHR* transcript abundances in liver

Expression of *GHR* transcripts in fetal liver are shown in Figure 5.7. Abundance of the *GHR* general transcript was significantly lower in BB fetuses. Other genotypes (AA, BA and AB) had almost 50% higher *GHR* mRNA expression in liver. *GHR* mRNA tended to be higher in liver of hybrids compared to purebreds, but this increase was not significant.

The liver specific variant of *GHR* (1A) showed a similar trend to the general transcript with significantly lower expression in liver of BB individuals of 2.5 to 3-fold. Hybrids showed significantly higher expression than purebreds with heterosis of (37.25 %) and a significant

sex by heterosis interaction that was due to higher expression levels in male individuals. *GHR-1B* expression was significantly higher in AA genotypes, compared to other genetic groups. Abundance of *GHR-1C* mRNA was not affected by genetics or sex and showed no heterosis effects in liver.

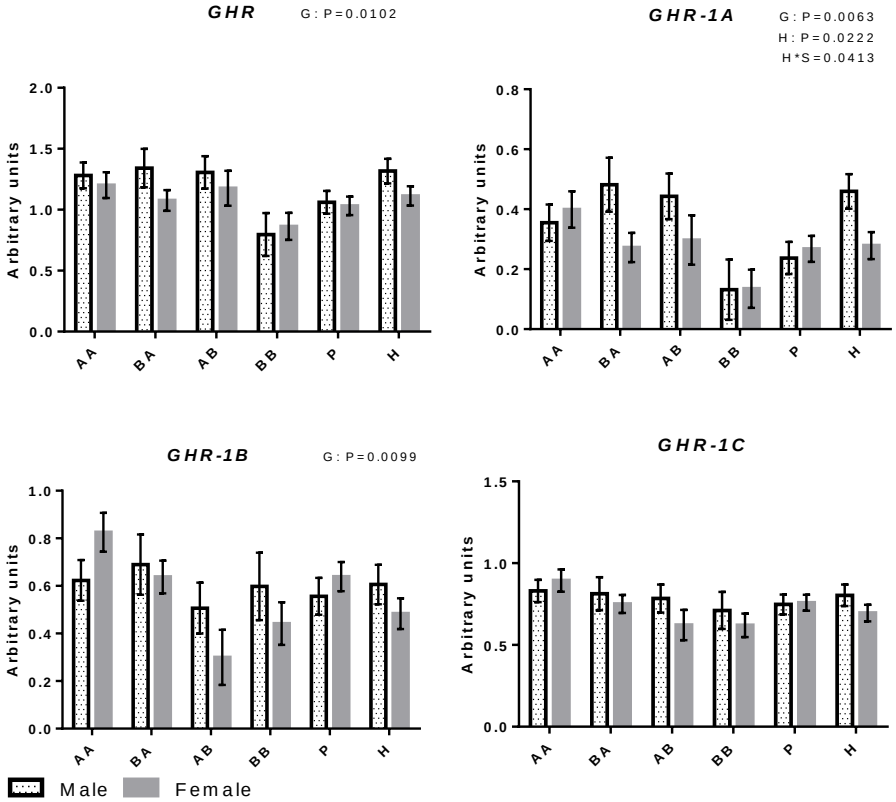

Figure 5.7 Least square means and associated standard errors of means for relative transcript abundance of *GHR*, *GHR-1A*, *GHR-1B* and *GHR-1C* in liver of D-153 fetuses. Means for Angus (AA) (n=23), Brahman (BB) (n=15) and reciprocal crossbred genetics (BA, n=13 and AB, n=22, sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. Effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) in ANOVA are indicated when significant (P<0.05).

5.3.2.2 *GHR* transcript abundances in fetal brain, heart and skeletal muscle

Transcript abundance of *GHR* transcripts in fetal brain, heart and skeletal muscle are shown in Figure 5.8. *GHR* mRNA level was higher in brain of AA fetuses in comparison to other genotypes. GHR-1B transcript abundance was significantly higher in AA and BA genotypes rather than AB or BB. *GHR-1C* was not affected by heterosis, genetic or sex effects in brain.

In fetal heart, *GHR* transcript showed a significant sex by genotype interaction. Hybrid fetuses overall, and female individuals, showed significantly higher levels of *GHR-1B* transcript in heart (42 %). *GHR-1C* also showed a very similar expression pattern to GHR-1B among individuals with high level of heterosis (33 %), except that there was no effect of fetal sex. *GHR, GHR-1B* and *GHR-1C* mRNA abundance were not affected by heterosis, genetic and sex effects in fetal skeletal muscle.

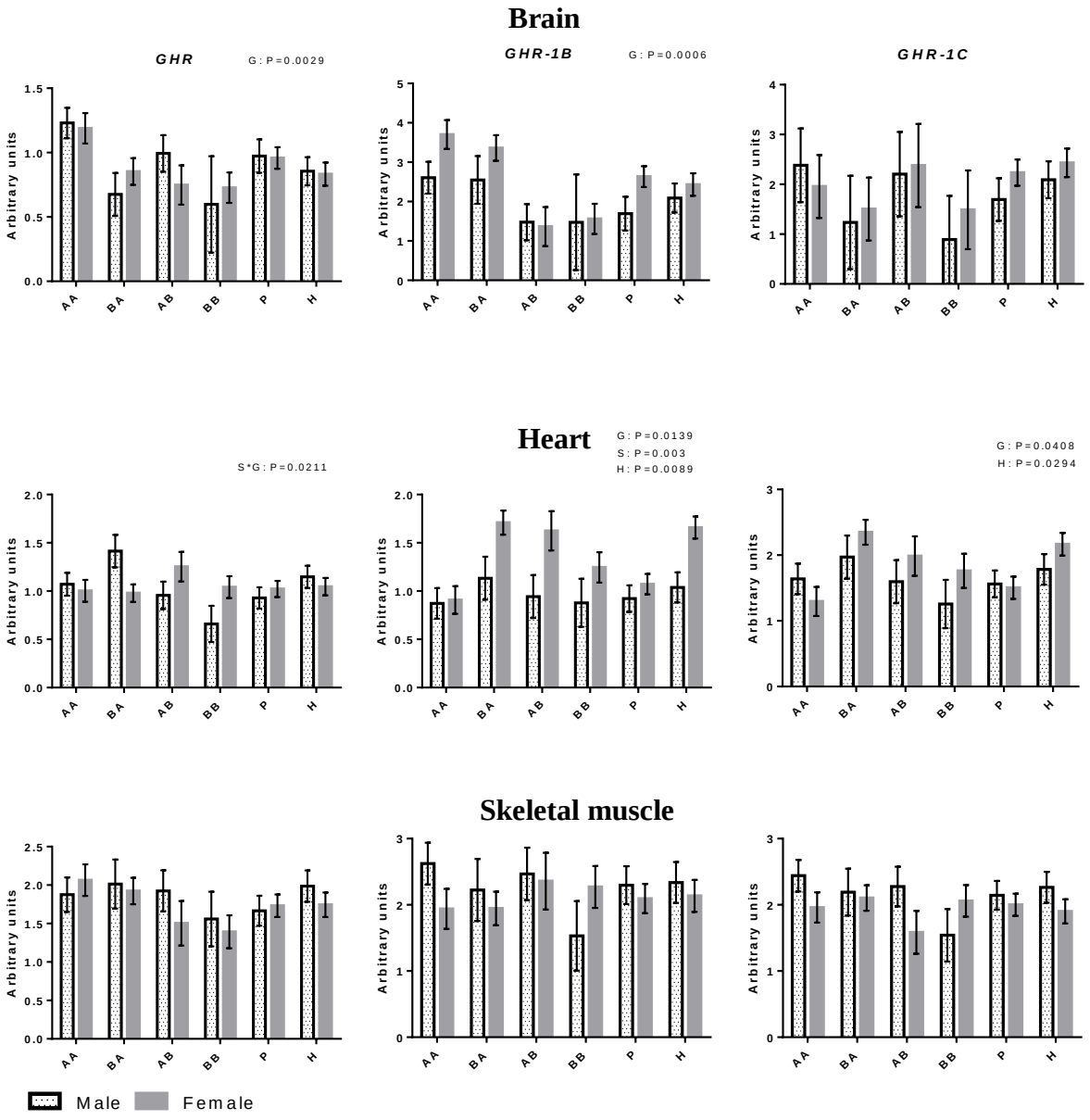

Figure 5.8 Least square means and associated standard errors of means for relative transcript abundance of *GHR*, *GHR-1B* and *GHR-1C* in brain, heart and skeletal muscle (*M. semimembranosus*) in D-153 fetuses. Means for Angus (AA) (n=23), Brahman (BB) (n=23) and reciprocal crossbred genetics (BA, n=13, and AB, n=22, sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. Effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) in ANOVA are indicated when significant (P<0.05).

5.3.3 Heterosis, genetic and sex effects on tissue-specific transcript abundances of *GHR* transcripts relative to fetal and organ weights

5.3.3.1 Liver tissue

Relative expression of liver *GHR* transcripts to fetus and liver weights are shown in Figure 5.9. Relative expression of liver *GHR* general transcript to fetus weight and liver weight showed a similar pattern. However, only *GHR*/liver weight was affected significantly by heterosis and sex effects. Hybrid fetuses had a significantly higher ratio of liver *GHR* to liver weight than other groups. Also *GHR-1A* relative to fetus and liver weights showed very similar patterns. Both ratios were affected by sex and heterosis effects in the same manner. BB genotype and purebreds showed significantly lower ratios.

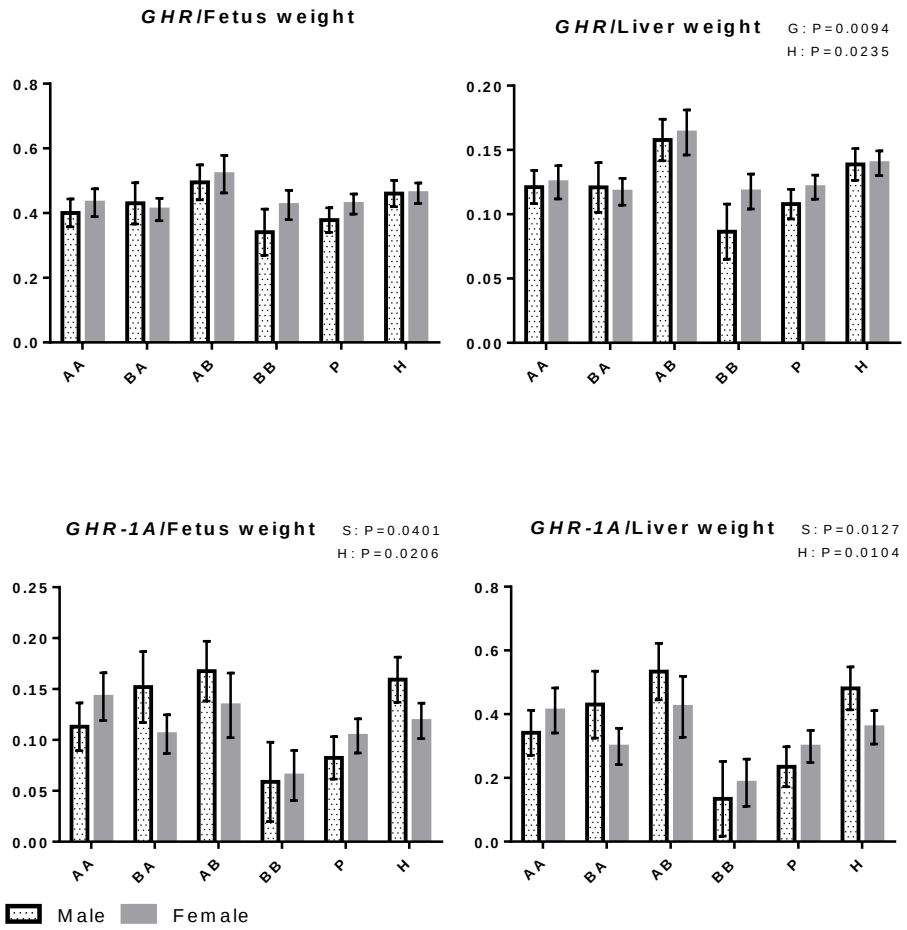

Figure 5.9 Least square means and associated standard errors of means for the ratios of liver *GHR transcripts* relative to fetus and liver weights in D-153 fetuses. Means for Angus (AA) (n=23), Brahman (BB) (n=15) and reciprocal crossbred genetics (BA, n=13 and AB, n=22, sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. Effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) in ANOVA are indicated when significant (P<0.05).

5.3.3.2 Brain, heart and skeletal muscle tissues

Abundance of tissue-specific *GHR* transcripts, relative to brain, heart, combined weights of four skeletal muscles and fetus weight, are shown in Figure 5.10. Relative expression of brain *GHR* transcript to fetus weight was not affected by heterosis, genetic and sex effects. The AA

genotype had a significantly higher *GHR* to brain weight ratio. Purebreds showed higher ratios, however, it was not significant.

Ratios of heart GHR mRNA to fetus and heart weights showed very similar patterns. Females with AB and BB genotypes showed significantly higher ratios than other genetic × sex combinations. In general, females showed higher ratios of heart GHR to fetus and heart weights, but this was only significant in the ratio of heart GHR expression to fetus weight.

Both skeletal muscle GHR ratios to fetus and combined skeletal muscle weights were not significantly affected by heterosis, genetics and sex effects.

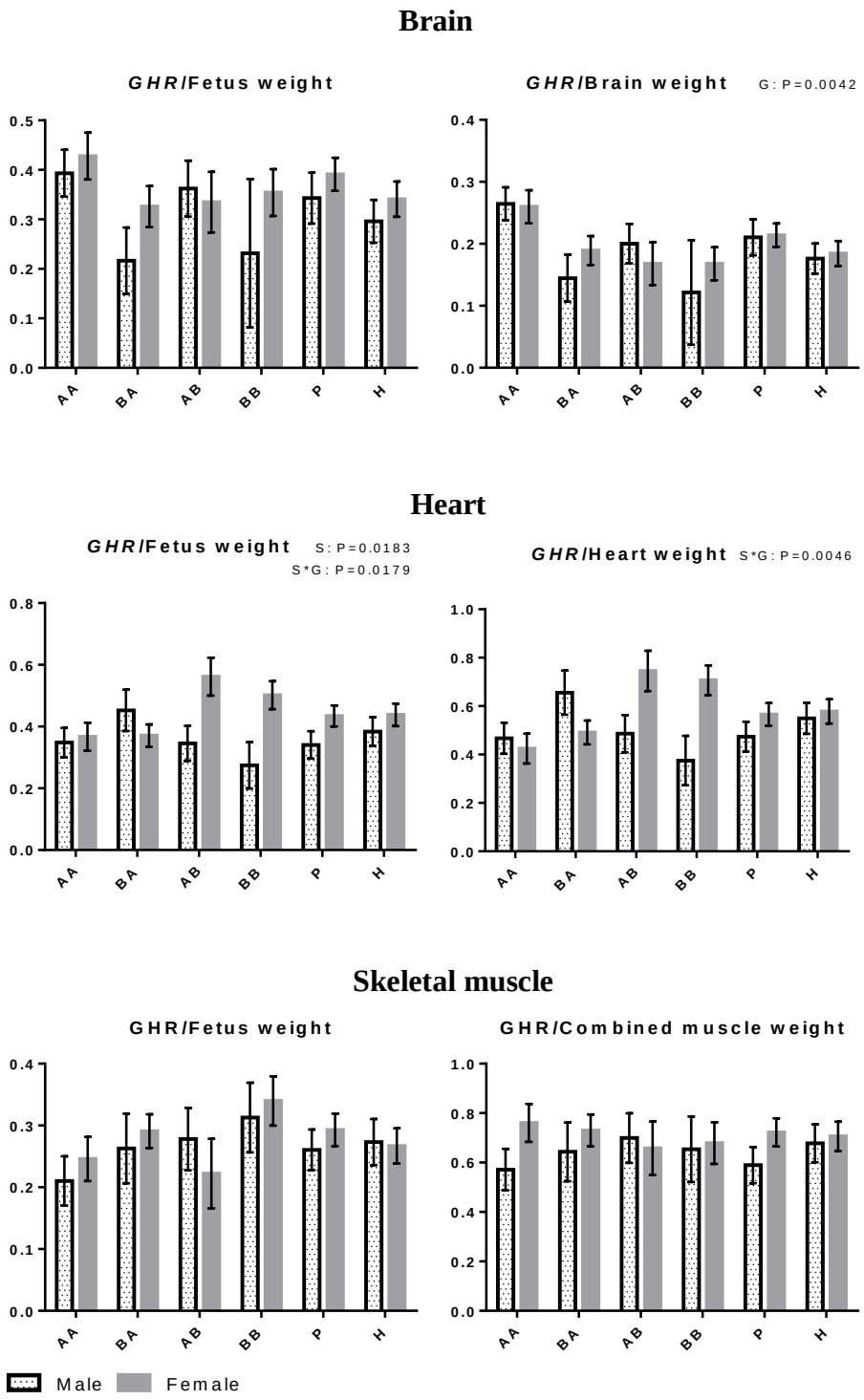

Figure 5.10 Least square means and associated standard errors of means for the ratios of tissue-specific *GHR transcripts* relative to brain, heart, combined skeletal muscle and fetus weights in D-153 fetuses. Means for Angus (AA) (n=23), Brahman (BB) (n=15) and reciprocal crossbred genetics (BA, n=13 and AB, n=22, sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. Effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) in ANOVA are indicated when significant (P<0.05).

5.3.4 Molecular heterosis of GHR transcript abundances in fetal tissues

In general, *GHR* general transcript showed positive heterosis among fetal tissues. Liver-specific *GHR* transcript showed a very high level of heterosis (37%) (Table 5.1). Both *GHR-1B* and *1C* mRNA showed a high level of heterosis in fetal heart. *GHR-1C* showed relatively high positive heterosis in brain and heart tissues, although it was not significant. Only heart and skeletal muscle showed positive heterosis levels for all of *GHR* transcripts.

Table 5.1 Overview of molecular heterosis effects in organs of bovine D-153 fetuses (n=73). Positive and negative deviations of the reciprocal F_1 mean from the parental mean as estimated in linear models are shown. The significance level considered as $P<0.05$ and indicated with *. *P*-values are from ANOVA (see methods).

Organs / Transcripts	Liver	Brain	Heart	Skeletal Muscle
GHR	14.9	14.29	9.6	7.31
GHR-1A	37.25*	-	-	-
GHR-1B	-13.11	-3.96	42.03*	1.97
GHR-1C	-2.65	22.38	33.01*	0.7

5.4 Discussion

Full length active bovine L1 is approximately 6-8 kb and contains two open reading frames (ORFs) which are necessary to initiate an actual retrotransposition (Girardot et al., 2006). The presence of an L1 insertion into the bovine *GHR* gene was first reported by Lucy et al. (1998b) who showed this insertion was specific to *Bos taurus*. We confirmed that inserted L1 in promoter of bovine *GHR* was specific to *Bos taurus* (Angus breed) and also consisted of a partial (5' truncated) L1 element which only had a portion of the second ORF. Therefore, it

cannot act as an active transposable element. However, it could potentially affect downstream gene expression. In human, 5' truncated L1 are widely distributed in the genome and it is believed that they are not capable of moving and replicating in the genome (Boissinot et al., 2001). However, Han et al. (2004) showed that even a partial L1 element integrated into an intron can down-regulate gene expression significantly. Further experiments, including allele specific expression levels and in vitro experiments are needed to establish potential effects of the L1 insertion in bovine *GHR* on liver-specific *GHR* transcription.

It has been reported that GT microsatellite variation in bovine *GHR* promoter as a short allele with 11 repeats ($(GT)_{11}$) is present in cattle without L1 insertion and as a long allele with 16-20 repeats ($(GT)_{16\text{-}20}$) is present and always linked with the L1 element (Lucy et al., 1998b). This experiment was conducted on five cattle breeds, including Brahman, Nellore, Hereford, Limousin and Santa Gertrudis (Lucy et al., 1998b). Subsequently, a number of studies have shown a significant association between the length of this specific GT microsatellite and production traits in cattle, including average daily milk production, weaning weight and carcass weight (Muhaghegh-Dolatabady et al., 2012; Curi et al., 2005; Hale et al., 2000). It was demonstrated that individuals with $(GT)_{11}$ allele had lower milk production than individuals with $(GT)_{16\text{-}20}$ allele (Muhaghegh-Dolatabady et al., 2012).

Some microsatellites are known to have important functions in the genome such as AC/TG (Gemayel et al., 2010). The AC/TG motif is able to absorb negative supercoiling through the formation of Z-DNA, which can affect nucleosomes by displacement and subsequently affect downstream transcription (Xu et al., 2011). A $(GT)_n$ microsatellite can increase gene activity and if a GT repeat is closer to promoter sequences it can be more effective as a transcriptional enhancer (Stallings et al., 1991).

We showed that the GT repeat in the bovine GHR is very close (90 bp) to the liver-specific promoter when considering the length variation between Angus and Brahman individuals. It may be worth considering this GT repeat variation as one of the factors affecting liver specific transcription of GHR in our experiment.

We showed that, generally, males had higher levels of *GHR* mRNA in liver, although it was not significant. It has been shown that pituitary GH and hepatic expression of some transcripts including *GHR* and *STAT5* are differentially expressed in adult males and females of several species including rodents and human (Rando and Wahli, 2011; Mode and Gustafsson, 2006). We demonstrated that hybrid males showed significantly higher *GHR-1A* mRNA compared with females. Since expression of *GHR* is regulated by a variety of factors such as steroid hormones (Horseman and Yu-Lee, 1994; Cosman et al., 1990), fetal male testosterone might be responsible for elevated levels of liver *GHR-1A* transcript.

We showed that BB genotypes had significantly lower *GHR general transcript* in liver and liver specific *GHR-1A* transcript. Since *GHR-1A* comprises a significant proportion of *GHR* transcript in liver (Jiang et al., 1999), a similar expression pattern to the general transcript in liver can be expected. This pattern in expression was not observed for *GHR-1B* or *GHR-1C* transcripts in liver.

We showed high levels of heterosis in liver *GHR* overall transcript abundance and *GHR-1A* transcripts. This is mainly due to lower levels of *GHR* mRNA expression in fetal liver of BB animals, which resulted in a lower level of expression of the GHR transcript in liver of purebred, compared to hybrid fetuses.

In the present experiment, purebred Brahman individuals (BB) showed the lowest level of GHR transcription in liver. In addition, they

- did not have the partial L1 element insertion in the liver-specific promoter of *GHR* gene, and

- had a short GT microsatellite allele with 11 repeats.

In conclusion, the GT microsatellite repeat variation and/or the partial L1 insertion could be important factors affecting the differential liver *GHR* transcription in BB individuals versus other genotypes. However, further investigations including *in-vitro* transcription studies need to be conducted to prove the enhancing/regulatory properties of the GT repeat and the partial L1 insertion on expression of liver-specific *GHR*.

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Chapter 6

MicroRNAs and their target sites in the *IGF* system and *GHR* genes: a potential role in heterosis of bovine fetal traits

6.1 Introduction

MicroRNAs (miRNAs) are 21-23 nucleotide long regulatory RNAs, first discovered in 1993 in *Caenhorhabditis elegans* (Liu et al., 2009). MiRNAs are initially transcribed as pri-miRNAs, can be several kilobases in length and contain a characteristic hairpin loop structure (Lee et al., 2004; Lee et al., 2002). The pre-miRNA stem-loop structure is exported to the cytoplasm where it is then cleaved by Dicer enzyme to produce the mature form of the miRNA (Lee et al., 2002). Two currently recognised mechanisms by which miRNAs impose translational regulation on their specific mRNA target(s) are repression and cleavage/degradation (Wang et al., 2007). Currently, based on the latest version of miRBase (www.mirbase.org), more than 760 bovine miRNA specific to *Bos taurus* have been identified. The majority of these miRNAs have been identified based only on sequence similarity to known vertebrate miRNA orthologs and have not been confirmed experimentally (Griffiths-Jones et al., 2007).

In mammals, miRNAs have been shown to regulate a large number of pathways including B-cell differentiation (Chen et al., 2004), adipocyte differentiation (Esau et al., 2004) and insulin secretion (Poy et al., 2004). Knockout of the miRNA-producing, Dicer1, in mice leads to lethality early in development which is a clear indicator of crucial roles of miRNAs in development (Bernstein et al., 2003). MiRNA profiling in D-30 bovine embryos showed that these regulatory small RNAs are differentially expressed between tissues and are also

differently expressed in cloned and IVF embryos (Castro et al., 2010; Coutinho et al., 2007). It has been shown that miRNAs regulate several genes in the IGF system and GH-IGF1 axis. It was demonstrated that let-7b is a major regulator of *GHR* expression in chicken (Lin et al., 2012). Elia et al. (2009) revealed a critical role of miR-1 in mediating the effects of the IGF1 pathway in human heart and skeletal muscles and demonstrated a feedback loop between miR-1 expression and the IGF1 signal transduction cascade. Ge et al. (2011) demonstrated that IGF2, as a critical regulator of skeletal myogenesis in mouse, is a direct target of miR-125b. A target site for miR-125 is located in the 3'UTR of the mouse *IGF2* and biogenesis of this myogenic miRNA is negatively correlated with production of IGF2. It has been shown that miR-223 has a functional target in the *IGF1R* UTR and suppresses cell proliferation in HeLa cells (Jia et al., 2011). Interestingly, knockdown of *IGF1R* has been shown to mimic the effects of miR-223 inhibition, resulting in decreased cell viability. Let-7 and Lin28 miRNAs have been shown to regulate glucose metabolism pathways in mouse models. Overexpression of these miRNA can repress multiple components of the insulin pathway including IGF1R and IR (Zhu et al., 2011).

Single nucleotide polymorphisms (SNPs) can potentially create or eliminate target recognition sites for miRNAs in target mRNAs. A SNP inside a pre-miRNA sequence (stem and loop) or the seed region can change secondary structure and consequently impact on the maturation process of a miRNA. New target sites for a miRNA, or elimination of an existing target site, have been linked to human diseases and phenotypic variation in farm animals (Farazi et al., 2013; Clop et al., 2006). Hiard et al. (2010) introduced "The Patrocles database" (http://www.patrocles.org/), a database which consists of SNPs that are predicted to perturb miRNA gene regulation. A G to A mutation in the 3'UTR of the ovine myostatin gene generated a new target site for two miRNAs in Texel sheep (Clop et al., 2006). It has been shown that these two miRNAs (miR-1 and miR-206), which are strongly expressed in muscle

tissue, downregulate transcription of the myostatin gene. This transcriptional inhibition of the myostatin gene is responsible for muscle hypertrophy in Texel sheep (Bingel et al., 2010).

Experimental identification of miRNA targets is difficult and time consuming. Therefore, computer software programs have been developed to predict target sites. Watson–Crick pairing of miRNA seed region with its target mRNA has been shown to be a reliable strategy to predict targets based on perfect complementary pairing (Lewis et al., 2003). There are different types of seed matching including 6nt, 7nt and 8nt match (Bartel, 2009) (Figure 6.1). Longer seed matchings are predicted to have higher target efficiency. There are two different types of 7nt match which are 7mer-m8 (6mer with an additional match to nucleotide 8 of the miRNA) and 7mer-A1 (6mer followed by an A at target position 1, see figure 6.1). The order of seed match type efficiency is: 8mer > 7mer-m8 > 7mer-A1> 6mer (Grimson et al., 2007). 8mers followed by 7mers are correlated more strongly with target efficiency (Lewis et al., 2005).

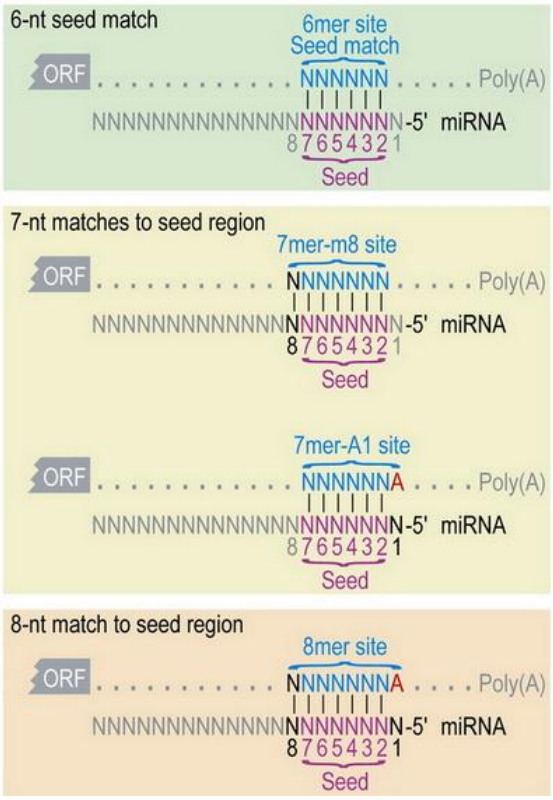

Figure 6.1 Different types of matching of miRNAs to their targets including 6, 7 and 8 nt long seed matchings. Nucleotides represented as N and Watson–Crick pairing indicated with vertical dashes (Bartel, 2009).

Predicted target sites are usually conserved among a wide range of organisms and conservation has different levels. Target sites can be broadly conserved (conserved among vertebrates), conserved (conserved across most placental mammals) or poorly conserved (only conserved among closely related species), and usually the vast majority of miRNA target sites are conserved among different species (Friedman et al., 2009; Lewis et al., 2005; 2003). Figure 6.2 is an example of broadly predicted conserved vertebrate miR-let-7 target sites in *IGF1R* 3'UTR (Targetscan.org).

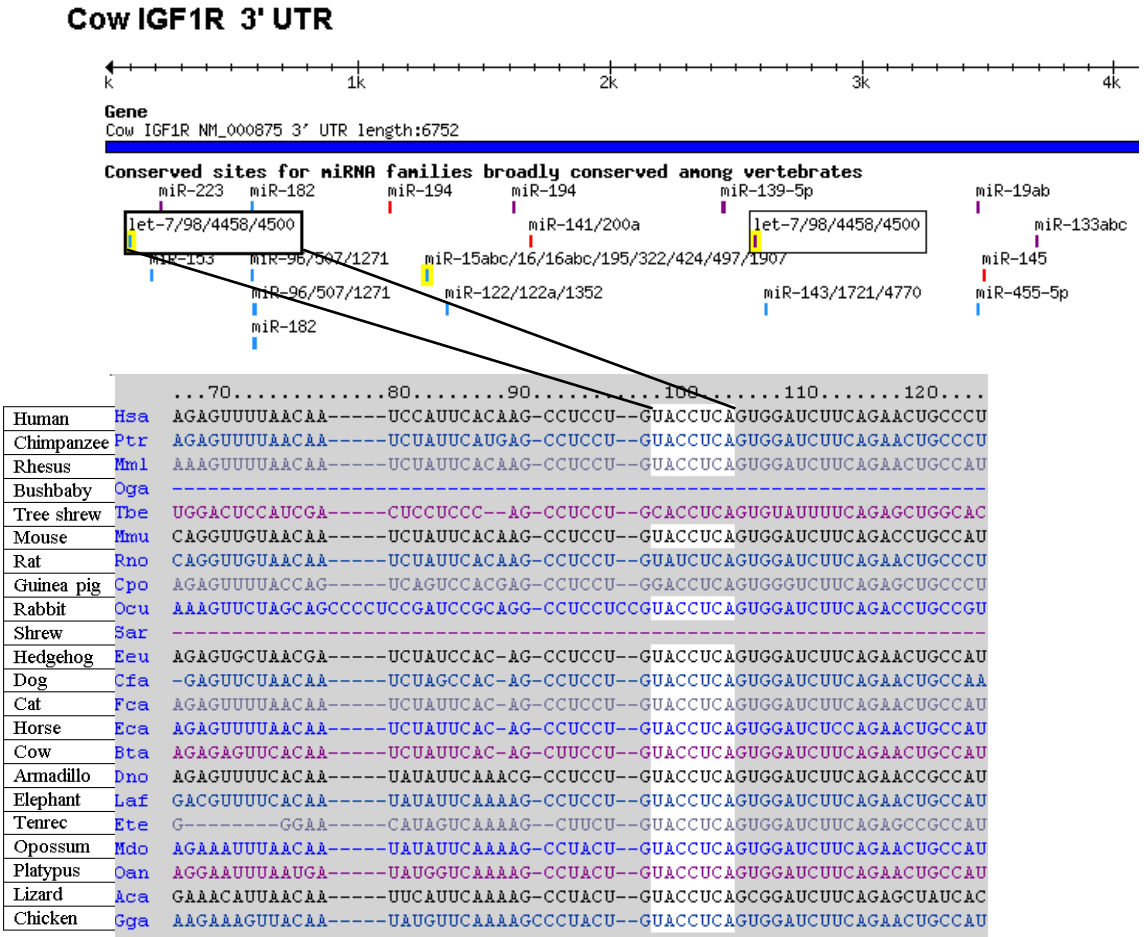

Figure 6.2 Two conserved target sites of miR-let-7 (highlighted in white) in the 3'UTR of bovine type 1 insulin-like growth factor receptor (*IGF1R*) and a comparison with multiple species (Targetscan.org). Abbreviated species names are presented at left side of sequences.

Several web-based algorithms are available for computational prediction of miRNA targets, including TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org) and PicTar (http://www.pictar.org). Experimentally validated microRNA target sites are available at TarBase (http://diana.cslab.ece.ntua.gr/tarbase/), miRecords (www.mirecords.biolead.org) and miRTarBase (www.mirtarbase.mbc.nctu.edu.tw) (Kozomara and Griffiths-Jones, 2011).

The above algorithms are valuable tools to predict and understand miRNA functions (Nam et al., 2009; Alves-Junior et al., 2009). Every tissue at each developmental stage has a specific

miRNA profile (Gu et al., 2013, Liang et al., 2007; Barad et al., 2004). Therefore, knowledge of experimentally validated miRNA target sites should be applied carefully.

A number of studies showed the regulatory effects of miRNA on the *IGF* system and *GH-IGF1* axis transcripts (Lin et al., 2012; Jia et al., 2011; Ge et al., 2011; Zhu et al., 2011). However, the potential role of miRNAs, as regulators of *IGF* system and *GHR* transcripts, in a bovine heterosis model remain unknown.

The aims of this chapter were to:

- investigate the sequence variation of miRNA loci including miR-483, Let-7-f2 and miR-98 and their target sites located in the 3'UTR of *IGF1* and *IGF1R*,
- profile fetal liver miRNA, and
- estimate heterosis effects in expression of miRNAs involved in regulation of the IGF-system and GH-IGF1 axis in fetal liver of hybrid and purebred Brahman and Angus cattle.

6.2 Material and methods

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). The two breeds used in this research are subspecies of domestic cow *(Bos taurus taurus*, Angus, A and *Bos taurus indicus*, Brahman, B), commonly referred to as *Bos taurus* (or taurine) and *Bos indicus* (or indicine) cattle, respectively (Hiendleder et al., 2008). Four different groups of D-153 fetuses were used in this study: purebred Angus and Brahman and the two reciprocal crosses. Fetal liver tissue was collected as described in Chapter 2, section 2.1. Genomic sequence variation of miRNA loci including miR-483, Let-7-f2 and miR-98 and

their target sites were investigated using PCR amplification of fetal liver DNA followed by agarose gel electrophoresis and standard Sanger sequencing technology. Nucleotide sequence data was obtained from purebred Angus (n=10) and Brahman (n=10) fetuses. Details of reactions and methods are described in Chapter 2. Sequenced fragments were subjected to sequence alignment with the computer program BioEdit version 7.0.0 (Hall, 1999).

Total RNA was extracted as described in Chapter 2, section 2.2. In order to ensure a balanced and cross classified experiment we selected 24 samples in total for miRNA profiling. Selection criteria were RIN value of RNA samples and representation of all sires used in the experiment. Three individuals of each genetic group and sex have been selected, including: AA males, AA females, AB males, AB females, BA males, BA females, BB males and BB females. Reduction in number of samples analyses also significantly reduced the costs of this experiment. GeneChip® miRNA 3.0 (Affymetrix, Santa Clara, CA, USA) platform was used to profile microRNAs in fetal liver samples. This chip contains 19724 miRNA probes including mature and pre-miRNA belonging to 153 organisms including bovine. Probe design of this chip is based on miRBase version 17 (http://www.mirbase.org). Cell data were normalised and transformed by Affymetrix® Expression Console™ Software 1.3 (Affymetrix, Inc.). Data was analysed by BRB-Array tools software package version 4.3 (Simon et al., 2007). Predicted (TargetScan and miRNAmap) and experimentally validated (TarBase, miRecords and miRTarBase) miRNA target sites belonging to the IGF system and GH-IGF1 axis were used to filter microarray data output.

MiRNA expression data and fetal phenotype including fetus and liver weights were analysed by Univariate Analysis of Variance (ANOVA) using the general linear model procedure of JMP statistical package version 4.0 (SAS Institute Inc.).

Data were fitted into the following linear models:

1- To analyse the effects of fetal genetics, sex and interactions between these factors:

$$Y_{ijk} = G_i + S_j + (G*S)_{ij} + e_{ijk}$$

where Y_{ijk} is the normalised miRNA 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), $(G*S)_{ij}$ is fetal genetic by sex interaction and e_{ijk} is the residual effect.

2- To estimate heterosis effects on gene expression:

$$Y_{ijkl} = H_i + G_j(H_i) + S_k + (H*S)_{ik} + e_{ijkl}$$

where Y_{ijkl} is the normalised miRNA expression level or fetal phenotype, Hi is heterosis effect (i= purebred, crossbred), $G_j(H_i)$ is fetal genetic effect nested within heterosis effect (j= AA, BB nested within purebred, j = BA, AB nested within crossbred), S_k is fetal sex (k= male, female), $(H*S)_{ik}$ is heterosis effect by sex interaction and e_{ijkl} is the residual effect.

Associations between fetal phenotypic traits and miRNA expression levels were analysed using simple linear regression models using JMP 4.0 (SAS Institute Inc.). Values are presented as least square means and their associated standard errors.

To investigate a potential relationship between liver transcript abundances of miRNAs with fetus and liver weights, associations were analysed by ANOVA using the general linear model procedure of JMP statistical package version 4.0 (SAS Institute Inc.). Same models as described above were used.

[†] A is Angus, B is Brahman and the first and second letters in a genotype indicating the breed of sire and dam, respectively.

Heterosis for fetus and liver weights was calculated as heterosis percent:

Heterosis % = [(crossbred average-purebred average)/purebred average] ×100

The significance level was $P<0.05$. Pearson correlation between transcript abundance of miRNAs and mRNAs (from Chapter 4) in fetal liver was determined using JMP version 4.0 (SAS Inc.).

6.3 Results

6.3.1 Selecting miRNAs within IGF system and GH-IGF1 axis genes

6.3.1.1 Predicted miRNAs

MiRNAs targeting the 3'UTR of the *GHR* and *IGF* system genes were predicted using TargetScan (Figure 6.3). In this study, only conserved 7-8mer seed match types were considered as potential targets including 8mer, 7mer-m8 and 7mer-1A which correlate more strongly with targeting efficiency (Lewis et al., 2005). In total, 158 miRNA target sites were found. Interestingly, some of the miRNAs targeted more than one gene (Figure 6.3). With the exception of miR-Let-7-f2 and miR-98, which are clustered together on the bovine X-chromosome, the rest of the miRNAs are not clustered together.

Gene	8mer binding site	7mer-m8	7mer-1A
GHR	miR-15abc miR-16abc miR-195 miR-322 miR-424 miR-497 miR-1907 miR-101ab	miR-135ab miR-212	miR-Let-7/98 miR-4458 miR-4500
IR	miR-Let-7/98 miR-4458 miR-4500 miR-27abc	miR-15abc miR-16abc miR-195 miR-322 miR-424 miR-497 miR-1907 miR-137ab	miR-15abc miR-16abc miR-195 miR-322 miR-424 miR-497 miR-1907 miR-128ab miR-133abc miR-135ab miR-205ab
IGF1	miR-Let-7/98 miR-4458 miR-4500 miR-454 miR-3666 miR-4295 miR-1ab miR-206 miR-29abcd miR-130ac miR-141 miR-148ab miR-152 miR-192 miR-215 miR-221 miR-222ab miR-301ab miR-483 miR-613 miR-721 miR-1928	miR-454 miR-3666 miR-4295 miR-18ab miR-33 miR-26ab miR-128ab miR-130ac miR-301ab miR-365 miR-721 miR-1297 miR-4465	miR-27abc miR-190ab miR-196abc miR-212 miR-499
IGF1R	miR-Let-7/98 miR-4458 miR-4500 miR-7ab miR-19 miR-99ab miR-100 miR-133abc miR-139 miR-194 miR-223	miR-Let-7/98 miR-4458 miR-4500 miR-141 miR-145 miR-194 miR-220a	miR-143 miR-4770 miR-96 miR-122 miR-153 miR-182 miR-194 miR-455 miR-507 miR-1271 miR-1352 miR-1721
IGFBP3	miR-19ab	miR-9ab	miR-34abc miR-499abc
IGFBP4		miR-33 miR-365	
IGFBP5	miR-143 miR-4770 miR-24ab miR-137ab miR-193ab miR-1721	miR-454 miR-3666 miR-4295 miR-19ab miR-101ab miR-130ac miR-138ab miR-139 miR-183 miR-301ab miR-721	miR-1ab miR-206 miR-101ab miR-144 miR-204 miR-211 miR-613
IGFBP7	miR-516 miR-518 miR-552	miR-206 miR-31 miR-34 miR-382	miR-424
IGFBP8		miR-18ab miR-19ab miR-132 miR-212 miR-124ab miR-4735	miR-143 miR-4770 miR-1 miR-21 miR-26ab miR-133abc miR-1297 miR-4465

Figure 6.3 TargetScan and miRNAMap predictions of bovine miRNAs targeting the 3'UTRs of insulin-like growth factor (*IGF*) system and growth hormone receptor (*GHR*) genes.

6.3.1.2 Experimentally validated miRNAs

Among 158 predicted mammalian miRNA target sites on *IGF* system and *GHR* genes, 28 were experimentally validated based on TarBase, miRecords and miRTarBase databases (Table 6.1). MiR-1 was recognised to target multiple members of *IGF* system genes including *IGF1*, *IGF1R* and *IGFBP7*.

Table 6.1 List of experimentally validated mammalian conserved miRNA target sites within insulin-like growth factor (*IGF*) system and growth hormone receptor (*GHR*) genes.

Target Gene	MiRNA
IR	miR-26b-5p
IGF1	miR-1, miR-27a, miR-206, miR-483
IGF1R	let-7d-5p, miR-1, miR-7-5p, miR-30a-5p, miR-99a miR-100, miR-122-5p, miR-133ab, miR-138, miR-145-5p miR-183-5p, miR-192-5p, miR-194, miR-196a-5p, miR-223 miR-335-5p, miR-378, miR-675
IGFBP1	miR-29c
IGFBP5	miR-140
IGFBP7	miR-1, miR-124-3p
IGFBP8	miR-18, miR-26a, miR-124

6.3.2 Sequence variation of miR-483, miR-let-7f and miR-98 loci and their target sites

A 500 bp DNA fragment harbouring miR-483 precursor was amplified and sequenced from fetal bovine liver. No sequence variation was observed inside the precursor miRNA loci among *Bos taurus* and *Bos indicus* individuals (Figure 6.4).

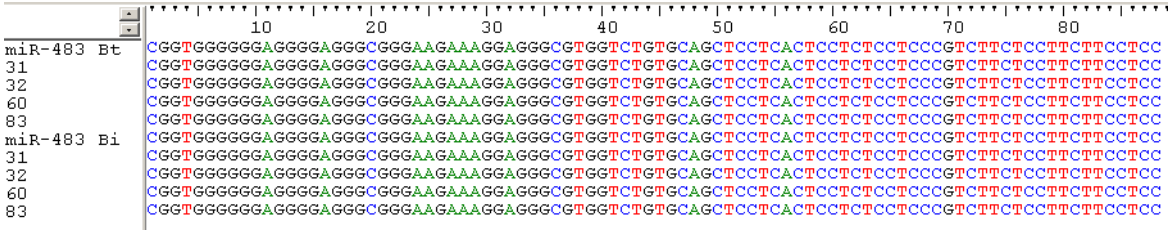

Figure 6.4 Alignment of miR-483 precursor locus sequences of purebred *Bos taurus* (Angus, Bt) and *Bos indicus* (Brahman, Bi) individuals.

A fragment with length of 1354 bp was amplified containing the loci for both miR-let-7f and miR-98 precursor loci miRNAs (Figure 6.5). No sequence variation was observed inside the precursor miRNA loci among *Bos taurus* and *Bos indicus* individuals (Figure 6.6).

```
   1  TATTTGCCTC ACACTACATA TCACCTGTCC CCTTCCACCT CCTTTTTCTT GCAGTCCTAA TTATCCAAGT
  71  AGCATCTACA ACTCTGATGG AAGCAACAGT GTTAAGACTA AATGTGAATG TGCTACACAC CAGGGAAAGT
 141  AGTAAGTTGT ATAGTTATCT TCAAATTGGG GCCTAAAATC CCTACCCCAC AACAATACAA CTTACTACCT
 211  CACCCCAGCA TGAGCAGAGT CCTCTAGCAA CAGCAGCAGG CAAAGGGCCA TTTTACCTGA TGACAGACCC
 281  TTTCAATGAC CTTCTGCCAG ATTCAGGGCA CCAGGACTCC TACCCTCTTT CACTGCTTTA AAAAATATTG
 351  TGTAGGCTTT AGTACAATCA TTTCCCAACT CAAGTGCCGA AGGAGACAGA AAGTATAGGG TAGCAGAAGT
 421  AAGTTCTGAG GAAATAGGGG TAGGGGAGAC ATATAATACC ACATAGACCA GTTATTAGAA TCACAGTCAC
 491  AAAATAGCTA CATTAGGCAC ATTAGAAGGA GGAATCAAAA GTATTTAGTA GTAATACCAT TTAGCCCACA
 561  GAGCTACCCT CCCCCAAAGC AGACTGCCCA AGGCAACATT CCTAGACTTT TAGATTCCAC AGACCCATGA
 631  AATTTATATA AAAACAAAAA ATAAAAAGAG ACTAACACAG GGTTACCAAC TTTTAATTTA ACCAAGAAAA
 701  AAAATCCATT AATATCATTT AATAAAAATA TTGTGATACC AAAGAAGTAG GAAATACATA ATCTCTGTAA
 771  TTTATAACAG TTCTTTAAAT TGAACGCATT CAGCTTTCTG AAAAAGTCAT TAAAGCTCCC TTATTCTGTT
 841  AACTTCCACC AAGGACTAGC ATTTAGGAAC CACTGTCAGG CGTTCTCATG AAGCACCATA CCACAATGGT
 911  CACTAAAAGG GACAGGACAC ACAGCCTCAC TACACATGCC AGAATGCAGT ACAGCATAAT CAGAATGAGA
 981  ACCTGTAAAC AGAGTACGTG GAAGGGAAG TGCACCACCG TGGGAAAGAC AGTAGACTGT ATAGTTATCT
1051  CCAAGATGGG GTATGACCCT AAAACTATAC AATCTACTAC CTCATCCCAC AGAGCACCAG TGTTCATCTT
1121  CAGTCTACTT GGGCCAGCTA CTTGGGTCAG GAAAAAGAAT CAACCAATAA TTAGCAAGAA GGAAAAATGG
1191  GAAGGAAAGT TACCTGAGAG GTTCTGTTCA GTGGAGTCAT TTGTCTTAGA TGCAGCACAC TGCAAAGAAA
1261  GGGACAGACC AATGTGGGAC TTTGCAAGCA CATGAGATAG AGGTCAACTC TAATAGTCGC CATCTCCCAC
1331  ATATGACCTG GATGGAGGAT AAGC
```

Figure 6.5 Fragment with sequences of miR-98 (highlighted at top) and miR-let-7-f2 precursors (highlighted at bottom) located on bovine X-chromosome.

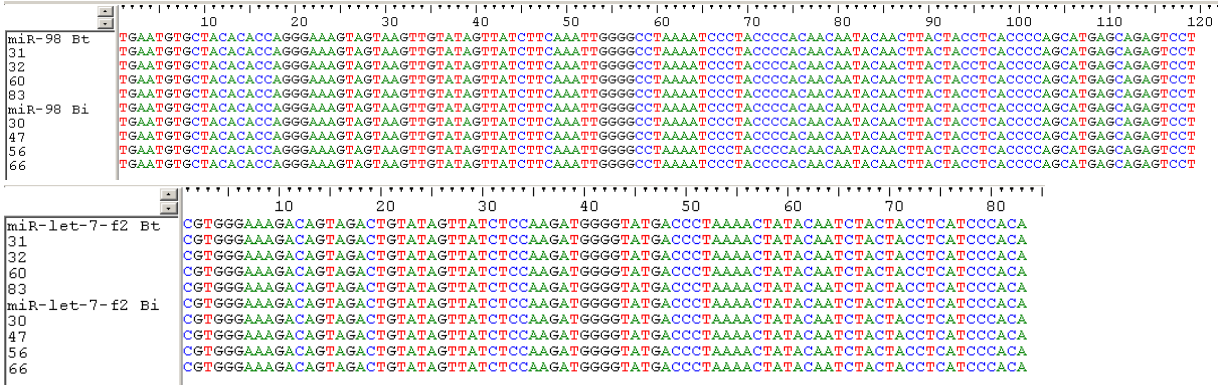

Figure 6.6 Alignments of miR-98 (top) and miR-Let-7-f2 (bottom) precursor sequences of purebred *Bos taurus* (Angus, Bt) and *Bos indicus* (Brahman, Bi) individuals.

One target site of miR-Let-7/98 in the 3'UTR of the bovine *IGF1* gene and 2 target sites in

the 3'UTR of the bovine *IGF1R* gene were amplified and sequenced (refer to Figure 6.3). No sequence variation was observed between or within purebred Brahman and Angus individuals (Figure 6.7).

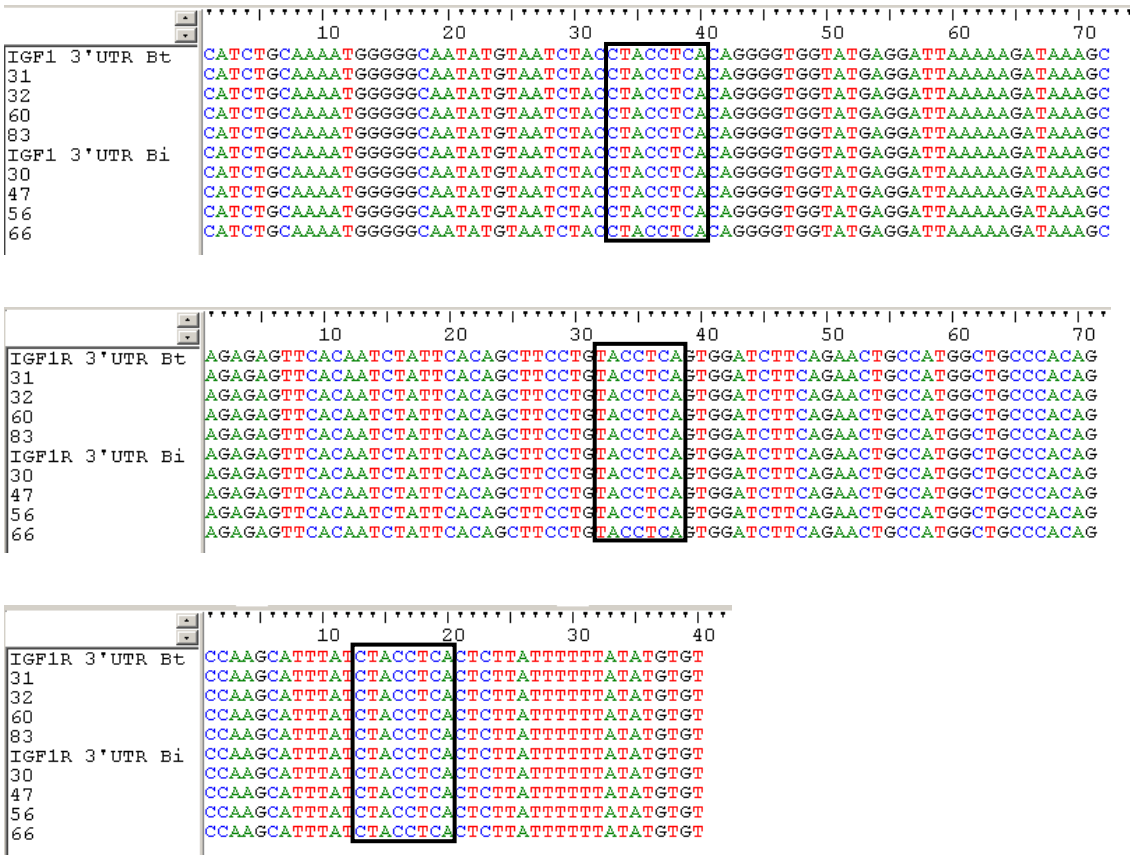

Figure 6.7 Alignments of miR-Let-7/98 target sites in the 3'UTR of the bovine *IGF1* gene (top), and first (middle) and second (bottom) target sites in the 3'UTR of the bovine *IGF1R* gene in *Bos taurus* individuals (Angus, Bt) and *Bos indicus* individuals (Brahman, Bi). Black rectangle is placed on miRNA target sites.

6.3.3 Heterosis, genetic and sex effects on miRNA transcript abundances

Among 158 predicted and experimentally validated miRNAs target sites, only 11 showed a significant (P<0.05) heterosis, genetic, sex or interaction effects in fetal bovine liver (Figure 6.8). Bta-miR-483 was also affected by interaction of heterosis by sex which was approaching significance (P=0.09) (Figure 6.8).

MiR-18a, miR-30a and miR-183, miR-29c, miR-124a, and miR-483 were among experimentally validated miRNA target sites on *IGF* system genes and have been demonstrated to have regulatory effects on *IGFBP8*, *IGF1R*, *IGFBP1*, *IGFBP7* and *IGF1* expression, respectively (TarBase, miRecords and miRTarBase). However, there was no predicted target site for miR-29c in the 3'UTR of the bovine *IGFBP1* gene. It was predicted that both miR-29a and miR-141 have target sites on the 3'UTR of the bovine *IGF1* gene, miR-132 on *IGFBP8*, miR-141 and miR-675 on *IGF1R*, miR-183 and miR-193a on *IGFBP5* and miR-195 on both *GHR* and *IR* genes.

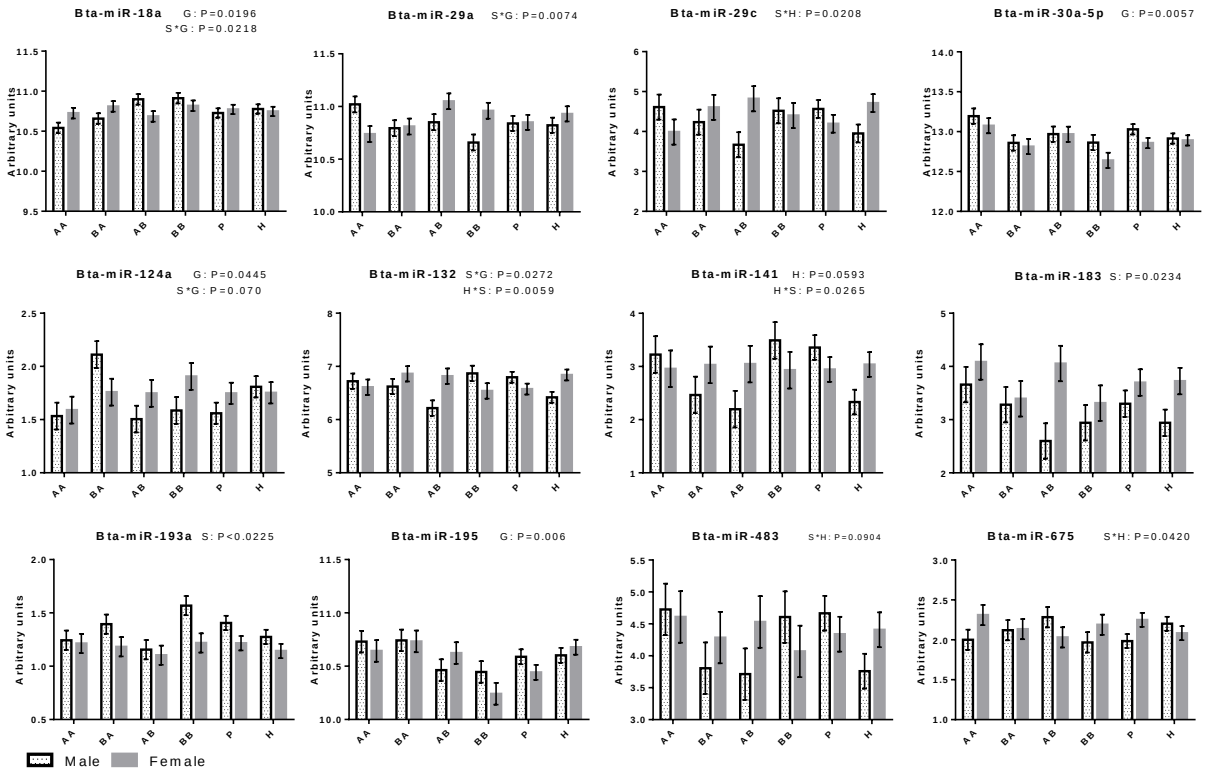

Figure 6.8 Least square means and associated standard errors of means of the abundance of mature miRNA transcripts and experimentally validated target transcripts in the liver of D-153 fetuses. Means for Angus (AA), Brahman (BB) and reciprocal crossbred genetics (BA and AB, sire given first), as well as for purebred (P, including: AA and BB) and crossbred (H, including: BA and AB) genetics are shown. ANOVA effects of heterosis (H), genetics (G), sex (S), interactions of sex by heterosis (S*H) and sex by genetics (S*G) are shown where significant (P<0.05).

6.3.4 Correlation between miRNA and corresponding mRNAs

Correlation coefficients of miRNA expression with their relevant mRNA are shown in Table 6.2. A negative correlation is an indication of the degradation effect of miRNA on its target mRNA.

Generally, there was not a strong negative correlation between miRNAs and mRNAs in fetal bovine liver in this study. The highest negative correlation was found between miR-424 and *IGFBP7* (r= -0.66 and $P<0.05$). MiR-424 was predicted to have a target site on the 3'UTR of the *IGFBP7* gene. MiR-483 and miR-1 showed negative correlations with IGF1 (r= -0.53 and r= -0.50, respectively) (for both $P<0.05$). These two miRNA were experimentally validated to target the *IGF1* gene. MiR-1 also showed negative correlations of r= -51 and r= -49 with *GHR* and *IGFBP8* genes, respectively. However, only *IGFBP8* showed a predicted target site for miR-1. Also, miR-124a showed negative correlations with *IGFBP1* (r= -0.52) and *IGFBP2* (r= -0.53), although there were no predicted target sites for this miRNA in *IGFBP1* or *2*.

Table 6.2 Correlation coefficients between miRNA and their target mRNA within insulin growth factor system (*IGF*) and growth hormone receptor (*GHR*) transcripts in liver on D-153 fetuses. The significance level considered as $P<0.05$ and indicated with *.

	IGF1	*IGF1R*	*IR*	*GHR*	*IGFBP1*	*IGFBP2*	*IGFBP3*	*IGFBP4*	*IGFBP5*	*IGFBP6*	*IGFBP7*	*IGFBP8*
Bta-miR-1	-0.50*	-0.15	-0.06	-0.51*	-0.06	-0.26	-0.3	-0.19	0.02	-0.01	-0.10	-0.49*
Bta-miR-9-star	0.19	0.22	0.18	0.06	-0.276	-0.16	0.03	0.10	-0.08	-0.33	-0.08	-0.23
Bta-miR-18a	-0.33	-0.31	-0.45*	-0.25	-0.13	0.19	-0.16	-0.15	0.25	0.06	0.31	-0.04
Bta-miR-26c	0.25	0.14	0.36	0.09	-0.01	-0.24	-0.07	-0.08	-0.23	-0.13	-0.16	-0.22
Bta-miR-29a	0.25	0.59	0.51	0.19	-0.06	-0.07	-0.09	0.31	-0.33	-0.02	-0.16	0.02
Bta-miR-29c	-0.16	0.17	0.13	-0.38	-0.14	-0.32	0.07	0.17	0.03	0.11	-0.01	-0.19
Bta-miR-30a-5p	0.24	0.23	0.06	0.50	0.35	0.27	-0.11	-0.05	-0.27	0.11	-0.26	0.26
Bta-miR-33a	0.16	-0.23	-0.2	0.09	-0.19	0.05	0.31	0.20	0.11	-0.07	0.01	-0.09
Bta-miR-101	0.42	0.63	0.47	0.17	-0.05	0.03	-0.30	0.01	-0.24	-0.27	-0.54*	-0.08
Bta-miR-124a	-0.07	-0.03	-0.01	-0.32	-0.52*	-0.53*	0.00	-0.1	-0.11	0.06	-0.00	-0.22
Bta-miR-132	-0.11	0.29	0.19	-0.00	-0.02	-0.13	0.29	0.36	0.20	0.3	0.01	0.17
Bta-miR-133a	0.02	0.28	0.31	-0.26	0.09	-0.08	0.05	0.15	-0.04	-0.05	-0.25	0.02
Bta-miR-133b	0.24	0.44	0.60	-0.06	-0.25	-0.51	-0.01	-0.04	-0.36	-0.25	-0.19	-0.08
Bta-miR-135b	0.23	-0.11	0.27	0.34	0.36	0.25	-0.01	-0.07	0.06	0.14	0.18	0.09
Bta-miR-141	-0.31	0.05	0.10	-0.00	0.27	0.05	0.22	0.30	0.17	0.21	0.14	0.07
Bta-miR-153	-0.15	0.06	-0.07	-0.18	-0.17	-0.13	0.11	0.25	0.29	0.49	-0.11	-0.32
Bta-miR-182	0.08	0.1	0.17	0.08	0.05	-0.25	0.20	0.34	-0.19	-0.07	-0.27	0.10
Bta-miR-183	0.03	0.34	0.30	-0.07	-0.00	-0.45*	0.21	0.42	-0.17	0.01	-0.25	0.12
Bta-miR-193a	-0.19	-0.4	-0.08	-0.10	0.18	0.05	0.33	-0.35	0.26	0.12	-0.10	-0.37
Bta-miR-195	0.13	-0.16	-0.10	0.31	0.45	0.26	0.4	0.37	0.04	0.36	0.32	0.28
Bta-miR-205	-0.41*	-0.42*	-0.49*	-0.32	-0.03	0.07	-0.31	-0.33	0.06	0.01	0.30	-0.21
Bta-miR-206	-0.19	-0.16	-0.22	-0.17	0.03	0.03	0.14	0.12	0.42	0.54	0.1	-0.17
Bta-miR-211	0.18	-0.24	-0.24	0.08	0.15	0.32	0.01	0.03	-0.01	0.19	0.20	0.26
Bta-miR-212	-0.37*	-0.01	0.02	-0.13	0.02	0.15	0.13	0.09	0.43	0.42	0.42	0.09
Bta-miR-424	0.23	0.42	0.39	-0.06	-0.42*	-0.50*	-0.04	0.00	-0.41	-0.51*	-0.66*	-0.27
Bta-miR-483	-0.53*	0.44	0.15	-0.02	0.16	0.07	-0.01	0.20	0.12	0.03	-0.36	0.18
Bta-miR-675	-0.10	-0.25	-0.35	-0.12	-0.09	0.09	-0.21	-0.27	-0.05	-0.23	-0.05	0.05
Bta-miR-1721	-0.05	-0.55*	-0.23	0.08	0.21	0.2	0.29	0.21	0.45	0.56	0.56	-0.15

6.4 Discussion

It was shown that the presence of a SNP in miRNA target sites can cause disease or a dramatic phenotype effect in an individual (Gong et al., 2012; Georges et al., 2006). Sequence analysis of the miRNAs selected for investigation in this study did not show any variation among bovine fetuses with *Bos taurus* or *Bos indicus* genetics. MiRNAs are essential for normal growth and development (Alvarez-Garcia and Misk, 2005) and miRNA target sites are

highly conserved across different species of mammals (Friedman et al., 2009). This may explain why there were no sequence variations either in miRNA loci or their target sites.

Based on the correlations between miRNAs and mRNAs, the majority of miRNAs did not show any strong evidence of a possible regulatory effect on fetal liver *IGF* system and *GH-IGF1* axis transcripts in our experimental model. However, this does not exclude potential regulatory effects of these miRNA on *IGF* system and *GH-IGF1* axis genes in other tissues or developmental stages. Another aspect of miRNA regulatory effects that needs to be considered is "mRNA translation inhibition" which is caused by imperfect matching of miRNA with its target mRNA (Filipowicz et al., 2008). It has been shown that a miRNA can inhibit protein translation without causing a reduction in mRNA levels of the target gene (Das et al., 2012). Therefore, not only mRNA transcription, but other measurements at the level of protein abundance are needed to validate a miRNA-mRNA targeting system.

In this experiment, miR-1 and miR-483 showed expression patterns which suggest a regulatory effect on its target gene, *IGF1,* in bovine fetal liver. However, negative correlations were not strong. MiR-483 was first detected in human liver and showed high expression in human embryonic, fetal and adult liver tissues (Liu et al., 2010; Fu et al., 2005). A number of studies have reported that aberrant expression of miR-483 is associated with various types of human cancer (Jain et al., 2012; Zsippai et al., 2011). Interestingly, this miRNA locus is located in intron 2 of the *IGF2* gene in human, mouse, rat, sheep (Ma et al., 2012, Fu et al., 2005) and cattle (*Bos taurus* genome at http://www.ncbi.nlm.nih.gov/mapview/). Intragenic miRNAs have a likelihood of being co-expressed with the host gene. It has been shown that miR-483 expression is correlated with *IGF2* expression (Ma et al., 2011; Meyer-Rochow et al., 2010). In contrast, Church et al. (2012) demonstrated that *IGF2* mutant mice showed up-

regulated miR-483 expression. Contrasting co-expression results may be due to imprinted and/or different methylated status of *IGF2* (Le et al., 2013).

Mir-675 is located in the first exon of *H19* gene. *H19* is a non-protein coding imprinted gene located near the bovine *insulin-like growth factor 2* (*IGF2*) gene (Zhang and Tycko, 1992). It was shown that *H19* is tightly co-expressed with *IGF2* during embryonic development in mouse (Varrault et al., 2006). The H19 Antisense RNA regulates tissue-specific *IGF2* transcription in mouse myoblast cells (Tran et al., 2012). MiR-675 is exclusively expressed in the placenta during development and controls fetal growth probably by targeting and downregulating the expression of *IGF1R* transcript (Keniry et al., 2012).

The regulatory role of miR-483 and miR-675 on *IGF1* and *IGF1R* mRNAs has been predicted (TargetScan.org) and experimentally validated (Ni et al., 2013). Therefore, we may suggest *IGF1* and *IGF1R* mRNA expression may be indirectly impacted by *IGF2* expression in other tissues via miR-675, or possibly co-expressed miR-483.

We showed that miR-124a had negative correlations with *IGFBP1* (r= -0.52) and *IGFBP2* (r= -0.53), also miR-1721 showed a negative correlation with IGF1R (r= -0.55). However, there were no predicted target sites for these mentioned miRNAs in the above genes. This indicates that an approximate negative correlation value of 0.5 observed between miR-1 and *IGF1* may not be considered as a strong/informative negative correlation in this study. Significant threshold of 0.05 introduces the possibility of having some of the significant P values as false positives. However, we have previously hypothesised that the different (epi)genetics in hybrids changes epigenetic mechanisms such as miRNA interference. Correlation analysis within genetics rather than across genetics as in the current limited data set could therefore reveal different correlation coefficients. Another explanation is there might not be a simple

pathway of regulating one transcript by one miRNA. Therefore, an increased sample size for miRNA profiling might be required to assess this further. Further experiments including miRNA profiling of other tissues with larger sample size are required to validate regulation of this complex network of high importance in fetal growth and development.

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Chapter 7

General discussion

7.1 Developmental changes in transcript abundance of *IGF* system and *GH- IGF1* axis genes in bovine tissues

In this study, transcript abundance of *IGF* system and *GH-IGF1* axis genes was assessed in bovine embryonic, fetal and postnatal tissues. Quantitative real-time PCR was used to measure mRNA expression of *IGF1* overall transcript, *IGF1 class 1* and *class 2*, *IGF1R*, *IGFBP1* to 8, *IR* overall transcript, *IR-A, IR-B, GH, GHR* overall transcript, and *GHR-1A*, *GHR-1B* and *GHR-1C*. To our knowledge, this was the first comprehensive study reporting the developmental expression of the *IGF* system and *GH-IGF1* axis genes in different bovine tissues, across pre- and postnatal development.

The IGF system is crucial for prenatal tissue growth and development, cell proliferation and differentiation (Firth and Baxter, 2002; Hossner et al., 1997; Jones and Clemmons, 1995;) and the GH-IGF1 axis plays a key role in postnatal growth and development (Skottner, 2012; Nakae et al., 2001; Allan et al., 2001). In the current study, abundance of transcripts from most genes decreased postnatally in studied tissues, except in liver which showed an increase for *IGF1*, *GHR* and *IGFBP4* and did not show any change for *IR*, *IGFBP1*, 3 and 6 transcripts. This was a demonstration of generally higher *IGF* system transcript abundance in bovine prenatal tissues.

We demonstrated that transcripts from the IGF system and GH-IGF1 axis had a specific pattern of expression in each tissue that differed across developmental stages. We showed that

most transcripts were expressed in all studied tissues and only two transcripts showed tissue-specific expression: *IGFBP1* was not expressed in bovine lung and *IGF1 class 2* transcript was not detected in bovine kidney. However, these transcripts showed some developmental stage-specific patterns as well. The *IGF1 class 2* was not expressed in adult heart and brain or embryonic brain, and *IGFBP1* was not detected in adult brain, heart or skeletal muscle.

In accordance with our results, tissue specific expression of *IGF1 class 2* mRNA was reported in cattle (Wang et al., 2003), human (Jensen et al., 1991) and rat (Shemer et al., 1992). In human and mouse, *IGFBP1* has been shown to be liver-specific (Han et al., 1996; Cerro et al., 1993) which is in contrast with our results. However, our findings in bovine regarding expression of *IGFBP1* in different tissues were similar to rat (Ooi et al., 1990).

Developmental expression of *IGFBP4* and *IGF1* showed differences between bovine and rodent in some tissues. In contrast to our results, Batchelor et al. (1995) showed that *IGFBP4* mRNA increased in lung tissue of rat postnatally in comparison to the prenatal stage. It was shown that in mouse, IGF1 hormone level increased postnatally until two weeks after birth and decreased after D-20 (Calikoglu et al., 2001; Breese et al., 1994). These differences regarding prenatal and neonatal IGF system expression were not observed in bovine and other studied mammals, e.g. human and sheep. However, differences in the timing of development of key organs between rodents and cattle could impact on the comparison of gene expression between these species (Otis and Brent, 1954).

Postnatal GH is an important regulator of IGF1 production (von Horn et al., 2002). *GH* gene expression is mainly from the pituitary gland, although GH expression is not confined to the pituitary gland. It is also present in some postnatal extra-pituitary tissues, in which it may act as an autocrine or paracrine growth factor (Harvey, 2010). Placental GH can be important

for growth and development of the fetus. Since placenta GH cannot be detected in fetal circulation and only presents in maternal blood and amniotic fluid, the direct role of placental GH in prenatal development is controversial (Skottner, 2012). Fetal pituitary gland is able to produce GH from the second trimester in some species, including human, cow and sheep (Waters and Kaye, 2002). However, mice deficient in GH, due to mutations in the genes encoding GH-releasing hormone receptor, showed normal birth weight (Efstratiadis, 1998). Spencer et al. (1995) measured umbilical blood concentrations of IGF1 and GH in normal and growth retarded human newborns. They found that only IGF1 is significantly lower in growth retarded individuals and GH level is not correlated with fetal growth in human.

There was no detectable *GH* mRNA in the bovine tissues studied. Since *GH* transcript from the pituitary gland as a primary source of GH was not measured, the potential roles of *GH* transcription in bovine prenatal growth and development remains inconclusive. However, significant and tissue and genotype-specific *GHR* expression raises the possibility of *GH* actions (see below).

The GHR mediates the biological actions of GH on target cells by transducing the stimulating signal across the cell membrane and by inducing the transcription of many genes, including *IGF1* (Kobayashi et al., 1999; Rotwein et al., 1991). It has been shown that *GHR* transcripts are present in fetal tissues of rat (Edmonson et al., 1995), cattle (Lucy et al., 1998a) and human (Hill et al., 1988). Knockout mouse models showed no significant body size or weight differences between controls and *GHR* knockout newborns. However, *GHR* deficient mice (Laron mice) were significantly smaller three weeks after birth (Zhou et al., 1997). Laron mice showed 90% less circulating IGF1 and these mice could live significantly longer (up to 50%) than normal mice (Coschigano et al., 2000).

The present data showed that *GHR* transcripts can be found in a variety of bovine tissues during pre and postnatal growth and development. This study confirmed that *GHR-1A* transcript is specific to liver and that its expression can be detected from mid-gestation in the bovine fetus. *GHR-1A* is the equivalent of liver-specific transcript in human (GHR-V1), mouse (GHR-L1), rat (GHR-V2) and sheep (GHR-1A) (Edens and Talamantes, 1998). A limited number of studies have investigated fetal liver *GHR-1A* expression in cattle. In contrast to our results, it has been reported that *GHR-1A* mRNA cannot be detected in the 8-month old cattle fetus (Lucy et al., 1998a). In addition, liver-specific transcript of *GHR* was not detected in human fetal liver (Kenth et al., 2011), although *GHR* overall transcript was found in both cattle and human fetal liver (Lucy et al., 1998a; Kenth et al., 2011). However, liver specific transcript of the *GHR* was detected at late gestation (D-145) in fetal sheep liver (Pratt and Anthony, 1995; Klempt et al., 1993; Adams et al., 1990).

We demonstrated that *GHR* mRNA was expressed in a variety of bovine fetal and embryonic tissues. The biological reasons for high levels of *GHR* expression in bovine embryonic and fetal tissues are not clear, in particular since the bovine placenta does not express *GH-V* (Skottner, 2012).

GHR is the main mediator of GH actions, although the role of the GH-IGF1 axis in prenatal growth and development has not been identified clearly (Osafo et al., 2005). It has been shown that infusion of GH in late-gestational fetal sheep did not alter fetal growth or fetal IGF1 and IGFBP3 concentrations (Bauer et al., 2000).

There is a high degree of similarity between GH and prolactin (PRL) and their receptors (GHR and PRLR), with homology in amino acid sequences and gene structures, in addition to physiological functions (Goffin and Kelly, 2001). Bovine GHR can be a receptor for PRL and

placental lactogen (PL) hormones, and interestingly, GHR and PRLR can form a functional heterodimer receptor (Goffin and Kelly, 2001; Vashdi et al., 1992). The affinity of ruminant PL to GHR and PRLR has been shown to be high (Byatt et al., 1992). Upon binding either of GH, PRL or PL to GHR, signal transduction initiates with phosphorylation of cytosolic Janus kinase 2 (JAK2) which activates STAT pathways (Hwa et al., 2011). Maternal bovine PL is in fetal circulation with peak concentration in mid-gestation (Kappes et al., 1992). In sheep, fetal serum concentration of PL is correlated with fetal weight in singletons (Schoknecht et al., 1991). Interestingly, infusion of ovine PL into fetal sheep increased serum IGF1 concentrations significantly (Schoknecht et al., 1992). The binding ability of other ligands such as PRL and PL to GHR can trigger STAT5 signal transduction (Hwa et al., 2011; Goffin and Kelly, 2001) and high correlations detected between *IGF1* and *GHR* transcripts in the present study could be explained by a role of GHR-IGF1 axis in prenatal growth and development in bovine.

7.2 Heterosis, genetic and sex effects on transcript abundance of *IGF* system components and *GH- IGF1* axis genes

Heterosis, or hybrid vigour, is the superiority of first generation hybrids over the average of the purebred parents (Dickerson, 1952). Heterosis has an important role in improving the productivity of plants and animals and has been used for over a century to obtain benefits in animal production industries (Hochholdinger and Hoecke, 2007). Heterozygosity is a necessary condition to observe changes in transcription and consequently phenotypic variation in hybrid individuals. Heterosis results from interactions between the paternal and maternal genome (Birchler, 2013).

The level of heterosis is known to be correlated with the genetic distance between parents (Chen, 2010). In addition to the parental genetics, various levels of heterosis have been noted due to different environmental circumstances. A heterosis × environment model was proposed by Frisch (1987). Effects of restricted feed, ticks and worms on the magnitude of heterosis showed that heterosis measured as the deviation from the mid-parent mean will increase continuously with the level of environmental stress (Frisch and Vercoe, 1984).

Despite the long history of using heterosis in industry, the molecular mechanisms underlying this phenomenon remain unclear. There are several hypotheses proposed from quantitative genetics, including dominance, over dominance and epistasis, but none fully explain the mechanism of heterosis (Alexander et al., 2009; Birchler et al., 2003; Cunningham, 1982). Bowman (1959) suggested that "it is highly probable that there is no single genetic explanation for heterosis, but dominance, whether partial or complete, and all types of genetic interaction combined in different proportions in different situations result in heterosis".

It was demonstrated that plasma IGF1 level is positively correlated with heterosis in cattle (Caldwell et al., 2011) and mouse (Adamo et al., 2006). It was also shown that plasma IGF1 is positively correlated to fetal growth and development in cattle (Hiendleder et al., 2006) and human (Leger et al., 1996). GH is a key stimulator of IGF1 production and also affects IGF2 production in liver postnatally (von Horn et al., 2002). GH actions are mediated through growth hormone receptor (GHR). Binding of GH to GHR initiates the transcription of many genes including IGF1 gene via STAT5 signal transduction (Hwa et al., 2011; Jiang et al., 2007).

The current study evaluated heterosis, genetic and sex effects on transcript abundance of *IGF* system and *GHR* genes in bovine fetal tissues. There were no heterosis effects on fetus weight

or organ weights. However, fetus weight and organ weights were affected significantly by genetics and sex, with the exception of brain and fetal placenta.

We demonstrated that hybrid males showed significantly higher *GHR-1A* mRNA than females. Since expression of *GHR* is regulated by a variety of factors such as steroid hormones (Horseman and Yu-Lee, 1994; Cosman et al., 1990), fetal male testosterone might be responsible for elevated levels of liver *GHR-1A* transcript. These findings suggested that male androgen may contribute to higher expression levels of *GHR* mRNA in fetal bovine liver and could explain the observed higher organ weights in males compared to females. However, more detailed research is needed to proof effects of fetal male androgen on transcription of *GHR-1A* in liver.

Considering the fact that the major proportion of circulating IGF1 is produced in the liver (Yakar et al., 1999), our results confirmed that the molecular heterosis observed in *IGF1* transcript of the bovine fetal liver is in accordance with the heterosis of circulating IGF1 levels reported in hybrid calves (Caldwell et al., 2011).

The *IGF1 class 2* transcript showed a higher level of heterosis in fetal liver and skeletal muscle than *IGF1 class 1* transcript. The *IGF1 class 2* transcript was reported to be more strongly associated with heterosis in circulating IGF1 hormone in hybrid mice in comparison to *IGF1 class 1* transcript (Adamo et al., 2006). It was also demonstrated that both *IGF1 class 1* and *2* transcript levels increased in liver after administration of GH to adult cattle and sheep. However, *IGF1 class 2* transcript showed a significantly greater increase in expression than *IGF1 class 1* transcript (Wang et al., 2003; Pell et al., 1993). Infusion of GH into sheep fetuses showed no increase in IGF1 hormone (Bauer et al., 2000). This may be an indication of a different role of the GH-IGF1 axis in postnatal, compared to prenatal life.

The present data showed high levels of molecular heterosis in liver *GHR* overall transcript abundance and liver-specific *GHR-1A* mRNA. This was the first report of heterosis in bovine *GHR* mRNA. Since we demonstrated a correlation of 0.99 between *GHR-1A* and *IGF1 class 2* transcripts and as GH actions are mediated through GHR, it is reasonable to conclude that the observed heterosis in liver *IGF1 class 2* transcript is due to increased liver *GHR-1A*.

The data also showed that all liver IGFBP transcripts showed negative or no heterosis. This may indicate that modulators of IGF1 (i.e., IGFBPs) are less expressed in liver of hybrid fetuses and therefore more free IGF1 (and IGF2 which was not investigated in this study) is available to bind to its receptors and initiate its wide range of actions.

There was no heterosis in weight of D-153 fetuses. However, a number of previous studies detected heterosis in birth weight of cattle (Elzo et al., 2012; Johnston et al. 2001; Kress and Nelsen, 1998; Brown et al., 1993). The present data suggest that mechanisms controlling heterosis in bovine at term and postnatally, i.e., liver *GHR* in addition to *IGF1* mRNA, are initiated in the fetal stage and can be readily detected in mid-gestation.

The higher correlation of *IGF1 class2* transcripts with *GHR-1A* transcript, and higher levels of molecular heterosis observed in fetal liver in the current study, suggest that liver *IGF1 class 2* and *GHR-1A* transcripts had higher contributions to heterosis in the bovine fetal model than other IGF system and GH-IGF1 axis transcripts.

Among studied tissues, fetal liver appears to be the most important tissue to study the molecular mechanisms of heterosis. Fetal liver was shown to express a higher diversity of transcripts of the GH-IGF1 axis which were also expressed at higher levels in fetal liver compared to other fetal tissues.

7.3 Micro RNAs and other potential regulators of *IGF* system and *GH-IGF1* transcripts

Experimental data and computational prediction of miRNA target sites suggested that each miRNA potentially targets multiple, ten to several hundred, mRNAs, which indicated that over 30% of all mammalian genes may be regulated by miRNAs (Rybak et al., 2009). By targeting the mRNA of protein-coding genes, miRNAs play a critical role in a variety of biological processes such as development, cell growth, proliferation, lineage determination and metabolism (Morris, 2008). In mammals, miRNAs have been shown to regulate a large number of pathways including B-cell differentiation (Chen et al., 2004), adipocyte differentiation (Esau et al., 2004) and insulin secretion (Poy et al., 2004). Knockout of the miRNA-producing enzyme Dicer1 in mice leads to lethality early in development. Dicer1 deleted embryos died before D-8 of gestation and did not develop any cell lineage which is a clear indicator of the crucial roles of miRNAs in early development (Bernstein et al., 2003).

In the present study, we used databases to select miRNAs which have been experimentally validated or predicted to have target sites in the *IGF* system and *GHR* transcripts. Heterosis, genetic and sex effects on transcript abundance of fetal liver mature miRNAs were evaluated. We showed that among 158 miRNA which were predicted or experimentally validated, only 11 were significantly affected by heterosis, genetic or sex effects. Correlation of miRNA expression with the transcript abundance of the *IGF* system and *GHR* transcripts revealed that some of the predicted or validated miRNA have a notable negative correlation with transcript abundance of the *IGF* system and *GHR* transcripts in fetal liver. Among those miR-483 and miR-1 with *IGF1* (r=-0.53 and -0.50, respectively) and miR-424 with *IGFBP7* (-0.66). A high negative correlation indicates the degradation effect of miRNA on their targets. MiR-1 also

showed negative correlations of r= -51 and r= -49 with *GHR* and *IGFBP8* genes, respectively. However, only *IGFBP8* showed a predicted target site for miR-1. Also miR-124a showed negative correlations with *IGFBP1* (r= -0.52) and *IGFBP2* (r= -0.53), although there were not any predicted target sites for this miRNA in *IGFBP1* or *2*. These observations indicated that an approximate negative correlation value of r= -0.5 which was observed between some miRNA and mRNAs in this study may not be considered as a strong correlation. However, we have previously hypothesised that the different (epi)genetics in hybrids changes epigenetic mechanisms such as miRNA interference. Correlation analysis within genetics rather than across genetics as in the current limited data set could therefore reveal different correlation coefficients. Another explanation is there might not be a simple pathway of regulating one transcript by one miRNA.

The present data showed some miRNAs, as gene expression regulators, were differentially expressed among different genotypes. It may be concluded that some miRNAs may have a potential regulatory role in transcription of *IGF* system and *GHR* genes in bovine fetal liver. However, more experiments with an increased sample size for miRNA profiling are required to assess this further. It is important to consider possible sequence variation in miRNA loci as they may impact any comparison between miRNA expression of *Bos taurus* and *Bos indicus*.

In this study, sequence variation of miRNA loci including miR-483, Let-7-f2 and miR-98 and their target sites located in the 3'UTR of *IGF1* and *IGF1R* were investigated. Sequence analysis did not show any variation among bovine fetuses with *Bos taurus* or *Bos indicus* genetics. Single nucleotide polymorphisms (SNPs) can potentially create or eliminate target recognition sites for miRNAs in target mRNAs. A SNP inside a pre-miRNA sequence (stem and loop) or the seed region can change the secondary structure and consequently impact on the maturation process of a miRNA. New target sites for a miRNA, or elimination of an

existing target site, have been linked to human diseases and phenotypic variation in farm animals (Georges et al., 2006; Gong et al., 2012).

The present data confirmed a retrotransposon insertion in the liver-specific promoter of the bovine *GHR* (Lucy et al., 1998a). This insertion was found to be specific to *Bos taurus* (Lucy et al., 1998b; Ohkubo et al., 2006). A similar insertion was reported in mouse (Moffat et al., 1999) and goat (Maj and Zwierzchowski, 2005), but has not been reported in other mammals, including human and sheep. The inserted retrotransposon, with a length of 1.2 kb, is a long interspersed nuclear element (LINE-1 element or L1) and belongs to the non-long terminal repeat (non-LTR) family of retrotransposons (Furano et al., 2004). Full length active bovine L1 is approximately 6-8 kb and contains two open reading frames (ORFs) which are necessary to initiate an actual retrotransposition (Girardot et al., 2006). However, any L1 insertion in genome can impact on regulation of transcription, especially when a gene is disrupted by an L1 insertion (Britten, 1997).

We confirmed that L1 inserted in the promoter of bovine *GHR* was specific to *Bos taurus* (Angus) and consisted of a partial (5' truncated) L1 element which contained a portion of the second ORF. Therefore, it cannot act as an active transposable element. However, it could potentially affect downstream gene expression. In human 5' truncated L1 are widely distributed in the genome and it is believed that they are not functional (Boissinot et al., 2001). However, Han et al. (2004) showed that even a partial L1 element integrated into an intron can downregulate gene expression significantly. Further experiments, including methylation analysis and allele specific expression levels and *in vitro* experiments, are needed to establish potential effects of the L1 insertion in bovine *GHR* on liver-specific *GHR* transcription.

Previous reports indicated that a GT microsatellite in bovine *GHR* promoter has a short allele with 11 repeats $(GT)_{11}$ in cattle without L1 insertion and a long allele with 16-20 repeats $(GT)_{16\text{-}20}$ with the L1 element (Lucy et al., 1998b). These data were obtained on five cattle breeds, including Brahman, Nellore, Hereford, Limousin and Santa Gertrudis (Lucy et al., 1998b). Subsequently, a number of studies have shown a significant association between the length of this specific GT microsatellite and production traits in cattle, including average daily milk production, weaning weight and carcass weight (Muhaghegh-Dolatabady et al., 2012; Curi et al., 2005; Hale et al., 2000), demonstrating that individuals with $(GT)_{11}$ allele had lower production than individuals with $(GT)_{16\text{-}20}$ allele.

Some microsatellites are known to have important functions in the genome such as AC/TG (Gemayel et al., 2010). The AC/TG motif is able to absorb negative supercoiling through the formation of Z-DNA, which can affect nucleosomes by displacement and subsequently affect downstream transcription (Xu et al., 2011). A $(GT)_n$ microsatellite can increase gene activity and if a GT repeat is closer to the promoter sequence it can be more effective as a transcriptional enhancer (Stallings et al., 1991).

We showed that the GT repeat in the bovine *GHR* is very close (90 nucleotide) to the liver-specific promoter even when considering the length variation between Angus and Brahman individuals. It may be worth considering this GT repeat variation as one of the factors affecting liver specific transcription of *GHR* in our experiment.

Interestingly, there is another GT microsatellite with 11 repeats located 800bp upstream of bovine (*Bos taurus*) *IGF1* (Sawaya et al., 2012). We did not perform sequencing among individuals. However, there might be variations between Angus and Brahman individuals.

The analyses also showed that there are genomic sequence variations in some transcriptional regulatory elements between parental breeds of Angus and Brahman resulting in heterozygosity in hybrid offspring. It is believed that heterozygosity is a necessary condition to observe changes in transcription and consequently phenotypic variation in hybrid individuals. The level of heterosis was shown to be correlated to the genetic distance between parents (Chen, 2010). In fact more genetic diversity will result in higher level of differences between transcriptional regulatory elements, including microsatellites or retrotransposons between purebred animals.

Heterosis mainly results from interactions between the paternal and maternal genome (Birchler, 2013). We showed that the differentially expressed genes and sequence variation in some regulatory elements in addition to differentially expressed miRNA contribute to molecular heterosis. As suggested by Bowman (1959), Chen (2010) and Birchler (2013), there is no simple explanation for hybrid vigour.

7.4 General conclusion

We demonstrated that the *IGFs* and *GHR* transcript profiles associated with developmental growth in the present study are to a significant extent in agreement with those seen in other species, including sheep, human and pig and appeared be less similar to patterns observed in rodent.

We demonstrated that the developmentally important *IGF* system and the *GHR* gene, contributed to heterosis in a bovine fetal model. Based on our bovine model we propose that liver *GHR-1A- IGF1 class 2* transcripts can be considered as an important axis involved in molecular heterosis which might be more important in late gestation growth and development

in bovine. Additionally, some investigated transcriptional regulatory elements, such as retrotransposons, microsatellites and miRNAs, might contribute to observed bovine molecular and phenotypic heterosis.

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